Aspects of Wood Decay and Preservation of Timber

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ABSTRACT

A number of species of wood decay fungi important for the damage they cause to timber and timber products in Korea were cultured. *Trametes versicolor*, which is one of the most important, was also cultured from a collection made in the UK and likewise the ascomycete *Daldinia concentica* was obtained for comparative studies. In the initial testing of the effectiveness of the two wood preservatives, ammoniacal copper quaternary (ACQ) and copper azole (CuAz) preserve injected blocks of Japanese Red Pine and Yellow Poplar were inoculated with *T. versicolor*, *Pleurotus ostreatus* and *D. concentrica*. Weight loss(%) of the wood blocks showed that Japanese Red Pine possessed greater natural resistance to decay by the white rot basidiomycete fungus *T. versicolor*, than to the white rot ascomycete *D. concentrica*. The results for Yellow Poplar were the opposite. It was also found that both preservatives had an inhibitory effect on all three test fungi regardless of tree species. Furthermore ACQ was the most effective preservative in relation to *T. versicolor*, which is the most damaging wood decay fungus in Korea. It has also been found that the absorption of the preservatives by the two different wood types differed with Yellow Poplar exhibiting a slightly greater absorption than the Japanese Red Pine, which might be a result of differences in the anatomical structure of the woods.

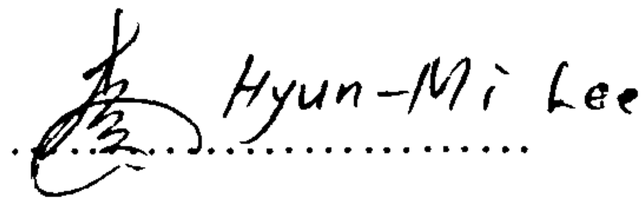
Fungal biomass was also determined using chitin and ergosterol assays. The results regarding levels of decay caused by *T. versicolor*, *P. ostreatus* and *D. concentrica* are in close agreement with the weight loss determinations. The assays also confirmed the effectiveness of the copper based preservatives.

The application of Scanning Electron Microscopy (SEM) has allowed

observations on the damage caused by the test fungi to the untreated blocks of the two wood species and the reduction in damage on blocks treated with the preservatives. Linked studies using the SEM and Atomic Force Microscopy have demonstrated differences in the micromorphology of the hyphal tips of the test fungi.

DECLARATION

I declare that while registered as a candidate for the degree of Doctor of Philosophy I have not, in the duration of this research programme, been registered as a candidate for another award from any other academic or professional institution.



(Hyun-Mi Lee)

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ABBREVIATIONS

ACQ	Alkaline Copper Quaternary
AFM	Atomic Force Microscope
cm	Centrimetre
CuAz	Copper Azole
°C	Degree Celsius
CPD	Critical point dryer
DAC	*Daldinia concentrica*
DW	Distilled Water
et al.	*et alia*
EtOH	Ethanol
Fig	Figure
Figs.	Figures
g	Grams
HCI	Hydrochloric acid
HPLC	Hight Performance Liquid Chromatography
Kg	Kilogram
L	Liter
LM	Light Microscope
M	Molar
MeOH	Methanol
min(s)	Minute(s)
mg	Milligram
ml	Millilitre
mm	Millimetres
µg	Microgram
µl	Microlitre
µm	Micrometre
NaOH	Sodium hydroxide
nm	Nanometre
PDA	Potato dextrose agar
PLO	*Pleurotus ostreatus*
ppm	Parts per million
pH	Power of hydrogen ion concentration
rpm	Revolutions per minute
SEM	Scanning Electron Microscope
spp	Species
TRV	*Trametes versicolor*

v/v	Volume by volume
w/w	Weight by weight

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CHAPTER 1

INTRODUCTION

Wood structure and composition

Our understanding of the role of fungi in wood decomposition is remarkably recent and effectively only began in the latter part of the nineteenth century with studies on the fungi involved in the decay of wood. The literature on comparative wood anatomy and decay prior to 1900 was summarized by Solereder (1908). Several decay patterns, such as white rot, brown rot and soft rot were recognized, but their pattern of decay is rather different (Zabel & Morrell, 1992). White and brown rot fungi are the two most effective wood-decay fungi and are mainly species of Basidiomycota (Valmaseda *et al.* 1990; Jin *et al.* 1990).

White rot fungi attack wood cells from inside to outside and cause decomposition of the brown-pigmented lignin resulting in the decayed part of the wood turning white. Brown rot fungi form hyphae in the cell walls at the early stages of decay, attacking the rings of cellulose and finally decomposing the lignin. Wood in contact with the ground frequently contains distinct microscopic cavities within their secondary cell walls caused by soft rot fungi (Hale & Eaton, 1985; Blanchette *et al.* 2004) and is more common in hardwoods than in softwoods (Birkinshaw, 1999).

Wood used for commercial purposes is derived from either gymnosperms or angiosperms. Both are highly vascular plants, producing water conducting tissues consisting of xylem tracheids (gymnosperms) or xylem vessels which make up the majority in angiosperms. Photosynthates are translocated in the phloem. These are

perennial plants which exhibit secondary thickening. The annual division of the meristematic cambium forms new conducting tissues which are inserted between the older wood and bark.

Fig. 1. 1 Wood cell wall layering showing microfibril orientation and relative size of the various layers from R. J. Thomas, In: Goldstein (eds), *Wood structure and Chemical Composition in Wood Technology Chemical Aspects.* ACS Symposium Series 43, 1977.

Plant cells have a primary wall formed during cell division and a secondary wall deposited after the cell enlargement phase has been completed. Conventional light microscopy, particularly with sections stained for lignin with phloroglucinol, reveals a lignin-rich area between contiguous cells. This area composed of the

middle lamella and primary walls of the contiguous cells is called the compound middle lamella. The lignin in the middle lamella or intercellular layer bonds the cells together. Since the primary wall also contains a high amount of lignin and is very thin, it is very difficult to detect: thus, the use of the term compound middle lamella (Thomas, 1991). Although the secondary wall appears as a rather homogenous layer with conventional light microscopy, polarizing light microscopy reveals a three-layer structure (Fig. 1.1.). This differentiation of the secondary wall is due to the different orientation of the crystallite within the three layers. Only about 25% of the total lignin is accounted for by this layer since it is very thin. Whereas the primary wall is a thin layer containing randomly oriented cellulose microfibrils, the secondary wall contains most of the wood substance and about 75% of the lignin. It consists of three parts: thin inner and outer layers with helical cellulose microfibrils oriented almost perpendicular to the fiber axis, and a thick central layer with helical cellulose microfibrils oriented nearly parallel to the fiber axis (Thomas, 1991). Cell walls comprise of as much as 95% of the mass of the woody plants (Goldstein, 1981). The chemical constituents of woody plant cell walls are cellulose (45-50), hemicelluloses (20-25), lignin (20-30), and extractives (0-10) (Thomas, 1991).

Another difference observable in xylem tissue is that of sapwood and heartwood (Figs. 1.2-1.6). Sapwood contains both living and dead cells and functions primarily in the storage of food. In general, heartwood consists of inactive cells that do not function in either water conduction or food storage (Miller, 2007). In durable woods, the heartwood consists of toxic extractives which contribute to the colour change and are responsible for the resistance of this wood to attack by microorganisms (Thomas *et al.* 1991). Sapwood, lacking these extractives, is not resistant to degradation even in those tree species with durable heartwood. The structure of softwoods is less complex than that of hardwoods, which have more cell

types (Nicholas, 1973).

Anatomy of soft and hardwoods - Gross morphological features (Thomas *et al.* 1991).

Fig. 1. 2 - 1. 6

Softwoods and hardwoods

The wood of hardwoods (angiosperms) contains xylem vessels that are responsible for water conduction and fibres that perform the support role. Softwoods (gymnosperms) contain cells called longitudinal tracheids that have the dual roles of conduction and support and they exhibit a range of specific gravities (softwoods 0.29 - 0.69; hardwoods 0.32 - 0.81). Thus, some softwoods are harder than some hardwoods and some hardwoods are softer than some softwoods (Goldstein, 1981; Thomas *et al.* 1991).

Softwood structure

Wood is distinguished from all other craft materials on the basis of cell structure. It is organic, contains cells, resin canals, pits, rays, and there are differences between early-wood and late-wood (Schmidt, 2006).

There are only two types of cells in softwood, wood fibers (longitudinal tracheids) and ray cells. About 95% of the softwood is made up of these wood fibers and short strips of radially aligned ray cells make up the remainder. Resin canals are

found in only a few softwoods, like pine, spruce, larch, and Douglas-fir, where special resin-producing cells called epithelial cells are formed.

Normally, the pit of one cell aligns with the pit of the adjacent cell to form a pathway. Rays are basically roadways for transporting food and other materials inside a living tree. The overall variation in wood fibers between early-wood and late-wood determines whether the grain is even or uneven (Peters, 2000).

One of the four longitudinal cell types listed in Table 1.1, the longitudinal tracheid, is the most important as it is found in all softwood species, occupies the greatest volume, and is the largest cell.

Hardwood structure

The cell structure of hardwoods is considerably more variable than in softwoods consisting of a combination of vessels, wood fibres and rays. Most of a hardwood's volume is comprised of at least four types of cells. These are vessel elements, wood fibers, ray cells, and parenchyma or storage cells. Some hardwood has only 10% of the volume taken up by rays, with vessels and fibers taking up 60% and 30%. Wood fibers in hardwoods tend to be shorter than in softwoods (Thomas, 1991; Peters, 2000).

Vessels occur in virtually all hardwoods, but never in softwoods. The vessels

in some woods form tyloses as sapwood changes to heartwood (Fig. 1.6.). Tyloses are bubble like membranes that develop inside the cavity of a vessel as it dies, occluding the lumen (Thomas, 1991).

Ring-porosity is found in many hardwoods which have a high concentration of vessels in their early-wood that are much larger in diameter than late-wood vessels. Diffuse-porous woods occur in both early-wood and late-wood.

Parenchyma serves primarily as storage cells and they are very useful for wood identification. Rays in hardwoods vary considerably in size and appearance (Peters, 2000)

Cells composing the longitudinal system consist of vessels, fibers, tracheids, and parenchyma (Table 1.2); in addition, longitudinal parenchyma occurs which is rare in softwoods.

Wood decomposition by fungi

The first report of fungi causing wood decay was in 1863 by Hermann Schacht, who described the decomposition phenomena of wood (Schacht, 1863; Blanchette, 1991). Experiments on wood decay fungi involve, e.g., *Pleurotus ostreatus* (Jacq.). P. Kumm, *Trametes versicolor* (L.) Pilat, *Phanerochaete*

chrysosporium Burds (Trojanowski & Hüttermann, 1984; Nutsubidze *et al.* 1990; Bartholomew *et al.* 2001).

Wood damage is influenced by various physical, chemical and biological influences on the wood-inhabiting fungi (Table 1.3).

Biological influences arise because of reciprocal effects between different organisms as anatagonism, synergism, and symbiosis.

Wood-inhabiting microorganisms use carbon present in woody tissues not only from enzymatically digested substrates, like simple sugars, peptides, fats but also from the storage material starch in the parenchyma cells. Additionally they utilize the main components of the woody cell wall, cellulose, hemicelluloses, and lignin.

Brown rot, white rot and soft rot fungi are groups of wood-decay organisms, among these three groups, brown rot fungi are highly potent wood-decay fungi

(Courtois, 1963; Barry *et al.* 2008). Fungal decay in wood is strongly influenced by environmental conditions such as presence of oxygen, moisture content and temperature (Williams & Hale, 2003). Temperatures between 2 °C to 38 °C, over 25 % moisture content and presence of oxygen are the most favourable conditions for wood decay caused by fungi (Green & Highley, 1995). Both brown rot and white rot fungi utilize the basic structural carbohydrates and certain extra cellular materials of wood as nutritional resources for growth (Wilcox, 1970).

Several studies including field experiments strongly support the view that nitrogen content present in wood is extremely important to the fungal decay of wood. Mechanisms such as autolysis, re-use of own mycelial nitrogen and lysis of other fungi in wood during decay are involved (Merrill & Cowling, 1966). In addition, thiamine is an essential vitamin for the growth of most wood-decay fungi (Jennison, 1952), later several alkaline treatment methods were developed to suppress the thiamine content in wood in order to control fungal growth in wood (Baechler, 1959). Alkaline treatment of wood also raises the pH of wood with increased resistance to the growth of brown-rot fungi (Highley, 1973). Different mechanisms have been recognized for wood decay caused by brown-rot and white-rot fungi (Green & Highley, 1995).

White - rot fungi

White rot decay has been divided into two groups. One group breaks down lignin and cellulose, or hemicelluloses, at the same time while the other group breaks down lignin first. Some white rot fungi are of the simultaneous type and are able to degrade the major wood cell wall structural polymers, lignin, hemicelluloses and cellulose and belong to a rather heterogeneous taxonomic group of organisms (Zabel & Morrell, 1992). Basidiomycetes and certain ascomycetes can cause white rot decay in wood (Ward *et al.* 2004). Degradation of lignin, cellulose and hemicelluloses are

common features for white rot fungi. However, the relative rates of degradation of lignin and cellulose vary depending on the fungal species and type of wood infected (Ward *et al*. 2004). White rot fungi can create different zones within the annual ring of the wood and this character specifically differs from brown rot fungal decay (Highley, 1999).

White rot decay is a multiple pattern wood decay. The wood decay pattern is based on the adaptation of white rot fungi to the much more heterogeneous structured wood of angiosperms, plus their ability to degrade all the cell wall constituents extensively. Two broad divisions are widely accepted according to their ability to selectively de-lignify with simultaneous rot (Rayner & Boddy, 1988; Zabel & Morrell, 1992). Oxidative processes are possible mechanisms involved in the degradation of lignin by white rot fungi involving phenol oxidases, such as laccase, tyrosinase and peroxidise (Szklarz *et al*. 1989; Grőngvist *et al*. 2005).

In general terms, wood tissues are made up from celluloses (polymer of D-glucose), hemicelluloses (xylans and mannans), lignins (polymers of phenylpropane), other polyaccharides and glycoporoteins. There polymers are laid down in close associations and the specific mix of neighboring components may influence the accessibility components for microbial enzyme degradation (Isaac, 1997). Cellulose is a major component of plant materials and is composed of microfibrils (chains of molecules complexed together). It is a polymer of D-linked glucose and is hydrolyed by the action of cellulases. In this process, cellulose fibrils are degradaed by a series of enzyme reactions (Isaac, 1997) in Fig 1. 7.

In order to degrade lignin, the white-rot fungi have developed an unspecific ligninolytic system consisting of peroxidises and laccases (phenol oxidases; LAC), which degrade lignin in an oxidative process (Hatakka, 1994) in Fig 1. 8.

Fig. 1. 8. Structure of lignin (reproduced with permission from "Real-World Cases in Green Chemistry," copyright 2000 the American Chemical Society).

White rot fungi degrade the cellulose less severely than brown rot fungi because their cellulolytic enzymes attack the molecules only from the ends and split off glucose or cellobiose units (Highley & Dashek, 1998). At the initial stage of decay lignin is broken down more than hemicelluloses or cellulose in a selective delignification process (Highley & Dashek, 1998). Usually hyphae penetrate the cells and delignify the middle lamella initially, however, in a selective delignification process the lignin is dissolved out of the adjacent cell wall due to the hyphae growth in the cell wall (Schwarze & Fink, 1998).

The rates of cellulose degradation are slower than in brown rot fungi and result in a slow loss of strength of the wood. This could be due to two reasons, firstly the result of glucose and cellobiose split off from the ends of the cellulose. A second reason could be the longer persistence of radial structure in the S_2 layer which is responsible for the strength of wood.

Simultaneous white rot decay by fungi occurs mostly in angiosperms and rarely appears in gymnospermous wood (Stubblefield & Taylor, 1986). The extreme flexibility of tracheid S_3 layer is the main reason for this selection process (Gartner, 2006). This character mainly distinguishes white and brown rot decay where the enzymes involved in the selective de-lignification and low molecular weight substances are easily diffused through the S_3 layer into the secondary wall (Schmidt, 2006). The resistant nature of S_3 layer in conifer tracheids is greater than the wood fibres in angiosperms.

The metabolites secreted from brown rot fungi can easily diffuse into the cell wall and cause degradation. However, for simultaneous rot, the S_3 layer is a considerable barrier. This validates the reason for appearance of this kind of decay type in angiospermous wood while gymnospermous wood is predominantly attacked by brown rot fungi (Schmidt, 2006).

Brown - rot fungi

Brown rot fungi generally decompose the cell wall and attack cellulose along with hemicelluloses in the same ratio without extensively changing the lignin. The hyphae are not readily evident on the surface of the wood. In nature coniferous trees are found to be most frequently decayed by brown rot fungi. In the early stages of degradation the fungi de-polymerize cellulose faster than the degradation products are utilized. The brown rot fungi begin the degradation process from the cell lumen and they then first degrade the S_2 and, later the S_1 wall as shown in Figures 1. 2 - 1. 6. This is consistent with the hypothesis that the initial depolymerization of cellulose characteristic of brown rot, is caused by a diffusible agent (Douglas *et al.* 1991). Brown rot fungi are mostly considered as very damaging towards wood (Schmidt, 2007). Brown rot fungi are unique in character among all other decay fungi and directly degrade the cellulose and hemicelluloses without removing lignin (Green & Highley, 1997).

The degradation of cellulose and hemicelluloses occurs in different stages, probably involving the penetration of hydrogen peroxide into the wood cell wall along with iron ions (Goodell *et al.* 1997). This decay mechanism also includes the action of various cellulose degrading enzymes; the major enzymes involved in the decomposition of wood are cellulases, ligninases, phenol oxidases, and lignin peroxidises, manganese oxidases, laccases and other oxidases (Cullen & Kersten, 1992; Andre & Marzull, 1997; Grőnqvist *et al.* 2004). The degradation mechanism originates from the degrading of hemicelluloses surrounding cellulose followed by the degradation of big chain cellulose molecules to small fragments (Highley & Kirk, 1979).

The actual loss of strength of the wood occurs only after the degradation of cellulose molecules. The initial loss of strength of wood is based on the degradation of hemicelluloses because the establishment of cellulose degradation at an early stage

is not easily determined (Highley & Kirk, 1979).

In addition production of hydrogen peroxide, production of oxygen free radicals, oxalic acid metabolism and the role of metals all play vital roles in brown rot decay (Evans & Hedger, 2001). Two chronological mechanisms are involved in the degradation of cellulose by brown rot fungi, these are oxidation and hydrolysis (Evans & Hedger, 2001). These two reactions seem to be tightly coupled reactions. Oxidation is most likely non-enzymatic with hydrolysis catalyzed by a complex of enzymes (Ritschkoff, 1996).

The low molecular weight compounds produced by brown rot fungi are believed to directly generate oxygen radicals or to initiate a chain reaction resulting in their production (Ritschkoff, 1996). Under the specific cultural conditions, such as nitrogen and carbohydrate limitation, brown rot fungi are able to produce more extracellular oxidative materials (Ritschkoff, 1996). The hydroxyl or other oxygen radicals are generated as a result of an oxidative mechanism. If hydrogen peroxide is the substrate for hydroxyl radical production it is most likely to be produced in small amounts at an early stage of hyphal growth. It is quickly scavenged and does not accumulate. The association between the cellulolytic enzymes and hyphal sheath components could also facilitate the hydrolysis of wood cellulose (Highley & Illman, 1991).

Soft - rot fungi

Soft rot has been defined anatomically as the spiralling growth of fungal hyphae within the S_2 layer of a wood cell wall and is caused by fungi belonging to the ascomycota and the deuteromycota (Savory, 1954; Hale & Eaton, 1985; Hale & Eaton, 1985a) and result in a softened layer of wood on all exposed surfaces, the damage progressively increasing in depth at a very slow rate (Richardson, 1998).

There is a softening of the surface layer of the wood when it is attacked by this group of fungi. Soft rot differs from other types of decay in several respects. It is caused by a different group of fungi, the physical and chemical character of its attack on the wood cells differs, and prevalently only the outer wood is severely damaged. Soft rot typically results in secondary wall cavities which when observed in transverse section the decay appears as numerous small holes of varying diameter within the secondary walls. In advanced stages of soft rot most of the secondary wall is removed and only the remnants of the outer secondary wall and the middle lamella between cells remains (Blanchette *et al*. 2004). Soft rot fungi have been reported to cause lignin degradation but with differences in degradation between hard and soft woods (Savory & Pinion, 1958; Hale & Eaton, 1985). Soft rot fungal decay generates more significant economic loss in wood industries compared to other types of fungal decay in wood (Pataky, 1999).

The preservative treated woods (e.g chromated copper arsenate (CCA) treated woods blocks) can also become infected by soft rot fungi, especially by deuteromycetes and ascomycetes (Schmidt, 2006). When the preserved wood blocks which have had contact with soil containing soft rot fungi there is great economic loss in preservative treated wood blocks and buildings constructed by wooden blocks (Schmidt, 2006). Microscopic studies are useful for the detection of soft rot fungal decay in wood (Schmidt, 2006).

Cavity formation helps in the detection of decay. Cavity formation commences when a hypha, lying in the wood cell lumen, makes a fine hyphal branch which penetrates through the S_3 layer and into the S_2 layer of the wood cell wall (Markham & Bazin, 1991). In the S_2 layer, the penetration hypha branches either bidirectional (T–branch) or bends in one direction (L-bending), with each branch growing parallel to cellulose microfibrils and enzymatic dissolution of the S_2 layer ensues (Markham & Bazin, 1991; Preston, 1979).

This enzymatic dissolution results in the formation of a diamond shaped cavity with conical ends (Preston, 1979). From the initial cavity further cavities may occur from lateral branches developing from the mature cavity or by a fine "proboscis hypha" which grows vertically from the tips of existing cavities (Hale & Eaton, 1985).

The SEM and TEM observation of cavity appearance shows that hyphae are normally associated with a variety of granular and fibrillar materials including extracellular slime (Table 1. 5), melanin and lignin break down products (Schmidt, 2006).

The residual materials will remain even after the death and lysis of cavity hyphae forming a skeleton in a highly degraded S_2 cell wall matrix. The Type 2 soft rot erosion causes a characteristic thinning of wood fibre walls from the cell lumen similar to that caused by white rot decay and higher ascomycete fungi like *Hypoxylon, Xylaria* and *Daldinia* (Rayner & Boddy, 1988, Zabel & Morrell, 1992).

Table 1. 5. Suggested possible functions for fungal slime layers.

Substrate recognition
Adhesion to and establishing contact
Covering the S_3 layer of the wood cell wall during the decay process
Conditioning of the substrate for decay
Modification of the extracellular ionic environment and pH-value
Transport vector for low - molecular decay agents and enzymes to the wood
Transport vector for degradation products to the hypha
Storage, concentration and retention of decay agents
Regulation of the decay process, e.g., by controlling the glucose level
Microenvironment for H_2O_2 maintenance needed for lignin degradation
Storage of nutrients
Permitting a film of liquid water to surround the wood cell wall
Protection of the mycelium against dehydration and adverse environmental conditions
Increase of surface area for aerobic respiration
Storage of copper or CCA from attack of impregnated wood

Wood preservation

The first successful preserving process by Kyan (1832) in which wood, rope, and canvas were soaked in dilute water solutions of mercuric chloride was used in the first treatment plant in the United States. Development of more effective preservatives demanded an explanation of how the chemicals protect wood from fungi. Bateman proposed in 1920 that any preservative must be soluble in water at least to the extent of producing a toxic water solution (Graham, 1973).

The biological problems relating to developing effective preservative systems for wood are often not completely recognized by the chemist and do not always relate to their chemical properties. Furthermore, biologists have in contrast always considered the relationships between the organisms and their environment. Therefore in studying wood decay ecological aspects should be considered so that a more complete understanding of the decay process is achieved (Dickinson, 1991). Such an approach to understanding the decay process will lead to the development of preservatives specifically designed for particular end uses. This should result in preservative systems which are better suited for specific ecological situations (Dickinson, 1991).

Wood decay fungi cause serious wood damage and create a significant economic loss to wood industries worldwide (Velmurugan *et al*. 2009). Many control strategies are used to inhibit fungal decay on wood in wood industries, including chemical and biological protection methods (Velmurugan *et al*. 2009). Several chemicals are used as preservatives to control fungal decay of wood. While using the chemical preservatives the nature of the chemical is extremely important because of potential hazardous problems to the environment. This validates the discovery of environmentally friendly wood preservative materials (Highley *et al*. 1994). Due to environmental restrictions the use of broad-spectrum biocides for wood preservation

is limited, mainly because of disposal problems (Green *et al*. 1997). Thus, there is an urgent need for the development of new, sharply targeted wood preservatives. Among major wood decay types brown-rot fungal decay is a major decay which results in considerable economical loss to the wood product industries (Kirkpatrick & Barnes, 2006).

New waterborne preservative-treated wood evaluated in relation to fungal decay of wood, including ACQ and CuAz, indicated that such treatments were effective in reducing wood decay. (Honglin *et al.* 2005). Wood composites should be protected against microbial and insect attack when used outdoors, especially in construction applications with prolonged exposure to moisture (Gardner *et al.* 2003).

The correct application of chemical treatment can protect wood from fungal decay and chemical treatment effectiveness mainly depends on the chemical formulation, method of application, moisture content of the wood, amount of preservative retained and depth of chemical penetration and distribution (Thomasson *et al.* 2006). Various kinds of methods are used for the application of preservatives in the wood industry and may include peeling, drying, conditioning, incising, cutting and framing (Chirra, 1995).

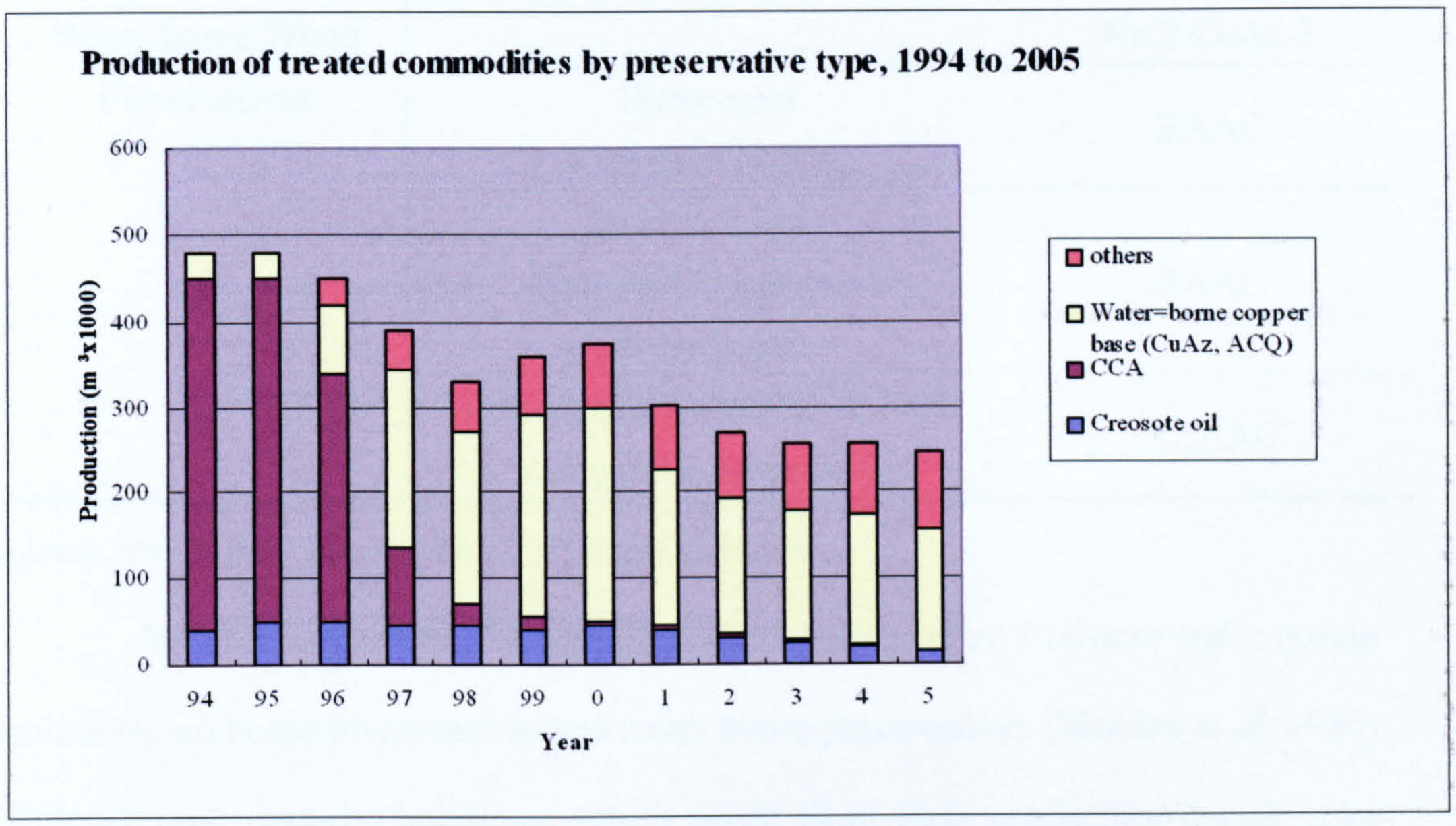
Environmentally-friendly wood preservatives

Wood preservation is a much more complicated process than is usually recognized and involves a wide range of chemicals which have been tested for this purpose. Wood products that are used under conditions that expose them to attack by decay fungi and insects are usually impregnated with the wood preservatives - creosote, pentachlorophenol, chromated copper arsenate (CCA) and ammoniacal copper arsenate (ACA) (Nicholas & Schultz, 1997). However, in due course treated products become unserviceable either as a result of mechanical damage or failure, biological deterioration, or obsolescence or are potentially very toxic (Nicholas & Schultz, 1997).

Currently the increased use of ammoniacal copper quaternary (ACQ) and copper azole (CuAz) as wood preservatives for residential construction has lead to concerns about the corrosion performance of fasteners (Zelinka & Rammer, 2006) but they are, however, considered to be environmentally friendly wood preservatives

(Peek *et al.* 1993). Reduction in wood decomposition using new treatments and corrosion protection of fasteners is becoming increasing important (Zelinka & Rammer, 2006). In North America oil based preservatives were most widely used during the first part of the 20 century but were later replaced by water based preservatives (Winandy, 1995).

Fig. 1. 9. Wood preservation in Japan, 2005.



Many Japanese companies were reluctant to use CCA-treated wood, due to the water pollution prevention regulations enforced by the government of Japan (1997). Thus, these companies treated wood with water-borne copper based preservatives (CuAz and ACQ). Details of preservative chemicals registered by the Japanese JIS K 1570 (2004) are given in Fig. 1.9.

Table 1. 7. Wood preservatives registered by JIS K 1570 (2004) for pressure treatment.

Category	Types	Abbreviation
Water-borne Wood Preservatives	Quat alkyl ammonium compound wood preservatives	No.1 AAC-1 No.2 AAC-2
	Ammoniacal copper quat wood preservatives	No.1 ACQ-1 No.2 ACQ-2
	Copper azole wood preservatives	No.1 CuAz-1 No.2 CuAz-2 No.3 CuAz-3
	Borne quat wood preservatives	BAAC
	Quat non-ester Pyrethroid compound Preservative	BAAC
	Azole quat non-ester pyrethroid compound preservative	AZAAC

notes: non-copper based (BAAC), Quat-Quaternary.

Wood preservatives mainly fall into three categories: Creosote and creosote solutions, oil borne preservatives and water borne preservatives (Madden *et al.* 1984). Creosote and creosote solutions, which result from their production during coke production from bituminous coal are widely used as preservatives for products such as railroad ties, large timbers, fence posts, poles and pilings (Hunter & Rossouw, 2005). The major advantage of creosote is that it is highly toxic to wood-destroying fungi, insects, and marine borers, low volatility, insolubility in water, ease of handling and application (Kimmel *et al.* 1994). The disadvantages of creosote solutions are that they are dark in colour, have a strong odour, are oily, result in an un-paintable surface, and have a tendency to bleed or exude from the wood surface and should not be used in homes or other living areas because of toxic fumes (Thomasson *et al.* 2006). The second major preservative group of oil borne preservatives is generally insoluble in water. They are usually dissolved in petroleum

or other organic solvents in order to penetrate wood (Loferski, 2000).

Research developments have recently made available oil borne preservatives formulated as water in oil emulsions or dispersions in water (Loferski, 2000). The major advantages of these preservatives are that they are toxic to fungi and insects, and can be dissolved in oils, have a wide range of viscosity, vapour pressure and colour, low solubility and can be glued depending on the diluents or carrier and also have ease of handling and use (Roll, 2003). The major disadvantages of these preservatives include an oily, un-paintable surface; depending on the carrier. For some applications these provide somewhat less protection to wood than creosote. Furthermore, they should not be used in homes or other living areas because of fumes which are toxic and irritating to plants, animals and humans (Roll, 2003). Pentachlorophenol is the most commonly used oil borne preservative. It is used commercially to treat poles, lumber, crossarms, timbers, and fence posts (Ormrod & Dalfsen, 1993).

The third types of wood preservative are the water borne preservatives which includes various metallic salts and other compounds (Freeman & McIntyre, 2008). The principal compounds used are combinations of copper, chromium, arsenic and fluoride (Freeman & McIntyre, 2008). Water borne preservatives have gained increasingly wider usage for lumber, plywood, fence posts, poles, pilings and timbers (Hunter & Rossouw, 2005). The major advantages of these preservatives are that treatment presents no hazard from fire or explosion, the wood surface is left clean, paintable and free of objectionable odours, safe for interior use and treatment of play ground equipment and they are leach resistant (Roll, 2003). The disadvantages of water borne preservatives are as follows: the wood is subject to warping and cracking (unless re-dried after treatment) and they do not protect the wood from excessive weathering (Freeman & McIntyre, 2008).

Most water-borne wood preservatives in commercial use are based on the

readily available and cost effective biocide copper (Preston & Jin, 1991). Copper-based wood preservatives, such as ammoniacal copper quaternary (ACQ) and copper azole (CuAz) compounds, have commonly been used for pressure treatment of wood for building construction but some decay fungi are known to be copper tolerant. CuAz and ACQ have been used in treatment of wood blocks in a reduced-pressure method to test their treatable potential for use as environmentally friendly wood preservatives with okara-based wood preservative and can protect wood against fungal attack as effectively as CuAz (Ahn *et al*. 2008).

Copper-based preservatives have been widely and successfully used for more than a century (Humar *et al*. 2001) because copper exhibits good biocidal activity (Nicholas & Schultz, 1997). A major requirement of any formulation of copper-based wood preservatives is efficacy against copper-tolerant fungi (Peek *et al*. 1993; Nicholas & Schultz, 1997). Several eastern hardwoods, such as red maple, are currently under-utilized for exterior structures as they are more susceptible to decay and therefore the development of the appropriate preservative treatment is necessary for new market opportunities in the timber industry. Smith *et al*. (1996) examined the efficacy of several preservatives against a brown-rot fungus; *Postia placenta* (Fr.) M. Lars. et Lomb, a filamentous basidiomycete, which rapidly depolymerize the cellulose in wood without significant lignin removal. This type of decay differs sharply from that caused by white rot fungi such as *Phanerochaete chrysosporium* which simultaneously degrade lignin and cellulose. Both white and brown rot fungi are common inhabitants of forest litter where they play an important role in carbon cycling. A white-rot fungus *Trametes versicolor* and a soft-rot fungus *Chaetomium globosum* Kunze ex Fries, a dematiaceous filamentous fungus found in soil, air and on plant debris, are common in nature as well as being wood decay fungi. *Chaetomiun* spp, are also encountered as causative agents of infections in humans (Guarro *et al*. 1999). Some species are thermophilic and neurotropic in natural.

Extensive decay is caused by *T. versicolor* but less than with *C. globosum* and *P. placenta* (Smith *et al.* 1996). Substantial differences in resistance to decay were found between heartwood and sapwood (Smith *et al.* 1996).

Most wood preservatives contain copper because of its fungicidal characteristics but copper is toxic at low concentrations to aquatic organisms. Therefore it is important to balance the amount of toxicity to aquatic organisms resulting from the leaching of copper when compared to the leaching of other chemicals used in the treatment of wood. This is an important environmental issue when developing an effective but environmentally acceptable preservative and the amount of leaching of copper needs to be compared to the leaching of other environmentally acceptable preservatives when trying to meet society's need for environmentally safe technologies to increase the durability of wood against biodeterioration (Gardner *et al.* 2003).

Copper based new preservatives

Many products use ACQ and CuAz as preservatives for treatment of wood in residential construction and this has led to concerns about corrosion performance of fasteners. Information on the effects of these preservatives on fastener corrosion rate is limited although Simpson Strong Tie (2005) has published a technical bulletin indicating corrosion by CCA and recommended fastener types for a given environment and preservative. ACQ and CuAz have a higher percentage of copper than other preservatives and therefore there is greater concern for corrosion protection. The effect of new wood preservatives on corrosion and a critical review of test methods used to measure corrosion in wood is given in a review by Zelinka & Rammer (2005).

Interactive antagonism

Interactive antagonism appears to be widespread in wood destroying basidiomycetes. Interactions between decay wood fungi may be significant in determining their distribution, growth pattern, ecological roles within wood tissues and the most common type of wood-decaying higher fungi. The majority of wood blocks under decay demonstrated interspecific interactions and antagonism between the different fungi with the white-rot fungi responding to isolates of three S*cytalidium* species (Rayner & Todd, 1979; Cease *et al.* 1989; Boddy, 1999).

In general mycelial morphology changes during interactions between *T. versicolor* and the ascomycete *D. concentrica.* Interspecific interactions not only effect changes in fungal community composition but also in fungal community development and decomposition processes, and in relation to biological control to preventing establishment of wood decay fungi (Boddy, 1999). Rayner &Todd (1979) reported on mutual relationships in the competitive ability of some wood-decomposing fungi in the interactive antagonism tests on 3% malt agar, wood lengths and on naturally decomposing wood.

Most white-rot fungal experiments have used *T. versicolor* and *P. ostreatus* for environmental biodegradation and biotechnological examination. Baldrian (2004) examined the effects of interspecific interactions between white-rot fungi and microorganisms on laccase activity in cultures of *T. versicolor* and *P. ostreatus* which increased significantly after the introduction of soil microorganisms.

Fungal structure

Chitin

The cell walls of many fungi contain chitin, a polymer of *N*-acetyl-D-glucosamine (Forster, 1949; Kent & Whitehouse, 1955; Lezica & Quesada-Allué, 1990), and the chitin assay has been widely used for the estimation of the chitin content of fungi and its conversion to mycelial biomass (Blumenthal & Roseman, 1957; Swift, 1973; Braid & Line, 1981; Goldstein, 1981) in Fig. 1. 10.

Chemically, the fungal cell wall is 80 to 90 per cent polysaccharide with most of the remainder consisting of protein and lipid (Bartnicki-Garcia, 1968). The amount of fungal biomass in wood can be used to determine the amount of growth of fungi in wood, in lignin modification, and degree of degradation. Various methods have been used to quantify fungal biomass in wood (Nilsson & Bjurman, 1998).

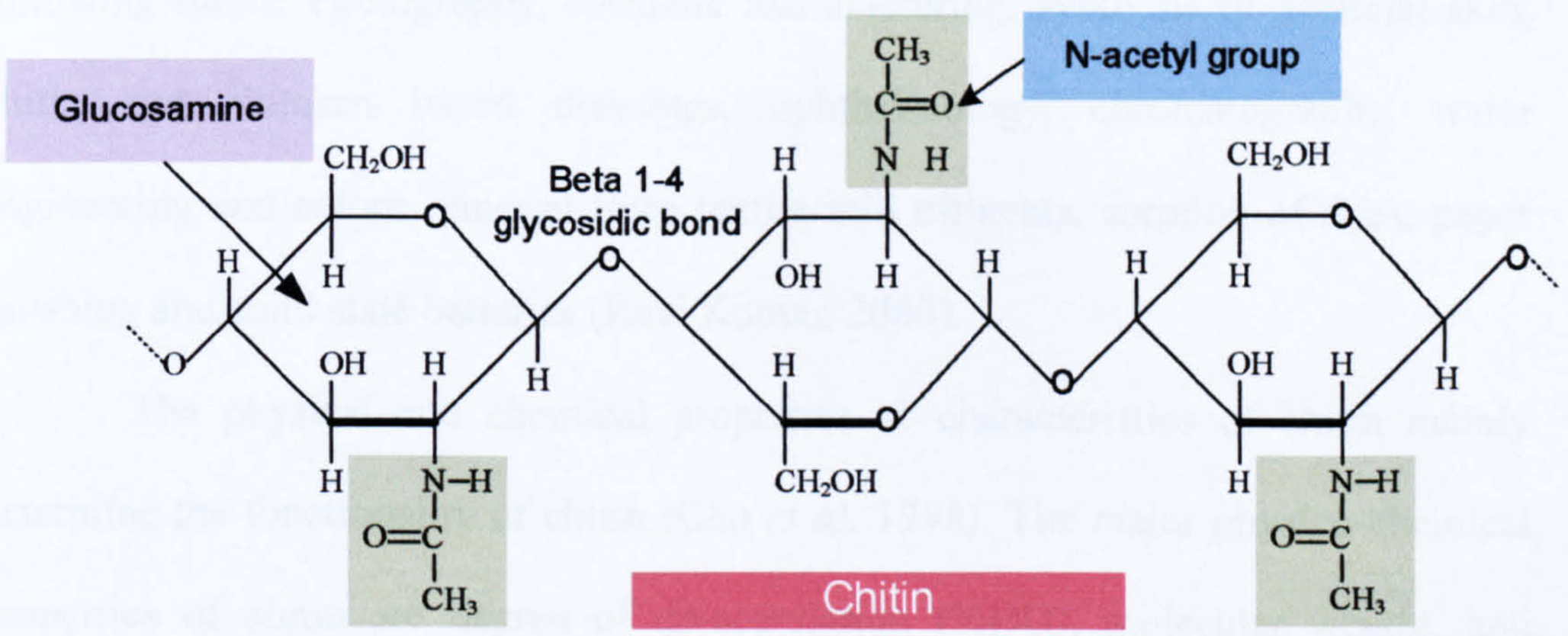


Fig. 1. 10. Structure of chitin

The chitin assay technique is one effective method to quantify the fungal biomass in degraded wood (Nilsson & Bjurman, 1998). Chitin is a highly hydrophobic and water insoluble natural resource material (Ravi Kumar, 1999).

Chitin is composed of an insoluble β (1-4)-linked polymer of N-acetyl glucosamine and is soluble in very dilute acids like acetic acid and formic acid (Synowiecki & Al-Khateeb, 2003). Chitin is the second most abundant polysaccharide in nature and a major constituent of cell walls of many fungi, insect exoskeletons and crustacean shells (Peter, 2002). Chitin is mainly used for chemical and pharmaceutical intermediates manufacturing and in the manufacturing of various food products (Ravi Kumar, 2000).

In terms of biomedical applications chitin acts as a good carrier to transfer various drugs and oligonucleotides to target cells in gene therapy. Binding capacity, cationic nature and biodegradability of chitin make possible chitin usage in drug delivery (Hasirci, 2007). Chitin can also be used for wound healing and burn treatment. Chitosan also has the capacity to reduce serum cholesterol level and to certain degrees, chitin has also been shown to stimulate the immune system (Andrade *et al.* 2003). In addition, chitin is a valuable resource commonly used in the following fields: Photography, cosmetic manufacturing, synthesis of artificial skin, chitin and chitosan based dressings, ophthalmology, chromatography, water engineering and colour removal from textile mill effluents, sorption of dyes, paper finishing and solid state batteries (Ravi Kumar, 2000).

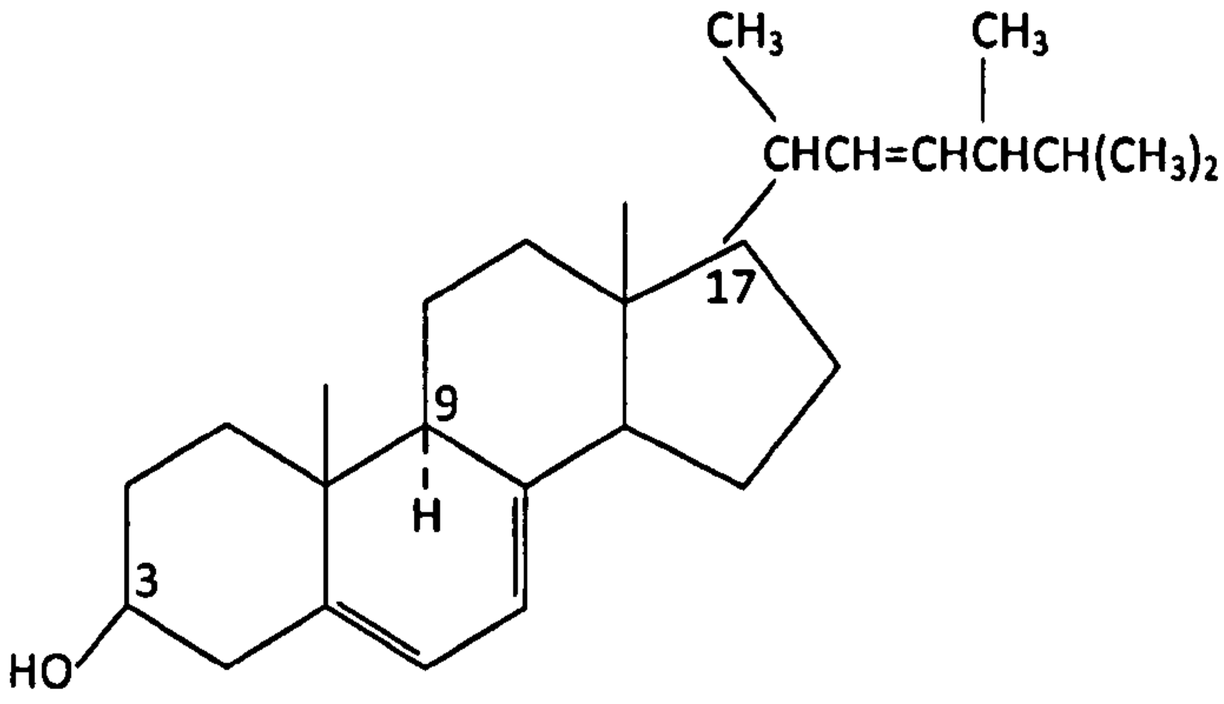
The physical and chemical properties or characteristics of chitin mainly determine the functionality of chitin (Cho *et al.* 1998). The major physico-chemical properties of chitin are degree of de-acetylation (%DD), molecular weight, and solvent and solution properties (Min *et al.* 2004). Besides, dry matter percentage, ash percentage, protein percentage, viscosity in centipoise and intrinsic viscosity are also general parameters for chitin and chitosan. Chitin de-acetylation degree is an important parameter and closely determines the function of specific chitin and directly denotes the purity of chitin (Min *et al.* 2004). The degree of de-acetylation can be determined by a number of analytical methods (Stevens, 2005).

There are several assay methodologies which have been developed specifically to determine the chitin and chitosan content in various materials (Khan *et al*. 2002). The major methods used for chitin determination are IR spectroscopy, ^{1}H NMR spectroscopy, ^{13}C solid state NMR (Chuen-How, 2005), thermal analysis, various titration schemes, acid hydrolysis, high pressure liquid chromatography, HPLC, acid hydrolysis-HPLC, separation spectrometry methods and infrared spectroscopy (Khan *et al*. 2002). The chitin content of freeze-dried and milled materials was successfully measured by HPLC according to (Ekblad & Näsholm, 1996; Ekblad *et al*. 1998). The condition of the acid hydrolysis may considerably affect the amount of hexosamine recoverable from chitin and other polysaccharides. Stronger acid but higher temperatures increase the yield but destruction of the hexosamine products may occur. Tests on purified chitin indicated that glucosamine yield is in the 70 - 80 per cent range under the hydrolysis conditions described from wood tissue decayed by fungi (Swift, 1972).

Many researchers have reported on the chitin content in basidiomycete fungi, especially *T. vesicolor*, *P. ostreatus* and *D. concentrica*. Mario *et al* (2008) reported the chitin content in *T. vesicolor, P. eryngii* and *P. ostreatus* etc Table 1. 8 below.

Ergosterol

Ergosterol concentrations vary widely among different fungal species (Antibus & Sinsabaugh, 1993; Sung *et al*. 1995; Pasanen *et al*. 1998). The ergosterol assay is commonly used to measure and estimate fungal biomass in wood (Mohebby *et al*. 2003; Niemenmaa *et al*. 2008). Basidiomycetous white-rot fungi are the only organisms that can efficiently degrade and mineralize polymeric lignin in wood (Hatakka, 2001) and ergosterol is a fungus-specific component of membranes (Weete, 1980).



Ergosterol

A precursor of

Vitamin D

Fig. 1. 11. Structure of ergosterol.

Ergosterol is a lipid and a prominent membrane component of most fungi and has been used as a fungal-indicator molecule in natural substrates as it is not found in native wood (Weete, 1980; Karen, 1997; Encinas & Daniel, 1999; Mohebby *et al*. 2003). Ergosterol is a crystalline sterol synthesized from sugars by yeasts and in filamentous fungi found as a major compound in fungal cell membranes and it also plays an important role in fungal growth (Pérez-Torrado *et al*. 2005). The free state form of ergosterol is mostly found in the phospholipid bilayer of the cell membrane and ergosterol shares the same functions as cholesterol in animal cells (Tabas, 2002). The chemical structure of ergosterol differs from cholesterol in having unsaturated carbon atoms at three positions and consisting of a methyl group at 28^{th} position instead of hydrogen atom at 24^{th} position (Cournia *et al*. 2007).

Major functions of ergosterol validate its importance in the field of research. Ergosterol is a main target for some antifungal agent (like amphotericin B), the mode of action of antifungal agent is mainly based on the binding capacity between the agent and ergosterol. Probably all antifungal agent developed based on attacking ergosterol first bind with fungal cell membrane ergosterol and create holes on the

fungal cell membrane, which generate the unusual passage and transfer of ions between the outer surface and inner part of the fungal cell. This unusual ionic transfer will kill the fungi (Young, 2003; Czub & Baginski, 2006).

Ergosterol can be used to determine fungal biomass in soil, mycorrhizas, forest litter, sediments, plant materials in contaminated building materials, and the indoor environment and in the detection of fungi in feedstuff, e.g, fungal contaminants of grains also cereals and it is found as a major component of edible mushrooms (Niemenmaa *et al*. 2008).

Quantification of fungal biomass based on ergosterol analysis is a multi-procedure method. The general techniques involved in the ergosterol assay in order are: sample extraction, saponification, partitioning, purification and determination of ergosterol by chromatography, usually HPLC (Manoharachary & Kunwar, 2002). Samples are usually extracted by using methanol and saponification is carried out with a petroleum ether extraction method (Noordkamp *et al*. 1997). Purification of compounds is commonly carried out on silica gel columns and purified compounds are analyzed by HPLC with a porasil column for analysis of ergosterol. However, different methodologies have been developed for ergosterol analysis (Nakamura, 1986).

In the common procedure the sample is first frozen by using liquid nitrogen followed by homogenization with a suitable solvent, usually ethanol centrifuged and the resultant supernatant is collected and saponification is carried out with either KOH, or alternatively hydroxide-ethyl alcohol-water the reaction mixture is then incubated in a boiling water bath (Gessner & Newell, 2002). The reaction is stopped by addition of neutralization solution, usually NaOH. Further extractions are carried out with various solvents e.g. hexane and the extracted product is partially purified by silica gel in the first purification stage with the second and final purifications carried out by HPLC (Still & Mansager, 1975; Barry *et al*. 1989; Wang *et al*. 2007).

Nowadays, GC-MS (Gas chromatography-Mass spectrometry) is mainly used to determine ergosterol (Magalhães *et al.* 2007). The presence of the elements Carbon, Hydrogen and Oxygen in ergosterol can be determined using an elemental analyzer. ANOVA analysis is commonly used as the statistical package for analysis of the final results. The extracted ergosterol can be stored at 4 °C for a week but is stable at -20 °C for 4 to 6 months without losing its activity (Cecilia *et al.* 2004).

Montgomery *et al* (2000) reported a range for total dry biomass and amounts of ergosterol from mycelial mats of fungal species harvested during the exponential and stationary growth phases. They reported 180-1648 µg ergosterol found in 53-445 mg of *Fusarium oxysporium* (Schlecht.: Fr.) ssp. *cannabis* biomass, 420-2104 µg ergosterol in 140-486 mg of *Trichoderma harzianum* Rifai, 663-2178 µg ergosterol in 199-595 mg of *Alternaria alternate* (Fr.) Keissl, 394-1024 µg ergosterol in 106-284 mg of *P. chrysogenum*, 229-675 µg ergosterol in 61-209 mg of *Chaetomium globosum* Kunze: Fr. and 87-345 µg ergosterol in 17-118 mg of *Rhizopus stolonifer* var (Montgomery *et al.* 2000).

Microscopy

Wood-degradation by fungi and other organisms can be observed by different methods of microscopy and these can be used to examine their relationships, determine their numbers, and measure the rates of physiological processes. Although wood has unmatched aesthetic qualities the most important attribute of wood is its mechanical properties. Thus techniques to follow changes in the wood are fundamental in the wood decay processes.

Many microscopic techniques have been used to understand the relationship between fungal growth in wood. The major microscopic techniques used in the field of wood microbiology are Light Microscopy, Scanning Electron Microscopy,

Transmission Electron Microscopy, Atomic Force Spectroscopy, Cryo Scanning Electron Microscopy, Environmental Scanning Electron Microscopy, and Dual Beam Scanning Electron Microscopy (Kaminskyj & Dahms, 2008).

The development of new images of fungal interactions in wood offers the possibility to address new answers to many questions in wood microbiology (Pringle & Taylor, 2002). However, technical complexity and instrument cost for many of these analytical imaging methods necessitates multidisciplinary experimental teams (Kaminskyj & Dahms, 2008). Microscopic techniques are providing a wide range of data to understand the surface structure, rigidity, chemical composition, and penetration of fungal hyphae of wood.

The establishment of new data abont fungal penetration of wood provides more information for a better understanding of wood-fungal interactions (Highley *et al.* 1994). Dual beam-cryo SEM-EDS is particularly suited to studying the wood-fungal interactions. Furthermore an exciting development for AFM is its integration with optical microscopes which allows the researcher to collect all of the data as available to AFM, while simultaneously tracking a specific target at high resolution (Kaminskyj & Dahms, 2008). This data can be combined to produce a multidimensional image embedded with chemical, biochemical, topographical and physical data (Kaminskyj & Dahms, 2008).

Fungal entry into wood may proceed through direct penetration via enzyme effects or mechanical pressure or indirectly through wounds or natural opening such stomata (Roustaee *et al.* 2000). Different tissues of wood may be penetrated differently by the same fungus. Each species of fungus may display different modes of penetration under different conditions (Roustaee *et al.* 2000).

Fungal degradation of lignocellulose is probably the most important process for recycling carbon in nature. The true nanostructure of the highly complexed biopolymer, lignocellulose is still unknown (Kluczek-Turpeinen, 2007). Under

aerobic conditions, some basidiomycete fungi are able to completely mineralize the wood lignin and wood polysaccharides to CO_2 and H_2O (Gutiérrez *et al.* 1995). The current detail on In-Situ decay is however relatively poor, particularly regarding the spatial distribution of enzymes and the non-enzymatic mechanisms involved in relation to the native wood structure (Daniel *et al.* 2004). The process is known to involve a range of physiological and biocatalytic activities during which the fungi colonize the wood structure and secrete a variety of hydrolytic and oxidative enzymes, as well as non-enzymatic steps (Daniel *et al.* 2004). Singh and Dawson (2006) examined the wood coating interface of saw-textured plywood using a range of microscopic techniques including light microscopy, confocal microscopy and scanning electron microscopy. They found that the surface tissues were greatly distorted with the applied coating closely conforming to the contours of the surface texture to produce a film of variable thickness. Scanning electron microscopy was used to evaluate the surfaces produced by different saw tooth types in normal and tension wood of maple (Vazquez-Cooz & Meyer, 2006) indicating that fuzzy grain in tension wood is responsible for increased power consumption by causing friction against the machining tool.

The earlier observations of weight loss caused by the growth of basidiomycetes in wood can be confirmed by microscopic techniques, including, light microscope, SEM, TEM, FS and Cryo-SEM. These studies can provide the information in the nature of degradation caused to cells/tissues, as well as describing cell to cell hyphal penetration. Observations using Cryo-SEM and FS can provide novel morphological details on the manner of fiber wall attack and component removal as well as on the spatial involvement (Kaminskyj & Dahms, 2008).

Light Microscopy

Wood anatomy, ultrastructure and wood microbiology research requires extensive background in all aspects of microscopy: light, scanning electron microscopy, transmission electron microscopy, video-light microscopy and image analysis (Anagnost, 1998; Hoppert, 2003).

The field of ultrastructure is very broad with applications in many biological, chemical and materials sciences, Applied to wood it emphasizes the light microscopic structures (smaller than 0.2 micrometers, 200nm) found in this natural material, either in the mature form or in its formative stages where various organelles of the living cell may be studied for their roles in producing the mature wood cells. The behaviour of wood in its many applications can be observed and explained via microscopy and related instrumentation such as EDXA (energy-dispersive x-ray analysis).

Light microscopy is briefly discussed by many researchers for its principles, advantages and applications (Hamm, 1948). Several studies have examined defect formation of wood on planed surfaces at the microscopical level (Hamm, 1948). Many researchers have investigated wood in all aspects using light microscopy as a tool, for example, Stehr and Östlund (2000) investigated crack formation in planed longitudinal wood surfaces of pine and found that cracks form at early wood/late wood boundaries exposed at the planed surface.

Scanning Electron Microscopy

Fungi producing white rot wood decay mainly belong to the basidiomycetes and they are particularly active in forest ecosystems. Hardwood is more susceptible to white rot fungal attack than softwood and untreated wood is more readily attacked than treated wood. The micromorphology is variable depending upon the multitude of, and, wood cell types attacked (Holmer *et al.* 1997; Daniel & Nilsson, 1998).

The structural organization of the wood cell wall is highly complex with few

materials having a unidirectional microstructure, and most fungal degradation patterns are distinctive and easily recognizable (Blanchette *et al.* 1990; Anagnost, 1998). However, it is not easy to distinguish between erosion type soft rot fungi and cell wall erosion by simultaneous white rotters. The tunnel within wood cell walls produced by hyphae of wood decay fungi (Singh & Wong, 1996) appears to be a unique form of fungal decay different from any of the well known fungal decay type (Kim & Singh, 2000). Soft rot fungi are particularly active under conditions that discourage the activity of white rot and brown rot fungi as well as in the presence of high preservative and moisture content.

The SEM has been used to observe degradation of cell wall components by a white rot fungi during decay of coniferous wood (Blanchette *et al.* 1978). A FE-SEM combination made it possible to obtain a visualized overview of the lignin distribution in various cell wall layers of the cell wall (Sarker *et al.* 2009). During the degradation of birch wood by the white rot fungi as *P. radiata* (*Phlebia radiate* Fr.) deterioration is across the cell wall through progressive delamination and removal of concentric orientated aggregates from the secondary S2 cell wall (Daniel *et al.* 2004).

