Molecular, Phylogenic, Mass-Spectrometry and Decay Analyses of Copper Tolerant *Phialophora* Species Causing Soft Rot of Wood

Hasanthi Karunasekera
Faculty of Forest Sciences
Department of Forest Products/Wood Science
Uppsala

Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2017

Acta Universitatis agriculturae Sueciae
2017:48

ISSN 1652-6880
ISBN (print version) 978-91-576-8869-9
ISBN (electronic version) 978-91-576-8870-5

Print: SLU Service/Repro, Uppsala 2017

Abstract

Soft rot decay of treated wood in ground contact and loss of service life is an important economic issue worldwide. One of the most important and cosmopolitan fungal groups causing soft rot belong to the imperfect fungal anamorph genus *Phialophora*. An aim of this study was therefore to determine the molecular identity and phylogeny of several economically important *Phialophora* species isolated from copper-treated and untreated wood using PCR combined with sequencing. Strain compatibility and copper detoxification of *Phialophora* species/strains were also assessed *in-vitro* from both liquid and solid cultures. The identity of a number of so far unclassified *Phialophora* spp. A strains isolated from treated wood in-service were found as *Phialocephala dimorphospora*.

The ability of strongly (*P. malorum*, *P. mutabilis*) and weakly (*Chaetomium globosum*) copper tolerant soft rot fungi to degrade $CuSO_4$ and micronized-Cu (MC) treated wood (birch/pine) was assessed using a modified ENV 807 decay test with vermiculate and sterile soil. A direct relationship between Cu-tolerance *in-vitro* and soft rot decay of Cu-treated wood was not found. Rather, the additive effect of copper binding to the wood and the chemical nature of the wood material (lignin type/level) appeared more important with the less Cu-tolerant fungi causing similar/greater mass losses than Cu-tolerant strains.

LC-MS/MS and MALDI-TOF MS/MS allowed characterization of total and extracellular (wall-bound) upregulated proteins produced by *P. malorum* while growing in Cu-supplemented growth medium. Despite *P. malorum* lacking a sequenced genome, it was possible using modern proteomics to characterize changes in global proteins and detect a number of unique as well as up-regulated and down-regulated proteins when grown in Cu-supplemented liquid media. Using MALDI-TOF MS/MS a number of cell wall/slime bound proteins were also shown up-regulated in response to growth in copper media and thereby likely involved in Cu-tolerance.

Keywords: copper tolerance, LC-MS/MS, MALDI-TOF MS/MS, *Phialophora molarum*, *Phialophora* spp., proteomics, soft rot decay

Author's address: Hasanthi Karunasekera, SLU, Faculty of Forest Science, Department of Forest Products, P.O. Box 7008, SE-75007 Uppsala, Sweden
E-mail: hasanthi.karunasekera@ slu.se

To my loving two sons Dinura & Venuja!

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Karunasekera, H. and Daniel. G. (2013) Molecular identification and phylogenic analysis by sequencing rDNA of the copper-tolerant soft-rot *Phialophora* spp. International Biodeterioration & Biodegradation 82, 45-52.

II Karunasekera, H. and Daniel. G. (2015) Phylogenic, molecular and decay analysis of *Phialophora* species causing soft rot of wood International Wood Products Journal 6 (4), 189-197.

III Karunasekera, H., Terziev. N. and Daniel. G. (2017) Does copper tolerance provide a competitive advantage for degrading copper treated wood by soft rot fungi? International Biodeterioration & Biodegradation 117, 105-114.

IV Karunasekera, H., Pettersson, J., Mi, J., Bergquist. J. and Daniel. G. (2017) Aspect of copper tolerance of the soft rot fungus *Phialophora malorum* grown *in-vitro* in copper supplemented media: Light, electron microscopy and global protein expression studies through proteomics approach (Submitted to International Biodeterioration & Biodegradation).

V Daniel, G., Volc. J., Halada, P. and Karunasekera, H. (2017) Aspects of copper tolerance of the soft rot fungus *Phialophora malorum* grown in *in-vitro* in copper supplemented media: Cell wall bound copper and detoxification (Manuscript).

The contribution of I-V to the papers included in this thesis was as follows:

I HK participated in the design of the experiments, carried out practical work and analysis of the experiment.

II HK participated in the design of the experiments, carried out practical work and analysis of the experiment

III HK participated in the design of the experiments, carried out practical work in the laboratory

IV HK participated in the design of the experiments, carried out practical work at SLU and participated in the experimental work carried out at Uppsala University.

V HK participated in the laboratory work at SLU.

Abbreviations

BLAST	Basic Local Alignment Search Tool
CCA	Chromated copper arsenate
DNA	Deoxyribonucleic acid
ITS	Internal transcribed spacer
LC-MS/MS	Liquid chromatography-mass spectrometry
LM	Light microscopy
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MC	Micronized copper
MEA	Malt extract agar
PCR	Polymerase chain reaction
SEM	Scanning electron microscopy

1 Introduction

1.1 Background

Wooden buildings and construction materials are continuously exposed to microorganisms especially to wood degrading fungi and insects. Among the three major groups of rot fungi namely white-, brown- and soft rot, the latter is the second most important group causing decay in economically important wooden structures e.g. telegraph poles, marine installations and cooling towers (Daniel 2014).

In such situations, accurate identification of decay fungi is one of the most important and primary steps for formulating strategies for remedial measures and further research studies. Even though there are fungal identification methods available including advanced techniques like light-, and electron microscopy and molecular identification tools to screen down to species and strain level, there is still a lack of easy, rapid and accurate identification methods available for soft rot fungi. This for example motivated the application of molecular identification techniques using PCR (polymerase chain reaction) based methods during the current research studies on soft rot fungi described in this thesis. Since wood is the main feed stock for wood decaying fungi, understanding wood structure (from macro-, micro- to ultrastructure) is important and provides the basis for studying and elucidating decay processes including the fungal decay mechanisms involved.

With increasing use of hazardous and toxic chemicals for protecting wood and the current knowledge and awareness among the general public, there has been a tremendous concern on the toxic effect of chemicals traditionally used as wood preservatives. For example, even though chromated copper arsenate (CCA) has been the most successful wood impregnation chemical for decades, it is now prohibited in many countries worldwide including USA and Europe. Since copper has a proven “broad spectrum” activity against most of the wood degrading fungi, it is still used as an active ingredient in many commercial wood preservative formulations. Therefore focus during research work in this thesis is on copper as a preservative against soft rot fungi.

A major drawback with copper containing wood preservatives is the occurrence of copper tolerant soft rot fungi species/strains, like for example *Phialophora* species as reported by Nilsson and Henningsson (1978) and Daniel and Nilsson (1988). Studies in this thesis are therefore concentrated on copper tolerant soft rot *Phialophora* species and their strains, including their molecular identification, biodegradation mechanisms and copper tolerance ability. Their copper tolerance mechanisms are not well known and a range of morphological and analytical methods are applied here for studying aspects associated with this process.

1.2 Structure of wood

Except for monocotyledonous species (e.g. palms, bamboo), all commercially important tree species originate from the dicotyledons and are divided into hardwoods and softwoods. Hardwoods are angiosperm, broad-leave species while softwoods are gymnosperm coniferous species (e.g. Norway spruce and Scots pine; Fig. 1a) having needle-shaped leaves. The majority of hardwoods in temperate zones (e.g. birch, oak; Fig. 1b) are deciduous (shed leaves) and softwoods are evergreen although there are exceptions. For example Eucalyptus is a hardwood with the majority of species evergreen, while larches are softwoods which shed their needles in winter.

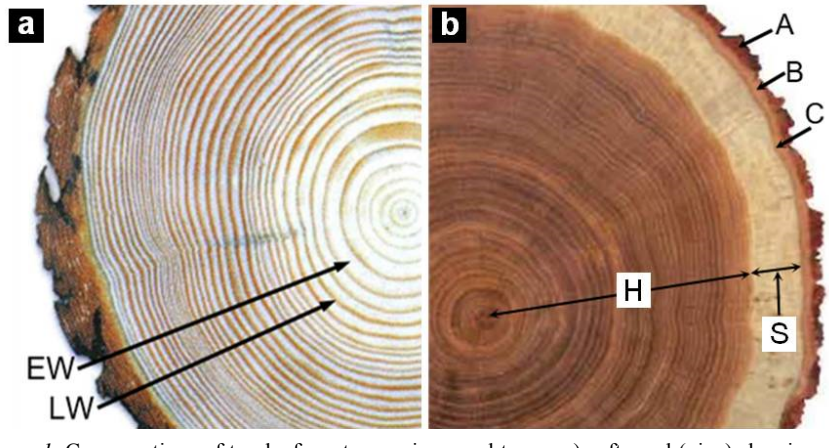

Figure 1. Cross sections of trunks from two major wood types; **a**) softwood (pine) showing clear growth rings where light bands are earlywood (EW) and dark bands are latewood (LW) and **b**) hardwood (oak) showing sapwood (S), heartwood (H), outer bark (A), inner bark (B), cambium (C) (From Nylinder et al., 2005).

In all trees, wood (i.e. secondary xylem) is formed by a living cell layer called the vascular cambium (Fig. 2) and its repeated cell division produces phloem cells to the outside and secondary xylem cells/wood cells to the inside. The xylem outer part (sapwood) contains both living and dead cells while the inner

part (heartwood) contains only dead cells. Due to deposition of phenolics and other extractives, the heartwood is less permeable and normally more durable than sapwood.

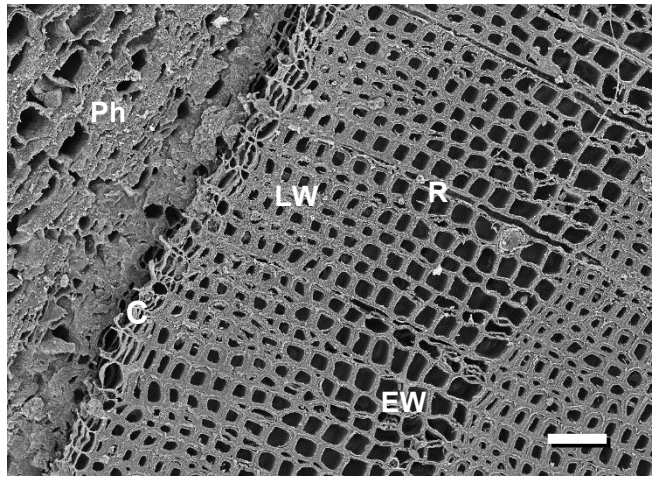

Figure 2. SEM micrograph of a transverse section of Norway spruce through phloem and xylem regions. C, cambium; EW, earlywood; LW, latewood; Ph, phloem; R, ray parenchyma. Bar: 40 µm (From Fernando, 2007).

Softwood structure is rather simple and more or less uniform compared to hardwood due to having a lesser number of cell types (Fig. 3a). Softwood xylem is mainly composed of longitudinal tracheids (90-95% of the total cell volume) and a smaller amount of ray parenchyma (5-6%), ray tracheids and epithelial cells surrounding resin canals (Sjöström, 1993).

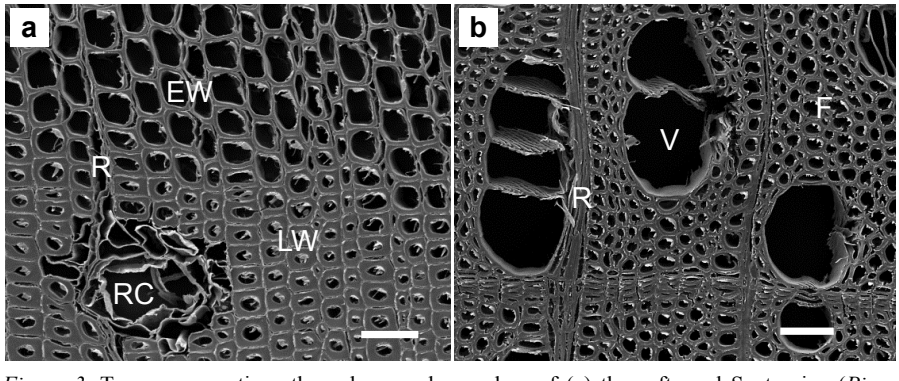

Figure 3. Transverse sections through secondary xylem of (a) the softwood Scots pine (*Pinus sylvestris*) showing simple cellular structure with ordered arrangement of earlywood (EW) - and latewood (LW) tracheids, rays (R) and a resin canal (RC) compared with (b) the more complex structure of hardwood xylem of birch (*Betula verrucosa*) showing the large vessel pores (V), thick rays (R; multi-seriate rays) and fibres (F) etc. Bars: a, b 20 µm.

In contrast, hardwoods like birch are complex and advanced in their cellular organization where hardwood xylem normally contain many cell types including fibres, vessels, tracheids and different types of parenchyma cells (Fig. 3b).

Trees in temperate regions contain concentrically oriented growth rings visible in cross sections of wood, due to the variation in the growth patterns in different seasons of the year (Fig. 1). Growth of a tree occurs during spring and summer and the cells formed in spring constitute early wood (EW) having thin cell walls and large cell lumina while that formed at the end of summer is latewood (LW) (Figs. 1, 2, 3) having thick cell walls and small lumina. In softwoods and ring-porous hardwoods these growth layers are visible due to differences in cell size and variation in cell wall thickness.

When considering the performance of wood against wood destroying micro-organisms it is necessary to understand the three dimensional structure of wood (Fig. 4). This allows recognition and understanding of the pathways of microbial entry and colonization into wood and to perceive the pathways of wood preservative penetration. The wood anatomy and three-dimensioned structure of wood varies greatly between wood species and represents a basis why certain species are more easily impregnated with preservations and why others are more easily degraded. A major controlling factor are the ratios between the wood cell types and presence and abundance of pits.

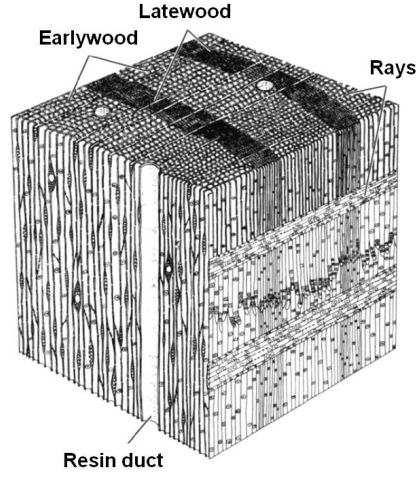

Figure 4. 3D cellular structure of softwood xylem with tracheids in early- and latewood regions including rays and resin canals (From Eriksson et al., 1990).

In this thesis work, both softwood (Scots pine; *Pinus sylvestris* L.) and hardwood (birch; *Betula verrucosa* Ehrh.) were used to study wood biodegradation by soft rot fungal species.

1.2.1 Wood cell wall ultrastructure and chemical composition

Wood cell walls which provide nutrients for microorganisms are complex and mostly composed with a hierarchical structure are organized into thin primary and thicker secondary cell wall layers which are laid down successively (Fig. 5). This double cell wall layering is universal for the majority of normal mature wood cell walls. It is built up of several layers, i.e. middle lamella (ML) which binds cells together, the primary cell wall (P) ca. 0.1-0.2 µm, and a secondary cell wall composed of three layers; the outermost and thinner S1 layer, the thick middle S2 and innermost thin S3 layer which in turn is frequently coated with a warty layer (W) (Fig. 5) (Sjöström, 1993). These layers vary depending on their structure and chemical composition. The middle S2 layer forms the main part of the wood cell wall with a thickness of ca 1-5 µm while the thin S1 and S3 layers are ca 0.2-0.3 µm and 0.1 µm in thickness respectively.

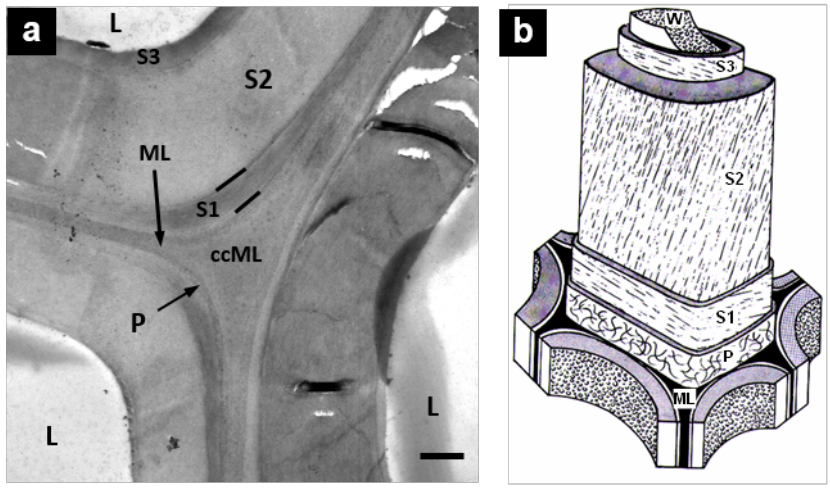

Figure 5. Ultrastructure (**a**; from Fernando, 2007) and the simplified schematic model (**b**; from Côté, 1980) of wood fibre cell walls showing the major wall layers. Dark lines in **b** (P and S1-S3 layers) indicate orientation of cellulose microfibrils. ccML, cell corner middle lamella; L, cell lumen; ML, middle lamella; P, primary cell wall; W, warty layer with warts. Bar: 0.5 µm.

