BIOLOGICAL CONTROL OF THE DRY ROT FUNGUS *Serpula lacrymans*

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THIS THESIS IS PRESENTED TO UNIVERSITY OF ABERTAY DUNDEE IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY

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I certify that this thesis is the true and accurate version of the thesis approved by the examiners

Signed [redacted] Date 2/11/98.

(Director of Studies)

This manuscript is dedicated to my Mum and Dad, without whom I would never has made it this far.

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ALAN JAMES SCORE

ABSTRACT

Serpula lacrymans causes wood decay, which is of major economic importance in many areas of the world and in the U.K. alone £400 million pounds worth of damage is caused on an annual basis. Conventional treatment regimes for wood decay caused by the wood decay basidiomycete *Serpula lacrymans* involve the removal of infected timber, the removal of non-infected timber around the infection site and the use of powerful fungicides. Depending upon the scale of the infection the treatment can be expensive and misidentification of the fungus causing the decay is not unknown. Health and environmental problems can arise with the use of the fungicides and removal of timber, in particular that of historical or artistic value, is not always desirable.

Biological control of wood decay fungi has been proposed for a number of years and previous investigations have indicated that control of decay fungi is possible. However, there were a number of problems reported, in particular, the extrapolation of the laboratory work into the field situation. It has been shown that *Trichoderma* isolates are antagonistic towards *S.lacrymans* on several media types and wood, with wood colonised initially by *Trichoderma* isolates resistant to infection by *S.lacrymans*. Screening experiments, carried out on different media and wood, provided a *Trichoderma* isolate that would be used in the medium-scale and field trial experiments. The screening experiments indicated that nutrient composition is important for the effectiveness of the *Trichoderma* isolates, with a high nitrogen level increasing the antagonistic effectiveness. Through the development of a small scale wood interaction system it was determined that certain *Trichoderma* isolates could prevent the decay of wood by *S.lacrymans*. However, the *S.lacrymans* developed specialised hyphal structures (strands) to bypass the *Trichoderma* colonised portions of the wood.

The medium-scale and field trial experiments indicated that *Trichoderma* could be used to protect wood from decay and prevent the spread of *S.lacrymans*. However, the existing *S.lacrymans* colony could not be killed although it could be induced to produce fruit-bodies by the direct application of the *Trichoderma* to the *S.lacrymans*. A PCR system was developed to detect the spread of both *S.lacrymans* and *Trichoderma* through wood. Although the *Trichoderma* could not be detected, the *S.lacrymans* could be detected in wood sections not visually infected with *S.lacrymans*.

Extracellular enzyme studies indicated that the phenoloxidase enzyme laccase was important in the defensive strategy of *S.lacrymans* and in the offensive strategy of *Trichoderma* isolates. These studies also showed that *S.lacrymans* may use laccase as a general stress enzyme as laccase was detected during antagonistic, nutritional and temperature stress situations.

"It was the vile smell of the hideous, insidious fungus that swallows houses alive like a python. It was Merulius Lacrymans. Dry rot".

Quote taken from the short story

'Superstitious Ignorance' by Michael Cornish

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Introduction

1.1 The importance of wood and timber

Wood is the hard, fibrous tissue that comprises the major part of stems, branches and roots of trees (and shrubs) belonging to the plant groups known as gymnosperms and the dicotyledonous angiosperms. It functions by providing mechanical support, food storage, liquid transport and secretion production (e.g., resins and tannins). Virtually all of the wood of economic significance is derived from trees and, as timber, it becomes a versatile material with a multitude of uses.

Timber is one of the oldest building materials known to Man and, today, nearly all houses have substantial quantities of timber in their construction, e.g., in roofs, doors, windows, and internal fixings and fittings. Although some large commercial and public buildings are constructed using combinations of steel, concrete and plastics as the main materials, it would be unusual not to find wood present in some form. With the development of new construction methods timber is again becoming widely used in the manufacture of support structures, e.g., frameworks. However, construction is not the only use to which timber can be put. Timber can be used as a means for transport (land, sea and air), for producing weapons, tools and utensils, and as a source of chemicals for industrial, domestic and pharmaceutical uses (Eaton and Hale, 1993). Wood is also the basic raw material in the papermaking industry, is used in the textile industry and in the manufacture of wood composites and panel products (Eaton and Hale, 1993). There are a number of chemical products derived from wood (Table 1.1) and these can form a substantial part of a country's production capability.

Timber is an important natural resource for many countries and the annual world production of timber is about 2.4×10^9 tons, with 10^9 tons used as industrial roundwood (e.g., sawlogs, veneer logs, poles and pilings, and pulpwood) and the remainder (about 53%) used as fuelwood and charcoal (Schniewind, 1989). The world production of roundwood is of the same order of magnitude (by weight) as the production of steel and iron. Unlike steel,

Bark Products	Phenolic acids
	Waxes
Cellulose	Rayon
	Cellophane
	Cellulose esters
	Cellulose ethers
Extractives	Rubber
	Tannins
Lignin products	Alkali lignin
	Lignosulphonates
	Dimethyl sulphide
	Dimethyl sulphoxide
	Vanillin
Naval stores	Pine oil
	Rosin
	Turpentine
	Tall oil
	Tall oil fatty acids
	Tall oil rosin
Pyrolysis products	Charcoal
	Methanol
	Acetone
	Acetic acid
Wood sugars	Glucose
	Xylose
	Mannose
	Ethanol
	Yeast

Table 1.1 : Historical chemical products from wood (Goldstein, 1989).

however, timber represents a renewable resource as long as the rate of removal does not exceed the rate of growth. Other countries, such as the United Kingdom, need to import timber to satisfy their requirements. In 1993, the value of timber imports in the United Kingdom was £6.3 billion with the value of timber exports being only £2.1 billion (The Forestry Industry Committee of Great Britain, 1992). Although the area of land in the United Kingdom that is under forest/woodland is increasing, the shortfall between timber imports and exports is unlikely to be reduced significantly in the near to medium future. Therefore, it is important that the timber be used in such a way that the likelihood of its decay is diminished and, therefore, will be in service for as long a period as possible.

Wood can be a very durable material, e.g., wooden furniture sealed in Egyptian tombs has survived for nearly 4000 years and Japanese wooden temples are still in existence after 1300 years of service (Schniewind, 1989). However, wood is subject to biodeterioration and can be broken down by a number of degradative organisms and this is important in the various nutrient cycles such as those for carbon and nitrogen. Problems for timber users arise when in-service timber (e.g., building timber, transmission poles) becomes the target for decay. The key to durability is the proper use of the wood and the proper understanding of the factors that destroy wood.

There are a large number of organisms, both micro- and macroscopic, that can attack wood, whether it is in the form of living trees or housing timber. The key to preservation is to create conditions that are unfavourable to the organisms that cause biodeterioration, principally fungi and insects. For example, in the case of termites, contact of housing timber with the soil must be avoided, and with fungi, the timber must be kept dry. In both cases preservative treatments can be used, although the trend nowadays is to move away from the use of chemically intensive treatments to procedures that depend more on the alteration of the immediate environment than on chemical usage.

The increase in the knowledge of the effects that certain chemicals can have on the environment has led to the demand that traditional chemically based treatments be used on a

less frequent basis. Therefore, new methods of preserving in-service timber against biodeterioration are being researched and developed. Amongst the methods being researched is the use of antagonistic fungi to eradicate wood decay fungi, i.e. biological control.

1.2 Wood structure

There are two basic categories into which wood species can be placed, these being the softwoods and hardwoods. All wood species have cells which transport molecules longitudinally (i.e. from root to crown) and radially (i.e. from bark to heartwood) and it is the arrangement and design of these cells that determine the type of wood.

In softwoods, support and longitudinal transport is achieved by tracheids and radial transport by ray tracheids, which also contain pits that allow transport between neighbouring cells. Food reserves, such as starch, are stored in parenchyma cells that move radially from their point of formation to the bark. Together with the ray tracheids, the parenchyma cells constitute the wood rays. Some of the softwood species, e.g. pine, also contain channels that run longitudinally and radially. These channels, known as resin canals, are lined with specialised parenchyma cells that secrete resin into them.

In hardwoods, support is provided by cells known as fibres and transport by vessels. Tracheids are present in some hardwood species in association with the vessels but they are different from those found in softwoods. Ray parenchyma cells provide storage although the less common longitudinal parenchyma also performs this role. Wood rays in hardwoods are composed of ray parenchyma almost exclusively. Structures similar to resin canals are also present and these are known as gum canals. Although both radial and longitudinal canals do exist, they are rarely both present in the same wood.

Wood is composed of a number of complex compounds such as sugars, phenols and carbohydrates but the three main constituents of wood are cellulose, hemicellulose and lignin (Sjöstrom, 1981). The main structural component of wood is cellulose which comprises 40-50% of the dry weight of wood (Kirk, 1973). Cellulose is a linear polysaccharide composed of approximately 10,000 β-D-glucopyranose units held together by β (1-4)-glycosidic bonds

(Allsopp and Seal, 1986). The cellulose chains are grouped together into bundles called microfibrils which contain highly ordered (crystalline) and less ordered (amorphous) cellulose regions, and it is the arrangement of these that determines how the cell wall is structured.

Hemicellulose is also a structural component of the wood cell wall and comprises 12-35% of the dry weight of wood (Kirk, 1973). Hemicelluloses are also polysaccharides with the individual units linked by β (1-4)-glycosidic bonds. However, unlike cellulose there is more than one basic unit that can be polymerised to form the hemicelluloses. The five main hemicelluloses are polymers of D-glucose, D-galactose, D-mannose, L-arabinose, D-xylose and 4-*O*-methyl-D-glucuronic acid (Kirk, 1973). The composition and nature of hemicellulose varies between hardwoods and softwoods and even within individual trees with hemicellulose molecules commonly containing less than 300 units (Kirk, 1973).

Lignin also plays a part in the structure of the cell wall and comprises 15-35% of the wood by dry weight (Kirk, 1973). The lignin polymer is comprised of highly branched oxyphenylpropane units derived from three substituted cinnamyl alcohols: ρ-coumaryl, coniferyl and sinapyl alcohols. The proportions of these precursors vary greatly between softwoods and hardwoods and between the individual hardwood species (Kirk, 1973).

There are other wood constituents, for example, pectin and extractive compounds that play an important part in wood structure. Pectin occurs in the middle lamella and is a polymer of glucuronic acid. The glucuronic acid subunits are often methylated and calcium crosslinks help to provide strength and stability. Wood extractives consist of a variety of structural compounds and phenolic and terpenoid extractives play an important part in the decay resistance of wood.

The woody plant cell wall is composed of a large number of microfibrils and the wall can split into different layers depending upon which way the microfibrils are arranged. The cell wall is divided into the primary wall and the secondary wall, which is further subdivided into three layers (see Figure 1.1)(Desch and Dinwoodie, 1981). The primary wall is characterised by being very thin with a random arrangement of microfibrils. The secondary

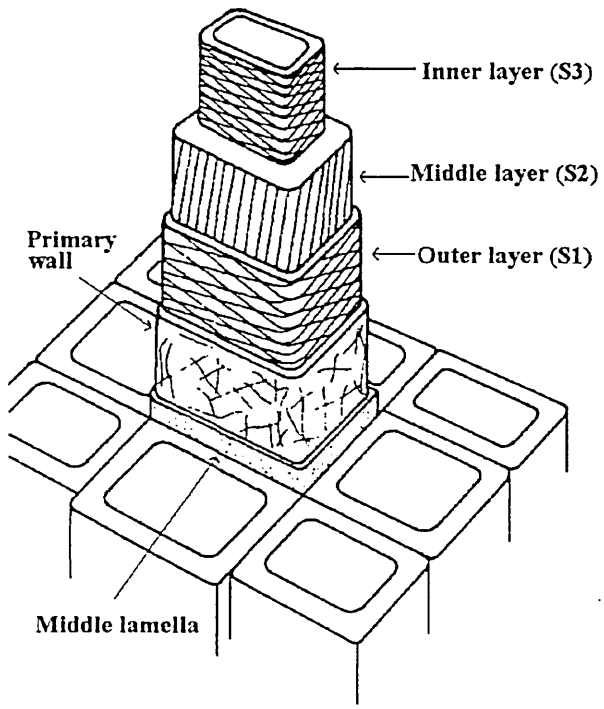


Figure 1.1: Structure of the wood cell wall (From Desch and Dinwoodie, 1981).

wall is split into the S_1, S_2 and S_3 layers. The outermost layer, S_1, is thin comprising less than 10% wall thickness, and has microfibrils lying parallel to one another in two distinct spirals, one right-handed and the other left-handed, but both with a pitch of 50° to 70° to the vertical axis. The middle layer, S_2, comprises 85% of the wall thickness and has microfibrils lying parallel to each other in a spiral with a pitch to the vertical axis of 10° to 30°. The innermost layer, S_3, comprises only 1% of the wall thickness and has a similar arrangement to the S_1 layer. In some species the S_3 layer is overlaid with a thin warty layer.

The lignin, cellulose and hemicelluloses are not uniformly distributed across wood cell walls, and differences exist in the distribution in hardwood and softwood cells. In *Betula papyrifera*, the secondary wall contains 16-19% lignin, the middle lamella contains 34-40% lignin, and the cell corner regions contain 72-85% lignin (Kirk, 1973). Cellulose makes up approximately 41% of the polysaccharides in the middle lamella-primary wall layer of *Betula verrcosa* cells, the remainder being hemicelluloses. Cellulose makes up between 48 and 60% of the polysaccharides of the secondary cell wall and is in highest concentration in the region nearest the lumen. Hemicelluloses comprise 40-52% of the polysaccharides and are in lowest concentration nearest the lumen.

In *Picea mariana* the percentage of lignin is approximately 22% although it is distributed differently in early- and latewood, with more lignin being present in the middle lamella and cell corners in latewood compared to earlywood. Approximately 33% of the polysaccharides in the middle lamella primary wall region of *Picea abies* is cellulose, the remainder being composed of hemicelluloses. In the secondary wall, cellulose accounts for approximately 55-64% of the polysaccharides, the highest concentration being nearest the lumen. Hemicelluloses comprise 36-45% of the polysaccharides of the secondary walls, the lowest concentration being nearest the lumen.

1.3 Deterioration of timber

The deterioration of timber can be brought about by a number of different causes and it is important to identify the cause accurately to enable the proper course of remedial action

to be taken. The principal causes of timber degradation are listed below and summarised in Table 1.2 :

1. Weathering
2. Mechanical wear
3. Thermal decomposition
4. Chemical decomposition
5. Insect attack
6. Marine organisms
7. Bacteria
8. Fungi

Deterioration of wood in the first four of the above categories is generally easy to identify as such although in certain circumstances mechanical wear can be mistaken for fungal decay. Weathering of wood consists of photochemical damage (by short- and long-wave ultraviolet radiation) of the cell wall constituents, oxidation of the break-down products, leaching of the soluble decomposition products and mechanical damage resulting from the constant swelling and shrinkage associated with surface wetting and drying (Zabel and Morrell, 1992). When wood is exposed to the weathering process it begins to change colour and texture, and after several years of exposure weathered wood is grey in colour and has a roughened texture. Although weathering in itself may not cause significant damage to the wood, it can make the colonisation by degradative organisms more likely.

The thermal decomposition of wood occurs at temperatures above 100°C and as such is only important in the process of kiln-drying of the wood to remove the moisture. It is unlikely that the householder will see the effects of thermal decomposition unless the wood has been subject to fire. The appearance of burnt wood resembles that of a brown cubical rot and one could be mistaken for the other.

Type of Damage	Causal Agents	General Descriptions	Prevention or Control
Weathering	UV. light, oxidation, swelling and shrinkage, leaching, and fungi	Unprotected surfaces develop a grey colour and roughened texture	UV. light-resistant coatings
Thermal decomposition	High temperature	<200°C, uniform surface brittleness >200°C, charcoal in absence of oxygen, combustion around 275°C	Fire-retardent chemicals
Chemical decomposition	Caustic chemicals	With acids wood turns brown, chars, and becomes brittle, with bases wood bleaches and defibrillates	Chemically resistant woods
Mechanical damage	Mechanical forces rupturing surface tissues	Selective surface erosion in heavy friction zones	High-specific-gravity woods, edge grain, or chemically hardened woods
Insect Damage	Termites Borers Ants	Localised honeycomb cavities, wood soiled and filled with frass Tunnels, cavities, pinholes Localised honeycomb cavities, wood channels clean	Insecticides or keep wood dry
Marine borer damage	Shipworms Pholads Gribbles	Interior tunnels with lime-coated walls Large interior tunnels near surface Surface tunnelling in tidal zone	Protective surface barriers or use wood preservatives
Decay	Fungi	*White* fibrous pockets or punky texture; *Brown* fibrous pockets or cubical checking pattern; *Soft* surface embrittlement and exfoliation in small fragments	Keep wood dry or use wood preservatives
Moulds	Fungi	Coloured spores or mycelium on the wood surface	Dry wood or use protective chemicals
Stains	Fungi	Sapwood discoloured grey, black, brown, blue and intensified in ray parenchyma	Dry wood or use protective chemicals
Ray cell and cell-wall damage	Bacteria	Soft surfaces, ray cells destroyed, microscopic tunnels in cell walls	Keep wood dry or use wood preservatives

Table 1.2 : Major types of wood damage and their description

Chemical decay is most notably caused by strong acids or alkalis and such decay is usually only a factor when such chemicals are accidentally spilled or when wooden containers or implements are used when dealing with these chemicals. Sulphur dioxide can also cause damage to exposed timber, as can prolonged exposure to running water (although the water is more likely to have an important effect with regards to raising the moisture content of the wood thus making it more amenable to fungal infestation). The degradation of wood by acids can resemble brown rot decay and alkali degradation can resemble white rot decay.

Insect damage has been reported to cause billions of dollars of damage, and one class of insect, the termites, cause annually $1.5 billion in damage in the United States alone (Zabel and Morrell, 1992). The three insect orders that cause the most damage are the Isoptera (termites), Coleoptera (beetles) and Hymenoptera (wood wasps, carpenter ants and carpenter bees) (Zabel and Morrell, 1992). Insect attacks can usually be recognised as such by the presence of narrow tunnels excavated by the larvae or mature insects as they burrow through the wood. The signs of insect attack and fungal decay are often seen together in the same area as many wood-boring insects, e.g., the death-watch beetle, attack only wood that has been partially decayed by fungi (Cartwright and Findlay, 1958). Insects usually only attack wood that has a lower moisture content than that necessary for fungal growth, however, certain groups of insects, e.g., the *Sirex* wood wasps, live in close association with wood-rotting fungi (Cartwright and Findlay, 1958).

Decay of wooden ships and structures by marine organisms causes $500 million of damage annually (Zabel and Morrell, 1992). There are two phyla involved, the Mollusca (molluscs) and Crustacea (crustaceans). The life-cycles of these organisms are generally poorly understood and there is a reliance on the use of highly toxic, broad-spectrum chemicals to combat these organisms. These organisms do not utilise the wood as a food source but mostly for protection by forming tunnels within the wood.

The decay of wood caused by fungi (a more detailed description is given in section 1.4) can be serious and economically damaging. Recognition of a fungal infestation of building timber depends upon the species of fungus involved, the kind of timber infested

and the stage at which the decay is at. There are, however, a number of visible or physical indications that can point to fungal infestation of timber.

1. Change of colour - wood that has been attacked by *S.lacrymans* (dry rot) takes on a dark brown appearance, whereas wood attacked by *Coniophora puteana* (wet rot) appears almost black in colour.

2. Softening of the wood - wood that has been decayed will become soft and with the use of a knife it will be found that it will not be possible to prise loose long splinters as the wood fibres will break prematurely.

3. Change in wood density - as wood decays it will lose weight and the lightness of the wood may indicate an advanced stage of decay. However, the change in density is not a clear indication of fungal attack as a rapidly growing tree may produce a light wood density and the moisture content of the wood will also affect the density.

4. Change in odour - advanced stages of decay may result in the production of a 'mushroomy' smell, e.g., *S.lacrymans*. This is not an accurate assessment of fungal attack as a musty or mouldy smell may indicate the presence of mould organisms that infest damp timber but do not decay it. However, the presence of a musty smell will indicate that conditions for dry rot or wet rot infestation are present.

More sophisticated and accurate methods of detection of fungal infestation have been developed and these will be discussed in greater detail in section 1.7.

1.4 Fungi and wood decay

Despite the many forms of wood, e.g., standing trees, logs in transit and storage, timber after conversion, during seasoning and in-service, they can all be attacked by fungi (Wilkinson, 1979). Fungi require a nutrient source, a suitable temperature, an oxygen and moisture supply. An understanding of how these factors interact helps to determine why wood rots. For example, wooden posts rot at ground level because the wood above ground is rarely wet long enough for fungi to become established and below ground the oxygen level (and perhaps the temperature level) will be too low. It is the region of the stake around ground level that has the conditions necessary for fungal growth and decay.

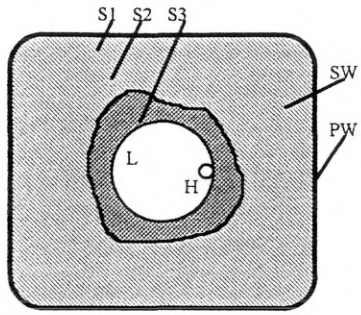
There are a large number of micro-organisms that can live within wood, many of which are fungi. The detection of these organisms and their potential for wood decay must be carried out as quickly as possible to determine whether the application of fungicides and/or wood preservatives is merited. Table 1.3 lists the characteristics and features of the major types of wood-inhabiting fungi. Wood decaying fungi can be grouped according to the type of decay that they produce, with the three main types being white-, brown- and soft-rots (Figure 1.2). White- and brown-rot fungi belong to the ascomycetes and basidiomycetes, whereas the soft-rots belong to the ascomycetes. White-rot fungi (e.g., *Coriolus versicolor*) destroy all the major constituents of the wood cell wall, i.e., the lignin, cellulose and hemicelluloses. Hardwoods are particularly susceptible to white rot fungi and some, e.g., beech (*Fagus sylvatica*) and ash (*Fraxinus* species) show characteristic dark zone lines around the infected areas of the wood.