Since commercial introduction of the scanning electron microscope in 1965 (Hollenberg & Erickson, 1973), wood anatomists have eagerly depicted the structure of wood with a clarity frequently lacking in micrographics obtained by other sources. As with the light microscope the complete picture of wood's anatomical structure is usually obtained from individual images of the transverse, radial, and tangential surfaces (Boonstra *et al.* 2006). In some cases, however, it is useful if all three surfaces of a single sample are displayed in one presentation. For example, the structure of the composite may be more readily visualized when the cellular elements are in proper spatial relationship with each other (Stoffler & Aebi, 2006).

The relationship between the quantitative characters of wood structure and habitat factors has received considerable attention in ecological wood anatomy (Lens

et al. 2008) However, because of the complicated roles that environmental factors play the data are far from sufficient to offer an overall understanding of this relationship (Sun & Lin, 1997). How fungal hyphae affect the quantitative characters of vessels is one of the most important problems in the wood microbiology field (Blanchette, 1984).

Fungal species (over 200) are the main cause of damage to objects of wood. Many fungal species have at least a partial celluloytic action (Lynd *et al.* 2002). The colonisation of cellulose substrata by specific groups of micro-organisms often poses more than a simple aesthetic problem (Pournou & Bogomolova, 2009). In fact, the microbial activity affects not only the strength of the wood, but often consists of a deep modification of the wood chemicals and physical structure (Blanchette, 2000). The study of fungal damage on wood using the SEM is the best tool to completely understand the mechanisms behind the damage (Pathan *et al.* 2008). The SEM has proved to be particularly useful because it allows direct observation of a non-conductive material and its chemical characterization without the need for surface metallization (Chen *et al.* 2006).

Numerous studies on fungal penetration and fungal damage on wood are based on the SEM. The main milestones in this field are as follows: weight loss and cell wall degradation in rubber wood caused by the sap stain fungus *Botryodioplodia theobromae* (Pat.) (Florence *et al.* 2002). SEM technique for three-dimensional structure of wood (Mcmillin, 1977), wood structure of *Aegiceras corniculatum* (Linnaeus) and its ecological adaptations to salinities (Sun & Lin, 1997), occurrence and possible role of endophytic fungi associated with seed pods of *Colophospernnum mopane* (Fabaceae) in Botswana (Jordan *et al.* 2006), SEM-FTIR spectroscopic evaluation of deterioration in a historic coffered ceiling (Genester & Palou, 2006). Ultrastructural studies of the mode of penetration by *Phoma macdonaldii* Boerema with in sunflower seedlings (Roustaee *et al.* 2000), Cryo-FE-SEM & TEM immuno-

techniques revealed new details on white-rot decay of lingo-cellulose (Daniel *et al.* 2004; Schwarze, 2007).

Atomic Force Microscopy

The first AFM was used in 1986 (Binning *et al.* 1986) to solve processing and material problems in a wide range of technologies affecting the electronics, telecommunications, biological, chemical, automotive, aerospace, and energy industries. The materials being investigating included thin and thick film coatings, ceramics, composites and biological membranes. In fact, without the breakthrough in tip manufacture, the AFM probably would have remained a curiosity in many research groups. New technologies are enabling industry to produce more advanced and nano scale-based science research. There are many variations of scanning probe microscopy, including AFM (Atomic Force Microscopy), electroachemical force microscopy and magnetic force microscopy that can have useful applications to microbiological systems.

Rodlets have been shown to occur on the spore surface of *P. chrysosporium* by AFM and comparsion of wall surface structures of both wild-type and mutant *Aspergillus nidulans* (Eidam) Wint. Var. offers insight into the events associated with fungal spore germination and wall remodelling (Ma *et al.* 2006). The nanoscale structure of softwood tracheid cell walls was determined by a combination of various preposition and different high resolution microscopy techniques.

Confocal Microscopy

Confocal microscopy is a valuable tool for examining wood structure and 3D construction of wood anatomy. Three-dimensional imaging of wood structure and wood based products has been carried out using a number of techniques. The three-dimensional morphology of wood tracheids can be briefly explained by confocal

microscopy. Tracheids are shown to have a characteristic shape composed of five different morphological zones. The visualization of the different morphological zones was accomplished with the use of computerized three dimensional (3D) reconstructions by confocal microscopy. 3D reconstructions could be generated from stacks of micrographs of wood blocks. The volumetric changes in relation to the hydration state of wood tracheid segments can be studied by confocal images. According to confocal images it is found that tracheid tips swell less than central regions and that as consequences of swelling the cell walls of wood tracheids expand inward towards the luman (Bardage *et al.* 2002).

Confocal microscopy has been used for 3D reconstruction of developing xylem (kitin *et al.* 2003; Möller *et al.* 2003), paper fibres (Jang *et al.* 1991; Jang *et al.* 1995) and wood structure in a range of species (Donaldson & Bond, 2005). Serial sectioning combined with light microscopy and scanning electron microscopy has also been used to construct virtual models of wood structure, individual spruce tracheids and pulp fibres (Bardage *et al.* 2002).

Confocal microscopy using safranine staining produced detailed images of tracheid distortion and damage but images were largely restricted to the outer surface of the wood (Donaldson *et al.* 2007). Because tracheids are folded and compressed to form a dense light absorbing surface it was not possible to image the internal structure of wood surface (Donaldson *et al.* 2007). Wood cell walls are good light absorbers (Donaldson & Lausberg, 1998) and this is apparently enhanced by the densification at the outer margin of the sawn surface. Wood individual tracheids had torn edges and were compressed into flaps of double cell wall that were then folded across each other horizontally (Donaldson *et al.* 2007). Wood small cell wall fragments filled the spaces between the folded flaps making a dense compact surface. Early wood was highly fibrous with the amount of roughness varying from place to place (Donaldson *et al.* 2007).

Three-dimensional reconstruction using either physical serial sections or confocal optical sections can produce virtual models of wood or fibre structure (Bardage, 2001; Bardage *et al.* 2002; Aronsson, 2002; Chinga *et al.* 2004; Donaldson & Bond, 2005). Such models can be represented as projections with perspective view (Aronsson, 2002; Chinga *et al.* 2004; Donaldson & Bond, 2005), as animations (Donaldson & Bond, 2005) or as interactive objectives suitable for display on web pages (Bardage, 2001).

The advantage over conventional 3D images provided by Scanning Electron Microscopy, for example (Core *et al.* 1979; Butterfield & Meylan, 1980), is the ability to view from any orientation and to digitally cut away parts of the structure to reveal internal details. Donaldson *et al.* (2007) reported that 3D images of wood sawn surfaces demonstrated the usefulness of 3D reconstruction in understanding the complex internal structure of the sawn surface. This confocal technique could be useful for other investigations of machined wood surfaces, decayed or weathered wood and for wood anatomical investigations (Bardage, 2001; Bardage *et al.* 2002).

Confocal microscopic computerized reconstruction and measurement of cross-sectional compactness has been used to analyze the collapse behaviour of fibers in a kraft-cooked fiber bundles containing early- and transition wood fibers. The confocal results showed that the collapsed behaviour of de-lignified fibers may be determined by fiber structure and dimensions and how these are affected by the action of external forces (Bardage *et al.* 2002). Nevertheless some deformation may also arise from internal stresses during drying of wood. Confocal images of cross-sectional compactness were shown to correlate with the collapse resistance of fiber walls (Bardage *et al.* 2002).

Bardage *et al.* (2002) reported that fibers with thin cell walls showed lower values of cross-sectional compactness, which seem to decrease towards the middle of the fiber and cross-sectional compactness seems to increase towards the fiber tips.

Bardage *et al.* (2002) reported that fiber ends may become flattened after delignification independently of high values of cross-sectional compactness. Computerized 3D reconstruction techniques may be a valuable tool in understanding the behaviour of collapse as wood fibers.

Aims and objectives

This study was undertaken to investigate the effects of more environmentally friendly copper based wood preservatives for the prevention of wood decay. The following aspects formed the focus for the study.

1. To isolate and culture three species of wood decay fungi, *Trametes versicolor*, *Pleurotus ostreatus* and *Daldinia concentrica*.
2. To treat wood blocks of Pine, Poplar and Cypress with ACQ (Alkaline copper quaternary) and CuAz (Copper azole) to determine their effectiveness in reducing wood decay.
3. To follow wood decay in treated and untreated wood blocks after inoculation with the test fungi and following a suitable incubation period. Weight loss, chitin content, ergosterol content and direct microscopical examination were used to assess levels of decay and therefore effectiveness of the preservatives.
4. To test treated and untreated wood blocks for decay following burial in a deciduous forest.

CHAPTER 2

MATERIALS AND METHODS

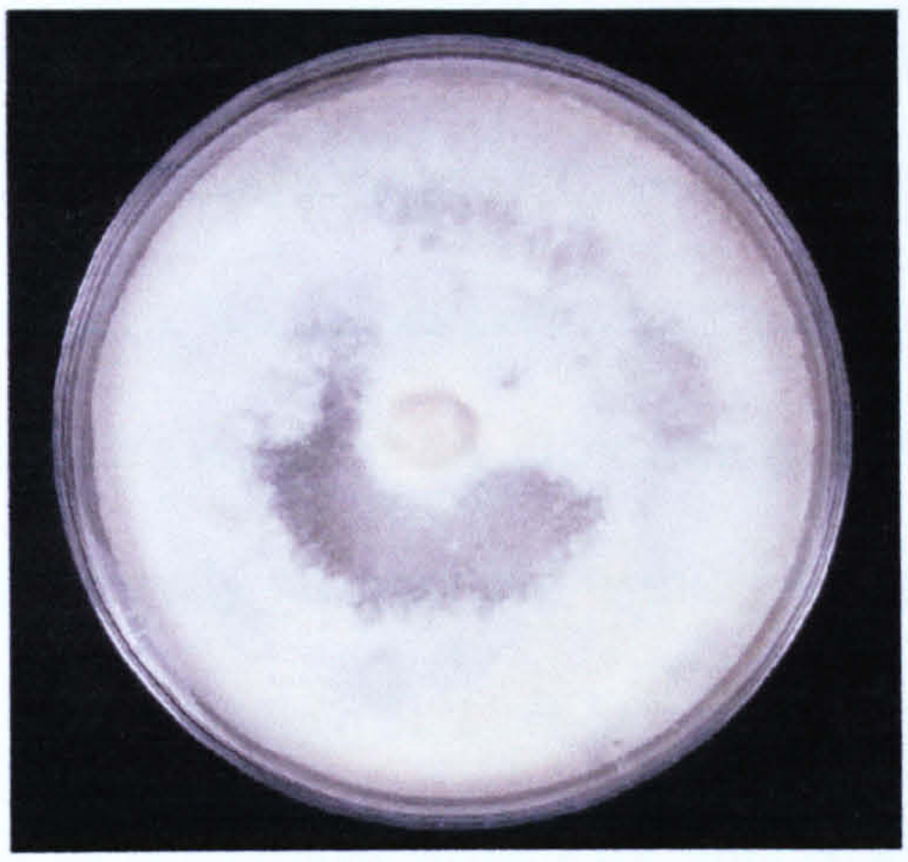
Anti-fungal efficacy of the Copper-based preservatives

The experimental protocols include collection, identification and culture of the three test wood decay fungi. This is followed by treatment of wood blocks for further experimentation.

Collection of wood samples and test fungi.

Wood samples and decay fungi were obtained in 2006 from the Forest Research Institute in South Korea. The sapwood specimens were Japanese Red Pine (*Pinus densiflora* S.et Z.), Yellow Poplar (*Liriodendron tulipifera* L.) and Bold Cypress (*Toxodium distichum* L. *Rich*) and the species of fungi tested were *Trametes versicolor* (KACC 26203), *Pleurotus ostreatus* (KACC 42310) and *Daldinia concentrica* (KCTC 26198). Figure 2. 1. Additional material and cultures of *T. versicolor* (HML1) and *D. concentrica* (HML2) were obtained from Ness Gardens, Wirral Merseyside and Northop Hall, Flintshire respectively.

Fig. 2. 1. Cultures and fructifications of the test fungi.

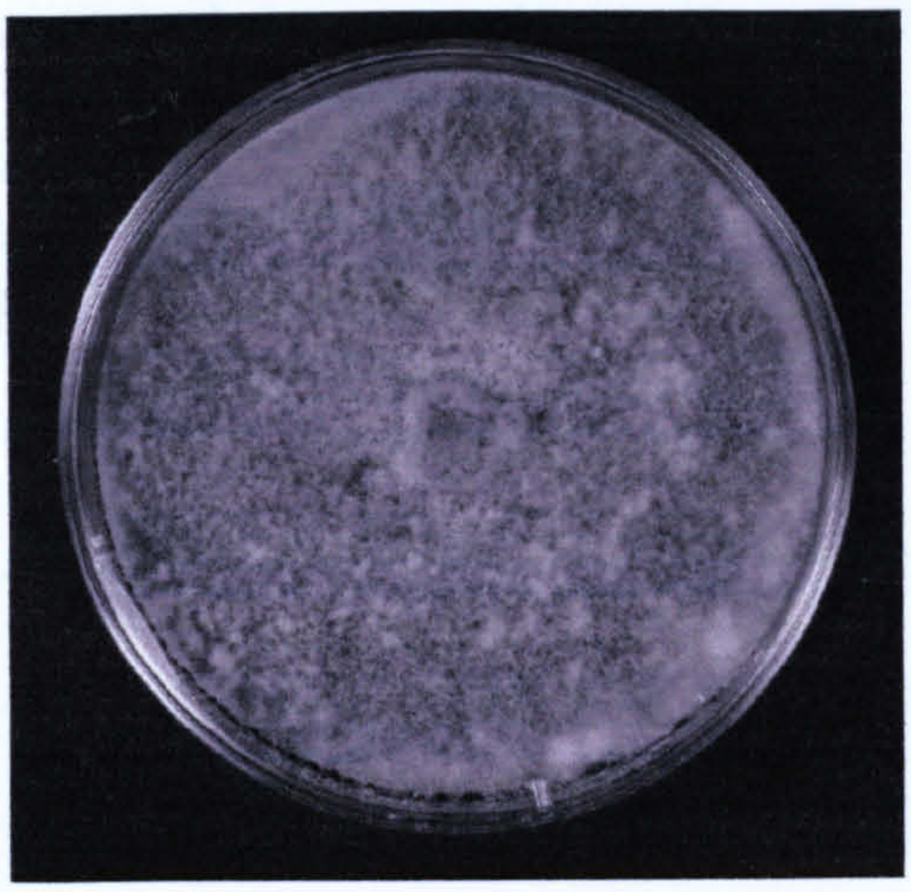


(a) Culture of *Trametes versicolor*

(b) Basidiomes of *Trametes versicolor*

(c) culture of *Pleurotus ostreatus*

(d) Basidiomes of *Pleurotus ostreatus*



(e) sporulating culture of *Daldinia concentrica*

(f) ascoma of *Daldinia concentria*

Trametes versicolor (L.) Pilat, formerly known as *Coriolus versicolor* Quel., is a common polypore fungus and the epiphet *versicolor* refers to its many coloured nature. Collections of *T. versicolor* from S. Korea and the UK display a variety of colours, which can even vary between basidiomes from the same trunk or stump. The species is one of the most commonly encountered polypores in Europe and is found practically all over the world (Gilbertson & Ryvarden, 1987; Hansen & Knudsen, 1997; Bougher, N. L. & Syme, K. 1998; Bernicchia, 2005).

This is a typical white - rot fungus appearing widely in the temperate regions where it usually grows on dead broadleaf trees or stumps and occasionally on coniferous trees.

The **Oyster mushroom,** or ***Pleurotus ostreatus*** (Jacq.). P. Kumm. is a common mushroom prized for its edibility. Long cultivated in Asia, it is now cultivated around the world for food. It is related to the similarly cultivated king oyster mushroom. Oyster mushrooms can also be used industrially for mycoremediation purposes (Moore & Chiu, 2001).

The fan like or oyster shape of the basidiomes, is characteristic hence its common English name, oyster fungus (Käärik, 1992). This fungus is saprotrophic on broadleaf trees and the optimal growing temperature is between 25℃ and 27℃. *Pleurotus ostreatus* has also been observed growing on chipboard wood in construction sites. It is also artificially cultured for food (Weil, 1983; Moore & Chiu, 2001).

Daldinia concentrica (Bolton) Ces. & De Not. is known by several common names, including King Alfred's Cakes, cramp balls and coal fungus (Dennis, 1981). It is generally widely distributed and is a common saprotroph on *Fraxinus* in the UK (Whalley & Watling, 1980).

Table 2. 1. An overview of experimental stages.

Wood species	Preservatives	Experimentation		
		Fungi		
		TRV	PLO	DAC
Pine (P)	Untreated CuAz ACQ	•Weight loss •Preservatives test •Interactive antagonism		
Poplar (Po)	Untreated CuAz ACQ	•Fungal biomass −Chitin-spectrophotometer −Ergosterol-HPLC		
Cypress (C)	Untreated CuAz ACQ	•Wood decomposition in soil •Microscopical examination −LM, SEM, AFM and confocal		

Preservative treatment of wood blocks.

Wood samples (2x2x1cm) were dried at 60 °C and the samples were impregnated with the test preservatives at 25℃ and 70 % humidity under vacuum for 30 min. After treatment, the moisture content was adjusted to 10-15 % w/w in a controlled environmental chamber and the samples were equilibrated at room temperature for 20 days. Preservatives used were copper azole (CuAz), and ammoniacal copper quaternary compound (ACQ).

Alkaline copper quaternary (ACQ, KS M 1701, 2005) is a preservative made up of a fungicide, copper, and quaternary ammonium compound (quat), an insecticide which also augments the fungicidal treatment (Zelinka & Rammer, 2006). Since it contains high levels of copper, ACQ-treated timber is five times more corrosive to common steel, according to the American Wood Preservers Association (AWPA) test results. It is therefore necessary to use double-galvanized or stainless steel fasteners in ACQ treated timber. Uses of fasteners which meet, or exceed, requirements for ASTM A 153 Class D are additional requirements for fastener durability. The U.S. began mandating the use of non-arsenic containing wood preservatives for virtually

all residential used timber in 2004 (So *et al.* 2006). The compositon of ACQ are copper (53-59 %) and N-D-metylbenzylammoniumchloride (41-47%).

Copper azole preservative (CuAz, KS M 1701, 2005) (CuAz denoted as CA-B under the American Wood Preservers Association standards) is the other major copper based wood preservative that has come into wide spread use in the USA, Europe, Japan and Australia following restrictions on chromated copper arsenate (CCA). The copper azole preservative is based on alkaline amine copper complex similar to that in ACQ but incorporates organic triazoles such as tebuconazole or propiconazole as the co-biocide (Freeman & McIntyre, 2008). The general appearance of wood treated with copper azole preservative is similar to that of wood treated with either CCA or ACQ (Lebow, 2004). The compositon of CuAz are copper (98.6-99 %), boron (40-46 %) and tebuconazole (1.6-2.4%).

Percentage absorption and retention of chemicals for weight loss.

The percentage absorption is based on the initial weight of the dry wood specimens and their final weight following treatment.

Absorption (%) = (Da - Do) x 100 / Do where Da = weight of specimens after each preservative treatment. Do = weight of dried specimens before treatment.

Preservative test for fungi resistance.

A preservative test was performed on the three fungi, TRV (*T. versicolor*), PLO (*P. ostreatus*) and DAC (*D. concentrica*) using the two preservatives, CuAz and ACQ. For each fungus, agar plates were centrally inoculated. The preservative was added to 6.0 mm filter paper discs as shown in Figure. 2. 2. At positions 12 and 6 0.2 % v/v was added and at 9 and 3 0.5% v/v was added. This was carried out for all concentrations up to 3 % v/v. Plates were then incubated at 25-27℃ checked weekly for up to 12 weeks.

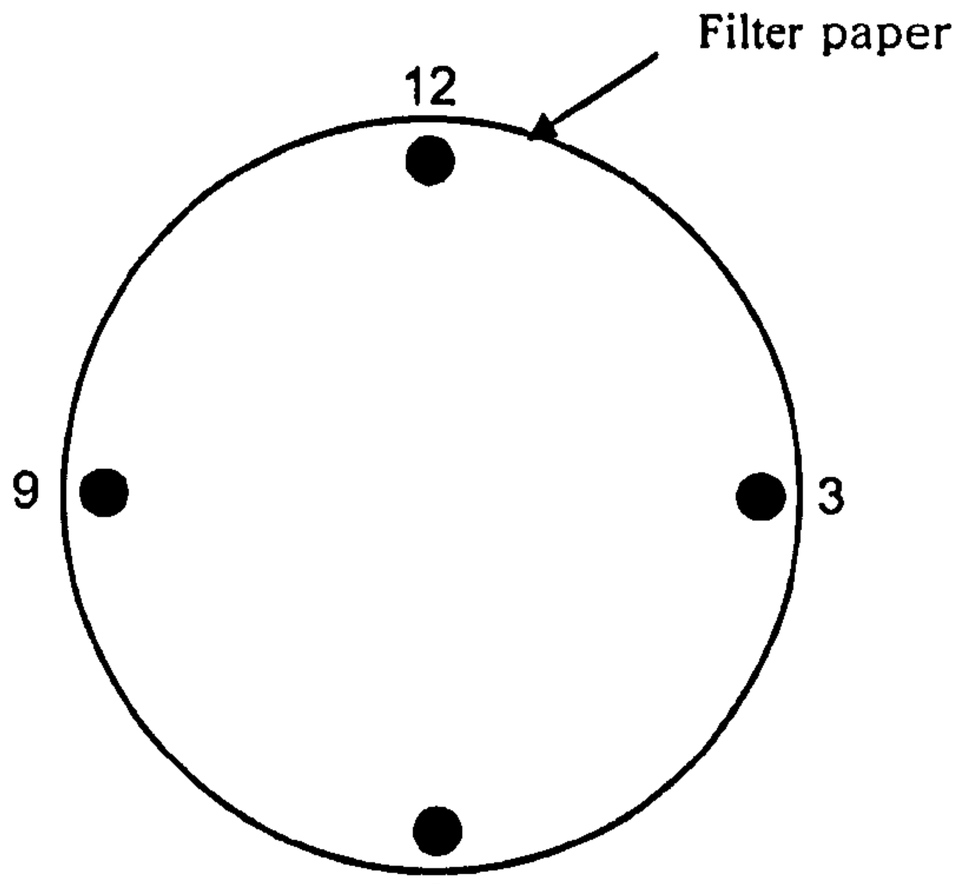


Fig. 2. 2 . A diagram of preservative test.

Anti-fungal activity test for wood after 60 days.

Each test fungus was cultured on 2 % malt extract agar medium. Five 1cm diameter agar plugs were removed from the growing margin of each of the plate cultures and transferred to sterile 1 L vessels (Fig. 2.2) containing 300 g of sand to which liquid malt extract medium (80 ml) was added. These were then inoculated with the test fungi and incubated at 25℃ (±3℃) in the laboratory under night and day light conditions. After 2-3 weeks incubation preservative-treated wood samples and untreated control wood blocks were placed on the surface of the sand cultures. After 60 days incubation at 70% humidity at 25℃ in a controlled environmental chamber (Jamison Door Co., Hagerstown, Maryland, USA) the wood samples were removed and dried at 60 °C for 24 hours. Weight loss as an index of decay resistance was determined as follows:

$$\text{Weight loss (\%)} = (Wo - Wd) \times 100 / Wo$$

Where, Wo = weight of sample before exposure to the fungus, Wd = weight of sample after number of days' exposure.

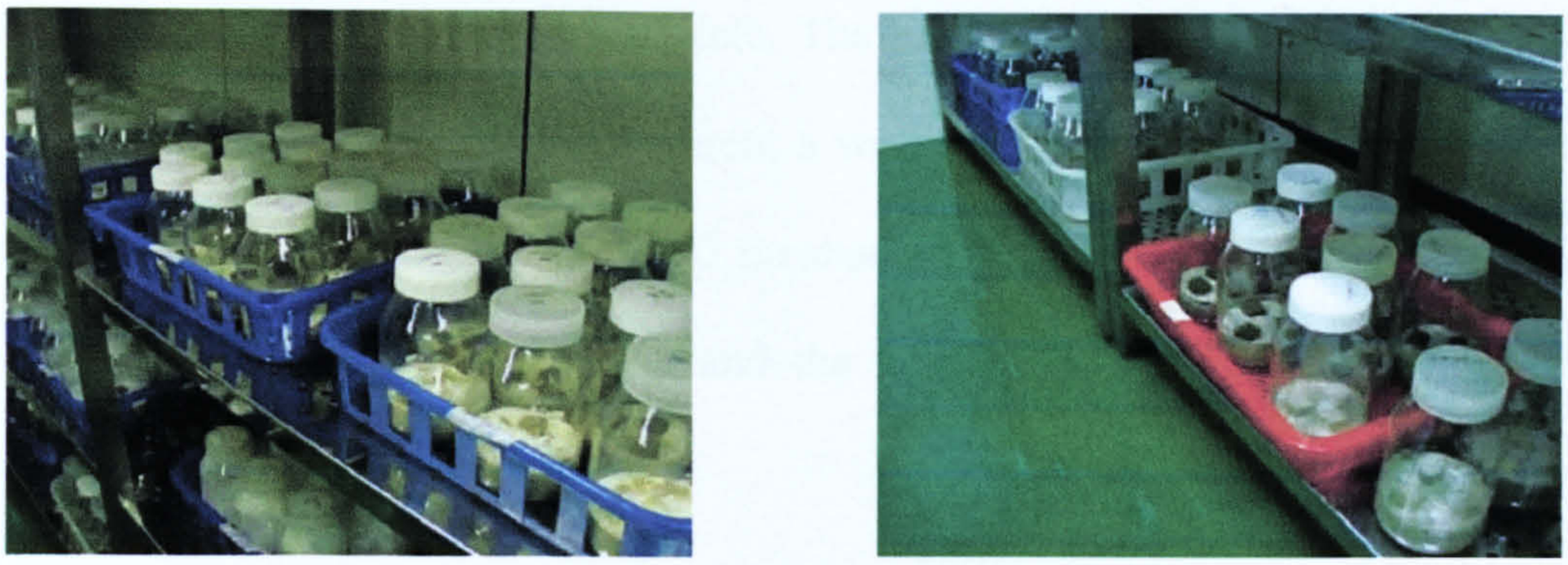


Fig. 2. 3. Sand culture vessels and wood block cultures inoculated with the test fungi.

Anti-fungal activity test for wood after 90 days.

Each test fungus was cultured on 2% malt extract agar medium. Five 1 cm diameter agar plugs were removed from the growing margin of each of the plate cultures and transferred to sterile 1 L vessels (Fig. 2.3) containing 300 g of sand to which liquid malt extract medium (80 ml) was added. These were then inoculated with the test fungi and incubated at 25℃ (±3℃) in the laboratory under night and day light conditions. After 2-3 weeks incubation preservative-treated wood samples and untreated control wood blocks were placed on the surface of the sand cultures. After 90 days incubation at 70 % humidity at 25℃ in a controlled environmental chamber (Jamison Door Co., Hagerstown, Maryland, USA) the wood samples were removed and dried at 60 °C for 24 hours. Weight loss as an index of decay resistance was determined as follows:

$$\text{Weight loss (\%)} = (Wo - Wd) \times 100 / Wo$$

Where, Wo = weight of sample before exposure to the fungus, Wd = weight of sample after number of days' exposure.

Interactive antagonism test of 3 fungi

2 % malt extract agar plates were divided into 3 sections. One fungus was inoculated onto each section of the plate. The plates were incubated for 3 days at 25 °C. When fungal growth was apparent a wood sample was placed in each Petri dish which were returned to the 25 °C incubator and checked weekly for up to 12 weeks. The interaction of the wood and the fungi was then determined and any antagonism between them was noted.

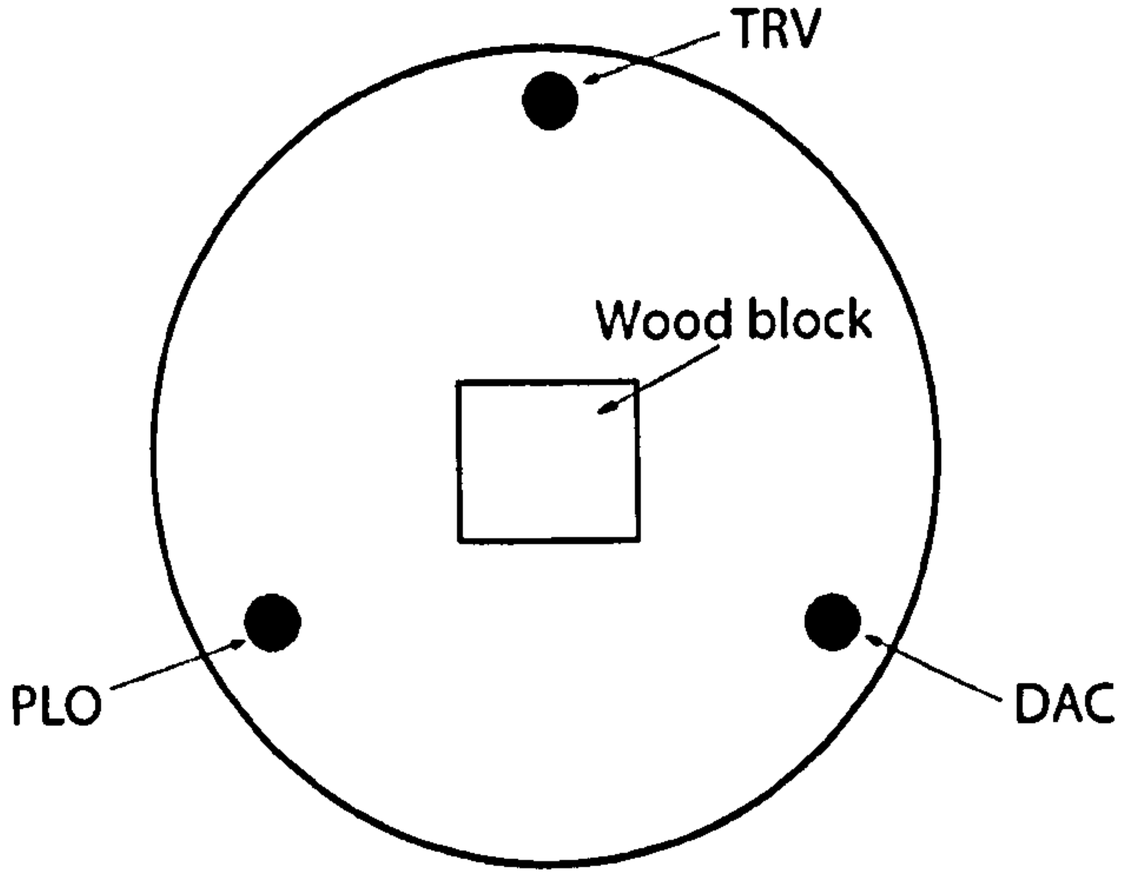


Fig. 2. 4. Testing interactions between fungi and the wood blocks.

Wood decomposition in the soil

The treated and non treated wood samples were buried in the litter layer at approximately 12-15 cm depth below the surface of a mixed deciduous wood in North Wales (Figure. 2.5). A total of nine different types of wood and each wood type contained 9 samples were buried. The 9 wood samples were placed in hair nets and secured before being buried in the ground approximately 12-15 cm for a 6 month incubation period. From each wood type a sample was viewed with a microscope Olympus SZ60. The weight loss of all samples was determined in Figure 2. 4.





Fig. 2. 5. Wood samples buried in Cilcain Hall wood in North Wales.

Chitin assays

Preparation of samples of cell wall chitin from the wood decay fungi.

Chitin assays were performed on samples following preservative treatment and anti-fungal activity tests. Three species of wood decay fungi were chemically analyzed for chitin by the Elson-Morgan method for glucosamine analysis. The white-rot basidiomycete fungi (*T. versicolor and P. ostreatus*) and the ascomycete fungus (*D. concentrica*) were used to detect hydrolysates and to determine the hydrolysis time of the cell wall to obtain the maximum yield of chitin.

0.1 g of each sample was refluxed at 100 °C for 4 hrs with 5 ml of 6M HCL in a reflux condenser. After cooling samples for 15 mins the supernatant was removed and filtered. The remaining wood powder was washed with distilled water and filtered to make a final volume of 50 ml for each sample. The pH of each sample was adjusted to pH 6 with sodium hydroxide solid, agucmol (46.8 %).

N-acetyl-glucosamine standards were prepared in 15 ml conical flasks with a concentration range of 0.05 ppm to 5 ppm for each sample. To these flasks, 2 ml of sample and 0.5 ml 4% acetylacetone were added. After voltexing, samples were cooled and 4 ml of 95% ethanol was added and voltexed and 0.25 ml of Ehrlich's reagent was then added followed by 0.5 ml of dimethylaminobenzaldehyde and samples were again voltexed.

Absorbance at 530 nm (Chen & Johnson, 1983) was read for each of the samples using a Jenway 6300 spectrophotometer.

Ergosterol assays

The ergosterol assay was similar to that previously described (Seitz *et al.* 1979). A modified ergosterol analysis method, including a simultaneous saponification and refluxing extraction procedure along with HPLC quantification, was used to measure fungal colonization rate in wood. Developing better methods for wood protection and bioconversion requires accurate measurement of fungal biomass and extracellular metabolites that are released during fungal growth in wood (Seitz *et al.* 1977; Seitz *et al.* 1979; Geo *et al.* 1993; Young & Games, 1993; Niemenmaa *et al.* 2008).

Samples of untreated yellow poplar and samples following preservative treatment were collected. Fungal inocula from *T. versicolor* and *P. ostreatus* were grown on 2% malt extract agar plates and the fungal strains used were kept in the storge medium. Sterilized fresh wood powder (2 g) and lyophilized dry mycelia (5 g) from liquid were extracted. The activated fungal inocula were added to 1 L of malt extract medium and adjusted to 5 L with distilled water. The mixture was heated for 6 hours at 60 °C. The resulting saccharized content was measured using an 11Brix (degrees brix: a unit of measurement of sucrose in a liquid) and the pH was adjusted to pH 6. To obtain a high quality fungal sample 5% of the culture each fungus was inoculated into 5 L of sterile malt extract medium, continuously provided with sterile air and incubated at 25 °C for 5 days before being lyophilized.

5 g of lyophilized mycelium and 2 g of wood powder were dissolved in 4 ml of 98% agucmol sodium ascorbate (v/v), 50 ml of 95% ethanol (v/v) and 10ml of 100% potassium hydroxide. The mixture was centrifuged at 3000 rpm at 4 °C for 20 mins. The supernatant was filtered through a 0.22 μm filter. The ergosterol was extracted from the filtered supernatant using a modified method of Mau (1998) and

Choi (2005). All reagents used were HPLC grade analar.

· For sample was mixed with 4 ml of sodium ascobate (98%), 50ml of ethanol (95%), 10ml of 50% KOH and 1ml of internal standard (100μg cholecalciferol (sigma Co.)/ml MeOH).

· The mixture was saponified at 78 °C for 1 hour was then partitioned with 15ml of deionized water (water phase), and 50ml of diethyl ether (organic phase).

· The water phase was then extracted 3 times with 10ml of potassium hydroxide with ethanol and 50 ml of *n*-pentane, and finally with 20 ml of *n*-pentane.

· The organic phase pool was washed 3 times with 50ml of KOH in ethanol, and washed with deionized water to neutrality.

· The organic layer was evaporated (N-N Japan) under vaccum conditions in the dark and redissolved in 3 ml of MeOH.

· Ergosterol was quantified with a HPLC analysis.

Table 2. 2. HPLC conditions for ergosterol analysis.

Instrument	HPLC (Sycam S2100)
Column	Grom-SIL 120 ODS-4HE (4x250 mm, I.d. 10 μm)
Mobile phase	Methanol/Acetonitrile (25/75; vol), 0.8 mL/min
Detection	UV, 264 nm
Oven temperature	30 °C

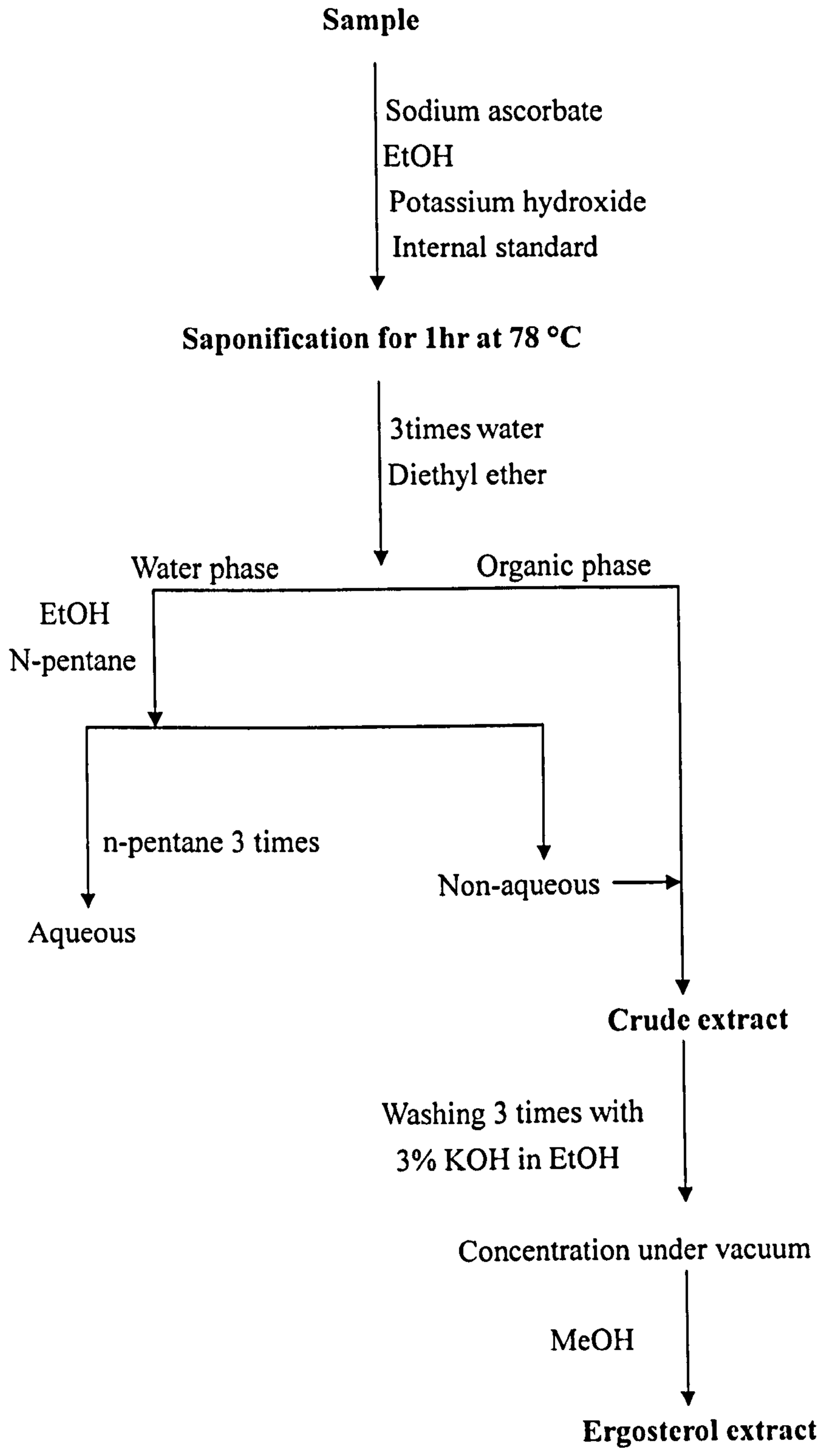


Fig. 2. 6. Ergosterol extraction methodology.

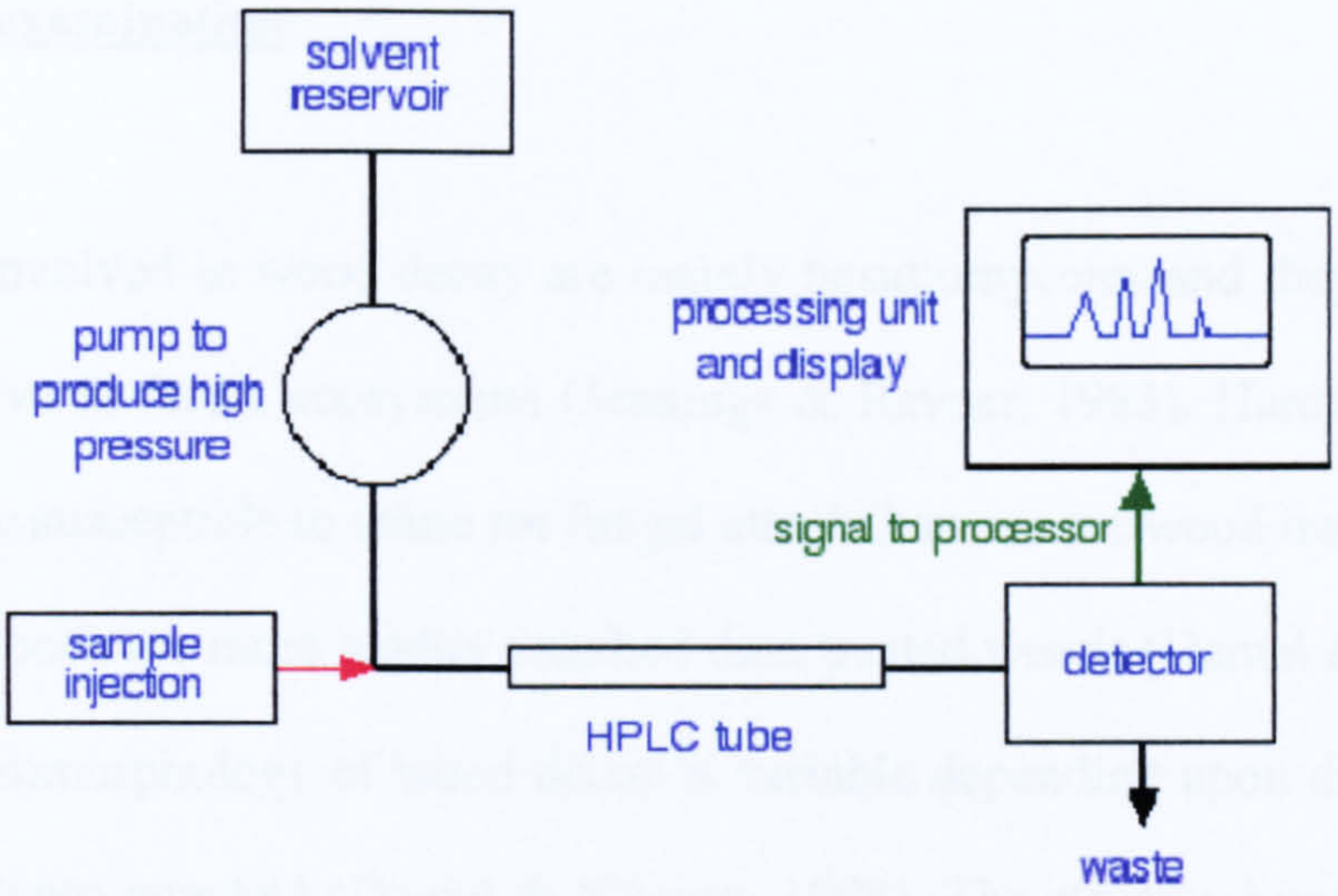


Fig. 2. 7. The components of an HPLC instrument.

Microscopical examination

Fungi involved in wood decay are mainly basidiomycetes and they are also particularly active in forest ecosystems (Jennings & Rayner, 1983). Hardwood tree species are more susceptible to white rot fungal attack than are softwood tree species, and untreated woods are more readily attacked than treated woods (Daniel & Nilsson, 1998). The micromorphology of wood decay is variable depending upon the density and wood cell types attacked (Daniel & Nilsson, 1998). The structural organization of the wood cell wall is highly complex and exhibits unidirectional microstructual hyphae of the fungus but most fungal degradation patterns are distinctive and are easily recognizable (Blanchette *et al.* 1990 & Anagnost, 1998). It is not always easy, however, to distinguish between the erosion type caused by soft rot fungi and cell wall erosion resulting from simultaneous white rot and soft rot although the tunnel within wood cell walls caused by hyphae of soft rot wood decay fungi appears to be a unique form of fungal decay type (Singh & Wong, 1996). Soft-rot fungi are particularly active under environmental conditions that discourage the activity of white and brown-rot fungi and also when there are high preservative and moisture contents of the timber (Daniel & Nilsson, 1998). It is therefore important to follow changes in the structure of different wood types by microscopic techniques and to compare the different patterns of rot caused by the different wood decay fungi and to assess the effect of different wood preservatives. The nature of the structure of native undecayed blocks of wood compared to wood blocks and preservative treated blocks inoculated with the test fungi incubated under controlled conditions was examined by light microscopy and with the scanning electron and atomic force microscopes.

The fungi tested were *T. versicolor*, *P. ostreatus*, and *D. concentrica* and the wood blocks inoculated were cut from Japanese Red Pine (*P. densiflora*), Yellow Poplar (*L. tulipifera*) and Bold Cypress (*T. distichum*). All the fungi for inoculation

were grown on 2% malt extract agar for 1 week at room temperature 25℃ to 27 ℃.

Light microscopy

Wood samples were selectively stained using a solution of phloroglucinol (4 g) in a mixture of alcohol (100 ml) and concentrated hydrochloric acid (50 ml). The solution once prepared was stable for several months when kept in the dark. This staining solution gives a bright red colour with mechanical wood or other lignified wood structures (Hoppert, 2003).

Sterilised circular coverslips 13 mm in diameter or square 18 x 18 mm (Chance Propper Ltd.) were then fixed to the floor of a Petri dish using small amounts of sterile agar. The coverslips were then overpoured with molten 2% malt agar and once it had solidified the agar covering the surface of the coverslips was removed using a flamed cork-borer or scalpel to remove a plug from above the coverslips. Plates with the circular coverslips were point inoculated at opposite positions. Sporulation can usually be observed by eye or with the aid of a binocular microscope and the agar surrounding the coverslips was then removed. The coverslips were then extracted from the plates and mounted directly onto microscope slides with lactophenol cotton blue (Nugent *et al.* 2006), and examined using an Olympus BH2 research microscope with images by a digital computer system.

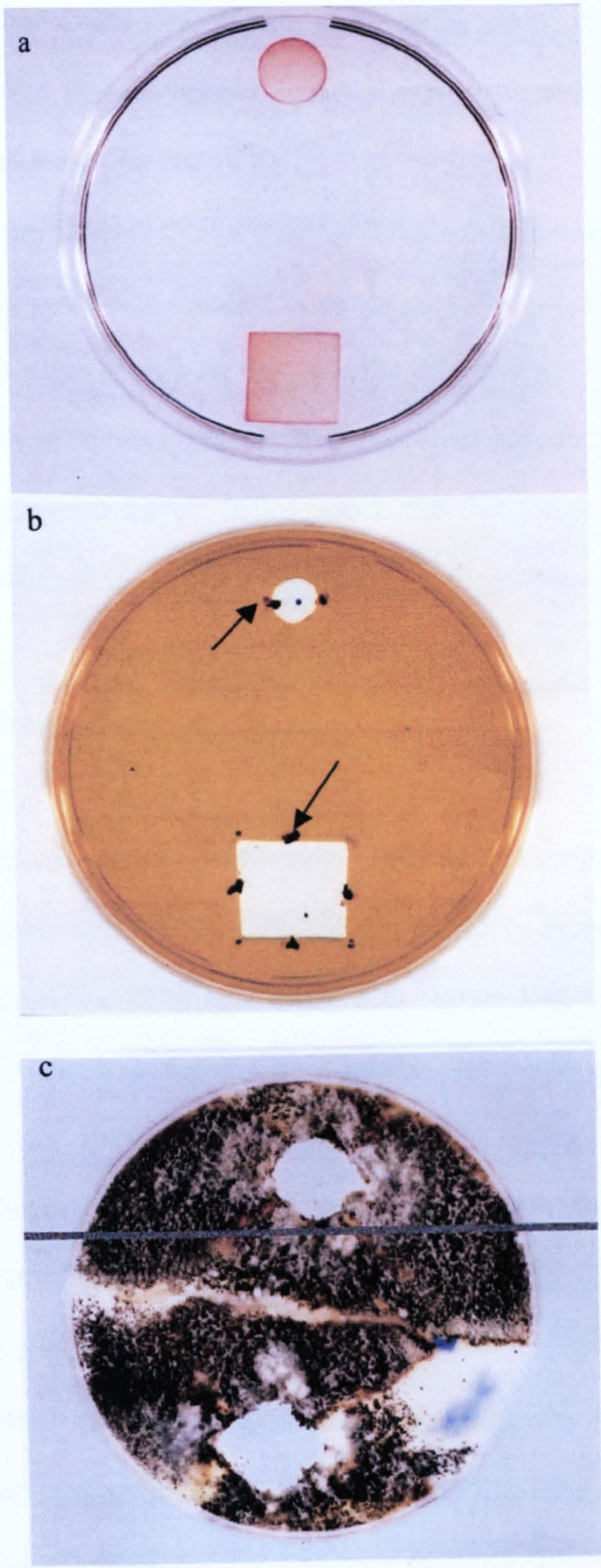


Fig. 2. 8. Coverslip culture technique.

a. Coverslips fixed to the floor of petri dishes using small amounts of agar, circular coverslip for SEM use and regular square coverslip for use with light microscope (agar has been coloured for clarity).

b. Agar was poured gently over the top of fixed coverslips and allowed to set. The agar is then cut away, using a scalpel or a cork borer for circular coverslip to create a wall above. The edges of well are then inoculated (arrowed).

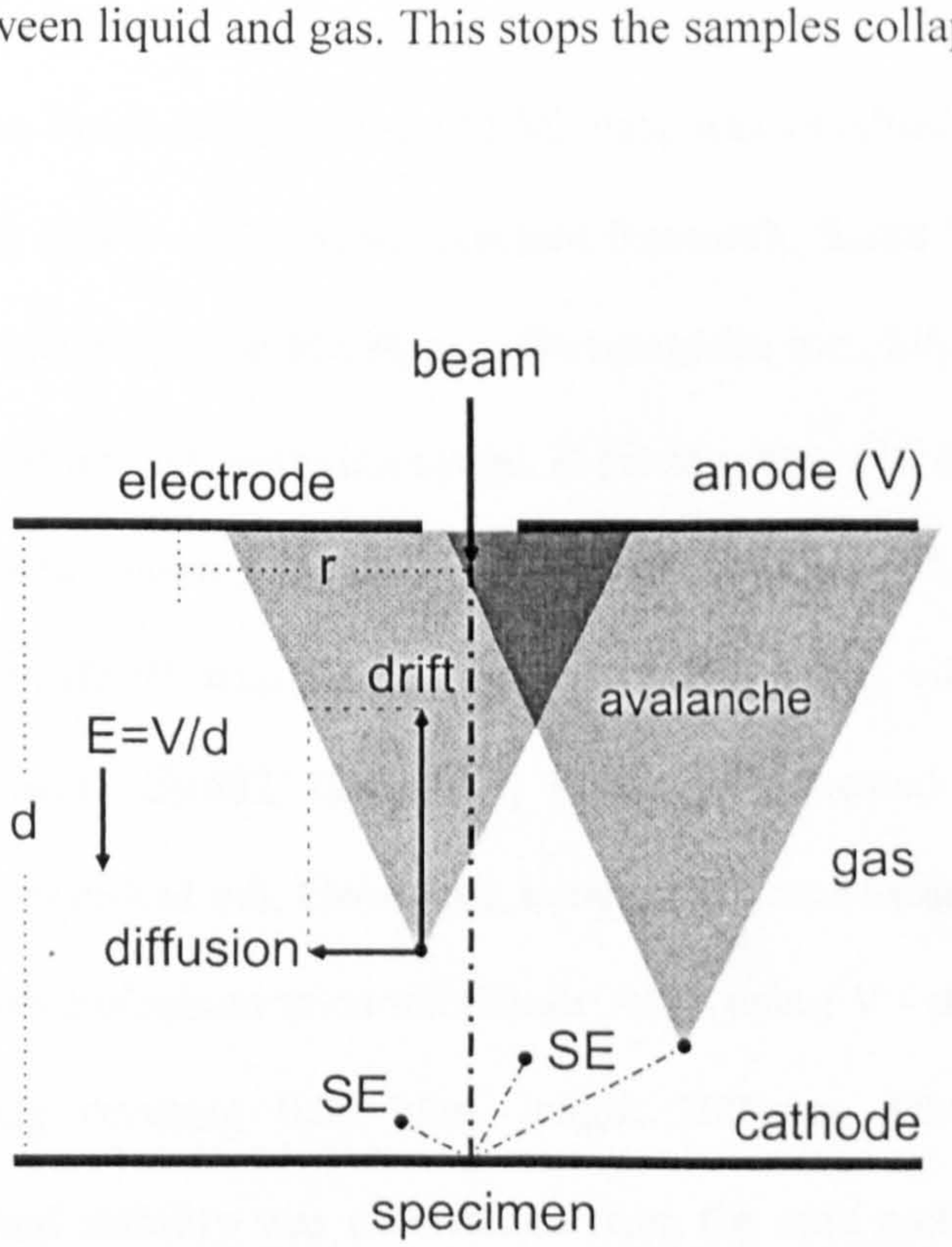
c. Shows growth of *D. concentrica* on the plate. Once growth is sufficient, the agar is cut away from around the coverslips which can then be removed and stained or prepared for examination.

Scanning electron microscopy

a. Circular glass cover slips on which the test fungi had grown on the surface using the modified cover slip culture technique developed by Nugent *et al.* (2006) were attached to aluminium stubs (pin type) with 'Silver Dag' conductive paint (Van Loenen Instruments). The samples were sputter-coated with gold using an Emitech K550X coating unit and examined using a JSM840 (Jeol UK Ltd) scanning electron microscope. Images were obtained using an image capture system (Oxford Instruments, INCA system, Oxford, UK).

b. The coating specimens then loaded into FEI (Quanta 200) ESEM (Environmental Scanning Electron Microscopy, 2008) for examination in Fig 2. 8 and Fig 2. 9. Specimens were also prepared by freezing in a Cryo-stage (Quorum technology) control unit before loaded into the ESEM. A chemical fixation is avoided; a cryo-SEM sample, rapidly frozen, is as close as possible to its natural state. No use of solvents, which can also remove sample components. No dehydration, delicate structures are maintained without shrinkage, Fast freezing means chemical balance is well maintained for microanalysis. Cryo-SEM is fast with a typical preparation time

of less than 10 minutes. The sample was mounted on to the appropriate sample holder and then plunge frozen, usually into "slushy" nitrogen. The sample holder was then withdrawn, under vacuum transfer device for transfer to the cryo-preparation chamber. After transfer to the (separately pumped) cryo-prep chamner the sample was maintained at a low temperature and low contamination conditions. Finally a thin conductive coating was usually applied to allow high resolution imaging or microanalysis in the SEM. Transfer to the SEM chamber was via an interlocked airlock and onto a cold stage module fitted to the SEM stage. Critical point drying (CPD) was also used by using acetone (100%) on sample to prepare for CPD. CPD uses liquid CO_2 to prevent collapse in ESEM. This is achieved by replacing acetone with liquid CO_2 and then the liquid CO_2 is taken to a critical temperature and pressure (34.5 °C and 1200 psi (pounds per square inch)). At these parameters, the shearing forces and surface tension on the samples are minimal and the CO_2 is in equilibrium between liquid and gas. This stops the samples collapsing whilst they are dried.



SE detection with GDD in ESEM - principle

Fig. 2. 9. ESEM gaseous detection device (GDD)-principle

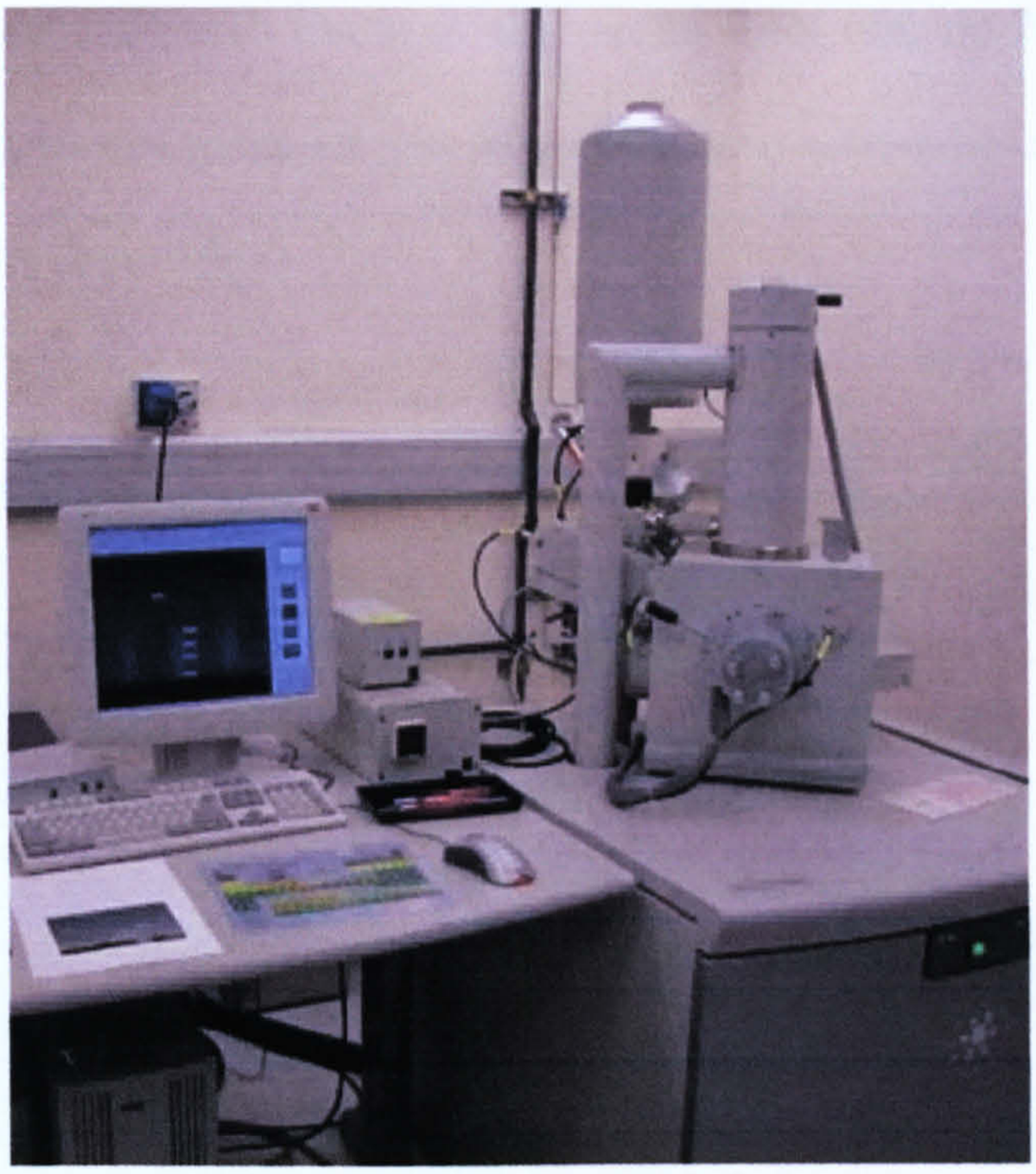


Fig. 2. 10. Environmental electron scanning microscope.