The major chemical composition of wood is cellulose, hemicelluloses and lignin. Cellulose is the main structural component of wood cell walls accounting for 40-55% of the total cell wall mass while hemicelluloses contribute 18-33% and lignin ca 22-28%. However the composition of lignin and hemicelluloses differs between hardwoods and softwoods. In addition the chemical composition of the individual layers in cells can vary considerably depending on both tissue (e.g. reaction wood) and cell type (e.g. fibre, parenchyma cell). The major hemicellulose type in softwoods is mannan (ca. 20% galactoglucomannan) and xylan in hardwoods (15-30% glucuronoxylan) (Sjöström, 1993). Lignin is a complex phenolic polymer made up of three basic monomers namely p-coumaryl, coniferyl- and sinapyl alcohols. The content of

these monolignols can vary between the two major wood types leading to differences in lignin composition/type where softwood lignin (i.e. guaiacyl lignin) consists almost exclusively of coniferyl alcohol and hardwood lignin (i.e. guaiacyl-syringyl lignin) is a copolymer of both coniferyl and sinapyl alcohols that can vary from being equal in proportion to being three times more than sinapyl alcohol (Boerjan et al., 2003).

Cellulose exists in the form of microfibrils in wood cells and their function is to convey strength to the cell wall, while hemicelluloses consist of different sugars and act to unite cellulose and lignin while the lignin acts as a matrix material. Cellulose and hemicelluloses are sugars that can provide the necessary energy for fungi to live and grow in wood and thereby demineralize its cellular structure, while lignin is a rather complex three dimensional network polymer of phenolic alcohols which can slow down fungal decay as generally seen with soft rot. However lignin can be used and degraded by white rot fungi and to a lesser extent by certain soft rot fungi.

1.3 Wood degrading organisms

Wood degrading organisms are found in quite diverse groups ranging from insects and molluscs to microorganisms like fungi and bacteria. In addition, mechanical damage to wood can also be caused by higher animals such as beavers and woodpeckers. Termites represent the most important group of insects specialized in living on wood while larval stages of other insects perform important decay. In marine environments; ship worms (molluscs) can cause rapid destruction of wood (Björdal and Nilsson, 2008). Biodegradation of wood due to microorganisms is a natural process where organic matter recycling takes place. Under natural conditions wood is often colonized rapidly by a variety of microorganisms that initiate biodegradation. Here the main wood structural components, i.e. polysaccharides and lignin, are demineralized and degraded into simpler molecules ending as CO_2 and water. If this process occurs on "man-made" products the service life can be reduced with considerable economic importance.

Microorganisms causing demineralization of wood include bacteria, blue stain and wood rotting fungi (i.e. brown- white- and soft rot). The manner by which wood demineralization occurs varies according to the different organisms involved, the substrate type and environmental condition as well as competition between the organisms (Daniel and Nilsson, 1988; Björdal and Nilsson, 2008).

Bacteria are early colonizers of wood and their effect on wood varies. Some types have little effect on wood cell walls and only degrade pectin rich pit membranes and parenchyma cells whereas others degrade wood cell walls. Bacteria can slowly degrade wood both in wet and moist environments (e.g. water saturated wood) and wood exposed in ground contact situations (when

subjected to drying and wetting) (Eaton and Hale, 1993). Wood degrading bacteria are divided in two groups tunnelling and erosion bacteria (Nilsson and Daniel, 1983; Daniel and Nilsson, 1986). Wood exposed in habitats (e.g. soil) is how ever generally colonized by a range of fungi and bacteria which may interact.

1.3.1 Wood degrading fungi

All wood degrading fungi are filamentous, meaning they form thin microscopic threads called hyphae (Rayner and Boddy, 1988). There are many different types of wood degrading fungi; higher (e.g. brown-, and- white rot) and lower order fungi (e.g. soft rot). Brown rot is possibly the most aggressive type of wood decay and can cause rapid depolymerization of cellulose and there by loss of wood strength. (Duncan, 1965). White - and brown rot fungi belong to the Basidiomycete family and soft rot fungi belong to the Ascomycetes and Fungi Imperfecti. Fungi are heterotrophic and consume preformed organic matter rather than producing their own food like plants. Fungi can also be unicellular as reflected by yeasts which frequently occur in wood but are unable to cause decay (Zabel and Morrell, 1992).

The vegetative and non-reproductive parts of filamentous fungi perform invasion and colonization of wood by apical growth of their hyphae. Hyphae are bound by a cell wall and growth of fungal cell walls occurs at the hyphal apex and regular intervals along hyphae where cross walls or septa of various types depending on taxonomic group are produced. This is a characteristic feature of major wood decaying fungi i.e. Basidiomycota, Ascomycotina and Deuteromycotina (Eaton and Hale, 1993).

In young apical hyphal regions, the septa are incomplete due to having a central pore in each septa, allowing the continuation and movement of cell contents into the adjacent cells. In older regions and depending on fungal taxonomic group, these central pores are plugged making the hypha truly compartmentalized containing vacuoles and storage materials. Filamentous fungi are commonly identified on the basis of whether their vegetative hyphae are septate or not (Eaton and Hale, 1993). Most of the higher Ascomycotina and Basidiomycotina can easily be identified morphologically by their characteristic fruit bodies, while the majority of other wood inhibiting fungi can also be identified from their asexual or sexual spores.

Among wood decay fungi, the white rot fungi are characterized by their unique ability to biomineralize not only polysaccharides (cellulose and hemicellulose) but also lignin to CO_2 and H_2O (Eriksson et al., (1990); Daniel, (2014). White rot fungi are the only wood decay fungi known with an ability to completely degrade and mineralize wood. They are also the only organisms capable of efficient lignin degradation and use cellulases and extracellular lignin modifying enzymes and chemical reagents to degrade wood polymers (Daniel, (2014).

Brown rot fungi also belong to the Basidiomycotina and these fungi are perhaps the most important agents in degrading wood products in-service (e.g. conifers). In the decay process, brown rot fungi degrade primarily the carbohydrate fraction of wood and only a minor portion of lignin in the wood, eventually leaving a chemically modified (i.e. demethylated) lignin residue. Wood strength losses are often very rapid in the early stages of decay due to the rapid depolymerization of the carbohydrate fraction. Heavily brown rotted wood typically shows a cubical crumby brown appearance Goodell et al., (2003). Goodell et al., (2003) postulates the wood biodegradation mechanism involves enzymatic and non-enzymatic processes, because they are not known to produce lignin degrading enzymes. Brown rot fungi colonize wood cells, when the wood moisture content is above the fibre saturation point and initially invade the wood structure by passing through cell lumens and colonize ray and axial parenchyma cells, which store food (starch, fatty acids etc.) that is easily accessible for readily available energy.

1.3.1.1 Soft rot fungi

The term soft rot was first coined by Savory (1954) to describe the soft appearance and texture of wood degraded by fungi producing characteristic chains of cavities within the secondary walls of wood cells when the wood is moist. The name soft rot was thus originally given due to the softening and greying of attacked wood surfaces (Savory, 1954; Hale and Eaton, 1993; Daniel, 2014). After brown rot, soft rot is the second most important wood decaying group of fungi in terrestrial and aquatic environments (Daniel and Nilsson, (1988). This type of decay occurs primarily under excessive moisture conditions and is caused by members of the Ascomycetes and Deuteromycetes (Fungi imperfecti) (Duncan, 1965). Decay occurs principally under conditions where growth of the more active and competitive Basidiomycete fungi is retarded due to extreme wetness, low aeration, and elevated temperatures or due to having high levels of soluble nitrogen (Duncan, 1965; Eaton and Hale, 1993).

The most characteristic feature of soft rot is the morphological appearance of attack in wood cell walls. The two main morphological characters of decay by soft rot are Type I and Type II (Fig. 6a) (Daniel and Nilsson, 1998; Blanchette et al., 2003; Daniel, 2014). In Type I attack (Fig. 6a), cavities are formed within secondary cell walls by hyphae aligned with the cellulose microfibrils (i.e. aligned along the microfibril angle; Daniel, 1994). In Type II attack, wood cell wall thinning occurs similar to that observed in higher Ascomycetes (e.g. *Hypoxylon*, *Daldina* spp.) and white rot fungi (Nilsson et al., 1989; Daniel and Nilsson, 1998). The main difference in attack between white- and Type II soft rot is that soft rot fungi do not degrade middle lamella regions even in advanced decay (Daniel and Nilsson, 1998; Kim and Singh, 2000; Daniel, 2014). Type I and Type II attack can occur even in the same wood

cells. In addition to these two decay forms another morphological form of attack has also been reported called Type III. Here, after penetration of soft rot hyphae and cavity formation in the secondary wood cell walls, solubilisation of polysaccharides takes place over large areas of the wood cell wall (Daniel, 2014).

The term "Soft rot" is also used to denote characteristic penetration and growth of the fungal hyphae within the secondary cell walls of wood either with or without softening of wood cell surface. Soft rot has aroused considerable interest especially in old timber (which has been exposed to moist conditions) because it causes decay under conditions not favourable for Basidiomycetes. Within the Fungi imperfecti group, the anamorphic genus *Phialophora* is one of the most documented soft rot biodeteriogens in preservative-treated as well as in untreated wood in Sweden (Nilsson and Henningsson, 1978) and in many other countries including Australia (Leightley, 1980) and USA (Zabel et al., 1985).

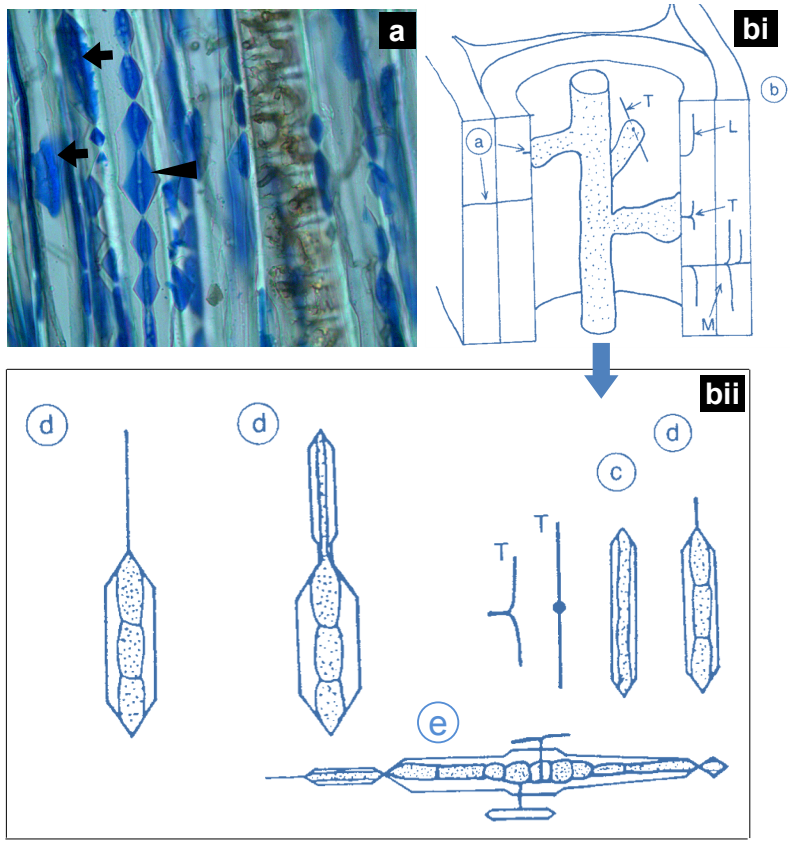

Figure 6. Soft rot decay types: (a) light micrograph showing both Type I (arrowhead) and Type II (arrows). (bi-bii) Hyphal extension models of soft rot fungi a- transverse penetration into and through cell wall, b- T-branching (T), L-branching (L) and continued penetration through cell wall with multiple branching (M); c- cell wall dissolution around T branch hypha to produce

cavity; d-proboscis hyphal growth and formation of a secondary cavity; e-repeated proboscis cavities resulting in chain of cavities (from Eaton and Hale, 1993).

Soft-rot fungi normally grow in wood in aquatic and damp environments and where wood is fully saturated with water, soft rot are the dominating microflora (Kim and Singh, 2000). They are often the most characteristic decay type found in fence posts, telegraph poles, window frames, cooling tower timbers and particularly wood in marine environments (Daniel and Nilsson 1998). After entering wood, the hyphae grow in the lumen of individual wood cells and produce fine penetration branches which grow through the S3 layer of the cell wall and gain access to the thick, cellulose-rich S2 layer. When penetration hyphae find a longitudinal plane of weakness in the S2 layer, they produce broader T-shaped hyphae (Fig. 6b) that grow along the cellulose microfibrils and secrete cellulase enzymes (Hale and Eaton, 1985). Due to the wood structure (i.e. orientation of cellulose) and defined diffusion of cellulase enzymes, characteristic rhomboidal cavities within the cell wall are formed, ultimately leaving the characteristic 'signature' of soft-rot even when the fungi are dead. Almost all soft-rot fungi need relatively high nitrogen levels for wood decay (ca 1% nitrogen content in the wood). If it is unavailable or insufficient in the wood, then nitrogen is often secured from the near environment (e.g. from soil at the bases of fence posts) by translocation through the hyphae.

Fungi causing soft rot primarily degrade the polysaccharides (cellulose and hemicellulose). The ability of decay fungi to produce enzymes to degrade cellulose varies depending on the rot type. In brown rot fungi a complete cellulase complex is only found in the *Coniophoraceae* (Nilsson and Ginns, 1979) and but many brown rotter's lack exoglucanases. In soft rot fungi, the majority of species produce endoglucanases (Nilsson, 1973) but many lack exoglucanases while others have both enzymes. In soft rot fungi, a variety of extracellular or cell bound carbohydrase enzymes are thought to break down polysaccharides. Nilsson (1974b) showed that many soft rot fungi have cellulases (56%), xylanases (75%) and mannanases (61%).

Soft rot fungi can cause extensive degradation of cellulose early in the decay process removing cell wall substances primarily from the S2 layer with the S3 layer adjacent to the cell lumen appearing morphologically unchanged (Nilsson, 1973). Even though the S1 layer is often attacked the middle lamella normally shows resistance due its high lignin content (Nilsson, 1973).

Some of the most important soft rot fungi belong to the imperfect anamorphic genus *Phialophora* from family Herpotrichiellaceae. *Phialophora* and its closely related genera *Cadophora* and *Lecythopora* are well known fungal species isolated from both treated (with copper) and untreated wood from ground contact situations. These fungi have been well documented internationally for soft rot attack of preservative (waterborne) treated wood in service (e.g. utility poles) including Sweden (Henningsson and Nilsson, 1976;

Nilsson and Henningsson, 1978; Daniel and Nilsson, 1998), Germany (Gersonde and Kerner-Gang, 1976) U.S. (Zabel et al., 1985; Zhong et al., 1995) and Australia (Leightley, 1979, 1980). Studies in this thesis concentrate on copper tolerant *Phialophora* soft rot fungi. These soft rot species/strains were selected mainly because they are reported as the most important fungi imperfecti causing soft rot decay of preservative treated timber in-service (primarily utility poles) (Daniel and Nilsson, 1998; Nilsson and Henningsson, 1978; Leightley, 1980). The occurrence of these fungal species is widespread in the world. Nevertheless, they represent one of the least studied wood rotting fungal groups.

1.3.1.2 Copper tolerant Phialophora spp.

Copper is arguably the most successful metal used in wood preservatives (Humar et al., 2005), however even wood treated with copper may be decayed by fungi which have developed mechanisms that allow them to survive and degrade copper- treated wood (i.e. referred often as copper tolerant). For some fungi (i.e. brown rot) copper-tolerance appears linked to oxalic acid production (Green and Clausen, 2005), but very little is known about copper tolerance mechanisms of soft rot fungi.

Phialophora species are the most well-known copper tolerant soft rot fungi isolated from copper preservative treated wood (Nilsson and Henningsson, 1978; Daniel and Nilsson, 1988; Daniel and Nilsson, 1989). For example *P. malorum*, *Phialophora mutabilis* and *Phialophora* spp. A (recently named as *Phialocephala dimorphospora*, Paper II) are copper tolerant *Phialophora* species identified from natural in-service environments. Among them, *Phialophora malorum* was shown to tolerate and grow on 10% w/v copper sulphate in agar (Daniel and Nilsson, 1988). Therefore during the present studies, *Phialophora* species and their strains were used as model organisms to investigate and elucidate aspects behind the mechanisms of copper tolerance of soft rot fungi. A brief description about the species and strains studied in this thesis is given in Table 1.

Table 1. *Details of the Phialophora species and strains used during the present studies.*

Fungal species	Strain	Culture collection/ Depositor	Origin/ Isolated from
1. *Phialophora malorum*	211-C-15-1	Dept. Forest Products	2% K33 impregnated poles, Sweden
	ATCC 66716	Wang 1990	CCA treated Douglas fir poles, New York
	CBS-245-60	Kidd & Beaumont	Eucalyptus poles, Denmark
	ATCC42795	Nilsson	Beech posts, Sweden
2. *Phialophora mutabilis*	24-E-1-1	Dept. Forest Products	CCA treated transmission poles, Sweden
	ATCC 42792	Dept. Forest Products	CCA treated transmission poles, Sweden
Lecythophora mutabilis	ATCC 44034	Leightley 1979, 1980	Treated Eucalyptus poles, Australia
3. *Phialophora* spp. A	35-1	Dept. Forest Products	Telephone poles, Sweden
	25M3	Dept. Forest Products	Telephone poles, Sweden
	204-1	Dept. Forest Products	Telephone poles, Sweden
	TS4M3	Dept. Forest Products	Telephone poles, Sweden

1.4 Identification of fungi

Traditionally, taxonomic keys have been used to identify fungi based on their morphology, colour and development of conidia. Fruit bodies of Ascomycetes and Basidiomycetes are normally used to identify species on the basis of macro- and microscopic characteristics that are specified in standard keys illustrated in books or on the internet containing specifically adapted databases (Huckfeldt, 2002). *In-vitro* growth on solid or in liquid medium can also be performed. In particular for Basidiomycetes, when only the mycelia is present, keys are based on microscopic characters of their hyphae as well as their growth parameters (Schmidt, 2006; Rayner and Boddy, 1988). *In-vitro* compatibility screening of mixed cultures can also be used for confirmation of strain discrimination (Molla et al., 2001).