Soft-rot decay of wood occurs when the wood is continually in contact with moisture such as pier pilings, cooling towers, and the buried portions of utility poles. Soft-rot fungi, e.g., *Chaetomium globosum*, primarily remove the polysaccharides (cellulose and hemicelluloses), although the lignin is removed albeit much more slowly than the polysaccharides (Kirk, 1973).

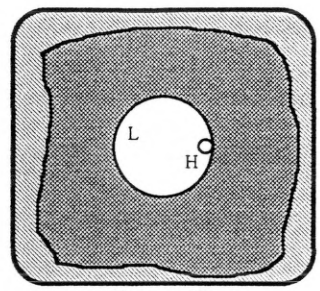
Brown-rot fungi include some of the most important destroyers of wood in-service (Wilkinson, 1979). Brown-rot fungi preferentially remove the polysaccharides, leaving the lignin largely undecayed but slightly modified. There are two groups of brown-rot decay, dry rot and wet rot. Dry rot is caused by *Serpula lacrymans* and occurs in buildings in temperate parts of the world. In North America, *Meruliporia* (*Poria*) *incrassata* causes a similar kind of rot (Wilkinson, 1979). *S.lacrymans* grows best in warm conditions (about 23°C) where there is poor ventilation and high humidity. It mainly attacks softwoods that have moisture contents between 20 and 40%. Wet rot is much more common than dry rot and is caused by a number of fungi but the most common is *Coniophora puteana*. This fungus grows on wood with moisture contents between 40 and 50% and can grow equally well indoors as well as outdoors.

Wood-inhabiting fungi	Cell-wall constituents used	Anatomical features	Causal agents
Decayers (Cell-wall erosion and/or large bore holes formed >2 μm)			
Simultaneous white-rotters	All	Cell walls attacked progressively from lumen surface	Basidiomycotina Some Ascomycotina
Sequential white-rotters	All, but hemicelluloses and lignin used selectively initially	Cell walls attacked progressively from lumen surface	Basidiomycotina Some Ascomycotina
Brown rots	Carbohydrates, but lignin modified	Entire wall zone attacked rapidly	Basidiomycotina
Type 1 soft rot	Carbohydrates	Longitudinal bore holes develop in secondary wall	Ascomycotina Deuteromycotina
Type 2 soft rot	Carbohydrates	Secondary wall erosion from lumen surface (in conifers mainly the S_2)	Ascomycotina Deuteromycotina
Nondecayers (No cell-wall erosion and occasional bore holes are minute < 1μm)			
Sapstainers	Wood extractives	Invade parenchyma cells in sapwood primarily	Ascomycotina Deuteromycotina
Moulds	Wood extractives	Surface growth on wet wood	Zycomycotina Ascomycotina Deuteromycotina
Scavengers	Wood extractives and decay residues	Penetrate wood cells primarily through pits	Zycomycotina Ascomycotina Basidiomycotina Deuteromycotina

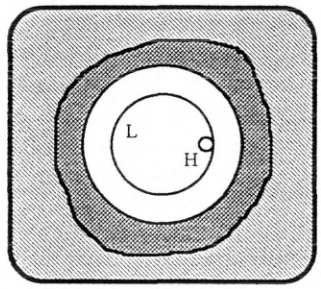
Table 1.3 : A summary of the anatomical and chemical features of the major types of wood-inhabiting fungi (taken from Zabel and Morrell, 1992).



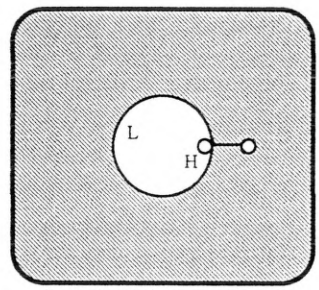
White Rot
(progressive erosion of all cell wall components from lumen surface)



Brown Rot
(rapid chemical attack of all cell wall carbohydrates)



Soft Rot (Type 2)
(progressive erosion of all cell wall components initially in the S2)



Soft Rot (Type 1)
(selective localised attack of secondary wall & formation of longitudinal bore holes)

Sound cell wall

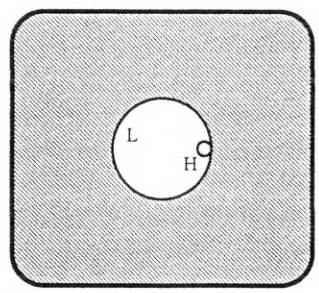
Decayed cell wall

PW - Primary Wall

SW - Secondary Wall (S1, S2, S3)

H - Hypha

L - Lumen



Mould/Staining Fungi
(no serious loss in wood strength but wood is discoloured)

Figure 1.2 : Brown-, white-, soft-rot and staining of timber (modified from Zabel and Morrell, 1992).

There are other fungi that can grow on wood by utilising the free carbohydrates present in the lumen after seasoning but do not cause decay of the wood. Mould fungi such as *Trichoderma*, *Aspergillus* and *Penicillium* species (deuteromycetes) colonise wood and cause some discolouration, although no serious loss in wood strength is observed. Staining fungi such as *Ceratocystis* spp. (deuteromycetes) and *Aureobasidium* spp (ascomycetes) cause unsightly discolouration of wood by the appearance of coloured spores and/or hyphae within the wood cells.

1.5 Brown-rot decay of timber

The initial colonisation of timber by brown-rot fungi depends upon their ability to utilise the non-structural carbohydrates but once they have gained access to these carbohydrates, they can rapidly colonise timber before any significant weight losses occur (Hulme and Shields, 1970). Although the level of nitrogen in wood is very low (about 0.03-0.10%) it plays a very important part in the colonisation and decay of wood by fungi (Merrill and Cowling, 1966). Work carried out by Levi *et al.* (1968) indicated that wood-destroying Basidiomycetes can function within this environment by the extremely efficient use of the nitrogen in their cellular metabolism and by obtaining nitrogen from a number of sources, for example, the wood or mycelia (from their own mycelia by autolysis or other fungal mycelia by hyphal lysis).

Brown-rot fungi degrade cellulose in a manner that differs from other cellulose degrading organisms. They cause a rapid and extensive depolymerisation of the cellulose and the degradation products are produced faster than they can be utilised (Cowling, 1961) and hence cause a great deal of damage in a short period of time (Figure 1.3). Brown-rot fungi decay wood by removing the hemicellulose and cellulose portions of the wood cell wall, whilst leaving the lignin modified but unused. The hemicelluloses surround the cellulose microfibrils and hence must be removed initially before the cellulose can be utilised (Green and Highley, 1995). Extracellular hydrolytic enzymes that can degrade microcrystalline glucose have been isolated from brown-rot fungi, however, the actual degradation of the cellulose can only proceed when the ligninolytic system is also being produced (Enoki *et al*, 1990).

Figure 1.3 : Brown-rotted wood.

The identity of the agent that produces the initial cellulose depolymerisation has been pursued for some time but still remains elusive. Observations of cell walls decayed by brown-rot fungi indicate that the S_2 layer is intensely degraded whereas the S_3 layer is largely untouched (Highley and Murmanis, 1985). Since the fungal hyphae are positioned within the lumen of the cell it is clear, however, that cellulolytic enzymes would be too large to penetrate the S_3 layer of the cell wall and reach the cellulose and hence, a small, diffusible agent must be involved. With the production of hydrogen peroxide by some brown-rot fungi (Koenigs, 1974) and the presence of iron in wood, it was suggested that an iron/peroxide oxidative system (similar to Fenton's reagent) was in operation. With the production of oxalic acid by brown-rot fungi (Takao, 1965), the acid could reduce the Fe^{3+} (the species normally found in wood) to Fe^{2+}, which is the active form in Fenton's reagent. This would result in the production of the hydroxyl radical that is a powerful oxidant that could rapidly depolymerise the cellulose.

Illman *et al.* (1988) have shown that the presence of the hydroxyl radical in wood samples inoculated with *P.placenta* was indicated, and that manganese levels were also being affected by the fungus. They suggested that cellulose-metal complexes and oxidation reactions by oxygen radicals were involved in the degradation of cellulose by brown-rot fungi.

Brown-rot fungi have a number of hemicellulose-degrading enzymes that attack the hemicellulose chains, reducing them to progressively shorter chains, which are then hydrolysed to simple sugars. Little work has been done on the hemicellulases of brown-rot fungi (Ilman *et al*, 1988) but the same problem of enzyme size as seen with the cellulose enzymes (with respect to gaining access to their target compounds) is evident.

The lignin in the cell wall is not utilised to any great extent although it is modified. The main effect on lignin is demethylation of aryl methoxyl groups (Kirk and Adler, 1970), although oxidative changes do occur including some cleavage of aromatic rings (Kirk, 1973). Harvey *et al.* (1986) suggested that brown-rot fungi may degrade lignin by a single-electron oxidation similar to that of white-rot fungi. In fact the electron oxidation that is used to degrade the cellulose could also be used to degrade the lignin (Enoki *et al*,

1990). However, it seems that the brown-rot decay does not result in large scale lignin loss because unstable intermediates in the process of lignin degradation can also by polymerised by single electron oxidations (Ilman *et al*, 1988).

The actual decay mechanism of brown-rot fungi is still in dispute. Green and Highley (1995) report that although the chemical changes in brown-rotted lignin are oxidative in nature, oxidative changes in cellulose as a result of brown-rot decay are not yet unequivocally demonstrated.

1.6 *Serpula lacrymans*

For hundreds, if not thousands of years, the dry rot fungus *Serpula lacrymans* has played a part in the affairs of man by causing the decay of wooden structures such as buildings and ships. There is some debate as to when the first mention of this fungus was made, however, some researchers have put forward the suggestion that the 'plague of leprosy in a house' as mentioned in the Old Testament (Leviticus 14.34) referred to an attack of dry rot. However, as the maximum growth temperature of *S.lacrymans* is 26°C (Hegarty, 1991) and the temperature in the Middle East reaches above 26°C on a regular basis this would seem unlikely. One of the earliest descriptions of *S.lacrymans* was made by Persoon in 1801 (Pegler, 1991), although Ramsbottom (1937) states that dry rot was a serious problem in wooden ships for some time before this date. In 1609 a naval Commission of Inquiry was appointed by James I to report on the conditions of the Royal Navy. The Commission suggested that timber used in ship building should be seasoned, however, this advice was repeatedly ignored and 'fleet after fleet rotted prematurely'. In fact, Ramsbottom suggested that dry rot must have been seen in wooden ships since the earliest times although there were no reports of its appearance.

One of the first reports on the fungal destruction of timber in a ship was made in 1812 by James Sowerby (Ramsbottom, 1937), when he inspected the Queen Charlotte, a first-rate warship. The Queen Charlotte had been constructed in 1810 at a cost of £88,534, however, she suffered a fungal infestation and repairs had to be made costing £94,499. The report made by Sowerby mentioned a number of fungi, including *Boletus lachrymans*, which is an early synonym for *Serpula lacrymans*. The decay aboard the Queen Charlotte

was never completely eliminated and repairs continued to be made to the ship until 1859 (when her name was changed to the Excellent) costing a total of £287,837.

Over the centuries there have been a number of methods described to eradicate outbreaks of *S.lacrymans*. These have ranged from the use of bird blood, cedarwood and hyssop (as described in Leviticus 14.34-14.53, Old Testament), to submerging infected timbers in water (a treatment for dry rot in wooden warships in the 19th century (Ramsbottom, 1937)) and finally to chemical treatment. Regardless of the treatment used, the removal of infected timber is carried out and the use of seasoned replacement timber is recommended. Given the right conditions, *S.lacrymans* can cause a great deal of decay in a short period of time even without the building owner realising there is a problem. Due to a lack of knowledge of the biology of the dry rot fungus and a certain amount of exaggeration from owners whose buildings have suffered a dry rot outbreak, there is a certain amount of mythology surrounding the activity of *S.lacrymans*. For example, it has been suggested that *S.lacrymans* can 'manufacture its own water', 'live on plaster', 'lie dormant in the walls', 'spring-up anew in a totally unrelated area', 'penetrate through stone and concrete' and 'grow without the need for wood' (Bravery, 1991). Although most of these statements are based on fact they have given *S.lacrymans* an almost demonic air that often leads to fear, despair and panic when the presence of dry rot is detected within a building. The present treatment methods for a *S.lacrymans* infestation can remove the fungus from a building if the procedures are carried out effectively. However, remedial companies can be somewhat overzealous with regards to what requires treatment (and to what extent the treatment needs to be carried out) and mistakes in identifying dry rot as wet rot (and vice versa) are not unknown.

The increased research into the biology of *S.lacrymans* will ultimately lead to the modification of existing treatment strategies or to the development of new ones.

1.7 Identification of fungal organisms

Identification of fungal species was traditionally based on the life cycle of the organisms and on morphology of the reproductive structures and spores produced (Moore-

Landecker, 1972). As understanding of the fungi has increased, physiological and genetic information is now included in fungal identification.

Onions *et al* (1981) gives a typical sequence of steps that can be followed to identify a particular fungal species. These steps are reliant upon the use of a pure fungal culture, which can be obtained by the use of specialised isolation media containing one or more inhibitory compounds, for example, basidiomycete fungi can tolerate the presence of benomyl in agar whereas mould organisms cannot. The organism must be grown on a medium that allows unchecked growth to occur and production of fruiting structures. Cultures should be examined on a frequent basis (this depends upon the rate of growth of the organisms, i.e., slow growing organisms need only be examined once a day whereas faster growing organisms need to examined two or more times a day) and the following details recorded.

1. Rate of growth – this should be carried out on several media types and actual measurements of the colony diameter can be taken.
2. Colony colour and colour changes.
3. Colour and colour changes of the reverse of the colonies.
4. Colour changes in the medium.
5. Texture of the colony surface.
6. Odour, if any.
7. Character of drops of transpired fluid often found on aerial hyphae.
8. Character of the submerged hyphae; colour, presence or absence of septa, approximate diameter, characteristics of special structures if any present.
9. The stage at which fruiting structures develop.
10. The character and disposition of the mature fruiting organs.
11. Colour, size and shape of mature fruiting organs or fruit-bodies.
12. Details of structure of the fruiting organs.
13. Full details of spores (colour, shape, septation, surface markings, size).

Date numbered 1-7 are obtained by examination of cultures with the naked eye or with a hand-lens, 8-11 by observations on living cultures with the aid of a low or moderate

power microscope. Numbers 12 and 13 necessitate the preparation of slides and the use of the highest powers on the microscope. The information recorded under number 8 and 10 should be sufficient to determine the correct Class and Order, with the other information gathered leading to the Family and then the genus. However, the correct identification of a fungal organism is dependent upon the accuracy of descriptions previously recorded. For example, The Compendium of Soil Fungi by Domsch *et al* (1980) lists descriptions of soil fungi, including *Trichoderma*. The following excerpt is a description of the *Trichoderma* genus :

'The Hyphomycete genus *Trichoderma* is characterized by fast-growing hyaline colonies bearing repeatedly branched conidiophores in tufts with divergent, often irregularly bent, flask-shaped phialides. Coniodiophores may end in sterile appendages with phialides only borne on lateral branches in some species. Conidia are hyaline or, more usually, green, smooth-walled or roughened. Hyaline chlamydospores are usually present in the mycelium of older cultures. If the phialides are strongly convergent the conidial states of otherwise similar cultures are place in *Gliocladium* (q.v.), while if they are straight and moderately divergent, in *Verticillium*.'

There is also a key to the species that is largely based on the colour and structure of the fruiting organs. However, even with this kind of information identification of fungal species can be difficult. Fungal identification is becoming increasingly accurate as more tools and techniques are added to the process. For example, the determination of carbon and nitrogen assimilation, enzymatic activity, protein electrophoresis and DNA analysis all provide detailed information. Such techniques have shown that there are variations in apparently morphologically identical isolates of *S.lacrymans* (Palfreyman and Vigrow 1991b ;Theodore *et al*, 1995).

1.8 Morphology of *Serpula lacrymans*

The morphology of *S.lacrymans* has been described more fully elsewhere (e.g., Nuss *et al.*, 1991) and what is written here is only intended as a brief description of the various stages of growth.

The life cycle of *S.lacrymans* starts with a spore, 9-12 μm in length and 4-6 μm in breadth (Schmidt and Moreth-Kebernik, 1990). The colour of the spores when produced by a fruit-body in a building is usually brick-red to brown-red and can be very useful in identifying the presence of *S.lacrymans*. There was some debate as to whether *S.lacrymans* could germinate upon wood as this could not be achieved experimentally (Nuss *et al*, 1991). However, it became apparent that although the spores could not germinate on healthy wood they could germinate on wood that had been previously attacked by other fungi (Nuss *et al.*, 1991). Upon the germination of the spore, monokaryotic hyphae are produced which form the primary mycelium (an amalgamated mass of hyphae), which can grow on the surface of the wood or within it. At this stage, the growth of the *S.lacrymans* is not visible to the naked eye.

When two or more monokaryotic hyphae join they produce dikaryotic hyphae which grow to form the secondary mycelium, which can be seen as tiny, snow-white patches on the wood surface. The dikaryotic and monokaryotic hyphae can be distinguished by the production of clamp connections by the former. The dikaryotic hyphae then grow away from the substrate independent of gravity and in doing so a tuft of radiating and shiny mycelium is produced. As the aerial mycelium develops it advances over the substrate and can be easily detached from it since there are few connections with the wood. If the conditions are favourable then the mycelium can reach large dimensions and two or more mycelia may join to form one large mycelium.

Favourable conditions are those of high humidity, stagnant air currents, low light levels and an appropriate temperature (optimum being around 23°C). If the air humidity is high then the mycelium produces liquid droplets, hence the name 'lacrymans' which means 'weeping'. However, if the mycelium is exposed to a draught (resulting in the reduction of the air humidity) the growth stops and the mycelium will die if it is exposed to air of medium humidity (Nuss *et al.*, 1991).

The young mycelium is always white although it does turn grey upon ageing. Injury or stresses (e.g., light, high temperature, food shortage, shortage of the vitamin aneurin or confrontation with moulds (Trichoderma, Penicillium, etc.) or their metabolites) cause a

colour change from white to yellow, burgundy or brown. The colour change is dependent upon the severity of the stress with a brown colour indicating hyphal lysis and death (Score and Palfreyman, 1994). Also, when under stress, *S.lacrymans* will show faster growth at discrete points along the mycelial front edge. This is termed 'point growth' and can be shown by both monokaryotic and dikaryotic mycelia.

The healthy mycelium has a smell like the common mushroom and the same smell is emitted by wood that is infected with *S.lacrymans*. This volatile production can be used to determine the presence of *S.lacrymans* and is used to train sniffer dogs to detect the dry rot fungus in buildings (Koch, 1990; Hutton, 1994).

The aerial mycelium growing on the surface of the substrate is morphologically different from the substrate mycelia growing within the substrate. The hyphae of the substrate mycelium are smaller in diameter, have simpler clamp connections and are irregularly branched. The differences between substrate and aerial mycelia have been interpreted as 'a response to environmental conditions' (Nuss *et al,* 1991).

The hyphae of the dry rot fungus attack the wood from the surface by entering the wood through pith rays. The hyphae then enter the cells via pits and holes made by enzymes released by the hyphal apex. The hyphae then passes through the hole (which is much smaller than the diameter of the hyphae) and the hyphal diameter becomes as large as it was previously once it has passed through the cell walls. The hyphae gain nitrogen from the wood cells and by recycling nitrogen from dead hyphae (which cannot be found in heavily rotted wood).

Hyphae in wood are found in grooves in the cell where enzymatic digestion has taken place resulting in the reduction of the cell wall thickness by up to 75%. Decayed wood is red-brown in colour with criss-cross fractures. Where the surface of the timber has been painted, decayed timber will bend convexly since the *S.lacrymans* can only decay the inner layers of the timber, which then shrinks causing the undecayed, painted portion to bend.

Serpula lacrymans produces two morphologically and physiologically different types of mycelium: one that has the potential to produce strands and the other that has the

potential to produce fruit-bodies. Environmental conditions, such as humidity, temperature and aeration, may play a part in triggering strand or fruit-body production. However, there may also be a genetic basis as to which type of mycelium is produced since different strains produced either strands or fruit-bodies when kept under identical conditions (Nuss *et al.*, 1991).

The strands of *S.lacrymans* are composed of a main leader hypha surrounded by its own branches and thin tendril hyphae (Cooke and Whipps, 1993). Autolysis and differentiation of these hyphae result in a series of tubes surrounded by fibrous material embedded in a non-cellular matrix. The formation of strands allows the *S.lacrymans* to advance over areas that are nutritionally deficient in search of new food sources. Strands are produced by aerial mycelia but never by substrate mycelia and may be round or ribbon-like, up to 1 cm wide and reach lengths up to 4 metres.

The first sign of the development of a fruit-body is a change in colour of the mycelium to flat chalky-white (Nuss *et al,* 1991). This mycelium becomes more dense, swells and hyphae aggregate to form the hymenium (or 'fruit-bearing layer'). The hymenium is composed of vertical hyphae that will become the basidia that produce the basidiospores. The surface of the fruit-body (hymenophore) becomes densely folded and changes colour to reddish yellow and flesh-coloured to different tints of purple-red. As the fruit-body matures the colour deepens to cinnamon-brown (Figure 1.4). The hymenal surface of the mature fruit-body is densely covered with basidia, with most of the basidia producing four basidiospores. The spores either ripen in sequence or simultaneously and are then released. The number of spores released is dependent upon the size of the fruit-body, the substrate and the age of the fruit-body. However, the number of spores released can range from 600,000 (Richards, 1953) to 50,000,000 (Nuss *et al,* 1991) per minute.

Under optimum conditions (good food supply and high humidity) fruit-bodies often grow out of the hymenophore forming one or more smaller fruit-bodies. Mycelia may form one large fruit-body or several smaller ones that join into one. Fruit-bodies can reach a diameter of 1 m or more and can be up to 3.3 cm thick. Fruit-bodies can be found all the year round but, generally, fruiting is more common between late summer and winter. The

Figure 1.4 : Fruiting body of *S.lacrymans*.

fruit-bodies do not have distinct form and size, as their shape and structure are dependent upon the form, structure and topography of the surface where they are produced. After spore release the basidia collapse and the fruit-body decays becoming the substrate for mould fungi.