Atomic force microscopy

All of the Atomic Force Microscopy (AFM) data was obtained using a Molecular Force Probe - 3D (MFP - 3D) AFM (Asylum Research, Santa Barbara, California, USA) with software written in IGOR pro (Wavemetrics inc., USA). The MFP-3D is equipped with a 90 μm x-y scanning range, Z-piezo range > 16 μm and was coupled to an Olympus IX50 inverted optical (IQ) microscope equipped with a colour CCD camera. The MFP-3D-IO was placed upon a TS-150 active vibration table (HWL Scientific instruments GmbH, Germany), which was located inside an acoustic isolation enclosure (IGAM mb, Germany), to help eliminate external noise.

Images were obtained in contact mode AFM using V - shaped silicon nitride cantilevers (spring constant 0.02 N/m, length 200 μm, OMCL - TR400PS-1, Olympus). Thermal stability was determined from the sum and deflection meter in the MFP-30 software. Once stable, the optical lever sensitivity was determined on a bare region of the glass substrate from force versus distance curves taken at 1 Hz, (1

force curve per second) with the scan size set to 0μm (*i.e.* no XP tip movement). Integral and proportional gains set just below the point where the cantilever started to oscillate (Cappella *et al.* 1997).

AFM Operation & Design

Fig. 2. 11. Atomic force microscope.

The AFM consists of a cantilever with a sharp tip at its end. The tip is brought into close proximity of a sample surface. The force between the tip and the sample leads to a deflection of the cantilever according to Hooke's law. Typically, the deflection is measured using a laser spot reflected from the top of the cantilever. If the tip were scanned at consistent height, there would be a risk that the tip would collide with the surface, causing damage. Hence, in most cases a feedback mechanism is employed to adjust the tip-to-sample distance to keep the force between the tip and the sample consistent. This can be achieved by mounting the sample on a piezoelectric crystal. The tip is then scanned across the sample surface and the vertical displacement is necessary to maintain a consistent force on the tip is recorded. The resulting map of a (x, y) represents the topography of the sample. Over the years several modes of operation have been developed for the AFM. Especially,

the dynamic mode which generates lower lateral forces on the sample and is widely used to image biological samples (Fig. 2. 11 and Fig. 2. 12).

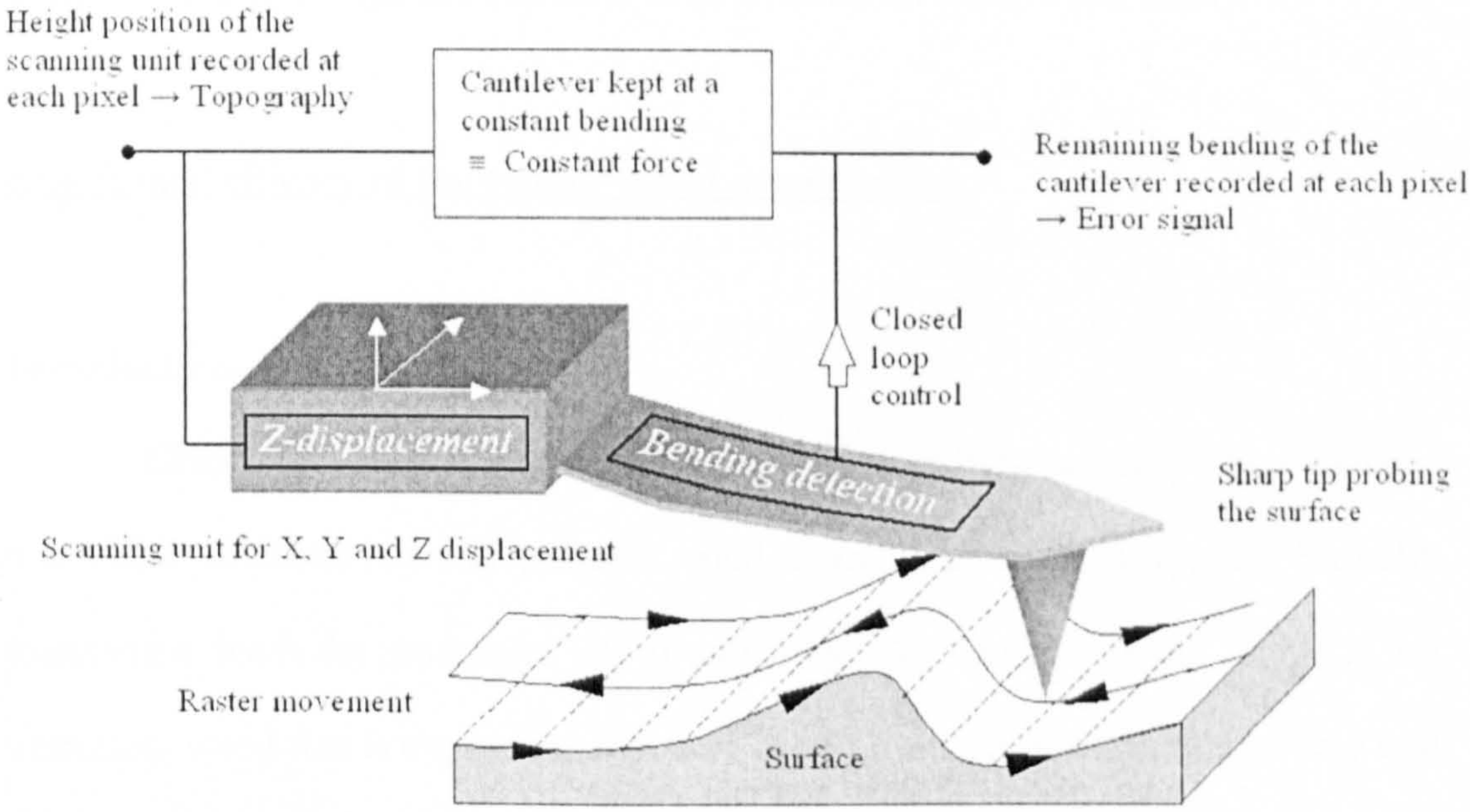


Fig. 2. 12. Illustrate the principles of the AFM.

CHAPTER 3

Weight loss and fungal interactions to determine levels of wood decay.

Anti-fungal efficacy of the copper-based preservatives

Introduction.

Chemical preservative treatment provides long term resistance to organisms that cause deterioration and protects wood from fungi. If it is applied correctly treatment extends the productive life of timber by 5-10 times (Loferski, 2000). If left untreated, wood that is exposed to moisture or soil for a sustained period of time will become weakened by various types of fungi, bacteria, insects and microorganisms. Development of effective new preservatives requires an explanation of how these chemicals impact resistance to wood degradation by fungi and bacteria (Graham, 1973).

The major commercially available water-based alternatives to chromated copper arsenate (CCA) are copper azole (CuAz)-based and alkaline copper quatenrnary (ACQ)-based products for the preservative treatment of timber for the protection from wood decay and fungi attack (Kear *et al.* 2005). The effectiveness of preservative treatment depends on the chemical selected, the method of application, the proportion of sapwood to heartwood, the moisture content of the wood, the amount of preservative retained, the depth of chemical penetration, and the distribution of the chemical in the wood. Sapwood of most commercial lumber tree species accepts preservatives much better than heartwood and softwood species generally can be treated more uniformly than hardwood species (Thomasson *et al.*

2006).

Results.

Water based preservative retention and impregnation of the wood blocks.

The testing focused on the CCA alternatives ACQ and CuAz timber preservative treatments. An important factor to consider is the quantity of copper present in treated wood samples and its tendency to be released and participate in the corrosion of steel fastners used in wood structures. The preservative in the wood that gave the best response for *P. ostreatus* was CuAz. Between the two preservatives most of all CuAz treated specimens were found to have good correlation in relation to the fitness of the particular preservative and retention and to the wood species.

Poplar and cypress treated specimens exhibited good retention, however, ACQ treated woods were more retentive than CuAz Table 3. 1. Thus with both preservatives the retention rate for the test specimens (142~154 kg/m^3) was not sufficient to get high preservative efficacy however bold cypress exhibited greater retention efficacy with both CuAz (238 kg/m^3) and ACQ (237 kg/m^3) treated wood.

Table 3. 1. Preservatives retention (kg/m^3).

Species	Preservatives	
	CuAz	ACQ
Pine	142	159
Poplar	154	178
Bold cypress	238	237

Table 3.1 shows preservative absorption based on the initial weight of dry wood specimens. Softwoods (pine and cypress) and hardwood (poplar) species were

impregnated with two different preservatives in the full section process (Thomasson *et al.* 2006). Bold cypress was very resistant because of the high percentage of heartwood as well as exhibiting greater absorption and permeability than pine and poplar, which probably results from anatomical differences between the three species. Additionally, ACQ treated wood samples were more absorptive than CuAz treatment for the three wood types. The effectiveness of a wood preservative depends on the depth of, and the wood species, in relation to penetration into the wood. Therefore, axial tracheids are the main cell type in softwood, comprising over 90% of cells in the woods, and in cypress the first formed tracheids are always wide-enough to develop inter-tracheid pitting.

Preservatives test of fungi resistance.

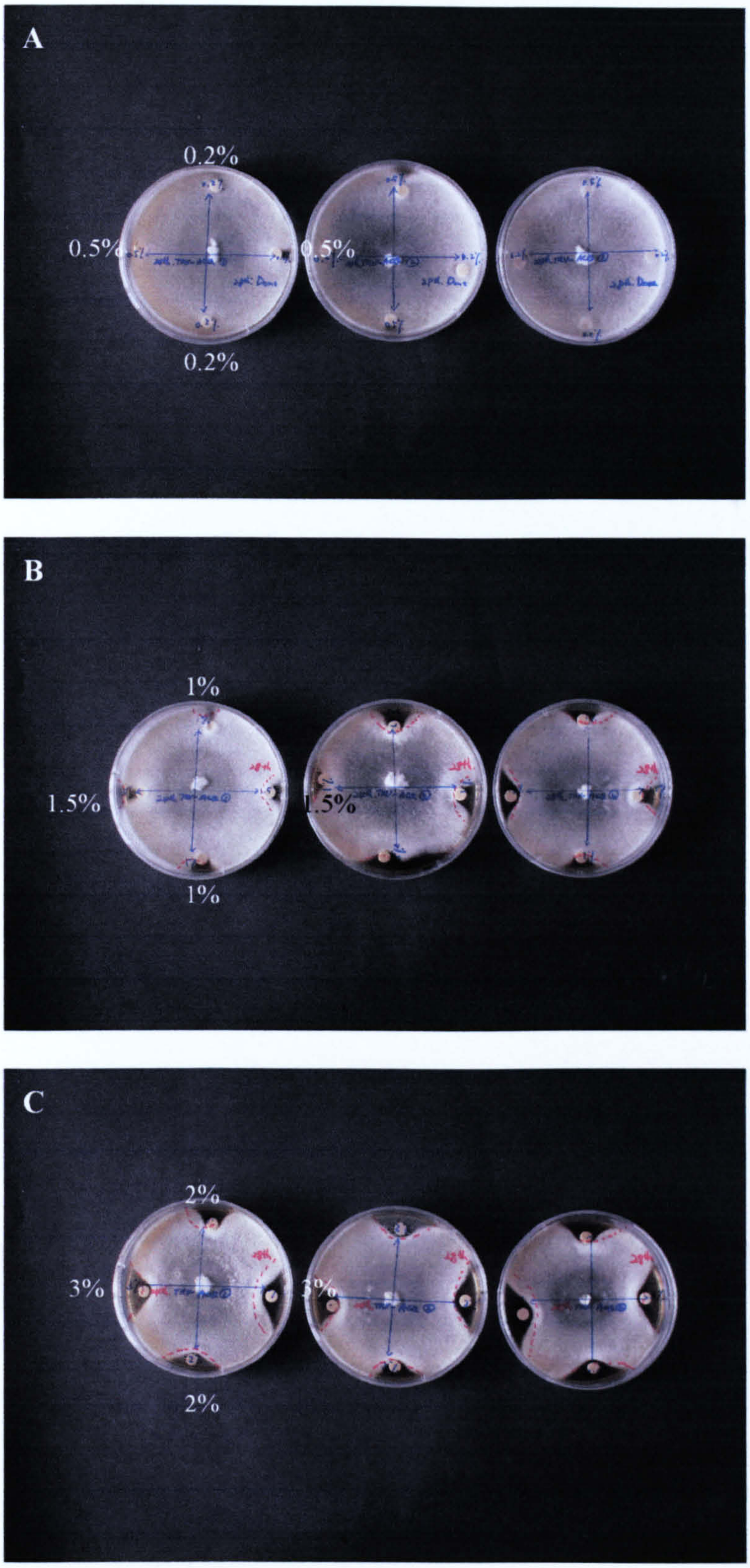
Table 3. 2 compares the two different preservatives and selected fungi on malt extract agar media with filter paper discs which had been impregnated with the chemicals after 7 days incubation. The species tested showed sensitivity to the two preservatives. No effectiveness was observed with 0.2 % and 0.5 % CuAz or ACQ with TRV, PLO and DAC. The fungal growth was however more restricted at 2 % and 3 % CuAz and ACQ.

Table 3. 2. Effect on selected fungi of different concentration of preservatives by agar diffusion.

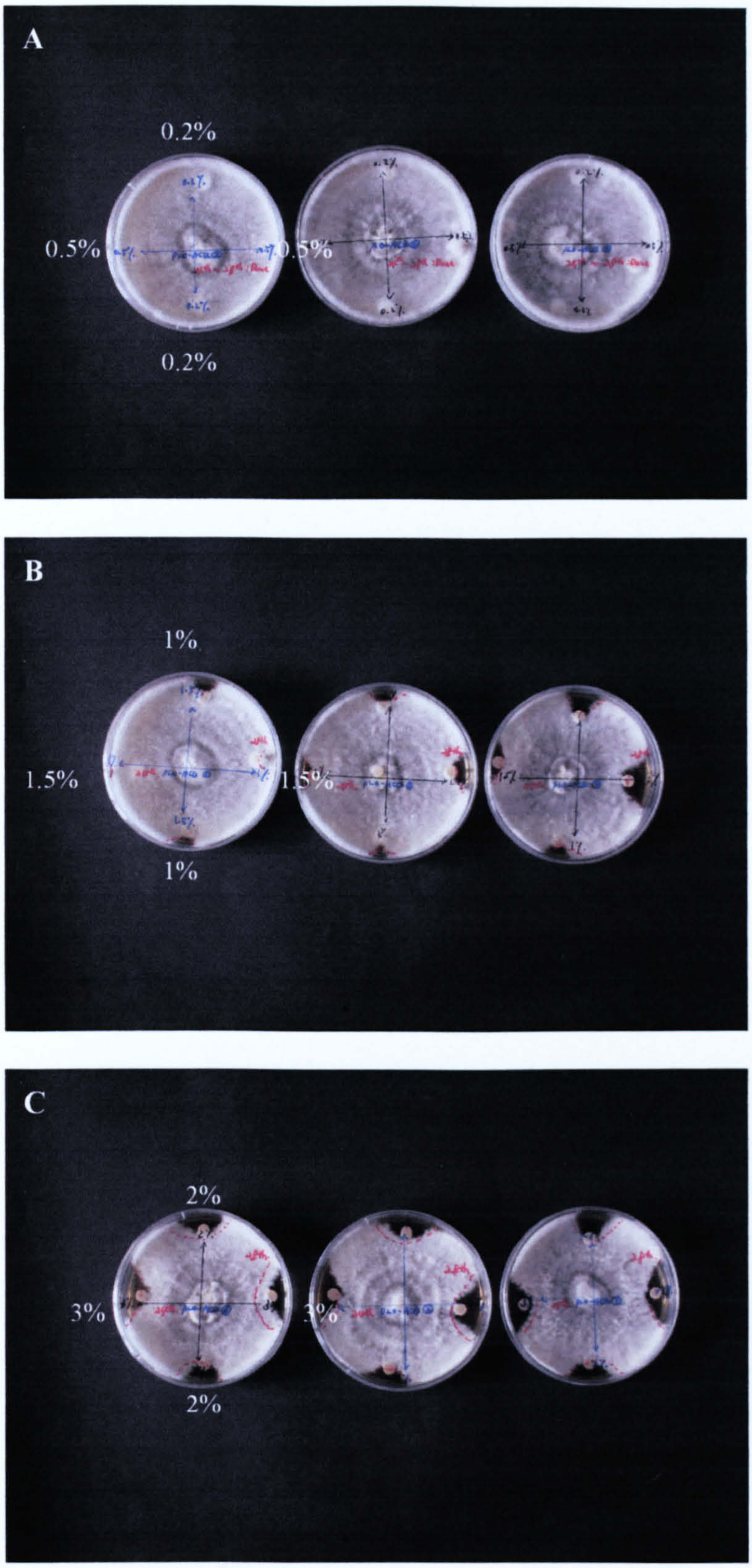
Species	Preservatives (concentration %)											
	CuAz						ACQ					
	0.2	0.5	1	1.5	2	3	0.2	0.5	1	1.5	2	3
TRV	−	−	−	−	+ +	+ +	−	−	+	+	+ + +	+ + +
PLO	−	−	−	−	+	+	−	−	+	+	+ + +	+ + +
DAC	−	−	−	−	+	+	−	−	+	+	+	+

Following by −: no effect, +: week effect, + +: moderate effect, + + +: strong inhibition.

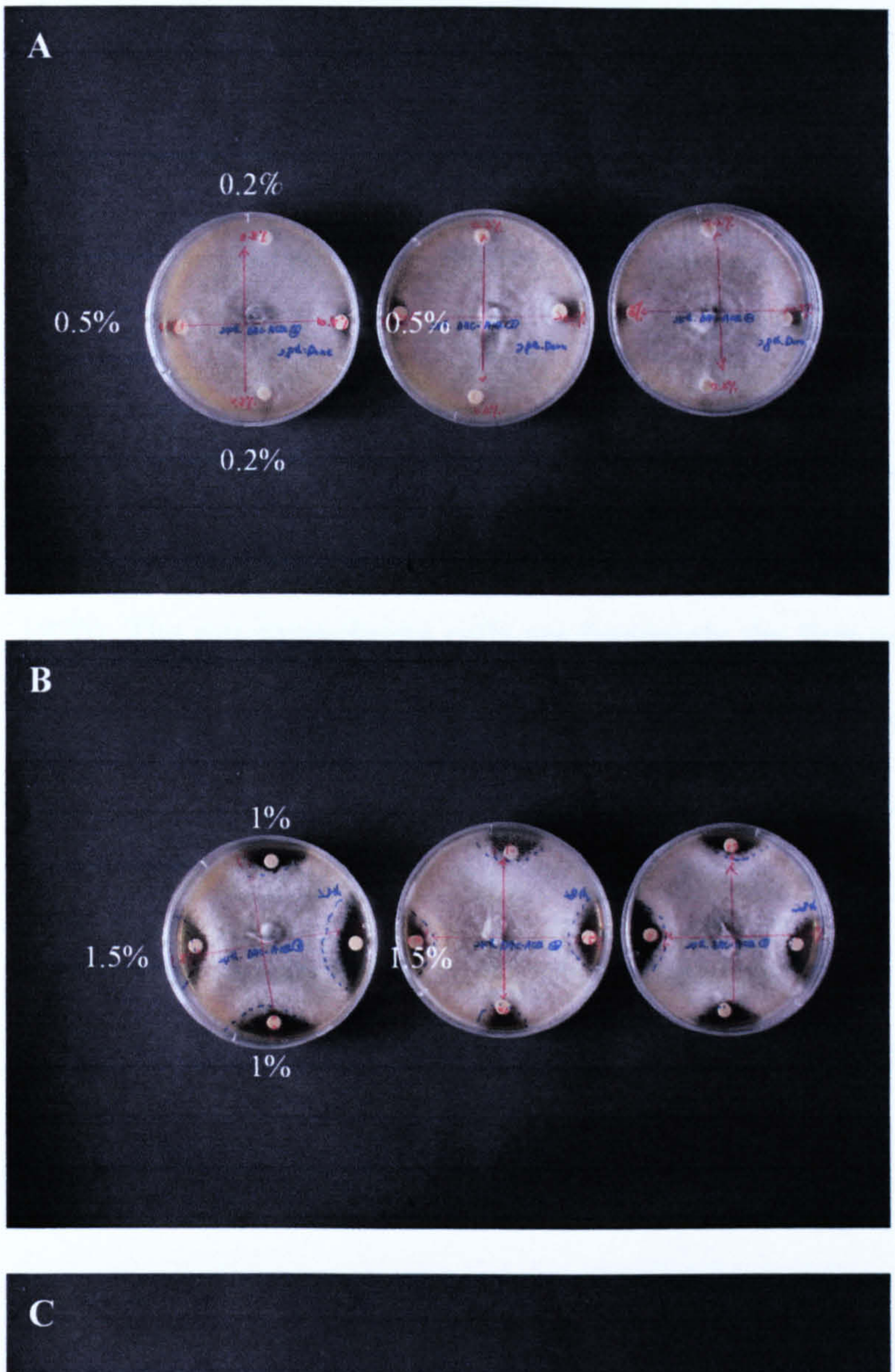
In terms of weight loss CuAz was the more effective preservative for the three wood species with regards to *P. ostreatus*. However, ACQ was more effective in the filter paper agar diffusion test for both *Trametes* and *Pleurotus*.



Figs. 3. 1. ACQ Preservative effect on *T. versicolor* after 7days incubation. It was found that for ACQ 2% and 3% proved to exert a strong preservative effect. A-C. Each test was performed in triplicate. Concentration of the preservative as detailed above.



Figs. 3. 2. ACQ Preservative effect on *P. ostreatus* after 7days incubation. It was found that for ACQ 2% and 3% proved to exert a strong preservative effect. A-C. Each test was performed in triplicate. Concentration of the preservative as detailed above.



Figs. 3. 3. ACQ Preservative effect on *D. concentrica* following 7days incubation. It was found that for ACQ 2% and 3% exerted a strong preservative effect. A-C. Each test was performed in triplicate. Concentration of the preservative as detailed above.

Weight loss of decayed wood.

Introduction.

The largest group of fungi that cause white rot belong to the Basidiomycotina with several thousand species causing decay in softwoods and hardwoods (Gilbertson, 1980). White rot fungi colonize wood quickly and become established in all cells of the xylem (Greaves & Levy, 1965; Wilcox, 1968; Liese, 1970). The ray parenchyma cells are frequently the first to be colonized. Bore holes may be numerous in early stages of wood decay and hyphal penetration from cell to cell via pit structures or directly through the wall is easily accomplished. The order in which various amounts of lignin, cellulose, and hemicellulose are degraded is different among species of white-rot fungi and may vary depending on the type of wood substrate being attacked (Kirk & Highley, 1973; Blanchette, 1984).

Results.

Weight loss of decayed wood (60 days).

The percentage weight loss caused by the individual species of wood decay fungi (Table 3.3) differed significantly and as a result the performance of preservative treatments could be assessed for each fungus. In the untreated and all treated species the greatest decay was caused by *T. versicolor*, in untreated poplar (32%). Untreated poplar also exhibited the highest weight loss for *P. ostreatus*. On the other hand, untreated pine was shown to have the strongest natural resistance to decay for all three test fungi plus high anti fungal efficacy for both CuAz and ACQ treated wood samples by *P. ostreatus*. Overall *D. concentrica* exhibited the smallest change the weight loss (0.5~8) for both untreated and treated wood blocks Fig. 3.4.

Table 3. 3. Average percentage weight loss in untreated and treated wood blocks after 60days incubation.

Preservatives	Untreated			CuAz			ACQ		
Species	Pine	Poplar	Cypress	Pine	Poplar	Cypress	Pine	Poplar	Cypress
TRV	17	32	18.9	13	18	4.1	4	13	7.9
PLO	3.9	9.1	8.1	0.9	2.1	3.5	1	1.7	1.5
DAC	8	2.5	7.2	3	0.5	3.4	3.9	1.4	1.5

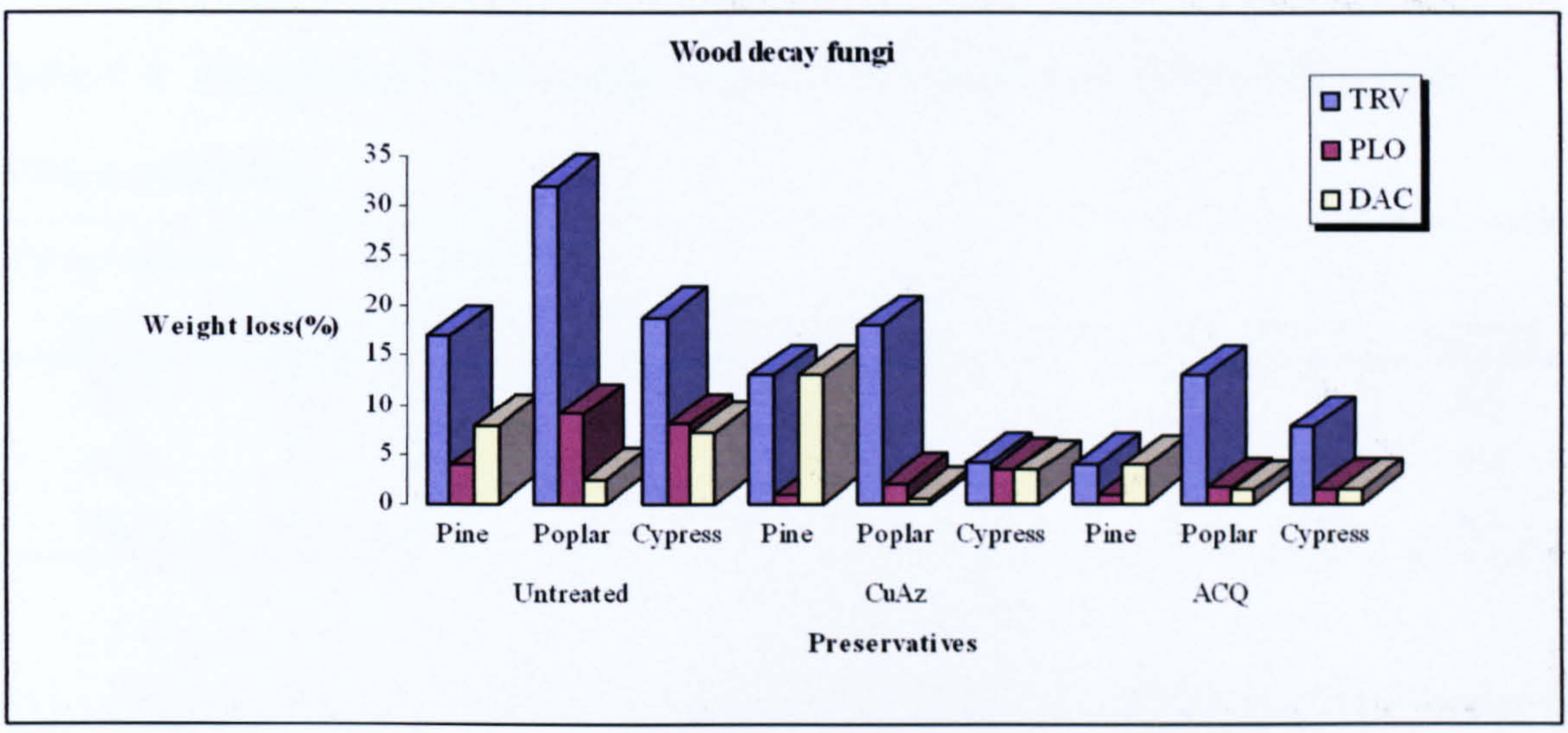


Fig. 3. 4. Chemical treatments of the various treated woods in relation to weight loss after 60 days incubation.

Weight loss of decayed wood.

The wood preservatives tested have been shown to decrease the amount of fungal colonization and to reduce the degradation of the timber, following impregnation by waterborne, copper-based preservatives, CuAz and ACQ (Figure 6-11).

The results show that cypress possessed greater natural resistance to decay by the wood decay ascomycete fungus, *D. concentrica* than to the white-rot basidiomycete fungi, *T. versicolor* and *P. ostreatus*. The converse was true, however,

for the poplar. Particularly, the weight loss of untreated pine was 35.4% and poplar was 32% caused by the white-rot fungus, *T. versicolor*. In addition, the most damaged wood was poplar as shown in Table 3. 4.

The results also show that both preservatives have an inhibitory effect on all three test fungi regardless of tree species. Both preservatives were effective against *P. ostreatus* and CuAz was the slightly more effective preservative for three species as shown in Fig. 3. 5.

Table 3. 4. Average percentage weight loss in untreated and treated wood blocks after 90days incubation.

Preservatives	Untreated			CuAz			ACQ		
Species	Pine	Poplar	Cypress	Pine	Poplar	Cypress	Pine	Poplar	Cypress
TRV	35.4	32	27.4	7.4	7.3	8.1	8	8	8.5
PLO	12	16.7	10.8	7.1	7.2	7.7	7.5	7.7	8.5
DAC	8.3	13.2	9.2	6.5	7.3	8.9	7.4	7.6	8.1

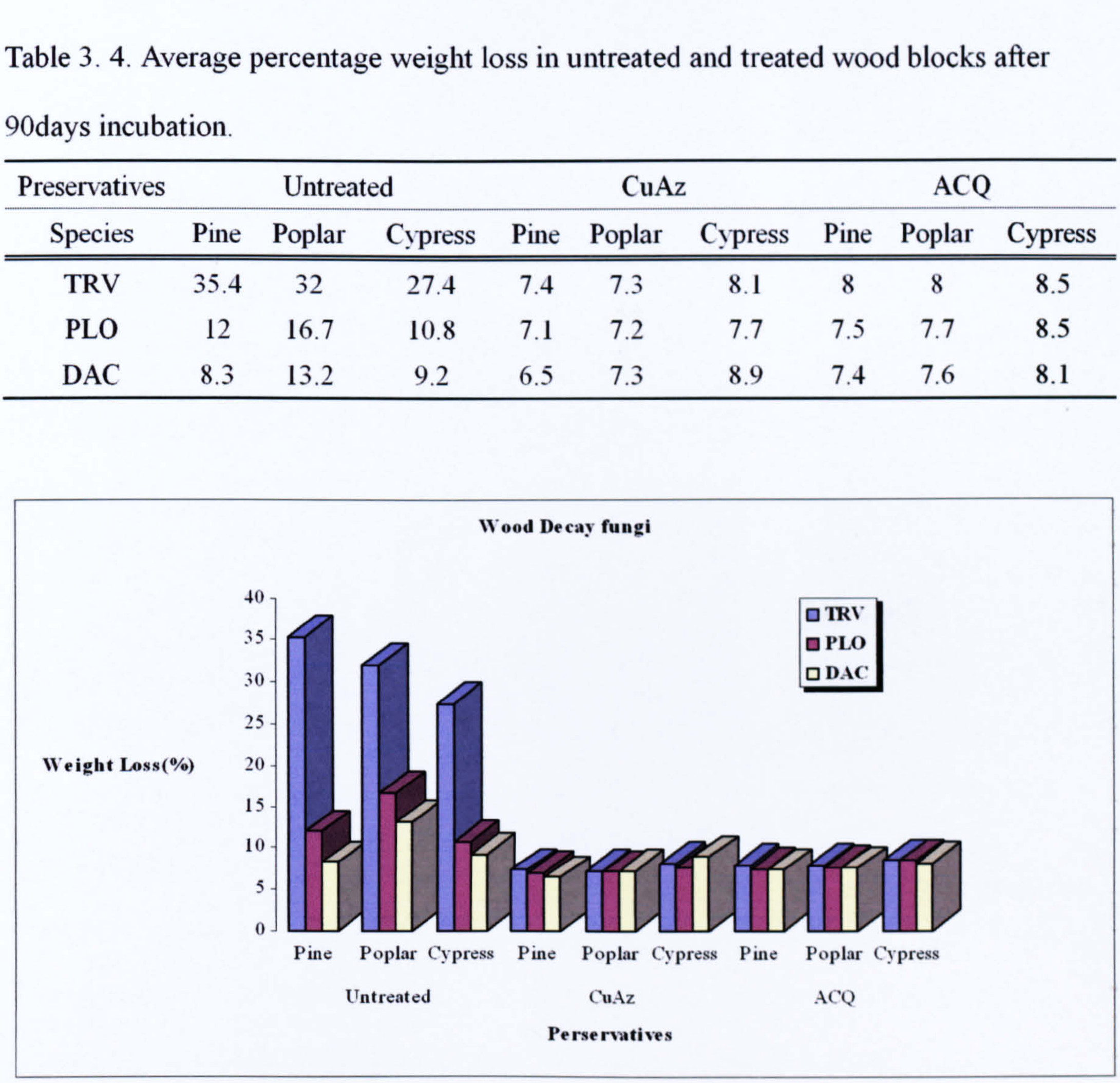


Fig. 3. 5. Chemical treatments of the various treated woods in relation to weight loss after 90 days incubation.

Table 3. 5. Preservatives effect of wood decay blocks.

Preservatives	Untreated			CuAz			ACQ		
Species	Pine	Poplar	Cypress	Pine	Poplar	Cypress	Pine	Poplar	Cypress
TRV	−	−	−	+	+ +	+ + +	−/+	−/+	−/+
PLO	−	−	−	+ +	+ + +	+ + +	−	−	−
DAC	−	−	−	+ +	+ +	+ +	−/+	−/+	−/+

Following by – no effect, +: week effect, + +: moderate effect, + + +: strong effect.

Anti-fungal activity Fig. 6~ 11.

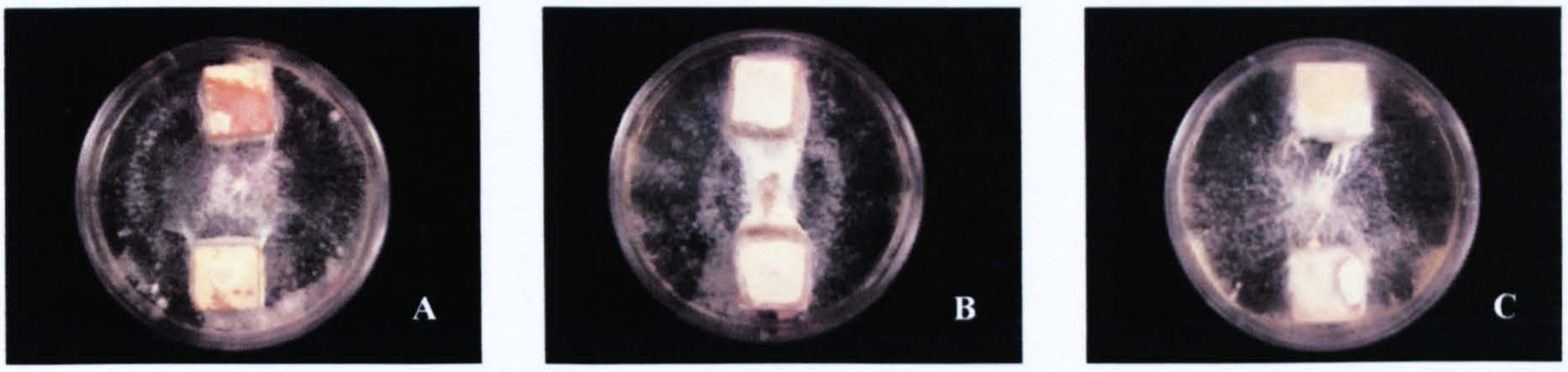


Fig. 3. 6. Anti-fungal activity. Mycelial growth of *T. versicolor* interacting with wood blocks treated with CuAz. Preservative treatment which had the most effect on protection against TRV was CuAz. A and D untreated pine tested against TRV, B and E untreated poplar tested against TRV, C and F untreated cypress tested against TRV, G and J CuAz treated pine tested against TRV, H and K CuAz treated poplar tested against TRV, I and L CuAz treated cypress tested against TRV.

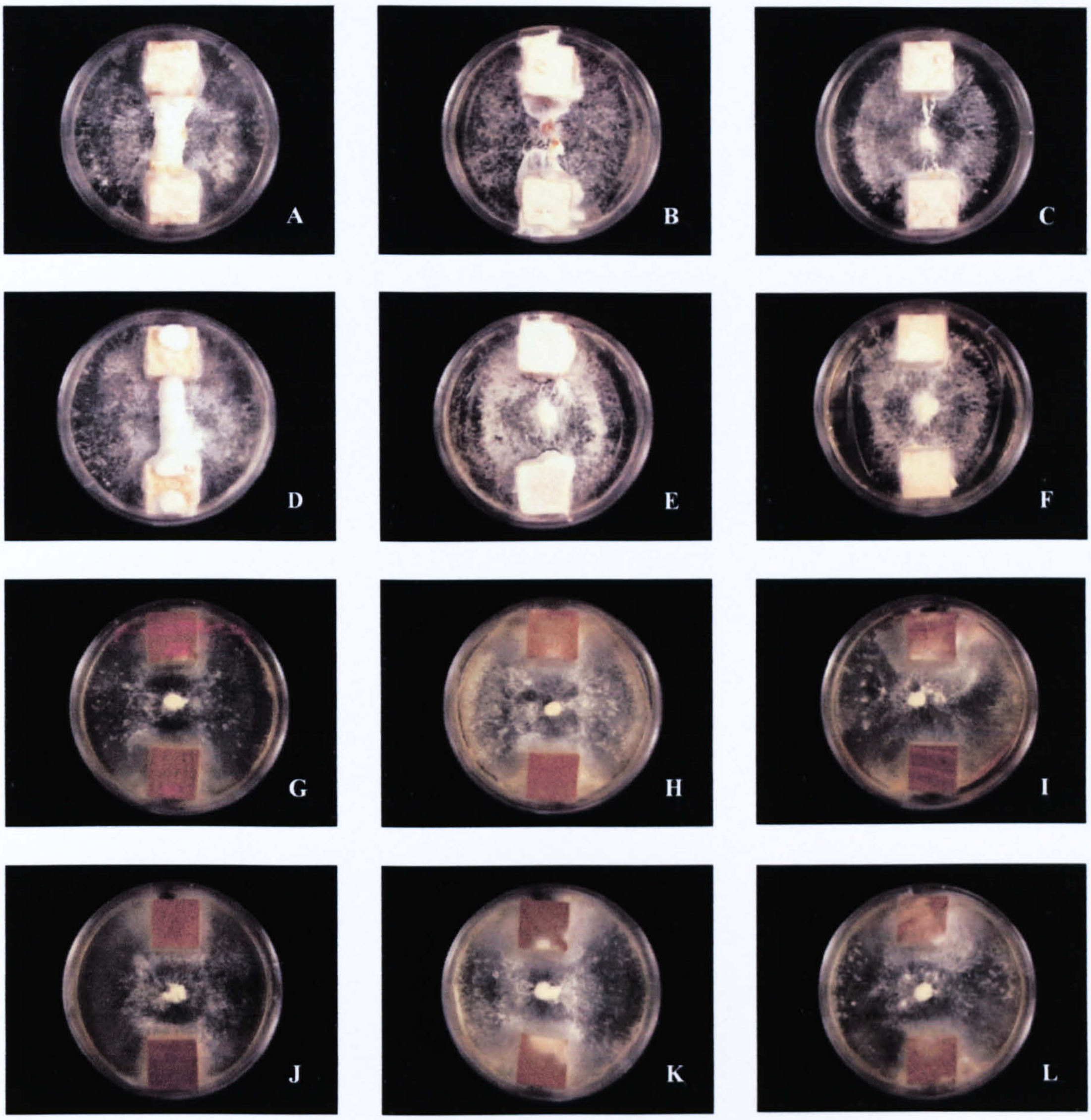


Fig. 3. 7. Anti-fungal activity. Mycelial growth of *T. versicolor* interacting with wood blocks treated with ACQ. Preservative treatment which had the most effect on protection against TRV was ACQ. A and D untreated pine tested against TRV, B and E untreated poplar tested against TRV, C and F untreated cypress tested against TRV, G and J ACQ treated pine tested against TRV, H and K ACQ treated poplar tested against TRV, I and L ACQ treated cypress tested against TRV.

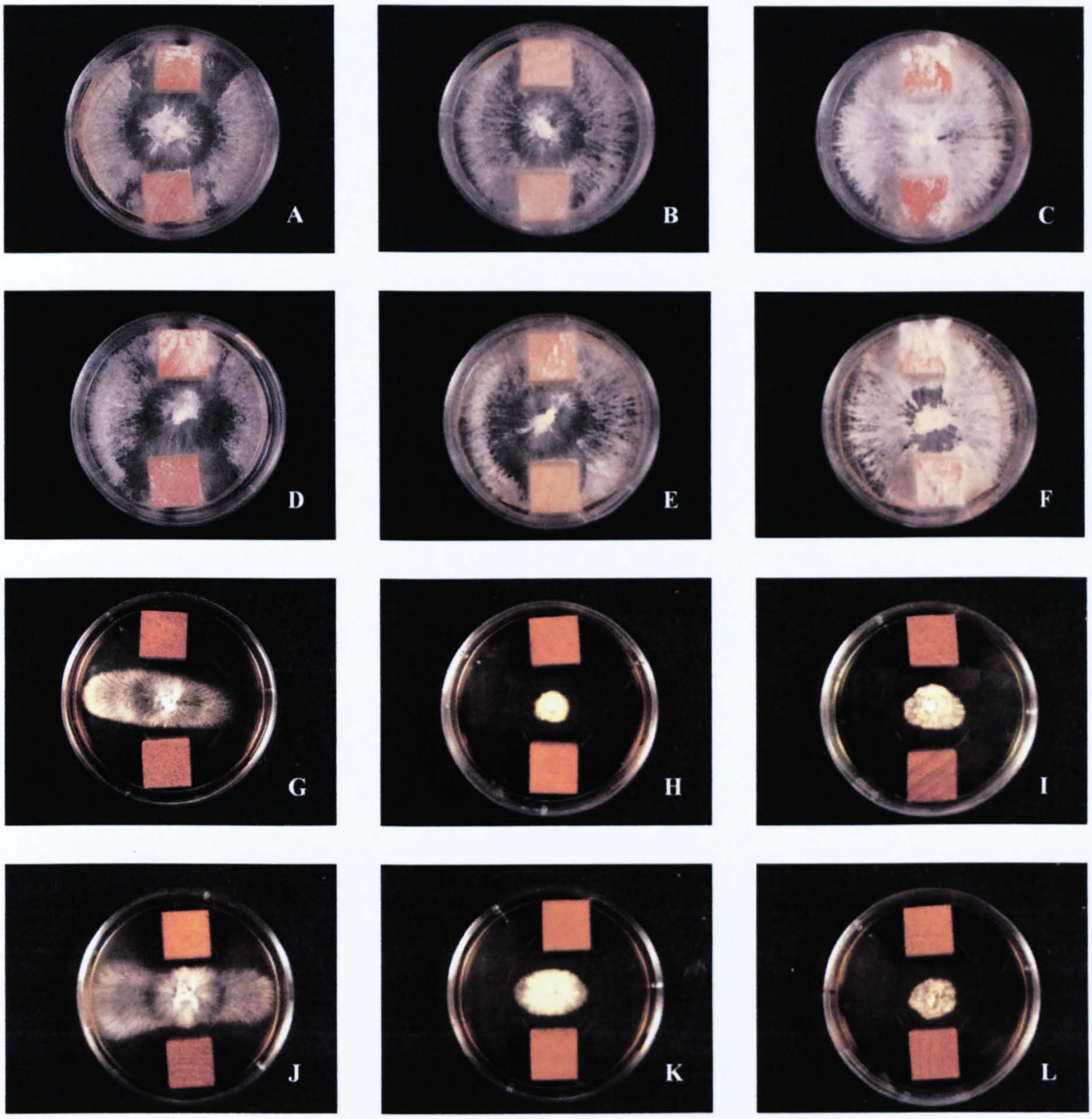


Fig. 3. 8. Anti-fungal activity. Mycelial growth of *P.ostreatus* interacting with wood blocks treated with CuAz. Preservative treatment which had the most effect on protection against TRV was CuAz. A and D untreated pine tested against PLO, B and E untreated poplar tested against PLO, C and F untreated cypress tested against PLO, G and J CuAz treated pine tested against PLO, H and K CuAz treated poplar tested against PLO, I and L CuAz treated cypress against PLO.

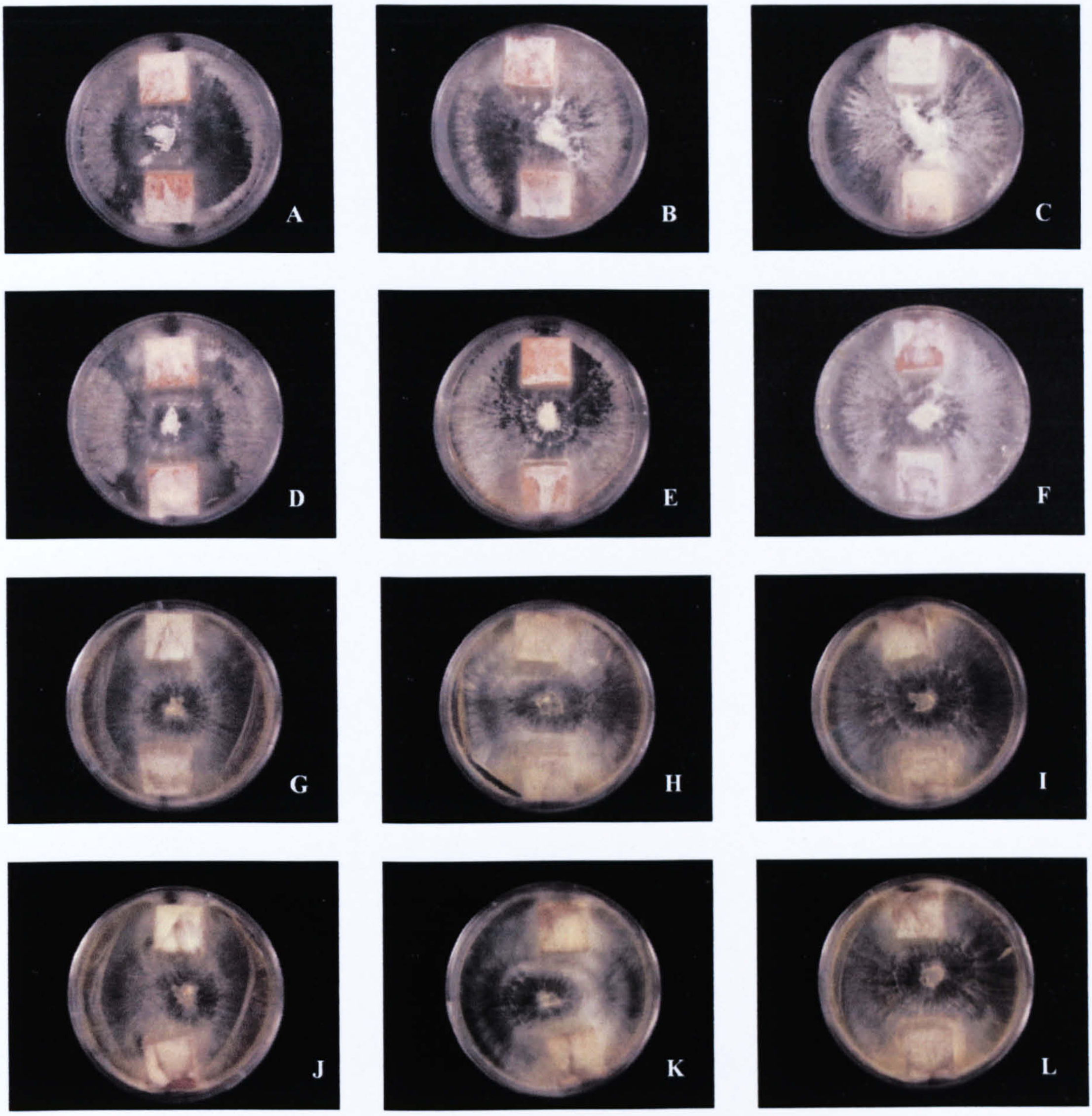


Fig. 3. 9. Anti-fungal activity. Mycelial growth of *P.ostreatus* interacting with wood blocks treated with ACQ. Preservative treatment which had the most effect on protection against TRV was ACQ. A and D untreated pine tested against PLO, B and E untreated poplar tested against PLO, C and F untreated cypress tested against PLO, G and J ACQ treated pine tested against PLO, H and K ACQ treated poplar tested against PLO, I and L ACQ treated cypress tested against PLO.

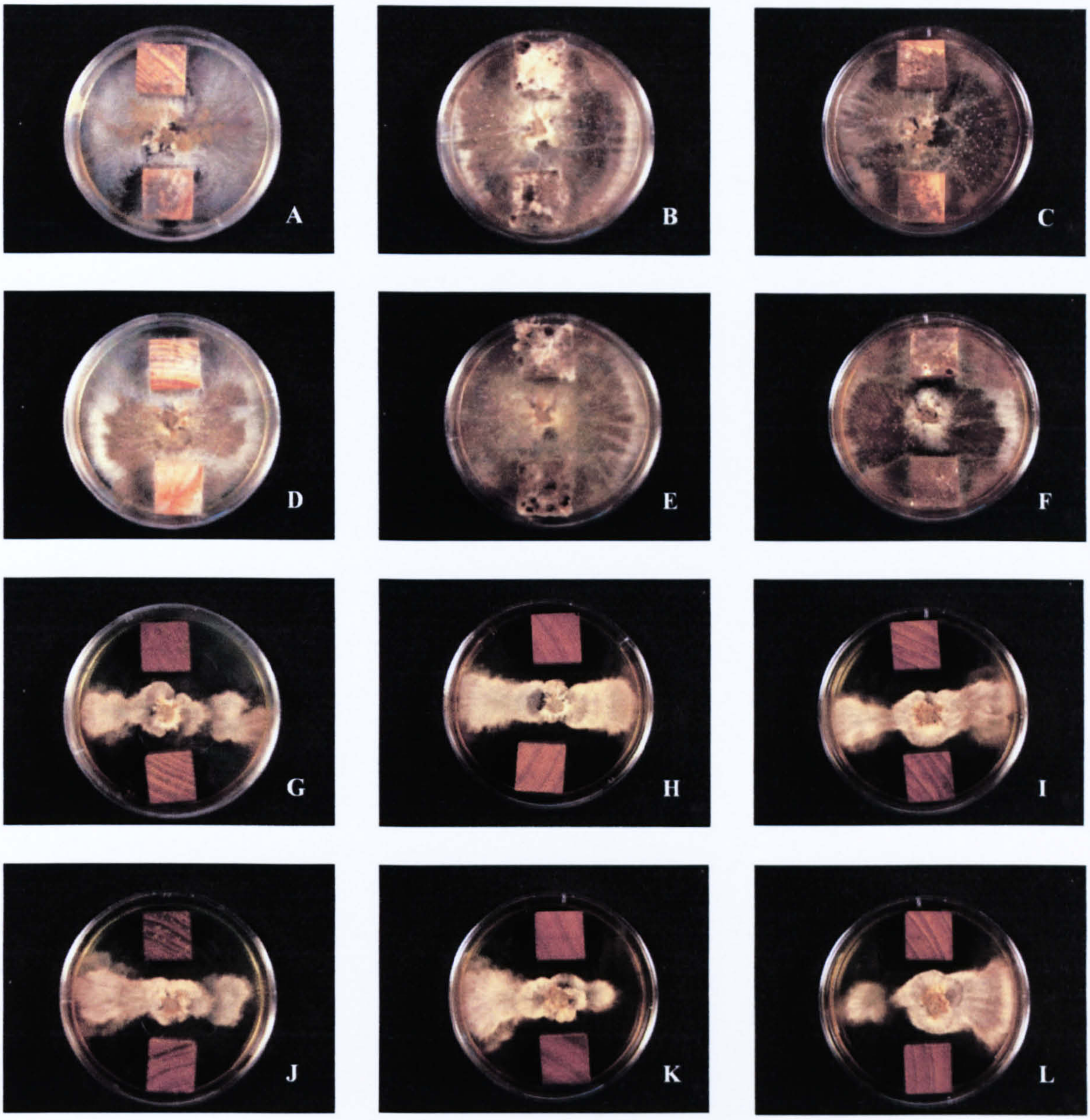


Fig. 3. 10. Anti-fungal activity. Mycelial growth of *D. concentrica* interacting with wood blocks treated with CuAz. Preservative treatment which had the most effect on protection against TRV was CuAz. A and D untreated pine tested against DAC, B and E untreated poplar tested against DAC, C and F untreated cypress tested against DAC, G and J CuAz treated pine tested against DAC, H and K CuAz treated poplar tested against by DAC, I and L CuAz treated cypress tested against DAC.

Fig. 3. 11. Anti-fungal activity. Mycelial growth of *D. concentrica* interacting with wood blocks treated with ACQ. Preservative treatment which had the most effect on protection against TRV was ACQ. A and D untreated pine tested against DAC, B and E untreated poplar tested against DAC, C and F untreated cypress tested against DAC, G and J ACQ treated pine tested against DAC, H and K ACQ treated poplar tested against DAC, I and L ACQ treated cypress tested against DAC.

The most important factor in protection against fungal colonization was found to be the concentration of the copper preservatives. The higher the concentration the greater the inhibition of the test fungi. Based on inhibition by CuAz and ACQ it was shown that at 2% concentrations of ACQ clear inhibition zones could be observed. Furthermore CuAz proved to produce a greater inhibition on all three test fungi than ACQ.

Interactive antagonism test of 3 fungi.

Introduction.

Most biodegradation experiments have used *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Pleurotus ostreatus* as the test organisms (Eggen & Majcherczyk, 1998; Bogan *et al.* 1999; Tuomela *et al.* 1999; Canet *et al.* 2001). Shearer and Zare-Maivan (1998) found antagonistic interactions to predominate among pairs of leaf and wood-inhabiting aquatic fungi and concluded that competitive interactions also influence fungal community structure in subaqueous wood. Competition is the most common type of interaction occurring between wood-decaying higher fungi. Since competition for nutrients in organic resources is effectively brought about by competition for space, the common division into interference and exploitation competition is not very appropriate as interactions can dramatically alter mycelial function, and have potential as biological control agents of fungal pathogens of trees and in service timber (Boddy, 1999).

Since the antagonistic action of actinomycetes against other micro-organisms was reported (Cavalcante & Eaton, 1981) many studies have illustrated this type of morphological and physiological changes (Bruce & Highley, 1991). Particularly, the interaction studies have been mainly carried out on fungal gowth (Porter, 1924; Boddy *et al.* 1985; Sharland & Rayner, 1986, 1989).

Results.

Many previous studies of fungal communities in decaying wood have relied on occurrence of external fruit-bodies (Käärik, 1975).

Antagonism between *Trametes*, *Pleurotus* and *Daldinia* fungi and influence of the preservatives are indicated in Fig. 3. 12. In the untreated wood blocks (Fig. 3. 12 A-C) the wood blocks were colonized and over grown by the test fungi but wood blocks treated with CuAz exhibited some resistance colonization although the effect of CuAz were minimal for *T. versicolor*. The samples treated with ACQ showed little effect on *T. versicolor* and *P. ostreatus* but there was some activity against *D. concentrica.*

The apparent greater inhibition effects of CuAz composed to ACQ could merely be due to greater diffusion rate of CuAz (D-F) composed to ACQ (G-I). In Fig. 3. 12 the mycelium was clearly preceded by a band of lysis often less 0.5mm wide in wood block and the three fungi. Fungal growth at the surface the blocks treated with ACQ and CuAz produced characteristic zone lines. Untreated wood block plates also characterization lines resulting from interactive antagonism between these three fungi.

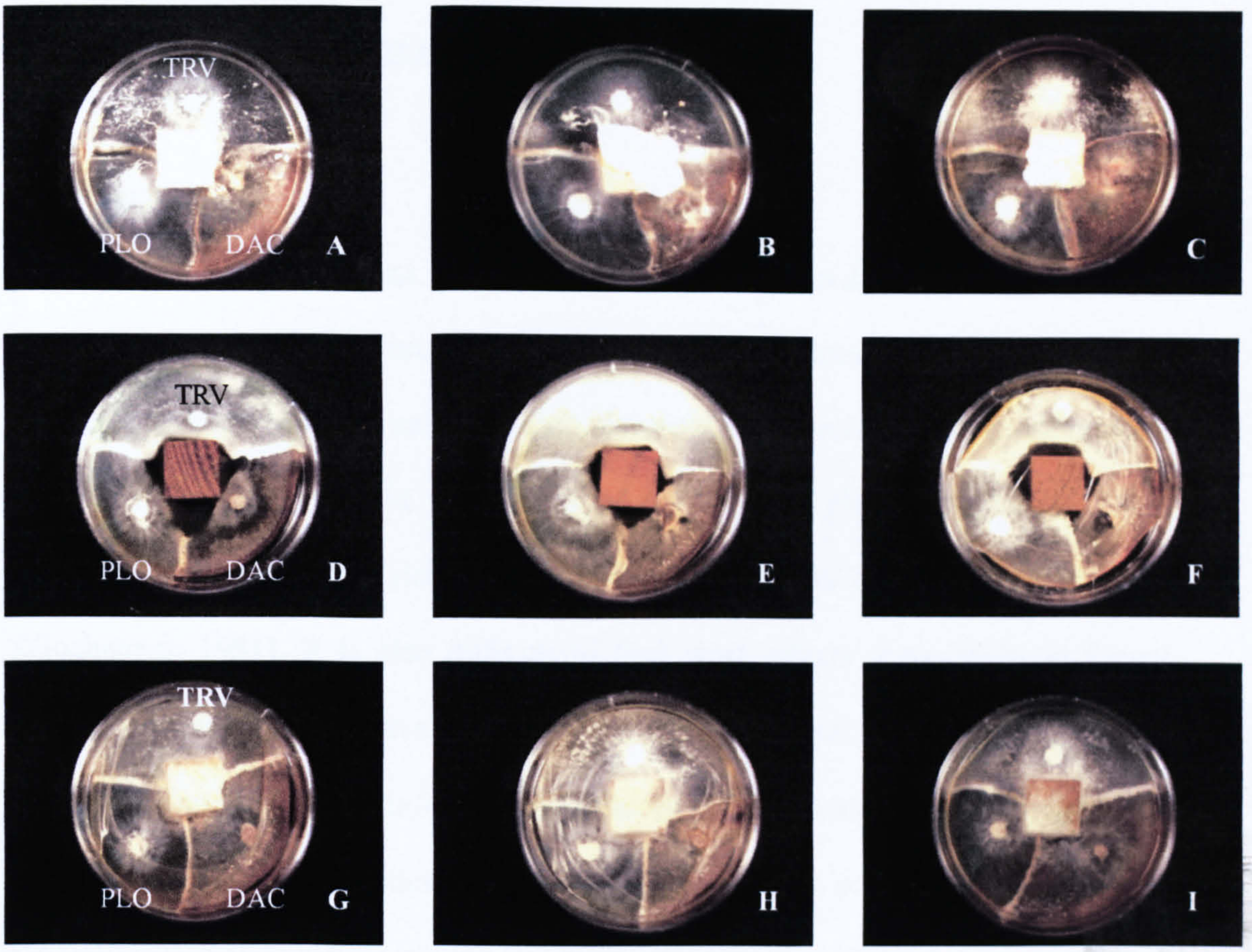


Fig. 3. 12. Interactive antagonism of three fungi (TRV, PLO, DAC) tested against three different types of wood blocks in agar plates and treated with ACQ and CuAz. Fungi growth was generally similar although CuAz treated Wood blocks exhibited the greatest inhibited effect. As the results, the most effect preservative of wood block was CuAz. A-C. Untreated-pine, poplar and cypress. D-F. CuAz treated-pine, poplar and cypress. G-I. ACQ treated-pine, poplar and cypress.