However soft rot species and strains often cannot be identified only by morphological characters since many species do not produce asexual spores and specific keys for microscopic features on hyphae like clamp connections seen on basidiomycetes do not exist. This emphasizes a great need for accurate and advanced techniques that use objective (i.e. molecular) information e.g. DNA based information for their identification.

1.4.1 Molecular identification of wood degrading soft rot fungi using DNA fingerprinting techniques

Molecular methods to characterize, identify and classify organisms do not depend on subjective judgment like classical identification methods but are solely based on objective information derived from the target organism. The most common molecular technique used today is Polymerase chain reaction (PCR) based methods (Schmidt, 2006; Jasalavich et al., 2000).

PCR techniques are very useful tools for fungal identification and can provide a reasonably definitive identification method for wood decay fungi (Schmidt, 2006; Högberg and Land 2004). Molecular identification by PCR amplification of the ITS rDNA sequence can be used as a sensitive and specific tool to detect fungi in wood (Jasalavich et al., 2000). Nuclear rDNA, particularly the ITS region (Fig. 7) is now perhaps the most widely sequenced DNA region in fungi, especially for identification and phylogenic studies (Gardes and Bruns, 1993; Schmidt, 2006). The ITS region has become a convenient target for molecular identification of fungi because the targeted region is short (between 600-800bp in length) (Schmidt, 2007) and can be readily amplified using universal primers (ITS 1 and ITS 4) (Fig. 7) that are specific for the rRNA gene (White et al., 1990). An advantage of the ITS region is also the high degree of variation present even between closely related species. The stranded ITS 1 and ITS 4 primers (Fig. 7) are the easiest and most widely used primers for amplification of ITS regions of fungal rDNA (White et al., 1990; Moreth and Schmidt, 2005; Schmidt and Moreth, 2000). In addition, the multicopy nature of the rDNA repeat makes the ITS region (Fig. 7) easier to amplify even with small diluted DNA samples.

DNA sequences by themselves can be used as a straight forward powerful means of identification of known and unknown fungi. The unknown/known sequence can be compared, through the Basic Local Alignment Search Tool (BLAST), to other sequences deposited in Gene bank, which is an annotated database of all available nucleotide and amino acid sequences (Schmidt, 2007; Altschul et al., 1990).

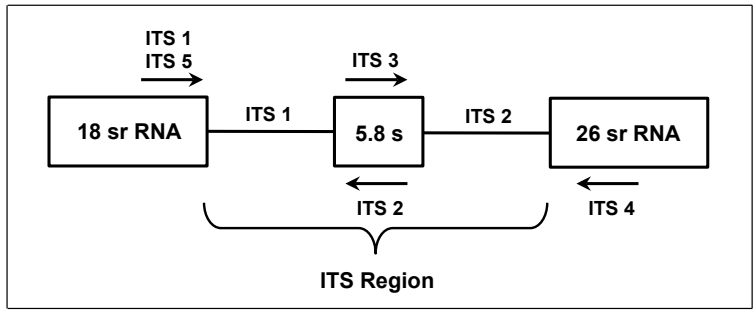

Figure 7. Internal transcribed spacer (ITS) region present in nuclear ribosomal DNA.

Although morphological characters can easily be used for microscopic identification of fungi, it is difficult to distinguish different *Phialophora* species and/or strains of the same species due to many of their complex morphological characters such as colony morphology, features in sexual spores and hyphae. This makes the PCR method a reliable and more accurate identification tool for *Phialophora* fungal species/strains identification.

One of the aims of this work was to use a reliable and sensitive DNA-based assay to detect and identify *Phialophora* spp. involved in wood decay.

1.5 Wood preservatives

Wood preservatives are designed to inhibit the activity of organisms destroying wood and wood-based products. Generally wood preservatives are pesticides which help to protect wood against fungi, bacteria or insects thereby prolonging service-life. Traditionally, wood has been protected by the use of broad-spectrum wood preservatives containing toxic substances with heavy metals (e.g. chromated-copper arsenate; CCA). Wood preservatives are generally divided into two classes; oil borne (e.g. creosote) and waterborne preservatives applied as water solutions (e.g. CCA, ACQ) (Rowell, 2005). The active ingredients used in wood preservatives include creosote, copper, zinc, chromium, arsenic, and other compounds. Depending on exposure condition, the effectiveness of the chemicals in preservatives vary. For example, heavy duty preservatives are used for wood in ground contact situations, and marine conditions due to the high decay that may occur (Ibach, 1999). Low hazarders preservatives are used for above ground situations.

1.5.1 Copper-based wood preservatives

In recent years, focus on copper-based wood preservatives have increased mainly due to increased concern on the environmental effects of preservatives. For example, there is a restriction and even total ban in some countries like Sweden on the use of the most successful wood preservative CCA (Freeman and McIntre, 2008). Copper has been one of the most effective biocides in wood preservatives for decades with copper proven to be algae-cidal, fungicidal, bactericidal and insecticidal (Humar et al., 2005). The fungicidal properties of copper were already recognized in the 1700s and since then has become the most widely used biocide. Although borates and organic biocides have also been shown to be important, copper remains the primary agent to protect wood used in ground contact situations or fully exposed to the weather (Lebow et al., 1999). With the US environmental protection agency's (EPA) and European decisions to phase out the use of arsenic-based wood preservatives, there has been great demand in developing new chemical formulations that are effective but will not cause harmful effects to environment and humans. Therefore, today there is priority for copper-based

and chromium and arsenic free wood preservatives as replacements to CCA. This has resulted in the development of different forms of copper treatments as the dominant form of wood protection. Ammoniacal and amine copper-based systems came to the market as a relatively less hazardous preservative to replace CCA, which includes quarts or azoles as co-biocides (Cookson et al, 2010). Recently micronized copper formulations with the same co-biocides were also developed (see chapter 1.5.2). This was highlighted by the introduction of Micronized-Cu (MC) in USA during 2006 (Archer and Preston, 2006; Freeman, 2002; Freeman et al., 2004; Freeman and McIntre, 2008).

Wood products treated with copper containing preservatives have been exponentially used since the 1970s and 80s and are at a high level of usage. The advantages of copper compounds as wood preservatives are: it is easy to create waterborne formulations, it is possible to determine penetration into wood and they are less effected by photo-degradation due to UV radiation (Freeman and McIntre, 2008). However, drawbacks of using copper containing wood preservatives are the occurrence of copper tolerant fungi species, corrosion of metal fasteners and aquatic toxicity caused by the leaching of copper based preservatives (Archer and Preston, 2006).

1.5.2 Micronized-Cu (MC)

When CCA was withdrawn from use it was replaced with organic co-biocides i.e. alkaline copper quaternary (ACQ) and copper azole (CA). ACQ and CA are also copper-based but instead of the heavy metals chromium and arsenic it contains organic co-biocides. The main disadvantage of ACQ and CA is its very corrosiveness to metal and thus only ceramic coated and stainless steel screws and nails can be used with such treated wood (Cookson et al., 2010; Freeman and McIntre, 2008). MC overcome the corrosive problem on metals (Evans et al., 2008). The preservatives used here are simply a modified version of existing formulations and the properties of the treated wood are similar. With this product instead of using dissolved copper in preservatives, small particles of copper (i.e. “micronized” copper) are impregnated into the wood.

MC was developed to address the issue of ionic copper formulations. The micronized formula is comprised of copper carbonate particles ranging in size from several microns to few nanometres in size (Evans et al., 2008). MC offers many advantages such as the reduced mass of copper needed to give the same level of protection as other systems provide (e.g. aqueous alkaline copper azole (ACA) to protect wood from fungi and less copper leaching from the treated material (Santiago-Rodrígue et al., 2015).

1.5.3 Mode of action of copper

Initial uptake of copper by fungal cells is thought to occur mostly through ion exchange followed by penetration into the cell. High levels of copper accumulation in cells is needed to have a fungicidal effect on fungi. When

there is excess copper inside fungal hyphae, cell damage occurs due to oxidation of proteins, enzymes or lipids leading to interruption of enzymatic processes in the fungal cells (Eaton and Hale, 1993). Copper can also interrupt the activities of some extracellular enzymes (e.g. enzymes involved in degrading lignocellulosic materials), binding to cell walls and block nutrient transport in and out of the cells. Denaturation of proteins and enzymes due to higher copper levels inside fungal hyphae leading to fungal cell death is thought to be the most common mode of action of copper-containing wood preservatives (Archer and Preston, 2006).

1.6 Copper tolerance

Wood decay fungi have developed mechanisms which allow them to survive and decay copper-treated wood and copper tolerance is thus known as the ability of an organism to succeed (continue to grow and reproduce) in the presence of copper ions. According to Gadd (1993), fungi that are able to grow on substrates containing copper concentration higher than 1.6 $mmolL^{-1}$ are considered as copper tolerant. Despite the fact that the phenomenon of copper tolerance has been known for many years the exact mechanisms of copper tolerance and copper toxicity is not completely understood especially for fungi (i.e. brown and soft rot fungi) degrading copper-treated wood.

Compared with other microbial groups fungi can be extremely tolerant to toxic metals even at high concentrations (Gadd, 1993; Schmidt and Ziemer, 1976). Copper tolerance in brown rot fungi is thought to occur by acid precipitation (oxalic acid) of copper into an insoluble form (oxalate form) rendering the copper fungicidally inert. Several brown rot fungi e.g. genus *Serpula* and *Postia* are known to show copper tolerance (De Groot and Woodward, 1999) and copper tolerant *Postia* species convert soluble copper into insoluble copper oxalate making it less toxic to the fungi (Clausen and Green, 2003).

1.7 Proteomics as a tool for characterizing fungal proteins induced during incubation with copper

Understanding the mechanisms of copper tolerance is of prime importance for providing insights into fungal interaction with copper wood preservatives and how the substrate will be affected. One way of approaching this is by identifying the global protein content produced by the fungi (i.e. external and internal) during growth in copper containing media.

Proteomics is a powerful tool for understanding cellular mechanisms in biology including fungal resistance against external forces and involves studying and characterizing total proteins expressed in a cell/organism under given conditions (Wilkins et al., 1995; Sharma et al., 2014; Bergquist et al.,

2007). During wood decay, fungi may express various proteins for both decay and general metabolism as well as for detoxification of preservatives in their surrounding environment (Kang et al., 2009). Proteomics can provide both qualitative and quantitative information of proteins expressed by an organism (Kang et al., 2009) and in the present study proteomic techniques were used to reveal proteins expressed by *P. malorum* when grown in culture supplemented with copper. Wood decay proteomics have been used previously in studies on other fungi including *Phanerochaete chrysosporium* (Martinez et al., 2004), *Fibroporia radiculosa* (Tang et al., 2013) and *Postia placenta* (Clausen and Green, 2003). The present application of proteomics for studies on copper tolerance by soft rot fungi (*Phialophora* spp.) with unknown genome seems to be the first of its kind.

1.8 Objectives of the present work

The major objectives of this study was to improve the current knowledge on copper tolerance by economically important soft rot fungi both *in-vitro* and in treated wood by investigating aspects of decay where tolerance may give competitive advantage. The work involved application of a range of mycological, molecular, biochemical and ultrastructural techniques as well as wood decay tests.

Specific aims targeting the major objectives of the work were:

(1) To investigate copper tolerance of *Phialophora* spp. grown under different copper levels in liquid- and on solid growth media, and at different temperatures (Papers I, II);

(2) To apply molecular techniques to determine the molecular phylogeny of a number of selected copper tolerant *Phialophora* spp. and their respective strains previously isolated from preservative-treated and untreated wood (Papers I, II);

(3) To determine whether copper tolerant *Phialophora* spp. as shown by an ability to grow on copper agar or in liquid cultures containing copper provides a competitive advantage for decay of copper pressure treated (as $CuSO_4$, or MC) birch and pine wood under standard test (i.e. ENV 807) conditions using both vermiculite and sterile soil (Paper III);

(4) To investigate copper detoxification mechanism(s) of soft rot fungi by identifying proteins in *Phialophora malorum* that may be either internally or externally up-regulated while growing in copper supplemented growth medium. Here focus was placed on biochemical tools for protein characterization such as proteomic techniques (LC-MS/MS method) and SDS electrophoresis and MALDI-ToF MS/MS for extracellular protein profile studies (Papers IV, V).

2 Material and methods

2.1 Fungal species and wood types used in the study

Three of the most commonly reported copper tolerant soft rot fungi *Phialophora* species (Henningsson and Nilsson, 1976; Nilsson and Henningsson, 1978; Daniel and Nilsson, 1988) namely *Phialophora malorum*, *Phialophora mutabilis* and *Phialophora* spp. A were used during the study. Apart from *Phialophora malorum* ATCC (American Type Culture Collection) 66716 and *Lecythophora mutabilis* ATCC 44034 that were obtained from CBS-KNAW (Fungal Biodiversity Centre Holland) culture collections, all other strains were obtained from the culture collection held at the Department of Forest Products, SLU. Further details of the species and strains are shown in Table 1 in the introduction.

Pine (*Pinus sylvestris* L.) and birch (*Betula verrucosa* Ehrh.) wood samples representing softwood and hardwood respectively were used as substrate materials throughout the studies.

2.2 Growth of soft rot fungi in solid- and liquid media

The *Phialophora* species were initially obtained from oil cultures and re-cultured on 2.5% w/v malt extract agar (MEA) (VWR International) in Petri dishes (as solid cultures) and maintained at 20^{o}C under dark conditions. After a second re-culturing on MEA, established fungal cultures were used for morphological and molecular identification.

Liquid cultures were prepared by inoculating fungal agar plugs removed from the edges of actively growing pure fungal cultures (5 plugs/flask), in 200 mL Erlenmeyer flasks containing 100 mL Abrams (1948) medium (pH 4.5), and incubated as stationary cultures at 20^{o}C. Similar liquid cultures were used to obtain initial (liquid) fungal substrate for all the experiments performed during the thesis, including growth analysis, wood decay testing, protein identification and for microscopy experiments (Papers I-V).

In addition to liquid- and solid cultures, decay by some selected soft rot fungi was checked *in-vitro* by growing them on birch and pine wood blocks, inserted in sterile soil as described in the AWPA wood block test (Paper II) and also in vermiculate (Paper III).

2.3 Investigation of copper tolerance on selected fungal spp.

To initially assess the growth of fungi on copper supplemented growth medium, two screening approaches were applied with growth checked in both liquid- and on agar media. Three *Phialophora* spp. selected for the study were grown on 2.5% (w/v) MEA incorporated with varying concentrations of sterile copper sulphate pentahydrate ($CuSO_4.5H_2O$) (i.e. 0.0, 0.064, 0.32, 0.064, 1.6, 3.2% Cu w/v). Agar cultures were inoculated with one fungal agar plug (1 plug/plate) from respective *Phialophora* spp. and plates incubated at 20^{o}C. Fungal growth on copper-containing medium was determined as total accumulated growth per week by measuring the increase in total diameter from the centre of the plate (Papers I, II).

In addition, liquid cultures were prepared using Abrams medium (pH 4.5) containing peptone (Sigma Aldrich, SW) (0.5 g/L) and copper sulphate giving final concentrations of 0.0, 0.032, 0.064, 1.6, 0.32, 0.064, 1.6, 3.2% w/v Cu. Two hundred m/L of Abrams medium was added to 500 mL Erlenmeyer flasks and autoclaved. Liquid cultures were then inoculated with 4-5 fungal agar plugs/flask and incubated as stationary cultures at 20^{o}C (Papers I, II).

2.4 Identification of fungal strains

Three approaches were used to identify the *Phialophora* species/strains: *i)* molecular identification by PCR amplification of the ITS region of rDNA sequence; *ii)* microscopic identification of morphological characters; and iii) *in-vitro* compatibility screening of mixed cultures for confirmation of strain discrimination.

2.4.1 Molecular identification by PCR

Genomic DNA was isolated from several of the copper tolerant *Phialophora* morphotypes from Sweden and related strains obtained from ATCC and CBS culture collections as described in Paper I.

DNA extraction was performed using a modified phenol/chloroform-extraction method (Larena et al., 1999). Agar plugs with mycelia were inoculated (5 plugs/flask) in 250 mL Erlenmeyer flask containing 2% w/v corn steep in 100 ml Abrams media (pH 4.5) and incubated for 4-5 days as stationary cultures at 20^{o}C. The resulting mycelia was harvested and freeze dried in an Edwards Freeze dryer, before grinding under LN_2. Mycelia powder suspended in extraction buffer (50 mM EDTA (pH 8.5), 0.2% SDS (dodecyl

sulphate sodium salt) was incubated at 65°C for 30 min, cooled to room temperature and centrifuged at 12 000 rpm for 15 min. The supernatant was separated and RNAse added to a final concentration of 50 µg/mL and incubated for 1 h at 30°C. After centrifuging for 15 min. at 10 000 rpm, the supernatant was collected and one tenth volume of 5 M potassium acetate (pH 5.2) was added and incubated on ice for 1 h. The solution was centrifuged (at 13 000 rpm 15 min) before extracting with phenol/chloroform/isoamyl alcohol (49/49/2). DNA was precipitated by 2 vol. of 95% ethanol and incubated at -20°C for 30 min. After final centrifugation at 12 000 rpm for 15 min, the recovered pellet was washed with 80% cold ethanol, vacuum-dried, re-suspended in 100 mL sterile water and stored at -20°C until further use (Papers I, II).