Despite the large amount of research carried out on *S.lacrymans* there are still many questions left to be answered in areas such as strand initiation and formation; spore release and discharge; functional interrelationships between hyphae; and basic fungal biochemistry.

1.9 Determining the presence and extent of an outbreak of *S.lacrymans*

1.9.1 Detecting wood decay

Even though the decay of structurally important timbers may have reached an advanced stage in a building, the presence of dry rot may or may not be readily apparent though it could be indicated by the presence of a fruiting body, spore dust covering an area of the floor, or by the collapse of joinery such as floorboards, skirtings, or door or window frames. However, the presence of wood-decay fungi may not be so easily determined and more sophisticated detection techniques may be required.

The simplest and traditional method for determining the presence of wood decay is with the 'penknife' test where a knife or other sharp instrument is pushed into the surface of the wood and twisted. If the resulting fracture is long and lifted up then the wood is sound, whereas in rotted wood the splinter will break prematurely. This can determine the presence of decay and give an estimate of the depth of decay. However, this is rather subjective and a more quantitative assessment can be obtained by the use of the 'pilodyn'. This hand-held instrument fires a pin into the wood and the depth of penetration is measured. If the wood is sound then the penetration depth will be small but this will increase if the wood is decayed. Although the moisture content of the wood can affect the results, the pilodyn can accurately measure the extent of decay at the wood surface.

Internal weakness of the wood as a result of decay can be measured by the use of a hand drill. The presence of decay can be detected by the decrease in resistance of the wood during drilling. Again this is a subjective form of measurement but this method has been

modified such that the changes in drill speed can be detected by computer and the areas of internal weakness mapped.

Sound waves can also be used to determine the status of wood. The traditional method is to strike the wood with a hammer - sound wood will produce a sharp ring, rotten wood a dull thud and a void will give a hollow sound. Instruments have now been developed that use sonic and ultrasonic sound waves, and acoustic resonance technology can now be used to detect internal wood decay (Eaton and Hale, 1993). However, the accuracy of these methods can be affected by the type of wood tested, e.g., sonic testing works well on Douglas fir and western red cedar but not as well on southern pine poles because of the high incidence of ring shakes (Eaton and Hale, 1993).

Detection methods measuring electrical resistance have also been developed, e.g., the Shigometer and the Conditionmeter. Decayed wood will register a reduced electrical resistance compared to sound wood. However, the accuracy of the results is dependent upon the moisture content of the wood and it has been suggested that readings can only be safely taken when the moisture content of the wood is above 45% (Eaton and Hale, 1993).

Radiation can also be used to detect internal wood decay. A method has been developed that uses gamma rays produced by a battery-powered emitter. This machine can map the internal regions of a pole within an hour, however, a computer must interpret the results. X-rays are commonly used to detect the decay caused by certain marine molluscs and insects (Eaton and Hale, 1993).

Collimated photon scattering (CPS), which was developed to investigate the quality of heating pipe insulation, has been modified to detect decay in buildings (Koch, 1990). As yet the system is not commercially available and, although it does have the advantage of directly detecting decay, it does not differentiate between decay caused by different types of decay fungus and it cannot detect active growing fronts where there will be little or no decay.

1.9.2 Detecting the presence of fungi

There are around 70 000 fungal species known and classification of these species is a huge and daunting task. Most species have received only limited study and their

classification is based mainly on traditional criteria based on readily observable morphological features (Carlile and Watkinson, 1996). There are, however, other species that have received more study and other features, such as nutrition, physiology and DNA base composition, have been used in classification. The correct identification of fungi is of great importance and, in contrast, to classification needs to be swift particularly when a pathogen is involved. Classical fungal identification tends to be based on morphology although the development of reagents (such as species-specific antibodies) that react with a particular species alone are becoming important in fungal identification.

Laboratory based systems have also been developed to detect the presence of fungi in wood. Fungal isolation allows the identification of the fungi and the determination of its decay capacity. However, this is very time consuming and requires skilled personnel to identify the fungi and determine their activity. Microscopic examination of the wood will also reveal the presence of decay, however, it is also time consuming.

There are an ever increasing number of methods being developed to detect the dry rot fungus directly. Dogs can be trained to find dry rot within the building environment (Koch, 1990; Hutton, 1994) by the detection of the volatiles released by the growing fungus. The dogs can detect dry rot before it is detectable by the naked eye (Hutton, 1994) and can find 90-100% of the dry rot in a building whilst making very few mistakes, i.e. undetected attacks and false positives (Koch, 1990). Although there are a number of limitations to the use of the dogs to detect dry rot, they can at least allow the surveyor to quickly map the approximate area of an outbreak, which can be confirmed by the use of moisture meters or a core sampler.

Laboratory based detection systems are also being developed and these are based on the use of specific polyclonal antibodies to detect the fungus in a wood sample. Two assay systems have been developed to detect *Poria placenta*, an enzyme-linked immunosorbent assay (ELISA)(Jellison and Goodell, 1986) and fluorescent antibody (FA) microscopy (Goodell *et al,* 1988). The ELISA could detect the decay fungus in wood before decay had been initiated and quantify the amount of fungus present. Although the FA microscopy technique could detect *P.placenta*, there was some cross-reaction with

other, non-decay fungi. The development of monoclonal antibodies would enhance the success of these systems and a detection system for *S.lacrymans* has recently been developed (Burge *et al*, 1994).

When the presence of dry rot has been detected, the first matter that should be dealt with is the extent of the outbreak and, if possible, to determine its source. However, determining the presence of a dry rot outbreak is not a simple process even for someone with a great deal of experience with this problem since the fungus could be present in timbers and/or brickwork that are located in positions that are very difficult, if not impossible, to examine accurately.

S.lacrymans requires a number of parameters to survive and grow within a building, not least of which is the presence of moisture. Therefore, the extent of a dry rot attack can be indirectly determined by the presence of moisture in timbers or brickwork. Bech-Andersen (1991) stated that during a survey of 60 houses, the mean distance from the moisture source to the hyphal tips was 2.7 metres varying from 0 to 6 metres. Knowledge of the location of timbers within the building, the location of potential or actual points of moisture ingress and the paths that moisture may take within a building are all important in predicting the extent of moisture movement within the building.

Richardson (1995) suggests that there are three main points in determining the presence and extent of dry rot within a building, and these are :

1. Carefully noting and recording defects in design, construction or maintenance that have allowed, or are likely to have allowed, moisture to penetrate into the fabric of the building.

2. Internally investigating the presence and extent of dampness caused by the defects previously noted.

3. Within the areas in which the presence of dampness has been established and in the areas where it is considered probable that moisture has penetrated :

(a) carefully investigating the condition of readily accessible timbers;

(b) assessing the probable presence of built-in and other concealed timbers, which are clearly at risk.

This strategy will allow the accurate assessment of moisture ingress and, hence, the likely extent of a dry rot outbreak. This will ultimately limit the cost of the treatment process, increase the likelihood of its success and reduce the possibility of an outbreak occurring again.

1.10 Current methods of eradicating an outbreak of *S.lacrymans* within a building

The measures that are taken to eradicate a *S.lacrymans* infestation have been designed to ensure that every part of the *S.lacrymans* colony is removed. The following section is a summary of a technical digest released by the Building Research Establishment at Princes Risborough (Digest No. 299, 1985). Once a detailed survey of the building has been carried out, and the extent of the dampness and/or decay has been determined then primary and secondary control measures designed to eradicate the dry rot attack should be implemented.

S.lacrymans cannot grow without a moisture source and so the primary aim must be to identify all sources of moisture ingress and eliminate them. Once this has been achieved the structure must be dried out as rapidly as possible, which can be achieved by increasing ventilation and introducing heating systems. These primary control measures, if thoroughly and effectively carried out, will bring a dry rot attack under control. However, the drying out process may take a long time, perhaps a number of years, to complete. Therefore, secondary measures, which are temporary but quick acting, need to be employed to prevent further fungal damage whilst the building is drying out.

The secondary measures include :

determining the full extent of the outbreak

removal of the rotted wood

containing any residual fungus within the wall

treating any timbers to be left in place

introducing preservative-treated replacement timber

application of additional support measures.

The full extent of the outbreak may or may not have been determined in the initial moisture survey and a secondary inspection, with a carpenter or joiner in attendance to

remove floorboards, skirtings, etc., may be necessary. A thorough inspection of the building must be made to determine the spread of the dry rot fungus and the boundary between decayed wood and sound wood must be determined. A sharp tool can be used to locate softened timbers and shrinkage or distortion can also indicate the presence of rot. If necessary, skirtings and floorboards can be removed to allow the inspection of joists and walls below and behind them. Adjoining rooms should also be inspected to determine if the fungus has spread. Removal of plaster is only necessary if timber embedded in the wall is at risk, although plaster around known infected timbers (a 300-450 mm zone) should be removed to ensure that the infection has not spread.

Wood that shows signs of softening or where the dry rot fungus has been identified should be removed. A margin of 300-450 mm around the area of visible infection/decay should also be removed to allow for the removal of fungus within the wood and hence not directly visible. However, this margin of safety is not hard and fast and the amount of timber removed is dependent upon the situation. For example, if lightly decayed wood is present within an area of dry wood then its removal (especially if it is of artistic or historical interest) is not strictly required. If the decay has affected structural timbers then a structural engineer must be consulted and the appropriate remedial action taken in accordance with the building regulations. All infected timber should be burnt, preferably on site. Such timbers should not be dumped where they could be used as hard-core or buried as both could be sources of re-infection of the original property or a different one. Where the decayed timber is of artistic or historical value, the timber can be retained and reused only if the timber is thoroughly dried and sterilised with an approved preservative.

Since *S.lacrymans* cannot obtain nutrients from brickwork, it is not necessary to treat the brickwork to kill the fungus provided that it cannot spread further and it has been severed from its nutrient source. Physical or ventilation barriers can be used to isolate wood remaining in contact with the wall. Fungicidal treatments should only be used where there is difficulty in drying out the structure. There are a number of methods to apply a fungicide to a wall - surface application by brushing or spraying, fungicidal renderings, preservative plugs or pastes, or irrigation.

Treatment of the timbers left in place with preservatives/fungicides is not strictly necessary although it may bring some reassurance to the building owner. It may be necessary to treat the timbers with a mild fungicide to prevent the growth of mould fungi that may infect the timber before it can be dried below 20% moisture content.

Replacement timber used in repairs should be resistant to infection by *S.lacrymans*. Naturally durable timber can be selected, however, it is likely to be more economical and practicable to treat the timber with a preservative. In damp situations, the timber should be treated with copper/chrome/arsenic or creosote, however, the creosote does have a strong odour and can stain materials in contact with the treated timber. Where damp conditions are not expected to persist, an organic solvent type of preservative can be applied.

Additional support measures can be introduced which are designed to prevent the movement of moisture from damp walls to timber by the introduction of ventilation barriers and damp proofing membranes.

1.11 The chemical preservation of timber

Methods for the prevention of timber decay have been practised for thousands of years, with the earliest timber boat builders (*circa* 1000 BC) using durable wood species and treating ships' timbers with an oily extract obtained from cedar (Zabel and Morrell, 1992). However, with the increase in ship building in the 18th and 19th centuries as a result of the imperialistic intentions of a number of European countries, the supply of durable wood species decreased rapidly and less durable species had to be used. This led to a vigorous search for effective methods to control the decay problem and increase the service life of the timbers in use. The development of wood preservation chemicals was started with the introduction of creosote in 1836 and the vacuum-impregnation process in 1839 (Zabel and Morrell, 1992). Further development of timber preservative chemicals was greatly enhanced with the rise of the chemical and petrochemical industries, with a number of the traditional timber preservatives being by-products.

The use of preservatives is dependent upon the nature of the timber, the location of the timber, the class of biological hazard, the type of biological agent involved and the timber moisture content (Table 1.4).

There are three major groups of wood preservative chemicals; tar oil preservatives, water-borne preservatives, and organic solvent-based preservatives. Tar oil preservatives are obtained as a by-product from the production of coke and from the distillation of tars and pitches. Water-borne preservatives are aqueous solutions of toxic salts and organic solvent preservatives are composed of biocidal compounds dissolved in a non-volatile, non-polar organic solvent (Eaton and Hale, 1993), with each type having advantages and disadvantages over the others (listed in Table 1.5)

Wood preservatives are powerful pesticides that are designed to kill insects and fungi and to remain active for as long a period as possible. A large number of wood preservatives have been developed, each with its own specialist action and usage. If the wood preservative is applied in accordance with the safety regulations detailed by the manufacturer and/or the Health and Safety Executive then there should be no problems. The particular regulations are specific for each individual wood preservative. However, this will not prevent the contamination of the environment with toxic chemicals in the form of vapour, treated sawdust/dust, wood shavings or treated wood that is removed from the building in a future alteration. In the case of the most toxic wood preservatives (e.g., pentachlorophenol) their use is either banned or restricted to professional or industrial users. Table 1.6 gives some examples of preservatives and any problems associated with them.

Perhaps the four most well known and often used wood preservatives are creosote, pentachlorophenol (PCP), copper-chrome-arsenic (CCA) and tributyltin oxide (bis[tri-n-butyltin] oxide). Creosote has a world-wide distribution of 16 million tonnes, CCA has a world usage of 100,000 tonnes (in 1988), PCP was one of the most widely used organic solvents since its development in the 1930s and tributyltin oxide is the most widely used organotin compound (Eaton and Hale, 1993). However, despite their well-known preservative qualities, each of these preservatives is now either banned or under restricted usage due to the toxic effects that they may have on humans and/or the environment.

Hazard Class	Situation in service	Description of exposure in service	Wood moisture content	Fungi			Insects		Marine borers
				Basidiomycetes	Soft rot	Others	Beetles[a]	Termites	
1	Above ground, covered (dry)	Permanently dry	Permanently <18%				*	+	
2	Above ground, covered (risk of wetting)	Exposed to occasional wetting	Occasionally >20%	*		*	*	+	
3	Above ground, not covered	Exposed to frequent wetting	Frequently >20%	*		*	*	+	
4	In contact with ground or fresh water	Permanently exposed to wetting; in contact with ground or fresh water	Permanently >20%	*	*	*	*	+	*
M	In salt water	Permanently exposed to wetting by salt water	Permanently >20%	*	*	*	*	+	+

* Biological agents present throughout Europe.

+ Biological agents present Europe-wide or only locally,

[a] The risk of attack from beetles may vary greatly from high to insignificant levels.

Table 1.4 : The definition of biological hazard classes for wood used in Europe (Eaton and Hale, 1993).

Preservative Type	Advantages	Disadvantages
Tar oil	• very effective in outdoor situations	• strong odour • cannot be used indoors • can leach out • wood cannot be painted after treatment
Water-Borne	• leave the wood clean for handling • odourless • can be transported in a concentrated form • leave the wood paintable after treatment • can be combined with fire-retardent chemicals	• treated wood swells, raising the grain • the wood requires redrying • some salt treatments will leach out • some treatments result in strength losses and embrittlement
Solvent-based	• the wood dries quickly after treatment • the active ingredient is not readily leached • no dimensional changes in the wood occur • treated wood can be painted and glued • water repellents, colouring agents and pigments can be incorporated	• the solvents are flammable • solvents more expensive than water

Table 1.5 : Advantages and disadvantages of the three principle types of wood preservatives.

Creosote is a very complex mixture of over 200 polynuclear aromatic hydrocarbons, but this is variable depending upon the distillation ranges used (Zabel and Morrell, 1992). The main constituents are tar oils (e.g. phenol), tar bases (e.g. pyridine) and 'neutral oils' (e.g. naphthalene). Creosote is a very effective preservative and pressure-impregnated timber can have a service life of 30-50 years. However, creosote is unsuitable for indoor use (due to its strong odour and ability to taint food), the surface of creosote treated timber is unpaintable and greasy to the touch, and oily tars can leach out (Eaton and Hale, 1993). Creosote is highly toxic but although it is known to contain compounds that are carcinogenic in animals (e.g. benzo-α-pyrene) studies have shown that the prolonged contact of workers in creosote treatment plants did not result in an increased cancer risk (Eaton and Hale, 1993). Creosote is still one of the most important preservatives in high hazard situations, although its use is declining with the use of other chemicals being preferred.

With its toxicity against both fungi and insects the ability to produce PCP in large quantities at relatively low cost led to this preservative replacing creosote for wood preservation in a number of situations. However, it soon became apparent that PCP was a very dangerous compound due to the presence of impurities, such as dioxins, in the formulation. Due to the effects that PCP has on humans (e.g. hyperthermia, coma and neurological damage) and the possible effects that it may have on the environment, this preservative has now been banned in many countries.

CCA is very effective against decay fungi, insects and marine borers although there are problems concerning hardwood decay by soft rot fungi. There are a number of possible reasons for this although incomplete distribution within the S_2 layers of the cell seems to be the major fault (Eaton and Hale, 1993). There is a certain amount of concern over the use of preservatives that contain chromium and arsenic, and research is being carried out to develop copper-based preservatives without the other two components.

Type of Preservative	Example	In-service Problems	LD_{50} (mg/kg; rat/oral)	Toxicity
Tar Oil Preservative	Creosote	Leaching; strong odour; treated timber cannot be painted		Highly toxic, corrosive, irritant, carcinogenic
Water-borne Preservatives	Copper-chrome-arsenic	Leaching; incomplete distribution within the wood		Irritation of mucous membranes, skin, and eyes; neurological damage; arsenic is a metabolic poison
	Alkyl ammonium compounds (e.g. alkyldimethylbenzyl ammonium chloride)	Leaching; inadequate distribution within the wood	280	Highly toxic, severe eye irritant
	Boron compounds	Leaching		Toxic
Organic solvent-based Preservatives	Pentachlorophenol	Contains highly toxic chemical compounds such as dioxins	50	Irritant of mucous membranes and skin; possible nerve damage; coma, hyperthermia.
	Copper naphthenate	Expensive; treated timber cannot be painted		Severe eye and skin irritant. Very toxic to fish
	Organotin compounds (e.g. tri-butyl tin oxide)	Chemical degradation; fungal detoxification	194	Highly toxic; skin irritant; nerve poison; causes damage to the immune system. Toxic to marine life.

Table 1.6 : Table of wood preservative and fungicides/insecticides added to preservatives with toxic data (Data from London Hazards Centre).

Tributyltin oxide is particularly effective against brown-rot fungi in above-ground situations and it was also used as an anti-fouling agent on ships' hulls. Although tributyltin oxide has been shown to be resistant to leaching, progressive loss of activity caused by chemical degradation has been demonstrated. Furthermore, certain wood-inhabiting fungi have also been shown to have the ability to detoxify this compound. Tributyltin oxide is highly poisonous and is known to be corrosive and produce neurological damage in humans. It may also be carcinogenic and teratogenic, and has now been banned as an anti-fouling agent because of its detrimental effects on marine life.

Organic solvent preservatives can also have insecticides added to their formulations to increase their broad-spectrum biocidal effectiveness. A number of chemicals that were used for this purpose have now been banned (e.g. aldrin, dieldrin) but new ones have replaced them, i.e. the organophosphorus compounds (e.g. chlorpyrifos) and the synthetic pyrethroids (e.g. permethrin). Both of these compound groups have low mammalian toxicity and low persistence in the environment, although permethrin is toxic to fish.

A number of chemical compounds have also been developed to deal with post-harvest deterioration of timber in particular mould and sap stain. Some of the more traditional biocides used for this purpose have either been banned (e.g. PCP), can be microbially degraded or are not fixed within the wood (e.g. 2-thiocyanomethylthio-benzothiazole and captafol) (Eaton and Hale, 1993). New formulations have been developed which can contain azaconazole, propaconazole, carbendazim, chlorothalonil, dichlofluanid, furmecyclox, the isothiazolones and IBPC (3-iodo-2-propynyl butyl carbamate). The effectiveness of these new formulations is still being determined and most do show fungicidal activity. However, the determination of effectiveness is no longer sufficient to allow the marketing of a preservative and the possible effects that the compound may have on the environment have also to be determined.

1.12 New methods for the eradication of *S.lacrymans*

Although the present treatment method for dry rot is effective if carried out in the proper manner, it can involve the use of toxic chemicals and may result in the wasteful

removal of uninfected timber. New methods for the treatment of dry rot which do not use chemical preservatives have been developed and these basically fall into three categories; 1) heat, 2) biochemical inhibitors, and 3) biological control.

A number of methods have been developed which use heat to kill the mycelia of *S.lacrymans*. It has been reported that *S.lacrymans* in wood will be killed if it is subjected to 40°C for more than 6 hours (Kjerulf-Jensen and Koch, 1992; Koch, 1990). The heat sensitivity of *S.lacrymans* is dependent upon the moisture content of the wood, with active mycelia in wood with a high moisture content being more sensitive than dormant mycelia in dry wood (Koch, 1990). Taking this into consideration, Miric and Willeitner (1984) reported that 40°C for 24 hours was the minimum level of heat treatment that should be used in the eradication of *S.lacrymans* in wood. Three methods have been developed to raise the temperature of a piece of wood to a high enough temperature to kill the dry rot mycelia. These use hot air (Koch, 1990), microwave radiation (Kjerulf-Jensen and Koch, 1992) or high frequency radio waves (Munck and Sundberg, 1994).

There are a number of problems associated with each of these methods. The hot air method uses oil or gas burners to generate hot air that is pumped into a building. The building or area to be treated is covered with insulation material to minimise heat loss. In order to observe the temperature change of the wood, heat sensors are placed in the treatment area. Using this method, it takes approximately 24-48 hours to raise the temperature of the timber/brickwork to the lethal temperature. However, this length of time is highly dependent upon the thickness of the timber/brickwork being treated, with thicker structures requiring a longer treatment time. It is not economic to treat small infections with this method (Kjerulf-Jensen and Koch, 1992) and the expense of the oil/gas burner fuel is dependent upon the size of the treatment area.