Wood decomposition in the soil.

Introduction.

Agricultural systems have usually been considered to represent artificial ecosystems as a consequence of cultivated land practices, nutrient inputs, and pesticide applications. Our understanding, however, of the roles of soil fungi in these systems is descriptive at best. Once a soil is cultivated and planted in a monoculture, declines in fungal species richness and changes in species composition take place (Gochenaur, 1981). It is also difficult to find what effects these shifts in fungal species composition may have on nutrient cycling and organic matter accumulation.

A further back of information exists on the interactions in agroecosystems among soil fungi, nematodes, microarthropods, and the concurrent processes of decomposition and nutrient cycling (Wicklow, 1973; Neher, 1999).

Results.

Weight loss for the untreated wood samples was significantly greater for poplar (26%) than for pine (12%) or cypress (11.4%) (Table 3. 6, Fig. 3. 13). There was also significant reduction in weight loss for wood blocks treated with ACQ and CuAz. This is clearly shown for poplar with little difference in effect between ACQ and CuAz. Weight loss in pine and cypress was not significantly reduced by treatment with either ACQ or CuAz.

Table 3. 6. The decomposition of wood samples buried in wood land soil.

Treatment	Weight loss (%)		
	Pine	Poplar	Cypress
untreated	12	26	11.4
ACQ	9.7	9	9.54
CuAz	10	10	10.2

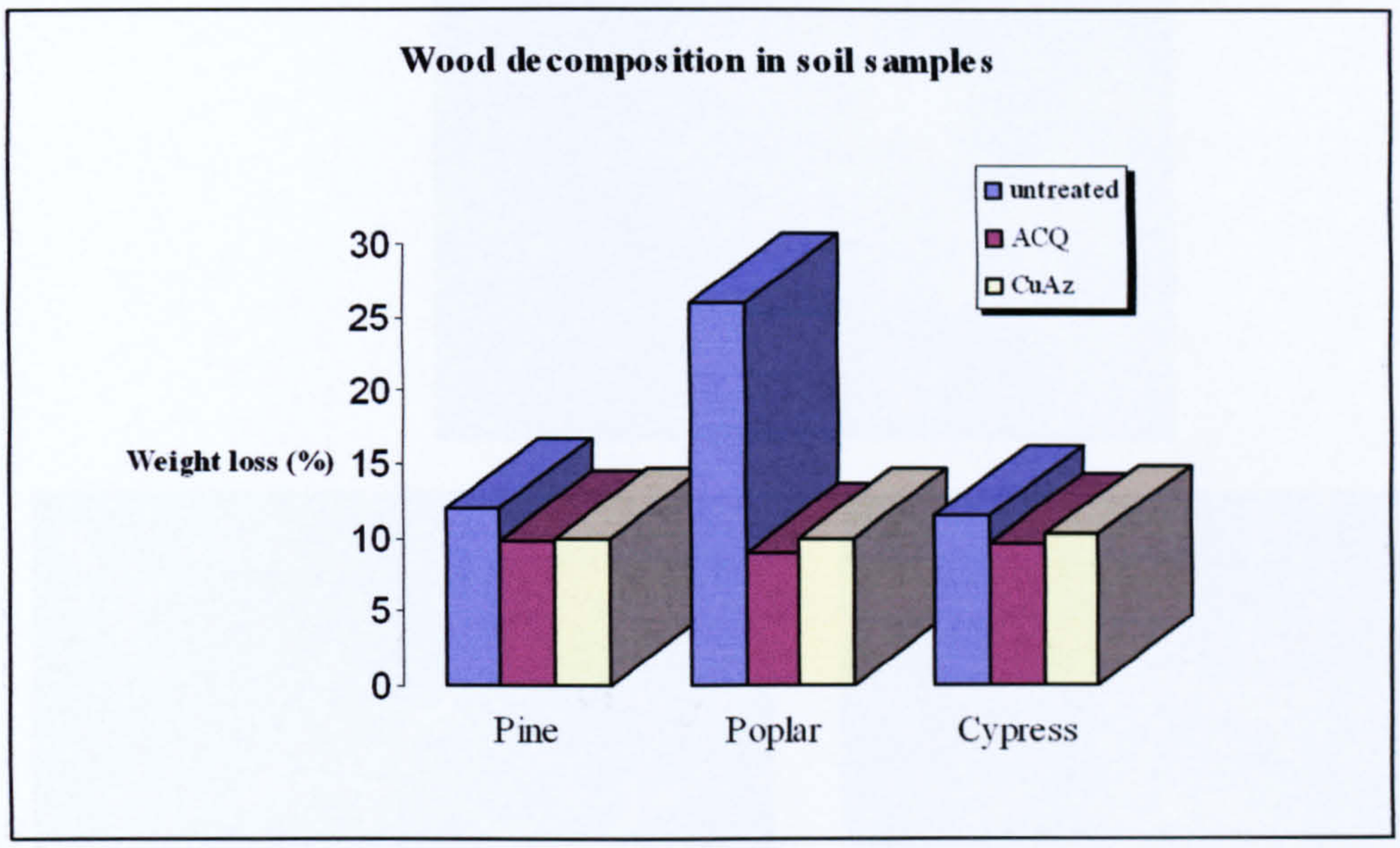
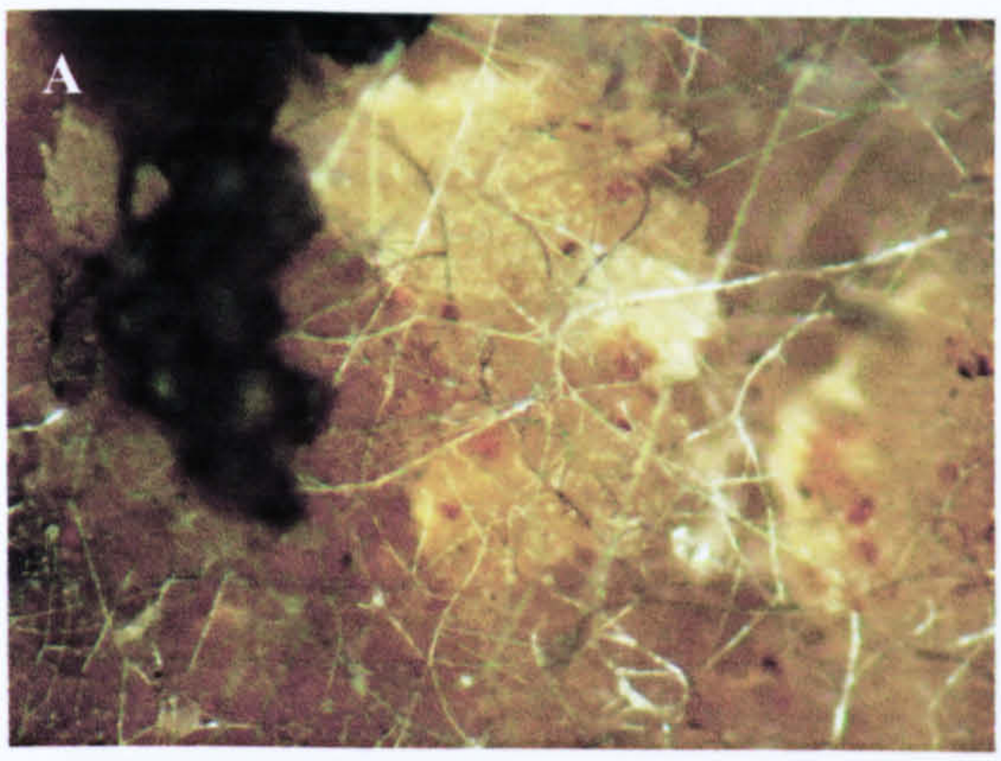
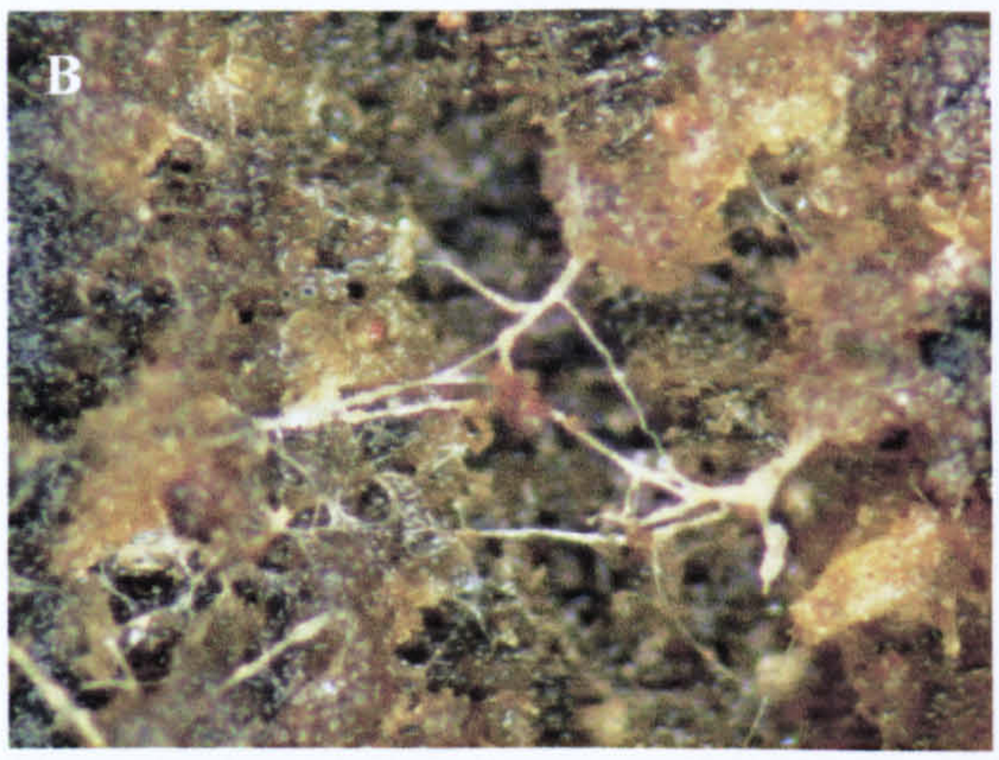


Fig. 3. 13. Wood decomposition in soil samples for 6 months.





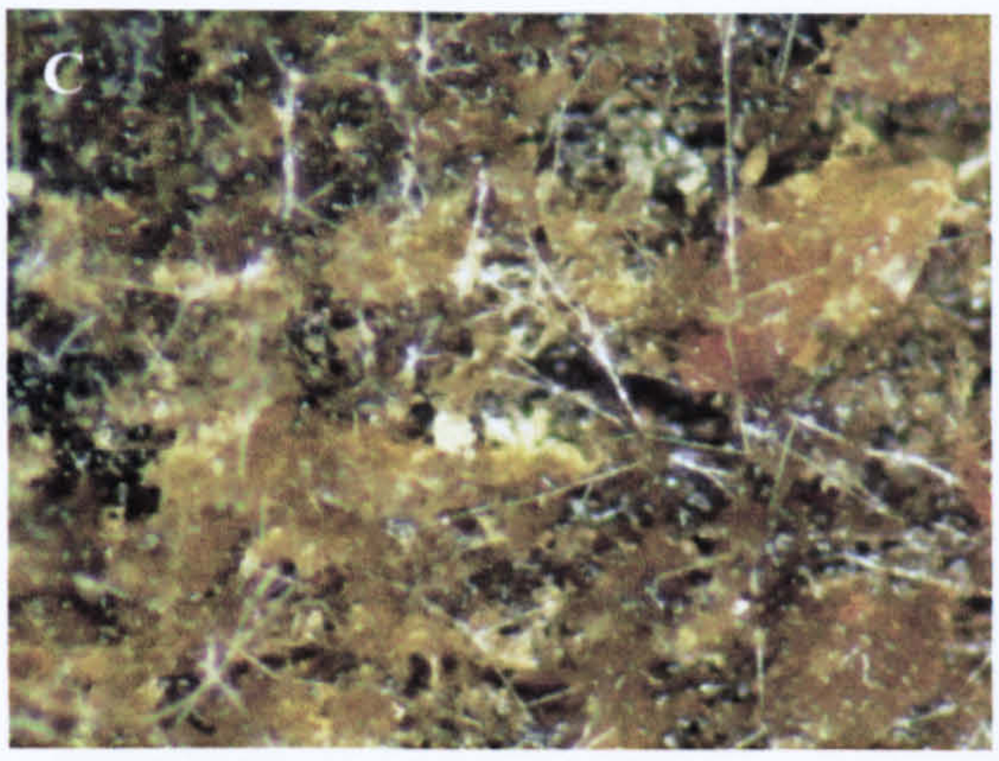


Fig. 3. 14. Appearance of pine blocks following burial in woodland soil with and without preservative treatment. A. Damage to the wood structure with significant mycelial growth on the surface of wood blocks. B and C. ACQ and CuAz treatment greatly reduced mycelial growth indicating good protection against degradation by wood decay.

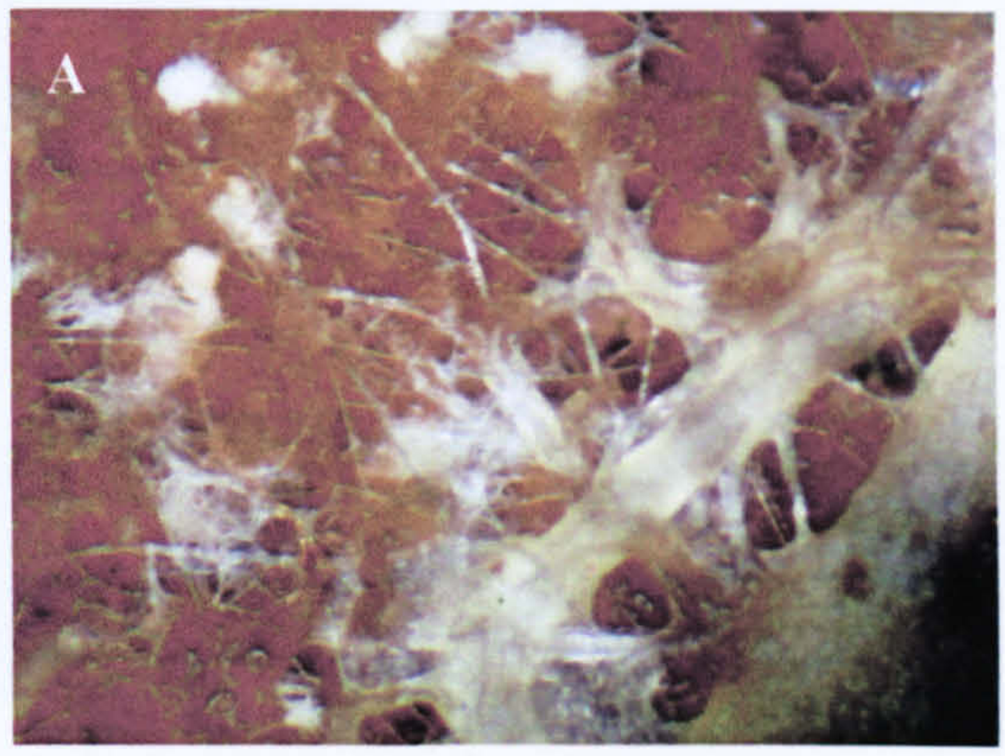


Fig. 3. 15. Appearance of poplar blocks following burial in woodland soil with and without preservative treatment. A. Damage to the wood structure with significant mycelial growth on the wood blocks. B and C. Showing typical hyphal growth. There was little evidence of fungal growth on the surface of treated wood blocks (D and E).

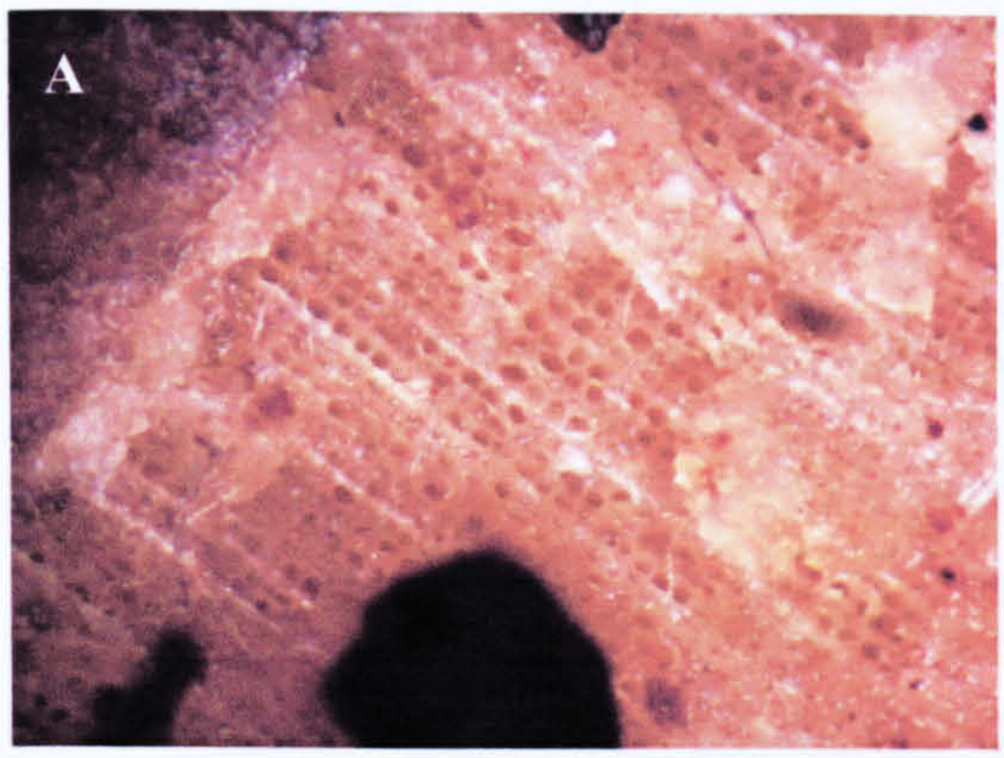


Fig. 3. 16. Appearance of cypress blocks following burial in woodland soil with and without treatment. In all cases there is little evidence for colonization of the wood blocks by fungi and therefore little evidence if any of wood decomposition.

It appears, therefore, that it is only poplar wood blocks which are significantly protected by the CuAz and ACQ preservatives following exposure to soil fungi in a native woodland. As discussed earlier there is no information on those fungi involved although the site chosen for the burial of the blocks was known to exhibit rapid wood decomposition.

Discussion

In this chapter the application of the more traditional method for assessing wood decay by weight loss over time is reported. Wood blocks of pine, poplar and cypress untreated and treated with CuAz and ACQ were inoculated with the wood decay fungi *T. versicolor*, *P. ostreatus* and *D. concentrica* and incubated for 60 and 90 days. Untreated poplar proved to be the least resistant to the decay fungi and cypress exhibited the greatest natural resistance to *D. concentrica*. It was shown that there were clear differences between untreated and treated wood blocks and that initially ACQ proved to be the most effective of the preservatives. After a 90 day incubation period the differences in weight loss between ACQ and CuAz were much less noticeable. It is also shown that weight loss caused by the ascomycete, *D. concentrica* was much less than the weight loss caused by the basidiomycetes *T. versicolor* and *P. ostreatus*. This was not surprising because although *D. concentrica* causes a white rot decay it is not considered to be a vigorous wood decay fungus (Burdekin, 1977; Whalley, 1996). Tests on the effect of the preservatives on inhibition of the test fungi using an agar diffusion technique also indicated that ACQ was more effective than CuAz. However for both preservatives a percentage concentration of 2 % or above was required to induce significant inhibition of *T. versicolor* and *P. ostreatus*. However, *D. concentrica* only exhibited slight inhibition even at 3 % for both preservatives. This is not so serious because this is not a major wood decay fungus. In testing of inhibition of the three fungi by preservative inpregnated wood blocks CuAz had by far the greatest inhibitory effect.

A series of interactive reactions between the test fungi on agar plates in the presence of treated or untreated wood block samples resulted in a range of interactions with *P. ostreatus* providing to be the most combative in the majority of

situations.

The effect of the preservatives on wood decay in a natural ecosystem was also tested and it was found that both ACQ and CuAz had similar protective qualities for all three wood types. However weight loss in poplar was significantly higher in the untreated, treated and wood blocks. This protection against wood decay could also be observed microscopically with little evidence of significant mycelial development on any of the treated wood block surfaces. On all of the untreated blocks fungal colonization of their surfaces was evident with considerable development on the surface of the untreated poplar blocks. This is in agreement with the weight loss results. It can be concluded that overall both copper based preservatives provided significant protection against fungal decay.

CHAPTER 4

Determination of biomass based on chitin and ergosterol assays.

Introduction.

Fungal decay of wood is traditionally assessed by determination of weight loss of wood block samples over time (Williams, 2005). This method is also used to determine the effect of various chemical preservatives in reducing wood decay (Tucker *et al.* 1997). Activation approaches include visual observation and/or determination of the amount of certain fungal specific compounds. Chitin, as a major component of fungal mycelium, has been successfully used to determine fungal biomass (Braid & Line, 1981; Bleau *et al.* 1999; Dutta *et al.* 2002). Additionally ergosterol which is exclusive to fungal membranes is the component which has gained popularity in the determination of fungal biomass in various ecosystems (Newell *et al.* 1987).

In this chapter the determination of chitin and ergosterol levels in the mycelium of the test fungi and in suitably inoculated wood block samples is presented. The application of these methods to assess the ability of two preservatives CuAz and ACQ to reduce wood decay is examined.

Chitin assays.

Results.

The fungi *T. versicolor*, *P. ostreatus* and *D. concentrica* and wood samples of pine, poplar and cypress were used to determine the chitin and ergosterol contents. All three wood samples were treated with the preservatives CuAz and ACQ and control samples were maintained without addition of preservatives for each wood

sample, respectively. The chitin content of different treated wood samples and untreated wood samples following inoculation with the test fungi and subsequent inoculation at 25 °C for 60 days are given in the Table 4.1. The chitin assay was determined after 60 days post inoculation by the test fungi. The chitin content of fungal biomass on both the treated and untreated wood samples was determined based on the method of Chen & Johnson, 1983 see also Chapter 2. Amounts of chitin were found to occur in the wood samples in the following order: pine, poplar and cypress. The results show that *P. ostreatus* produced higher quantities of chitin in poplar and lower quantities of chitin were produced by *T. versicolor* in cypress. A significant chitin content reduction was observed in the wood samples treated with the preservatives CuAz and ACQ. The greatest reduction in chitin content was observed with the wood samples treated with ACQ. Fungal biomass figures of 289.9, 373.4 and 321.5 mg/kg were obtained from the untreated pine, poplar and cypress wood samples, respectively (Table 4.1). Around a 50 % reduction in fungal biomass was obtained in all of the *P. ostreatus* wood samples which had been treated with CuAz (Table 4.1). Furthermore a 75 % reduction was observed in the same samples which had been treated with ACQ. Surprisingly the treatment of poplar wood blocks with CuAz and ACQ had little impact on the chitin levels in these samples resulting from growth of *D. concentric*. Following all preservative treatments, the production rate of chitin was significantly reduced (Table 4.1 and Fig .4.1).

Table 4. 1. Chitin content of fungal biomass from treated and untreated wood following inoculation by the test fungi.

Wood species	Preservatives	Chitin (mg/kg)		
		Fungi		
		TRV	PLO	DAC
Pine	untreated	199.5	286.9	203.1
	CuAz	114.1	148.7	85.3
	ACQ	90.8	68.0	91.0
Poplar	untreated	257.6	373.4	171.7
	CuAz	194.7	154.4	137.1
	ACQ	189.0	140.6	142.9
Cypress	untreated	131.4	321.5	258.1
	CuAz	79.5	141.4	73.7
	ACQ	62.2	104.0	96.8

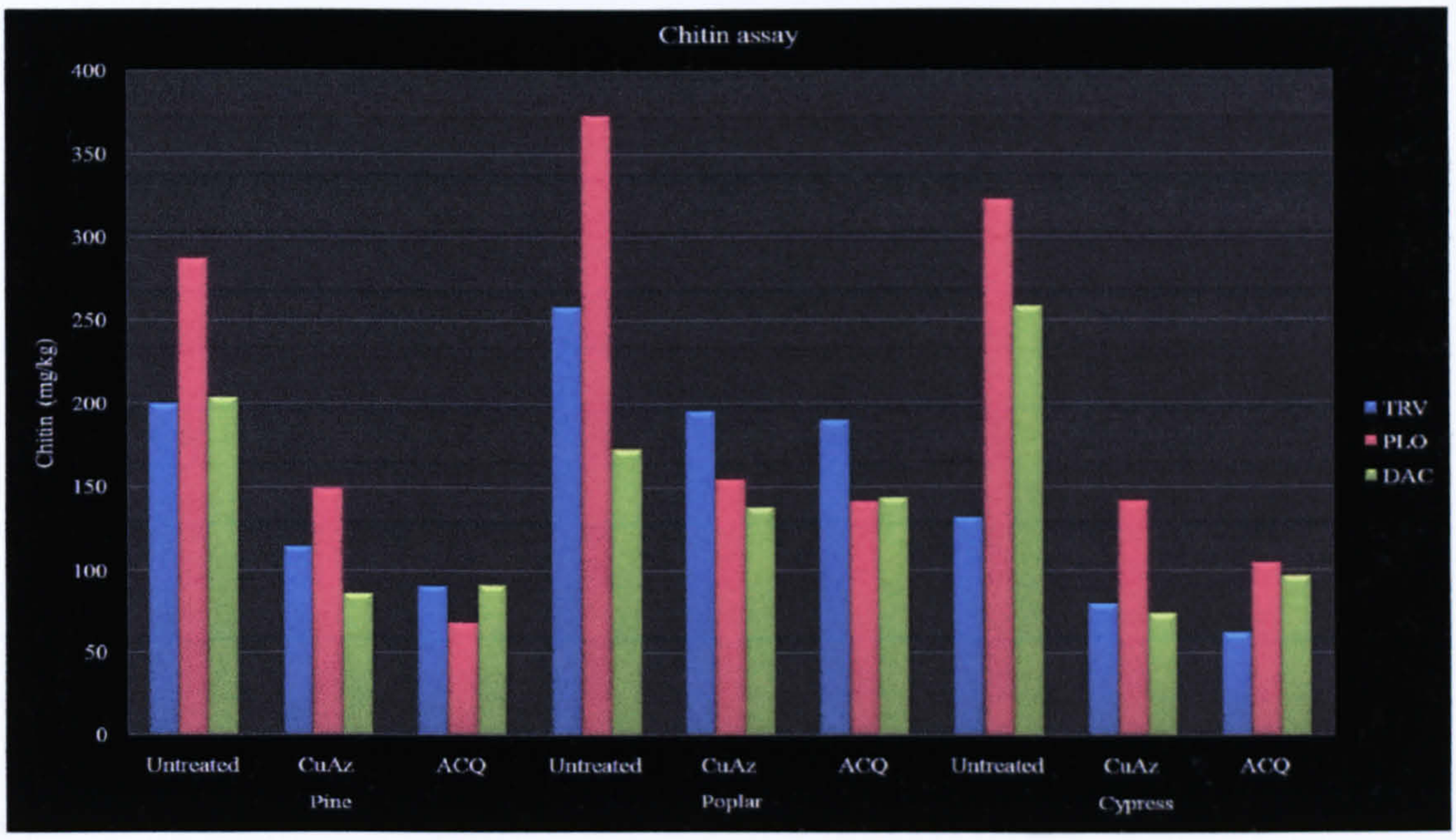


Fig. 4. 1. Comparative chitin assays of fungal biomass on the different wood types.

Discussion.

There is a strong requirement for the accurate determination of fungal biomass, for example, in different branches of ecology, food control, bioremediation and forestry. As chitin is found almost exclusively in fungi it is frequently used by environmental microbiologists as an indicator to measure the fungal biomass in various substances, especially in wood. When fungal biomass cannot be separated from a solid substrate, growth of the fungus can be monitored by measuring a specific chemical component. Chitin, a cell wall constituent of most fungi, is the component most commonly measured (Ride & Drysdale, 1972; Donald & Micocha, 1977). Comparison of the initial amount of a fungal component in a substrate with the increase in amount of that component caused by fungal growth determines the suitability of that component as an indicator of early or limited fungal growth. On that basis, the chitin assay method is a very useful method to determine fungal growth in wood samples (Braid & Line, 1981). Commonly colorimetric determinations are required for the chitin measurement but these involve multiple step processes which may interfere with the accuracy of the results. Thus, other chromatographic techniques have been developed to measure the chitin content, such as ion-exchange chromatography and amino acid analysis (Young & Games, 1993). Most of the methods for determining the chitin content include the liquid extraction and saponification to give total chitin. The methods for extraction typically require larger samples and larger volumes of reagents. Thus, it highlights the importance to develop a new method which requires small amount of samples. This investigation was also done by using HPLC and a development of an HPLC based chitin assay is very useful for the measurement of fungal growth in solid substrate materials, such as wood (Dawson-Andoh, 2002). The results from the current study indicate that biomass based on chitin estimation can be used to assess the fungal biomass present in the wood samples and natural products. The results of the three test fungi indicated

that the chitin content of all three fungi were different and that fungal biomass was significantly reduced when wood samples were pre- treated with preservatives.

Ergosterol assays

Results.

Table 4. 2 shows the levels of ergosterol found in a variety of wood samples treated with preservative compared with controls and native fungal samples. The wood samples pine, poplar and cypress and fungal samples *T. versicolor* and *P. ostreatus* were used to determine ergosterol contents. A significant variation in the level of ergosterol was found to occur between all treated samples. The samples treated with ACQ showed lower level of ergosterol content than CuAz treated samples. 0.99 µg/100 g and 4.71 µg/100 g of ergosterol content were found in the wood samples poplar treated with ACQ and inoculated with *T. versicolor* and poplar treated with ACQ and inoculated with *P. ostreatus*, respectively (Table 4.2). While, 1.65 and 5.94 µg/100 g of ergosterol content were found in the wood samples Poplar treated with CuAz and inoculated with *T. versicolor* and Poplar treated with CuAz and inoculated with *P. ostreatus*, respectively (Table 4. 2). 2.17 µg/100 g of ergosterol was extracted from the untreated poplar inoculated with *T. versicolor*, while, 21.17 µg/100 g of ergosterol was extracted from the untreated polar inoculated with *P. ostreatus* (Table 4. 2). High levels of ergosterol were recorded in both *T. versicolor* and *P. ostreatus*, 97.37 µg/100 g and 128.91 µg/100 g, respectively. In all the treated samples ergosterol content decreased significantly compared to the control wood samples and the native fungal samples (Table 4.2; Figs 4.2, 4.3, 4.4).

Table 4. 2. Ergosterol content of fungal biomass from wood.

Samples	Ergosterol (µg/100g dry weight)
T. versicolor	97.37
Poplar TRV untreated	2.17
Poplar TRV CuAz	1.65
Poplar TRV ACQ	0.99
P. ostreatus	128.91
Poplar PLO untreated	21.17
Poplar PLO CuAz	5.94
Poplar PLO ACQ	4.71

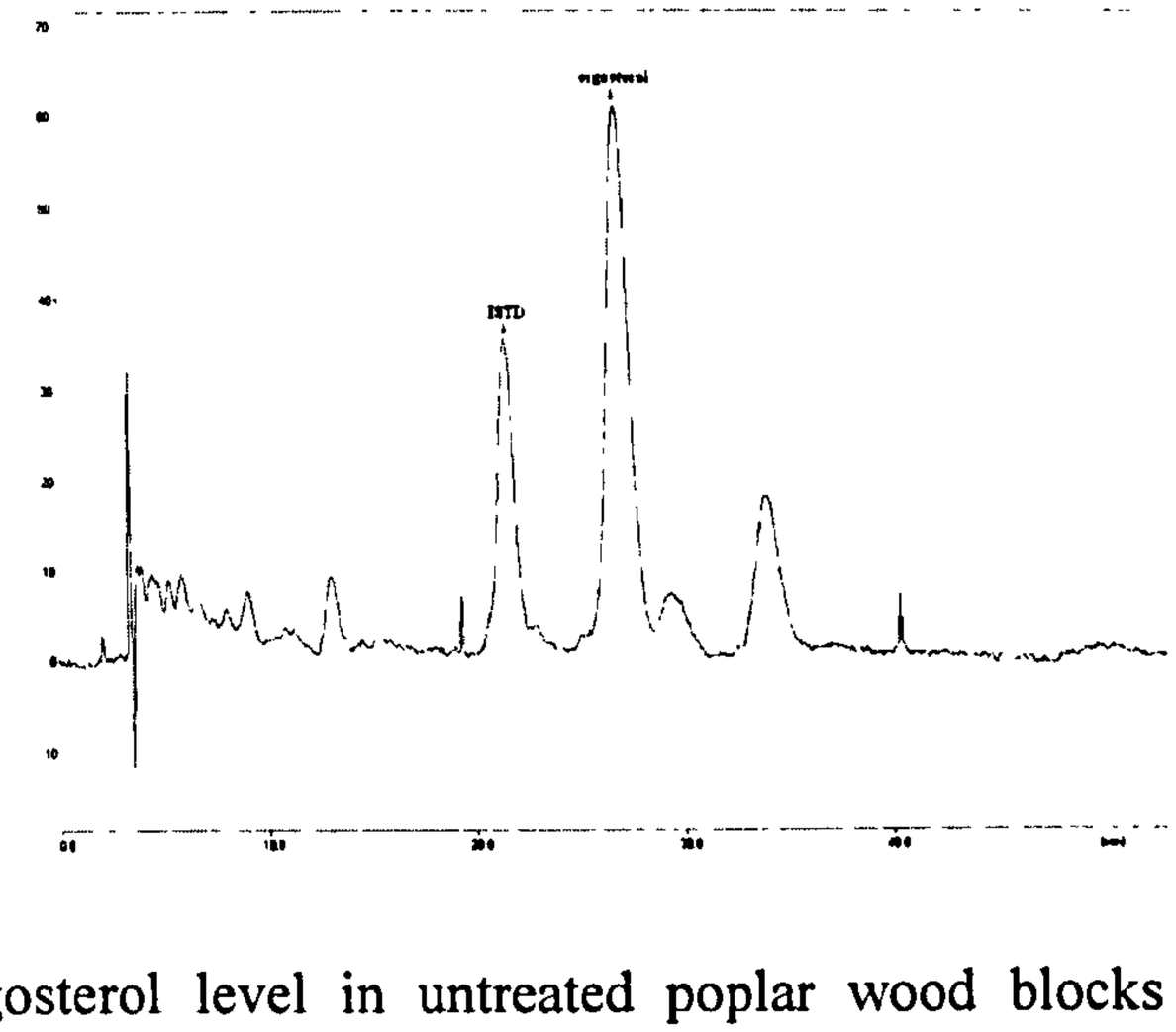


Fig. 4. 2. Ergosterol level in untreated poplar wood blocks inoculated with *T. versicolor*.

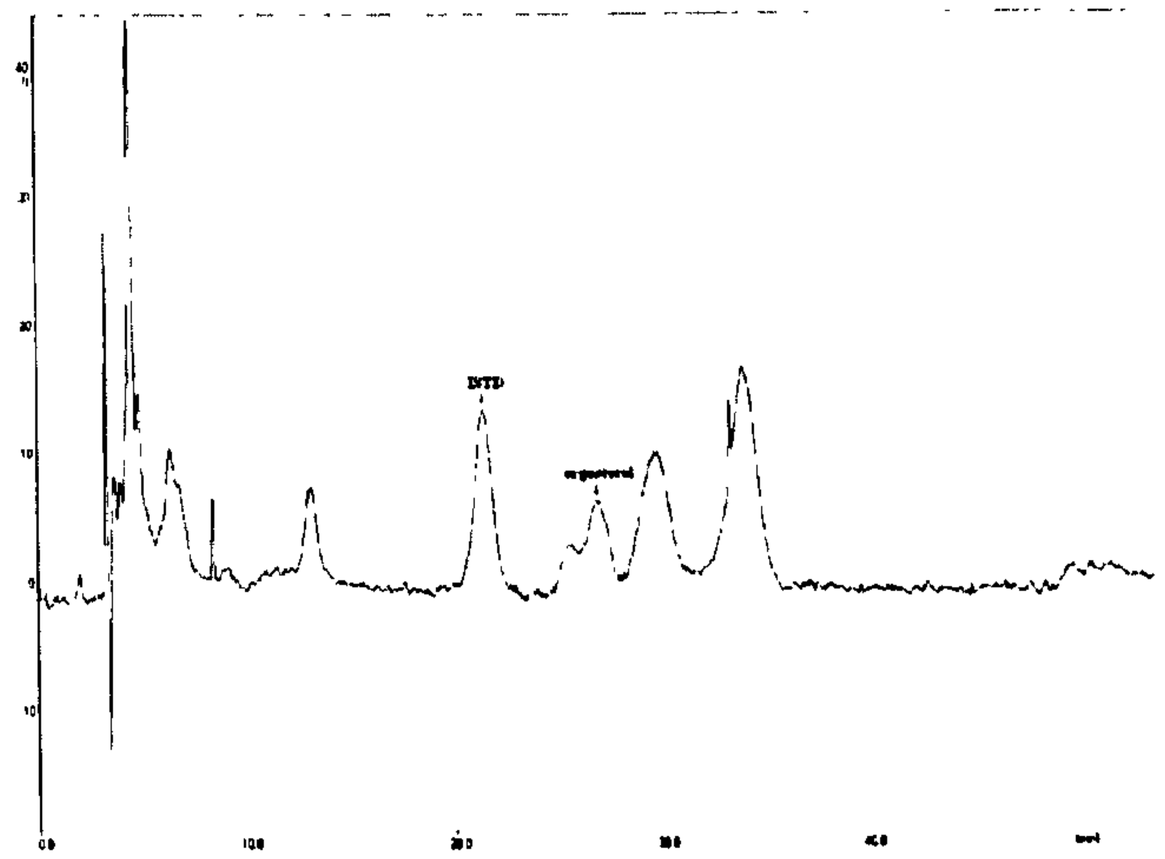


Fig. 4. 3. Ergosterol level in poplar wood blocks treated with CuAz and inoculated with *T. versicolor*.

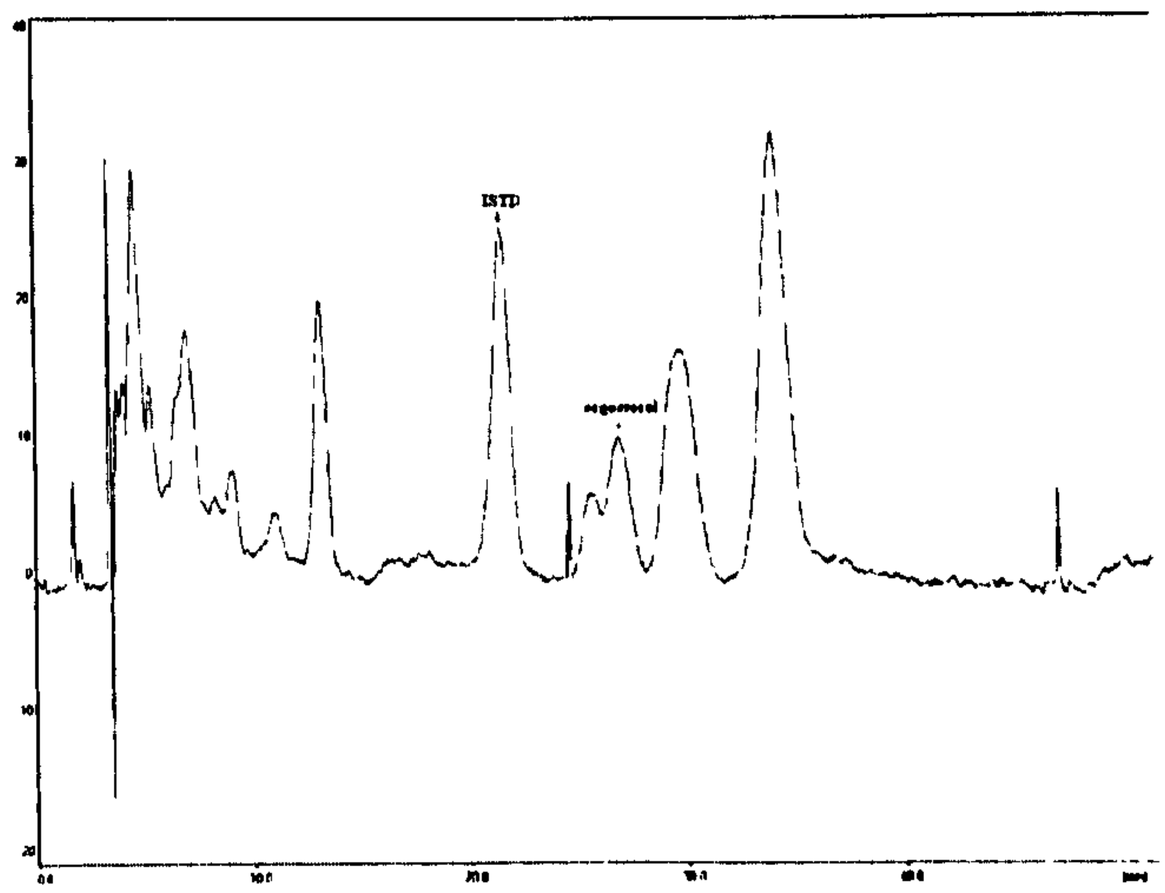


Fig. 4. 4. Ergosterol level in poplar wood blocks treated with ACQ and inoculated with *T. versicolor*.

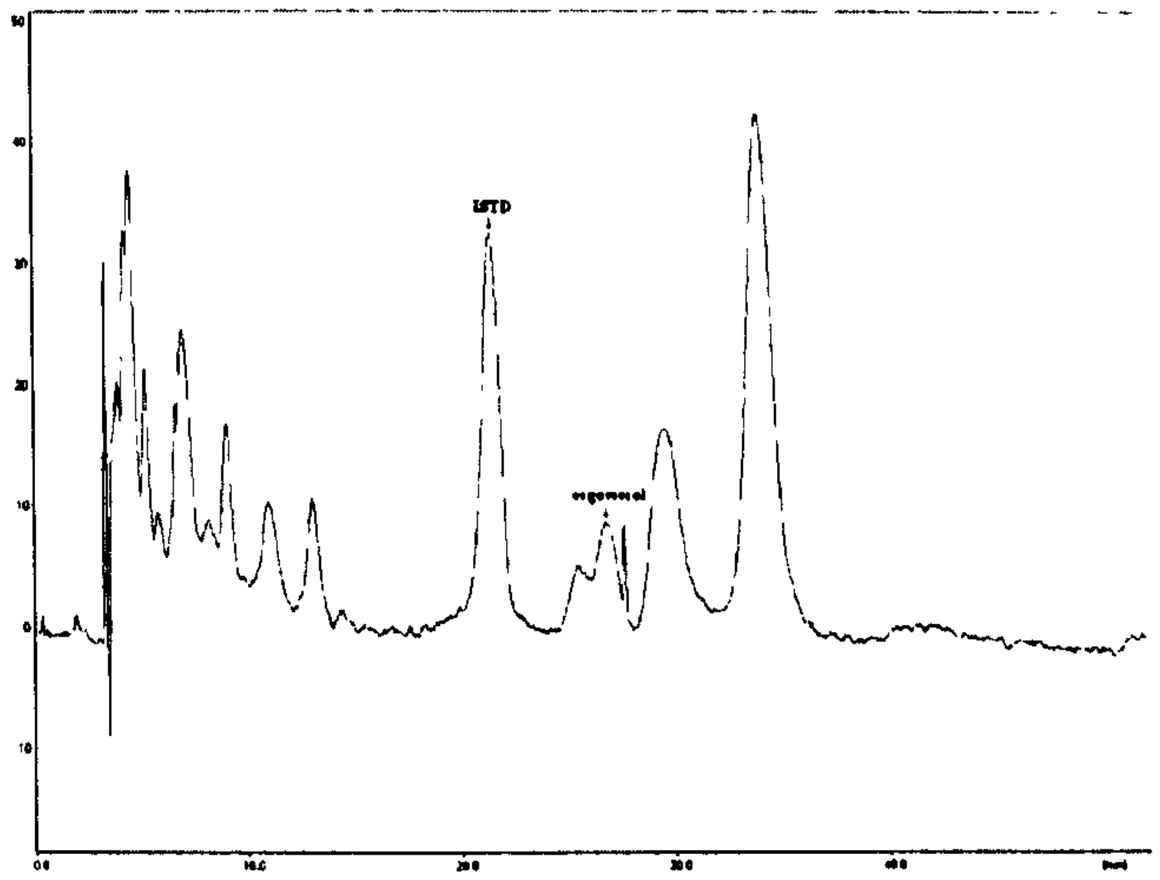


Fig. 4. 5. Ergosterol level in untreated poplar wood blocks inoculated with *P. ostreatus*.

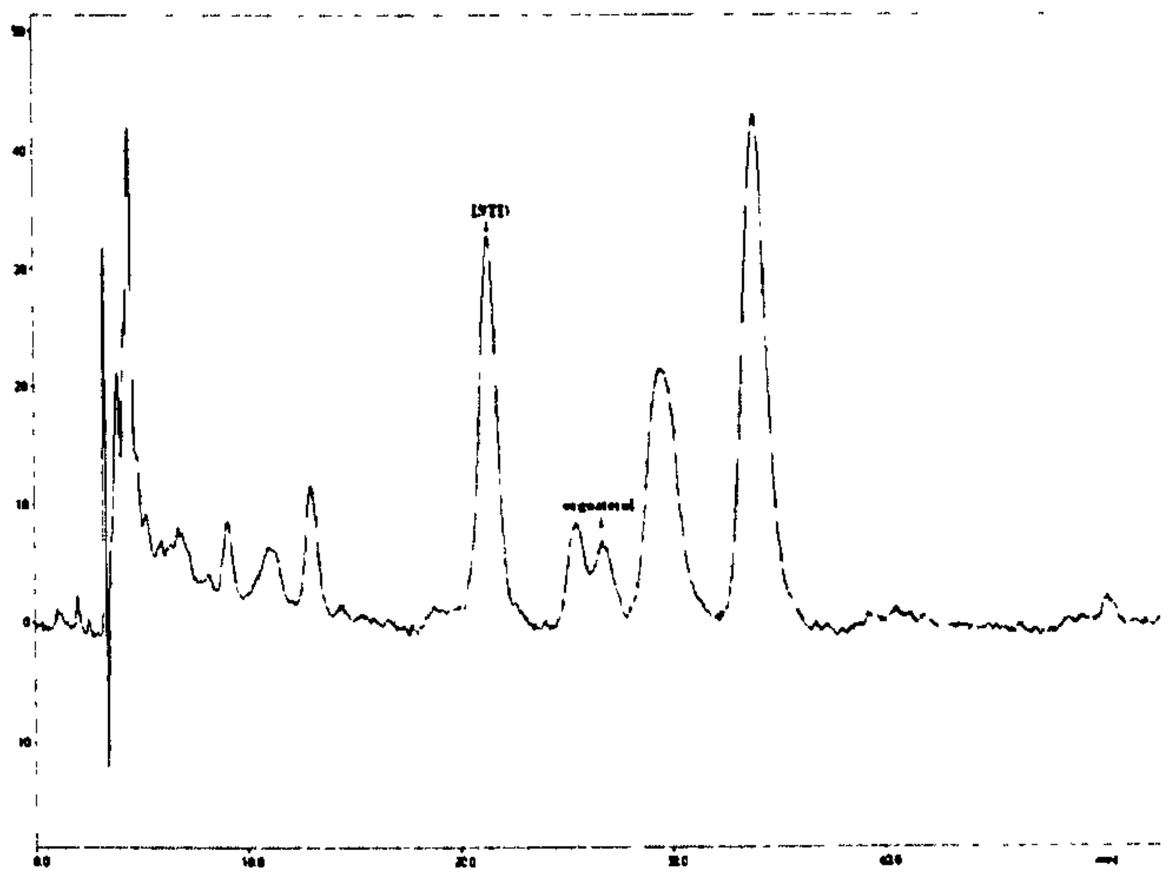


Fig. 4. 6. Ergosterol level in poplar wood blocks treated with CuAz and inoculated with *P. ostreatus*.

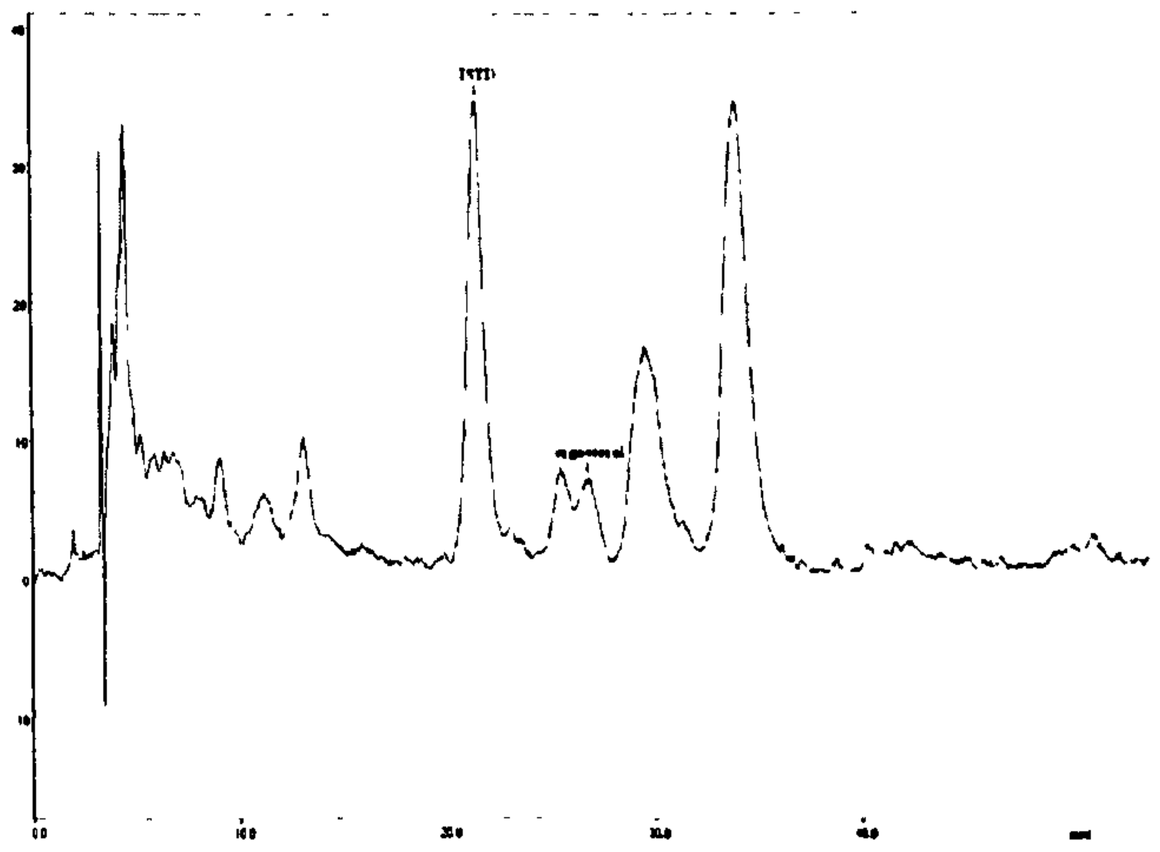


Fig. 4. 7. Ergosterol level in poplar wood blocks treated with ACQ and inoculated with *P. ostreatus*.

Discussion.

Ergosterol measurement is mainly analyzed by using the method of Seitz *et al.* (1977, 1979). This method involves methanolic extraction, alkaline saponification, and C18 reversed-phase high performance liquid chromatograpic (HPLC) separation with untraviolet (UV) detection at 282 nm. The ergosteol method is a major approach to the estimation of fungal biomass, and it depends on the assumption that ergosterol is rapidly degraded upon death of fungal hyphae. Throughout the years since the ergosterol method was developed, there have been indications that perhaps ergosterol is not degraded as rapidly as assumed. Schwadorf and Müller (1989) showed that ergosterol was stable during cereal storage, but fungal growth may have been possible in that study. Ergosterol levels in various fungi typically range from about 0.1 to 15 mg/g of dry weight (Newell *et al.* 1987). For a given fungal species, environmental factors such as age, medium, moisture and temperature may affect the yield of ergosterol produced per unit of fungal biomass. Thus, caution must be used when one attributes absolute amounts of fungal biomass on the bsis of measured ergosterol levels. According to the current investigation around 65 % of the ergosterol content was reduced in poplar inoculated with TRV which had been pre-treated with ACQ compare to untreated poplar. There was a 75 % ergosterol reduction in poplar inoculated with PLO and pre-treated with ACQ compared to untreated samples. While both CuAz treated wood samples showed higher ergosterol contents than ACQ preservative treated wood samples. Less than 30 % ergosterol content reduction was observed in poplar pre-treated with CuAz and inoculated with *T. versicolor* compared to untreated poplar inoculated with *T. versicolor*. West *et al.* (1987) suggested that it may be more appropriate to use ergosterol for measuring changes in fungal populations. Hence, our results should be of interest to biologists and environmental scientists in many different disciplines where fungi are involved. In addition the ergosterol assay's capability in detection early of growth would be

valuable in studying secondary metabolite synthesis in relation to fungal growth on solid substrates. The ergosterol rate is often correlated with fungal biomass in order to obtain specific biomass production. Since the biomass may be overestimated due to ergosterol in dead fungi specific biomass production by natural fungal communities may possibly be underestimated.

However, based on results obtained here it was found that ergosterol has several advantages over chitin in measuring fungal growth on wood samples. The ergosterol assay was more sensitive, easier and faster than the chitin assay. It was also demonstrated that ACQ preservative is more active compared to CuAz in reducing activity of *T. versicolor* and *P. ostreatus* which are the most important wood decay fungi tested and also the most important in Korea.

CHAPTER 5

Direct observation by light, scanning electron and atomic force microscopy

Introduction.

The anatomy of the wood has been described by e.g. Grosser (1977) and Carlquist (2001) and the relationship between wood anatomy and microbial colonization and wood decomposition has been reviewed elsewhere (Eriksson *et al.* 1991; Daniel, 2003). Softwood is relatively homogeneous in structure and consists primarily of tracheids, axial parenchyma and epithelial cells surrounding resin canals. Tracheids are dual purpose cells combining properties of both mechanical support and water conduction. By comparison, hardwood is more heterogeneous, and its water conducting functions are served by vessels, while fibres or fibre tracheids mainly supply mechanical strength and support. Parenchyma is a more prominent feature of hardwood than softwood, with varying amounts of axial parenchyma (Schwarze, 2007). Thus the structure and chemical composition of wood has a significant influence on its degradation and decomposition by microorganisms and the resulting patterns of decay. The type of cell, chemical composition, wood decomposition and cell wall morphology may all govern the effected of enzymes on the woody substrate (Eriksson *et al.* 1990).

Wood consists of an orderly arrangement of cells with walls composed of varying amounts of cellulose, hemicellulose and lignin (Blanchtte, 2000). Lignin is distributed throughout the secondary wall and compound middle lamella but the greatest concentration is in the middle lamella (Eriksson *et al.* 1990). These compounds are generally recalcitrant and only select microorganisms are able to cause degradation.

Results.

The impregnation of wood samples with preservatives is attended by certain difficulties which are, firstly, the extremely variable absorption of the different preservatives in relation to wood species, and, secondly, the non-uniform character of the penetration by fungi in softwood and hardwood. Some wood species are readily degraded by fungi and may also exhibit poor penetration by the preservatives.

Anatomical observations of pine by light and scanning electron microscopy.

The results of the wood decay testing of pine by *T. versicolor*, *P. ostreatus* and *D. concentrica*, and of wood blocks treated with ACQ and CuAz were observed by LM and SEM. Two types of cells shown in Figure 5. 1, 5. 3 (LM) and in Figure 5. 10, 5. 13 (SEM) occur in all softwood and these are parenchyma cells and epithelial cells. The nature of the epithelial cells provides a means of separating *Pine* from the other genera as the epithelial cells are mostly thick-walled in the fusiform rays. In Figure 5. 1 these cells differ from the longitudinal tracheids of pine has developed. There are pits in the walls of the ray parenchyma of softwood and bordered pits in the softwood tracheids (Figures 5. 2 and 5. 7). White rot decay by *T. versicolor* and *P. ostreatus* in pine shows degradation of all cell wall components. Advanced wood rot is seen with extensive degradation of tracheids ((Figures 5. 2 and 5. 7). ACQ is more effective then CuAz in reducing wood decay.

Anatomical observations of poplar by light and scanning electron microscopy.

The results of wood decay of untreated poplar by *T. versicolor*, *P. ostreatus* and *D. concentrica* and of wood blocks treated with ACQ and CuAz were observed by LM and SEM. Vessel elements found in poplar which is a hardwood are shown in Figures 5. 3, 5. 5 (LM), 5. 8 and 5. 14 (SEM). In Figure 5. 8 a diffuse-porous vessel element with scalariform perforation plates can be observed. Perforation plates provide pathways for liquid flow from vessel element to vessel element and

intervessel pits from vessel to vessel. Intervessel pitting can be seen and is very obvious and occurring mainly on the tangential walls of vessel elements (Figures 5. 4, 5. 5 (LM) and 5. 8, 5. 14 (SEM)). Figures 5. 5 and 5. 8A, 5. 11A, 5. 14A illustrate decay by white-rot fungi *T. versicolor*, *P. ostreatus* and *D. concentrica* in poplar. Hyphae can be seen within the damaged area in Figure 5. 4A. ACQ is more effective than CuAz in protecting poplar wood from decay.

Anatomical observations of cypress by light and scanning electron microscopy.

Wood decay of untreated cypress by *T. versicolor*, *P. ostreatus* and *D. concentrica* and of wood blocks treated with ACQ and CuAz were observed by LM and SEM. Figure 5. 6 (LM) and 5. 9 (SEM) illustrate axial parenchyma cell distribution and a further difference is the presence of bordered pits on axial parenchyma cells of cypress Figure 5. 15 (SEM). There are cupressoid pits which resemble piceoid pits but differ from them in that the aperture is included and elliptical rather than linear as in the piceoid type of cypress (Figure 5. 12A). Figures 5. 12. C and D, 5. 15. B and C (SEM) there are the longitudinal tracheids general-purpose cells that perform the dual roles of support.