After confirming the presence of DNA on 1% w/v agarose gels, the nuclear rDNA region (ITS1-5.8S-ITS2) was amplified by PCR using universal primers ITS 1 and ITS 4 (White et al., 1990). The PCR reaction mixture was prepared with DreamTaq Green DNA polymerase (Fermentas Life Sciences, Sweden) with slight modification of the manufacturer's protocol. A 25 µL reaction mixture was prepared by adding 2.5 µL of 2 mM dNTP mix, 1U of DreamTaq Green DNA polymerase, 1.25 µL of 10× diluted ITS 1 and ITS4 primers, 2.5 µL of 10× DreamTaq Green buffer and finally 1 µL of extracted DNA as the template.

The PCR reaction cycles were performed in a Thermal cycler, Unocycler (VWR International) and complete details of the procedure are discussed in Papers I and II. PCR amplified products were separated with 1 % w/v agarose gel electrophoresis on 1× TBE buffer (45 mM Tris-borate, 1mM EDTA, pH 8.0) and stained with ethidium bromide prior to visualization under UV-light.

PCR amplified products were purified using ExoSAP (GE Healthcare) according to the manufacturer´s protocol and sequenced with ITS 1 forward primer at Uppsala Genome Centre, Sweden. Sequencing results were analysed using sequencing scanner software from Applied Biosystems. Analysed sequences were compared with database references in NCBI using blast algorithm to recognize their identity. We aligned all sequences used in the study together with the downloaded reference sequences from GenBank, using the ClustalW algorithm of Megalign from the laser gene Package (version 3.08, DNA star, Inc., Madison, WI, USA) (Lindahl et al., 2010) and adjusted using Sequence Editor Se_Al (version 1.0a 1) (Menkis et al., 2004). Aligned sequences were used in its fasta format in Phylogeny.fr web server; http://phylogeny.lirmm.fr (Dereeper et al., 2008) for phylogeny analysis. Details are described in Papers I and II.

2.4.2 *In-vitro* compatibility screening for strain discrimination

Pure strains of *P. malorum*, *P. mutabilis* and *Phialophora* spp. A were grown together on 2.5% w/v MEA plates to study strain compatibility. Fungal plugs

(5 mm) were isolated using a cork borer from the actively growing edges of pure fungal cultures and placed opposite each other on fresh MEA agar plates. Compatibility was visually assessed as dual, direct, and opposition cultures at the position where the mycelia came in contact. Modes of interaction of the mycelial cultures were assessed according to Molla et al. (2001). This test was used to discriminate between strains that were difficult to distinguish morphologically.

Inter-compatibility tests and microscopic features of fungal hyphae were used as additional tools to confirm the PCR results.

2.4.3 Light microscopy (LM) identification of *Phialophora* spp. and strains

Key morphological features of fungal hyphae were examined after staining with 1% w/v aniline blue in lactophenol and observed using a DMBL Lecia microscope operated in bright field mode. Digital images were recorded with an Infinity X-32 digital camera (DeltaPix, Denmark) directly mounted on the microscope. In addition, asexual spores (when available) of studied *Phialophora* species and strains were also examined and digital images recorded.

2.4.4 Morphological analysis of decay using light microscopy

Samples were observed using LM to detect any changes in fungal colonization and morphology of decay pattern in wood induced by $CuSO_4$ and MC treatments. Since cavity formation in secondary wood cell walls is readily observed, emphasis was given to viewing transverse (TS) sections with radial (RLS) and tangential (tangential longitudinal; TLS) sections used only to confirm the attack process.

Sections (ca 10 µm) were cut from degraded wood blocks using a Lecia sliding microtome and placed on glass slides and left unstained or stained with drops of either 1% w/v aq. safranin or with aniline blue. Sections were subsequently mounted in 50% v/v glycerol in water before observations using the Lecia DMLB light microscope with digital images recorded using an Infinity X-32 camera (DeltaPix, Denmark).

2.5 Wood decay experiments

Three soft rot species *P. malorum*, *P. mutabilis* and *Chaetomium globosum* were used from the culture collection at the Department of Forest Products/Wood Science (details in Table 2). Both *Phialophora* strains were originally isolated from copper-treated wood poles and stakes (Henningsson and Nilsson, 1976) and their identity recently reconfirmed molecularly by Karunasekera and Daniel (2013). Previous studies report *P. malorum* as the most copper tolerant having an ability to grow up to 6.4% w/v Cu supplemented MEA medium, *P. mutabilis* as mildly copper tolerant

(Karunasekera and Daniel, 2013; Daniel and Nilsson, 1988) and *C. globosum* only as weakly copper tolerant (Daniel and Nilsson, 1988).

Table 2. *Fungal species, strains and their origin used for the test (Daniel and Nilsson,1988).*

Species/strain	Species origin	Cu-tolerance on agar/in liquid cultures	Cu-tolerance classification
Phialophora malorum 211-C-15-1	2% K33 impregnated poles, Sweden	10% w/v $CuSO_4$	High Cu tolerance
Phialophora mutabilis 24-E-1-1	CCA treated transmission poles, Sweden	5.0% w/v $CuSO_4$	Medium Cu tolerance
Chaetomium globosum F171-1	ATCC 34152	0.0<0.1% w/v $CuSO_4$	Poor Cu tolerance

Decay capacity of the three fungi were evaluated using a modified ENV 807 (2001) wood decay test with two wood species and three $CuSO_4$ and Micronized (MC) concentrations. Scots pine and birch wood samples with dimensions of 5×15×20 mm^3 were cut, cleaned (trimmed), and dried at 103^oC $\pm$ 2^oC for 18-24 h and cooled in a desiccator before obtaining absolute dry weight. After conditioning in a climate chamber (12^oC $\pm$ 2^oC, 65% RH) for 2-3 days, wood samples were weighed to obtain the 12% absolute dry weight before impregnating with copper solutions.

$CuSO_4.5H_2O$ (VWR International) and an emulsion of MC particles were diluted with deionized water to have formulations with 0.2, 0.4, 0.6% w/v pure copper. The conditioned wood blocks were vacuum/pressure impregnated using an autoclave full cell method (vacuum 200 mbar for 20 min followed by 6 bar pressure for 90 min) with the copper concentrations. After impregnation, wood samples were weighed for initial wet weight and conditioned at room temperature. Prior to testing, the wood blocks were kept in deionised water for 10 min. The wood blocks were not leached and thus both fixed and non-fixed copper was retained in the wood. Untreated wood samples were used as controls.

Treated and untreated wood samples were exposed to selected soft rot fungi in vermiculite and sterile soil tests and details of experiments including fungal inoculation, mass loss etc. are described in Paper III.

Even though birch is not normally impregnated with conventional wood protecting chemicals like copper for use in-service, in this study it was used to achieve sufficient mass loss within a reasonable time as the time needed for a similar results with pine would have been several years even under accelerated decay situations.

2.5.1 Morphological analysis of decay using light microscopy

Samples were observed using LM to detect changes in fungal colonization and morphology of the decay pattern induced by $CuSO_4$ and MC treatments. Emphasis was given for detection of cavity and erosion decay by using transverse (TS) sections as described in section 2.4.4.

2.6 Characterization of fungal proteins induced during incubation with copper

Initial reference mapping and characterization of proteins through LC-MS/MS and MALDI-TOF MS/MS were carried out aiming at identifying internal and external proteins that may be up-regulated or down regulated when *P. malorum* was grown in copper supplemented growth medium. Since *P. malorum* shows greatest copper tolerance (Daniel and Nilsson 1988; Karunasekera and Daniel 2013) it was chosen for the proteomic studies.

Liquid cultures of *P. malorum* were established using Abrams medium (pH 4.5) with $CuSO_4.5H_2O$ added to make final concentrations of 0.0, 0.064 and 0.64% w/v copper. Further details are given in Papers IV and V. Liquid cultures (100 mL) were prepared by adding 5 mL of mildly homogenized (Ultra-turrax) mycelia from 14-day old liquid cultures. Thereafter fungi were grown for six weeks at 25^{o}C on a VKS Edmund Buhler horizontal rotary shaker (80 rpm). Since growth analysis results (Karunasekera and Daniel, 2013) showed *P. malorum* mycelia mass in copper supplemented media was highest after six weeks, it was chosen as the time for harvest. For isolating mycelial proteins, mycelia were harvested using glass funnels, washed with deionized water and transferred to Falcon tubes after adding respective medium. Initial lysing and homogenization of mycelia was conducted under cooling (4^{o}C) with 30 mL of Tris-HCL (pH 7.8) buffer containing 1% (v/v) 2-mercaptoethanol and phenylmethanesulfonylfluoride (PMSF) (0.004%) using an Ultra-turrax. After centrifugation (1500 rpm, 4^{o}C, and 20 min.) the supernatant was transferred into new Falcon tubes and stored at 4^{o}C until mass spectrometry analysis was performed. The LC-MS/MS analysis was performed at Uppsala University and details of the procedure are described in Paper IV.

For insight into key components involved in potential copper extracellular regulation, peptide identification (i.e. peptide mass fingerprints) through MALDI-TOF mass spectroscopy was also performed using 10 day old cultures of *P. malorum* from control and 0.064% w/v Cu concentration. After isolating mycelia using glass funnels as described above, samples were stored with respective media at 4^{o}C and MALDI analysis performed at the Institute of Microbiology, Academy of Sciences, Praque, Czech Republic. Details of protein extraction and MALDI analysis is found in paper V.

2.7 Microscopy for morphological analysis of *Phialophora*

Electron microcopy (EM) can provide structural and chemical information over a range of magnifications down to atomic level. Various EM methods are available (e.g. Transmission electron microscopy, Scanning electron Microscopy (Daniel, 1994; Daniel, 2016) and the selection of the EM technique basically depends on the objectives of the experiment and the specimen being observed.

2.7.1 Scanning electron microscopy for morphological analysis of *Phialophora*

SEM was used in the present study to observe changes in copper tolerant fungal morphology due to the presence of copper. Mycelia from six week old liquid cultures (i.e. control, 0.064, and 0.64% (w/v) Cu were selected for the study. Mycelia samples were fixed in 3% v/v glutaraldehyde containing 2% v/v paraformaldehyde in 0.1 M sodium cacodylate (pH 7.2) at room temperature. Samples were subsequently dehydrated, critical point dried, coated with gold (e.g. gold; Emitech E5000 sputter coater) to prevent sample charging and observed using a Philip XL30 ESEM. Further details for sample preparation for SEM are discussed in Paper IV.

3 Results and discussion

Among the most common approaches used to identify fungi, molecular identification using PCR was recognized as the most reliable and accurate method to identify the species/strains of the copper tolerant *Phialophora* genus used in the present work. For example, a group of previously unidentified morphologically and ecological similar *Phialophora* strains (*Phialophora* spp. A) after DNA sequencing was identified as *Phialophora dimorphospora* (Paper II).

With the inter-compatibility tests conducted it was possible to discriminate between most of the *Phialophora* species/strains that could not be distinguished morphologically. As an example, different strains of *P. malorum* showed delimiting zones indicating that they were not compatible with each other (Paper I).

3.1 Molecular identification and phylogenic analysis of *Phialophora* spp. (Paper I)

3.1.1 PCR identification of different *Phialophora* spp./strains

PCR of the ITS region has been the most widely sequence DNA region for fungal identification (Högberg and Land, 2004) since it contains noncoding regions specifically having high interspecific variation making them suitable for species identification (Grades and Burns, 1993). Therefore ITS 1 and ITS 4 universal primers were selected for *Phialophora* species identification. ITS 1 and ITS 4 primer combinations successfully amplified the rDNA-ITS region of all the *Phialophora* strains used in the present study (Fig. 8) and a detailed discussion is found in Paper I.

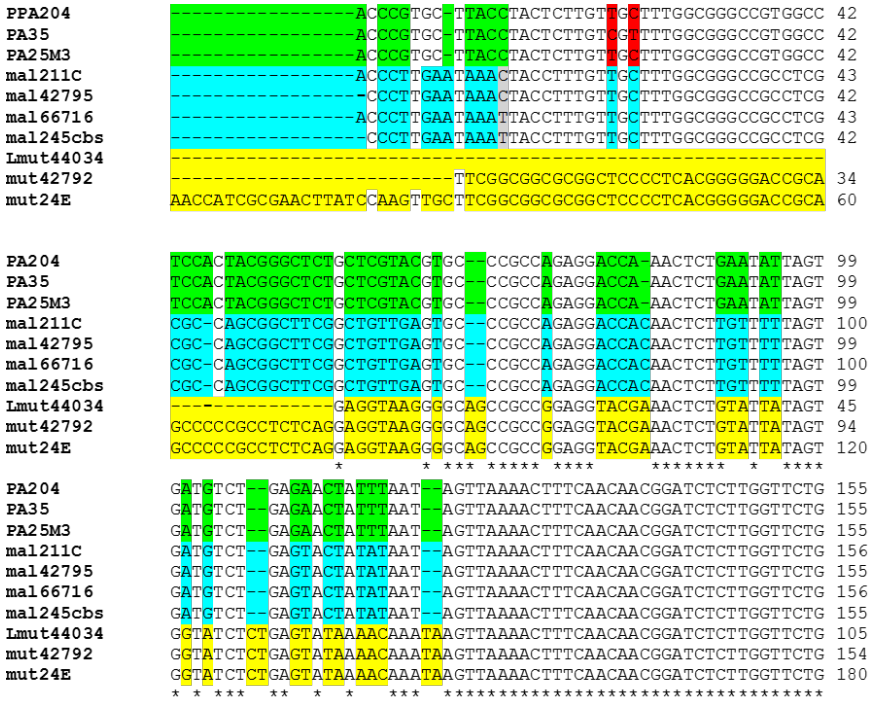

Figure 8. Aligned internal transcribed spacer (ITS) sequences of *Phialophora* species. Nucleotides at 42-180 show differences between *Phialophora* species and strains. Abbreviations: *Phialophora* spp. A strains PA25M3, PA 204, PA 35; *Phialophora malorum* strains mal211C, mal42795, mal66716, mal245cbs; *Phialophora mutabilis* strains Lmut44034, mut42792, mut24E. Differences between *Phialophora* spp. A strains are indicated in red and *P. malorum* in grey.

Figure 8 shows inter- and intraspecific variation between three *Phialophora* species and their respective strains. Even though interspecific variation between the three *Phialophora* species were clear, their intraspecific variation in the rDNA was comparatively low (i.e. only a few base pair differences in the ITS region were apparent (Fig. 8). However, it would still be possible to use this intraspecific variation for strain specific primer designing to identify the different *Phialophora* strains.

When the DNA sequencing results were BLAST searched in Gene Bank at NCBI, *P. malorum* and *P. mutabilis* strains (Table 1, see introduction) were identified to their closest matching sequences. After considering BLAST identities and relevance to wood decay, *P. malorum* strain 211-C-15-1 was first identified as *Cadophora malorum* but according to ISF species and Gams (2000), *C. malorum* is synonymous with *P. malorum*, therefore in this study *P. malorum* strain 211-C-15-1 molecular identity is considered as *P. malorum* not *C. malorum*. *P. malorum* strain ATCC 42795 was also identified with BLAST as similar to *P. malorum* strain 211-C-15-1 but ClustalW analysis indicated these two strains as intraspecific. *P. malorum* strain ATCC 66716 was

identified in NCBI as *P. malorum* with 93% homology. Similarly, CBS strain 245.60 was also identified as *P. malorum* with 87% identity.

Since *P. mutabilis* was not available in GenBank it was not possible to identify *P. mutabilis* strains 24-E-1-1 and ATCC 42792 with the same species name, however further ClustalW analysis indicated these two strains have identical sequences.

Phialophora spp. A. was identified only to genus level due to unavailability of homologue sequences in the GenBank. However it was later recognized with a homologue identity as *Phialophora dimorphospora* (Paper II).

3.1.2 Intercompatibility tests

Compatibility interactions were analyzed according to the description of Molla et al. (2001) as inhibiting, intermingling, invasion/replacement. Strains of *P. malorum* and *P. mutabilis* clearly showed delimiting zones when incubated as compatibility cultures indicating they were incompatible with each other demonstrating different strains (Fig. 9). Although molecular identification indicates *P. malorum* strain ATCC 42795 and 211-C-15-1 had similar identity at species level, the compatibility test showed they have inhibition at the touching zone (Fig. 9). Figure 9a shows that all *P. malorum* strains studied were incompatible with each other. *P. mutabilis* strain ATCC 42792 and 24-E-1-1 were intermingling but strain ATCC 42792 showed inhibition with *L. mutabilis* 44034.

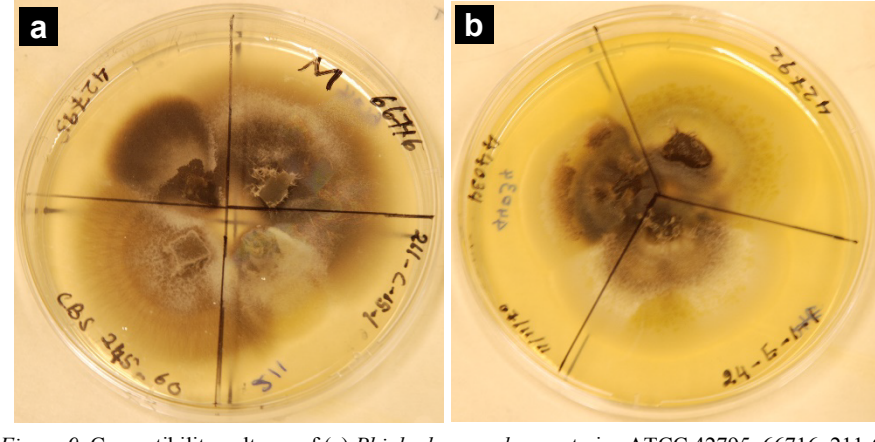

Figure 9. Compatibility cultures of (a) *Phialophora malorum* strains ATCC 42795, 66716, 211-C-15-1, CBS 245-60 and (b) *Phialophora mutabilis* strains 44034, 42792 and 24-E-1-1. The strains show delimiting zones when incubated as compatible cultures indicating incompatibility.