Both the microwave radiation and radio wave methods can be used on small areas. A problem seen with both types of methods is the high temperatures achieved, e.g., 70-80° C with the radio waves (Munck and Sundberg, 1994) and up to 120°C with the microwaves (Kjerulf-Jensen and Koch, 1992) with obvious implications concerning the fire risk of these methods. Depending upon its size a piece of timber in a building can have

a range of moisture contents, which means that the effectiveness of the heat treatment being used will vary along the length of the timber. This could result in the under- or over-treatment of certain portions of the infected timber, with under treatment resulting in re-infection and over treatment resulting in heat damage to the timber or even fire.

Whenever an organism is exposed to sub-lethal temperatures it produces a heat shock response resulting in the production of heat shock proteins which have been shown to give the organism the ability to survive lethal temperatures (Plesofsky-Vig and Brambl, 1990). When *S.lacrymans* is subjected to a sub-lethal temperature it will respond with a heat shock response, and subsequently, it can survive the lethal temperature of 40°C (White *et al*, 1995) which has implications in the heat treatment of *S.lacrymans*. In the treatment situation, if the heat transfer is rapid then there will not be enough time for a heat shock response and the fungus will be killed. However, in situations where the heat transfer is slow then the possibility that *S.lacrymans* can survive supposedly lethal temperatures will exist.

One area that is seeing an increasing amount of research is in the use of biochemical or metabolic inhibitors to control the growth of *S.lacrymans*. These compounds are not necessarily poisons but interfere with the uptake of certain vital nutrients such as amino acids or nitrogen. One such compound is α-aminoisobutyric acid (Watkinson, 1984; Elliot and Watkinson, 1989; Connor, 1990; Dobson *et al*, 1993), which is an analogue of the amino acid alanine. *S.lacrymans* can take-up α-aminoisobutyric acid but does not metabolise it or incorporate it into protein (Watkinson, 1984). This results in the exclusion of utilisable amino acids from the fungus' metabolism resulting in nitrogen starvation. Elliott and Watkinson (1993) have shown that the linear extension growth of *S.lacrymans* is reduced and that wood treated with α-aminoisobutyric acid (8.0% w/v) showed little weight loss. Dobson *et al*, (1993) showed that using α-aminoisobutyric acid applied to the mycelium reduced the linear extension rate of *S.lacrymans* on wood to 30% (as compared to the controls) and that almost complete inhibition was seen on brickwork. Elliott and Watkinson (1993) reported that α-aminoisobutyric acid was effective against other brown-rot fungi and at least two species of white-rot fungi. α-aminoisobutyric acid

could have an application in limiting the spread of *S.lacrymans* in buildings through both timber and brickwork. However, the minimum treated area to ensure that *S.lacrymans* cannot bypass the treatment, particularly in timber, must be determined.

Another relevant compound is heptadecenylsuccinic anhydride, although this compound is used to modify the cell wall long chain polymers (Codd and Banks, 1992). Wood modified with this compound showed resistance to decay caused by the brown-rotters *Coniophora puteana* and *Gloeophyllum trabeum.* Research with other anhydrides, such as acetic anhydride, indicate that almost complete resistance to wood decay can be achieved and that the probable mode of action is via the mechanical bulking of the sub-microscopic pores in the wood cell wall (Codd and Banks, 1992).

Biological control of wood decay fungi is receiving increasing attention as a method to reduce the amount of chemical preservatives/fungicides used to treat wood decay. Research has been carried out into the use of *Trichoderma* spp. to control the decay of creosoted transmission poles by *Neolentinus lepideus* (Bruce and King, 1986 a; Bruce and King, 1986 b; Bruce *et al*, 1990). In addition *S.lacrymans* can also be controlled by *Trichoderma* spp. under certain experimental conditions (Doi and Yamada, 1992; Palfreyman *et al*, 1991a). However, the experiments reported by these latter authors utilised enriched artificial media containing a range of nutrients at levels not normally found in wood. Biological control in general and of wood decay fungi in particular is further discussed in the following section.

1.13 Biological Control and its application to wood decay prevention

One definition of biological control is " the reduction of inoculum density or disease producing activities of a pathogen or parasite in its active or dormant state, by one of more organisms, accomplished naturally or through manipulation of the environment, host or antagonist, or by mass introduction of one or more antagonists" (Baker and Cook, 1974). When the microbial community of a particular ecosystem, e.g., soil, is altered such that the microbial population is reduced certain organisms can take advantage of the reduced competition and rapidly colonise a greater proportion of the ecosystem than that which would be achieved under typical conditions.

Biological control in agriculture has been practised in some form or another for thousands of years by manipulating the environment, either directly or indirectly, to favour one or more organisms that can control agricultural pests. For example, when fields were allowed to be flooded by rivers the microbial community was severely depleted thus allowing *Trichoderma* spp. to rapidly colonise the soil and suppress plant pathogens. Another example involves fruit trees that had branches infected with canker (caused by certain species of bacteria, e.g., *Pseudomonas*, *Erwinia* and *Corynebacterium*, and fungi, e.g., *Phomopsis* and *Nectria*). Austin (1657) suggested that the wounds left behind after removing diseased branches should be painted with washes of cow-dung and urine. This would raise the nitrogen content of the wound area favouring the growth of antagonistic organisms that would compete with the canker causing organisms.

Since the 1880's biological control has become an important method for dealing with certain pests in agriculture. However, it was not until 1926 that the possibility of using biological control to combat fungal plant pathogens was taken seriously (Faull, 1988). The resultant research indicated a number of biological control agents that could be used against many different diseases. However, the initial screenings were carried out in sterile or heavily amended substrates that favoured the biological control agent, and when the agent was transferred to the field trials the success was not repeated (Faull, 1988) due to disregard of the microbial community already present in the environment and the effect it would have on the introduced control agent. To succeed the environment must be altered in some way to allow successful colonisation of the introduced control agent. Research into the suitability of organisms for biological control must include screening experiments that take into account the nutritional status of the target environment, the method of control (e.g., competition, antibiosis, etc.), the effects that organisms naturally present in the environment have on the control agent and the method of propagation and spread of the agent in the environment.

There are successful examples of mycoherbicides and mycopesticides being used in agriculture, with their use increasing as the products are refined to suit a particular problem. The use of mycoparasites to control fungal plant pathogens will take more time

due to the complexity of the interactions involved (i.e., mycoparasite-plant, mycoparasite-fungal pest, mycoparasite-environment). There are, however, already several commercial examples of mycoparasites being used to control fungal plant pathogens. One of the earliest examples was the use of *Phanerochaete gigantea* to control the spread of *Heterobasidion annosum* which attacks pine and other coniferous tress producing a white heart rot of the wood (Jeffries and Young, 1994). Ricard (1981) has developed the use of certain *Trichoderma* species (*T.harzianum* and *T.polysporum*) to combat silver leaf disease of fruit trees. *Trichoderma* species can also be used in conjunction with chemical protectants to reduce the incidence of fungal soil pathogens. The chemical protectant is used to eradicate the pathogen from the soil (or other environment, e.g., a tree stump) initially. This also results in the reduction of all other susceptible soil micro-organisms. The *Trichoderma* species, whether introduced artificially or already present in the soil, rapidly grow throughout the soil and restrict the growth of other soil micro-organisms.

To date there are a large number of potential applications for *Trichoderma* species biocontrol being investigated. For example, *Trichoderma* biocontrol of *Aspergillus flavus* and *Fusarium moniliforme* (Calistru *et al.*, 1997), *Sclerotium rolfsii* (Mukherjee and Raghu, 1997), *Rhizoctonia solani* (Bertagnolli *et al*, 1998) and *Pythium ultimum* (Migheli, *et al.*, 1998) are all being investigated with varying degrees of success.

Trichoderma spp. are widely distributed and can be found in soil types taken from most parts of the world (Domsch *et al*, 1980, Papavizas, 1985), although individual species may be restricted geographically, e.g., *T.viride* and *T.polysporum* are adapted to low temperatures, and, *T.harzianum* is found in warm climatic regions (Papavizas, 1985). *Trichoderma* spp. are found in well-decomposed organic matter, and can be found on root surfaces, on decaying bark (especially when it has been damaged by other fungi) and on propagules (e.g. sclerotia) of other fungi (Papavizas, 1985). The natural soil population of *Trichoderma* spp. usually does not exceed 10^2 CFU/g of soil although this can be affected by the pH of the soil with acidic soils showing a higher population density (Chet, 1987).

The potential of *Trichoderma* species as biological control agents against fungal soil pathogens was first suggested more than 50 years ago (Chet, 1987). The interest in

Trichoderma species for biological control is due to the large number of antagonistic traits that they have. Their ability to rapidly colonise a substrate, even under environmental stress, coupled with the production of antifungal metabolites has resulted in several *Trichoderma* species being considered for control of wood decay fungi (Bruce *et al.*, 1995). However, it was only with the development of biotechnology coupled with financial and environmental concerns that serious interest in the use of organisms to control plant pathogens became evident. The antagonistic properties of *Trichoderma* species were investigated (Dennis and Webster, 1971 a-c) and soon after the first successful control of a plant pathogen in the field was reported (Chet, 1987). With the increase in the number of *Trichoderma* strains and the development of treatment formulations and delivery systems, the interest in biological control has resulted in the marketing of several commercial treatment systems (e.g. for the treatment of *Heterobasidion annosum* and silver leaf disease).

Although *Trichoderma* species have been used successfully as biocontrol agents in agriculture, their use as biocontrol agents in wood and wood-based products has met with limited success. The greatest success has been achieved by using the *Trichoderma* species as bioprotectants, i.e. they are used to prevent the initial colonisation of the wood products by the decay organisms, rather than kill the decay organisms already present in the wood prior to application (as would be the case in biocontrol). There has been a commercial product released for the treatment of creosoted distribution poles in order to prevent infection of the pole interiors with *Neolentinus* (*Lentinus*) *lepideus* (Jeffries and Young, 1994). This involved the use of a mixture containing *T.harzianum*, *T.polysporum* and *Scytalidium* FY. Although the control agents colonised the interior of the poles, the extent of colonisation was affected by the presence of other non-decay residents within the poles (Bruce and King, 1986b). However, those areas of the poles that were colonised by the *Trichoderma* species were protected against attack by the *N.lepideus* for periods up to seven years (Bruce *et al*, 1991).

To realise the full potential of fungi such as *Trichoderma* and *Scytalidium* species as bioprotection/biocontrol agents a number of factors must be fully investigated. The

mode of antagonism must be determined, in particular those modes that are utilised under the conditions in which the target fungus is found. Once this has been achieved then methods could be found to improve the effectiveness of the antagonist by enhancing the modes of antagonism. Another area that requires investigation is the development of formulations and methods of delivery by which the antagonist is applied to the area to be treated. The search for fungi with new or enhanced antagonistic attributes should continue, as should the development of new screening procedures that accurately reflect the environment in which biocontrol is to be practiced. One possible way to enhance the effectiveness of the existing mycoparasites is through the process of molecular biology. In this way a particular antagonistic attribute could be enhanced or the ability of a fungus to survive and colonise a particular substrate could be improved.

The use of *Trichoderma* spp. for the pretreatment of wood has been much researched, however, use in remedial treatment of, for example, dry rot has received little attention. The current project was designed to fill this information gap.

1.14` Aims of the project

There are a number of aims to the project and these are listed below.

- to screen a number of *Trichoderma* isolates for their antagonistic potential against *S.lacrymans*, to carry out the screening on several substrates including wood and upon analysis of the results chose one particular isolate to take forward to the next stage.
- to develop a medium-scale experiment which will test the effectiveness of the chosen *Trichoderma* species against *S.lacrymans* growing in medium-length pieces of timber, with the experiment being carried out in a semi-controlled environment.
- to develop a field site to enable the testing of the chosen *Trichoderma* species in a building which is infected with *S.lacrymans*.
- to investigate certain attributes of the biochemical interaction between *S.lacrymans* and *Trichoderma* species.

Chapter 2

Biological Control of the Dry Rot Fungus *Serpula lacrymans* by *Trichoderma* Species : The Effects of Complex and Synthetic Media on Interaction and Hyphal Extension Rates

2.1 Introduction

Research into the biological control of wood decay fungi has continued for the past thirty years. Fungal organisms such as *Trichoderma*, *Gliocladium* and *Scytalidium* have attracted interest as they are antagonistic towards decay fungi in the laboratory. For example, *T.harzianum* and *T.polysporum* were found to mycoparasitise several wood decay fungi including *Lentinus lepideus*, *Postia placenta* and *Gloeophyllum trabeum* (Murmanis *et al.*, 1988a) and prevent or reduce decay of southern pine wood blocks by brown rot fungi such as *Antrodia carbonica* and *Neolentinus lepideus* (Highley and Ricard, 1988). However, not all brown rot fungi could be controlled and the *Trichoderma* were ineffectual against white rot fungi.

Although there has been success within the laboratory concerning the use of *Trichoderma* isolates to control decay fungi, there has been some difficulty in duplicating that success within field systems. Bruce and King (1986a,b) showed that although *Trichoderma* could kill *N.lepideus* in agar studies, the control of the decay fungus within creosote-distribution poles was limited by incomplete colonisation of the poles by the *Trichoderma* isolate. However, the *Trichoderma* did protect those areas it colonised and remained viable for at least six years after inoculation.

Although the problems involved with the long-term protection of distribution poles have been noted by other authors (for example, Morris *et al.*, 1986), the successful use of biological control systems for the protection of wood has been indicated. For example, the control of blue stain fungi (*Aureobasidium pullulans*, *Ceratocystis coerulescens* and

Ceratocystis spp.) in *Pinus radiata* has been achieved by the use of the bacterium *Pseudomonas cepacia* (Benko, 1989). However, effective control of the stain fungi did decrease over time in the field situation.

The effective control of *Serpula lacrymans* by *Trichoderma* spp. has been shown in previous studies (Doi and Yamada, 1991; Palfreyman *et al.*, 1991a). However, the interactions carried out during these investigations were undertaken using enriched media containing a range of nutrients very different from those found in wood. Although the use of such enriched media is normal practice during the screening or potential control organisms, recent reports (Doi and Yamada, 1992; Srinivasan *et al.*, 1992a,b) have shown that the composition of the media can have a profound effect upon the nature of the interaction.

The use of enriched media for the screening of potential control organisms, whilst allowing rapid screening, can provide results which are not duplicated in the field because of the limitation of one or more nutrients normally found in these situations. Fungi require a number of macronutrients (for example, carbon, nitrogen and oxygen) and micronutrients (for example, iron, copper and zinc) for normal growth. If one or more of these nutrients were to be provided in excess then changes in the growth of fungi could arise. These changes could be observed either as a stimulation or inhibition of certain fungal processes, for example growth or reproduction. For example, copper is important as a metal activator of several fungal enzymes, particularly oxidases, however, at supraoptimal concentrations copper is a potent inhibitor of fungal growth and is a key component of several fungicides (Garraway & Evans, 1984).

New media are being developed which closely mimic nutrient conditions found in the natural environment of fungi. A medium has been developed which mimics the carbon:nitrogen ratio in wood (Hutterman & Volger, 1973). Using this medium the

screening of possible antagonists of wood decay fungi could provide results that may be duplicated within the field.

2.2 Materials and Methods

The following organisms were used : *S.lacrymans* (FPRL 12C, obtained from the Building Research Establishment, Garston, UK.), *T.harzianum* IMI 206040, *T.harzianum* 25, *T.viride* isolates 24, 40, 60, 70, 110, *T.hamatum* 150, *T.polysporum* 200 and *Trichoderma* 38 (for origins of the *Trichoderma* isolates see Srinivasan *et al.* 1992). All chemicals were obtained from Sigma unless otherwise stated.

Organisms were maintained at 4°C on 5% malt extract (Oxoid, L39)/2% technical agar (Oxoid, L13) (MEA). New stocks were prepared every six months by removing a core from the old plate and placing it on fresh agar. Interactions were carried out on this medium or one of the following media : (a) low nitrogen medium/normal iron (LNM-NFe) comprising minimal essential medium (MEM) containing nitrogen at 0.4 mM and iron at 0.1 mM; (b) high nitrogen medium/normal iron (HNM-NFe) comprising MEM containing nitrogen at 7.9 mM and iron at 0.1 mM; (c) high nitrogen medium/low iron (HNM-LFe) comprising MEM containing nitrogen at 7.9 mM and iron at 0.01 mM; (d) low nitrogen medium/low iron (LNM-LFe) comprising MEM containing nitrogen at 0.4 mM and iron at 0.01 mM. By comparison the MEA contained nitrogen at 40.0 mM and iron at 0.34 mM. In the MEM nitrogen was supplied in the form of L-asparagine and iron in the form of ferric sulphate. The constituents of MEM are described in Table 2.1.

Hyphal extension rates of all organisms were recorded on the various media by measuring colony diameters in two directions at right angles. A minimum of three measurements were taken for each sample and five replicates were used for the determination of each mean. Interaction experiments were carried out essentially as described in Rayner and Todd (1977). For the interactions, a core of *S.lacrymans* was placed into the agar at the edge of the plate with five plates set up for each interaction

Constituent	Amount
Glucose	5.000 g
L-Asparagine (LNM)	0.013 g
(HNM)	0.220 g
Potassium Dihydrogen Orthophosphate (BDH)	1.000 g
Magnesium Sulphate (Anhydrous)	0.500 g
Potassium Chloride	0.010 g
Iron Sulphate (NFe)	0.010 g
(LFe)	0.001 g
Manganese Acetate	0.008 g
Zinc Nitrate	0.002 g
Calcium Nitrate	0.050 g
Copper Sulphate (Anhydrous) (BDH)	0.002 g
Ammonium Nitrate (Fisons)	0.008 g
Technical Agar (Oxoid)	20.00
Distilled Water	up to 1.0 litre

Table 2.1 : The constituents of minimal essential medium (MEM). This medium was based on a nutrient solution devised by Hutterman and Volger (1973. The overall contribution to the C:N ratio by nitrogen sources in the salts was negligible.

study. The plates were incubated at 22°C ± 1°C for 8 days after which a colony radius of 22-40 mm was achieved. The relevant *Trichoderma* isolate was then subcultured (fungal core) onto the agar at the opposite plate edge to the *S.lacrymans*. The plates were then incubated at 22°C ± 1°C and inspected daily for visible changes. After interactions had reached a conclusion, i.e. no further visible changes had occurred for 4 days, or when the entire *S.lacrymans* had apparently been killed (assumed to have occurred when the mycelia changed colour from white through yellow to brown) cores were taken from the browned areas of the *S.lacyrmans* to check for viability. Viability was tested by subculturing the cores onto a medium containing MEA plus 4 ppm of benomyl, a concentration which inhibits the growth of *Trichoderma* spp. but has no effect on the growth of *S.lacyrmans* (unpublished observations). The types of interactions were classified as (1) replacement (unilateral antagonism), (2) deadlock (mutual antagonism), and (3) intermingling (no antagonism) (Cooke and Rayner, 1984).

2.3 Results

2.3.1 Hyphal extension rates

The hyphal extension rate (HER) of *S.lacrymans* was markedly affected by the various growth media used in this analysis, the mean hyphal extension rates on the five media were : MEA, 11.92 mm day^{-1}; LNM-NFe, 5.88 mm day^{-1}; HNM-NFe, 6.27 mm day^{-1}; LNM-LFe, 4.96 mm day^{-1}; HNM-LFe, 5.28 mm day^{-1}. Thus, all the minimal essential media, whatever the nitrogen or iron content, supported HERs of *S.lacrymans* approximately 50% of that on the MEA.

The effects of media constituents on the growth of the *Trichoderma* spp. were varied, with some organisms, such as *T.hamatum* 150, being relatively unaffected by medium constituents whereas others, such as *T.harzianum* 25, showed different HERs on the range of media used (Figure 2.1). Overall, nitrogen levels had the most consistent effects on extension rates (Figures 2.2 a, b) with low nitrogen media stimulating growth. However, low iron levels did influence the effect that the nitrogen levels had on certain *Trichoderma* species. For example, *Trichoderma* 38 had a greater HER on LNM than HNM (both with normal iron levels), however, on LNM/HNM with low iron the HERs were not significantly different. Iron levels, in general, had a lesser effect on the HERs compared to the nitrogen levels (Figures 2.3 a, b), although the level of nitrogen did have an effect on the ability of some *Trichoderma* species to deal with the low iron levels. For example, the iron levels had no significant effect on the HERs of *T.polysporum* 200 on the LNM media, however, this *Trichoderma* species grew faster on the HNM with normal iron levels rather than with low levels.