Pine

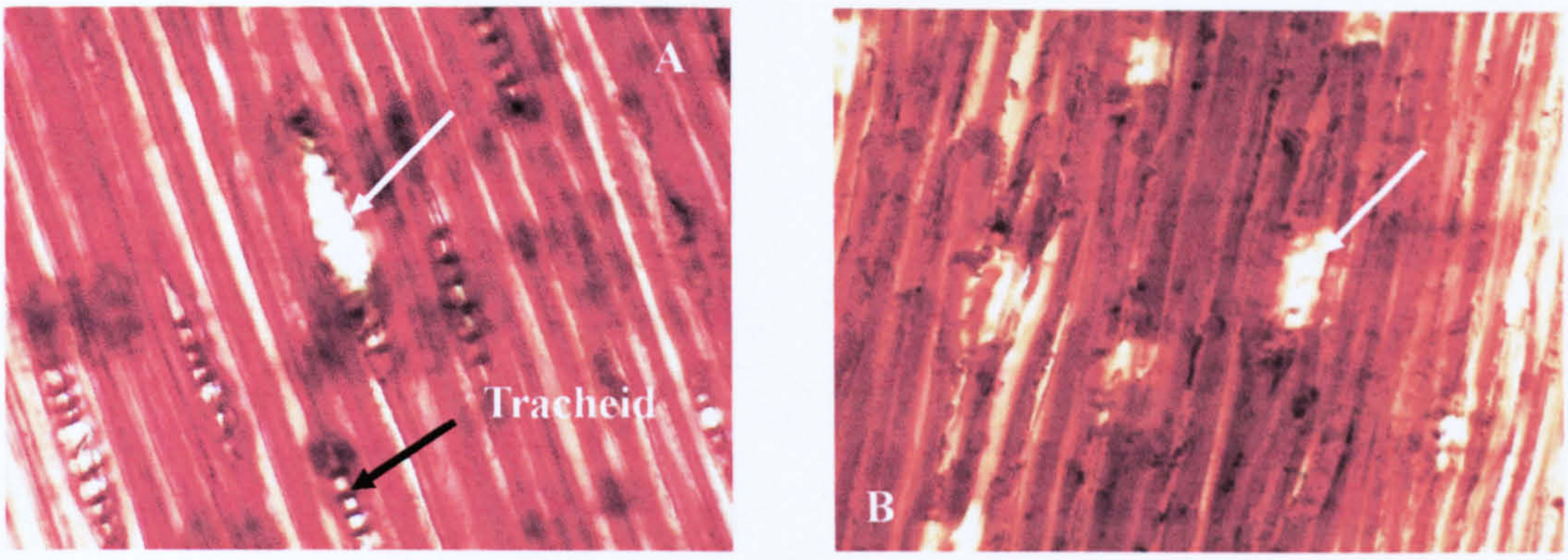


Fig. 5. 1. Examination of pine blocks following inoculation with the test fungi shows important differences. Figs. 1. Illustrates damage caused by *T. versicolor* and *P. ostreatus*. In Fig 5. 1. A the damage caused to parenchyma cells (arrowed). In Fig 5. 1. B. There is more severe damage caused by *P. ostreatus* In Fig 5. 1. A tracheids (dark arrow) can be seen and are still intact.

Pine

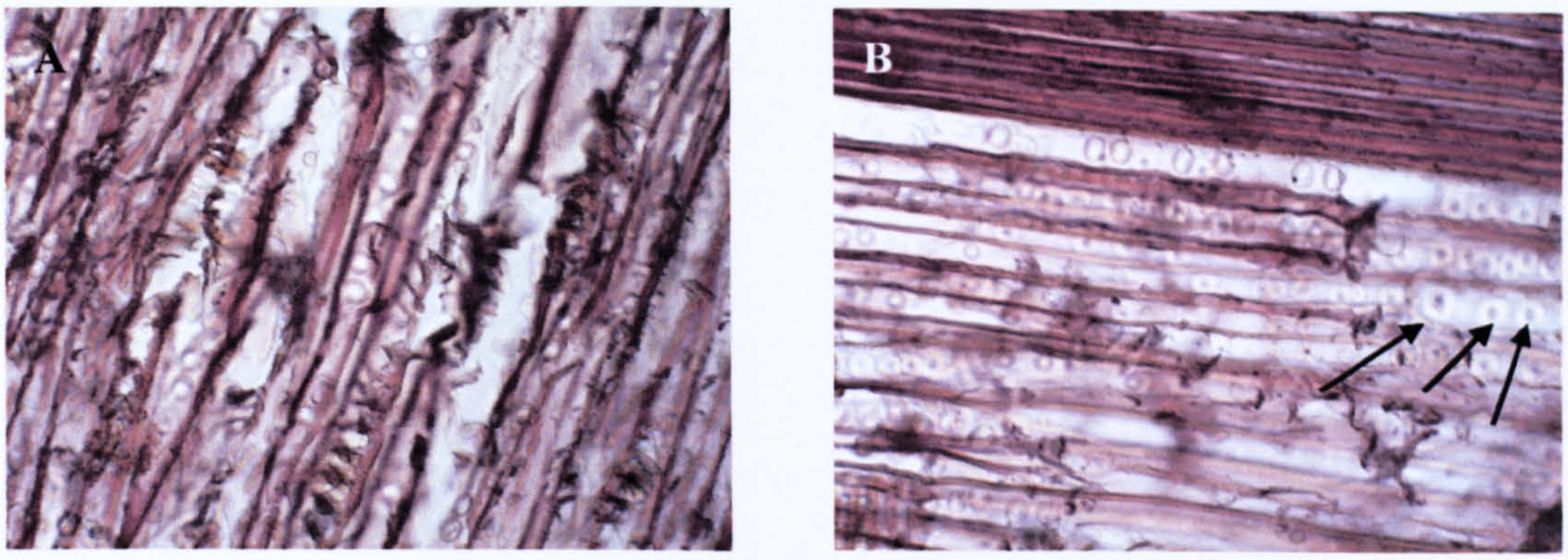
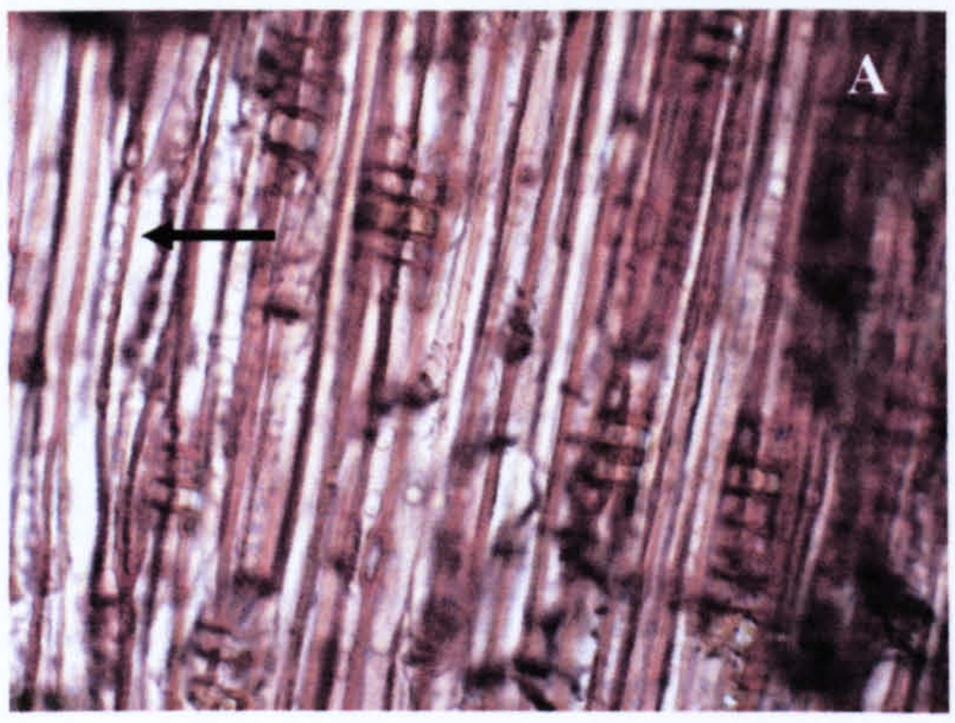


Fig. 5. 2. Examination of pine blocks following inoculation with the test fungi present important differences especially these treated with ACQ preservative compared to untreated wood blocks (Fig.5. 1). Fig. 5. 2. Illustrates cell wall attacked by *T. versicolor* and *P. ostreatus*. In Fig 5. 2. A there is damage caused to fibres of some of the bordered pits. In Fig 5. 2. B. Less damage is caused by *P. ostreatus*. In Fig 5. 2. B. The bordered pits (arrowed) with thickenings on pit membrances can be seen and there are intact.

Pine and Poplar



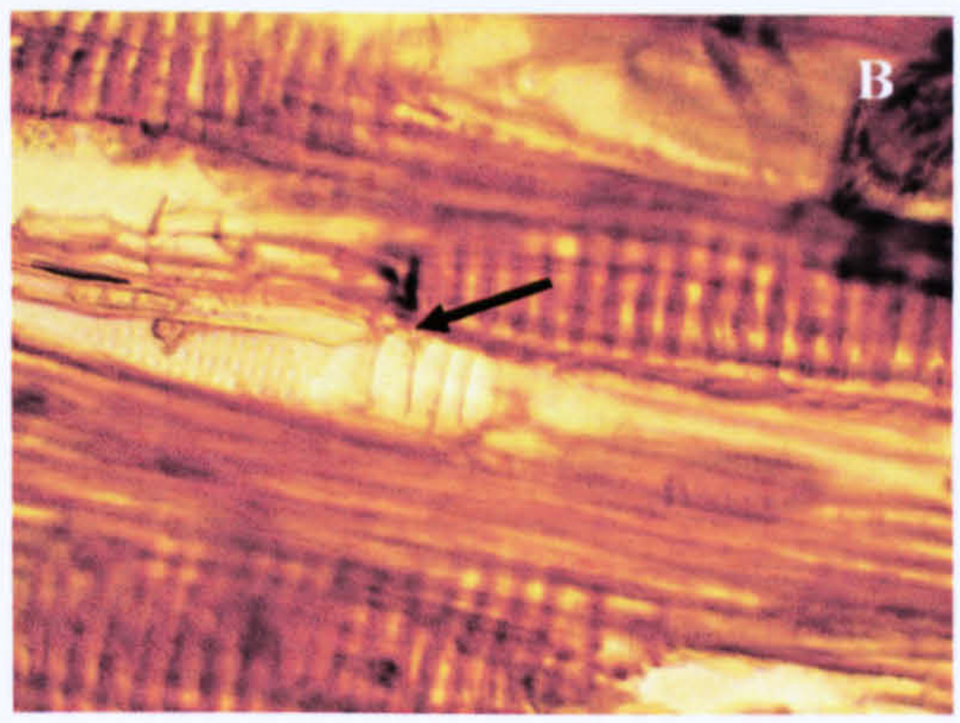


Fig. 5. 3. Examination of pine and poplar blocks following inoculation with the test fungi show important differences when blocks treated with CuAz preservative are compared with untreated wood. Fig. 5. 3. Little damage was caused by *P. ostreatus* and *D. concentrica*. In Fig 5. 3. A. Some damage was caused to parenchyma cells (arrowed). In Fig 5. 3. B. Less damage was caused by *D. concentrica*. In Fig 5. 3. B. The narrow-diameter vessels (arrowed) and rays can be seen and are intact and apparently undamaged.

Poplar

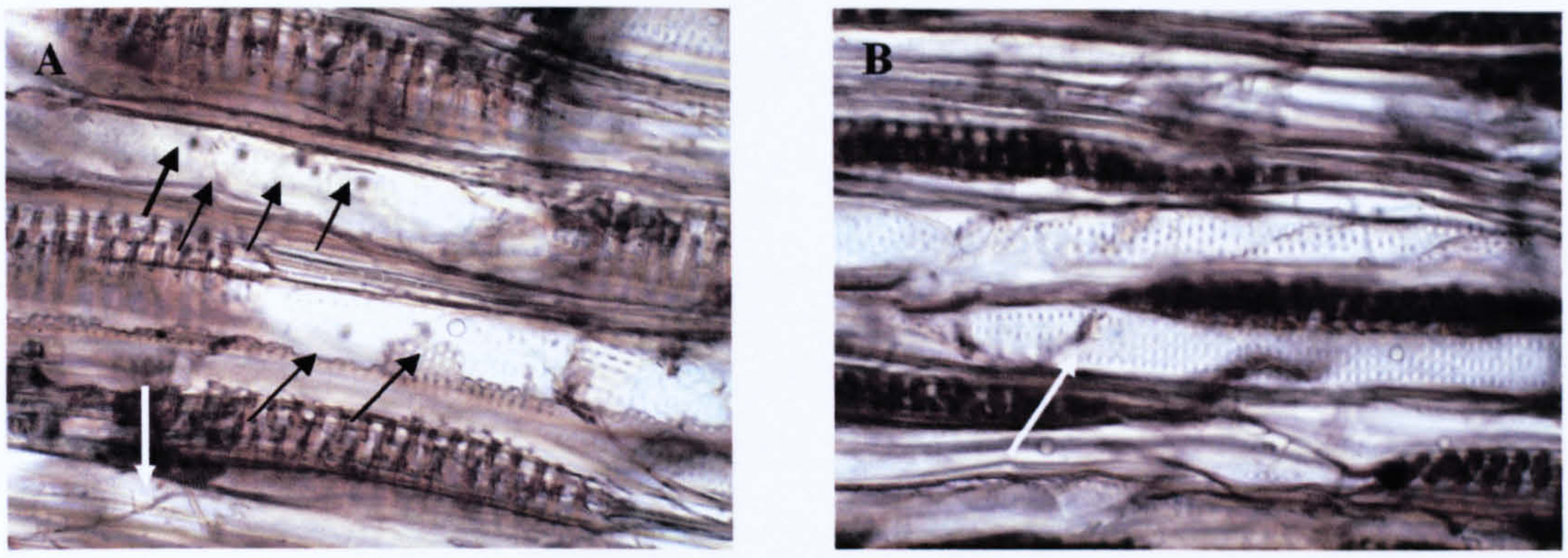


Fig. 5. 4. Examination of poplar blocks following inoculation with the test fungi show important differences when wood blocks treated with CuAz preservative are compared with untreated wood blocks. Fig. 5. 4. Little damage was caused by either *P. ostreatus* or *D. concentrica*. In Fig 5. 4. A. Small amount of damage was caused to the intervessel pitting (dark arrows) by *P. ostreatus*. Fungal hyphae are visible (white arrow). In Fig 5. 4. B. Little damage was caused by *D. concentrica* and the wood cells remain intact and in good condition. In Fig 5. 4. B. The ray structure and ray-vessel pitting (arrowed) is seen to be intact.

Poplar

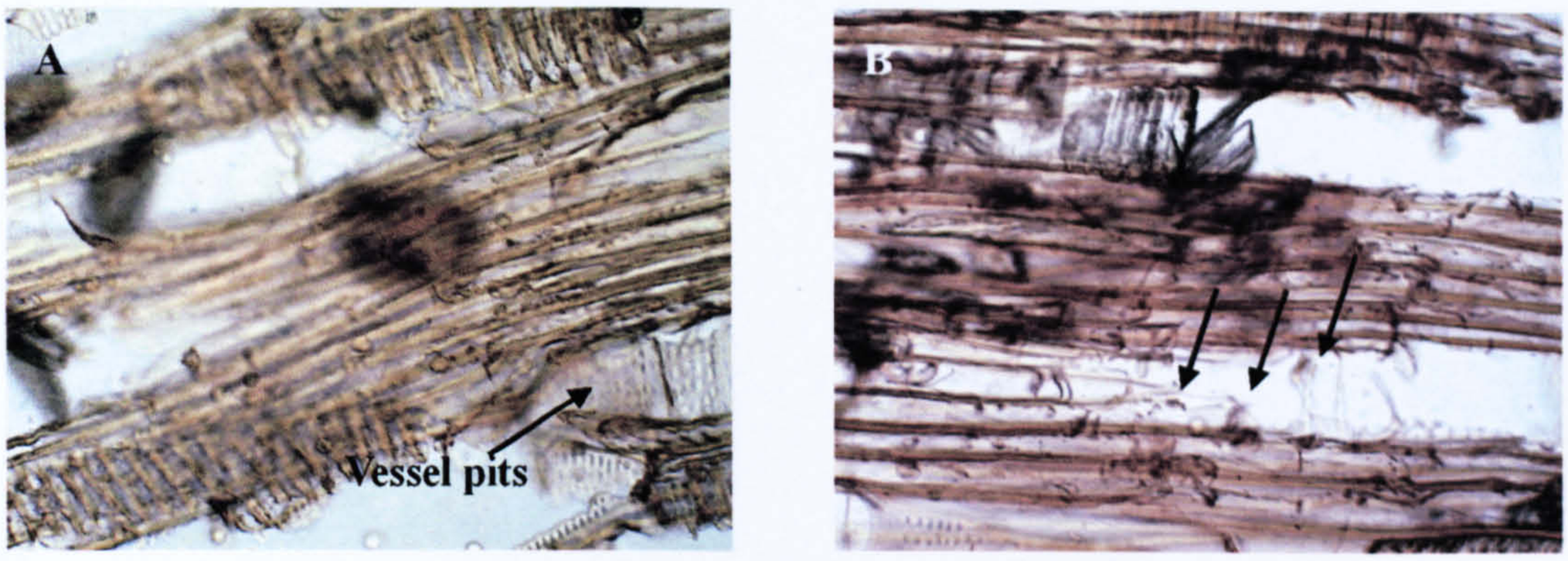


Fig. 5. 5. Examination of poplar blocks following inoculation with the test fungi present important differences when wood blocks treated with ACQ preservative are compared with untreated wood blocks Fig. 5. 5. Some damage was caused by *P. ostreatus* and *D. concentrica*. In Fig 5. 5. A. Damage was caused to vessels and vessel pits shown (arrowed) by *P. ostreatus*. In Fig 5. 5. B Damage was caused by *D. concentrica* which is also apparent as mycelium within cavities of the secondary walls of vessels (arrowed).

Cypress

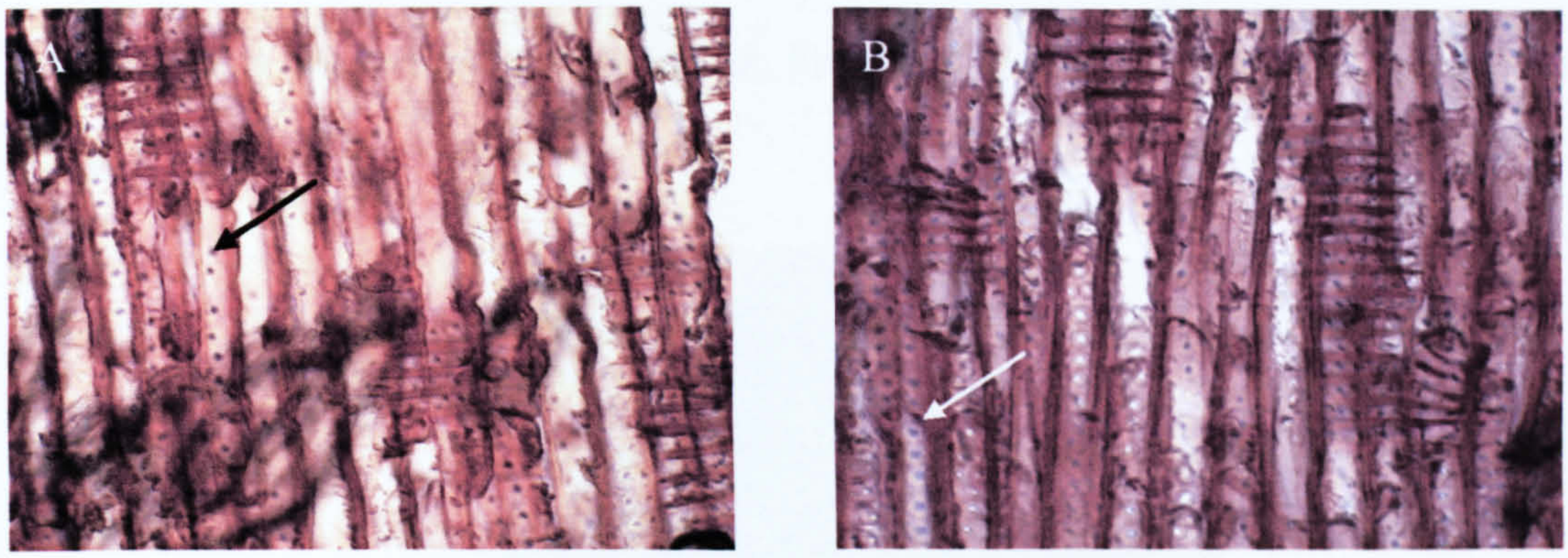


Fig. 5. 6. Examination of cypress blocks following inoculation with the test fungi show important differences when wood blocks treated with ACQ preservative are compared with untreated wood blocks. Fig. 5. 6. Little damage was caused by *P. ostreatus* and *D. concentrica*. In Fig 5. 6. A and B there are perforations in longitudinal view with axial parenchyma cell (arrowed).

Aspects of the interactions between rotted wood structures and fungal activity were investigated by high resolution scanning electron microscopy and a range of different effects of the wood decay fungi could be observed. The effects of the copper preservatives on protection against wood decay was also followed.

A considerable amount of variation exists between microscopic wood decay characteristics caused by the white-rot fungus *T. versicolor* in pine, poplar and cypress.

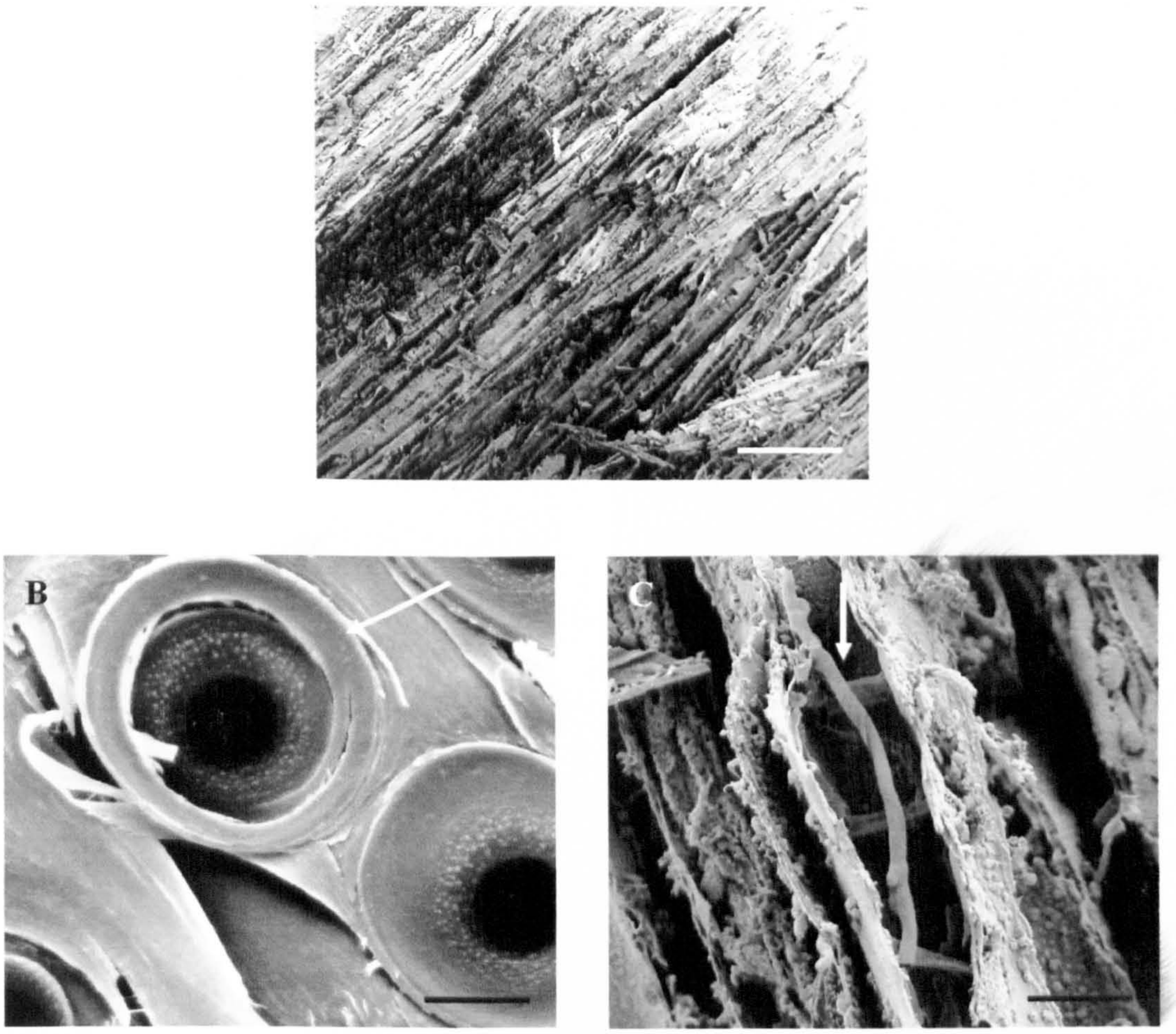
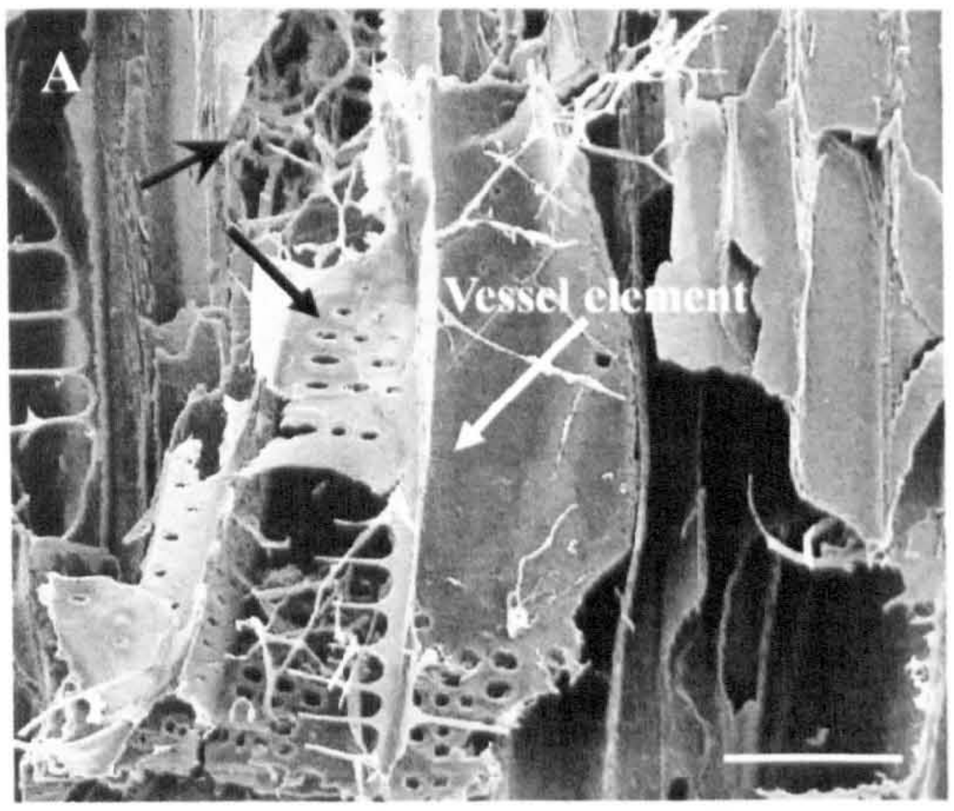
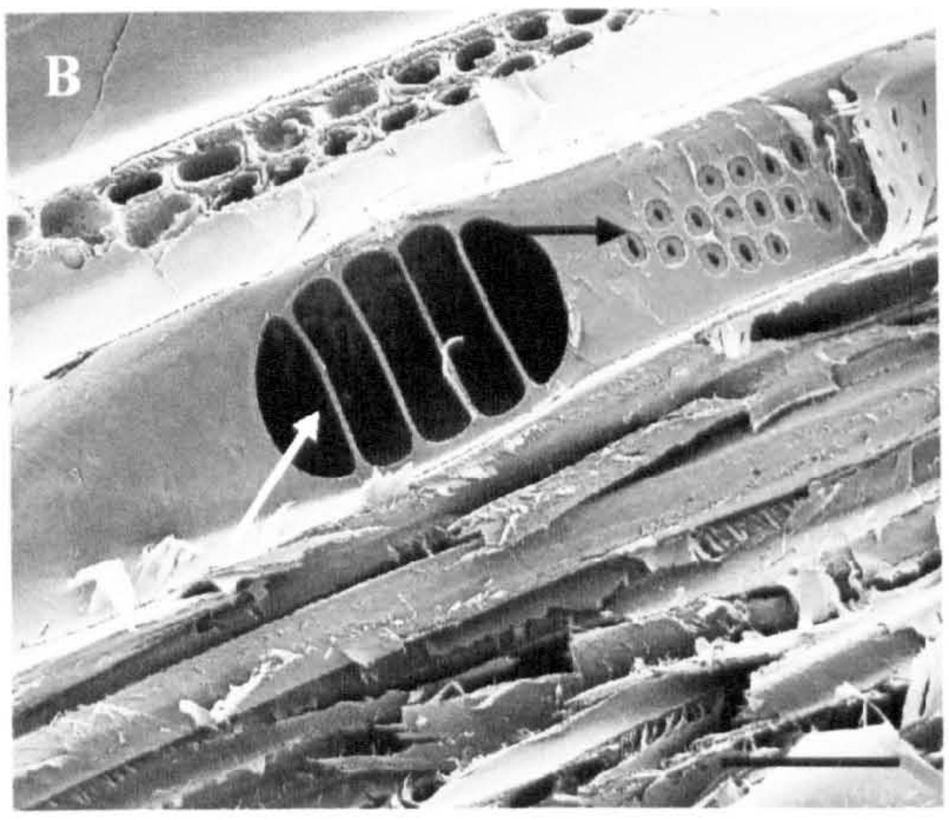


Figure. 5. 7. A-C. Examination of pine blocks following inoculation with *T. versicolor* show important differences between untreated wood blocks and the two preservatives treated wood blocks. Fig. 5. 7. Illustrates the appearance of untreated pine wood blocks, ACQ and CuAz treated pine wood blocks following inoculation with *T. versicolor*. In Fig 5. 7. A. The white-rot wood decay damage caused by *T. versicolor* is apparent. Fig. 5. 7. B. No obvious damage to the pits (arrowed) indicates good protection against *T. versicolor* when ACQ was used. In Fig 5. 7. C. No structural damage to wood treated with CuAz is apparent although there is hyphal growth (arrowed) in a small pocket of the wood where there was probably poor penetration of the preservative. The scale bars = 300 μm, 10 μm and 10 μm.





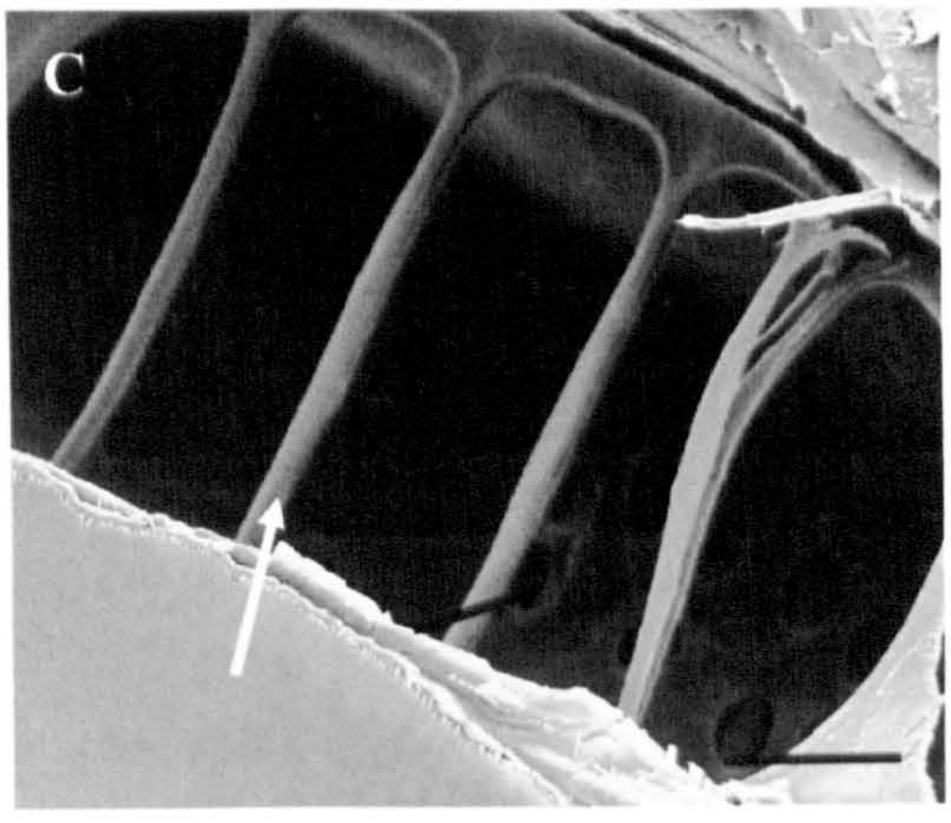
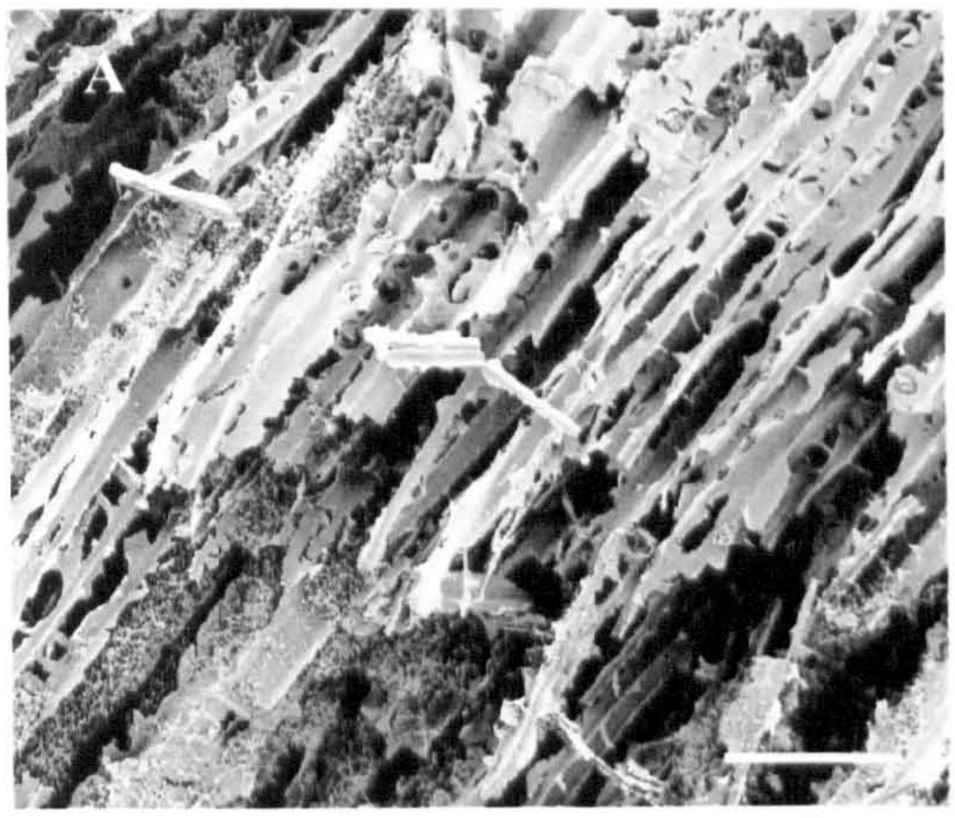


Figure. 5. 8. A-C. Examination of poplar blocks following inoculation with *T. versicolor* indicates important differences between untreated wood blocks and the two preservative treated wood blocks. Fig. 5. 8. Illustrates the appearance of untreated wood blocks, ACQ and CuAz treated poplar wood blocks following inoculation with *T. versicolor*. Fig. 5. 8. Illustrates damage caused by *T. versicolor*. Fig. 5. 8. A. Shows damage caused to a vessel element (white arrow) and vessel tips (black arrow) as well as fungal growth (short arrow). Figs. 5. 8. B and C. Indicates that less damage was caused by *T. versicolor* following treatment with ACQ and CuAz. Clear scalariform perforations (white arrows) and intervessel pitting (black arrows) and intact vessels are clearly visible. The scale bars = 10μm, 100μm, 30μm.



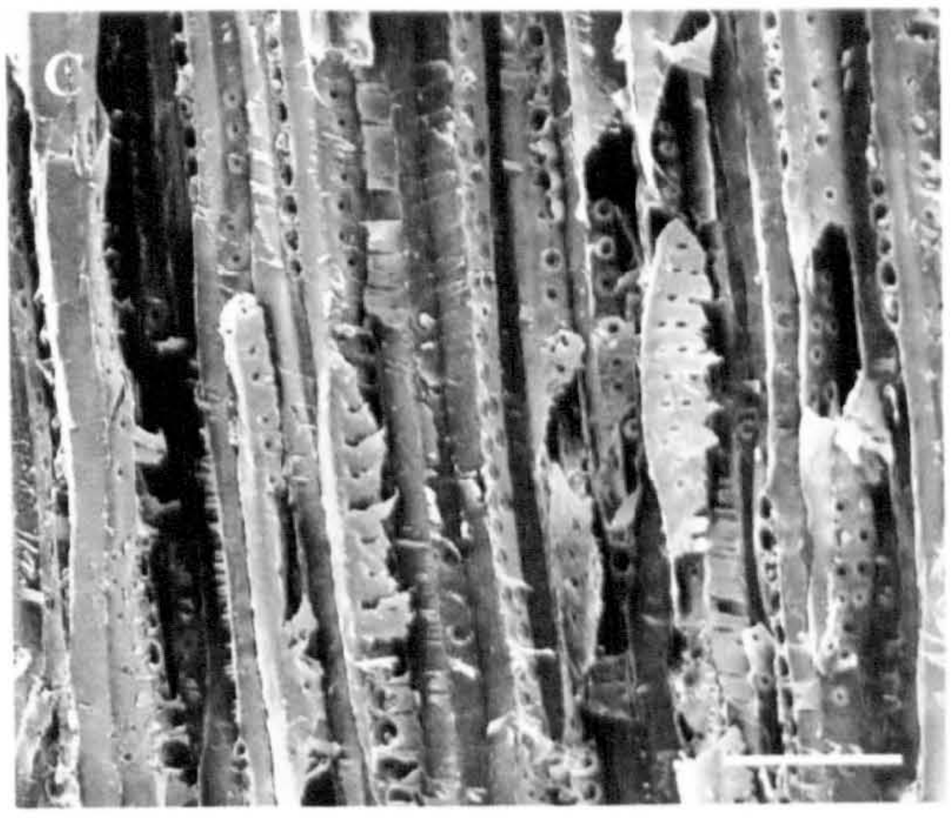


Figure. 5. 9. A-C. Examination of cypress blocks following inoculation with *T. versicolor* demonstrates important differences between untreated wood blocks and the two preservative treated wood blocks. Fig. 5. 9. Illustrates the appearance of untreated wood blocks, ACQ and CuAz treated cypress wood blocks following inoculation with *T. versicolor*. Fig. 5. 9. A. Structural damage with severe pitting and the presence of mycelium. Fig. 5. 9. B. There is less damage caused by *T. versicolor* and the structure of the tracheids and parenchyma cells with the plate area can be clearly seen following treatment with ACQ. Fig. 5. 9. C. A longitudinal view with axial parenchyma cells and perforations visible and these are intact following treatment with CuAz. However minor damage to the wood structure was caused by *T. versicolor*. The scale bars = 200 μm , 200 μm, 100 μm.

There is a considerable amount of the microscopic wood decay characteristics caused by *P. ostreatus* occurs in pine, poplar and cypress.

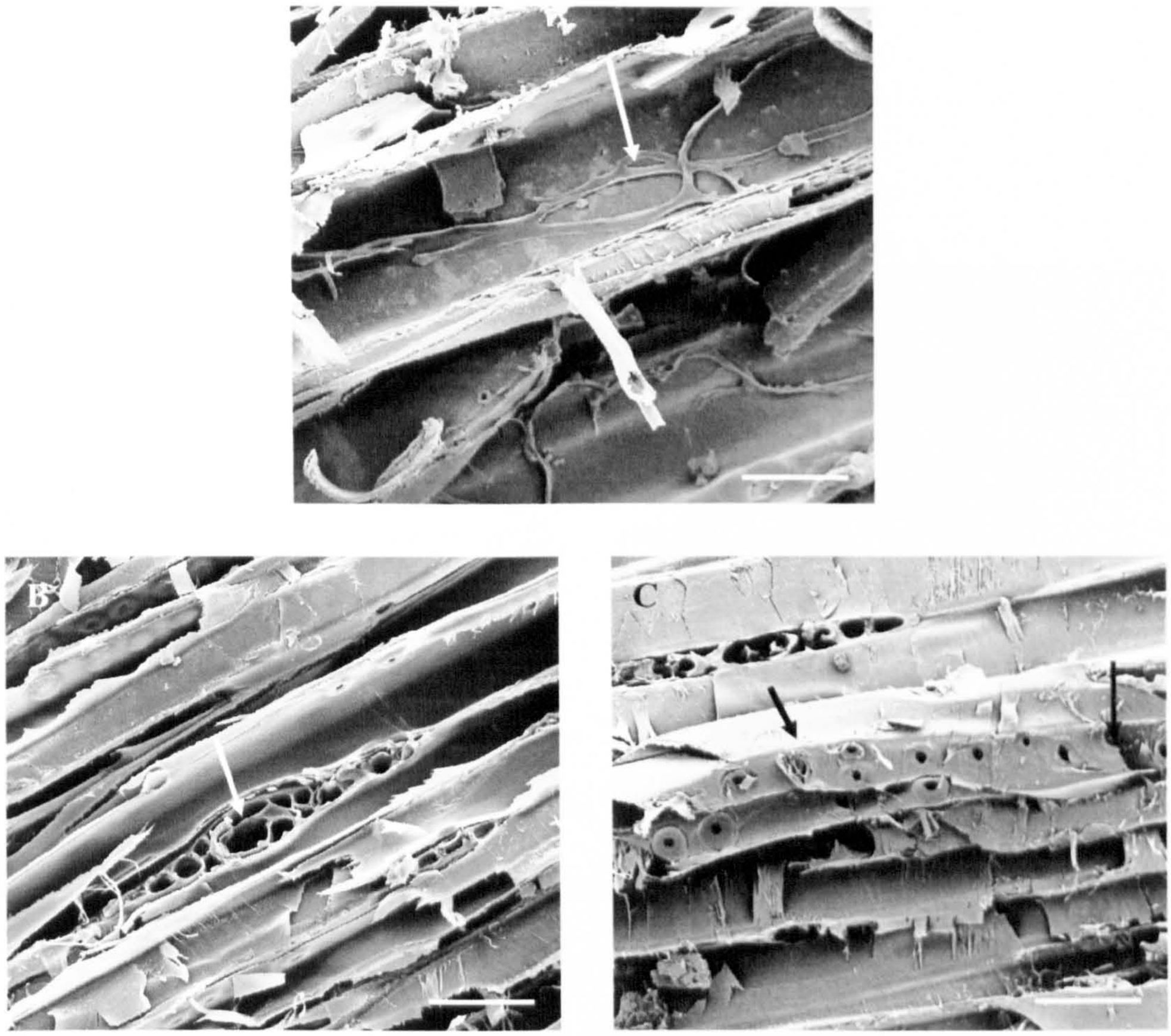
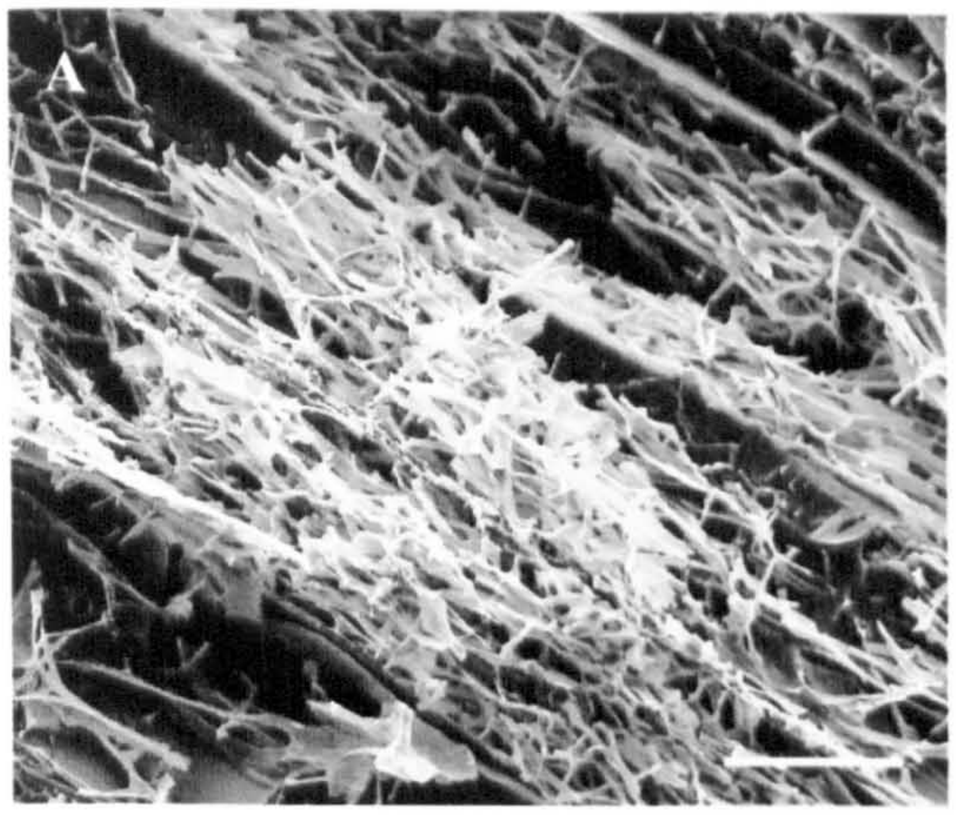
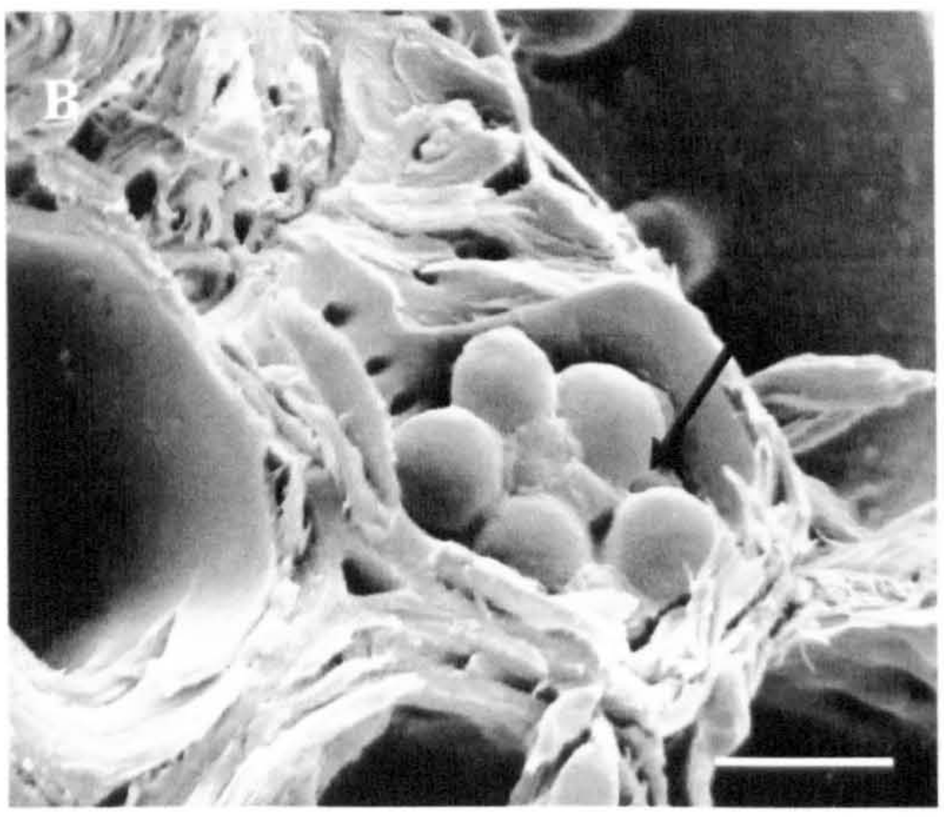


Figure. 5. 10. A-C. Examination of pine blocks following inoculation with *P. ostreatus* indicates important differences between untreated wood blocks and the two preservative treated pine wood blocks. Fig. 5. 10. Illustrates the appearance of untreated wood blocks and the effect ACQ and CuAz on preservation of pine wood blocks following inoculation with *P. ostreatus*. Fig. 5. 10. A. Damage caused by *P. ostreatus* is apparent and fungal hyphae can be observed (arrowed). Fig. 5. 10. B. Partial damage caused to a fusiform ray with a resin canal in the thin walled epithelial cells (arrowed) following treatment with ACQ. Fig. 5. 10. C. Damage was caused by *P. ostreatus* to the rays (arrowed) in wood treated with CuAz. The scale bars = 40 μm, 100 μm, 100μm.





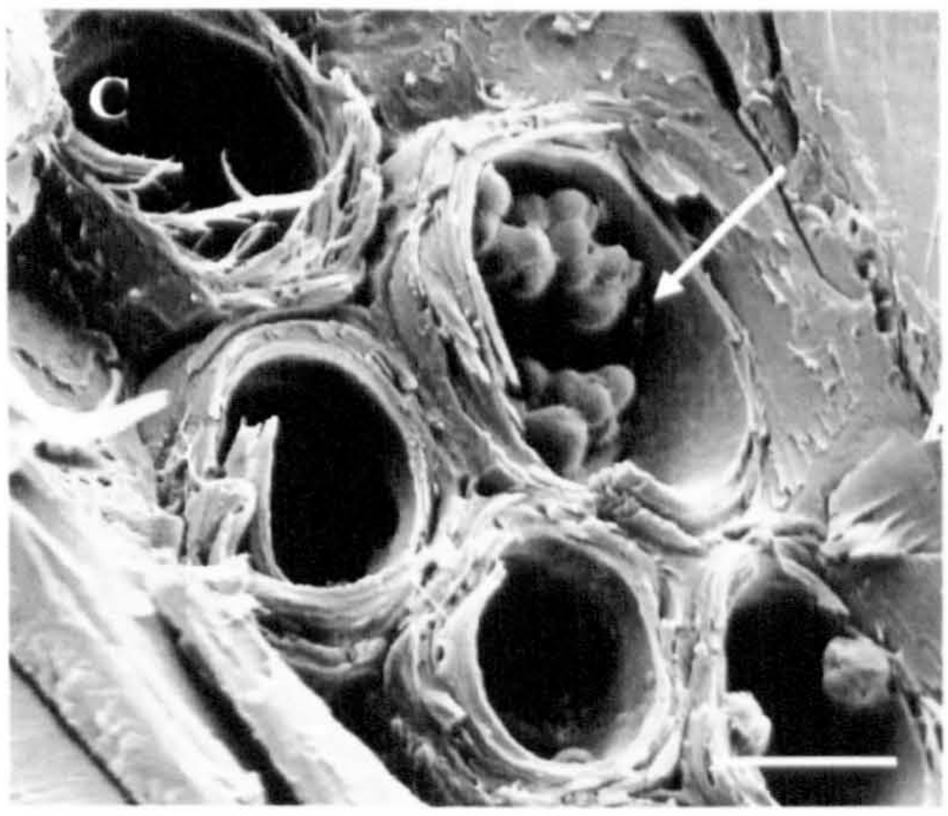


Figure. 5. 11. A-C. Examination of poplar blocks following inoculation with *P. ostreatus* indicates important differences between untreated poplar blocks and the two preservative treated poplar wood blocks. Fig. 5. 11. Illustrates the appearance of untreated wood blocks and the effect of ACQ and CuAz on preservation of poplar wood blocks following inoculation with *P. ostreatus*. Fig. 5. 11. A. Illustrates damage caused by *P. ostreatus* with its white bleached appearance and development of cavities in the wood. Figs. 5. 11. B and C. ACQ and CuAz preservative treatment has reduced the damage caused by *P. ostreatus* with mainly intact cells present. The presence of fungal spores (arrowed) was observed. The scale bars = 100 μm, 10 μm, 10μm.

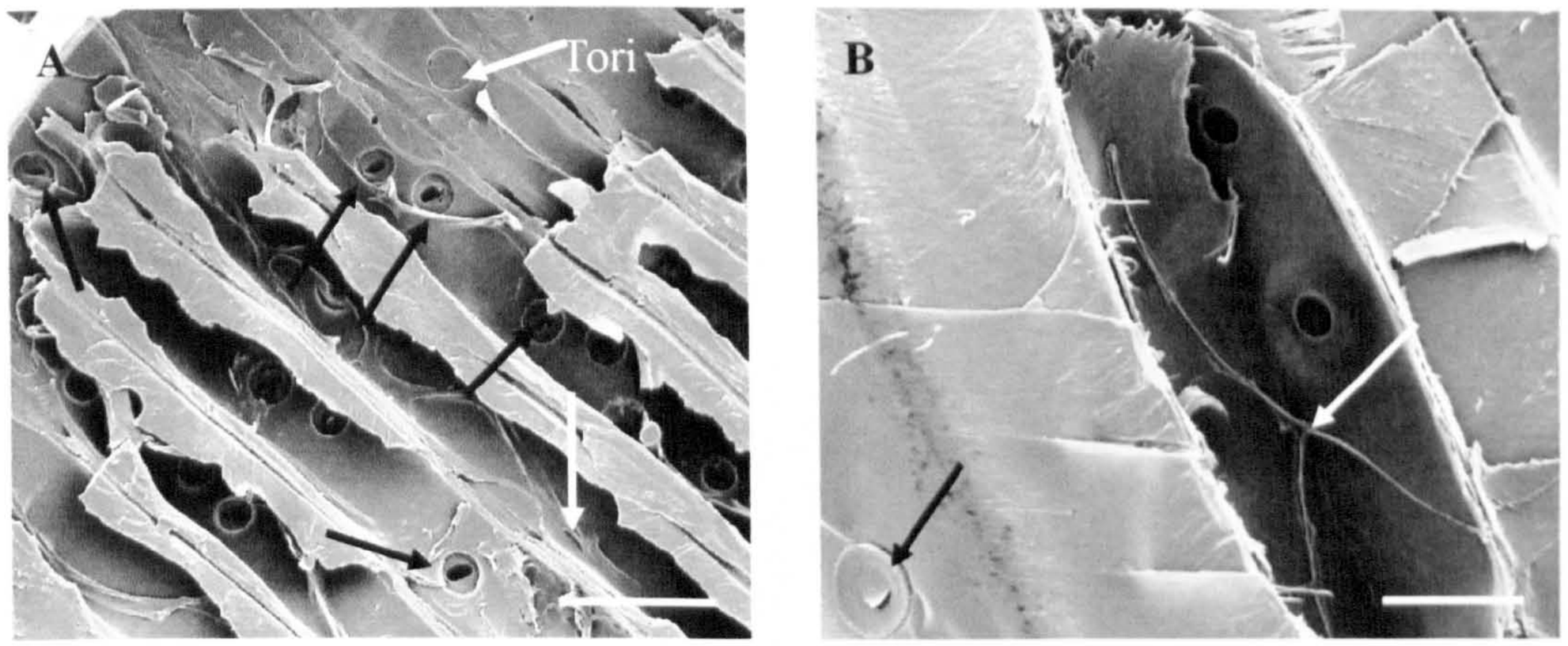
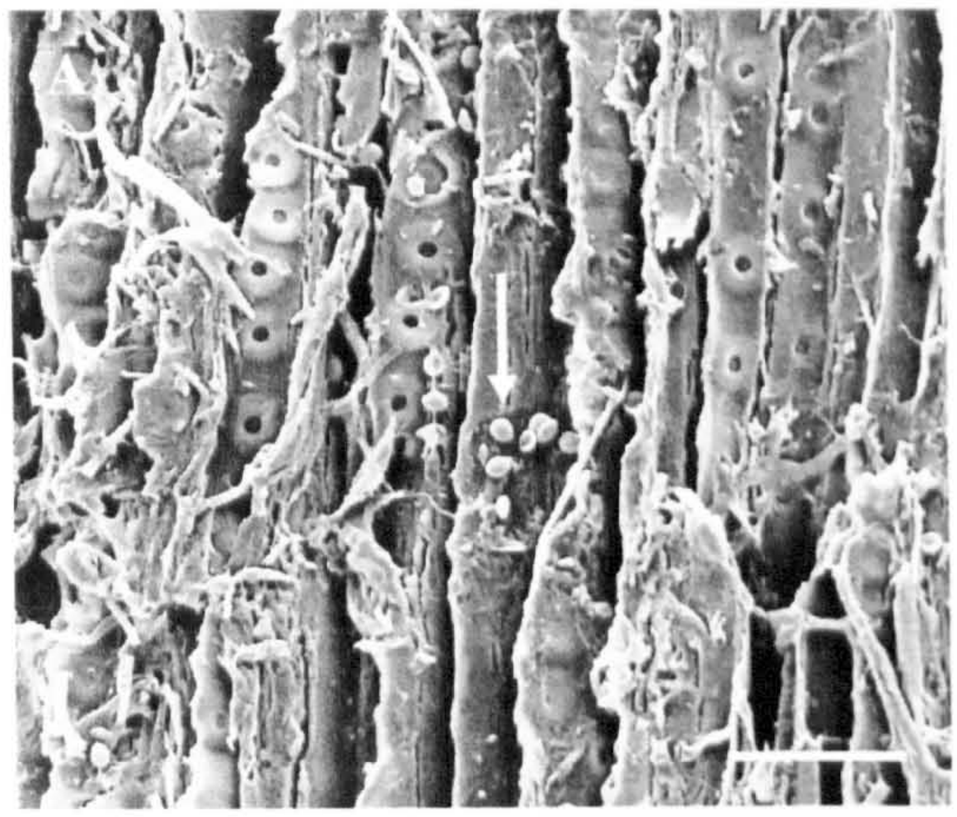
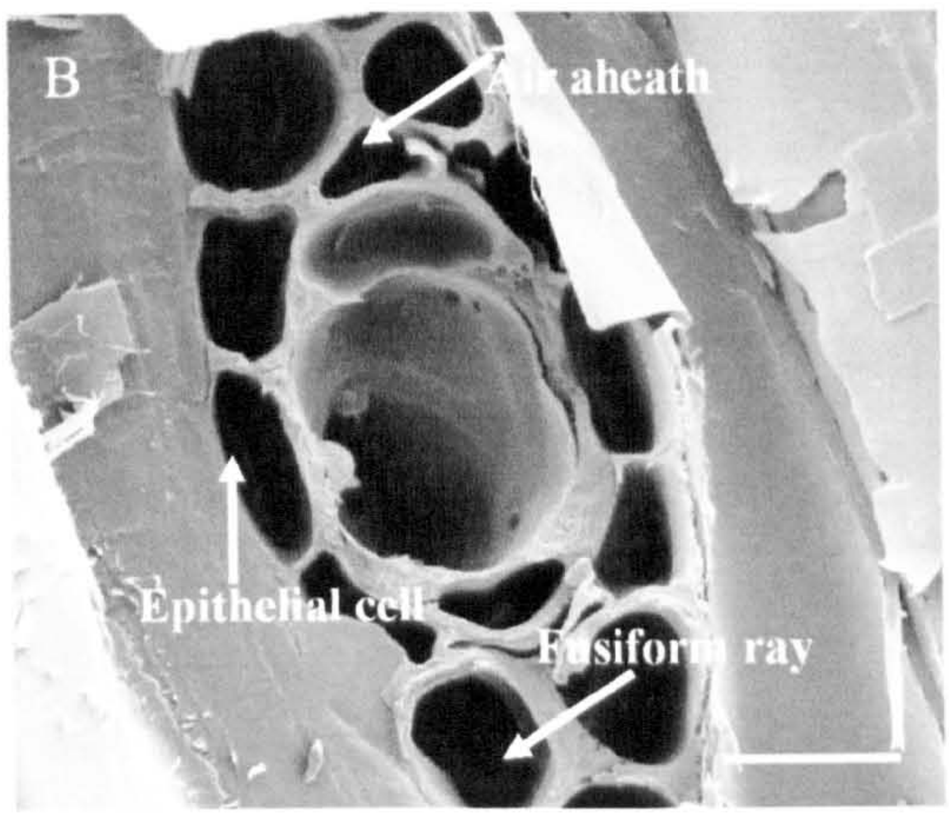


Figure. 5. 12. A-D. Examination of cypress blocks following inoculation with *P. ostreatus* indicates important differences between untreated cypress wood blocks and the two preservative treated wood blocks. Fig. 5. 12. Illustrates the appearance of untreated wood blocks (A) and (B), and ACQ (C) and CuAz (D) treated cypress wood blocks following inoculation with *P. ostreatus*. Fig. 5. 12. A and B. Shows the damage caused by *P. ostreatus*. Fig. 5. 12. A. Note degraded tori (white arrow) with bordered pit typical of white rot fungal decay with cupressoid cross field pitting (dark arrows) in a longitudinal tracheid. Fig. 5. 12. B. Damage caused to bordered pit (dark arrow) indicates partial to whole dissolution of pit membrances. Fig. 5. 12. C. There is no damage caused by *P. ostreatus* and the bordered pits in the longitudinal tracheids can be seen and are intact at the latewood stage in the ACQ treated wood. Fig. 5. 12. D. Rays can be seen and are intact in CuAz treated wood. The scale bars = 40 μm, 30 μm, 200 μm, 200 μm.