Phialophora spp. A strain discrimination was not clearly visible due to the dense black colour of the mycelia but it was evident from stereomicroscopy observations that they are mutually intermingling (Fig. 10). Incompatibility

tests were also done at the species level which showed inhibition indicating that they were different (Paper II).

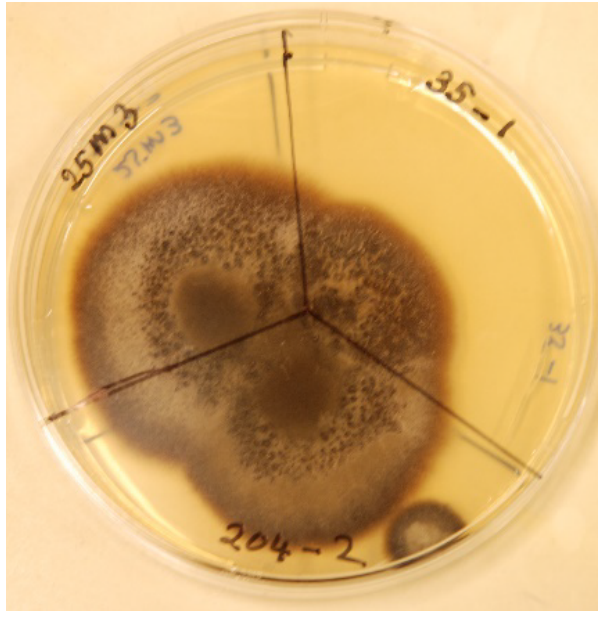

Figure 10. Compatibility cultures of *Phialophora* spp. A strains 35-1, 204-2 and 25M3 showing discrimination. With stereomicroscopy it was evident they were mutually intermingling (not visible).

3.1.3 Phylogenic analysis of *Phialophora* spp.

Phylogenic analysis of the *Phialophora* species studied (Table 1) and reference species from Gene Bank database showed they were mainly clustered into three clades (Fig. 11); i.e. one with *P. malorum* strains, one with *P. mutabilis* and another with *Phialophora* spp. A strains. In the phylogeny tree, the sequences with only a few nucleotide base differences between strains gave shorter branches or were branchless indicating they were closely related. All *Phialophora* spp. A strains branched from a similar location indicating a high level of homology among the strains (Paper I).

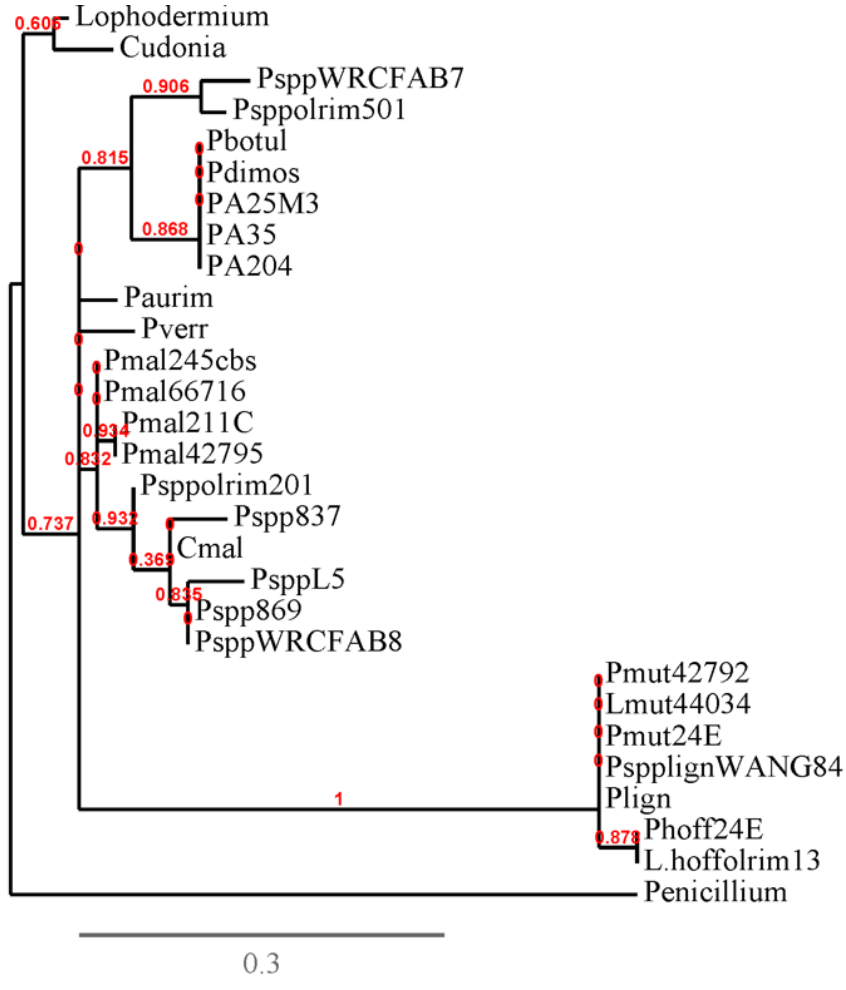

Figure 11. Phylogenetic relationship of *Phialophora* spp./strains listed in Table 1 and other sequences selected from GenBank. Phylogeny tree is constructed with 100 bootstrap values and percentages above branches are the frequency of each branch that appeared in the 100 bootstrap replicates. *Lophdermium pinastri*, *Cudonia monticola* and *Penicillium* spp. were used as outgroup for the tree. Abbrevations: *Phialophora malorum* as **Pmal**, *Phialophora mutabilis* as **Pmut**., *Phialophora* spp. A as **PA**, *Phialophora botulispora* as **Pbotul**., *Phialophora urium* as **Paurim**, *Lecythophora hoffmanii* as **L.hoffolrim13**, *Phialophora verrucosa* as **Pverr**, *Cadophora malorum* as **Cmal**, *Lecythophora mutabilis* as **Lmut**, *Phialophora hoffmanii* as **Phoff**, followed by strain description number. Unidentified *Phialophora* spp. as follows: *Phialophora* spp. abbreviated as **Pspp** followed by description number; **Psppolirim501** AY805587, **Psppolirim201** AY8055586, **PsppL5** Fj903340, **Pspp869** GU934601, **Pspp837** AY618680, **PsppWRCFAB8** AY618678, *Phialophora lignicola* **Plign** AY618677, **PspplignWANG84** AFO 83193.

3.1.4 Copper tolerance of *Phialophora* spp.

Results with $CuSO_4$ supplemented liquid- and solid cultures showed wide interspecific and intraspecific variations in the growth of different *Phialophora* species (Paper I). The high copper tolerance of *P. malorum* 211-C-15-1

(Daniel and Nilsson, 1988) was confirmed with growth on 6.4% w/v Cu in solid and 3.2% w/v Cu in liquid cultures.

The screening results indicated that some *Phialophora* strains displayed equivalent or occasionally slightly higher growth rates at lower copper concentrations (i.e. 0.032, 0.064%) than control, suggesting a stimulation effect of copper (Fig. 12; Paper I).

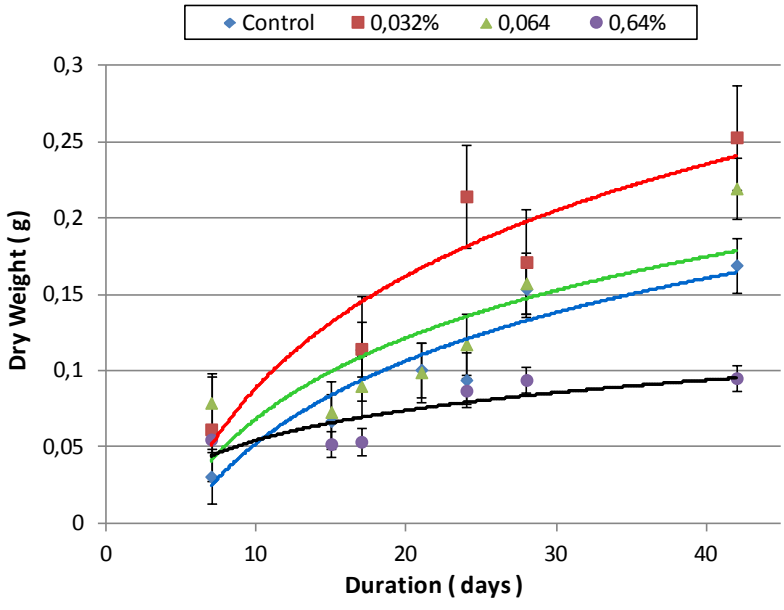

Figure 12. Growth curve (dwt/mean of 3 replicates) of *Phialophora malorum* strain 211-C-15-1 over time. Note stimulated growth in 0.032 and 0.064% Cu-supplemented cultures in comparison with control.

Liquid culture experiments performed with *P. malorum* 211-C-15-1 and *P. mutabilis* 24-E-1-1 showed the *P. malorum* strain produced greater amounts of mycelia (analyzed as dry wt.) than *P. mutabilis* at the three copper concentrations studied (0.032, 0.064, and 0.64% w/v Cu) with maximum observed in 0.032% w/v Cu cultures. Both *P. mutabilis* and *P. malorum* showed colour change of the liquid culture medium (in concentrations 0.032 and 0.064% w/v Cu), turning from light blue to green/yellow, suggesting release of extracellular components (e.g. chelators or low molecular substances) into the culture media. These two *Phialophora* species also showed differences in pH development in the culture filtrate with growth overtime where *P. malorum* decreased the pH from 4.5 to 3.7 in contrast to increased pH from 4.5 to 5.5 by *P. mutabilis*.

The differences observed in pH, discoloration of culture filtrate and mycelia dry weight development, may suggest presence of different mechanisms of copper interaction coupled with growth. Although the *Phialophora* strains used

belong to closely related *Phialophora* species the differences in growth rate in standard growth medium as well as in copper added to liquid or solid media cultures confirmed varying levels of copper tolerance.

3.2 Phylogenic, molecular and decay analysis of *Phialophora* species causing soft rot of wood (Paper II)

Since soft rot decay of treated wood in ground contact and loss of service-life remains an important economic issue worldwide, further studies were carried out on unclassified *Phialophora* spp. A strains 204-1, 35-1, TS4M3 and 25M3 isolated previously from wood in-service. Molecular identification, was performed as in earlier studies for *Phialophora* spp./strains (Paper I) using PCR and sequence analysis. Sequencing results for *Phialophora* spp. A showed a product of ca 53-487 base pairs in length (Fig. 13) and ClustalW2 sequence alignment showed close similarity between the four strains. Strains 35-1 and 25M3 showed differences at single base positions 34 and 37 and 370 and 374 respectively (Fig. 13). Although the four strains appeared morphologically similar, sequencing results show minor differences in their nucleotide sequences with strains 35-1 and 25M3 dissimilar to 204-1 and TS4M3 and 35-1 different from 25M3 (Fig. 13). Results from molecular analysis thus showed existence of minor differences in their nucleotide sequences although the four strains appear morphologically similar.

The Neighbour joining un-rooted phylogenetic tree (Fig. 14) showed all strains of *Phialophora* spp. A to cluster closely into a single clade with a bootstrap value of 87% and very close proximity (89% bootstrap value) placing them together with *P. dimorphospora*. From this it was concluded that the four *Phialophora* spp. A strains are similar and closely homologous to *P. dimorphospora*. This was further confirmed by NCBI BLAST local alignment results that gave 99% identity values for *Phialophora* spp. A strains 35-1, 204-1 and 98% identity for strains 25M3 and TS4M3 with *P. dimorphospora.* Neighbour joining phylogenic analysis further showed that *P. botulispora* and *P. lagerbergii* were less closely associated with *Phialophora* spp. A strains (Fig. 14).

```
PA204-1         -------GGTAGACCTCCACCCGTGCTTACCTACTCTTGTTGCTTTGGCGGGCCGTGGCC 53
PA35-1          ------GGGTAGACCTCCACCCGTGCTTACCTACTCTTGTGGCTTTGGCGGGCCGTGGCC 54
PA25M3          -------GGTAGACCTCCACCCGTGCTTACCTACTCTTGTTGCTTTGGCGGGCCGTGGCC 53
PATS4M3         CCCGTCGGGTAGACCTCCACCCGTGCTTACCTACTCTTGTTGCTTTGGCGGGCCGTGGCC 60
                       ********************************* * ******************

PA204-1         TCCACTACGGGCTCTGCTCGTACGTGCCCGCCAGAGGACCAAACTCTGAATATTAGTGAT 113
PA35-1          TCCACTACGGGCTCTGCTCGTACGTGCCCGCCAGAGGACCAAACTCTGAATATTAGTGAT 114
PA25M3          TCCACTACGGGCTCTGCTCGTACGTGCCCGCCAGAGGACCAAACTCTGAATATTAGTGAT 113
PATS4M3         TCCACTACGGGCTCTGCTCGTACGTGCCCGCCAGAGGACCAAACTCTGAATATTAGTGAT 120
                ************************************************************

PA204-1         GTCTGAGAACTATTTAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA 173
PA35-1          GTCTGAGAACTATTTAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA 174
PA25M3          GTCTGAGAACTATTTAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA 173
PATS4M3         GTCTGAGAACTATTTAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA 180
                ************************************************************

PA204-1         TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA 233
PA35-1          TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA 234
PA25M3          TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA 233
PATS4M3         TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA 240
                ************************************************************

PA204-1         TCTTTGAACGCACATTGCGCCCTGTGGTATTCCGCAGGGCATGCCTGTTCGAGCGTCATT 293
PA35-1          TCTTTGAACGCACATTGCGCCCTGTGGTATTCCGCAGGGCATGCCTGTTCGAGCGTCATT 294
PA25M3          TCTTTGAACGCACATTGCGCCCTGTGGTATTCCGCAGGGCATGCCTGTTCGAGCGTCATT 293
PATS4M3         TCTTTGAACGCACATTGCGCCCTGTGGTATTCCGCAGGGCATGCCTGTTCGAGCGTCATT 300
                ************************************************************

PA204-1         TAGACCACTCACGCTTGGCGTGGTATTGGGGCACGCGGTTCCGCGGCCCTCAAAATCAGT 353
PA35-1          TAGACCACTCACGCTTGGCGTGGTATTGGGGCACGCGGTTCCGCGGCCCTCAAAATCAGT 354
PA25M3          TAGACCACTCACGCTTGGCGTGGTATTGGGGCACGCGGTTCCGCGGCCCTCAAAATCAGT 353
PATS4M3         TAGACCACTCACGCTTGGCGTGGTATTGGGGCACGCGGTTCCGCGGCCCTCAAAATCAGT 360
                ************************************************************

PA204-1         GGCGGTGCCGGTAGGCCCTAAGCGTAGTAAATCTCCTCGCTATAGGGTCCTCTCGGTGGC 413
PA35-1          GGCGGTGCCGGTAGGCCCTAAGCGTAGTAAATCTCCTCGCTATAGGGTCCTCTCGGTGGC 414
PA25M3          GGCGGTGCCAGTAGGCCCTAAGCGTAGTAAATCTCCTCGCTATAGGGTCCTCTCGGTGGC 413
PATS4M3         GGCGGTGCCGGTAGGCCCTAAGCGTAGTAAATCTCCTCGCTATAGGGTCCTCTCGGTGGC 420
                ************************************************************

PA204-1         TCGCCAGAACCCCCAACTTCTCAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAAC 473
PA35-1          TCGCCAGAACCCCCAACTTCTCAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAAC 474
PA25M3          TCGCTAGAACCCCCAACTTCTCAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAAC 473
PATS4M3         TCGCCAGAACCCCCAACTTCTCAAGGTTGACCTCGGATCA-------------------- 460
                **** ***********************************

PA204-1         TTAAGCATAT------ 483
PA35-1          TTAAGCATATCAATAA 490
PA25M3          TTAAGCATATCAAT-- 487
PATS4M3         ---------------
```

Figure 13. ITS sequence of *Phialophora* spp. A strains. Nucleotides from 53-487 showed only four bp differences between the four strains. Abbreviations: *Phialophora* spp. A strains: PA204-1, PA35-1, PA25M3, PATS4M3. Inter-strain differences are marked in red and their locations indicated in green.

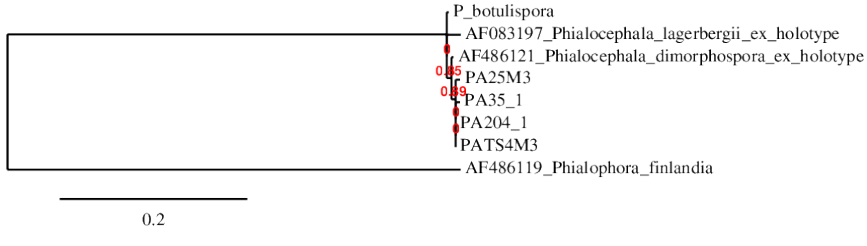

Figure 14. Phylogenetic relationship of *Phialophora* spp. A strains 204-1, 35-1, TS4M3, 25M3 and *P. botulispora* together with species selected from Genebank; *P. lagerbergii* (AF083197) and *P. dimosphospora* (AF486121). The phylogenetic tree was constructed with 100 bootstrap values and the frequency values of each branch are indicated on top of the branch.