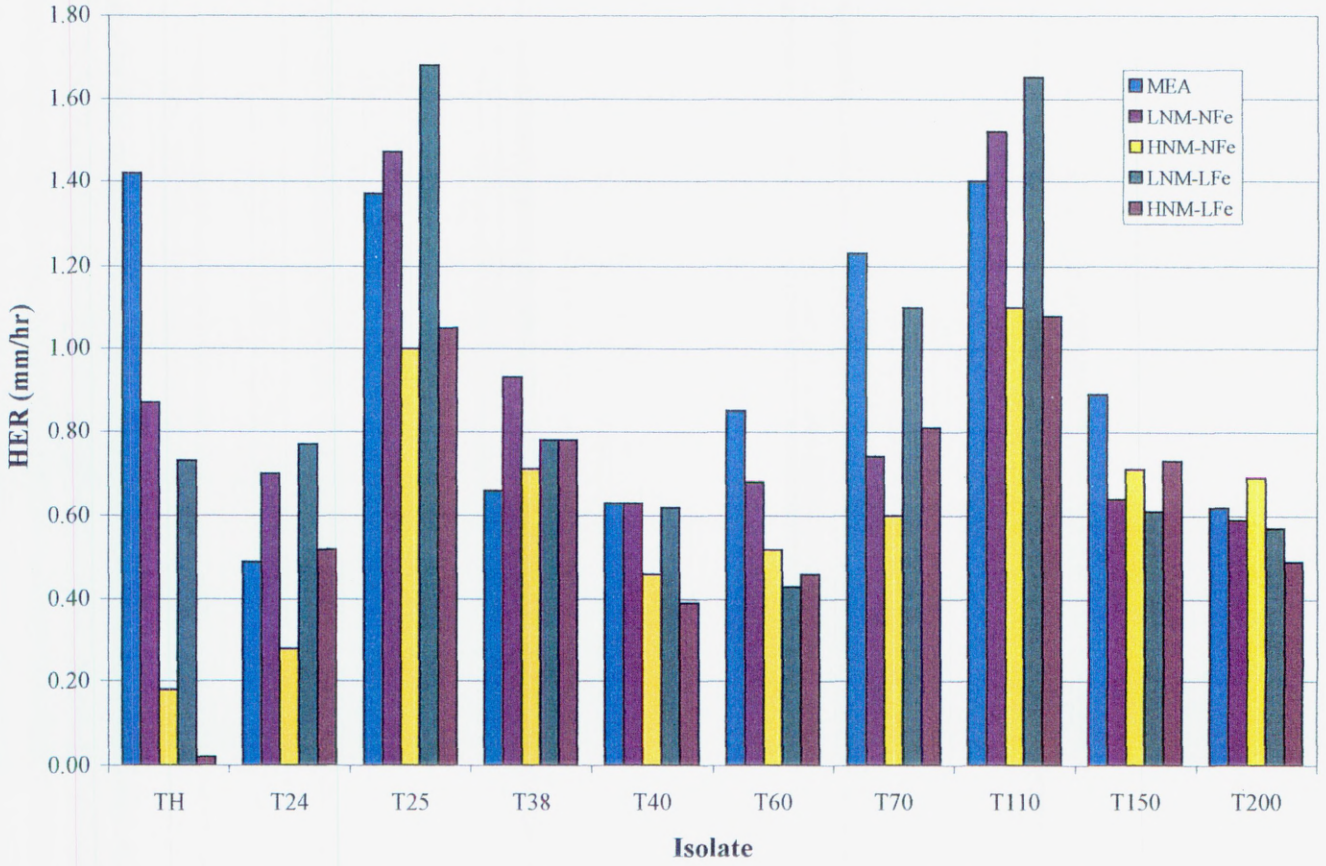
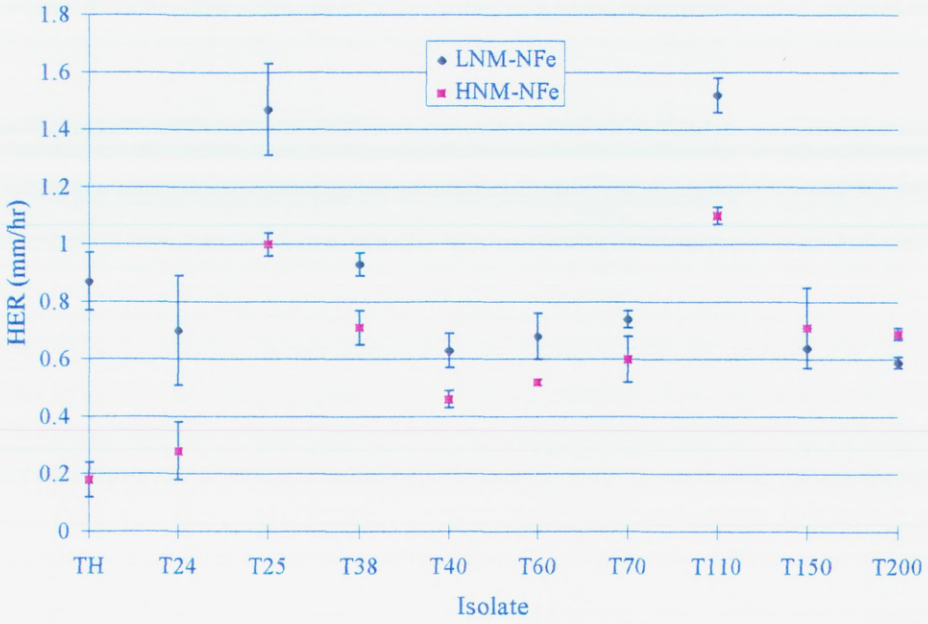
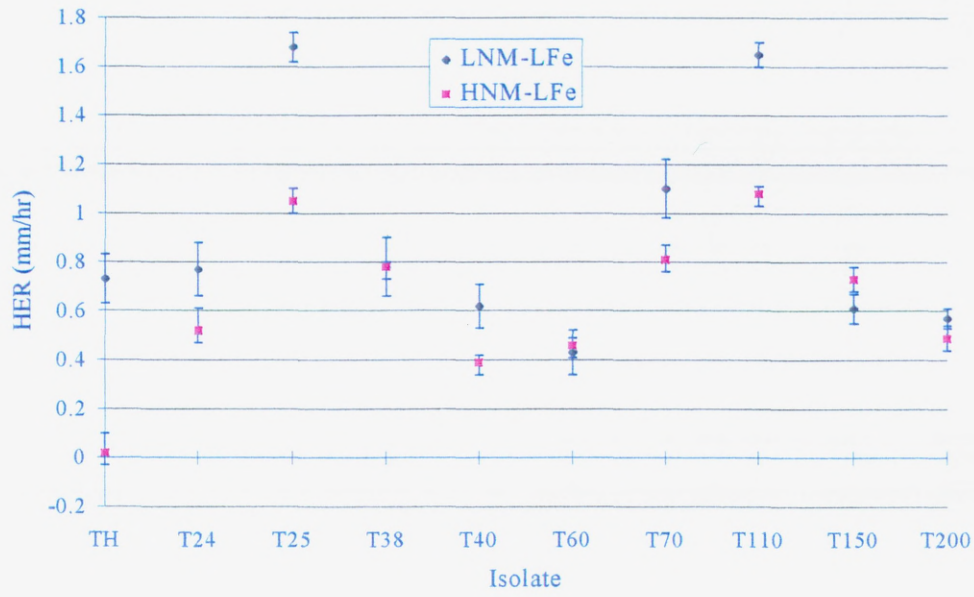


Figure 2.1 : The effect of the various media on the hyphal extension rates (HERs) of the *Trichoderma* isolates. HERs were recorded by measuring colony diameters in two directions at right angles over the period of time taken by the fungal isolate to cover the plates. Results shown are the mean and standard error of the mean of five replicates. Key : TH, *T.harzianum* 206040; T24, *T.viride* 24; T25, *T.harzianum* 25; T38, *Trichoderma* 38; T40, *T.viride* 40; T60, *T.viride* 60; T70, *T.viride* 70; T110, *T.viride* 110; T150, *T.hamatum* 150; T200, *T.polysporum* 200.

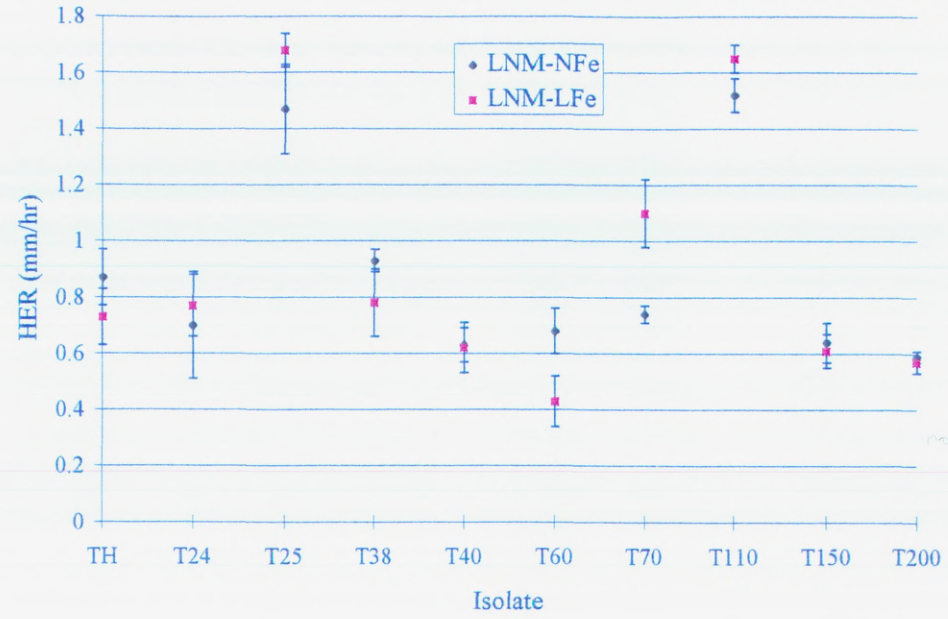


(a)

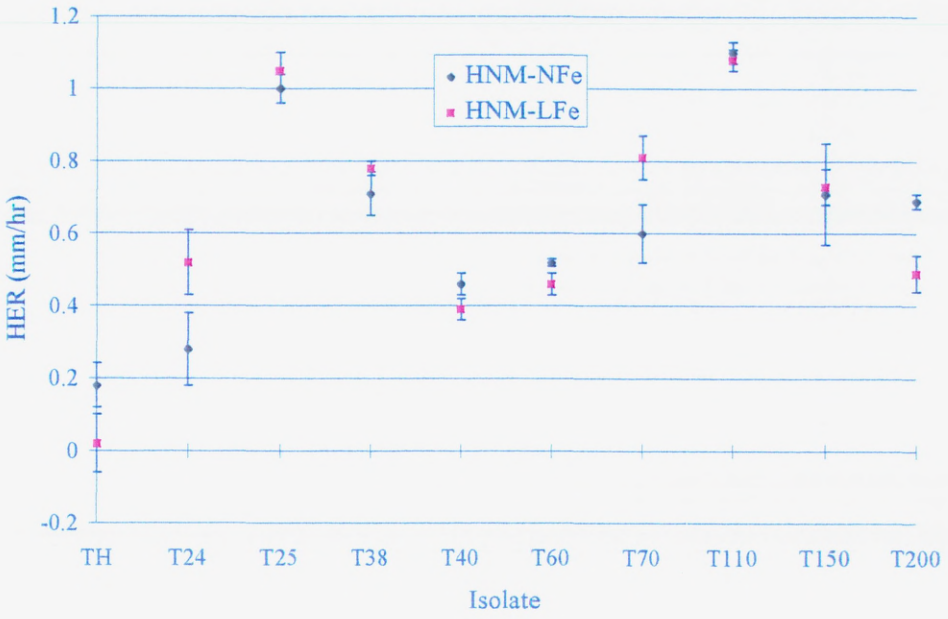


(b)

Figure 2.2 : Effect of nitrogen concentration on hyphal extension rates (HERs) of 10 *Trichoderma* isolates at normal (a) and low (b) iron levels. HERs were recorded by measuring colony diameters in two directions at right angles. The results represent the mean hyphal extension rates with standard error of the mean (bars).



(a)



(b)

Figure 2.3 : Effect of iron concentration on hyphal extension rates (HERs) of 10 *Trichoderma* isolates at low (a) and high (b) nitrogen levels. HERs were recorded by measuring colony diameters in two directions at right angles. The results represent the mean hyphal extension rates with standard error of the mean (bars). (a)

The change from MEA to the minimum essential medium had a variety of effects on organisms with a general stimulation of growth on the low nitrogen media. Most organisms grew equally well on the low nitrogen media and the MEA but more slowly on the high nitrogen media. Exceptions to this were *T.harzianum* 206040, *T.viride* 70 and *T.hamatum* 150 whose hyphal extension rate was greater on the MEA than on the LNM-NFe. Of particular interest because of the outcome of the interaction experiments (see section 2.3.2) are the effects of the media on *T.harzianum* 25 and *T.viride* 70. The HERs of *T.harzianum* 25 were highest on the low nitrogen media regardless of iron concentration. The HERs of *T.viride* 70 were similarly affected by nitrogen concentrations but in addition were iron sensitive with low iron levels increasing the HER. Also shown in Figure 2.2 are the data for organisms *T.viride* 40 and *T.viride* 110. *T.viride* 40 had a relatively low HER and was particularly insensitive to the medium constituents. *T.viride* 110, however, showed a very high HER, particularly on the low nitrogen media, but, as shown in Figure 2.4, was not especially effective at killing *S.lacrymans* on this type of medium.

2.3.2 Interactions

The nature of the interactions between all the isolates of *Trichoderma* in these studies and the type strain of *S.lacrymans* (FPRL 12C) was investigated. A summary of the interactions is given in Table 2.2. *T.harzianum* 25 and *T.viride* 70 efficiently killed *S.lacrymans* in all systems tested. By contrast, isolate T200 was only effective on the HNM-NFe medium. All other organisms showed intermediate efficiency against *S.lacrymans*, with all organisms being effective on the HNM-NFe medium and only two, *T.harzianum* 25 and *T.viride* 70, being effective on the LNM-NFe medium. Indeed, on the LNM-NFe medium the majority of *Trichoderma* isolates were overgrown by *S.lacrymans*. Deadlock reactions were produced in a number of cases, specifically on the LNM-NFe and

	Media Type				
	MEA	LNM-NFe	HNM-NFe	LNM-LFe	HNM-LFe
N (mM)	40.0	0.40	7.90	0.40	7.90
Isolate Fe (mM)	0.34	0.10	0.10	0.01	0.01
T.harzianum	T	D	T	T*	T
T24	T/D	S	T	S	T
T25	T	T	T	T	T
T38	S	S	T	S	T
T40	S	S	T	S	T
T60	S	S	T	S	T
T70	T	T	T	T	T
T110	T	S	T	T	T
T150	T	D	T	S	D1
T200	S	S	T	S	D2

Table 2.2 : Effect of media type on the outcome of the interactions between *S.lacrymans* and the 10 *Trichoderma* isolates. Three main types of interaction were seen : replacement of *S.lacrymans* by *Trichoderma* (T), deadlock (D) or replacement of the *Trichoderma by* the *S.lacrymans* (S). Exceptions were as follows : D1, deadlock reaction with *T.hamatum* 150 but browning of the *S.lacrymans* occurred; D2, deadlock reaction with *T.polysporum* 200 but there were morphological changes to the *S.lacrymans*; T*, most interaction plates showed replacement of *S.lacrymans* but regrowth occurred on one plate; and T/D, some interaction plates (two) showed replacement of *S.lacrymans* whereas some (three) showed a deadlock reaction.

the HNM-LFe media. The viability tests on benomyl agar showed that yellow mycelia taken from the *S.lacrymans* colony could regrow but mycelia taken from the brown areas of the colony did not show any signs of regrowth.

In the cases where interactions resulted in the killing of *S.lacrymans* there was a wide variability in the time taken to render the decay organism inactive (Figures 2.4). The most efficient organism was again *T.harzianum* 25, *S.lacrymans* being rendered non-viable after 7 days on the LNM-NFe medium and within 15 days on the HNM-LFe medium where *T.harzianum* 25 was at its least efficient. The results shown in Figures 2.4 (a, b) confirm that the *Trichoderma* isolates were generally most effective when the interactions were carried out on HNM-NFe rather than on the LNM-NFe.

Though not illustrated here, a wide range of specific and reproducible visual effects were seen during the interaction experiments. Every interaction was characterised by a common change in the colour of the *S.lacrymans* mycelia from white through yellow to brown. However, the degree of colour change was markedly different depending on whether the *Trichoderma* isolate produced a lethal effect or not. *S.lacrymans* interacting with lethal *Trichoderma* isolates (for example, *T.harzianum* 25 and *T.viride* 70) turned yellow on contact, and as time progressed this mycelia turned brown. During non-lethal interactions the *Trichoderma* isolates produced essentially the same colour changes, but the *S.lacrymans* recovered in these interactions producing near white mycelia (at the boundary of the browned mycelia) which then gradually overgrew the *Trichoderma* isolates. Microscopic work has indicated that yellowing of the *S.lacrymans* was accompanied by granulation of the hyphal cytoplasm and browning of the hyphae was linked to the loss of cytoplasm from the hyphae.

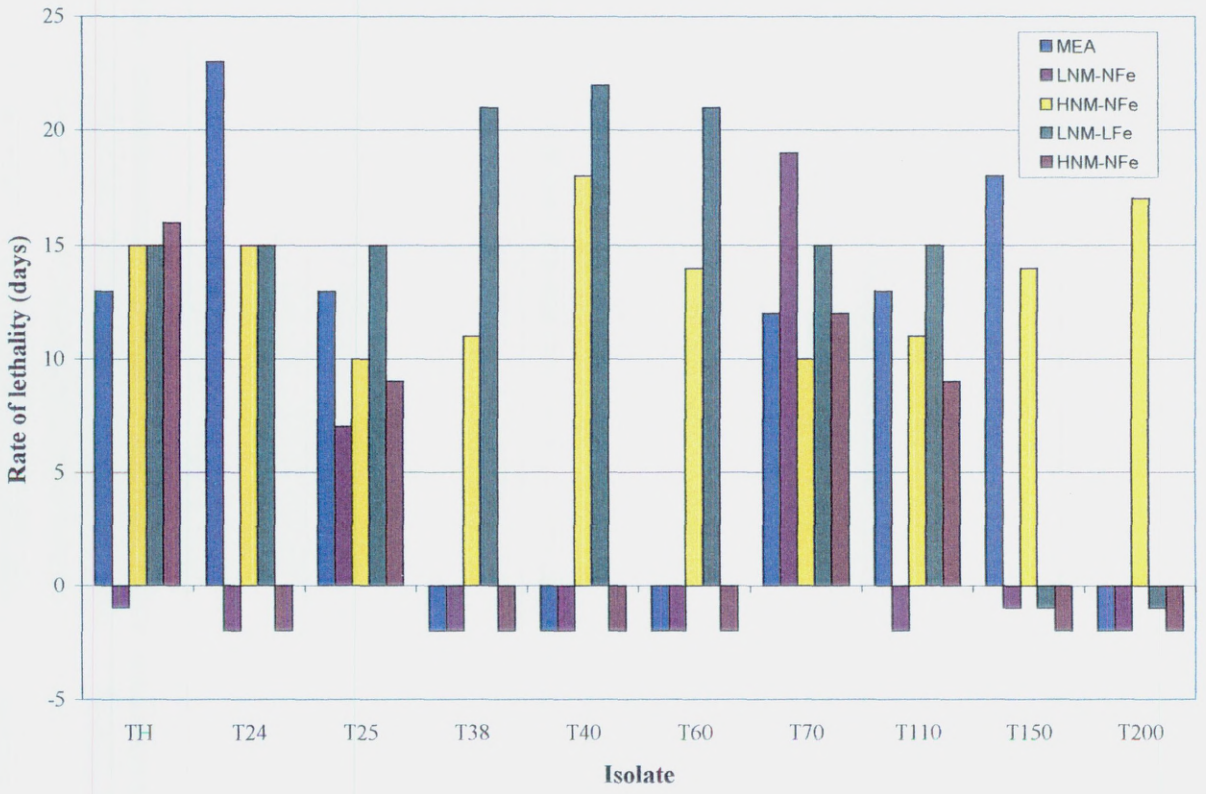


Figure 2.4 : Rate of replacement of *S.lacrymans* by the *Trichoderma* isolates during plate interactions. Interactions were carried out by plating *S.lacrymans* initially and then the relevant *Trichoderma* species onto the plates. The plates were then incubated until they had reached a conclusion, i.e., deadlock or overgrowth. Small bars below the line represent a deadlock reaction, with the large bars below the line indicating interactions where *S.lacrymans* overgrew a *Trichoderma* isolate. Bars above the line represent the time taken to render the *S.lacrymans* non-viable (mean of five observations).

Another effect, seen in the interactions with lethal *Trichoderma* isolates only, was the appearance of a narrow strip (1-5 mm in width) of white/off-white mycelia stretching from the edge of the *S.lacrymans* colony to the start of the yellow mycelial colouration. This strip of mycelia reacted in the same way to the *Trichoderma* isolates as other parts of the colony except that no brown colour change occurred. This effect was seen only on the MEA and the HNM-NFe media.

Changes in *Trichoderma* spore colour were also evident dependent upon the position of the *Trichoderma* mycelia during the interaction. Green spores were produced over the bulk of the *Trichoderma* colony, green/light green spores at the *S.lacrymans* colony edge (interaction zone) and yellow/white spores produced by the *Trichoderma* mycelia that was intermingled with the *S.lacrymans* colony. The pale-coloured spores turned to green over time. This effect was not limited to media type although the density of spores did decrease on the minimal media compared to the MEA. Another effect was the production of chlamydospores. The highest density and largest chlamydospores were produced on the MEA. The quantity of chlamydospores also increased in close proximity to the *S.lacrymans* colony.

A visual effect that was limited to the two low iron media (LNM-LFe and HNM-LFe) was the appearance of a green tinge to the agar under the *S.lacrymans* colony. This was seen in the interactions with *T.harzianum* 206040, *T.viride* 24, *T.harzianum* 25 and *Trichoderma* 38 on the HNM-LFe and *T.viride* 70 on the LNM-LFe. However, with *T.hamatum* 150 (on the HNM-LFe) instead of the agar colour change a small band of *S.lacrymans* mycelia behind the brown mycelia turned green (the green colouration

appeared approximately half-way through the interaction). Another colour change, seen with *T.harzianum* 25 only on HNM-NFe, was the agar turning orange.

2.4 Discussion

Agar screening experiments have traditionally been the most rapid way of determining whether one fungus is antagonistic towards another. If such results are to be used as models for natural antagonisms then the possible effect of the normally nutrient rich experimental media must be considered. In the experiments reported here the low nitrogen medium (0.4 mM nitrogen and 0.1 mM iron) was designed so that it mimicked the carbon:nitrogen ratio in wood, with a view to more realistic screening of *Trichoderma* species for use in a wood system.

Nitrogen is important in the growth of fungi as it is used in the formation of proteins and other cell components such as nucleic acids and chitin, whereas carbon is used as an energy source. In a balanced artificial medium the carbon level is ten times that of the nitrogen level (Oxoid, 1985). However, in wood, the carbon:nitrogen levels is often 500:1 or higher and as such the fungi that colonise wood must be able to use the available nitrogen very efficiently.

Whilst the change from MEA medium to the MEM media affected the hyphal extension rate (HER) of *S.lacrymans* neither the level of nitrogen or iron had any measurable effect on *Trichoderma* extension rates. Similar results have been reported indicating a lack of effect of nitrogen levels (Thornton and McConalogue, 1990) though iron has been reported to stimulate growth (Paajanen, 1993). However, in this latter paper iron levels 50 times those used in this current study were needed to stimulate growth.