Considerable diversity exists between the microscopic wood decay characteristics caused in *D. concentrica* for pine, poplar and cypress.





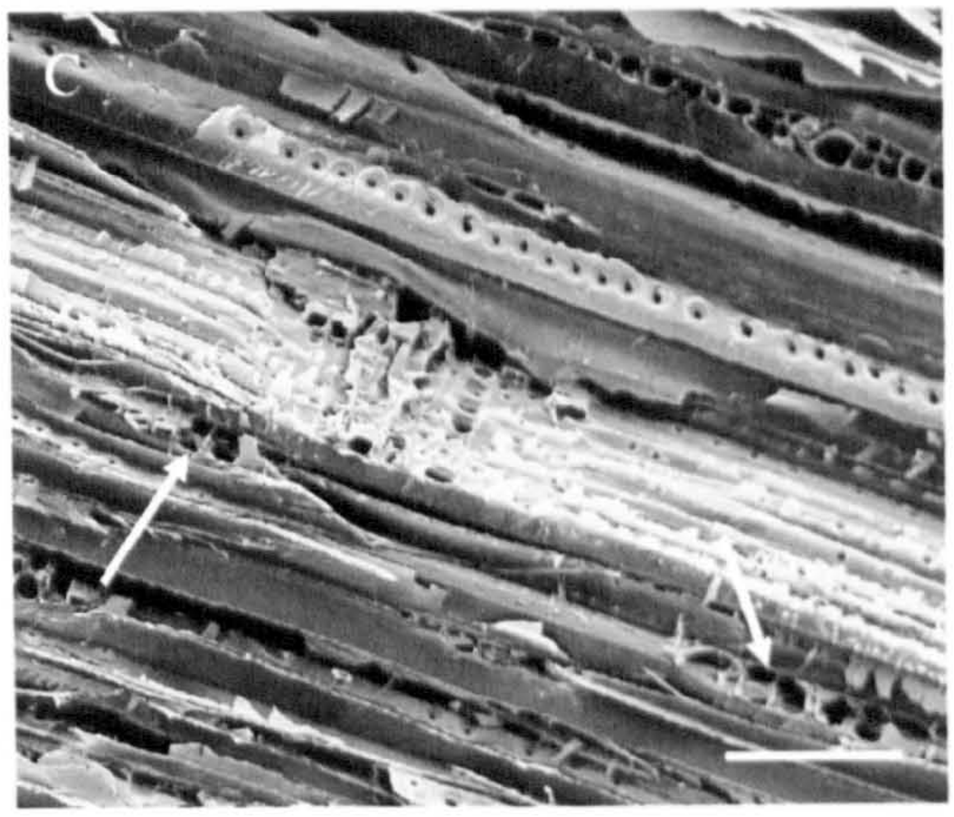


Figure. 5. 13. A-C. Examination of pine blocks following inoculation with *D. concentrica* indicates important differences between untreated pine blocks and the two preservative treated wood blocks. Fig. 5. 13. Illustrates the appearance of untreated wood blocks, ACQ and CuAz treated pine wood blocks following inoculation with *D. concentrica*. Fig. 5. 13. A. Limited damage caused by *D. concentrica*. The decay to the wood is restricted but there is extensive mycelial growth and production of ascospores (arrowed), with some damage to latewood tracheids. Fig. 5. 13. B. The positive effect of ACQ is clear with an undamaged fusiform ray with resin ducts in epithelial cells. Fig. 5. 13. C. Little damage caused by *D. concentrica* with CuAz preservative treatment with a long tracheid with fusiform ray and minor damage (arrowed) by *D. concentrica*. The scale bars = 50 μm, 30 μm, 100 μm.

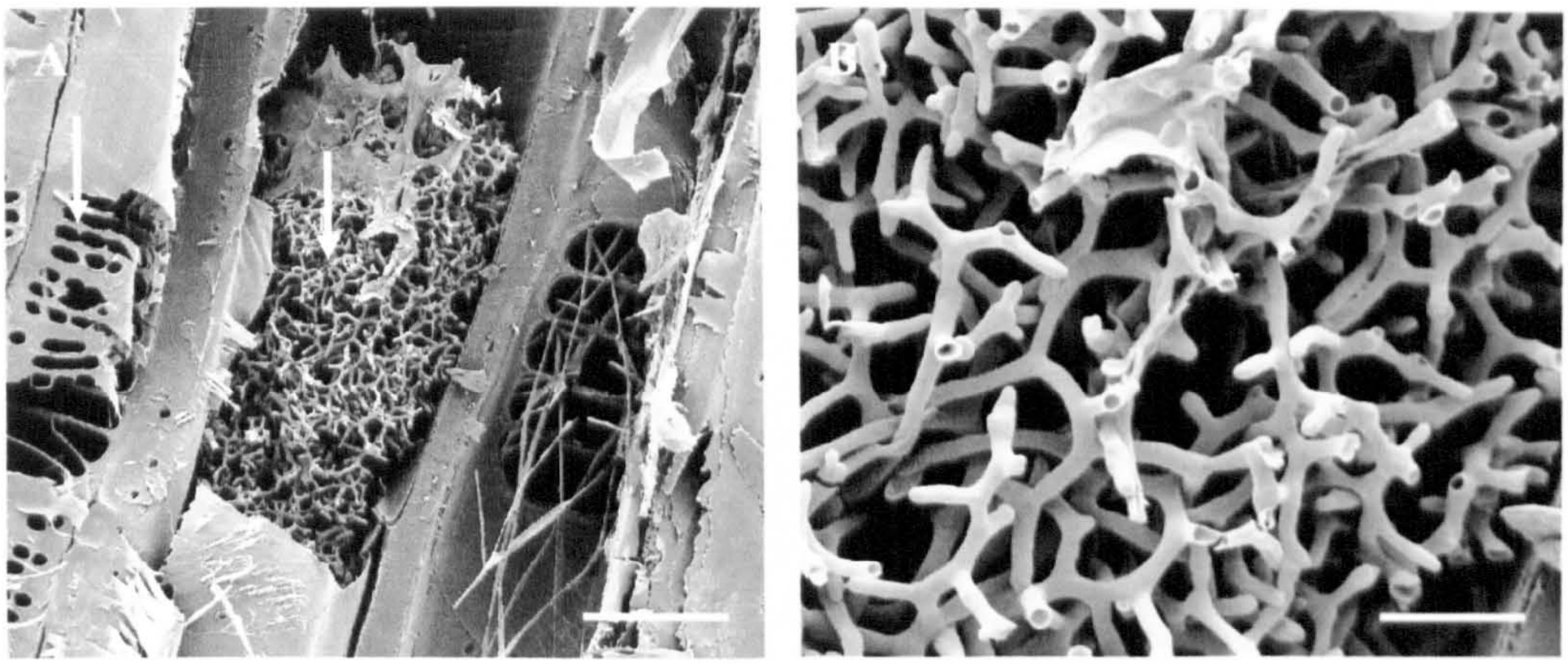
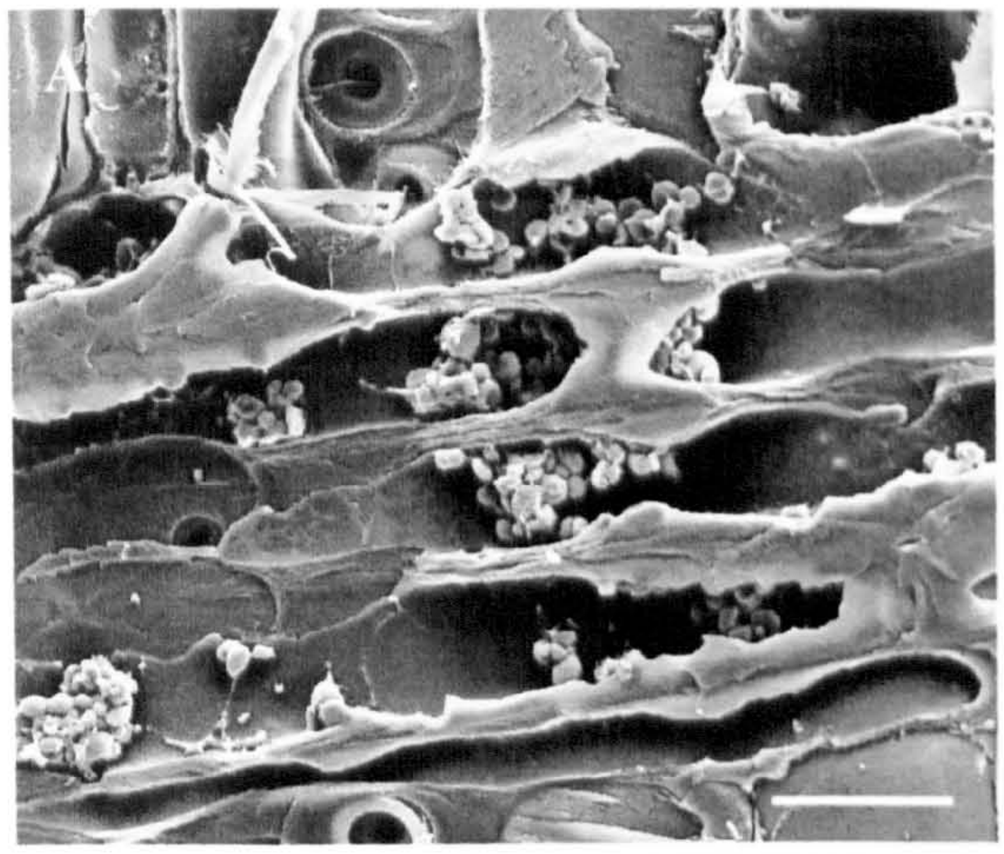
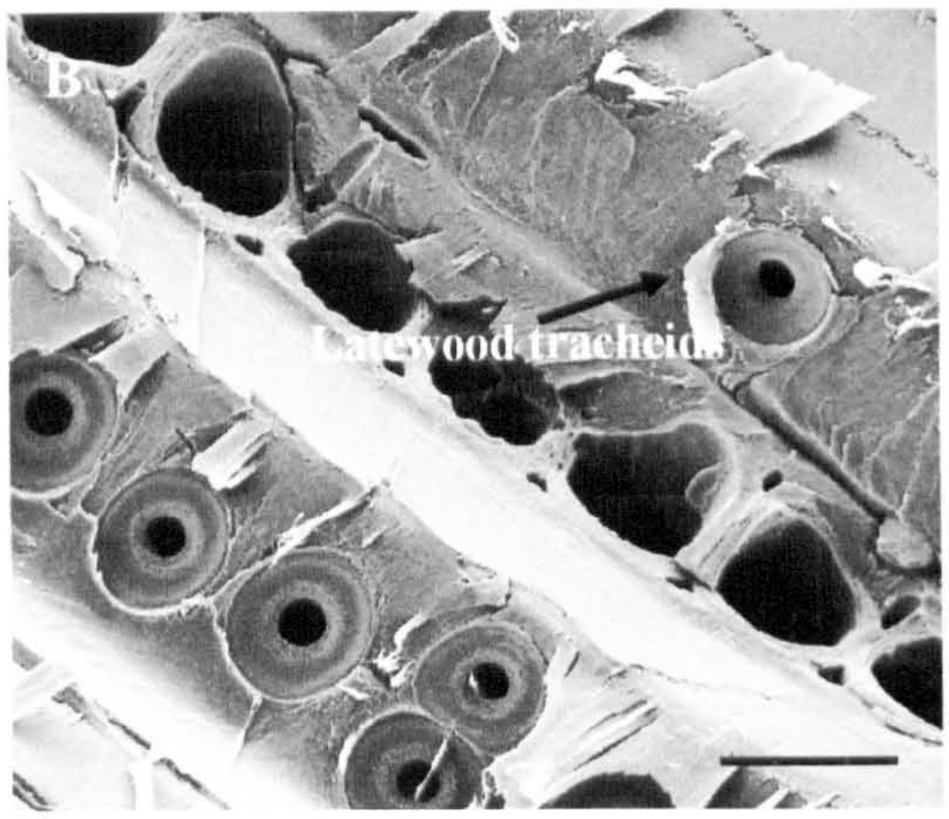


Figure. 5. 14. A-D. Examination of poplar blocks following inoculation with *D. concentrica* demonstrates important differences between untreated poplar blocks and the two preservative treated wood blocks. Fig. 5. 14. Illustrates the appearance of untreated wood blocks (A) and (B), and ACQ (C) and CuAz (D) treated poplar wood blocks following inoculation with *D. concentrica*. Fig. 5. 14. A illustrates damage caused by *D. concentrica* and damage to vessel elements and vessel tips (left arrow). The structural damage of untreated poplar is however limited but there is fungal growth in damaged vessel elements (middle arrow). Fig. 5. 14. B. The modified dichotomous hyphae of the ascocarp being formed is typical for *D. concentrica*. Figs. 5. 14. C and D. The effect of ACQ and CuAz preservatives indicates good protection against *D. concentrica* and an intact ray and vessel can be seen. The scale bars = 100 μm, 20 μm, 30 μm, 100 μm.





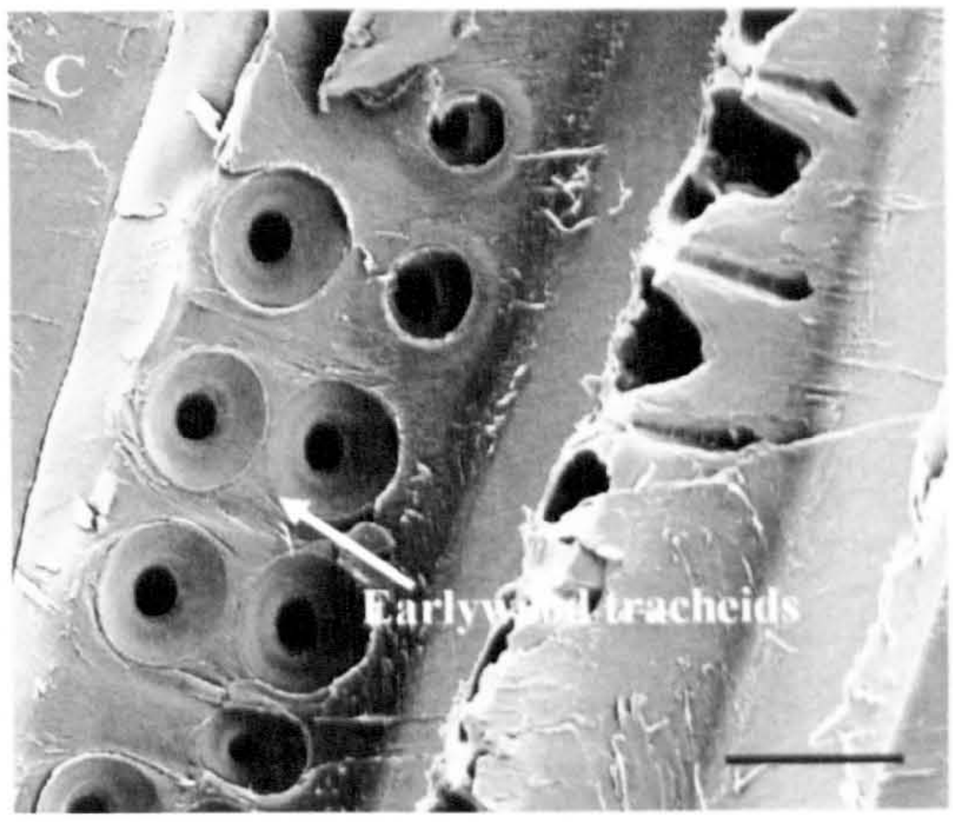


Figure. 5. 15. A-C. Examination of cypress blocks following inoculation with *D.concentrica* shows important differences between untreated cypress wood blocks and the two preservative treated wood blcoks. Fig. 5. 15. Illustrates the appearance of the untreated wood blocks and effect of the ACQ and CuAz treatment of cypress wood blocks following inoculation with *D. concentrica*. Fig. 5. 15. A. The damage caused by *D. concentrica* is shown with ray cells having cross-field pits and presence of spores. In addition, the structural damage of cypress is evident from disintegrated cell walls coupled with spore production in a longitudinal tracheid. Figs. 5. 15. B and C. Show bordered pits in the intact longitudinal tracheids as well as biseriate bordered pits which occurred in the wood blocks treated with ACQ and CuAz. The scale bars = 50 μm, 30 μm, 30 μm.

Hyphal tip topography

Extension of fungal hyphae is the result of primary wall growth at its tip (Trinci, 1978; Prosser & Trinic, 1979) the apical region involved in this growth is called the extension zone. Precursors required for primary wall growth are synthesized in a relatively long region of hyphae (the peripheral growth zone) distal to the tip and then transported to the extension zone where they are incorporated into the tip wall (Trinci, 1978). Fungal haphal tip growth requires the localized synthesis and expansion of the apical plasma membrance (PM) and cell wall. Tip growth of fungal hyphae is comparable to that shown by other similarly growing plant cells in that it is highly localized (predominantly the most apical ~50nm) and involves many separable, but highly integrated, processes. Regulation of tip extensibility can be attributed to the cell wall, components of the cytoskeleton associated with the PM or combination of both (Heath & Skalamera, 2001).

The nature and behavior of hyphal tips is important in relation to penetration and development of the decay fungi in wood. It is likely that it is a combination of physical and enzyme processes which are involved in the wood decay process (Rayner & Boddy, 1988).

Scanning electron microscopy of hyphal tips of the fungi.

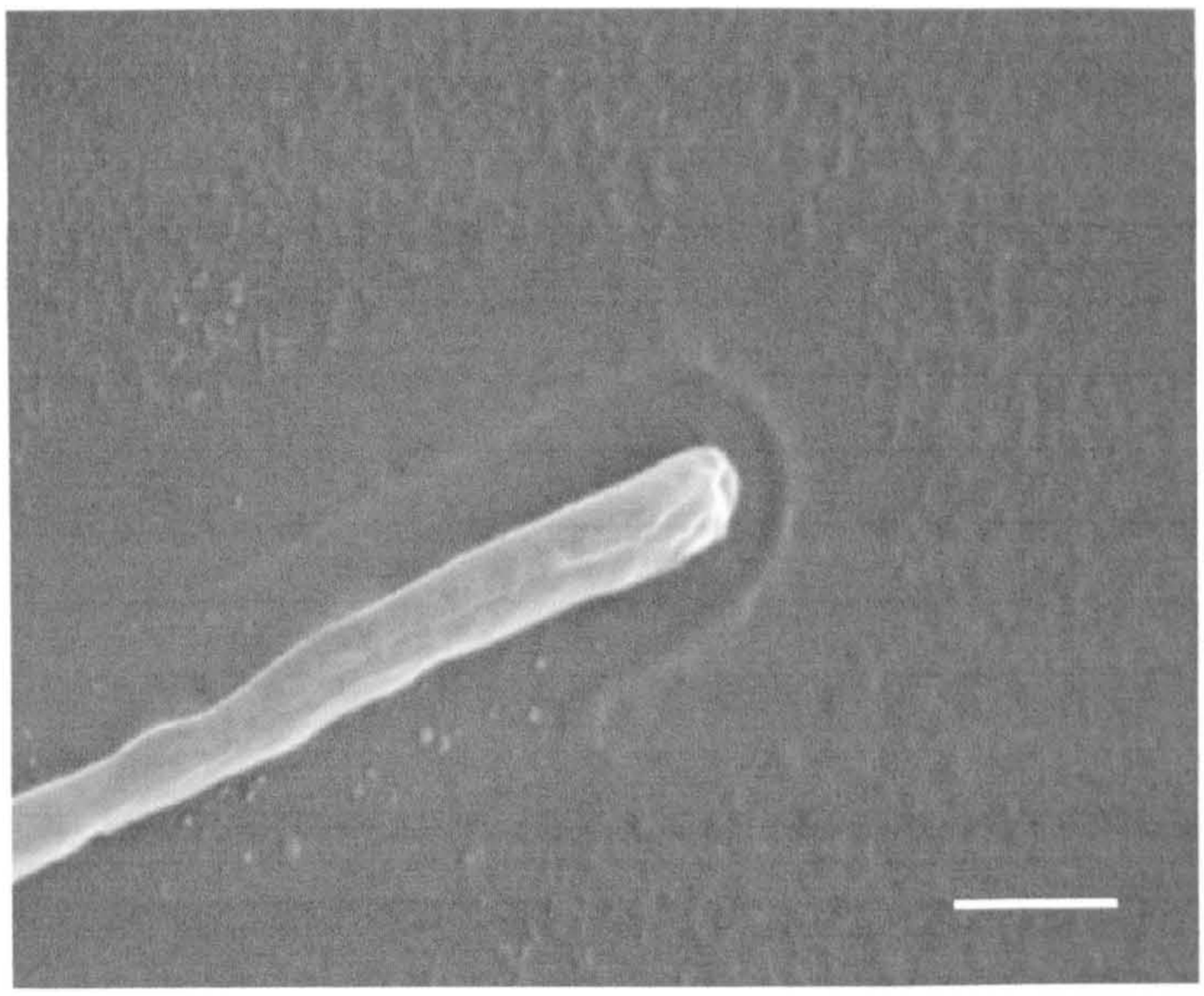


Fig. 5. 16. Structure of a hyphal tip in *Trametes versicolor*. The scale bars = 50μm.

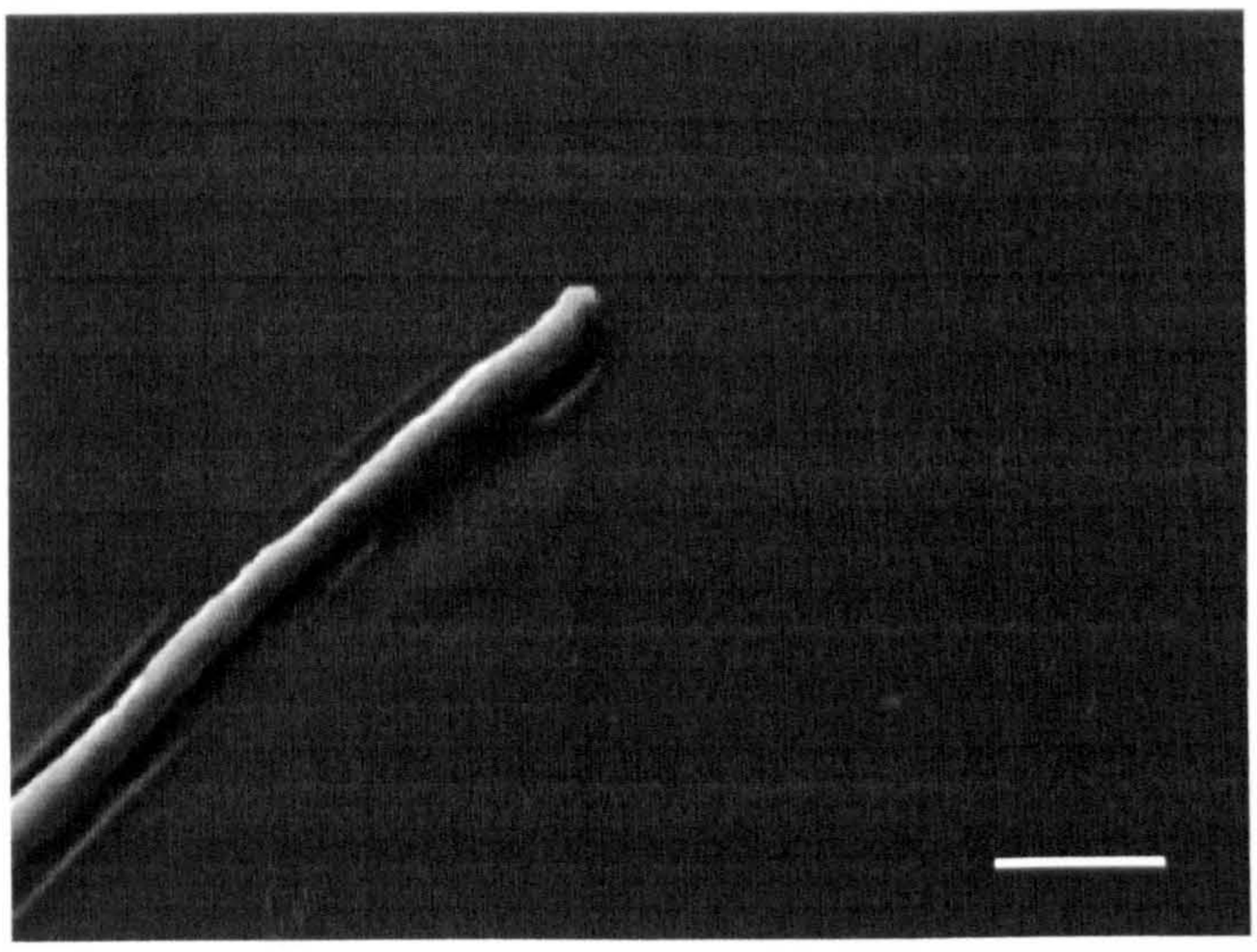


Fig. 5. 17. Structure of a hyphal tip in *Daldinia concentrica*. The scale bars = 20μm

Figures 5. 16 and 5. 17 show minor differences in the hyphal tips of the basidiomycete *T. versicolor* and the ascomycete *D. concentrica*. The basidiomycete hypha is of a slightly greater diameter. In both cases there is some evidence of a slime layer surrounding the hyphae with more produced in *T. versicolor* (Fig. 16).

Morphological aspects of *T. versicolor*, *P. ostreatus* and *D. concentrica* hyphae.

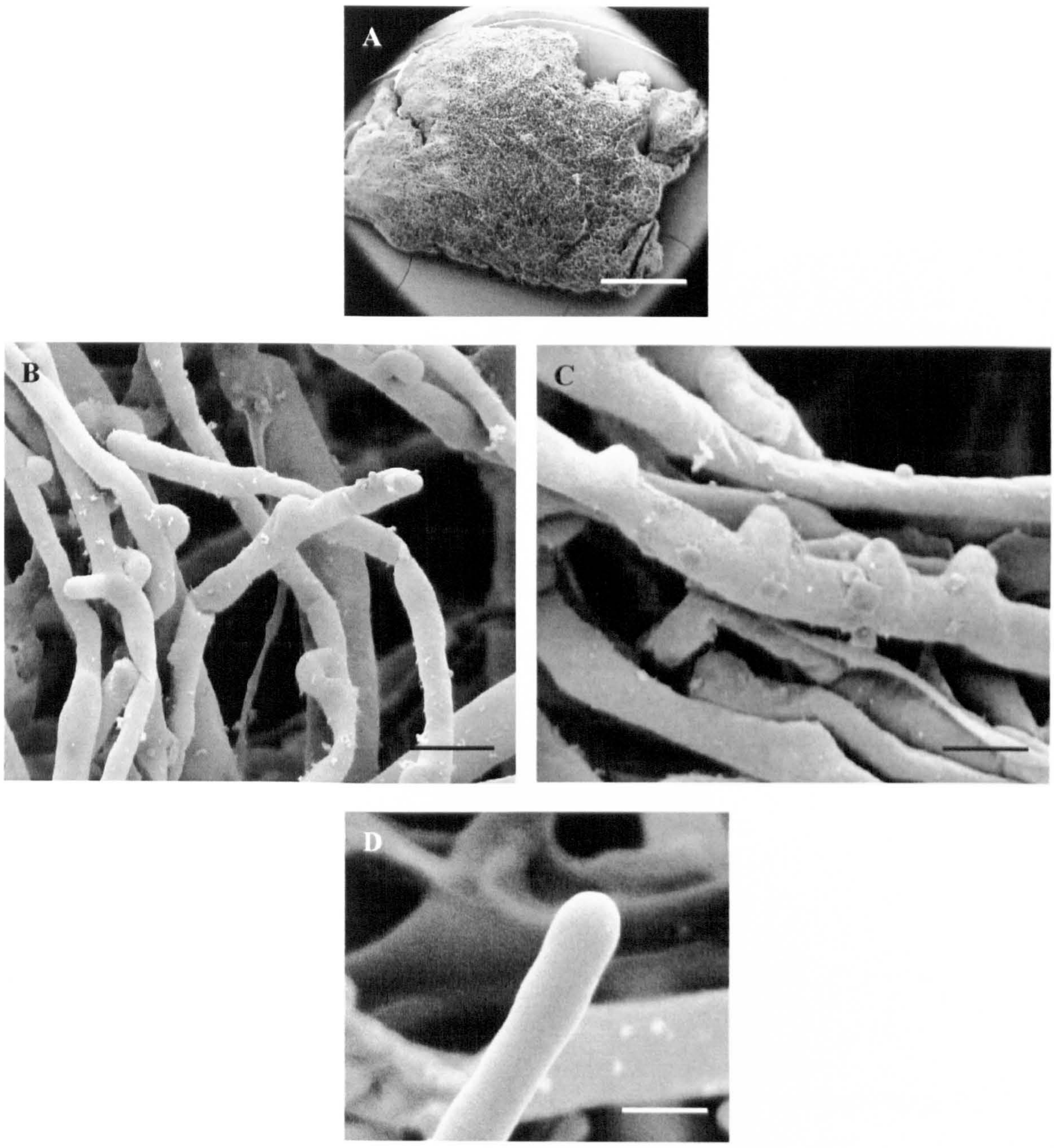
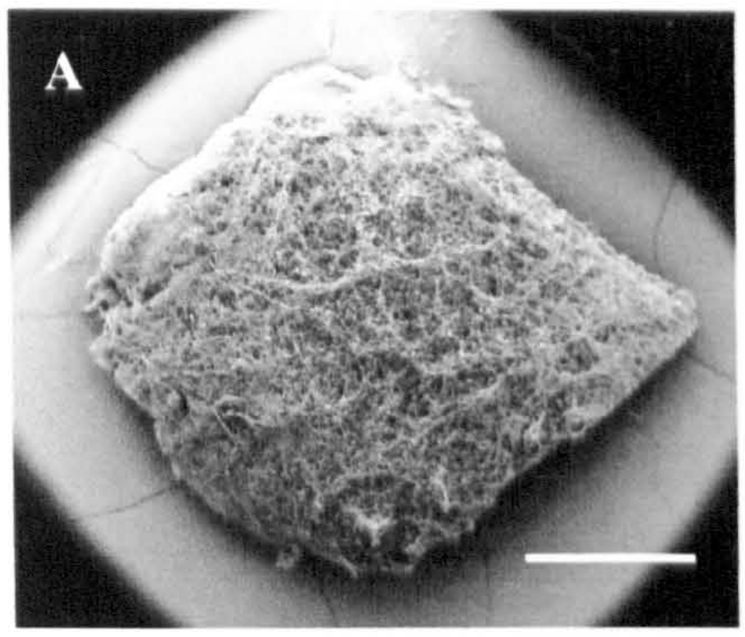
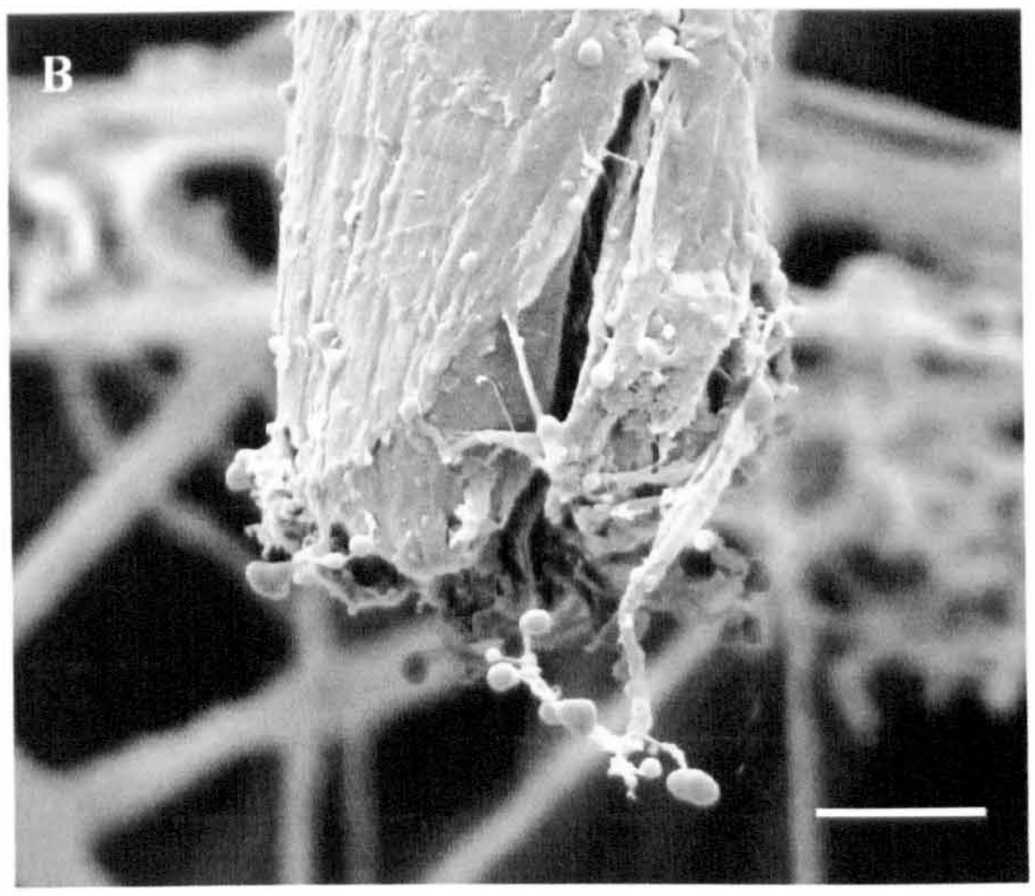
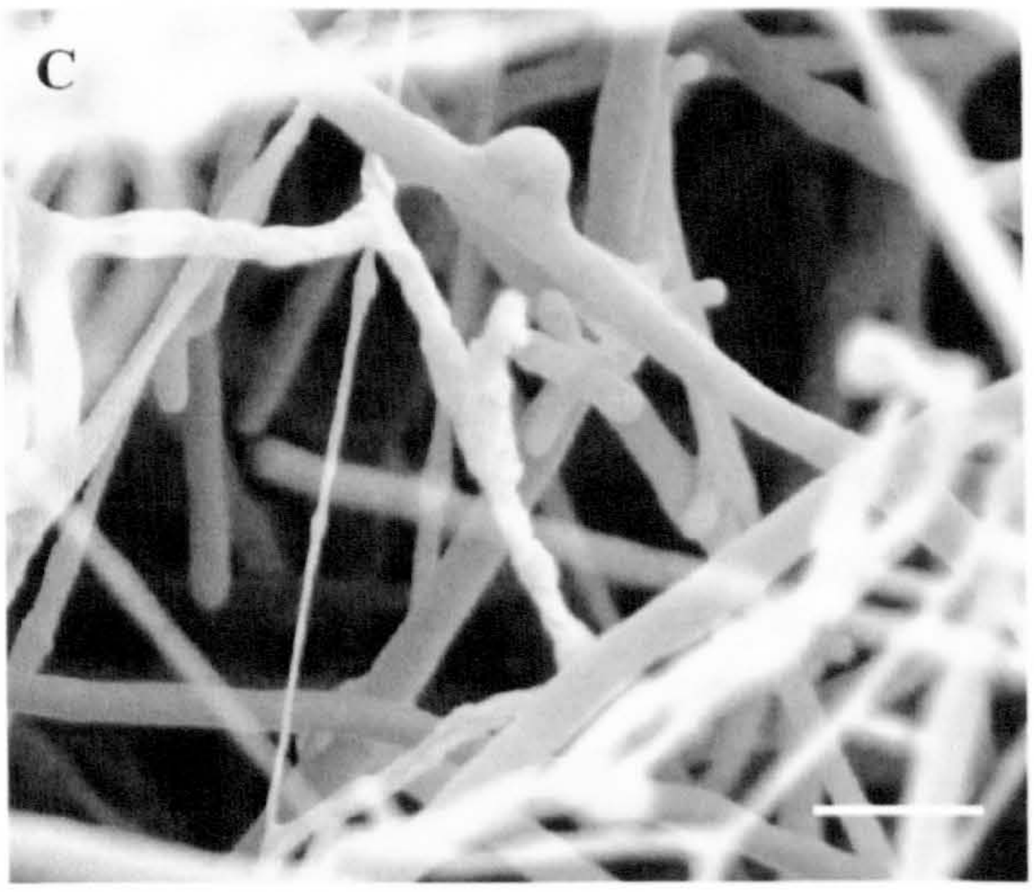


Fig. 5. 18. A. Mycelial mass of *T. versicolor*. B and C. Hyphae of *T. versicolor* with clamp connections. D. Hyphal tip. The scale bars = 2mm, 10μm, 5μm and 2μm.







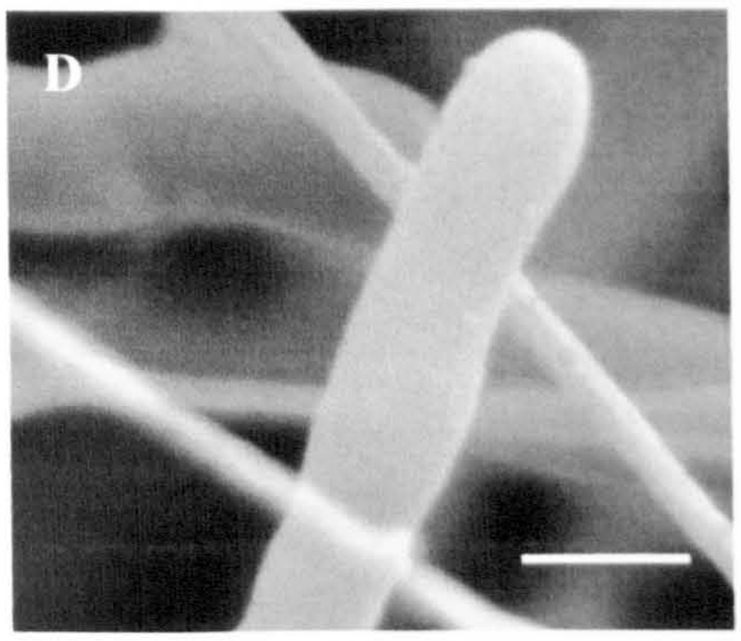


Fig. 5. 19. A. Mycelial mass of *P. ostreatus*. B. Hyphal growth and branching. C. Hyphae of *P. ostreatus* with clamp connections. D. Hyphal tip. The scale bars = 5mμ, 10mμ, 10mμ and 2mμ.

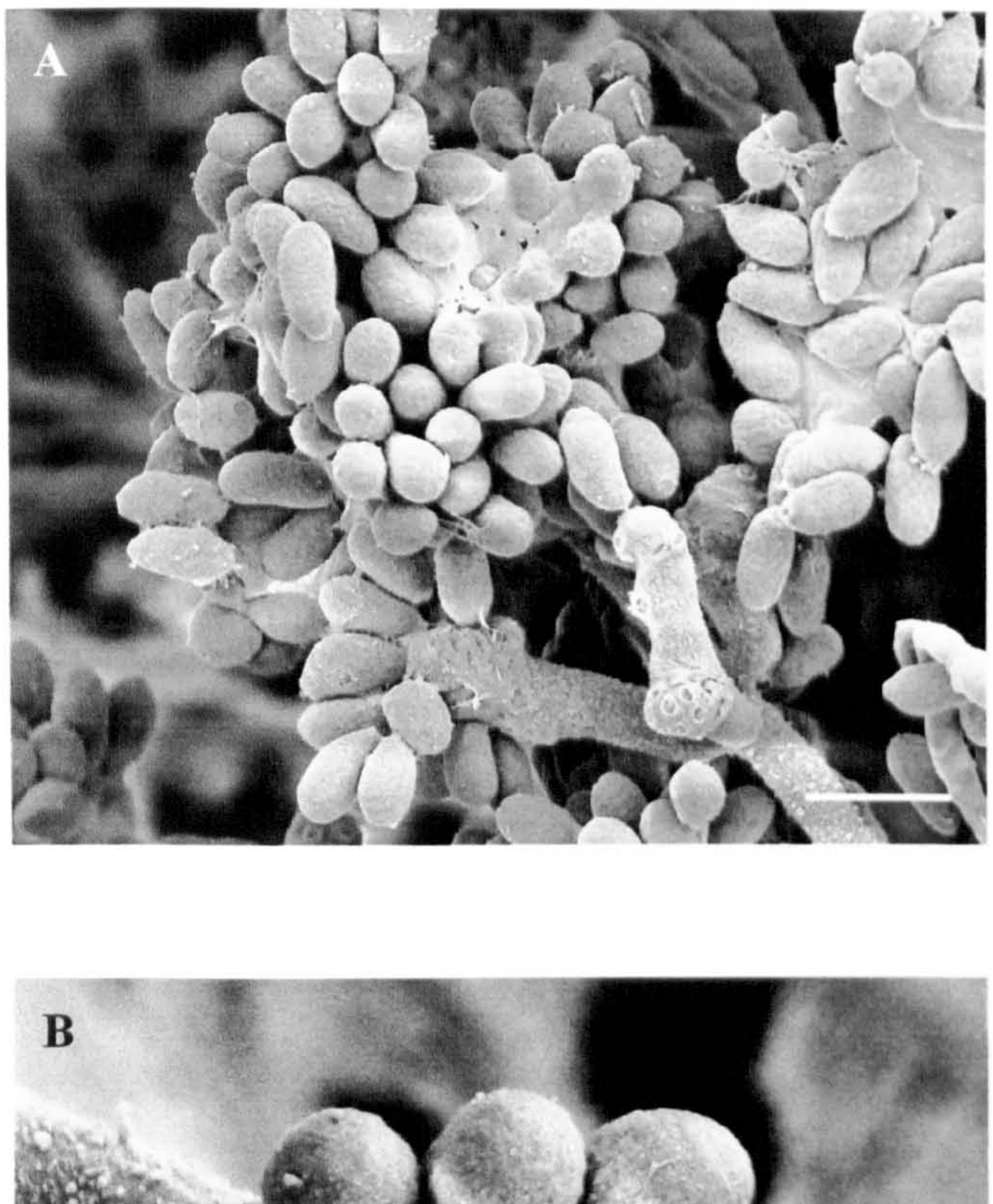


Fig. 5. 20. Conidiogenous structures of *D. concentrica*. A and B. Conidiophores showing the the conidiogenous cells with nodulose whorls of conidia. The scale bars = 10mμ, 5mμ.

The scanning electron micrographs indicate very similar hyphal tips in both *T. versicolor* and *P. ostreatus* (Figs. 5. 18 and 5. 19). In both fungi the hyphal tips appear rounded and even and there is obvious wall ornamentation. Clamp connections are clearly visible and appear at regular intervals in the more nature mycelium (Figs. 5. 18. C and 5. 19. C). There are indicatives that clamp connections are produced more frequently in T. versicolor (Fig. 5. 18.C). In *Daldinia concentrica* hyphae quickly become conidiogenous and produced nodulose branches of conidia (Fig. 5. 20).

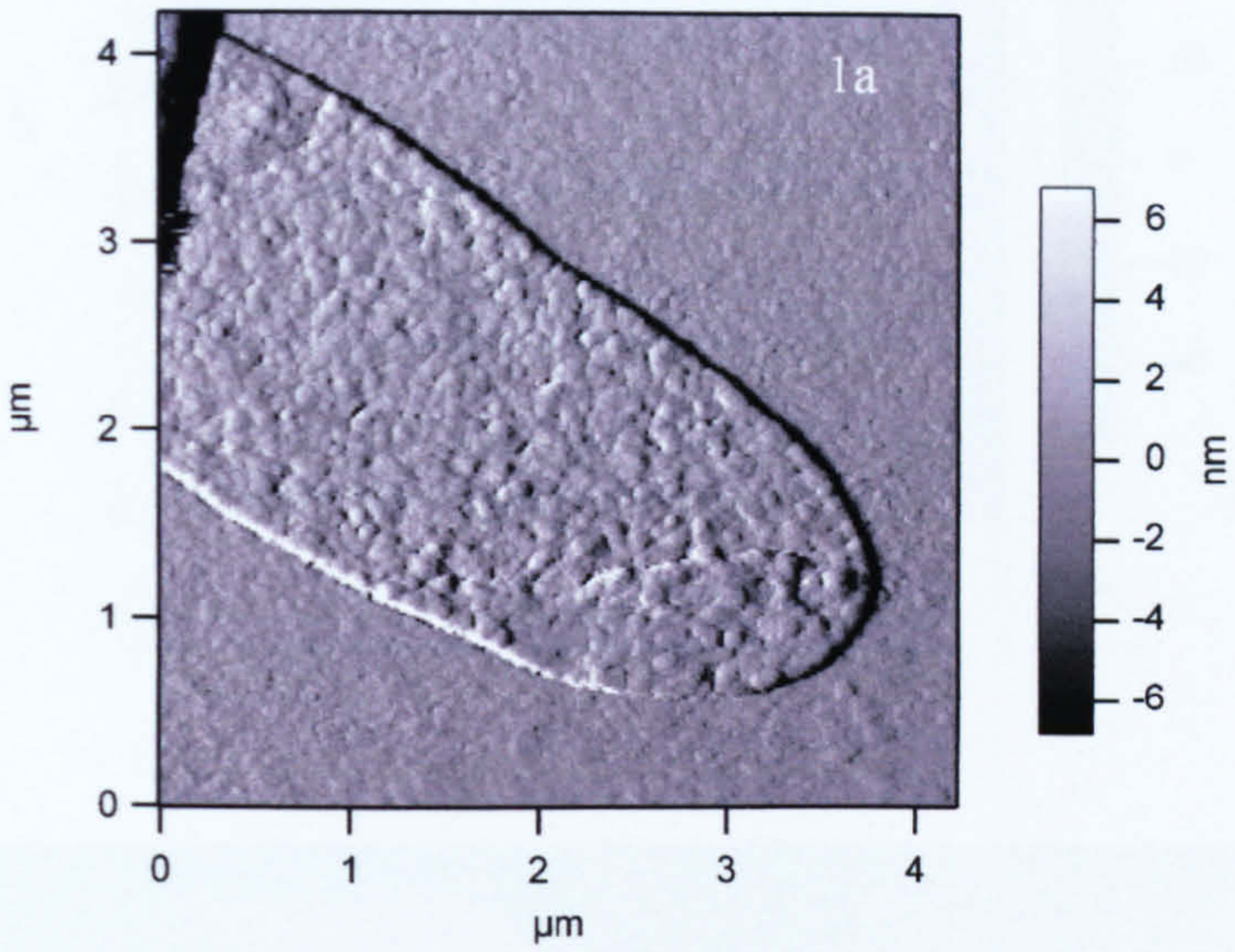
Another approach taken in the comparison of hyphal tips involved the atomic force microscope. This is a method which has been used to characterize surface properties of wood, pulp and paper (Niemi *et al*. 2002). However, as pointed out by Gustafsson *et al* (2002), the interpretation of the structures is still unclear (Koljonenk *et al*. 2003). The surface architecture of spores and cells has been imaged at high resolution using electron microscopy. In contrast, AFM can generate images of living and fixed cells with nanometer scale resolution, and force spectroscopy (FS) can probe their physical parameters (Dufrêne, 2004; Ma *et al*. 2006). Recently, AFM and FS have been used to examine growing *A. nidulans* hyphae. Our results support fungal tip growth models (Ma *et al*. 2006).

The initial examination of the hyphal tips by AFM shows a number of features common to the two wood decay basidiomycete species, *T. versicolor* and *P. ostreatus*. The general shape of the hyphal tip is similar with a hyphal diametre of approximately 2.3μm in *T. versicolor* and 1.9μm in *P. ostreatus*. In both fungi the AFM reveals a granular/rod shaped surface topography with a more pronounced granulation in the *T. versicolor*. The ascomycete *D. concentrica* appears quite different with a hyphal diametr of approximately 0.22μm and a much less noticeable surface ornamentation. Alternation to these structures and topography are likely once

contact with the chemical preservatives occurs. Atomic force microscopy will be able to detest small changes and is expected to indicate the effects of the chemicals on wood decay fungi.

AFM images of fungal hyphae tip growth and surface topography are shown in Figures. 5. 21 ~ 5. 23. These figures show mature hyphal tip surfaces. Figure 1a is a contact mode deflection image of scan size 4 x 4μm and Figure 1b shows a three-dimensional height image at approximately the 100nm height point. Figure 2a is a contact mode deflection image of scan size 3.5 x 3.5μm and Figure 2b is a three-dimensional height image at approximately the 120nm height point. Figure 3a is a contact mode deflection image of scan size 20 x 20μm and Figure 3b is a three-dimensional height image at approximately the 100nm height point.

Operating contact mode images for fungal hyphae tips by AFM.



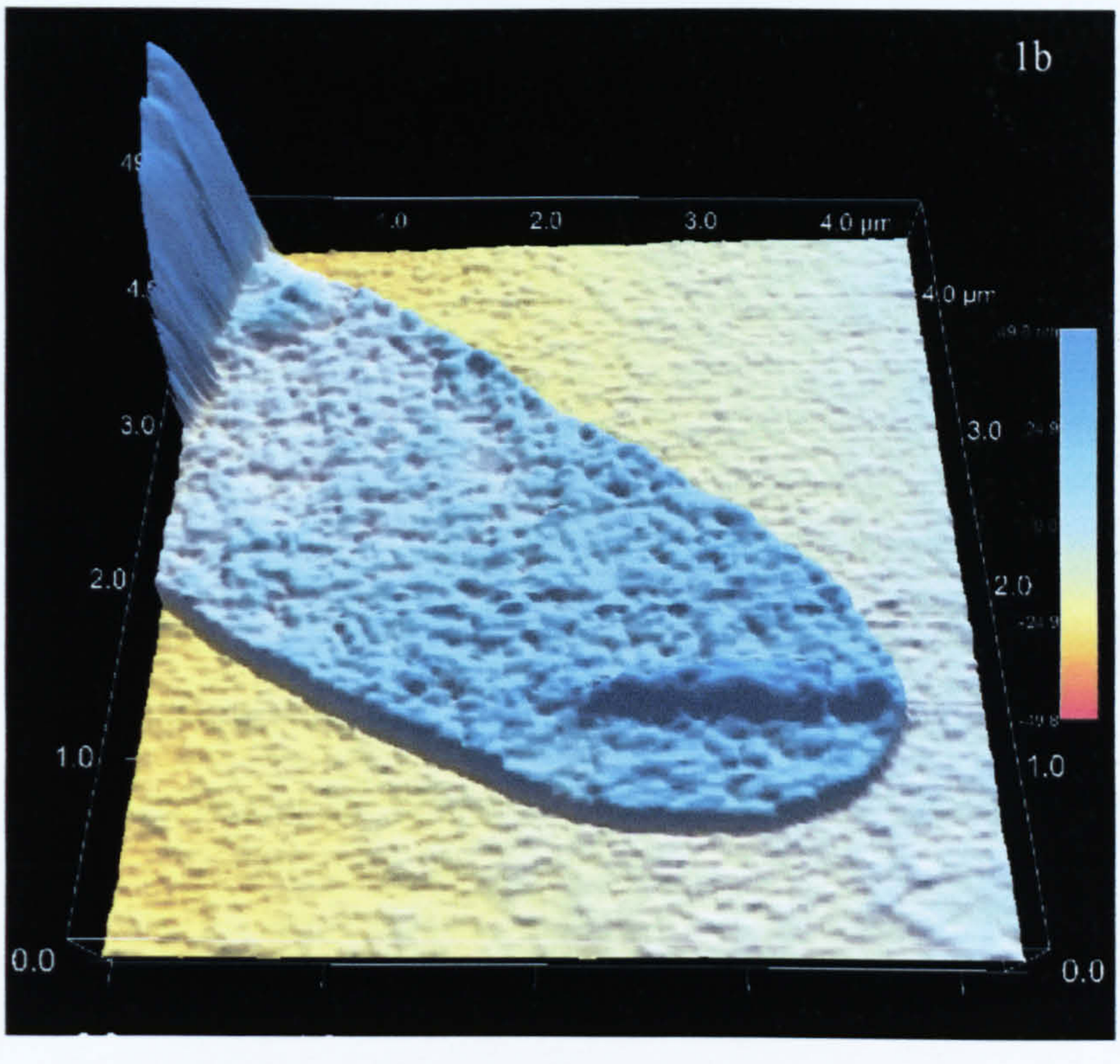
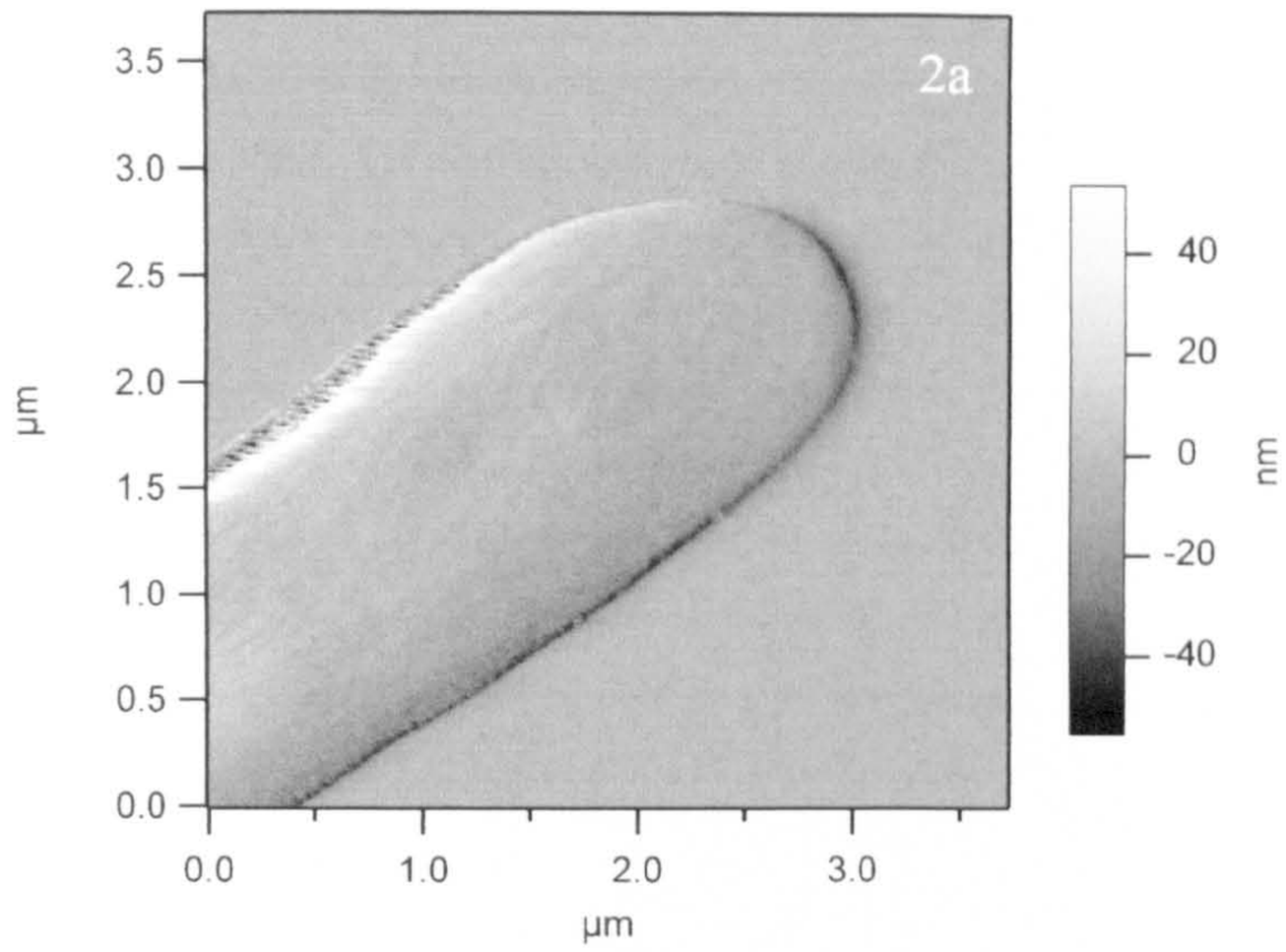


Fig. 5. 21. 1a. Deflection image. 1b. 3D reconstructed from AFM height image for *T. versicolor.*