When strain compatibility was analyzed visually, all *Phialophora* spp. A strains appeared compatible but due to their strong melanization it was difficult to distinguish between them (Fig. 15). However closer observations using stereo- and light microscopy indicated differences. *Phialophora* spp. A strains 25M3, TS4M3 and 204-1 grew into each other when they encountered, while strain 35-1 was not completely compatible with the other strains and produced a delineation line and stopped growth at the point of contact. According to Molla et al., (2001) the mechanism causing stoppage of growth can be identified as dead lock and friendly when hyphae grow into each other and intermingle. Therefore strains TS4M3, 25M3 and 204-1 which grew into each other at contact possess similarities, while strain 35-1 appears slightly different and not completely compatible.

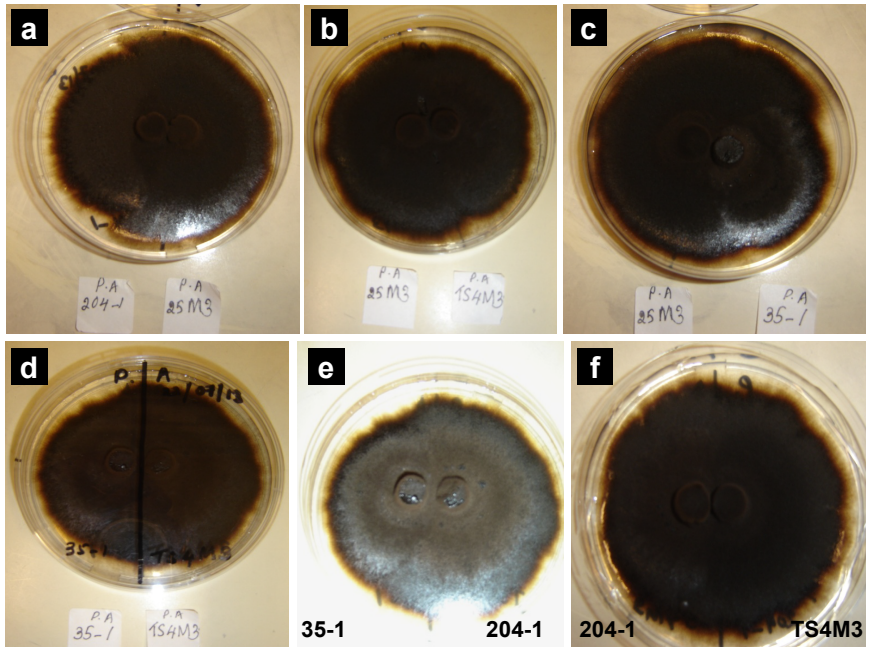

Figure 15. Compatibility cultures of *Phialophora* spp. A strains 35-1, 25M3, TS4M3 and 204-1 on MEA plates. All strain combinations are shown: (a) 204-1 vs 25M3, (b) 25M3 vs TS4M3, (c) 25M3 vs 35-1, (d) 35-1 vs TS4M3, (e) 35-1 vs 204-1 and (f) 204-1 vs TS4M3. Visually all strains appeared compatible apart from 35-1 which showed delineation with other strains (but was not clear due to the black colour of the cultures). Stereo- and light microscopy observations showed intermingling at the point of contact but no hyphal fusion was noted.

Variable growth of *Phialophora* spp. A, strains were observed on $CuSO_4$ supplemented agar over a period of 7 weeks variable growth was observed for all strains on 0.032 and 0.064% Cu (Table 3) Only strains 35-1 and TS4M3 grew on 0.32% w/v Cu. No growth was detected on 0.64% Cu for any strain. This indicated that strains 35-1 and TS4M3 have similarities in copper

tolerance ability. Similarly, strains 204-1 and 25M3 also appear to have similar growth rates but on a lower copper level. When *Phialophora* spp. A strain growth was observed on micronized copper incorporated into agar media, strains 25M3, 204-1 and 35-1 grew at 5.0% but not at 10.0% (w/v) and greatest growth was observed with strain 25M3 (Paper II).

Table 3. *Growth of Phialophora spp*. A. *strains on copper (as $CuSO_4$) incorporated into MEA plates at 20°C over 35 days. Phialophora spp. A strains 25M3 and 204-1 did not grow on 0.32% w/w $CuSO_4$ supplemented MEA plates. Results reflect mean of 3 replicates.*

Phialophora spp. A strain	Average growth rate (mm/day) % w/w $CuSO_4$ concentrations			
	0.0	**0.032**	**0.064**	**0.32**
P.A 35-1	0.53	0.30	0.37	0.13
P.A 25M3	0.65	0.26	0.09	-
P.A 204-1	0.72	0.27	0.11	-
P.A TS4M3	0.60	0.38	0.39	0.10

The AWPA (2008) soil block decay test was used to determine *Phialophora* spp. A decay of both birch and pine wood (Fig. 16). After 8 weeks exposure, wood blocks become typically black due to the highly melanized nature of the hyphae as seen from cultures grown on MEA plates (Figs 10, 16). Results from analysis of mass loss performed over a period of 5 months were consistent with that expected for soft rot fungi with greater mass loss on birch than pine by the four *Phialophora* spp. A strains (see Table 5 in Paper II). There were also variations in decay among the four strains with strains 204-1 and 25M3 the most aggressive with ca 17-19% mass loss on pine and 25-29% mass loss on birch. Strain 35-1 produced the lowest mass loss (i.e. 8% on pine; 11% on birch) after 5 months indicating it was the least aggressive. The difference in decay of pine and birch by soft rot is well known and related to both lignin type and lignin level of softwoods versus hardwoods and a detailed discussion can be found in Paper III. Even though the results of the wood decay test emphasize considerable variations in decay capacity of the different *Phialophora* spp. A isolates, light microscopy indicated that all four *Phialophora* spp. A produced similar morphology of decay. Initial colonization by the strains was via the rays in both wood species and by the vessels in birch. The characteristic decay feature for soft rot fungi with no decay of middle lamellae in pine and birch and vessel secondary cell walls in birch was evident. This reflects the higher guaiacyl lignin content and presence in birch middle lamellae and vessel secondary cell wall compared to fibre cell walls (Daniel, 2014).

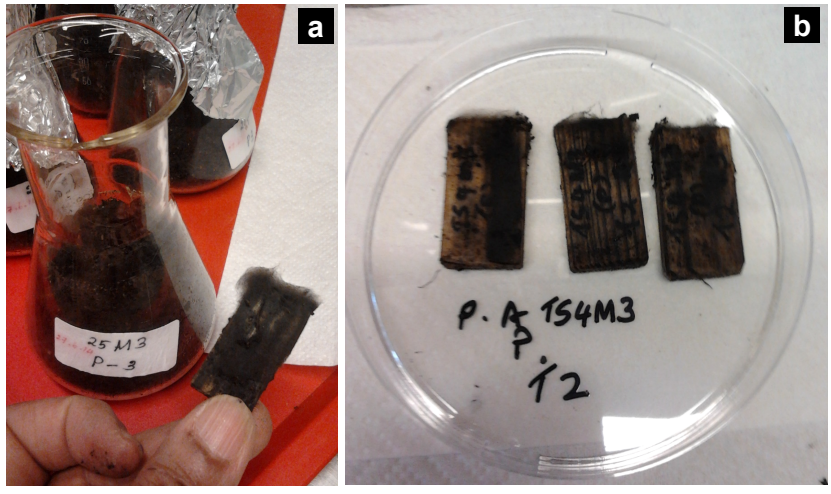

Figure 16. AWPA (2008) soil block decay test for *Phialophora* spp. A strains. (a) Mycelium colonization on pine wood blocks by strain 25M3. (b) Replicates of strain TS4M3 on pine wood blocks just before sampling.

3.3 Does copper tolerance provide a competitive advantage for degrading copper treated wood by soft rot fungi? (Paper III)

Even though several environmentally acceptable wood protection modifications like acetylation, furfurylation and impregnation with oil and silicones are available on the market, copper-based preservatives are likely to remain in the future as the major form of wood protection in the world. Moreover the introduction of micronized-Cu (MC) in USA has further emphasized the importance of copper-based wood preservatives.

Wood treated with copper-based chemicals can have short service life due to aggressive decay by fungi showing copper tolerance such as shown by the brown rot fungus *Antrodia vallantii*. The exact biochemical mechanism used by such brown rot fungi for degrading copper-treated wood are still poorly understood, but thought to involve both the depolymerisation of cellulose together with "binding-up" or detoxification of free copper. Previous studies by Daniel and Nilsson (1986) show the ability of soft rot fungi (i.e. *P. mutabilis)* to bind copper both extracellularly and intracellularly and for hyphae to penetrate high levels of copper precipitated on the cell lumina of fibres.

Since copper tolerant fungi are reported to degrade copper-treated wood (i.e. with commercial loadings) a modified ENV 807 (2001) standard wood decay test using both vermiculite and soil was carried out to test the decay ability of copper tolerant *Phialophora* species. Two copper tolerant soft rot fungi showing high (*P. malorum)* and medium- (*P. mutabilis)* copper tolerance (i.e. from *in-vitro* studies) and the weakly copper tolerant (*Chaetomium globosum)* decay capacity were evaluated using two wood species (pine and birch) and three $CuSO_4$ and MC concentrations (details in Paper III).

3.3.1 Mass loss

Mass loss for the three fungi for treated ($CuSO_4$ and MC) and untreated birch and pine wood blocks on vermiculite are shown in Table 2 (Paper III). For the three fungi the mass loss of controls after 36 weeks ranged from ca 36-55% i.e. in the order *C. globosum* > *P. mutabilis* > *P. malorum*. Mass loss was fairly similar in the beginning (i.e. after 24 weeks ca 39-44%) indicating that all three fungi were similarly aggressive and able to colonize the wood structure. For pine the order of decay was the same only the mass loss was very much lower and ranged from 2-33% (see Table 2 in Paper III).

The trends of mass loss for wood blocks and fungi exposed in soil were also the same as the vermiculite test, in the range ca 13-28% with the same order after 36 weeks. The difference between mass loss between birch and pine is consistent with the type and level of lignin present in wood and its effect on decay.

Birch wood is lignified with syringyl-guiaicyl moieties (ca 22%) and pine guiaicyl lignified (28%) with the higher lignin level and type (guiaicyl lignified) giving greater natural resistance in pine, no matter if treated or untreated with copper. Birch vessel secondary cell walls and middle lamellae show guiaicyl lignification and decay was not noted in vessels or in middle lamellae in untreated or treated wood with the three fungi using light microscopy after 36 weeks.

With all copper treatments and wood species, decay progressed over time. In general, the vermiculite test led to higher mass losses than in soil. Increasing copper (as $CuSO_4$ or MC) reduced decay in birch generally for all fungal species with *P. mutabilis* and *C. globosum* on the whole producing greater mass loss than *P. malorum* particularly at lower copper concentrations (i.e. 0.2%). Comparatively, in pine very little difference in mass loss were noted in the beginning (after 24 weeks) but later after 36 weeks, *C. globosum* showed slightly greater mass loss at 0.2 and 0.4% $CuSO_4$ than both *Phialophora* species, but for MC the mass losses were similar after both 24 and 36 weeks.

Although *P. malorum* possessed the highest copper tolerance *in-vitro* compared with *P. mutabilis* and *C. globosum*, this did not result in greater mass loss of the copper-treated samples. This indicates that copper tolerance did not provide *P. malorum* a greater ability for degrading copper-treated wood under the experimental system used.

3.3.2 Morphology of wood decay patterns for insights on the decay mechanism

Results from LM observations (Figs 17-18,) showed colonization of wood blocks (both pine and birch) by all three fungi regardless whether copper-treated (as $CuSO_4$ or MC) or non-treated in soil and vermiculite cultures. Fungal colonization pattern was found to be similar in both wood types and the decay progressed from the outside inwards primarily through the rays in

addition to the resin canals in pine. Fungi initially degraded parenchyma cells in both wood types (i.e. radial/axial parenchyma cells in pine and apotracheal and marginal parenchyma in birch) in addition to latewood tracheids of pine (details in Paper III). Basic characteristics of wood decay of the two wood types including chemistry, cell types and copper treatments are shown in the Table 3 in Paper III.

The complex wood anatomy of birch, which is a diffuse-porous hardwood, appeared to influence decay morphology (Fig. 17). The parenchyma cells were the first cellular elements to be degraded in both treated- and untreated wood blocks (Fig. 17a) indicating that none of the copper treatments prevented decay of unlignified cells (details in Paper III). Nevertheless guiaicyl lignified vessels remained ungraded in all wood samples (Fig. 17c).

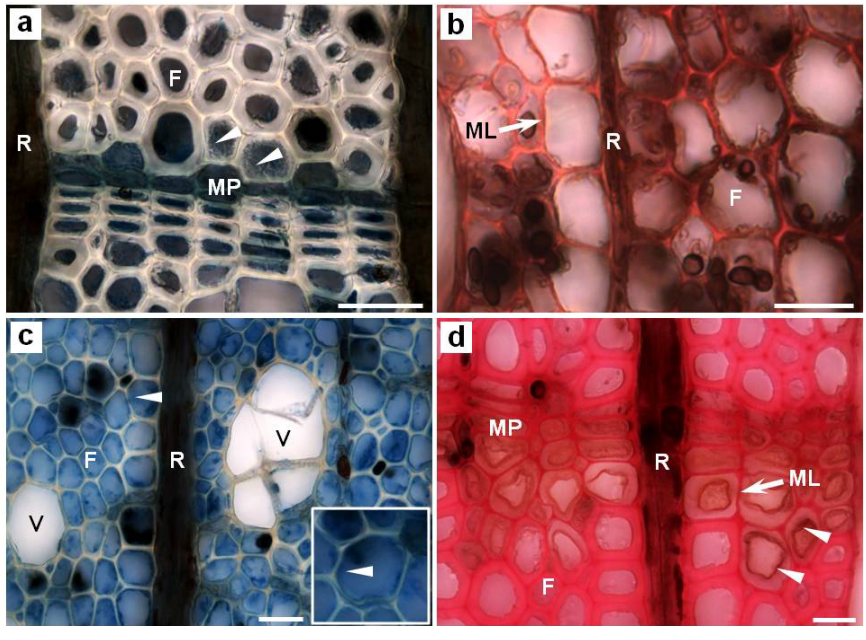

Figure 17a-d. Colonization and decay morphology in birch wood blocks by *P. mutabilis (a, b)*, *P. malorum (c)* and *Chaetomium globosum (d).* Arrowheads in a, c and d indicate attack of lumen side fibre cell walls, small hyphae causing erosion and fibre cell walls remained in advanced stage of attack, respectively. F, fibre ML, middle lamella; MP, marginal parenchyma; R, ray parenchyma; V, vessel. Bars: 25 µm.

A similar decay morphology shown by the three fungi was cell wall erosion (i.e. soft rot Type II) by which the birch fibres and parenchyma cells were degraded (details in Paper III). A progressive decay pattern was apparent where initial decay was close to the fungal hyphae but later developed into the entire secondary wall leaving the highly lignified middle lamella/S1 and S3 regions un-degraded (Fig. 17a-d). All three fungi produced typical cavities (i.e. soft rot Type I) within the secondary wall of untreated birch wood while true S2

cavities in treated samples were observed only with *P. mutabilis* (Fig. 1e in Paper III). Overall, microscopy observations suggest that the copper treatments ($CuSO_4$ and MC) neither inhibited hyphal colonization nor decay (Paper III) which was in agreement with results reported earlier by Daniel and Nilsson (1998).

The principle decay patterns differed between the two wood species (Fig. 18). All three fungi showed typical soft rot cavities (Type I) in pine control samples with latewood tracheids showing some extensive degradation (Fig. 18c, d).

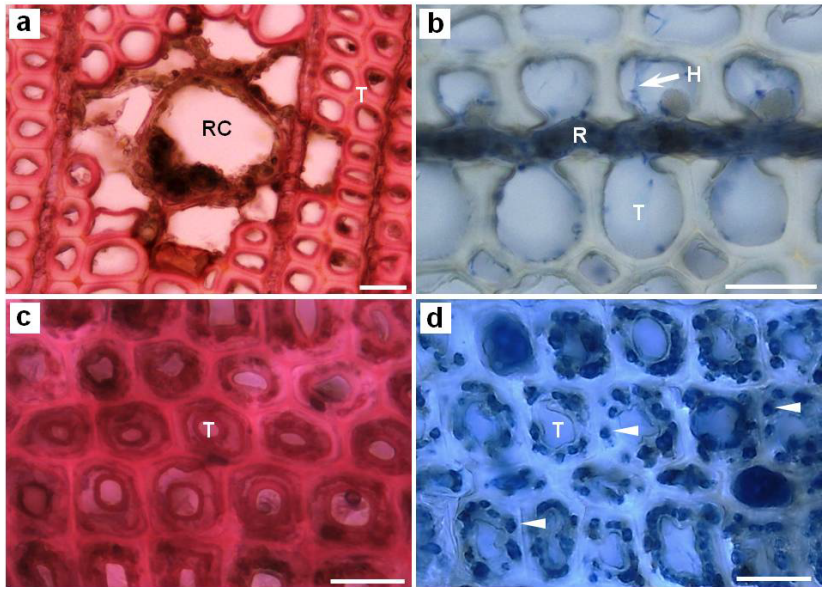

Figure 18 a-d. Colonization and decay of pine wood by *P. mutabilis* (a-c) and *P. malorum (d)*. Arrowheads in d indicate typical cavities in the secondary cell wall of tracheids. H, hyphae; R, ray parenchyma; T, tracheids; RC, resin canal. Bars: 25 µm.