Trichoderma species are widely distributed and can be isolated from soils taken from most parts of the world (Domsch *et al.*, 1980). For example, *Trichoderma* species are known to grow on a wide range of substrates (e.g. cork, waxes, wood, stainless-steel and other fungi); they can also survive high levels of CO_2 (up to 10%) and can live in basic and acidic soils (Papavizas, 1985); they can also produce a wide range of metabolic

products (e.g. polyketide, terpenoid and non-polypeptide metabolites (Taylor, 1986)). The variable effects of nitrogen and iron on *Trichoderma* growth are consistent with the variable nature found generally within the *Trichoderma* genus. The results presented in this paper apparently do not agree with previous reports (e.g. Fargasova, 1992), which indicate that the HER of *T.viride* species was reduced when nitrogen concentrations were reduced from 35 mM to 12.5 mM. However, it is possible that the very much lower nitrogen levels used in these current studies induced a different type of growth in the organisms used. The fast extension rate of *T.harzianum* 25 on both LNM-NFe and LNM-LFe indicates that not only is this organism able to grow on media containing a carbon:nitrogen ratio similar to that found in wood but also it is an effective scavenger of iron (Srinivasan *et al.*, 1992b).

Of all the organisms tested during the interactions, *T.harzianum* 25 was the most efficient antagonist of *S.lacrymans* on the range of media tested. In fact this organism was more effective in the low nitrogen medium than the malt extract agar medium despite a report (Doi and Yamada, 1992) that the poor supply of nutrients reduces the effectiveness of many *Trichoderma* species. It may be that the low nitrogen media is stressing the *S.lacrymans* making it more susceptible to antagonism caused by *T.harzianum* 25.

Iron could also be an important factor in the biocontrol of wood decay fungi since *S.lacrymans* may utilise the ion as part of a non-enzymatic complex used to initiate wood decay (Koenigs, 1974; Murmanis *et al.*, 1988 b). This is important since *Trichoderma* species release compounds known as siderophores which sequester iron (Anke *et al.*, 1991; Srinivasan *et al.*, 1992 b) and if the iron can be removed from wood by such a mechanism it will be unavailable for the decay processes of *S.lacrymans*. *T.harzianum* 25, along with a number of other organisms, namely *T.harzianum* 206040 and *T.viride* 70 and 110, was more effective at killing *S.lacrymans* on the low iron containing media than on those with

normal iron levels. The *Trichoderma* siderophores may be more effective at scavenging for iron than the *S.lacrymans* siderophores. Certain siderophores have been reported to be fungicidal/fungistatic (Anke *et al.*, 1991) which may also explain the results on the low iron media but it is also possible that *S.lacrymans* requires iron to protect itself from *Trichoderma* attack. In addition, as in the low nitrogen media, the *S.lacrymans* may simply be stressed by the lack of iron (since iron plays a central role in the energy metabolism of aerobic and semiaerobic micro-organisms (Schippers *et al.*, 1987)) and hence more susceptible to attack.

The experiments carried out on the minimal media provide several other points of interest apart from demonstrating the potential of *T.harzianum* 25 as a control organism. Interactions carried out in the high nitrogen media, with 7.9 mM nitrogen and 0.1 mM iron, indicate that all of the *Trichoderma* isolates could potentially kill *S.lacrymans*. The high nitrogen levels could be having an effect by enhancing one or more modes of antagonism employed by the *Trichoderma* isolates. Many *Trichoderma* species have been shown to produce lytic enzymes, for example, laminarinase and chitinase (Srinivasan *et al.*, 1992 b). Therefore, an increased availability of nitrogen may result in an elevated production of lytic enzymes giving an increase in lethal antagonism. For most of the isolates tested the iron levels had little effect in this media but for T150 and T200 the low level of iron resulted in a deadlock reaction rather than a lethal one. For these isolates it is probable that the *S.lacrymans* is being stressed by the high nitrogen concentration making it more susceptible to attack. However, increasing levels of nitrogen (at least as supplied in the form of glutamine) in agar have been shown to cause an increase in the linear extension rate of a *S.lacrymans* colony (Watkinson *et al.*, 1981).

Trichoderma species have also been shown to produce volatile metabolites (for example, 6-*n*-pentyl-2H-pyran-2-one and 6-*n*-pentenyl-2H-pyran-2-one, Claydon *et al.*,

1987) which can control the growth of certain fungi, for example, *Neolentinus* (*Lentinus*) *lepideus* (Bruce *et al.*, 1984) and *Sclerotinia sclerotiorum* (Claydon *et al.*, 1987). However, Claydon *et al.* (1987) suggested that these volatiles may only induce fungistasis or behave as paramorphogens (i.e. inhibit linear growth but not biomass accumulation), and are therefore unlikely to be able to produce the lethal effects reported in this paper. With the fast hyphal extension rates that the *Trichoderma* isolates in this study have shown, it is unlikely that volatile production would be sufficient for any effects to be seen before or during hyphal interaction.

It has been reported that glucose utilisation is dependent on available nitrogen, with high levels of nitrogen resulting in the complete removal of glucose from media by *Trametes versicolor* R-105 and *T.harzianum* Th2 (Freitag and Morrell, 1991). It is possible, therefore, that there is a competition for the available glucose in the media and that the effective *Trichoderma* isolates are utilising the glucose before the *S.lacrymans* thereby stressing the *S.lacrymans* and making it more susceptible to the modes of antagonism employed by the lethal *Trichoderma* species.

Fargasova (1992) showed that *T.viride* M-108 produced elevated HERs, conidiation levels and pigmentation when grown on media with elevated nitrogen levels (35 mM) compared to media with lower levels of nitrogen. On analysing the pigments, at least two anthraquinones (1-acetyl-2,4,5,7-tetra-hydroxyanthraquinone and 1,3,6,8-tetra-hydroxyanthraquinone) were detected. Anthraquinones have been shown to be inhibitory towards the basidiomycete fungus *Fomes annosus* (Donnelly and Sheridan, 1986). It is possible that the high nitrogen levels may have caused an increase in conidiation thereby causing an increase in the levels of anthraquinones, which may account for the increased effectiveness of the *Trichoderma* isolates. However, this explanation could not account for the results produced by isolates *Trichoderma* 38 and *T.hamatum* 150 since they did not

produce spores under the test conditions. For these two isolates at least, therefore, another mode of antagonism must have been employed. In any case, this increase in conidiation might not be an important factor in wood since *Trichoderma* species require light to sporulate effectively.

The colour changes of *S.lacrymans* observed during the interactions are consistent with those reported elsewhere (for example, Score *et al*, 1998). The yellow colouration of *S.lacrymans* has been linked to ageing (Cartwright and Findlay, 1958). The brown colouration has not been previously described in *S.lacrymans* and may be linked to the production of melanin or melanin-like compounds (see Chapter 6) and phenoloxidase enzymes (see Chapter 7).

Overall these experiments indicate that *T.harzianum* 25 was the most efficient organism tested for killing *S.lacrymans* on the low nitrogen medium. Its ability to antagonise *S.lacrymans* was compromised by the medium used for the interactions, although the efficacy of the antagonism (measured by speed of killing) was affected by the nitrogen and iron contents. The results for both *T.harzianum* 206040 and *T.viride* 110 seem somewhat paradoxical since both isolates were ineffectual on the LNM-NFe medium but effective on the others. Currently there is no explanation for this apparent anomaly. Much of the work reported in this chapter has previously been reported (Score and Palfreyman, 1994).

Chapter 3

Molecular Identification of *Serpula lacrymans*

3.1 Introduction

Over the past decade the application of molecular biology techniques, for example protein profiling and western blotting, to investigate *S.lacrymans* has revealed a number of characteristics that were not apparent using traditional mycological techniques. For example, some variation between apparently morphologically identical isolates has been found (Palfreyman *et al*, 1991b; Palfreyman and Vigrow, 1992) and differences have also been found in the molecular structure of young and old *S.lacrymans* mycelium (Vigrow *et al*, 1991).

Molecular techniques were extended to include the differentiation of *S.lacrymans* from other wood-inhabiting fungi. However, although *S.lacrymans* could be distinguished from other basidiomycetes in the laboratory, the testing of field samples resulted in ambiguities (Vigrow, 1992). By using the polymerase chain reaction (PCR) specific DNA patterns can be obtained for individual fungal species. Recently, RAPD PCR (random amplification of polymorphic DNA by the polymerase chain reaction) was shown to differentiate between *S.lacrymans* isolates and between *S.lacrymans* and other wood destroying fungi (Theodore *et al*, 1995). However, the work reported in this paper still relied upon the extraction of fungal DNA from pure cultures, although the authors did suggest that PCR amplification and identification of DNA obtained from mycelium *in situ* could be feasible. To date, no attempt has been made to detect the presence of one particular fungus from wood infected by a range of fungal species.

The polymerase chain reaction selectively amplifies a specific region of a DNA molecule (Brown, 1990). The target DNA sequence or at least the sequences that border the region of interest must normally be known. Targeting of the region is achieved by the

use of two short oligonucleotides (primers), of known sequence, that anneal to the DNA molecule. During successive steps of denaturation, primer annealing and amplification, the primers will anneal to the newly made strands and original DNA leading to an exponential amplification of the target DNA sequence. Figure 3.1 illustrates the steps in PCR. RAPD PCR is a modified version of the above system whereby a single, shorter, random oligonucleotide sequence is used. This means that the primer will anneal to a variety of sites in the target DNA. Although these primers are not specific for a particular fungal DNA sequence, they do produce a unique set of bands for the species' DNA used.

The polymerase chain reaction is increasingly being used for inter- and intra-species fungal identification. For example, a PCR-based procedure has been developed to identify different species of *Armillaria* (Harrington and Winfield, 1995) and a RAPD-PCR protocol has been shown to identify between *Verticillium dahliae* isolates with differential pathogenicity on cotton plants (*Gossypium* spp.; Ramsay *et al*, 1996). However, neither of these methods relies upon extracting fungal DNA from infected plant material and to date no protocol has been developed which identifies fungal species from DNA extracted from wood infected with a variety of fungal isolates.

The work reported in this chapter was designed to develop a protocol for identifying fungal species from DNA extracted from infected wood samples. DNA extraction and RAPD-PCR protocols developed for use with fungal species were utilised (see Brown, 1990, and Lee and Taylor, 1990 respectively).

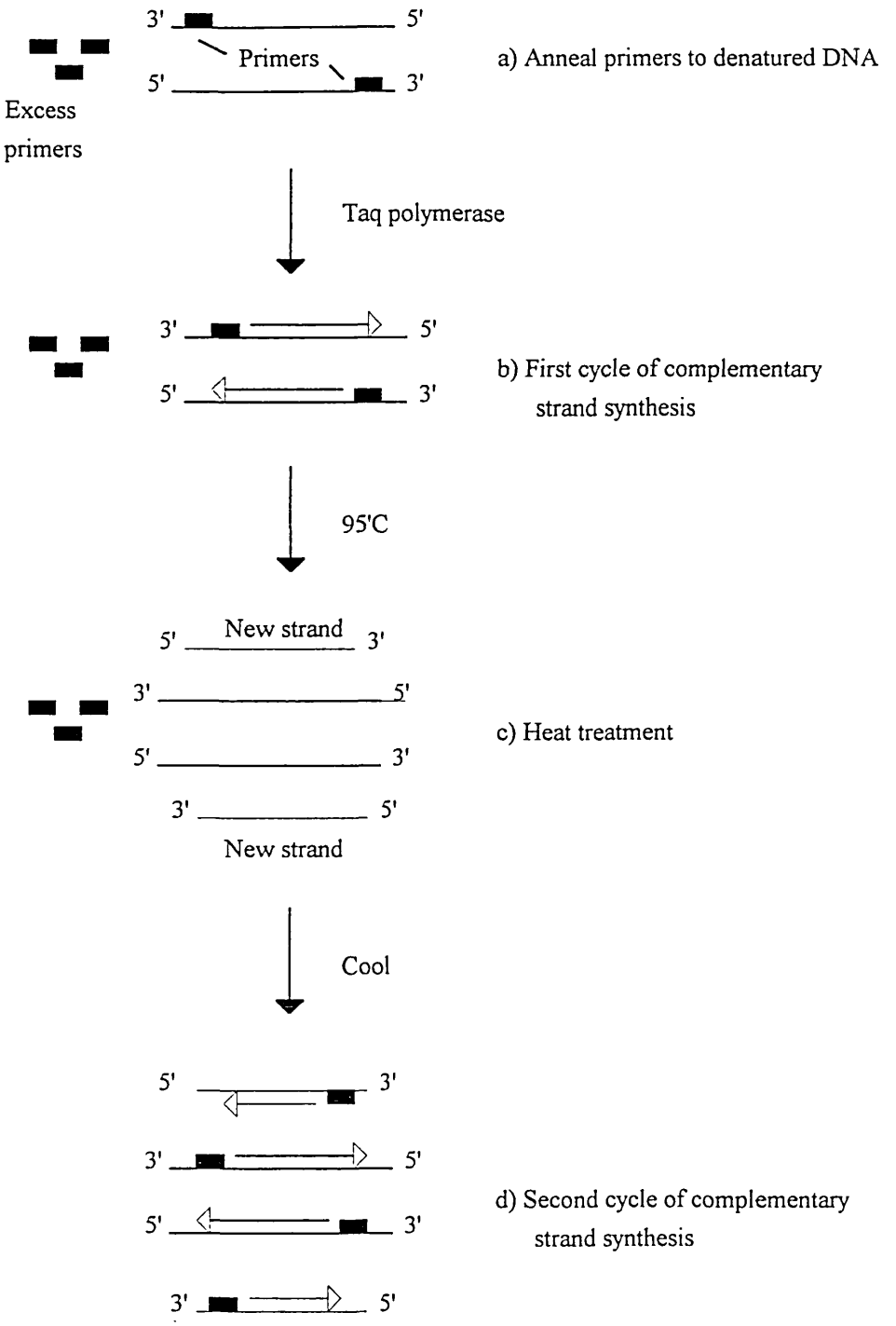


Figure 3.1 : Polymerase Chain Reaction (PCR) (Re-drawn from Brown, 1990)

3.2 Methods

3.2.1 Preparation of samples for DNA extraction

Fungal cultures were grown in liquid malt extract broth (3%) until the surface of the broth was covered by the fungal mycelia. The mycelia were separated from the broth by filtration through a Whatman No.1 filter and then washed with ultra-pure water. Two ml eppendorf tubes were then half-filled with the washed mycelia and stored at -20°C.

Wood samples were ground into sawdust by using a hammer mill. The mill was carefully cleaned between each sample to avoid DNA contamination. Each sample was placed into a self-seal polythene bag, labelled and stored at -20°C until required.

3.2.2 DNA isolation protocol (modified procedure of Lee and Taylor, 1990)

Ground sample (infected wood or frozen mycelia ground in a mortar and pestle) was placed into a 2 ml eppendorf tube up to the 0.5 ml mark and then lysis buffer (800 µl : 50 mM Tris-HCl [pH 7.2], 50 mM EDTA, 3% SDS, 1% β-mercaptoethanol) was added. The tube was dipped into liquid nitrogen and then placed into a water bath set at 65 °C. During the incubation period of 60 minutes, the tube was dipped in liquid nitrogen every 20 minutes to re-freeze the contents. Then phenol:chloroform:isoamylalcohol (24:20:1, 800 µl) was added and the tube vortexed briefly to mix the contents.

After centrifugation at 10,000 *g* for 15 minutes the aqueous phase was removed, in 100 µl aliquots, and placed into a sterile eppendorf tube. An equal amount of chlorofom:isoamylalcohol was added and mixed by vortexing. Once the phases had separated the aqueous phase was placed into a sterile eppendorf tube and 3 M sodium acetate (10 µl or 1/10 of the sample volume) and of isopropanol (0.54 volumes) were added to the tube, with mixing provided by inversion of the tube. After centrifugation at 10,000 *g* for two minutes the supernatant was poured off and the pellet washed once with 70% ethanol and left to dry at room temperature. TE (100 µl : Tris:EDTA; 10 mM Tris-

HCl, 0.1 mM EDTA) was added to the tube which was incubated at 22°C until the DNA had resuspended. The tubes were then stored at -20°C until required.

Prior to the addition of 3% SDS and 1% β-mercaptoethanol, the lysis buffer was autoclaved, as was the TE and 3 M sodium acetate. All eppendorf tubes and pipette tips were autoclaved prior to use.

3.2.3 Determination of the amount and purity of the DNA extracted

To determine the amount of DNA, a 1:100 dilution of the extracted DNA was prepared. The absorbance of this dilution was measured at wavelengths of 260 and 280 nm. At 260 nm an absorbance of 1.0 corresponds to 50 μg of double-stranded DNA (the result was multiplied by 100 to take into account the dilution factor) (Brown, 1990). The ratio of the absorbances at 260 and 280 nm for a pure sample of DNA is 1.8. If this ratio is less than 1.8 then this indicates contamination by protein and/or phenol (Brown, 1990).

3.2.4 Use of fungal DNA in PCR

The PCR protocol used was based on one developed for *S.lacrymans* by Theodore *et. al.* (1995). The preparation of the reaction mixture was carried out in an ultraviolet flow hood. All tubes and pipette tips were autoclaved prior to use. Stock solutions of the primers and nucleotides were prepared using sterile distilled water. The equipment was exposed to U.V. light for at least thirty minutes prior to the preparation of the reaction mixture that consisted of the following :

2.5 μl Reaction buffer [10x] ([1x] buffer : 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 200 mgl^{-1} gelatin in 67 mM Tris-HCl, pH 8.8)

1.1 U Taq polymerase

100 μM Nucleotides

0.2 μM Primer

5.0 µl DNA sample

10.03 µl Sterile water

25.0 µl Immersion oil

Control tubes were prepared which contained all of the above except that water was added to replace the DNA sample.

The PCR cycle consisted of four programs, each linked to each other in sequential order :

Program 1 : 94°C for 4 minutes (denaturation) 1 cycle

Program 2 : 94°C for 1 minute (denaturation) 45 cycles

36°C for 1 minutes (annealing)

72°C for 2 minute (extension)

Program 3 : 72°C for 10 minutes (extension) 1 cycle

Program 4 : 4°C until ready to be used.

3.2.5 The testing of different primers

Five different primers were tested to determine which would give the best DNA profiles for *S.lacrymans*, and these are listed as follows :-

Primer 1 : TGG TCC CTG C

Primer 2 : ACA ACG CCT C

Primer 3 : GGG ACG TTG G

Primer 4 : ACC GCG AAG G

Primer 5 : GGA CCC AAC C

Primers 1-3 were supplied by VHBio and primers 4 and 5 supplied by Operon Technologies Inc..

3.2.6. Visualisation of the PCR products

To visualise the PCR products, the products were run on an agarose gel and then viewed using an ultraviolet light source. 2.0% DNA agarose gels (150 ml) were prepared by dissolving the agarose in TBE (Tris : Boric Acid : EDTA) buffer (5[x] buffer : 54.0 g tris base, 27.5 g boric acid, 20 mls 0.5 M EDTA) which was then poured into the horizontal gel moulds, with the well combs in place. When the agarose was solid the gel moulds were placed into the horizontal buffer tank and 500 mls TBE running buffer (5 [x] TBE buffer diluted to 1 [x] with distilled water) were added with 8.5 µl of each sample being placed into individual wells. Ten samples were run on a single gel, with lanes for *S.lacrymans* and *T.harzianum* 25 DNA, PCR control samples and at least one lane for the molecular weight markers. The gel unit was then connected to the power pack that was set at 143 milliamps (0.8 milliamp per cm^2 of gel) and left for 1.2 hours. The gel was stained with ethidium bromide for 30 minutes after which time it was viewed using an ultraviolet light source. Photographs were taken using a Polaroid camera fitted with an ultraviolet filter to obtain a permanent record.

3.3 Results

3.3.1 Extraction procedure

Initially the extraction procedure gave varying results with the amount and purity of DNA extracted. Although *S.lacrymans* DNA could be isolated from broth grown cultures the purity was poor. For example, one extraction resulted in 970 μg of DNA being obtained but the purity was only 1.25. The cause of the low purity was identified as being the existence of a polysaccharide slime that developed on the outer hyphal surface of the *S.lacrymans* grown in liquid culture. To circumvent this problem, *S.lacrymans* was removed from the surface of a solid agar plate by scraping off the mycelia with a sterile spatula. After extraction, 875 μg of DNA was obtained with a purity of 1.8. Liquid *T.harzianum* 25 cultures provided large amounts of DNA (e.g. 3965 μg) at high purity (1.8).

The extraction procedure was initially modified by the addition of the freeze-thaw technique to maximise the number of fungal cells broken open and by including the chloroform:isoamylalcohol step to ensure that all phenolic substances (particularly in the wood samples) were removed as these could interfere with the amplification of the DNA. The technique was further modified by replacing the vortexing with mixing by tube inversion, which would minimise the shearing of the sample DNA.

3.3.2 Primer evaluation

The results for the individual primers are given in Figure 3.2. From these results Primer No. 3 gave the best banding pattern for *S.lacrymans* and this primer was used in the remainder of the PCR experiments.

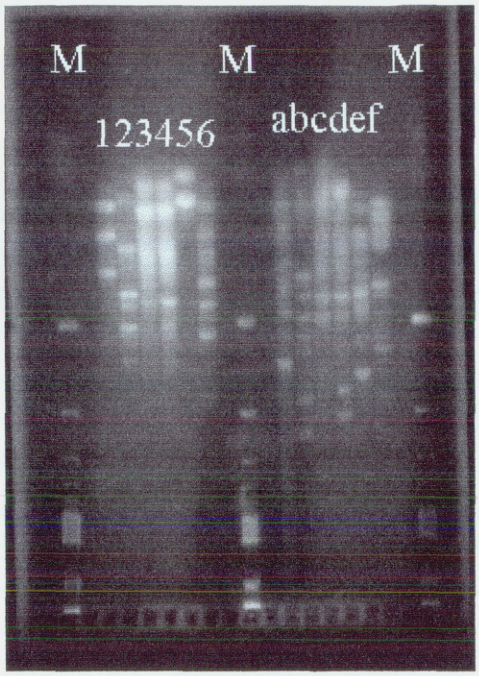


Figure 3.2 : Results using the primers listed in 3.2.5. Key : M-molecular weight markers; 1 - primer 1; 2 - primer 2; 3, 4 - primer 3; 5 - primer 4; 5 - primer 5; a-f represent the no DNA controls for primers 1-5.