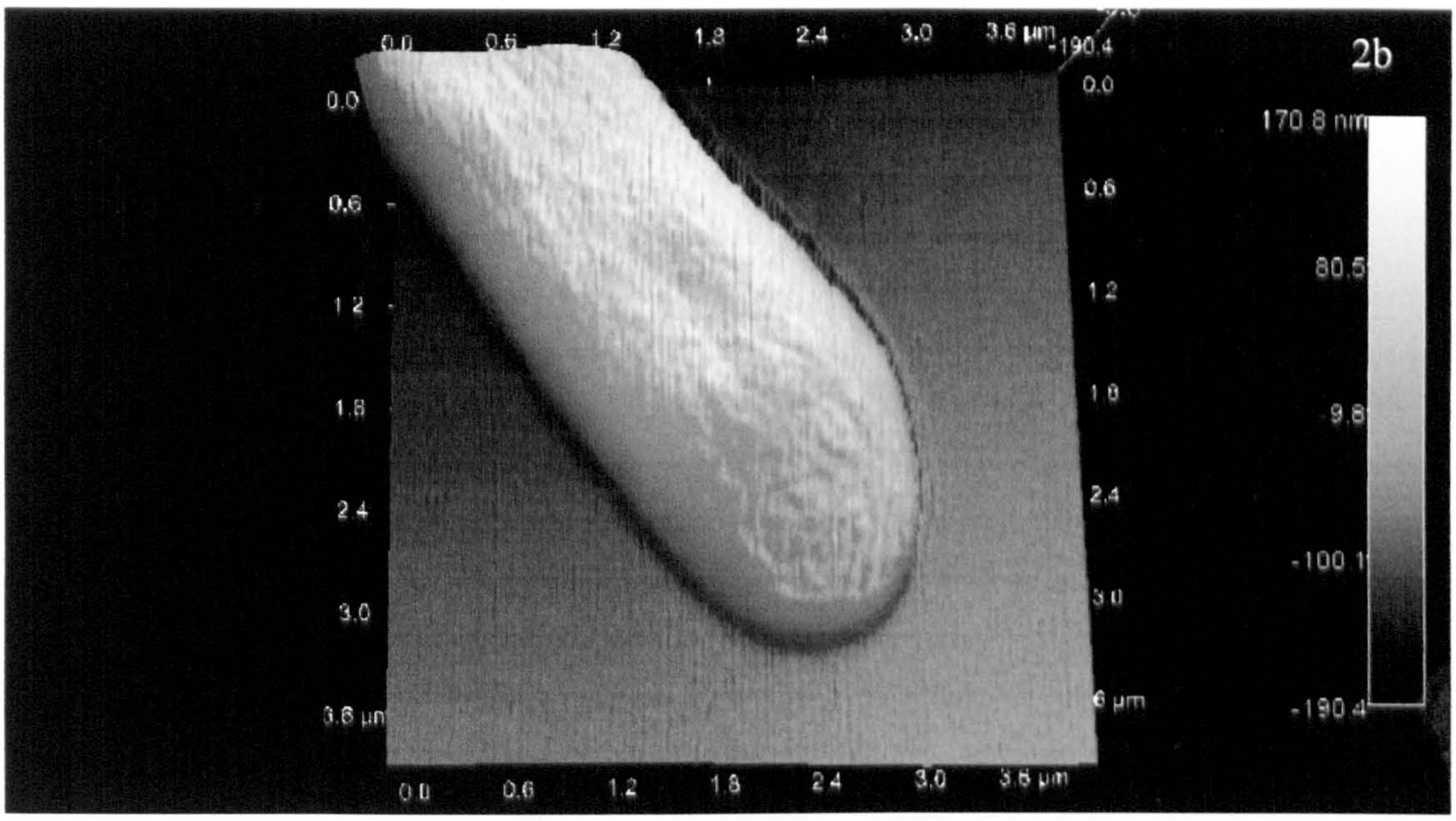
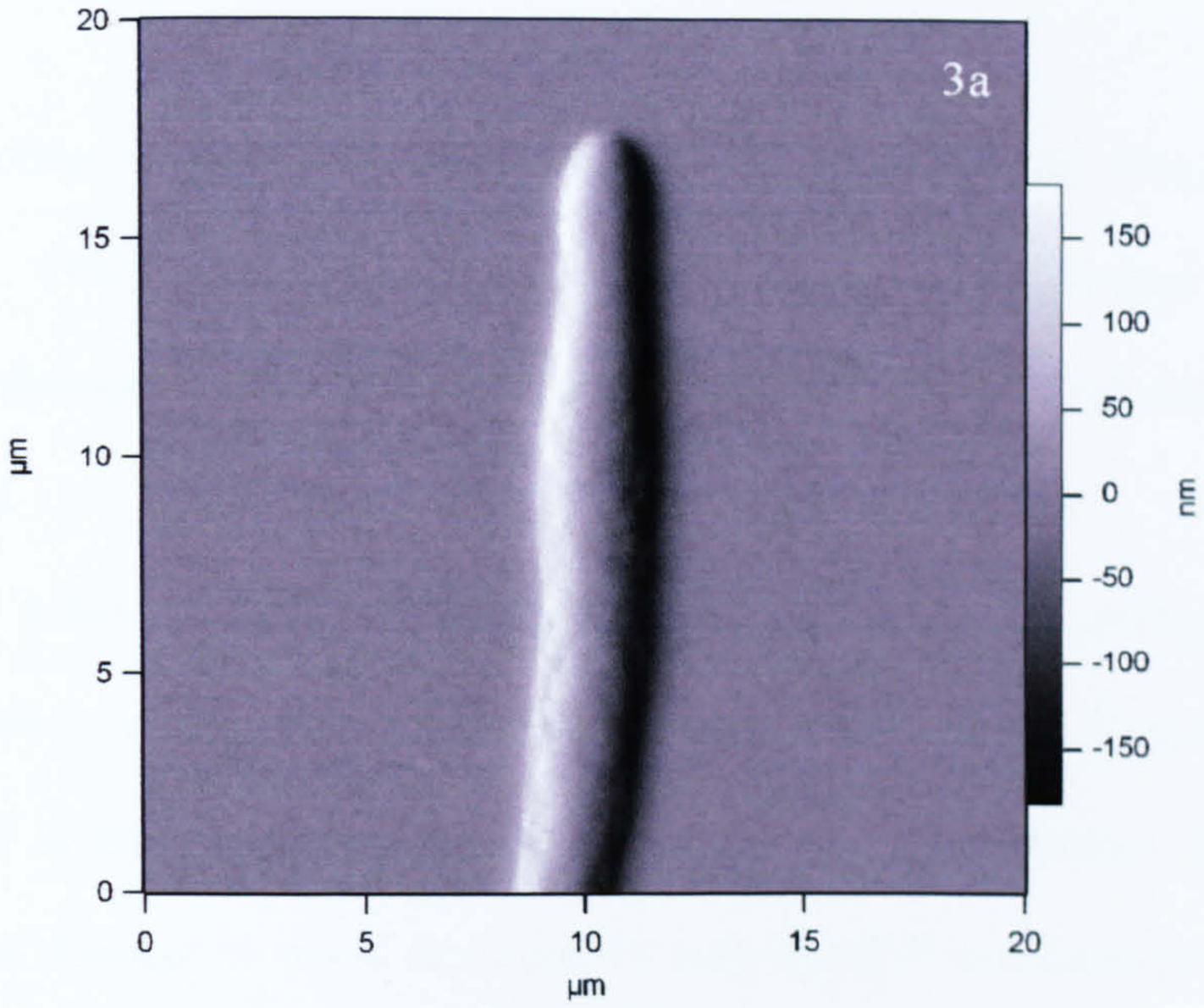


Fig. 5. 22. 2a. Deflection image. 2b. 3D reconstructed from AFM height image for *P. ostreatus.*



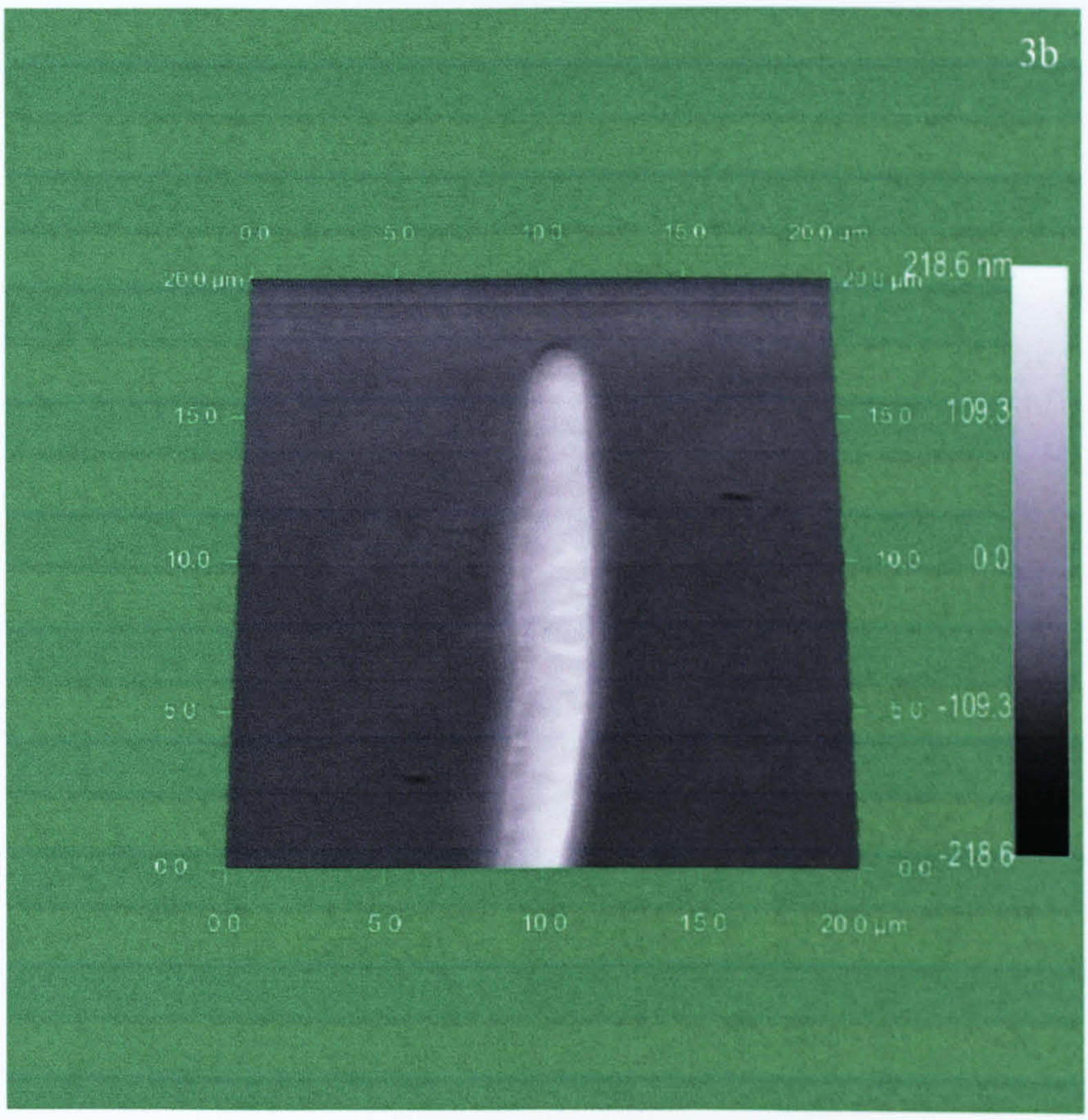


Fig. 5. 23. 3a. Deflection image. 3b. 3D reconstructed from AFM height image for *D. concentrica.*

Discussion

Direct observational methods can be used to assess fungal decay of wood (Eriksson *et al.* 1990) although there are many variables such as type of wood, hardwood or softwood, environmental situation, and the main wood decay fungal species involved.

Firstly it has been shown that the two preservatives tested ACQ and CuAz both greatly reduce visible damage to the wood samples tested. Examination of untreated wood blocks indicates that *T. versicolor* and *P. ostreatus* both cause significant visual damage to wood structure. In pine wood *P. ostreatus* proved to be the most destructive. As might be expected from the weight loss of wood blocks following inoculation and incubation *D. concentrica* caused by far the least damage although mycelia growth could be observed. This is not surprising as *D. concentrica* is not considered to be an important white rot decay fungus (Burdekin, 1977; Whalley, 1996). The scanning electron microscope with its great depth of field provides valuable insight into the development of the test fungi and their effect on wood structures. In contrast the application of atomic force microscopy produces images which are not easy to interpret. This has also been raised by other researchers (Bustafsson *et al.* 2002; Koljonenk *et al.* 2003). The current study does however show major differences between *T. versicolor* and *P. ostreatus* (Basidiomyceta) and *D. concentrica* (Ascomycota). *Trametes versicolor* and *P. ostreatus* both possess a granulated hyphal tip topography with this appearing as a short rod like surface. It is very clear in *T. versicolor* but *Daldinia concentrica* in contrast appears to have an almost smooth surface.

A major problem with using direct observation to assess wood decay is its subjective nature although the effects of the copper preservatives are readily seen.

CHAPTER 6

General discussion and conclusions

The current work was undertaken to investigate fungal decay of three wood species commonly used as lumber in the building trade in South Korea. Furthermore the effect of two water based copper derivative preservatives were evaluated for their ability to reduce wood decay in these timbers. Wood preservation has become an important issue because of economic loss through wood decay and more recently as a result of increasing legislation regarding preservatives which are no longer allowed because of environmental issues. In Chapter 1 the main aspects of the different type of wood decay, white, brown and soft rot, are reviewed together with the techniques used to assess decay. The application and success of the more environmentally friendly water based copper derivative wood preservatives was also reviewed.

Wood decay and protection offered by the preservatives was examined and assessed using three main approaches. The traditional method used is based on weight loss over time and this proved to be a useful measure although a long term solution (Chapter 3). A second approach determined fungal biomass based on estimation of chitin and ergosterol content (Chapter 4) and the results obtained reflect both advantages and disadvantages of the methodology. Direct observation using light, scanning and atomic force microscopy provided an alternative approach to follow wood decay and to examine the effect of the preservatives (Chapter 5).

The three wood types selected, Japanese Red Pine (*Pinus densiflora*), Yellow Poplar (*Liriodendron tulipifera*) and Bold Cypress (*Toxodium distichum*) are widely

used in South Korea and Asia by the building industry. They exhibit different properties and vary in susceptibility to fungal decay. It is well known that different wood species exhibit different degrees of natural resistance to decay (Rccb, 1997). Thus white pine and bold cypress were judged to possess moderate resistance and yellow poplar to have little resistance. Bald cypress has good resistance because of its high percentage of heartwood (Rccb, 1997). Two of the test fungi selected, which are both basidiomycetes, *Trametes versicolor* and *Pleurotus ostreatus* are important white rot decay fungi of timber in the region. *Daldinia concentrica*, an ascomycete, was selected for comparison since although it causes a weak white rot resulting in so called calico wood in ash (Fraxinus excelsior) it is not considered to be a serious problem (Burdekin, 1977).

The structure of the wood is fundamental in relation to fungal attack and decay and the effectiveness of the chemical treatment is also closely linked to absorbance and retention of the chemicals following treatment. Pine and cypress are both softwoods whilst poplar is a hardwood. Uptake and retention for all treated wood types indicated that ACQ was more readily absorbed than CuAz. The highest uptake and retention of both chemicals was found to occur in poplar and marginally the least in pine (Honglin *et al.* 2005; Zelinka & Rammer, 2006).

Based on weight loss, over a period of 60 days, the results for untreated wood blocks indicated that the ascomycete *Daldinia concentrica* was generally the least damaging and although it caused an average 8% weight loss in pine as opposed to 3.9% for *Pleurotus* this was unexpected. *Daldinia concentrica* grows on deciduous trees, mainly *Fraxinus* in the UK and Europe and is not reported to grow on coniferous tree species such as pine (Whalley & Watling, 1980). Untreated poplar recorded the highest weight loss for both *T. versicolor* and *P. ostreatus*. Overall

Trametes proved to have the greatest effect which is in general agreement with published records (Huntley & Walker, 1985). The two important white rot basidiomycetes, *T. versicolor* and *P. ostreatus*, tested here, were both susceptible to the copper preservatives and ACQ proved to be the most effective with a difference in % weight loss for pine of 4% (cf 17% untreated), for poplar 13% (cf 32% untreated) and for cypress 7.9% (cf 18.9% untreated). A similar pattern emerged for *P. ostreatus* (Chapter 3). CuAz was also effective in reducing weight loss in all of the three wood species tested. Overall *D. concentrica* exhibited the smallest change in weight loss with both ACQ and CuAz although for this fungus CuAz was slightly more effective. Since *T. versicolor* and *P. ostreatus* are the important wood decay fungi in Asia this is of lesser significance. Furthermore *D. concentrica* although a wood rot fungus is not generally considered to be a problem for decay of lumber or to the building trade (Burdekin, 1977; Whalley, 1996). It has, however, been reported as growing in unusual situations and has been found growing on a bathroom cabinet (Whalley & Watling, 1984). When weight loss was recorded following a 90 day post inoculation period for the two main white rot fungi the differences between ACQ and CuAz were now found to be minimal. A similar finding was obtained for *D. concentrica*. Therefore over the 60 day period ACQ proved to be the most effective preservative but a further 30 days of exposure resulted in similar results. This suggests that retention of the preservative is paramount to long term decay resistance and future investigations into absorption and retention are considered to be fundamental. The effect of rainfall on leaching of copper from wood was investigated by Che & Kim (2006) and they found that copper was readily leached from both ACQ and CuAz preservatives even after one month of exposure. After 6 months, however, the accumulative amount of copper leached at 345.3 ppm for ACQ was almost four times as great as the 92.9 ppm leached from CuAz. Che & Kim (2006) also found that the majority of the copper which leached out took place

during the first month of exposure to natural rainfall. The results here are suggestive of a slow leaching of copper from the wood blocks but in the present study the wood blocks were tested under laboratory conditions and were therefore not subjected to the additional leaching that would be expected from exposure to rainfall. This might explain why there was a markedly stronger preservative effect for ACQ after 60 days but after 90 days ACQ and CuAz proved to have similar fungicidal effects. Comparison of the three test wood species indicated that cypress again exhibited the greatest natural resistance to fungal decay. When the effect of CuAz and ACQ at concentrations ranging from 0.2% to 3% were tested against the three test fungi it was found that for CuAz a minimum concentration of 2% was required for clearly observable inhibition using impregnated filter paper discs. Even at 3% the inhibition against. *P. ostreatus* and *D. concentrica* was minimal. However, with ACQ all test fungi were inhibited by a concentration of 1% and at 2% and 3% they were strongly inhibited. *Daldinia concentrica* was weakly inhibited even at 3%. Thus based on weight loss over 60 days and the filter paper inhibition test ACQ proved to be the most effective of the water based copper preservatives.

It is realized that laboratory testing, although convenient and usually easily reproducible, has the major disadvantage of not testing the effect of preservatives under natural conditions. These are after all the conditions under which wood preservatives need to be effective. Therefore untreated wood blocks and wood blocks treated with ACQ and CuAz were buried in the litter/upper soil layer of a mixed deciduous wood and after 6 months burial they were examined for evidence of decay. As expected untreated wood blocks exhibited considerable weight loss with poplar losing 26% of its weight. Treatment with ACQ and CuAz reduced weight loss to around 10% for all three wood species tested demonstrating their preservative ability under natural field conditions.

Therefore both ACQ and CuAz provided protection against the fungi present in this environment when compared to the control (untreated wood). Although the wood blocks were not specifically exposed to the important white-rot fungi *T. versicolor* and *P. ostreatus* the wood site was chosen because previous studies had shown it to be active in wood decomposition (Nugent, 2006). It would in future be valuable to place treated and untreated wood blocks in a similar situation following pre-inoculation and establishment of the test white-rot fungi. This could be expected to provide a more natural test for evaluating the effectiveness of copper based preservatives.

In the natural environment, white-rot fungi interact with different types of wood and soil inhibiting fungi and compete with them for resources and space. The interactions among wood-rotting fungi have attracted considerable attention (Boddy, 1999) and some studies have focused on the effect of interactions on the production of extracellular enzymes by fungi. Most studies have centred around laccase which is sometimes produced during interspecific interactions (White & Boddy, 1992) and it has been suggested that it might be preferentially located in the interaction zones in the mycelia of wood-decaying basidiomycete fungi (Iakovlev & Stenlid, 2000). The induction of laccase activity is a typical reaction of a wide range of white-rot fungi to interspecific interactions (Baldrian, 2004). How wood preservatives interact or interfere with wood decay fungi could therefore be linked with fungal enzyme activity such as laccase or cellulase and future studies should investigate this aspect (Asiegbu *et al.* 1993; Martinez *et al.* 2008). Copper has been used for over one hundred years as an effective fungicide for the control of a wide range of plant diseases. Its use in the control of powdery mildew of the grapevine caused by *Erysiphe nector* established copper based compounds as fungicides to control a wide range of fungal plant pathogens (Richardson, 1997). Its mode of action is generally

considered to be through inactivation of enzymes.

Although weight loss over time is a well tested and popular method for determination of wood decay and/or effectiveness of preservatives it has the major disadvantage of the incubation time required. Therefore other methods such as amount of specific chemicals or direct observation have been examined.

One such approach to the determination of fungal growth and decay or fungal growth and effect of the preservatives is the determination of fungal biomass in the wood blocks. This was obtained during the current study by estimation of chitin content based on the amount of glucosamine present. This provides an estimate of total fungal mycelium and is therefore potentially a good measure of susceptibility or resistance to decay (Rayner *et al*. 1994). *Pleurotus ostreatus* produced the greatest amount of fungal biomass based on chitin content in all three wood species tested ranging from 287 mg/kg to 373 mg/kg. The highest amount being obtained for growth on untreated poplar. ACQ and CuAz both effectively reduced the amount of fungal biomass for both of the basidiomycete decay fungi (Chapter 4) which is in line with the results obtained for weight loss over a corresponding period of time. Surprisingly *D. concentrica*, which is not considered to be a strong white rot decomposer (Whalley, 1996), produced considerable biomass in all three wood species but this was again considerably reduced in the treated wood samples. However, in recording biomass based on amounts of chitin present % recovery of glucosamine following hydrolysis of chitin should be taken into consideration (Swift, 1992). It is also necessary to take in to account the % of chitin as a % of dry weight for the mycelium of different fungal species. Mario *et al*. (2008) reported a range of percentages for the fungi they investigated but *T. versicolor* (13.1%) and *P. ostreatus* (15.3%) do not differ significantly.

Ergosterol, another measure used, does not determine total biomass but provides an account of fungal activity at the time of sampling (Pronyk *et al*. 2006). Determination of ergosterol levels in mycelium of *T. versicolor* and *P. ostreatus* showed the amount in *P. ostreatus* to be considerably higher and this complements the results for glucosamine content. Estimation of ergosterol in treated and untreated poplar wood also demonstrated higher levels in the *Pleurotus*. Significantly the ACQ treated wood contained less ergosterol than the CuAz treated wood. Thus on the basis of dry weight loss, chitin and ergosterol levels ACQ has proved to be the more effective preservative. This is, however, based on a relatively short incubation time (60 days) and it is long term preservation that is important to the building industry. Determination of resistance to fungal decay using these preservatives and the methods above is, however, quantitative unlike the direct observational approaches. These do, however, provide a rapid visual assessment of fungal growth on the test samples and have the potential to enable a quick assessment of the effectiveness of chemicals in wood preservation. The SEM in particular can be used to follow colonization by fungal hyphae and resulting damage to the cellular structure of the wood (Chapter 5). Differences were observed between untreated and preservative treated wood blocks. To what extent this can be correlated with wood decay as estimated by weight loss or chitin content is difficult to assess. The application of the Atomic Force Microscope is relatively new and the results obtained here are amongst the first of their kind. Atomic force microscopy has been successfully used to characterize surface properties of wood, wood pulp and paper (Niemi *et al*. 2002). However interpretation is not easy (Koljonen *et al*. 2003). It was possible to demonstrate differences in the fine detail of hyphae of the three test fungi and future work could provide a means of rapidly assessing the effect of chemicals on the growth of hyphal tips.

Future work

1. Assessment of fungal growth and amount of wood decay after pre-inoculation with *T. versicolor* and *P. ostreatus* followed by burial in soil.
2. Determination of leaching of copper from ACQ and CuAz in natural and under laboratory conditions.
3. Determination of effect of ACQ and CuAz on fungal hyphae as observed by light. electron and atomic force microscopy.
4. Determination of living compared to dead mycelium in wood and after contact with preservatives use of vital stains.
5. Determination by confocal microscopy of the effect of ACQ and CuAz on living haphae.

CHAPTER 7

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- http://images.google.co.uk/images

Appendix I

Collection of test fungi.

Trametes versicolor (L.) Pilat:

The top surface of the cap shows typical concentric zones of different colours. Flesh 1-3 mm thick, leathery texture. Commonly grows in tiled layers applanate, upper surface velutinate and shiny, on stumps often forming clusters with a contracted base, 3-5 x 4-8 x 0.1-0.5 cm; upper surface variable in colour, greyish to brown, glabrous zones brown, reddish brown, bluish to black. Strongly zonate with glabrous zones; pore surface grey to white, context leathery tough; pores round, 3-5 per mm, Spores 5-6 x 1.2-2 um. Pore surface whitish to light brown, pores round and with age twisted and labyrinthine. 2-5 pores per millimetre (Vesterholt, 1997).

Pleurotus ostreatus (Jacq.). P. Kumm:

Basidiome fasciculate; stem felty only at the base; cap ash grey, dark brown, olivaceous, blue black or dark brown, 6-14 cm, glabrous, soft-fleshy; gills shortly decurrent, anastomosing; stem very eccentric to lateral, whitish with strigose base. Spores 7.5-11 x 3-4 um.

Daldinia concentrica (Bolton) Ces. & De Not:

Ascoma hemispherical to globose, sessile or occasionally stipitate, single or coalescing, up to 8 cm across, erumpent from bark or superficial on decorticated wood; *ectostroma* relatively thin, at first reddish brown, becoming black and either matt or shiny; *entostroma* massive, fibrous, dark purplish-brown with a large number of conspicuous darker concentric zones. Perithecia small, closely crowded, monostichous, oblong-ovate, 0.8-1.2 x 0.4-0.8 mm; ostiola umbilicate often inconspicuous; asci cylindric sp.p. 105-185 x 8-12 um with stipe 45-95 um long; ascospores uniseriate, inaequilaterally elliptic, dark brown, 11-21.5 x 5.5-9.5 um

(Whalley & Watling, 1980).

Appendix II

Chitin assays.

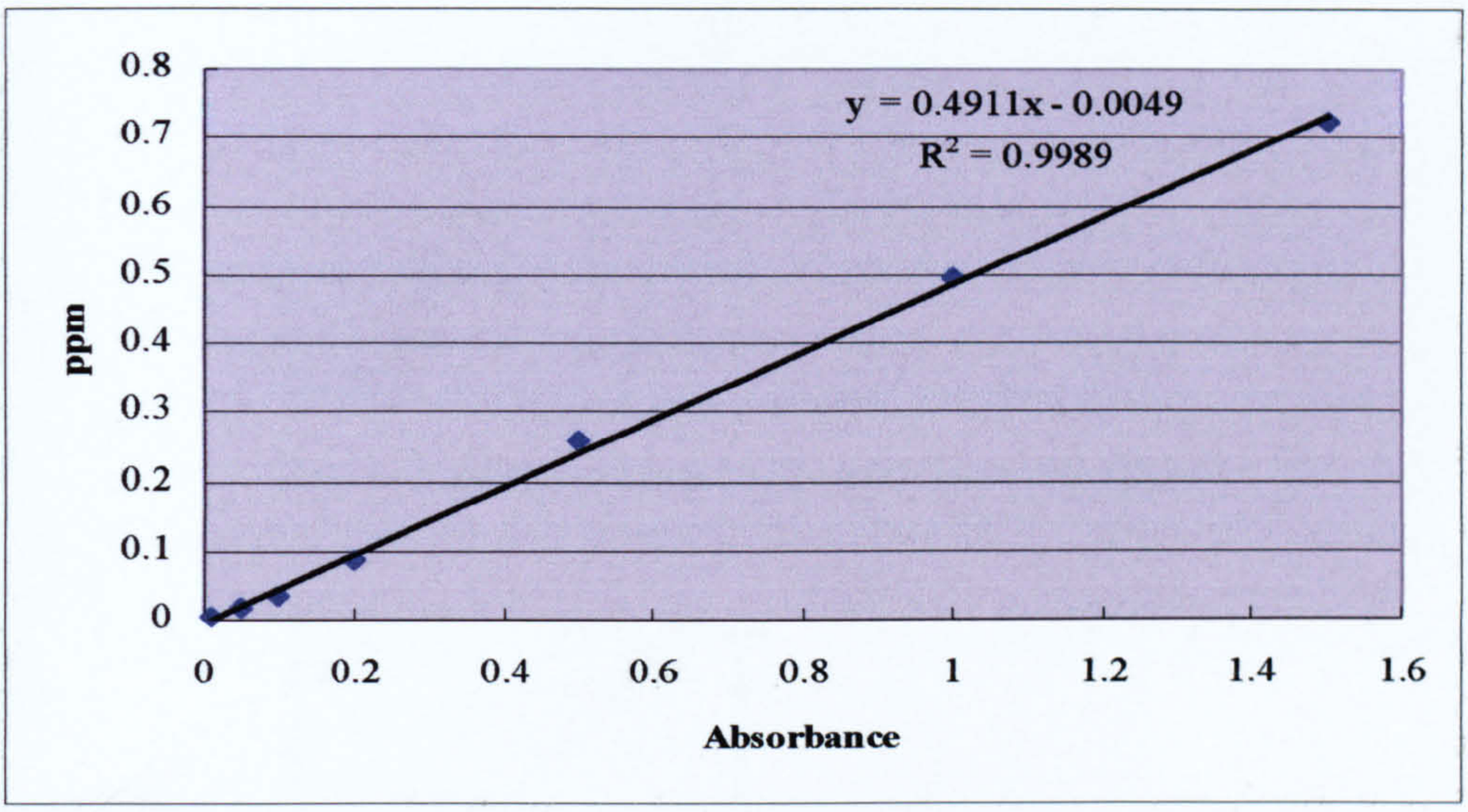


Fig. Relationship of absorbance at 530 nm to concentration of glucosamine hydrochloride. Each value is the mean of six determinations.

Appendix III

The HPLC chromatograms of ergosterol contents of wood decay fungi.

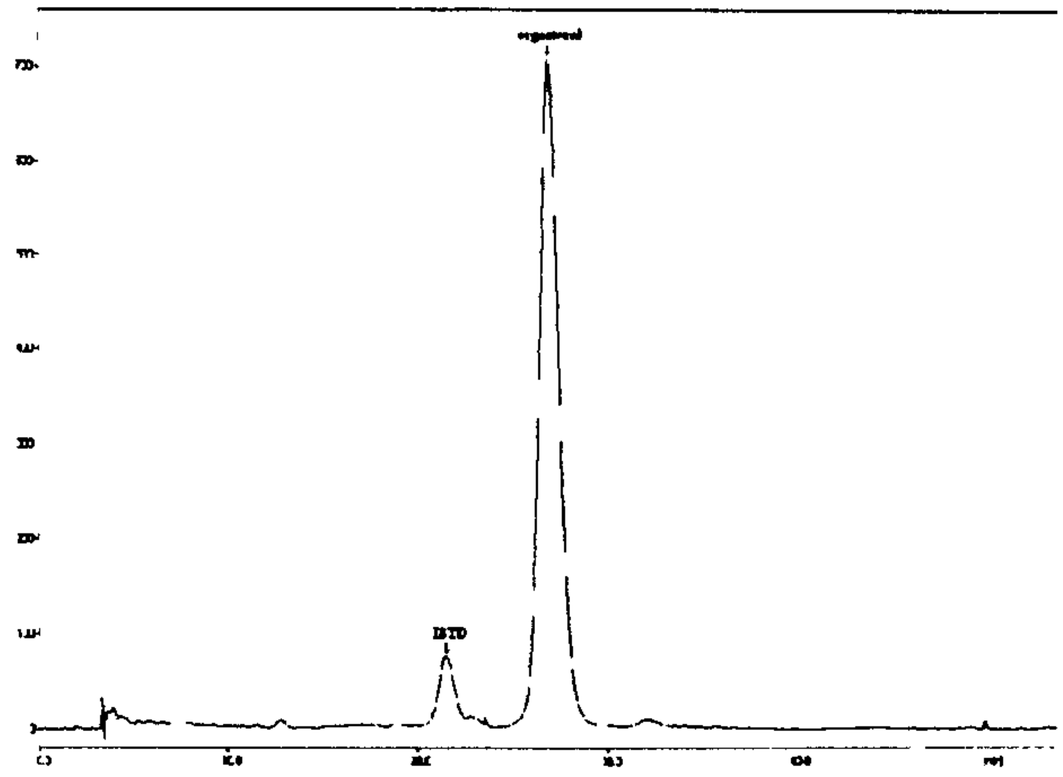


Fig. 1. Ergosterol level in *T. versicolor* mycelium.

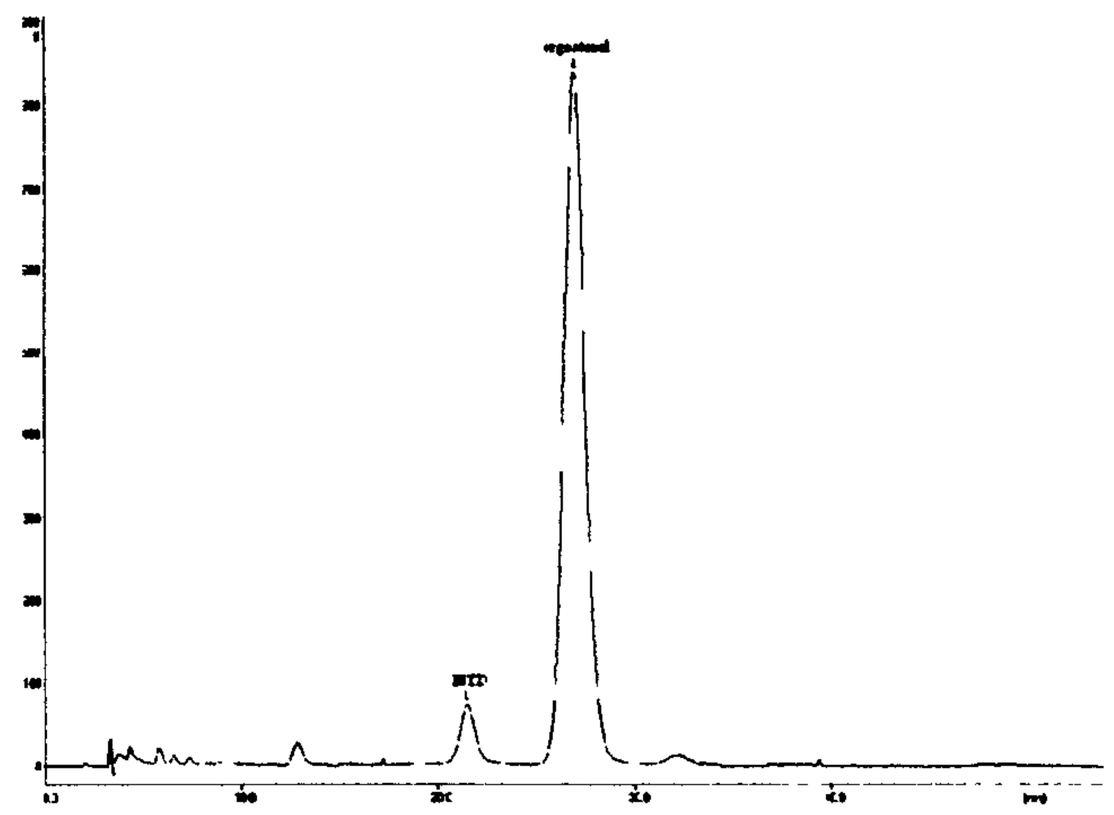


Fig. 2. Erogsterol level in *P. ostreatus* mycelium.

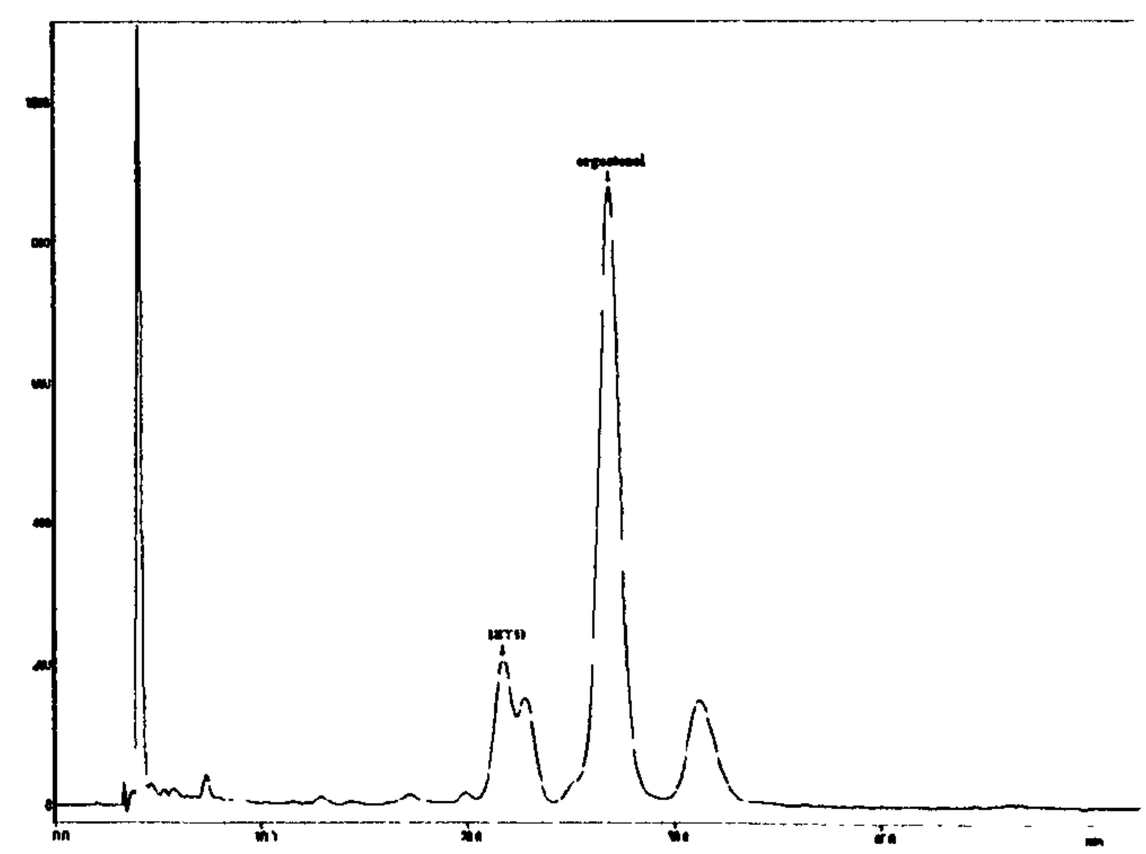


Fig. 3. Erogsterol level in *D. concentrica* mycelium.

Appendix IV

Additional images of fungi …..of wood decay.

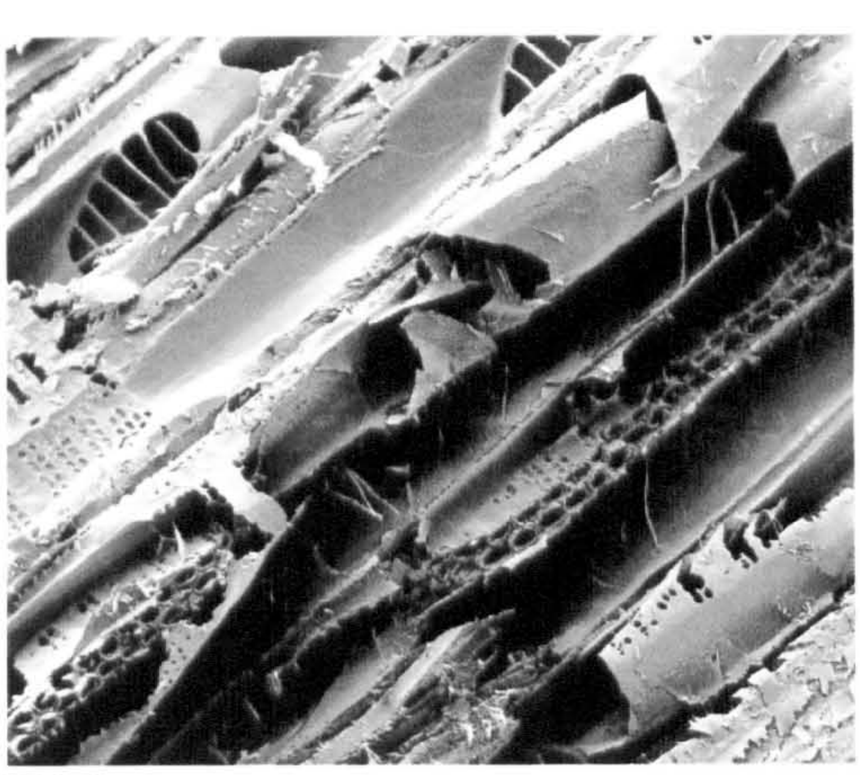
Wood decomposition in the soil samples for 6 months.

Pine-untreated

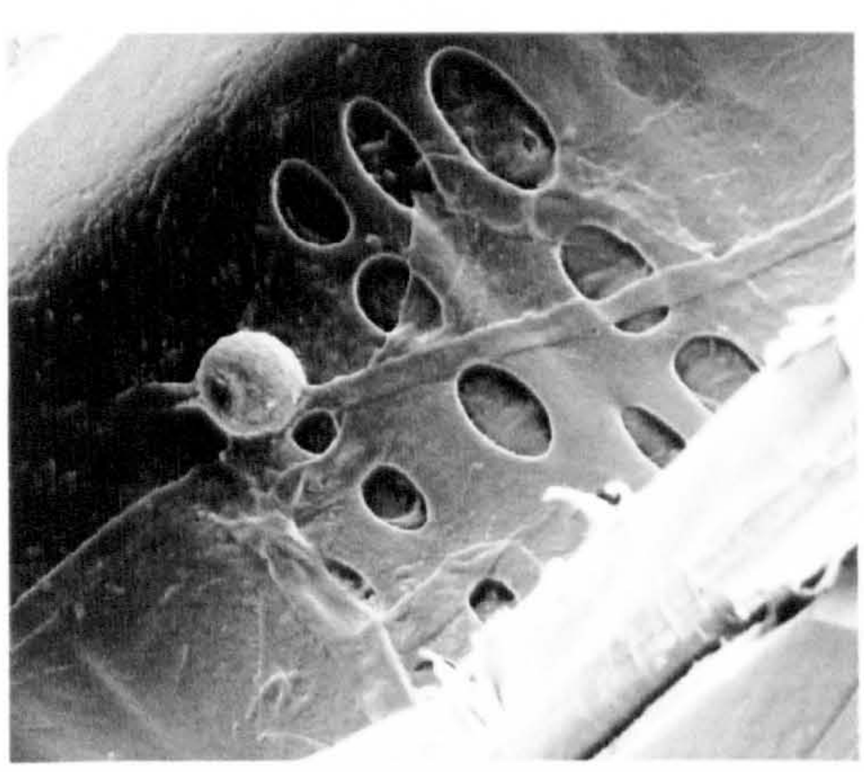
X200

x 100

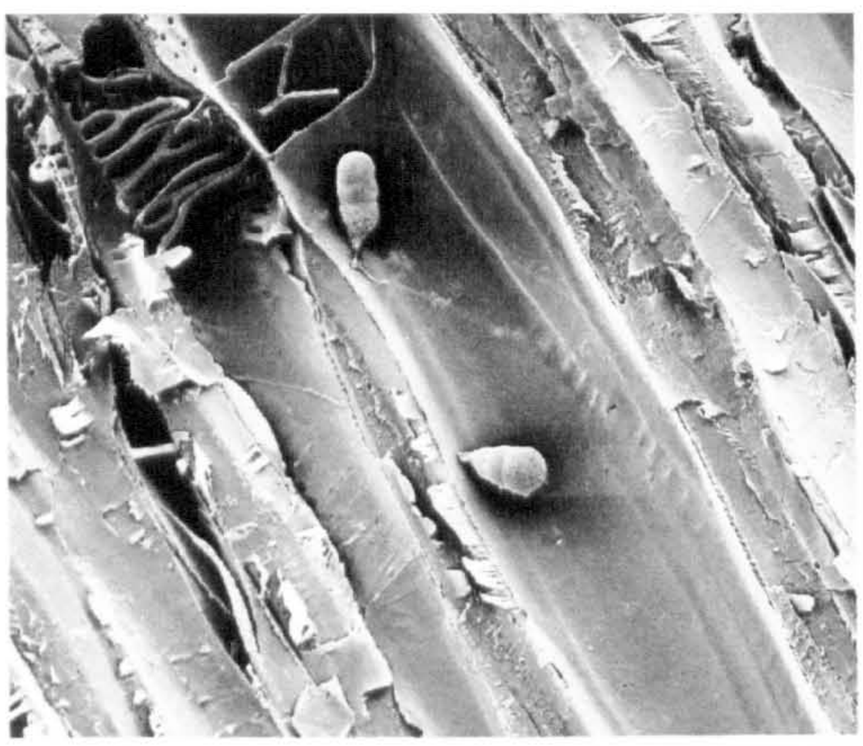
Poplar-untreated



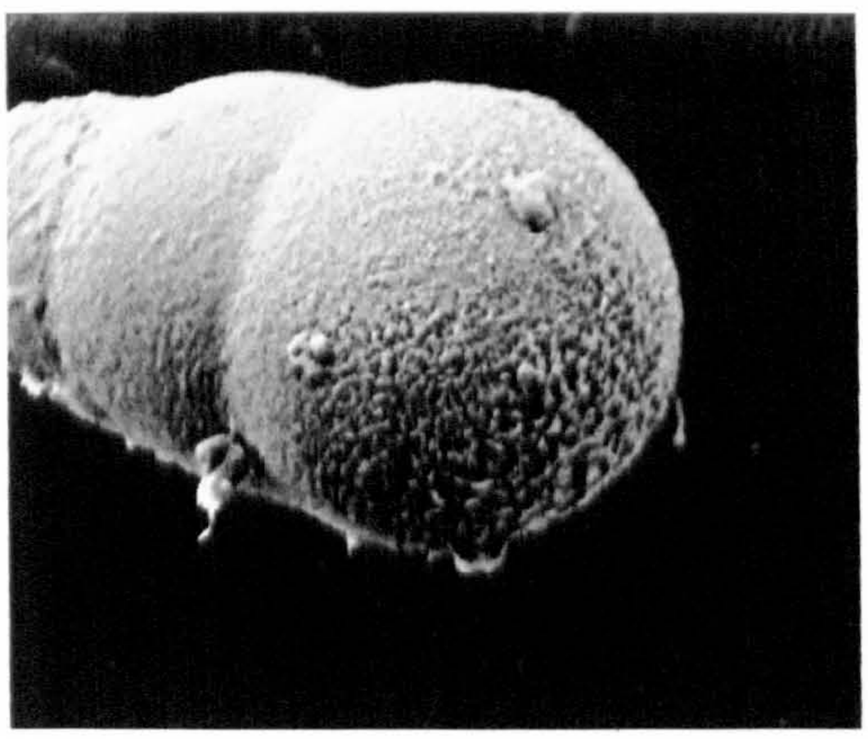
X200



x 20

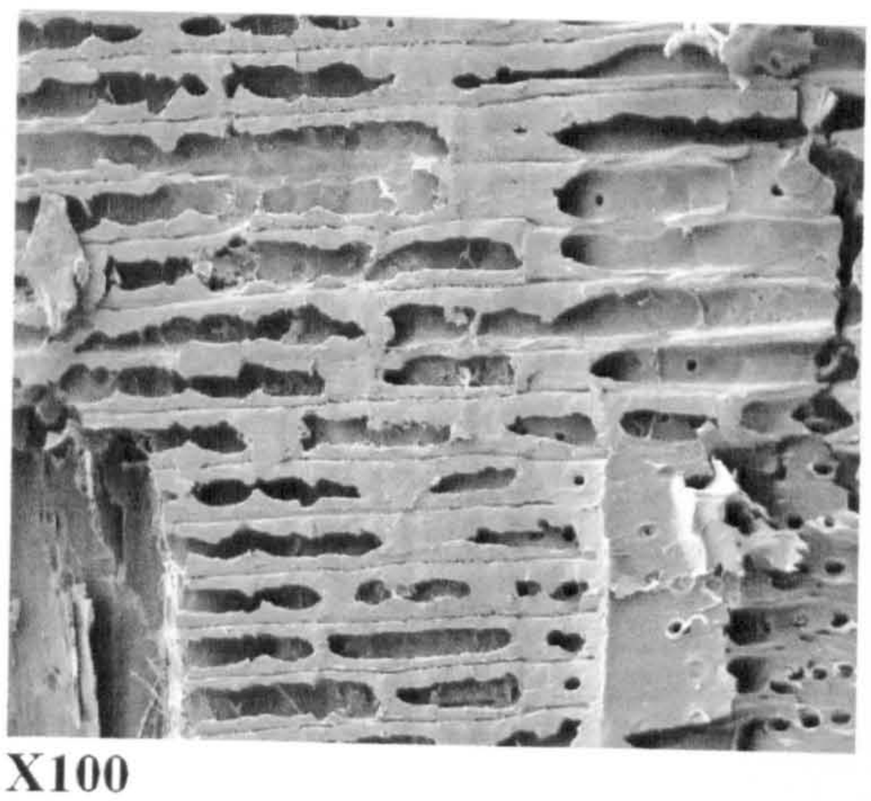


X 100

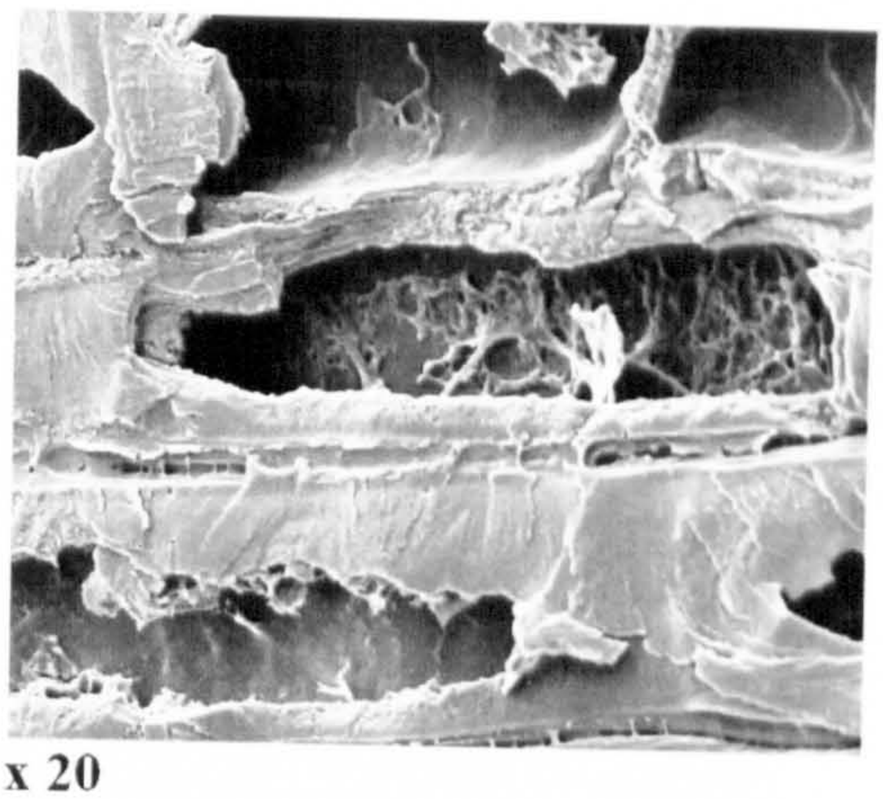


x 10

Cypress-untreated



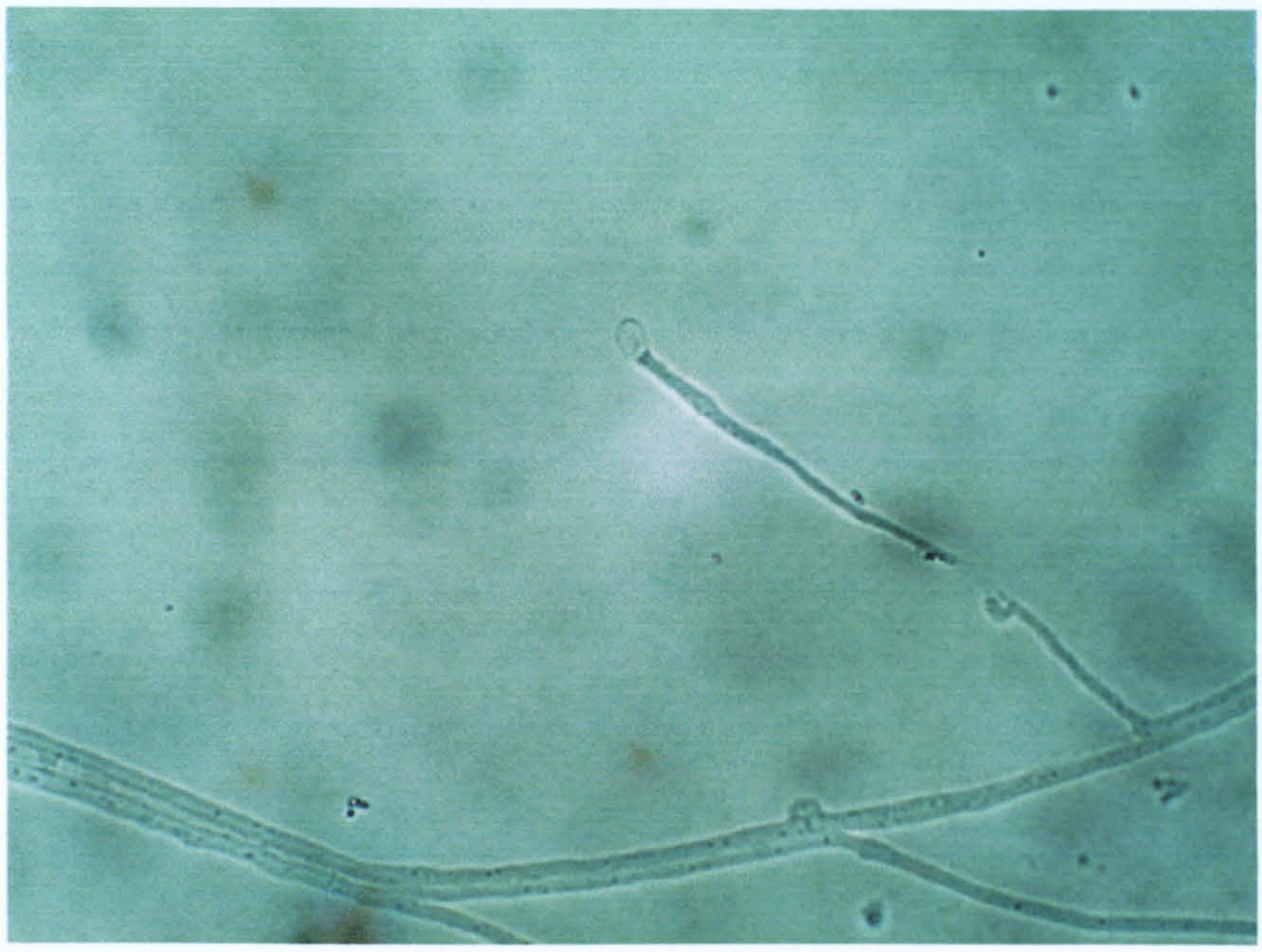
X100



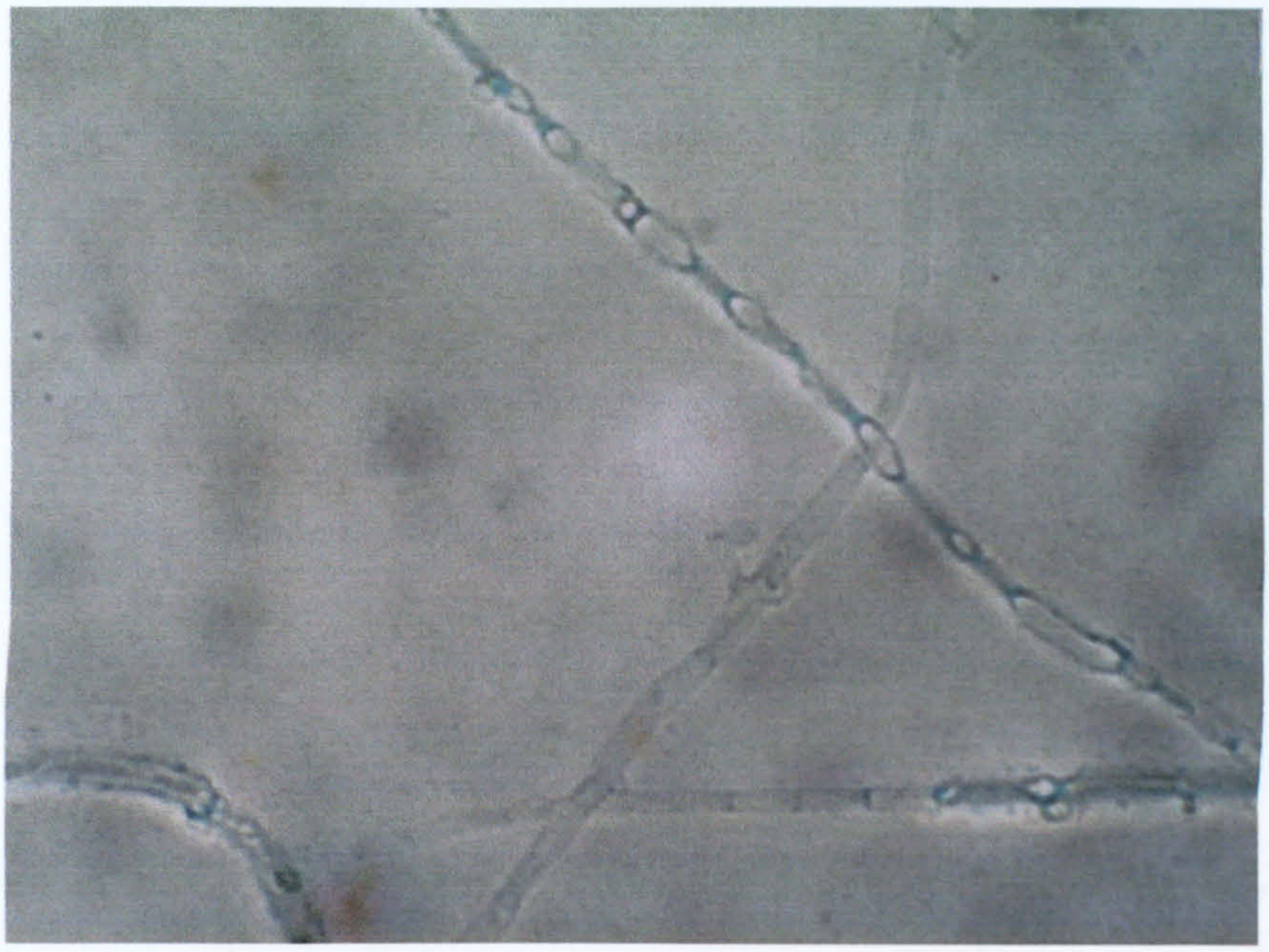
x 20

Appendix V

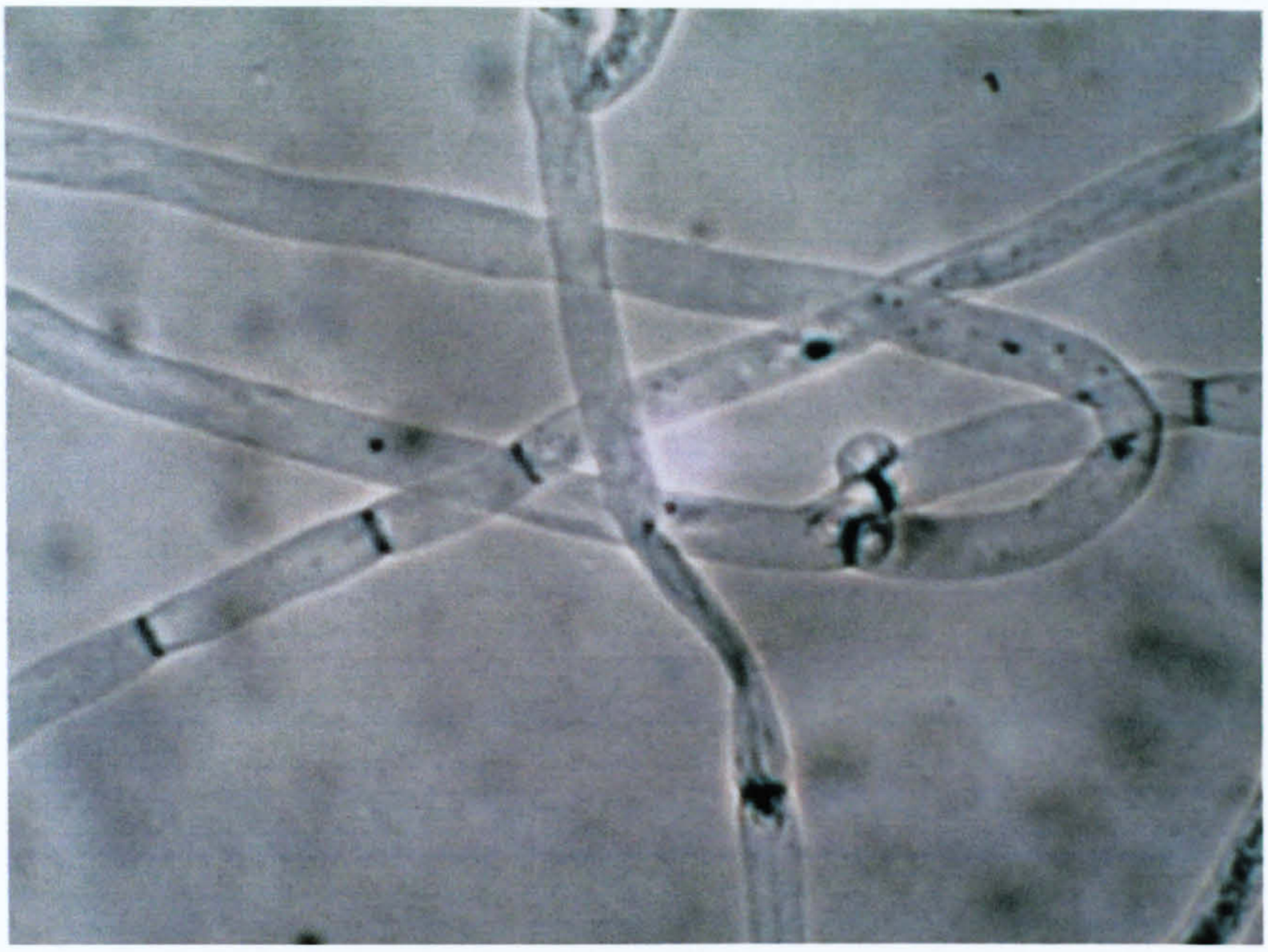
Light microscope images of fungi hyphal tip.