In contrast, few cavities were observed in secondary cell walls of copper-treated pine samples (Paper III). Nevertheless abundant hyphae within rays, resin canals and tracheid lumina were observed (Fig 18a, b). Overall, the microscopy results indicated weak erosion attack and poor cavity formation in copper-treated pine samples (Paper III) which was consistent with results from the mass loss studies (Table 2, Paper III).

The present study (Paper III) suggests that copper tolerance was probably not the most important criteria for degrading copper-treated wood and thus may not provide a competitive advantage for decay similar to that shown by brown rot fungi as discussed in detail in Paper III. For example, there was little difference in mass loss of copper-treated birch and pine wood between the fungal species. Furthermore *C. globosum* and *P. mutabilis* produced greater

mass loss in copper- treated wood than *P. malorum* which was the most highly copper tolerant fungus shown from *in-vitro* studies.

Therefore, studies provide support for the hypothesis that the probable governing aspect controlling soft rot decay in copper-treated wood is the type and level of lignin together with copper rather than level of copper.

It should be remembered that the present results are interpreted from decay tests using fungal monocultures which may not reflect totally natural situations where both fungi and bacteria may compete for wood substrates. The several previous worldwide reports showing the frequent isolation of *Phialophora* species from Cu-treated in-service onto Cu-agar however testifies to their importance. The isolation of fungi onto Cu-agar or for example use of molecular techniques to identify fungal species in homogenized wood extracts from Cu- treated wood from in-service does not mean they are however responsible for the main decay. In this context, a combination of copper tolerance and an ability to overcome the lignin barrier may be the most important and provide the best competitive advantage under natural conditions.

3.4 Studies on *Phialophora malorum* using proteomics (Papers IV and V)

In the following two studies, the research was orientated to looking at the mechanisms and possible involvement of proteins in the copper tolerance of the most copper tolerant soft rot fungus *P. malorum* studied. In general, a number of intracellular and extracellular mechanisms have been proposed involved to allow fungi to resist metal toxicity *in-vitro* and *in-situ*. These include both extracellular (exclusion) mechanisms of metal exclusion and binding as well as intracellular mechanisms where metals are bound to cellular components and rendered non-toxic. Some metal tolerant fungi are known to employ both mechanisms. In this respect proteins (e.g. metallothioneins/chelators) have frequently been implicated in fungal metal detoxification processes.

Therefore two approaches were employed in the following studies. The first approach (Paper IV) involved analysis and studies on the changes in the total cellular proteome of *P. malorum* mycelia when grown in copper-supplemented media. The second approach was developed to study the extracellular proteome and detect specific proteins up-regulated and weakly bound in the fungal cell wall and associated extracellular slime, i.e. that lying outside the fungal cell membrane. The two approaches can be seen therefore as complementing one another.

3.4.1 Aspects of copper tolerance of the soft rot fungus *Phialophora malorum* grown *in-vitro* in copper supplemented media: Light, electron microscopy and global protein expression studies through a proteomics approach (Paper IV)

During wood colonization and decay, fungi may express a variety of proteins involved in both cell wall breakdown and general fungal metabolism (Kang et al., 2009; Daniel, 2016). Changes in the dynamics of protein turn over can be studied using proteomics and provide quantitative and qualitative information on all the proteins expressed by an organism at the time of extraction (Kang et al., 2009). For example, proteomics approaches have been used previously to investigate copper response in a variety of plants including rice (Ahsan et al., 2009), *Arabidopsis* and *Cannabis sativa*. Here proteins have variably been shown involved in antioxidant and defense related processes (e.g. Cu-detoxification) as well as up-regulated for reduction of Cu II to Cu I (Smith et al., 2004, Ahsan et al., 2009).

In the present study, a proteomics approach was combined with light microscopy cytochemistry, SEM and ICP-AES to observe both the morphological changes in fungal hyphae and investigate changes in global protein expression by *P. malorum* when grown under normal and copper-supplemented culture conditions. *P. malorum* was grown in cultures supplemented with Cu and LC-MS/MS used to determine whether changes in global proteins were induced through copper stress.

Two experiments were conducted for LC-MS/MS analysis: *i)* an initial pre-screening study using *P. malorum* mycelia from control, 0.064 and- 0.64% Cu *and ii)* a second experiment using control and 0.064% Cu samples each with 3 replicates. Data from pre-screening were searched against the entire Swissprot database and in the second experimental data against both Swissprot and *C. globosum* databases for relative protein quantification. This was done because the genome of *P. malorum* is unknown while that of *C. globosum* is sequenced and should show some similarity to *P. malorum* (i.e. with respect to soft rot capacity, although not in copper tolerance). More details for sample preparation and data analysis are given find in Paper 4.

Results from the comparative pre-screening proteomic analysis are shown (Fig. 19). Sixty eight proteins were found common in all three samples (i.e. control, 0.064 and 0.64% Cu) types. Thirty three proteins were common between control and 0.064% Cu samples, 13 between control and 0.64% Cu and 26 between the two copper concentrations respectively. The highest number of proteins identified in one sample type was 50 in the 0.64% Cu sample. The pre-screening analysis also allowed separation of the proteins percentagewise into different functionally important groups (Fig. 20). However, very little difference was shown in the percentage of the different functional proteins between samples (Fig. 20), although a large numbers of

proteins could not be identified due to the lack of *P. molarum* genome suggests greater differences.

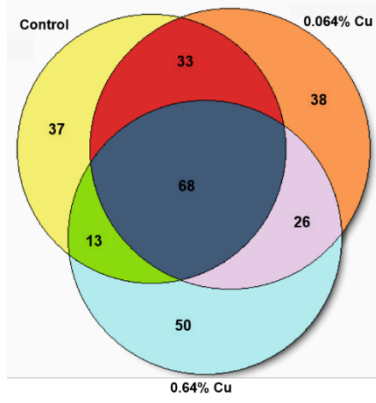

Figure 19. Venn diagram of proteins found in the control, 0.064% Cu and 0.64% Cu supplemented *P. malorum* samples.

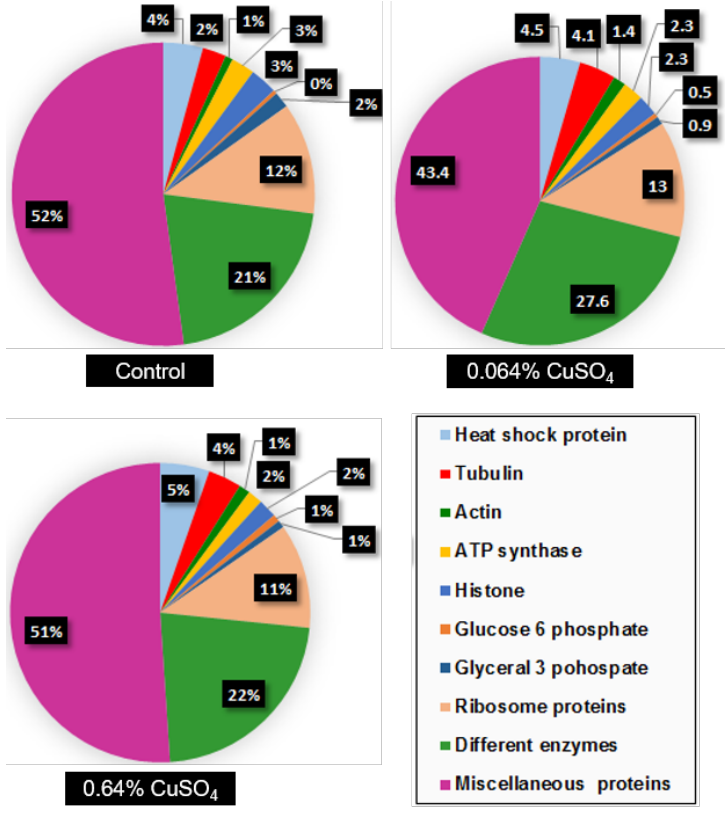

Figure 20. Proteins expressed by *P. malorum* when grown in control and copper-supplemented media. Results reflect percentage functional importance of the different proteins in the different media. Since the genome of *P. malorum* was not available, quantitative analysis of the proteins were not possible and only qualitative analysis was performed against the genome *C. globosum.*

An interesting aspect noted during the proteomic studies was the up-regulation of heat shock proteins (Paper 4; Table 2) in 0.064% Cu-treated liquid cultures. This coincides with the stimulatory effect (i.e. in dry mycelial wt.) of copper noted at similar levels (i.e. 0.032 and 0.064% Cu) in the liquid culture experiments (Paper 1; Fig. 4)

When the six *P. malorum* samples results were analyzed and searched towards all fungi and against the *C. globosum* protein sequence database and the following proteins were found as common proteins in both methods: ATP-citrate synthase α, ATP-citrate synthase sub-unit 1, ubiquitin and actin (Paper IV). Currently, it is not known what specific role these proteins have in copper tolerance.

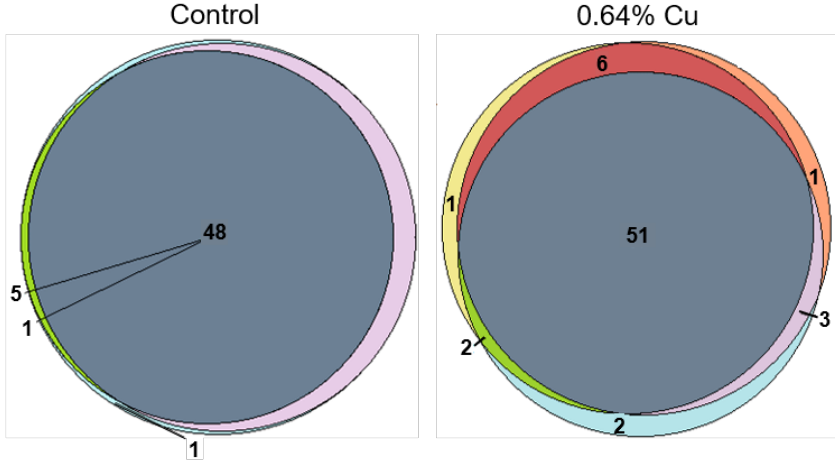

Figure 21. Venn diagrams for qualitative results of 3× control and 3× 0.064% Cu samples of *P. malorum* when searched against the *C. globosum* database. Colour key; yellow, orange and light blue indicate proteins found in one control/copper sample; grey is for proteins commonly find in all control/copper samples. Red, pink and green indicate proteins common between 2 samples.

This appears to be the first reported study using proteomics on *P. malorum* which shows great potential to unravel the proteins involved in copper tolerance. However, analysis are somewhat restricted by the lack of *P. malorum* genome.

3.4.2 Aspects of copper tolerance of the soft rot fungus *Phialophora malorum*; cell wall bound copper and detoxification (Paper V)

Fungi having ability to degrade waterborne preservative treated wood are generally recognized as exhibiting metal tolerance. A number of different mechanisms have been proposed that allow copper tolerant soft rot fungi to resist heavy metal toxicity. One mechanism involves extracellular complexation of heavy metals with the fungal cell wall and extracellular slime material and thereby control metal uptake. Another mechanism to control

uptake of metals is by the extracellular secretion of chelators. The aim of this study was to develop a more specific approach than the global approach used in Paper IV and to examine the extracellular proteins/peptides differentially expressed by *P. malorum* and bound to the fungal cell wall and extracellular slime when grown *in-vitro* in Cu-supplemented and control culture media.

The work involved developing a non-destructive approach using mild extraction of proteins from 10 day old *P. malorum* mycelia grown in Cu-supplemented (0.064 % Cu) and control cultures. The experimental setup for protein extraction, fractionation (on SDS PAGE gel), protein concentration, enzyme digestion and protein identification from MALDI-TOF MS/MS mass spectrometry are outlined in Paper V.

Using SDS PAGE electrophoresis and protein separation, it was possible to detect differences in electrophoretic banding and thereby in protein expression of *P. malorum* grown in control and Cu-cultures after 10 days growth i.e. in the logarithmic stage of development. Twelve characteristic bands were detected on SDS page gels for Cu-cultures that were absent in the controls (Fig. 22). MALDI-TOF MS/MS showed some of these bands to represent polypeptides from several hydrolytic enzymes that were over expressed/unique during growth under Cu-supplement conditions and presumably involved in copper tolerance. It should be noted that during cavity formation in wood, several of these hydrolytic enzymes would also be expected to be produced/released within the confines of the cavities and thereby in direct contact with copper released during wood cell wall breakdown.

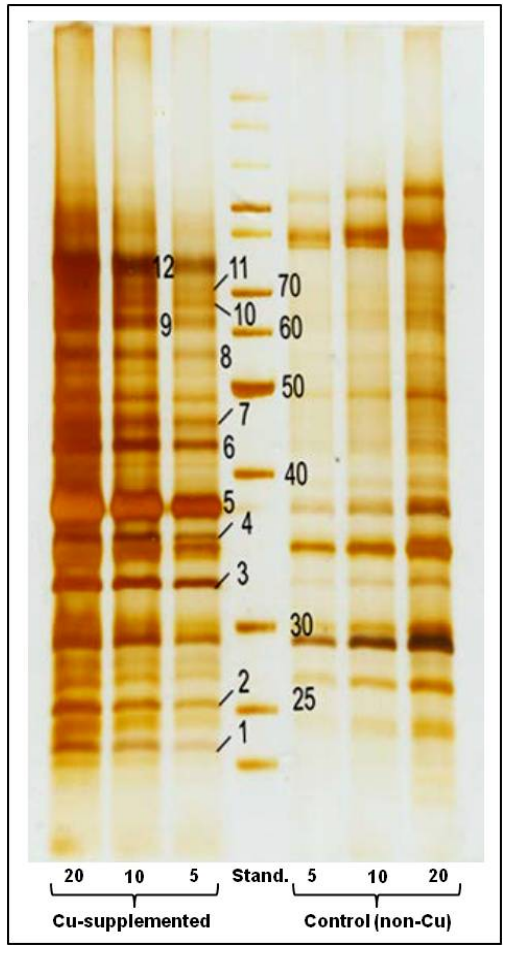

Figure 22. SDS PAGE electrophoresis gel picture for *P. malorum* (10 days old cultures). Loading scheme: from left 20, 10, 5 µL of protein extracts from Cu-supplemented samples, molecular weight standard (stand.) and 5, 10, 20 µL of protein extracts from control samples (i.e. non-Cu supplemented).

The present mild method developed for extraction of proteins represents a novel approach whereby extracellular proteins up-regulated and found associated with the fungal cell wall/extracellular slime can be characterized. With this navel approach 12 proteins (details in Paper V) either unique/overexpressed in Cu-cultures compared to control were produced. Interestingly several enzymes (i.e. peptidases, glucosidases) were detected and thus may be recognized as important for providing a first line (Paper V) defense against copper toxicity by preventing Cu-ions entering in to cell cytoplasm of *P. malorum.*

4 Conclusions and future perspectives

* Molecular identification with PCR amplification of ITS rDNA sequencing can be used as a successful and reliable technique for identification of soft rot (SR) fungi isolated from copper-preservative-treated and untreated wood. Molecular phylogenetic analysis differentiated *Phialophora* species/strains into three clades as *P. malorum, P. mutabilis* and *Phialophora* spp. A.

* Four unidentified strains of *Phialophora* spp. A previously isolated from preservative-treated wood were for the first time characterized to species level as *Phialophora dimorphospora* by molecular identification and phylogenetic analysis.

* There is considerable future potential for making species specific primers for *Phialophora* spp. /strains and use PCR techniques directly to identify soft rot fungal species more easily and accurately and use them on degraded wood.

* The present studies have led to an improved understanding of soft rot decay mechanism(s) in copper-treated wood. Total mass loss were related to copper loading corroborating results from previous studies in the laboratory and decay morphology was similar for both the copper tolerant and non-tolerant soft rot fungi tested. However, strong copper tolerance (i.e. with *P. malorum*) did not provide a competitive advantage and thereby greater mass losses under the experimental conditions used. The chemical nature of wood material, in particular lignin and its collective influence with copper treatment, was considered more important in preventing soft rot decay rather than an ability for fungal copper tolerance.

* Modern proteomics LC-MS/MS were used for the first time to characterize the proteome of the soft rot fungus *P. malorum*. Despite its genome being unknown, it was possible to identify some unique proteins in copper and copper samples when searched against the databases for all fungi. From the unique proteins identified from *P. malorum* grown in copper-treated samples,

ATP-citrate synthase subunit I was considered important since has been shown involve in hyphal cell wall changes (e.g. wall thickening) which may help copper tolerance.

* A novel extraction and SDS-PAGE protein separation approach with protein characterization using MALDI-TOF MS/MS was developed allowing for characterization of cell wall/slime bound extracellular proteins involved in copper binding that may provide a first line defense against copper toxicity.

* For further insights into the mechanisms of copper tolerance and involvement of proteins by *P. malorum*, the genome should be determined.

References

Abrams, E (1948). Microbiological Deterioration of Organic Materials: Its Prevention and Methods of Test. National Bureau of Standards Miscellaneous Publication 188. U.S. Department of Commerce, Washington DC.

Ahsan, N., Renaut, J. & Komatsu, S. (2009). Recent developments in the application of proteomics to the analysis of plant responses to heavy metals. *Proteomics,* 9(10), 2602-2621.

Altschul, S.F., Gish, W., Miller, W. Myers, E.W. & Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology,* 215 (3), 403-410.