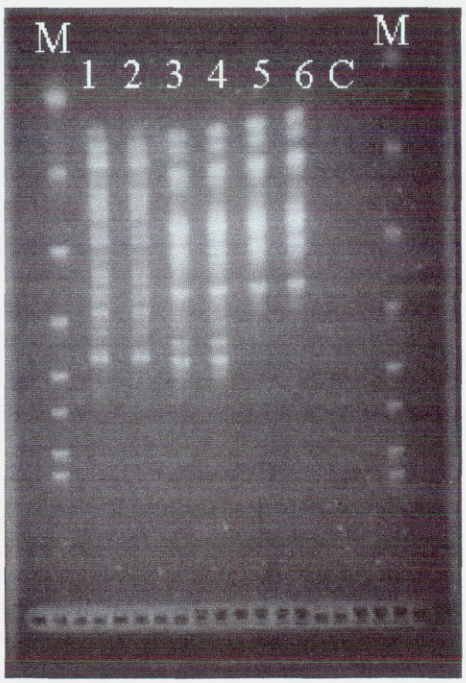


Figure 3.3 : PCR results for *S.lacrymans* and *T.harzianum* 25 DNA. Key : M - molecular weight markers; 1, 2 - *S.lacrymans* DNA; 3-6 - *T.harzianum* 25 DNA; C - control lane (no DNA).

3.3.3 Polymerase chain reaction

A number of modifications were made to the amplification procedure, and these are listed below :-

- the 10 [x] reaction buffer was replaced with the Taq buffer supplied by the manufacturer of the Taq enzyme;
- 0.5 µl of undiluted Taq enzyme (2.5 U) was added to the reaction mixture in place of dilute Taq;
- the reaction volume was increased to 100 µl;
- the nucleotide concentration was increased to 1.25 mM and the primer concentration increased to 20 mM.

In an attempt to identify the contamination source a series of reaction mixtures were prepared replacing each component with a new solution but minus any DNA. A comparison was also made between reaction mixtures composed of the old and new components. The results indicated that the contamination was present irrespective of which component was replaced. Although the water used to dilute the components was autoclaved three times for thirty minutes at 121°C ± 1°C, this was replaced with commercially available HPLC grade water and this eliminated the source of the contamination.

Using the experimental modifications detailed above another PCR was carried out using *S.lacyrmans* and *T.harzianum* 25 DNA, with the result being clear banding patterns for both sets of DNA with no obvious contaminating DNA in the control lanes (Figure 3.3)

3.4 Discussion

Molecular biology techniques have become more popular in biological research for the identification and detection of organisms within different environments. In particular, the PCR technique allows the identification of a particular organism from a small amount of starting DNA. Most systems involving the identification of fungi depend upon the culture of the fungal isolate in the laboratory from which the DNA is extracted. However, it is not always possible to culture an organism within the laboratory and hence the protocol described here provides a technique that could be used to identify an organism from situations where it is not possible to isolate either a specific organism or its DNA.

The polymerase chain reaction is being used more frequently in attempts to differentiate between fungal species and isolates. For example, PCR based methods have been developed to identify *Armillaria* species (Harrington and Wingfield, 1995), *Verticillium dahliae* isolates (Ramsay *et al*, 1996) and *Gaeumannomyces*, *Phialophora* and *Magnaporthe* isolates (Henson, 1992). Methods have also been developed that attempt to attribute genetic markers to pathogenicity (Arisan-Atac *et al*, 1995; Ramsay *et al*, 1996) although the results were not conclusive.

However, the success of the polymerase chain reaction is dependent upon a number of factors (for example, choice of primer, DNA extraction method and contamination source elimination) and each of the papers mentioned in the previous paragraph used different extraction procedures, primers and polymerase chain reaction conditions. The polymerase chain reaction has to be optimised for each procedure and organism used.

From the results it is clear that RAPD-PCR can be used to detect specific fungal DNA in infected wood if appropriate standard DNA can be obtained. Although this technique is not species specific, specific banding patterns are produced for each fungal species. The technique could be made more sensitive by the development of a specific

primer for the fungal species in question. With further research into the biochemistry and origins of *S.lacrymans* via techniques such as PCR the development of more successful control strategies would become easier.

Chapter 4

Wood Interaction Systems

4.1 Introduction

The successful biological control of wood decay fungi has been demonstrated in laboratory conditions by a number of researchers (see Chapter 2). However, the success of the biological control agent was dependent upon the substrate used to carry out the investigations. Although substrates have been developed to mimic the nutritional status of wood these have still led to the incorrect extrapolation of the results to the field situation.

Generally, the quickest and easiest method to determine whether one fungus is antagonistic towards another is to carry out an agar screening experiment. However, the nutrient composition of the agar will affect the final result. Score and Palfreyman (1994; see also chapter 2) reported that nitrogen and iron levels in different media types produced different results from interactions between *S.lacrymans* and *Trichoderma* species. Similar results were described by Score *et al* (1998) for interactions carried out on malt extract agar and sawdust agar. This is most likely caused by the stimulation or inhibition of certain biochemical pathways by nutrients within the media. For example, Srinivasan *et al* (1992a) reported that different media types resulted in different levels of extracellular enzymes (for example, chitinase and laminarinase) and siderophores. Although attempts have been made to develop media that reflect the nutritional status of wood (for example, minimal medium developed by Hutterman and Volger (1973)) this still does not fully reflect the nutritional and environmental status of wood.

A number of wood systems have been developed which investigate the decay qualities of wood rotting fungi and the protective qualities of antagonistic fungi. Wood blocks and wafers of various sizes have been used in conjunction with media and soil to carry out decay and antagonism efficacy tests (for example, Hulme and Shields, 1972;

Takahashi and Nishimoto, 1985; Doi and Yamada, 1991; Morrell, 1991). However, these systems do not permit the development of an interaction as seen on agar plates and they only use weight loss to determine the antagonist effectiveness. This could lead to misleading results as, for example, Palfreyman *et al* (1995) reported that *Trichoderma* spp. could continue the decay of partially brown-rotted wood.

The work reported in this chapter describes the development of small- and medium-scale wood interaction systems for the interaction of *S.lacrymans* with *Trichoderma* spp. These systems should indicate the potential of the biological control agent for preventing the decay of wood by *S.lacrymans*.

4.2 Methods

4.2.1 Small scale wood interaction system

The methodology behind this experiment was based on the system developed by Thornton (1989). 500 ml Duran bottles were filled with 90 g of soil (with the water holding capacity altered to 130%), with a pine sapwood feeder strip (2.5 cm^2) placed onto the soil surface. The bottles were then autoclaved at 121°C ± 1°C for one hour and after cooling, the bottles were inoculated with *S.lacrymans* FPRL 12 C and incubated at 22°C ± 1°C until the fungus had covered the feeder strip. Pine sapwood stick assemblies were constructed using three sticks (80 mm by 5 mm by 5 mm) held together by two pieces of plastic mesh (mesh size 5 mm^2). The assemblies were sterilised as for the bottles and after cooling the assemblies were aseptically placed into each bottle (Figure 4.1). The bottles were then incubated at 22°C ± 1°C until the *S.lacrymans* FPRL 12 C had grown approximately halfway up the sticks.

The partially infected sticks were then aseptically removed, inverted and inserted into fresh bottles, prepared as above but inoculated with an agar plug from a plate of one the following *Trichoderma* species : *T.harzianum* IMI 206040, *T.viride* SIWT 24, *T.harzianum* SIWT 25, *Trichoderma* SIWT 38, *T.viride* SIWT 40, *T.viride* SIWT 60, *T.viride* SIWT 70, *T.viride* SIWT 110, *T.hamatum* SIWT 150 or *T.polysporum* SIWT 200. The jars were then incubated (22°C ± 1°C) until either the *Trichoderma* species killed the *S.lacrymans* FPRL 12 C or the *S.lacrymans* FPRL 12 C reached the soil surface. Control bottles consisted of sticks incubated with *S.lacrymans* FPRL 12 C and transferred to uninoculated bottles and harvested at the end point. The sticks were then weighed to obtain the wet weight and oven dried at 103°C ± 1°C for three hours to obtain the dry weight. The sticks were then cut into two equally sized pieces and each piece was weighed.

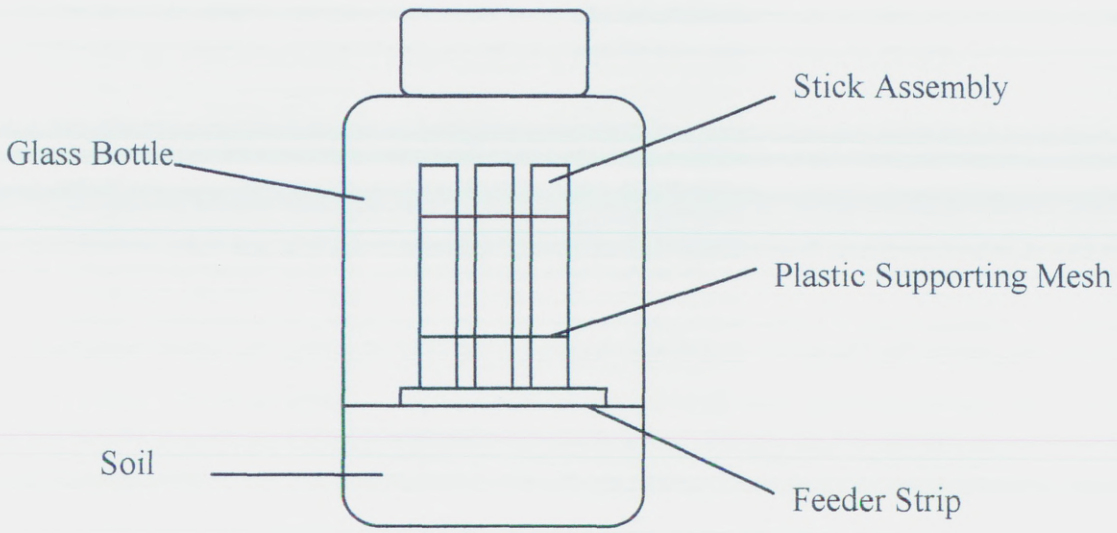


Figure 4.1 : Design of the small scale wood interaction system

Figure 4.2 : Set-up of the medium-scale stake experiment.

4.2.2 Medium-scale wood interaction system

The medium-scale wood interaction system was developed in conjunction with Dr. T. Hutton of Hutton and Rostron Environmental Investigations Limited, Guildford, Surrey and the cellar was located in the H+REI offices in Netley House, Surrey. The only environmental parameter controlled in this system was the humidity. A structure consisting of clay house bricks was constructed by placing the bricks in a 2 x 2 arrangement, such that a brick pile five bricks high was built (Figure 4.2). Pine sapwood stakes (1500 cm x 6 cm x 2 cm) were then inserted through the middle of the brick pile until they rested on the bottom pair of bricks. The whole assembly was situated on a plastic tray to minimise water loss from the bricks. Twenty such constructs, arranged in four rows of five, were prepared. Water was then poured onto the bricks on a daily basis until no more water was absorbed and free water was observed in the trays. The humidity of the room was elevated to >98% initially by the use of a humidifier, however, once this level was achieved it soon stabilised without the use of the humidifier. Air movement was kept to a minimum by reducing the number of times that entry to the room was required to inspect the system.

The stakes were inoculated by using a pine sapwood block (50 mm x 25 mm x 15 mm) infected with *S.lacrymans* FPRL 12 C. The blocks were inserted into the centre of the construct next to the stake on the bottom pair of bricks. The system was then left until the *S.lacrymans* FPRL 12 C had colonised the bricks and colonised approximately the bottom third of the stakes (approximately 3.5-4 months). Although the growth of the *S.lacrymans* FPRL 12 C was sufficient to achieve this, the *S.lacrymans* FPRL 12 C mycelia started to die back on 18 of the stake constructs. It was then decided to proceed with the inoculation of the stakes with the *T.harzianum* SIWT 25.

The inoculation procedure was carried out using two different methods. Spores were collected from 20 agar plates inoculated with *T.harzianum* SIWT 25 by flooding the

plates with sterile distilled water and knocking the spores off into the water with a sterile inoculation loop. The water/spore mixture from each of the plates was then poured into a sterile bottle and stored at 4°C, with the suspension prepared no more than four days before use. By using a haemocytometer, it was determined that the spore suspension contained 1.26×10^8 spores ml^{-1}. The spore suspension was applied to the ends of the stakes using a paint brush until the stakes could absorb no more liquid.

The second method utilised a bran mixture that was infected with *T.harzianum* SIWT 25. The bran mixture consisted of flaked maize (2 litres), perlite (15% v/v) and 400 ml of tap-water. The mixture was prepared in autoclave bags and sterilised by autoclaving at 121°C for 15 minutes on two occasions. The mixture was then placed into 500 ml Duran bottles such that half the bottles were filled. The bottles were inoculated with *T.harzianum* SIWT 25 agar plugs (three per bottle) and then incubated at 22°C ± 1°C for a minimum of 14 days. A pair of holes (5 mm) were drilled through the stakes 5 cm ahead of the *S.lacrymans* FPRL 12 C colony margin, with two more pairs of holes drilled at a further 5 cm distance from the first pair and each other. These holes were then filled with the bran mixture.

The stake constructs were checked every 2-3 weeks to determine whether water should be added to the bricks and photographic records were taken once every 10-12 weeks. After 4.5 and 9 months incubation the moisture content was taken half-way up and at the top of the stakes by using a moisture meter, and at 4.5 weeks only readings were also taken at brick level. After a period of nine months, the stakes were harvested and stored at 4°C until they were divided into 30 segments, each 5 cm in width (Figure 4.3).

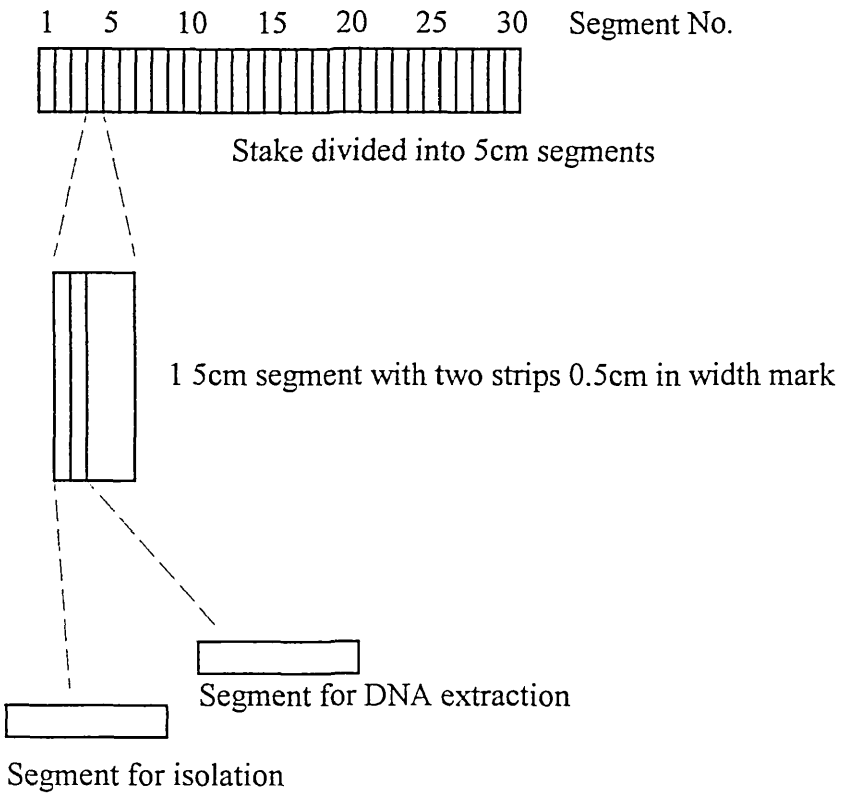


Figure 4.3 : Diagrammatic representation of the division of the stakes for analysis.

From each 5 cm segment a further 2 segments, each 5 mm in width, were removed from one side of the larger segment. One of these segments was used for fungal isolation and the other was stored at -20°C until it could be dried and ground into sawdust. The sawdust was also stored at -20°C until the DNA could be extracted for PCR analysis. A visual determination of the extent of decay of the smaller segments was recorded, with an estimation of the area of decay as a percentage of the surface area of the inner face of the segment being recorded.

4.2.2.1 Fungal Isolation Studies

To attempt to map the spread of both the *S.lacrymans* FPRL 12 C and the *T.harzianum* SIWT 25 through the stakes isolation studies were carried out. For *S.lacrymans* FPRL 12 C, half of one of the smaller segments was plated onto malt extract agar containing 4 p.p.m. benomyl and for *T.harzianum* SIWT 25, the other half of the segment was plated onto malt extract agar containing methyl orange. The plates were checked periodically to determine whether the fungi had been successfully isolated.

4.2.2.2 PCR Studies

In conjunction with the isolation studies DNA analysis by using the polymerase chain reaction was also used to map the spread of the fungi through the stakes. See Chapter 3 for a description of the DNA extraction and PCR procedures.

4.3 Results

4.3.1 Small scale wood interaction system

4.3.1.1 Visual Observations

The *S.lacrymans* FPRL 12 C colonised the stakes rapidly within 24 hours and formed a growth habit typical of that seen in buildings. The length of incubation within the bottles affected the visual appearance of the mycelia as the mycelia became dense and compact after a period of time (approximately 12 days). The *Trichoderma* isolates can be split into two groups depending upon their effectiveness against *S.lacrymans* FPRL 12 C. *T.harzianum* IMI 206040, *T.harzianum* SIWT 25, *T.viride* SIWT 70 and *T.viride* SIWT 110 all colonised the stakes and contacted the *S.lacrymans* periphery just below the mesh. This resulted in some yellow discolouration of the *S.lacrymans* FPRL 12 C periphery that stopped advancing. However, after 48 hours, cords were produced that advanced down the stake over the *Trichoderma* mycelia. Once in contact with the soil, the *S.lacrymans* FPRL 12 C produced mycelia that rapidly colonised the soil.

The second group of *Trichoderma* isolates (*T.harzianum* SIWT 24, *Trichoderma* SIWT 38, *T.viride* SIWT 40, *T.viride* SIWT 60, *T.hamatum* SIWT 150 and *T.polysporum* SIWT 200) colonised the stakes and met the *S.lacrymans* FPRL 12 C periphery just below the mesh. However, the *S.lacrymans* FPRL 12 C continued to advance down the stakes over the *Trichoderma* mycelia. There was little or no yellow discolouration although there was some compaction of the *S.lacrymans* FPRL 12 C periphery. Once the *S.lacrymans* FPRL 12 C reached the soil this was rapidly colonised.

4.3.1.2 Weight Loss Studies

Figure 4.4 indicates the weight losses calculated for each stick half. For those sticks incubated with *T.harzianum* IMI 206040, *T.harzianum* SIWT 25, *Trichoderma* SIWT 38,

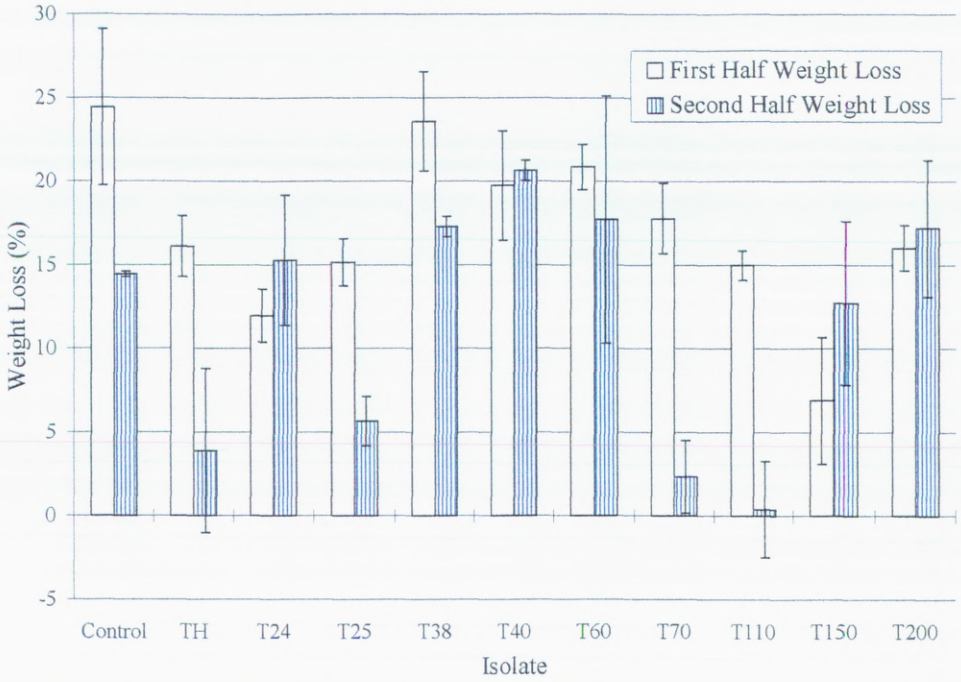


Figure 4.4 : Weight losses for the pine sapwood stakes. Weight losses were by infecting either end of the stakes with *S.lacrymans* and one of the *Trichoderma* isolates. The stakes were incubated until a deadlock or replacement result occurred. 'First half weight loss' refers to the weight loss in the first half of the stake infected with *S.lacrymans* only. 'Second half weight loss' refers to the weight loss in the second half of the stick infected with *S.lacrymans* and the Trichoderma species. The following organisms were used in this study, *S.lacrymans* FPRL 12 C (Control), *T.harzianum* IMI 206040 (TH), *T.viride* SIWT 24 (T24), *T.harzianum* SIWT 25 (T25), *Trichoderma* SIWT 38 (T38), *T.viride* SIWT 40 (T40), *T.viride* SIWT 60 (T60), *T.viride* SIWT 70 (T70), *T.viride* SIWT 110 (T110), *T.hamatum* SIWT 150 (T150) and *T.polysporum* SIWT 200 (T200).