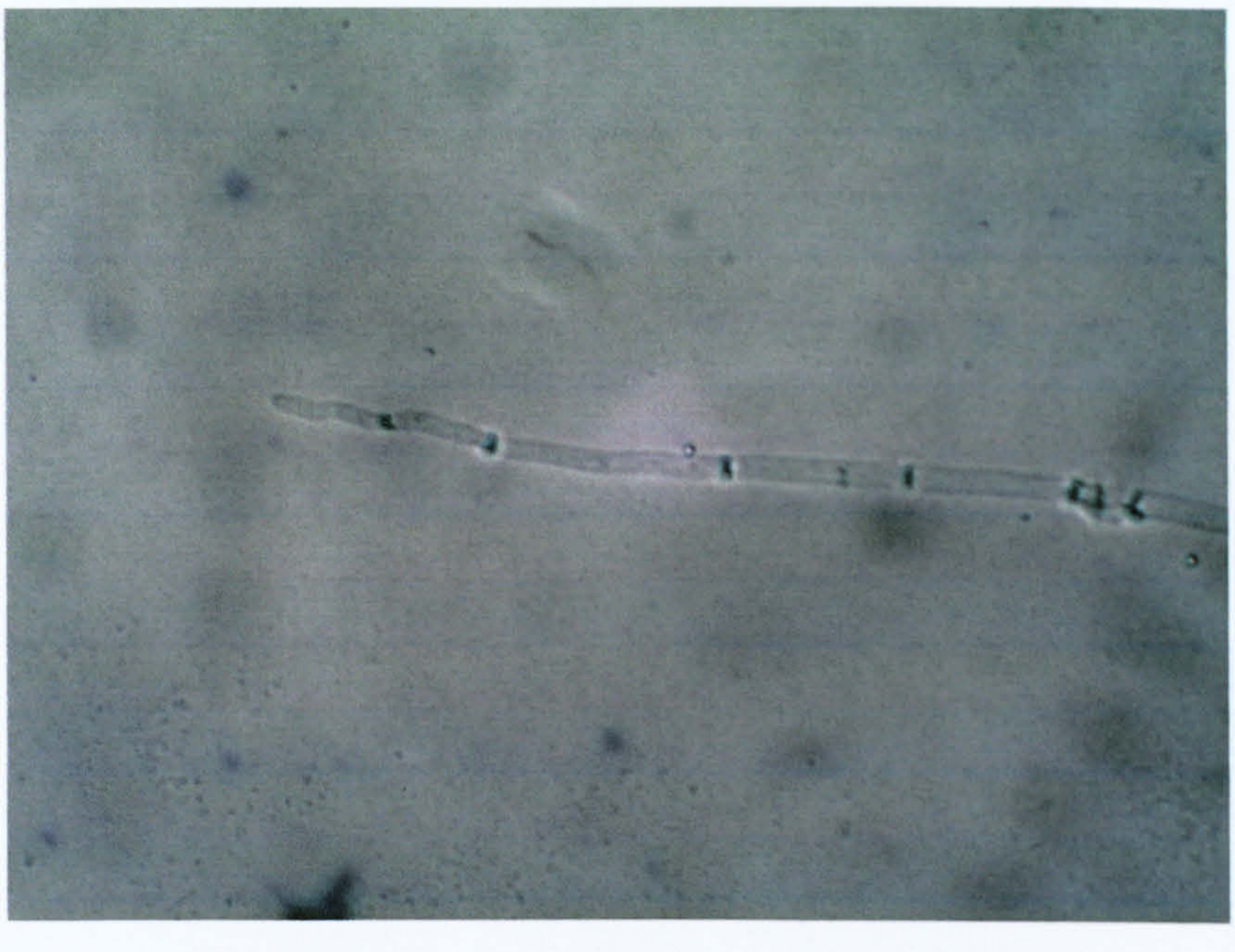
Swollen hyphal tip and hyphae of basidiomycete with clamp connections in subculture in distilled water *T. versicolor*.



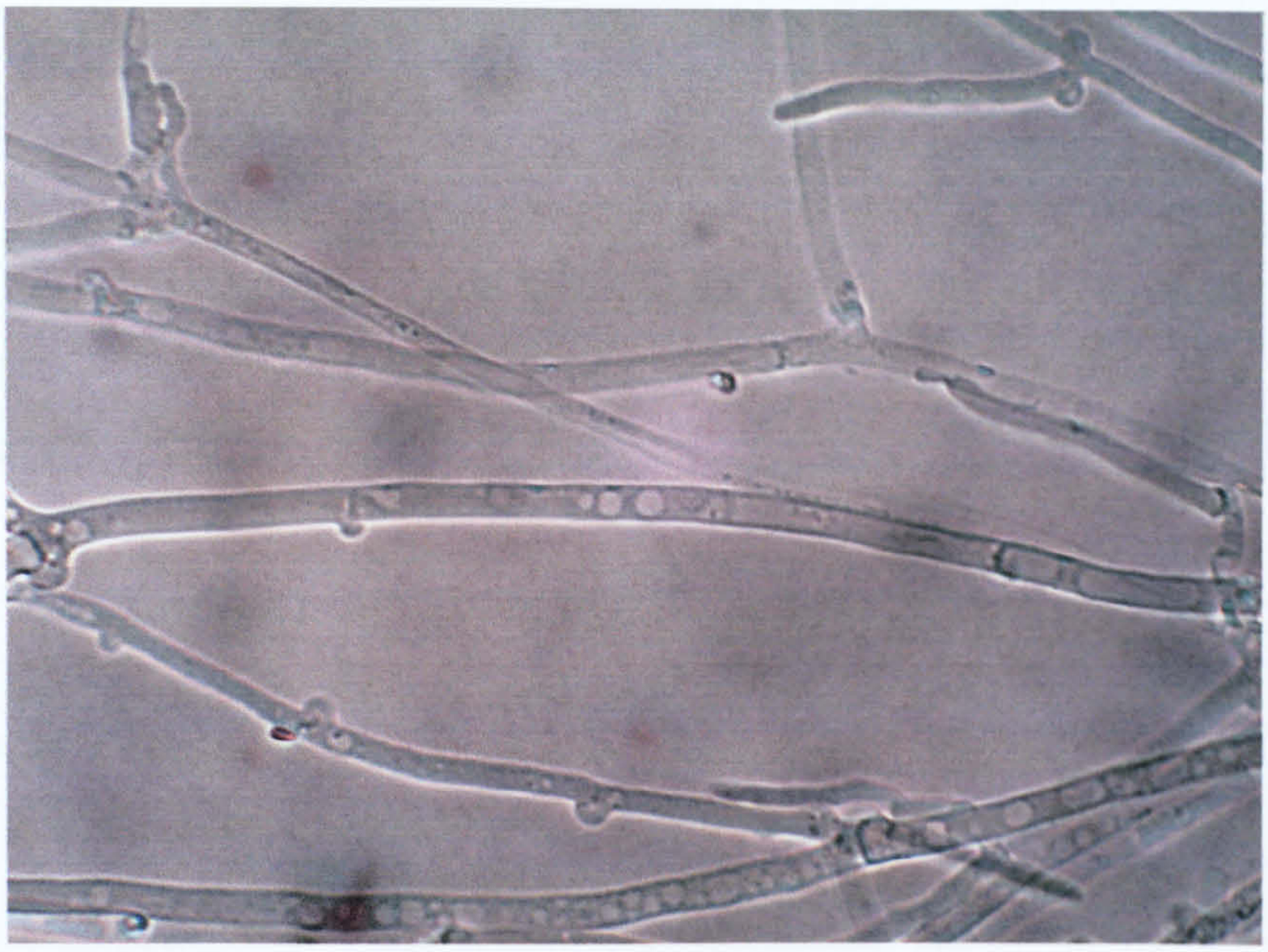
Hyphae of *T. versicolor* reacting to CuAz preservative 0.2% v/v



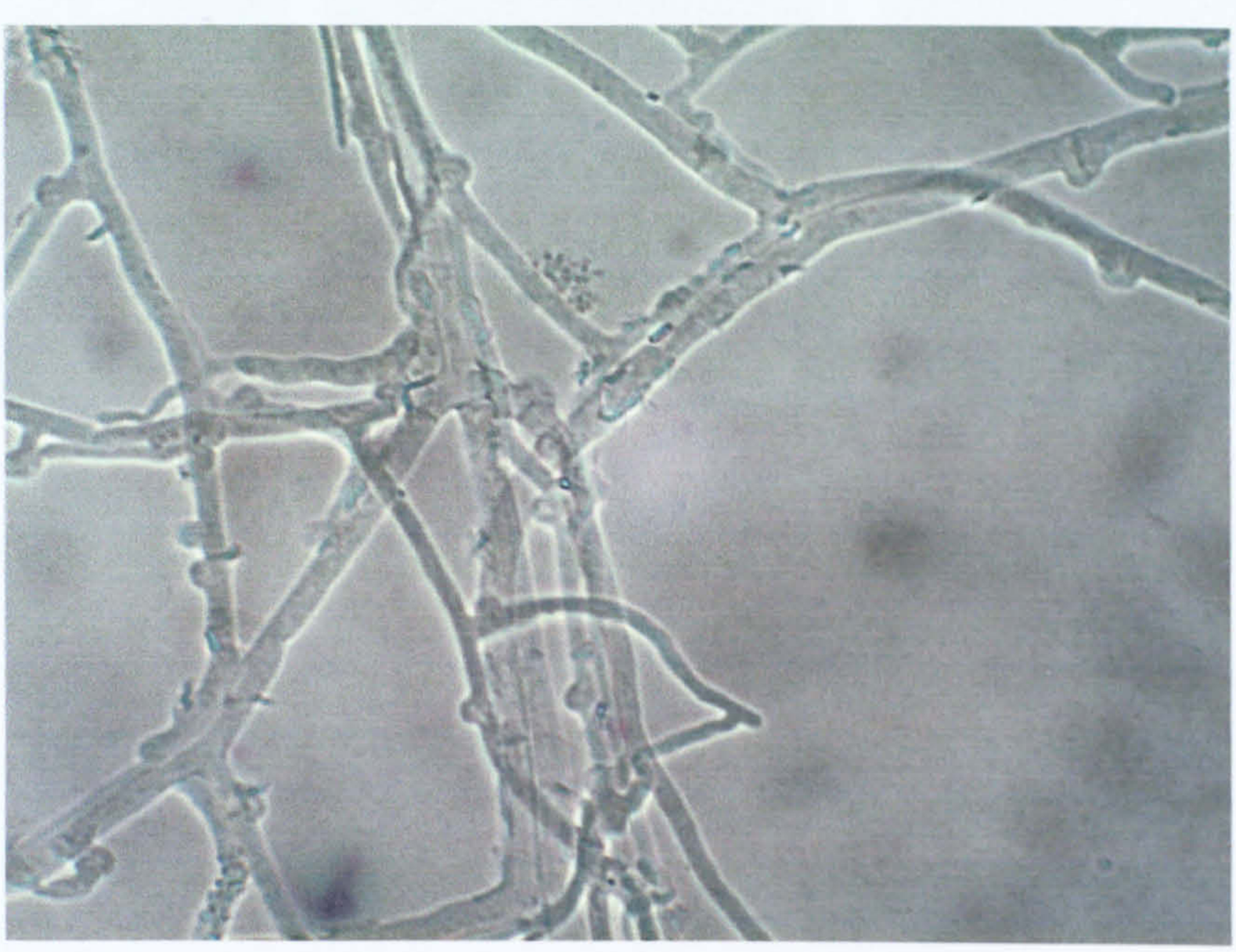
Hyphae of *T. versicolor* reacting to ACQ preservative 0.2% v/v, showing septate hyphae.



Hyphae of *P. ostreatus* was inoculated in distill water, showing septate hyphae.

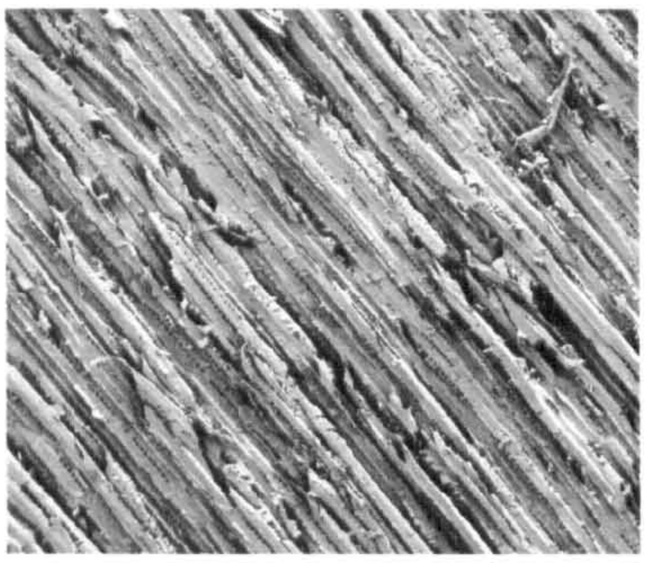


Hyphae of *P. ostreatus* reacting to CuAz preservative 0.2% v/v, showing clamp connections with septate hyphae.

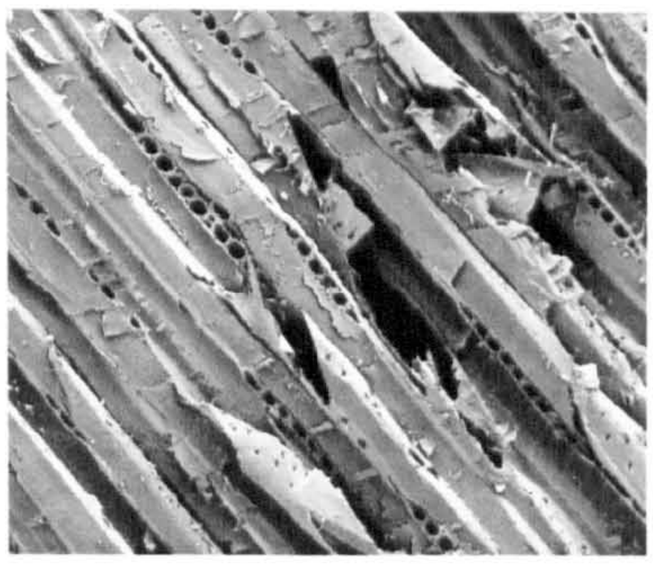


Hyphae of *P. ostreatus* reacting to ACQ preservative 0.2% v/v, showing clamp connections with septate hyphae.

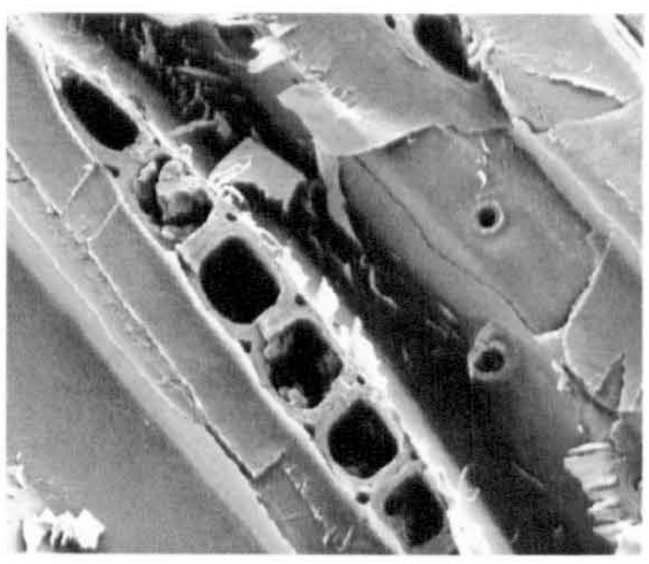
Wood anatomical observation of SEM.



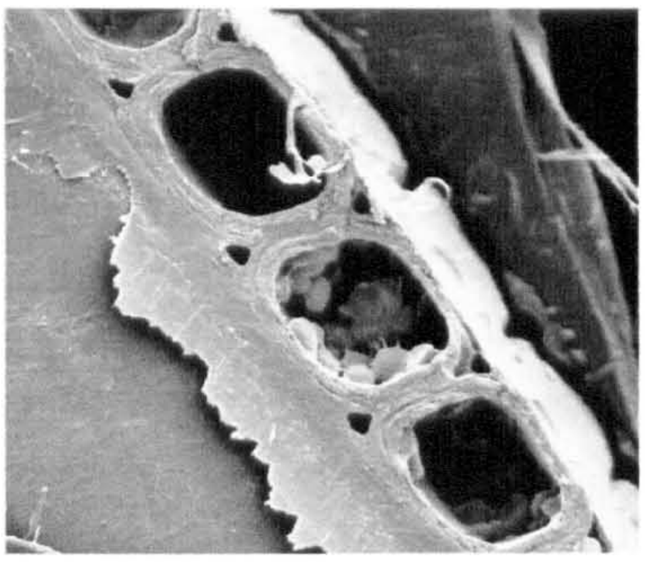
x 500



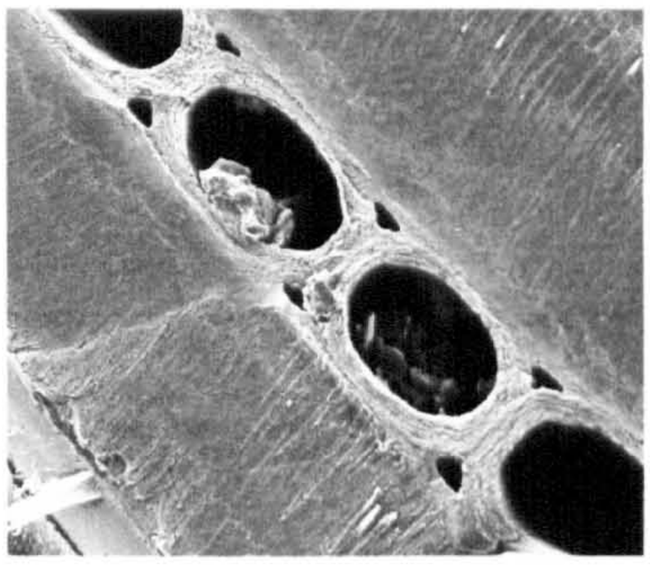
x 200



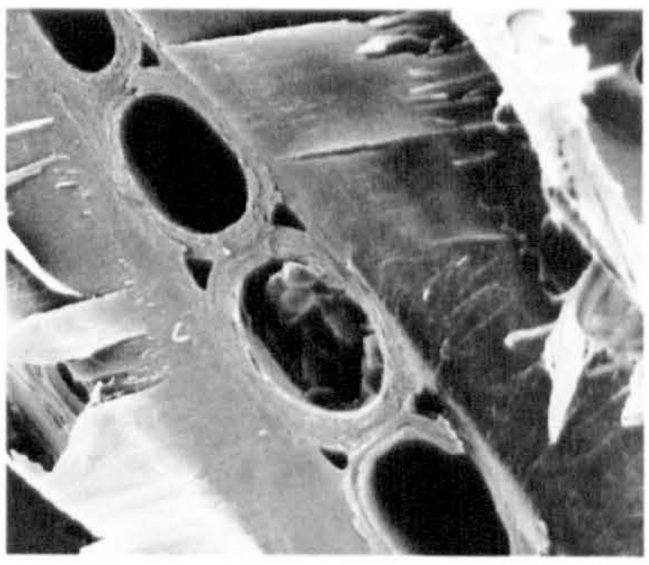
x 40



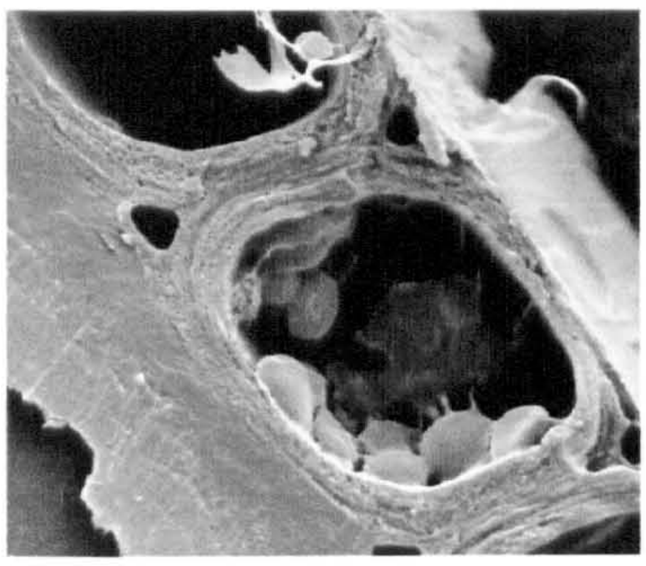
x 20



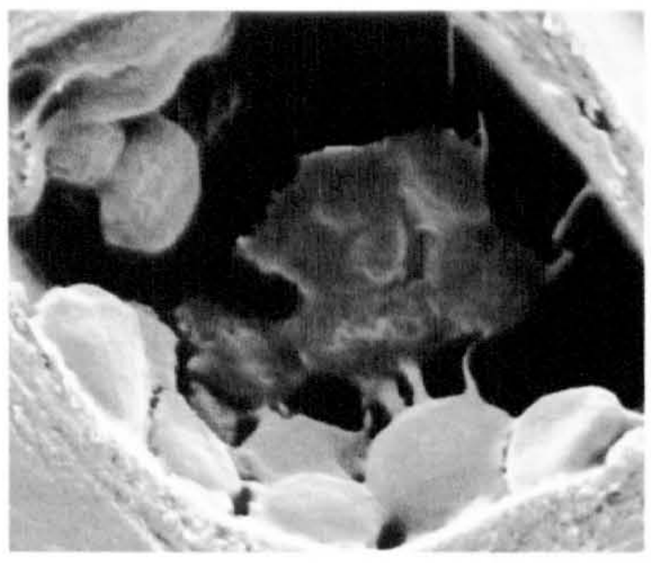
x 20



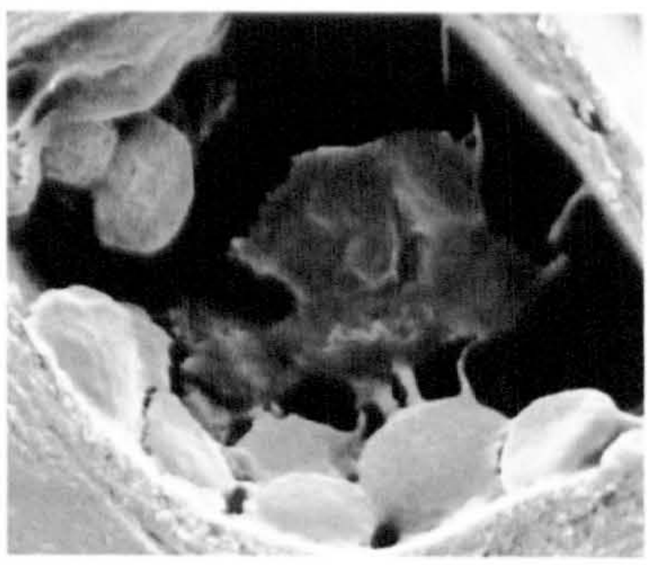
x 20



x 10



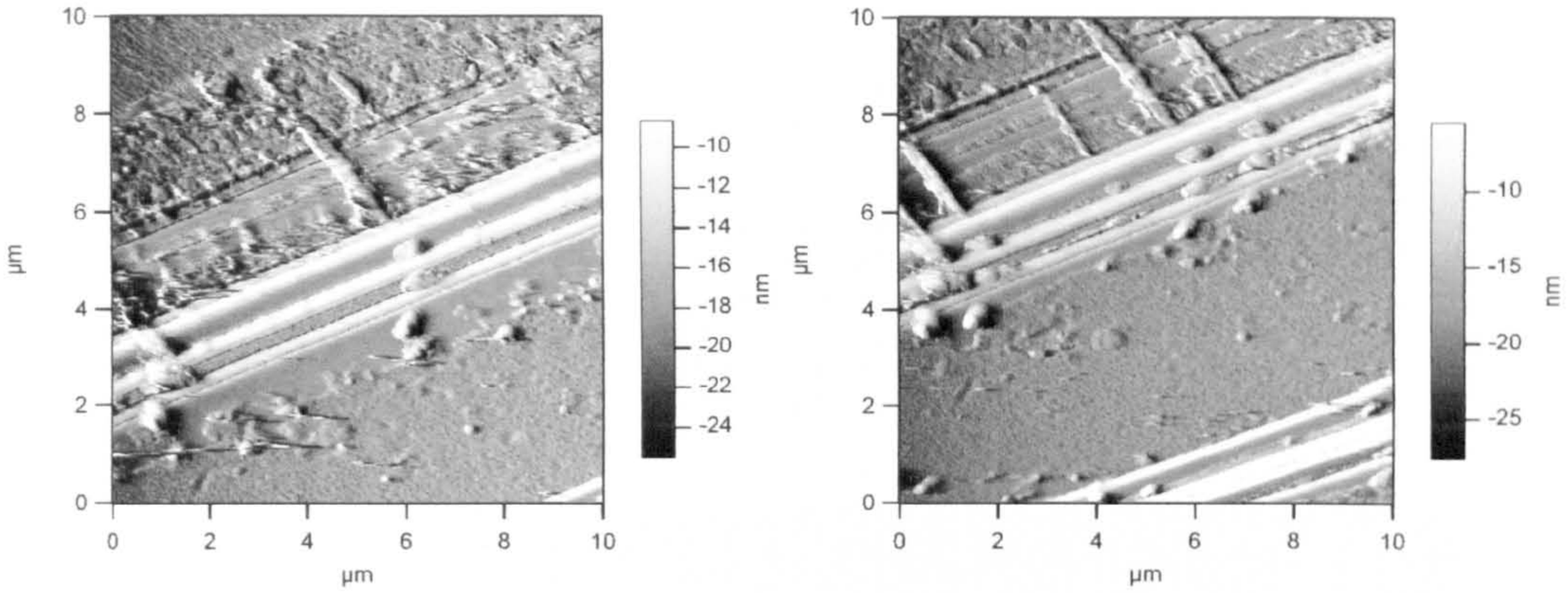
x 5



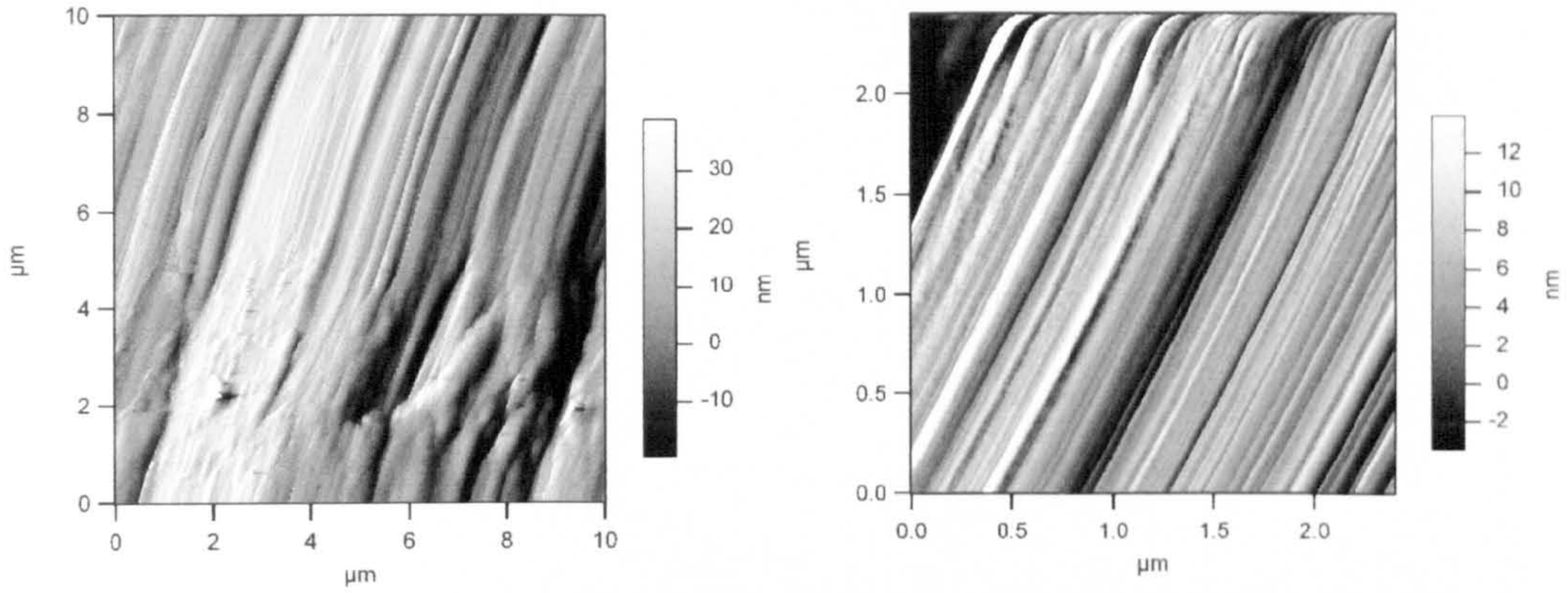
x 5

Untreated cypress wood blocks following inoculation with *D. concentrica.*

Wood anatomical observation of AFM.

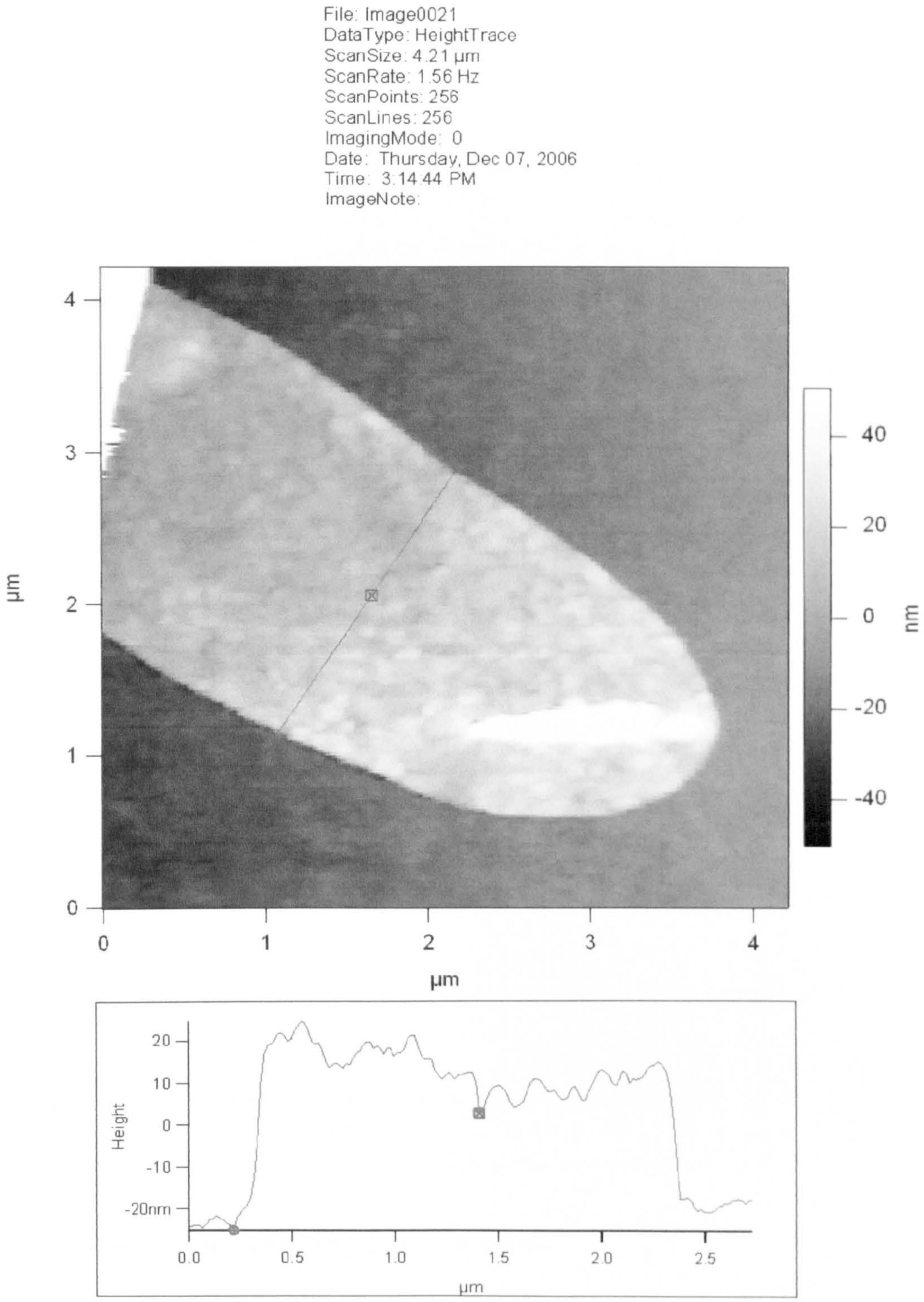


Untreated cypress wood blocks following inoculation with *D. concentrica*.

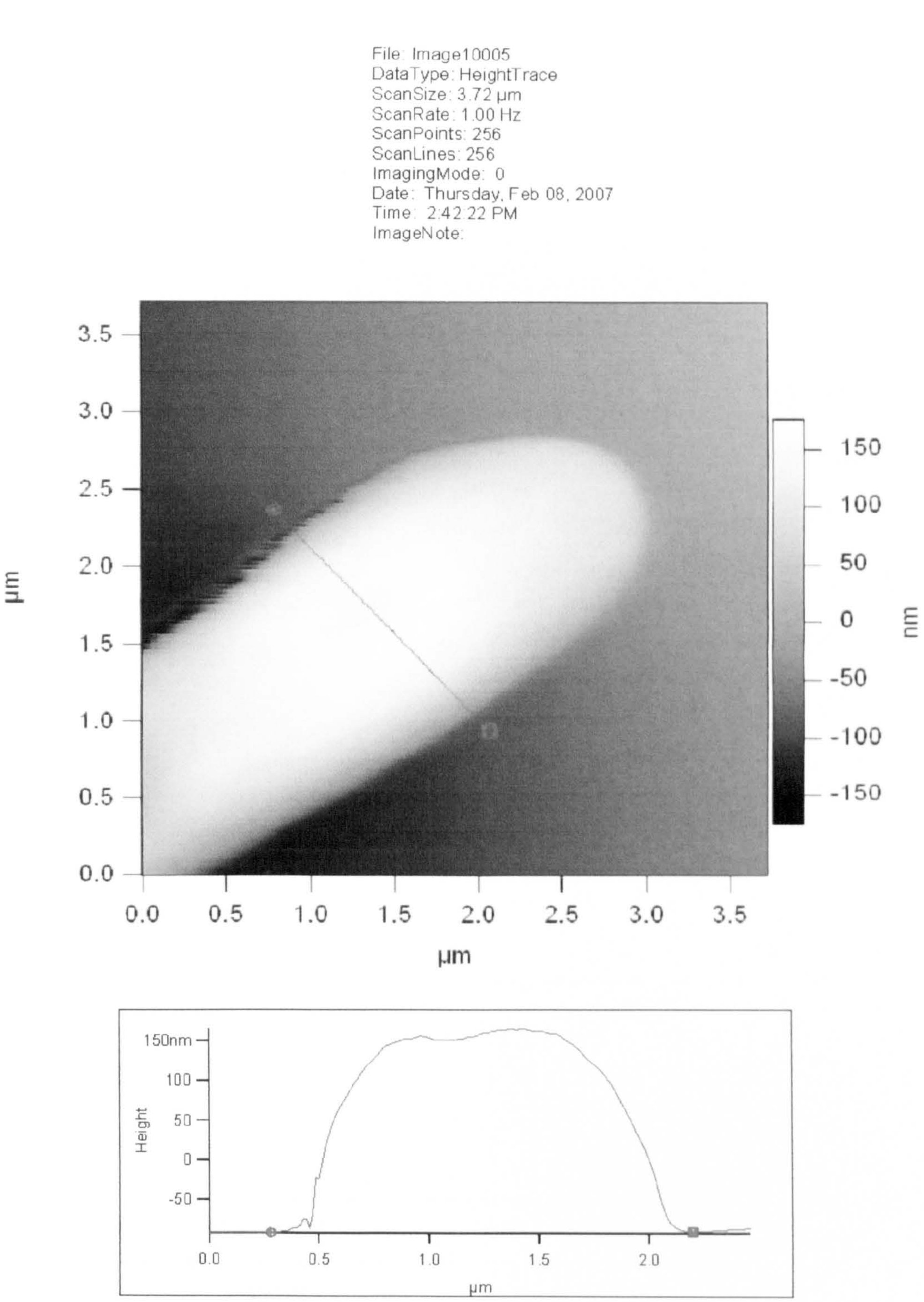


AFM deflection images for pine wood blocks following inoculation with *P. ostreatus* and ACQ treated pine wood blocks.

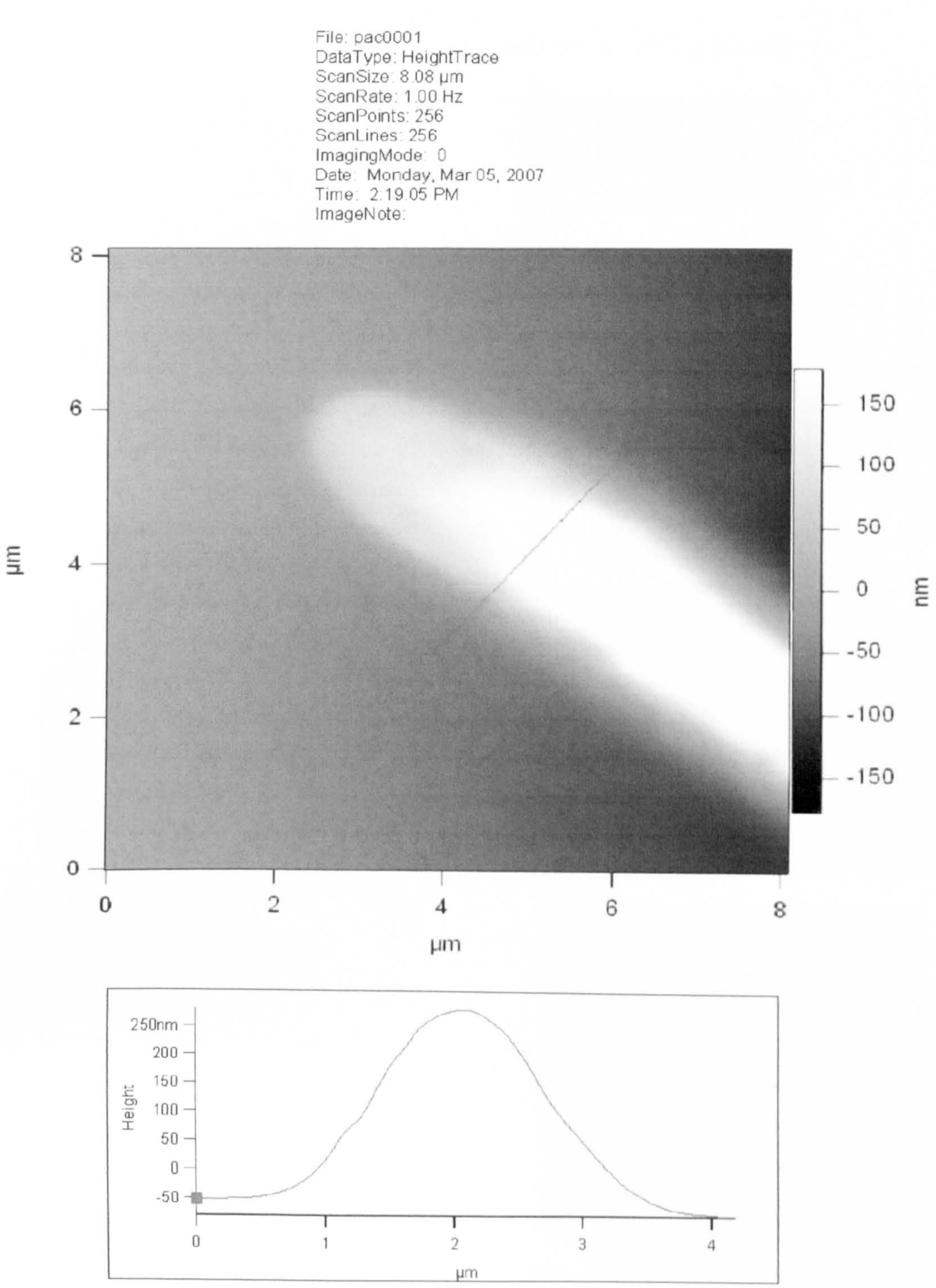
Atomic Force Micrographs of fungal hyphae tip.



AFM height image of fungal hyphal tip for *T. versicolor*.

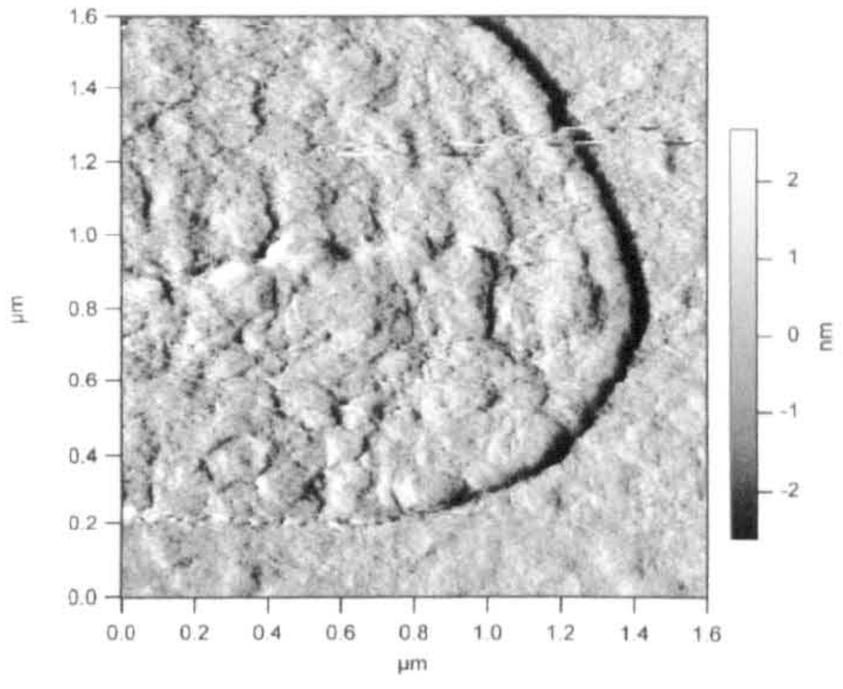


AFM height image of fungal hyphal tip for *P. ostreatus*.

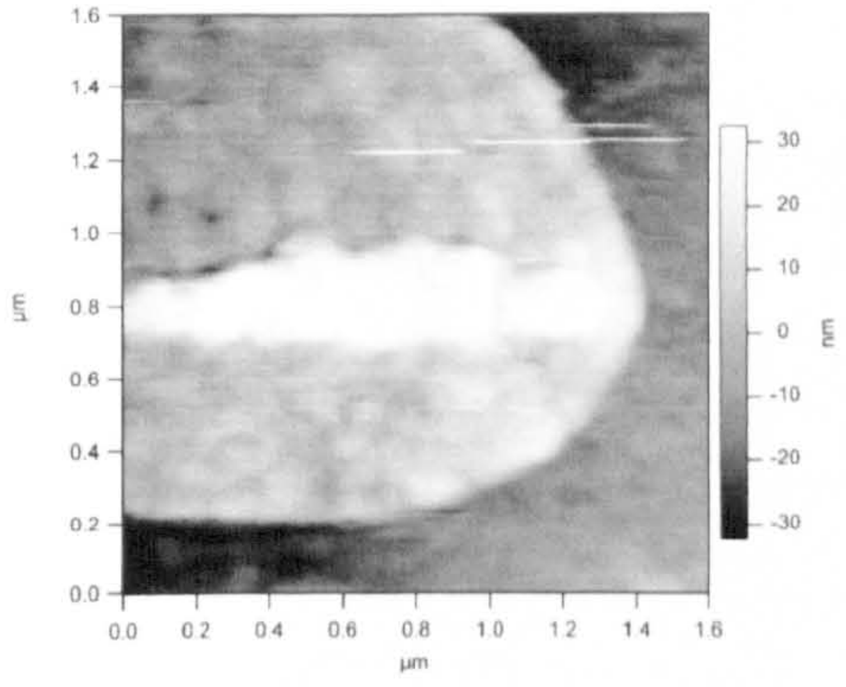


AFM height image of fungal hyphal tip for *D. concentrica*.

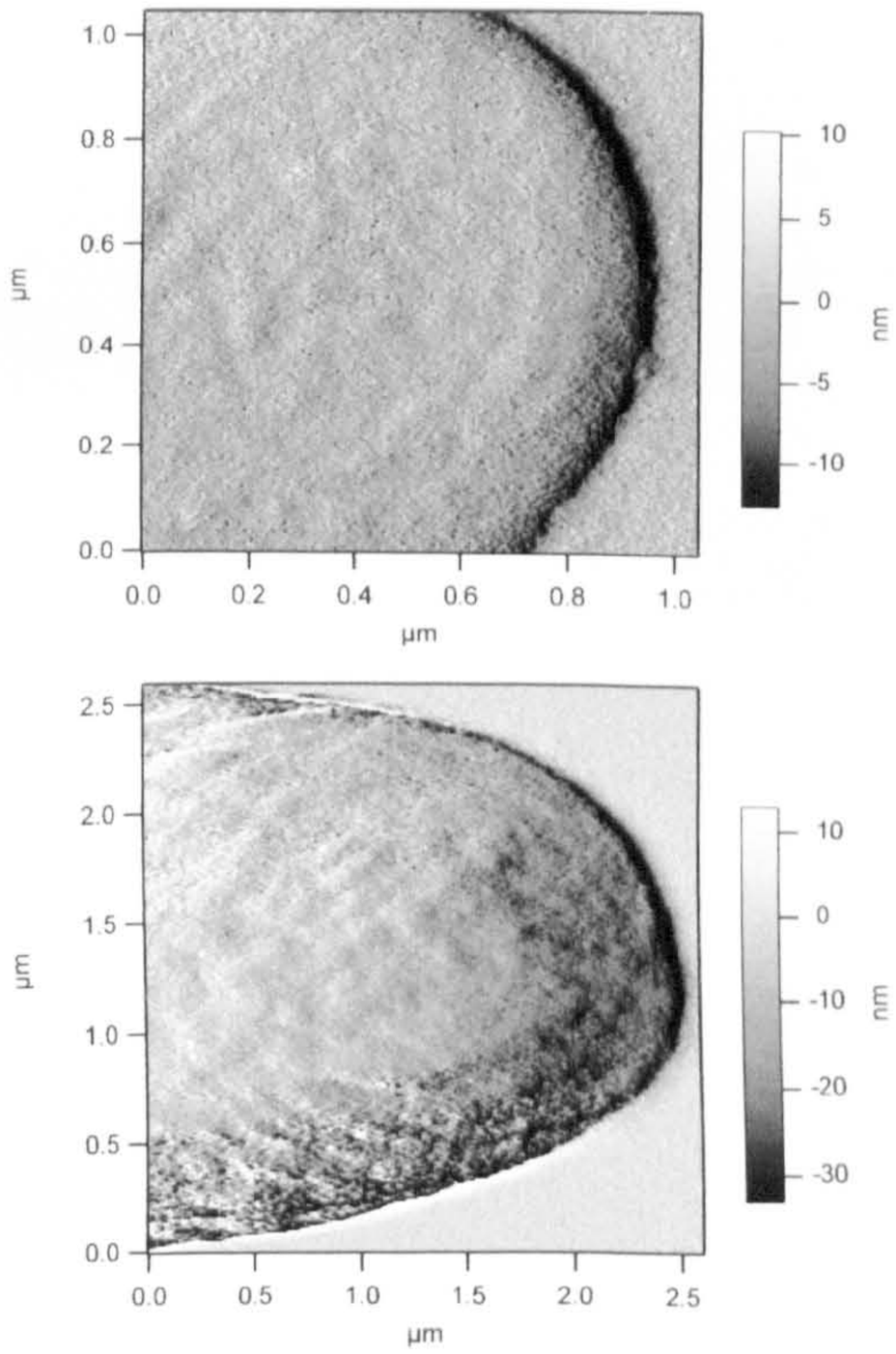
Phase of *T. versicolor*



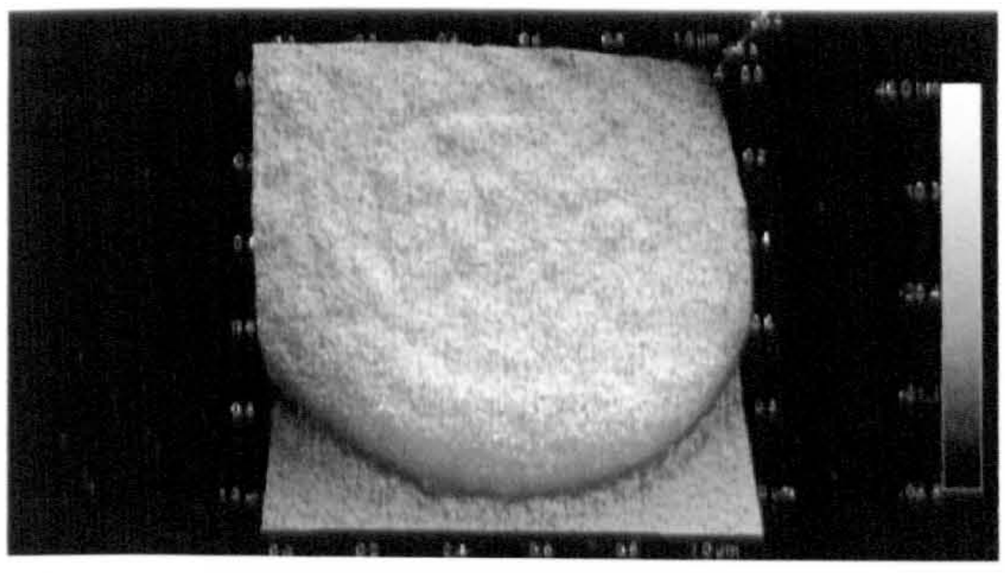
3D image of *T. versicolor*

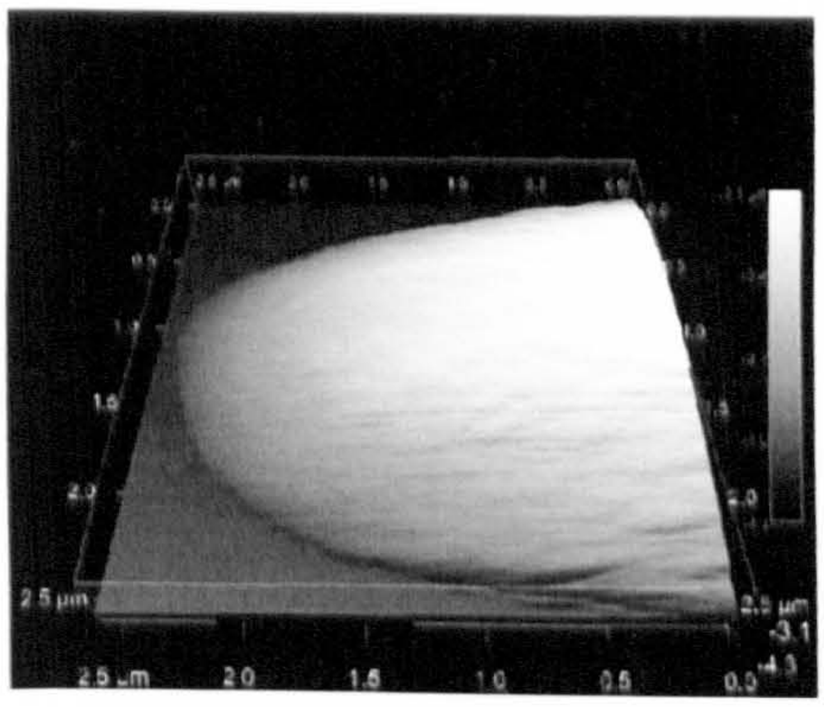


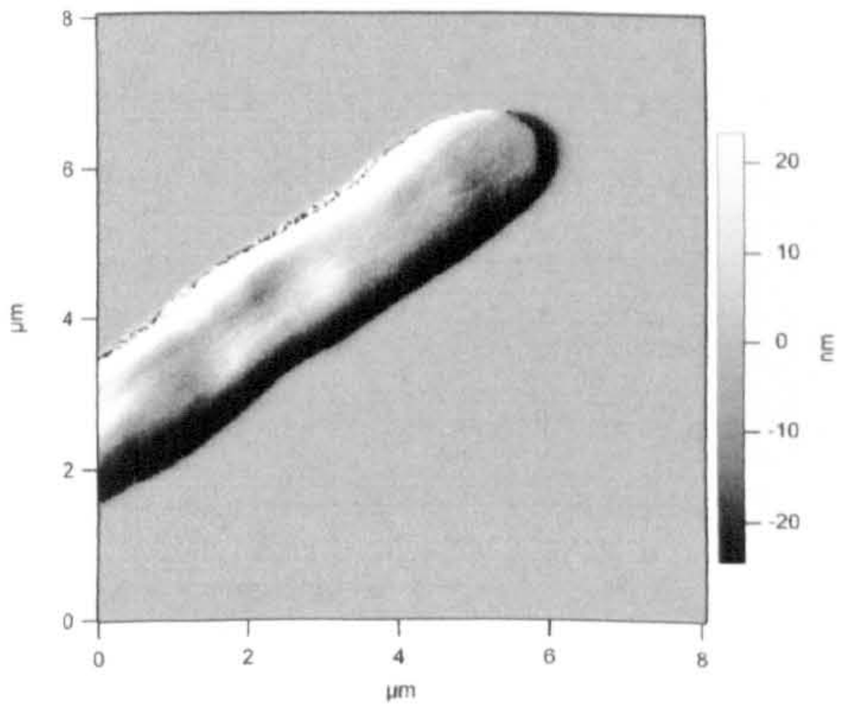
Phase of *P. ostreatus*

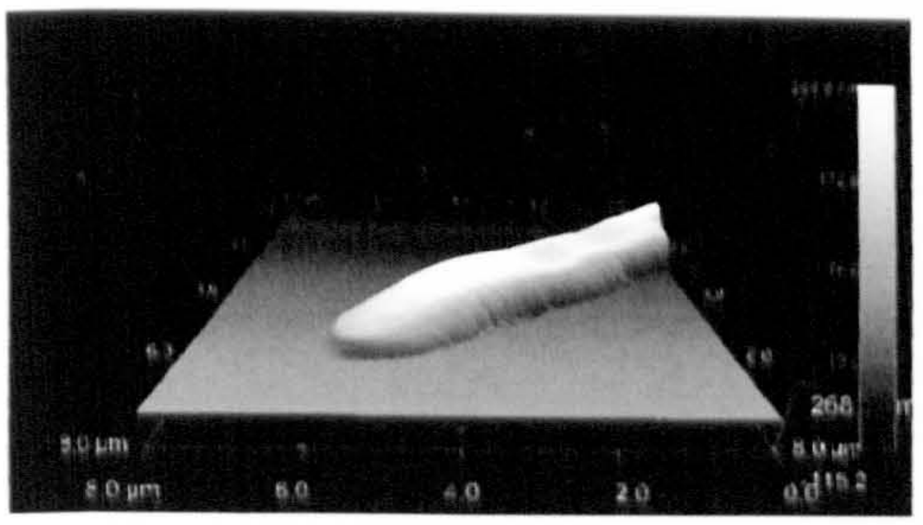


3D image of *P. ostreatus*

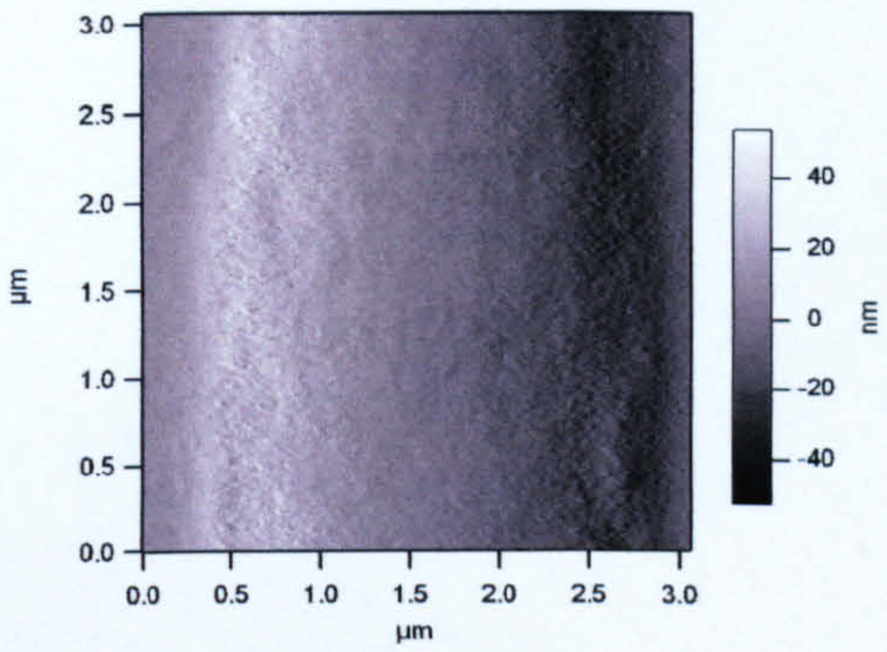








Phase of *D. concentrica*



3D image of *D. concentrica*