Archer, K. & Preston, A. (2006). An overview of copper based wood preservatives. Wood Protection 2006. http://www.forestprod.org/wood protection06archer.pdf. AWPA Technical Sub-committee P6. (2008). AWPA E10-08. Standard method of testing wood preservatives by laboratory soil block culture.. American Wood Preservers Association, Birmingham..

Bergquist, J., Hakansson, P., Sundqvist, B. & Zubarev, R. (2007). Mass spectrometry of proteins - Uppsala perspectives on past and present. *International Journal of Mass Spectrometry*, 268(2-3), 73-82.

Bjordal, C.G. & Nilsson, T. (2008). Reburial of shipwrecks in marine sediments: a long-term study on wood degradation. *Journal of Archaeological Science*, 35(4), 862-872.

Blanchette, R.A., Held, B.W., Jurgens, J.A., McNew, D.L., Harrington, T.C., Duncan, S.M. & Farrell, R.L. (2004). Wood-destroying soft rot fungi in the historic expedition huts of Antarctica. *Applied and Environmental Microbiology*, 70(3), pp.1328-1335.

Boerjan, W., Ralph, J. & Baucher, M. (2003). Lignin biosynthesis. *Annual Review of Plant Biology,* 54, 519-546.

Clausen, C.A. & Green, F. (2003). Oxalic acid overproduction by copper-tolerant brown-rot basidiomycetes on southern yellow pine treated with copper-based preservatives. *International Biodeterioration & Biodegradation*, 51(2), 139-144.

Cookson L.J., Creffield J. W., McCarthy K. J. & Scown, D. K. (2010). Trials on the efficacy of micronized copper in Australia. *Forest Products Journal*, 60(1), 6-12.

Côté, W.A. (1980). *Papermaking fibers: A photomicrographic atlas*. Syracuse, Syracuse University Press, New York,

Daniel, G.F. & Nilsson, T. (1986). Ultrastructural observations on wood degrading erosion bacteria. *International Research Group on Wood Preservation. (IRG/WP/1283)*, Stockholm, Sweden.

Daniel, G.F. & Nilsson, T. (1988). Studies on preservative tolerant *Phialophora* Species. *International Biodeterioration*, 24(4-5), 327-335.

Daniel, G.F. & Nilsson, T. (1989). Effect of hydrofluoric-acid pretreatment on the degradation of wood by soft rot fungi. *Material Und Organismen*, 24(2), 121-138.

Daniel, G. (1994). Use of electron-microscopy for aiding our understanding of wood biodegradation. *FEMS Microbiology Reviews*, 13(2-3), 99-233.

Daniel, G., & Nilsson, T. (1998). Developments in the study of soft rot and bacterial decay. In: *Forest Products Biotechnology, (*Eds.Bruce, A., Palfreyman, J.W .) pp. 37-62.

Daniel, G. (2014). Fungal and Bacterial Biodegradation: White rots, Brown rots, Soft rots, and Bacteria. In: *Deterioration and Protection of Sustainable Biomaterials*, Vol.1158 (Schultz, T.P. Goodell, Nicholas, D.D. (Eds.), pp. 23-58 American Chemical Society, and Washington.

Daniel, G., (2016). Fungal degradation of wood cell walls. In: secondary xylem biology (Kim, Y.S., Funada, R., Singh, A.P., Eds) Ch. 8., pp 131-167. ISBN: 978-0-12-802185-9. Elsevier Academic Press, Amesterdam.

De Groot, R.C. & Woodward, B. (1999). Using copper-tolerant fungi to biodegrade wood treated with copper-based preservatives *International biodeterioration & biodegrad* 44 (1), 17–27

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.F., Guindon, S., Lefort, V., Lescot, M. & Claverie, J.M., (2008). Phylogeny. fr: robust phylogenetic analysis for the non-specialist. *Nucleic acids research*, *36*(suppl. 2), W465-W469.

Duncan, C.G. (1965). Determining resistance to soft-rot fungi. *Res. Report FPL-48*, U.S. Department of Agriculture, Forest Service, Forest Products Laboratory, Madison, WI, U.S.

Eaton, R.A. & Hale, M.D.C. (1993). *Wood Decay, Pests and Protection*. London, Chapman & Hall, London.

Eriksson, K.E.L., Blanchette, R.A. & Ander, P., (1990). Morphological aspects of wood degradation by fungi and bacteria. In *Microbial and enzymatic degradation of wood and wood components* (pp. 1-87). Springer Berlin Heidelberg.

ENV 807. (2009). Wood preservatives-determination of the effectiveness against soft rotting micro-fungi and other soil inhibiting micro-organisms. European committee for Standardization, Brussels.

Evans, P., Matsunaga, H. & Kiguchi, M. (2008). Large-scale application of nanotechnology for wood protection. *Nature Nanotechnology*, 3, (577).

Fernando, D. (2007). *Ultrastructural characterization (morphological and topochemical) of wood pulp fibres; Effects of mechanical and kraft processes*. Diss. (PhD thesis) Swedish University of Agricultural Sciences (SLU) Uppsala, Sweden.

Freeman, M.H. (2002). Waterborne copper naphthenate- a chromium and arsenic free wood preservative. Proceedings of the 2002 FPS Wood preservation workshop, Orlando, Florida. FPS, Madison, Wisconsin.

Freeman, M.H., Kamdem, P. & Brient, J.A. (2004). Waterborne copper naphthenate a chromium and arsenic free preservatives for wood and composites. Poster presented at Environmental Impacts of Preservative Treated Wood Conference, Orlando, Florida.

Freeman, M.H. & McIntyre, C.R. (2008). A comprehensive review of copper-based wood preservatives. *Forest Products Journal*, 58(11), 6-27.

Gadd, G.M. (1993). Interactions of fungi with toxic metals. *New Phytologist*, 124(1), 25-60.

Gams, W. (2000). *Phialophora* and some similar morphologically little-differentiated anamorphs of divergent ascomycetes. *Studies in Mycology*,(45), 187-199.

Gardes, M. & Bruns, T.D. (1993). ITS Primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology*, 2(2), 113-118.

Green, F. & Clausen, C.A. (2005). Copper tolerance of brown-rot fungi: Oxalic acid production in southern pine treated with arsenic-free preservatives. *International Biodeterioration & Biodegradation*, 56(2), 5-79.

Goodell, B. (2003). Brown rot degradation of wood: our evolving view. ACS Symposium Series 845, Washington pp 97–118

Hale, M. D. & Eaton, R. A. (1985). The ultrastructure of soft rot fungi. I. fine hyphae in wood cell-walls. *Mycologia*, 77(3), 447-463.

Henningsson, B. & Nilsson, T. (1976). Some aspects on microflora and the decomposition of preservative-treated wood in ground contact. *Material and Organismen*, 3, 307-318.

Hogberg, N. & Land, C.J. (2004). Identification of *Serpula lacrymans* and other decay fungi in construction timber by sequencing of ribosomal DNA - A practical approach. *Holzforschung*, 58(2), 199-204.

Huckfeldt, T. (2002) Hausfäulepilze. http://www.hausschwaminfo.de.

Humar, M., Kalan, P., Sentjurc, M. & Pohleven, F. (2005). Influence of carboxylic acids on fixation of copper in wood impregnated with copper amine based preservatives. *Wood Science and Technology*, 39(8), 685-693.

Ibach R. E. (1999). Wood preservation. Wood handbook: Wood as an Engineering Material.(General Technical Report FPL-GTR.113) USDA Forest Service, Forest Products Laboratory, Madison, pp . *l* 14.1-14.27.

Jasalavich, C.A., Ostrofsky, A. & Jellison, J. (2000). Detection and identification of decay fungi in spruce wood by restriction fragment length polymorphism analysis of amplified genes encoding rRNA. *Applied and Environmental Microbiology*, 66(11), 4725-4734.

Kang, Y.M., Prewitt, M.L., & Diehl, S.V. (2009). Proteomics for biodeterioration of wood (*Pinus taeda* L.): Challenging analysis by 2-D PAGE and MALDI-TOF/TOF/MS. *International Biodeterioration & Biodegradation*, 63(8), 1036-1044.

Karunasekera, H. & Daniel, G. (2013). Molecular identification and phylogenic analysis by sequencing the rDNA of copper-tolerant soft-rot *Phialophora* spp. *International Biodeterioration & Biodegradation*, 82, 45-52.

Kim, Y.S. & Singh, A.P. (2000). Micromorphological characteristics of wood biodegradation in wet environments: a review. *IAWA Journal*, 21(2), 135-155.

Larena, I., Salazar, O., Gonzalez, V., Julian, M.C. & Rubio, V. (1999). Design of a primer for ribosomal DNA internal transcribed spacer with enhanced specificity for ascomycetes. *Journal of Biotechnology*, 75, 187-194.

Leightley, L.E. (1979). Soft-rot decay in copper-chrome-arsenic treated eucalypt power transmission poles in Queensland, Australia. Isolation and identification of soft-rot fungi. *International Journal of Wood Preserv*ation 1, 143-150.

Leightley, L.E. (1980). Rapid screening method for determining soft-rot decay ability. *Mycologia*, 72(3), 632-637.

Lewbow, S.T. Nilsson, T. & Morrell, J.J. (1999). Performance of copper-based wood preservatives in soil bed exposures. *Material Und Organismen*, 33, 235-244.

Lindahl, B.D., de Boer, W. & Finlay, R.D. (2010). Disruption of root carbon transport into forest humus stimulates fungal opportunists at the expense of mycorrhizal fungi. *ISME Journal*, 4(7), 872-881.nam,

Martinez,D., Larrondo,L.F., Putnam, N., Gelpke, M.D.S., Huang, K., Chapman., J., Helfenbein, K.G., Ramaja,P., Detter, J., Larimer, F., & Coutinho, P.M. (2004) Genome sequence of the

lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nature Biotechnology, 22(6), 695-700.

Menkis A., Allimer, J., Vasiliauskas R., Lygis, V., Stenlid J. & Finlay R. (2004). Ecology and molecular characterization of dark septate fungi from roots, living stems, course and fine wood debris. *Mycology Research,* 108(8), 965-973.

Molla, A.H., Fakhrul-Razi, A., Abd-Aziz, S., Hanafi, M. M. & Alam, M.Z. (2001). *In vitro* compatibility evaluation of fungal mixed culture for bioconversion of domestic wastewater sludge. *World Journal of Microbiology and Biotechnology* 17, 849–856.

Moreth, U. & Schmidt, O. (2005). Investigations on ribosomal DNA of indoor wood decay fungi for their characterization and identification. *Holzforschung*, 59, 90–93.

Nilsson, T. (1973). Studies on wood degrading and cellulolytic activity of microfungi. Studia Forestalia Suecica, 104, 1-40.

Nilsson, T. (1974b). Microscopic studies on the degradation of cellophane and various cellulosic fibres by wood-attacking micro fungi. Studia Forestalia Suecica, 117, 1-37.

Nilsson, T. & Henningsson, B. (1978). *Phialophora* species occurring in preservative treated wood in ground contact, *Material und Organismen*, 13, 292-313.

Nilsson, T. & Ginns, J. (1979). Cellulolytic activity and the taxonomic position of selected brown-rot fungi. *Mycologia,* 71(1), 170-177.

Nilsson, T. & Daniel, G. (1983) Tunneling bacteria In: Proceedings of *International Research Group on Wood Preservation*, Document Stockholm, Sweden.

Nilsson, T., Daniel, G., Kirk, T.K. & Obst, J.R. (1989). Chemistry and microscopy of wood decay by some higher ascomycetes. *Holzforschung*, 43(1), 11-18.

Nylinder, M., Woxblom, L., & Fryk, H. (2005) Ädellöv: Virke och förädling (Broadleaved: wood and processing.), Tierps Tryckeri AB, Sweden.

Rayner, A.D.M. & Boddy, L. (1988). *Fungal Decomposition of Wood: Its Biology and Ecology*. Chi Chester, New York, USA: John Wiley.

Rowell, R.M. (2005). Chemical Modification of Wood. In: *Handbook of Wood Chemistry and Wood Composites.* (Ed.Rowel, R.M.) CRC Press LLC New York. pp.537-598.

Santiago-Rodrígue, L., Grigg, J.L., Bradham, K.D., Nelson, C., Luxton, T., Platten W.E., & Rogers, K.R., (2015). Assessment of the bioaccessibility of micronized copper wood in synthetic stomach fluid. *Environmental Nanotechnology, Monitoring & Management* 4, 85–92

Savory, J.G. (1954). Breakdown of timber by Ascomycetes and Fungi Imperfecti. *Annals of Applied Biology*, 41(2), 336.

Schmidt, O. & Ziemer, B. (1976). On the inhibitor tolerance of fungi, *Material Und Organismen*, 11, 215–230.

Schmidt, O. & Moreth, U. (2000). Species-specific priming PCR in the rDNA-ITS regions as a diagnostic tool for *Serpula lacrymans*. *Mycological. Research*., 104 69-72.

Schmidt, O. (2006). *Wood and Tree Fungi biology: Damage, Protection*, *and Use,* Berlin. Springer. 1-329.

Schmidt, O. (2007). Indoor wood-decay basidiomycetes: damage, causal fungi, physiology, identification and characterization, prevention and control. *Mycological Progress*, 6(4), 261-279.

Sharma, S., Aggarwal, R., Yadav, A. & Gupta, S. (2014). Protein mapping of *Chaetomium globosum*, a potential biological control agent through proteomics approach. *Journal of Plant Biochemistry and Biotechnology*, 23(3), 284-292.

Sjöström, E. (1993). *Wood Chemistry: Fundamentals and Applications*. 2nd ed. Academic Press, San Diego, CA, USA.

Smith, A.P., DeRidder, B.P., Guo, W.J., Seeley, E.H., Regnier, F.E. & Goldsbrough, P.B. (2004). Proteomic analysis of *Arabidopsis* glutathione S-transferases from benoxacor- and copper-treated seedlings. *Journal of Biological Chemistry*, 279(25), 26098-26104.

Tang JD, Parker LA, Perkins AD, Sonstegard TS, Schroeder SG, Nicholas DD & Diehl, S.V. (2013). Gene expression analysis of copper tolerance and wood decay in the brown rot fungus *Fibroporia radiculosa*. *Applied and Environmental Microbiology* 79(5), 1523-1533.

Wang, C.J.K. (1990). Ultrastructure of percurrently proliferating conidiogenous cells and classification. *Studies in Mycology* 32, 49-64.

White, T. J, Bruns, T, Lee S, Taylor J W. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M A, Gelfand D H, Sninsky J J, White T J, editors. PCR protocols: a guide to methods and applications. New York, N.Y: Academic Press, Inc.; 1990. pp. 315–322.

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: a Guide to Methods and Applications*. (Eds. Innis M A, Gelfand D H, Sninsky J. J, White, T. J) Academic Press Inc., New York. pp. 315-322.

Wilkins, M.R., Sanchez, J.C., Andrew, A.G., Ron, D.A., Humphery-Smith, I., Hochstrasser, D.F., & Williams, K.L. (1995). Progress with proteome projects: Why all proteins expressed by a genome should be identified and how to do it. *Biotechnology and Genetic Engineering Reviews,* 13 (1), 9-50.

Zabel, R.A., Lombard, F.F. & Wang, C.J.K. & Terracina, F. (1985). Fungi associated with decay in treated southern pine utility poles in the eastern United States. *Wood and Fiber Science*, 17(1), 75-91.

Zabel, R.A.,& Morrell, J.J., 1992. *Wood Microbiology, Decay and its Prevention*. Academic Press, Orlando, 476 pp.

Zhong H.Y., Rogers, S.O., & Wang, C.J.K., (1995) Assessment of *Phialophora* species based on ribosomal DNA internal transcribed spaces and morphology. *Mycologia* 87, 72-83.

Acknowledgements

I am grateful for the Dept. of Forest products SLU for providing me with this Opportunity to pursue my PhD studies. I thank Formas for providing me financial grants for this PhD study.

I would like to express my sincere gratitude to my main supervisor Professor Geoffrey Daniel for giving me the opportunity pursue my PhD studies introducing me to world of wood science and microscopy. I am grateful for your tireless efforts in guiding me throughout the study, teaching me microscopy techniques, constructive comments, and suggestions to improve my work and specially my writing. Most of all you always had the time for me my endless problems in lab, research questions and support my lab work all the time. Your humble and friendly attitude was a blessing to me during my whole PhD period especially during my stressful days of thesis writing. I appreciate your inspiring leadership.

I would like to thank my second supervisor Prof. Nasko Terziev for support and guidance in practical things and encouraging me to complete my studies and correcting my thesis and paper.

Thank you Dr. Gunilla for helping in lab experiments and all support at lab. I also gratefully to all our department people Kim, Mohamed, Gabriella for constant support. Shengzhen and special thanks to Jie for sharing our nice room and being a lovely, helpful friend all the time.

I would like to thank you Prof. Jonas for all guidance and support during my proteomic work at Uppsala university, thank you Dr. Margarita and Katarina also for the great support during the research work at your lab.

Finally Dinesh my loving husband I am grateful from bottom of my heart for all the help and support you provided me during this harsh long PhD path. Your kind, helpful generous support especially when I was writing the thesis reading and correcting word by word so many times ultimately brought me to this end. I don’t get words to thank you Dinesh you light finally this task which I thought will not possible me to achieve. Thank you my loving two sons for your care, love, encouragement and sharing my tears gave me strength and courage all the time. No words to thank you my Father, mother, sister and

brother for encouraging all the time. Also thank you my father- mother in-laws and in all in-laws for your support!

Also thank you all my friends in Sweden and especially in Uppsala and Stockholm for your great support always.