T.viride SIWT 70, *T.viride* SIWT 110 and *S.lacrymans* FPRL 12 C only, the differences in weight loss between the two halves were significant at a 5% level. In comparing the weight losses with regard to infecting organism, those sticks incubated with *T.harzianum* IMI 206040, *T.harzianum* SIWT 25, *T.viride* SIWT 70 and *T.viride* SIWT 110 showed a decrease in the weight loss of the second half of the sticks compared to the weight loss shown by the control sticks. However, those sticks incubated with *Trichoderma* SIWT 38 and *T.viride* SIWT 40 showed a weight loss increase over the control, with the other *Trichoderma* species showing no significant difference in weight loss.

4.3.2 Medium scale wood interaction system

4.3.2.1 Visual observations

The *S.lacrymans* FPRL 12 C colonised the bricks rapidly and was observed on the outer surface of the brick assembly within twelve weeks after inoculation (Figure 4.2). The *S.lacrymans* FPRL 12 C continued to grow on the stakes until it appeared above the level of the bricks by which time the stakes had sufficient moisture to allow colonisation by mould organisms. Initially, the moulds provided some resistance to the spread of the *S.lacrymans* FPRL 12 C as the advancing colony front turned yellow on contact with the moulds. Initially the advance of the *S.lacrymans* FPRL 12 C periphery was halted but after approximately 4-7 days the colony began to advance over the mould growth on the stakes (Figure 4.5)

Figure 4.5 : *S.lacrymans* growing on a medium-scale stake infected with mould organisms.

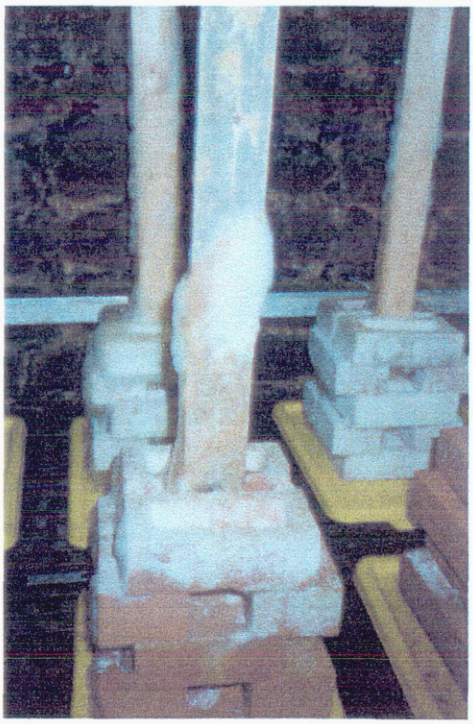


Figure 4.6 : *S.lacrymans* mycelium dying from brick level upwards.

This growth continued but when approximately 30-50% of the stakes were covered the *S.lacrymans* FPRL 12 C appeared to die from brick level upwards on most of the stakes (Figure 4.6). The surface growth on two of the stakes (4 and 19) died completely but on one of the stakes (5) it appeared unaffected. However, by the end of the experiment the *S.lacrymans* had begun to show another flush of growth (Figure 4.7). Other stakes showed the formation of cords where the main colony hyphae had died off (Figure 4.8). The moisture contents of the stakes are shown in Table 4.1. The moisture content of the stakes at brick level varied between 23% and 100%, with those stakes showing signs of decay having the higher moisture contents (see Table 4.2). The moisture contents of the stakes levelled off around 22-25% approximately half-way up the stakes. Upon closer inspection of the stakes strands were seen where the *S.lacrymans* FPRL 12 C had largely died off. The strands were seen above the level that the surface mycelia had reached and had grown over the wet areas of the stakes.

The growth of the *S.lacrymans* FPRL 12 C was enhanced by the presence of the bran mixture even when it was inoculated with *T.harzianum* SIWT 25. The *S.lacrymans* FPRL 12 C did not show any of the colour changes normally associated with the presence of *T.harzianum* SIWT 25 (see Chapter 2), however, on two of the stakes (4 and 19) the growth of the *S.lacrymans* FPRL 12 C was inhibited over part of the stake where the bran/*T.harzianum* SIWT 25 mixture was placed. There was no obvious visual evidence for the colonisation of the stakes by the *T.harzianum* SIWT 25 with the exception of the *S.lacrymans* FPRL 12 C growth inhibition.

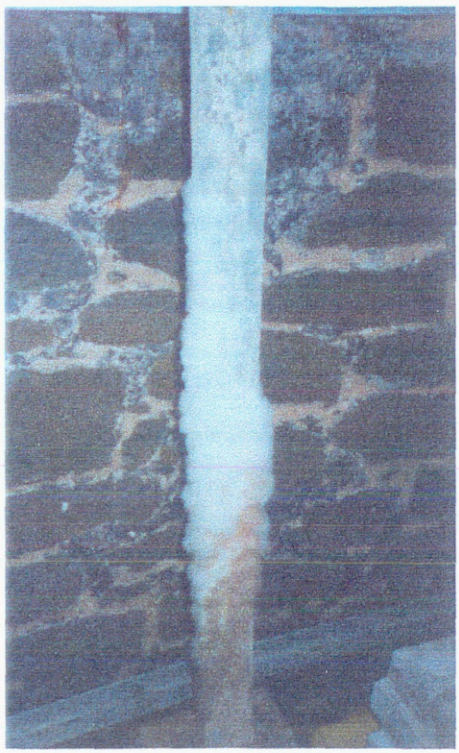


Figure 4.7 : *S.lacrymans* showing signs of regrowth.

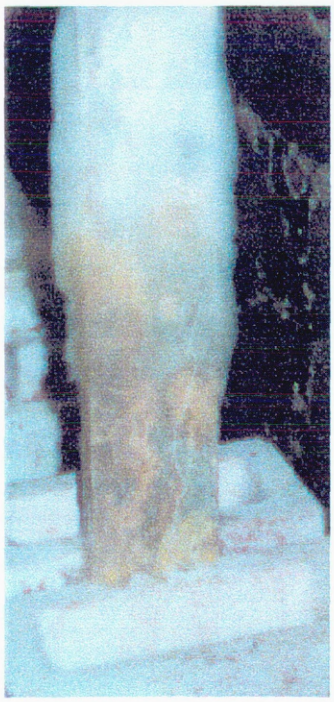


Figure 4.8 : Formation of cords by *S.lacrymans*.

Stake No.	Moisture Readings					
	Site 1		Site 2		Site 3	
	4.5 wks	9 wks	4.5 wks	9 wks	4.5 wks	9 wks
1	50	*	24	40	23	24
2	25	*	24	28	23	24
3	24	*	24	24	21	24
4	50	*	24	40	21	23
5	24	*	22	22	22	22
6	50	*	22	28	22	21
7	80	*	22	80	21	23
8	60	*	24	24	24	24
9	27	*	22	30	21	24
10	40	*	23	25	27	28
11	24	*	24	22	25	25
12	23	*	22	24	24	24
13	100	*	24	40	25	25
14	100	*	24	40	23	24
15	30	*	22	24	22	22
16	50	*	23	23	24	23
17	24	*	24	24	24	24
18	24	*	24	24	24	24
19	24	*	25	25	27	26
20	25	*	28	30	25	27

Table 4.1 : Moisture readings (%) of the stakes at 4.5 and 9 weeks. Site 1 was at brick level, Site 2 was half-way up the stakes and Site 3 was at the top of the stakes.

* - indicates no result.

	Treatment											
	Bran + *T.harzianum*				Bran Only				*T.harzianum* spores		Water	
Section	1	4	16	19	6	15	17	18	8	20	7	9
1	100	100	50	30	100	100	80	80	50	0	0	80
2	75	65	100	10	100	Wet	75	75	40	5	0	30
3	60	50	100	20	100	Wet	60	50	40	10	0	30
4	60	100	75	10	100	10	50	75	40	5	0	40
5	80	100	90	20	100	Wet	25	40	50	5	0	30
6	50	100	-	20	100	40	50	40	30	5	0	20
7	50	100	30	25	100	30	40	75	20	5	0	20
8	50	80	30	25	90	20	75	75	10	5	0	20
9	80	80	40	25	90	30	60	80	0	5	0	30
10	80	90	45	25	100	33	50	70	0	5	0	25
11	90	-	45	25	90	30	50	75	0	5	0	40
12	75	75	0	25	75	10	40	60	0	10	0	30
13	0	75	0	10	75	5	**40**	75	0	10	0	30
14	0	30	0	**0**	60	5	**40**	25	0	5	0	40
15	0	50	**0**	**0**	45	0	**20**	33	0	0	10	30
16	**0**	70	**0**	**0**	**30**	**0**	-	25	0	5	0	5
17	**0**	70	**0**	0	**5**	**0**	20	20	0	0	0	0
18	**0**	60	0	0	**0**	**0**	0	**0**	0	0	0	0
19	0	0	0	0	0	0	0	**0**	0	0	0	0
20	0	**0**	0	0	0	0	0	**0**	0	0	0	0
21	0	**0**	0	0	0	0	0	0	0	0	0	0
22	0	-	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	0	0	0

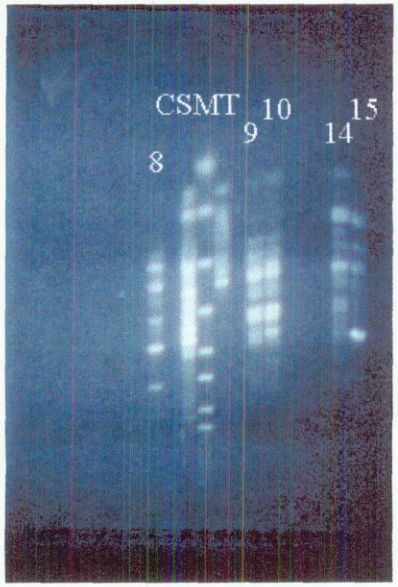
Table 4.2 : Visual decay (%) estimation of each stake segment. Those values underlined and in bold indicate to which segments the bran mixture was applied. 'Wet' indicates that no decay was observed in this segment and that the segment contained a high moisture content. '-' indicates no sample taken.

4.3.2.2 Isolation Studies

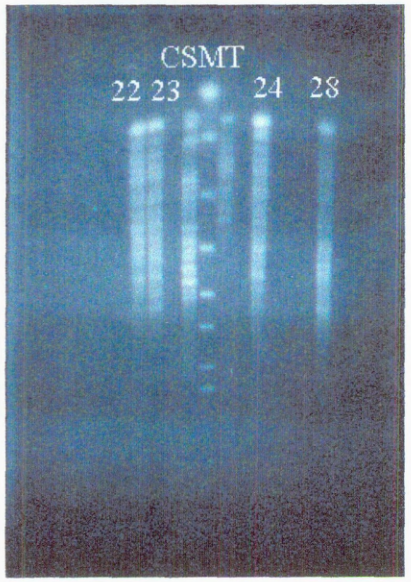
The isolation studies proved inconclusive regarding the viability of the *S.lacrymans* and the spread of the *T.harzianum* 25. Isolation media for *S.lacrymans* (malt extract agar with benomyl) which contained benomyl up to 50 p.p.m. was not sufficient to stop all the mould organisms from growing thus preventing the *S.lacrymans* from growing. *T.harzianum* 25 could not be positively isolated as there were other *Trichoderma* spp. present in the stakes. From the isolation studies attempted it was noted that there were several *Trichoderma* species present as well as *Mucor* and *Penicillium* species.

4.3.2.3 PCR Studies

Some results were obtained which may indicate the spread of the *S.lacrymans* through some of the stakes. For example, in stake 19 (Figure 4.12) samples23-29 have bands that match those in the *S.lacrymans* controls. Evidence for the spread of *T.harzianum* 25 within the stakes is somewhat less clear although matching bands were found in samples 17-22 from stake 19 (Figure 4.11). Overall, the PCR results are not clear it is difficult to discern any spread of either the *S.lacrymans* or *T.harzianum* 25 through the planks. The PCR procedure needs to be further optimised for these samples as at present unknown experimental factors lead to smearing of the DNA (for example, Figure 4.10b) or block the DNA replication process (for example, Figures 4.9 and 4.10a).

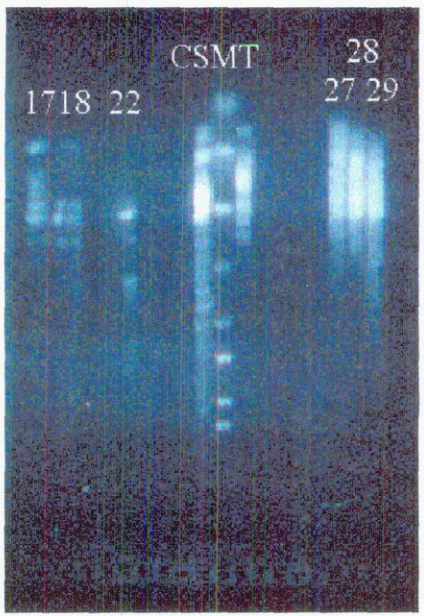


(a)

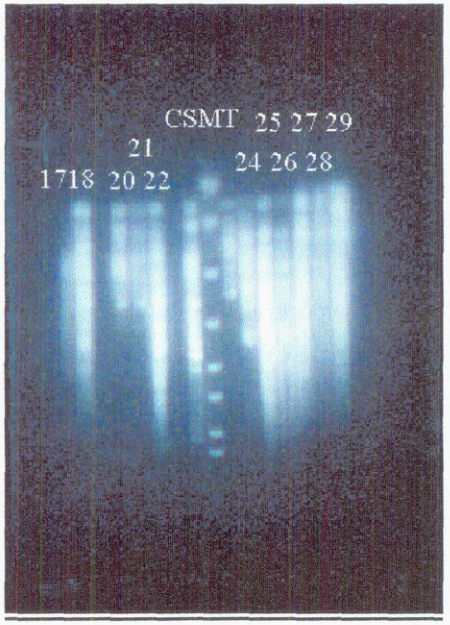


(b)

Figure 4.9 : PCR results for Stake 17. Key : C - control lanes (no DNA); S - *S.lacrymans* DNA; M - molecular weight markers; T - *T.harzianum* 25 DNA; numbers correspond to the stake sample.



(a)



(b)

Figure 4.10 : PCR results for Stake 18 (a) and Stake 4 (b). Key as for Figure 4.8.

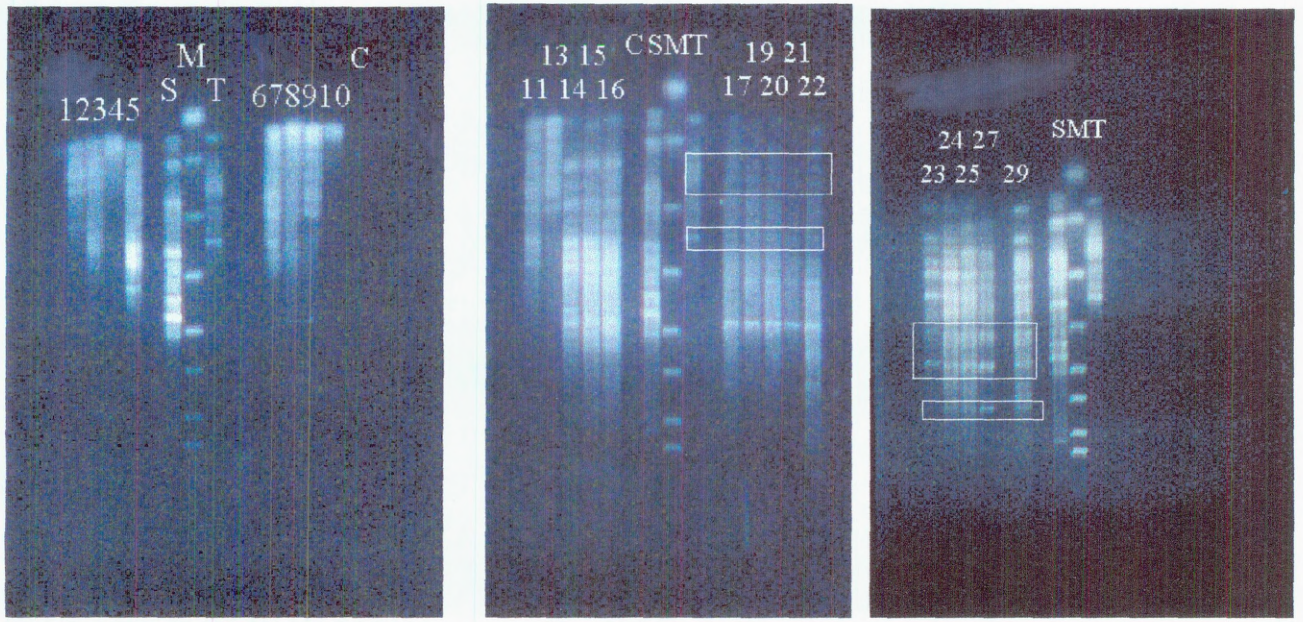


Figure 4.11 : PCR results for Stake 19. Figure as for Figure 4.8.

4.4 Discussion

The use of biological control in timber preservation has been proposed by a number of authors. However, despite successes in the laboratory the transfer of the potential control agent to the field situation has led to the discovery of inadequacies in the use of the control agent, for example, incomplete colonisation of the timber to be protected or control efficacy decrease over time. The experiments detailed in this chapter were designed to determine whether *Trichoderma* spp. could control the decay of timber caused by *S.lacrymans* initially in a small-scale laboratory-based system and then in a medium-scale system in a non-sterile, semi-controlled environment.

Both experimental systems showed that the *Trichoderma* isolates used could not inhibit the spread of the *S.lacrymans*. However, certain *Trichoderma* isolates could enforce a morphological change, i.e. the production of cords, upon the *S.lacrymans* in order for it to continue to advance. For example, confrontation between *S.lacrymans* and *T.harzianum* 25 in the small stakes led to the stalling of the ordinary colony hyphae including colour changes in the *S.lacrymans* colony periphery indicative of antagonism (Score *et al.*, 1998). After a short period of time the *S.lacrymans* produced cords that advanced over the Trichoderma infected portion of the stakes and down into the soil. This action would seem to suggest that the production of cords was due to the deadlock between the two colonies. Score *et al.* (1998) reported that *S.lacrymans* produced cords against *T.reesei* 192656 during agar interactions, apparently induced by the formation of a deadlock between the colonies. Further evidence for this hypothesis is found in the medium-scale results where the advance of *S.lacrymans* was inhibited over areas where the *T.harzianum* had colonised the stakes. However, no cords were produced because the *T.harzianum* had not fully colonised the breadth of the stake thus leaving part of the stake unprotected allowing the *S.lacrymans* colony to grow past the treated area.

Prior to application of the *Trichoderma* in the medium-scale experiment, cord formation by the *S.lacrymans* was observed near or at the top of the stakes, even though the nearest visible appearance of the *S.lacrymans* colony was still only half-way up the stakes. This would seem to indicate that the *S.lacrymans* was spreading up through the centre of the stakes. Cords were also evident where the surface *S.lacrymans* growth had apparently died back which could indicate that the cords, in this particular instance, were produced in response to an unfavourable environmental condition.

S.lacrymans cords, which may have roles in exploration, exploitation and survival of the fungus (Rizzo *et al.*, 1992), are composed of a main leader hypha surrounded by its own branches and thin tendril hyphae (Cooke and Whipps, 1993). Autolysis and differentiation of these hyphae results in a series of tubes surrounded by fibrous material embedded in a non-cellular matrix. The formation of cords allows the *S.lacrymans* to advance over areas that are nutritionally deficient in search of new food sources. One function attributed to strand formation is in the conservation of nitrogen in the mycelium (Jennings and Watkinson, 1982; Coggins, 1976). Mycelium between the strands breaks down and the breakdown products, in particular nitrogen, are re-utilised by the growing hyphae (Coggins, 1976). A very high carbon-nitrogen ratio (such as that exists in wood) may favour the production of strands as they then become an important nitrogen source (Jennings and Watkinson, 1982). The presence of such fibrous material and/or the non-cellular matrix may prevent hydrolytic enzymes, such as chitinase and laminarinase considered to be a significant mode of antagonism of *Trichoderma* species against wood decay basidiomycetes (Srinivasan *et al.*, 1992b), from reaching their targets in the cell wall of *S.lacrymans*.

Another possibility is that the cords contain some unknown compound that protects the cords against attack by the *Trichoderma*. Rhizomorphs of *Armillaria* spp., which are

essentially more complex versions of strands, are known to incorporate melanin into their structure (Rizzo *et al*, 1992) which protects them from environmental stresses and antagonistic attack by hydrolytic enzymes. Therefore, the *S.lacrymans* cords may contain melanin or melanin-like compounds that protect them from the *Trichoderma* isolates. The melanin may then be incorporated into the mycelia that is produced when the cords reach the soil in the small-scale system thus enabling the *S.lacrymans* to colonise the soil despite the presence of the *Trichoderma*. However, it has been reported by Doi and Yamada (1991, 1992) that *Trichoderma* control of *S.lacrymans* in autoclaved soil was ineffective.

S.lacrymans cords can be up to 4 metres in length (Nuss *et al*, 1991) although it is not known whether cord formation would remain unaffected by the presence of the *Trichoderma*, however, it does indicate that *Trichoderma* treatment alone is unlikely to be sufficient to inactivate the basidiomycete in the field.

Despite the continued spread of the *S.lacrymans*, certain *Trichoderma* isolates did prevent the decay of the stakes (both small- and medium-scale). This may be due to the *S.lacrymans* being unable to colonise the interior of the *Trichoderma* colonised portion of the stakes. This does not appear to be the case at least in the medium-scale stake experiment as *S.lacrymans* cords appeared some distance from the colony periphery without any obvious surface connection. However, it is also possible that the *S.lacrymans* was able to colonise the interior portion of the stake but the presence of the *Trichoderma* spp. may have prevented the initial depolymerization of the cellulose. *S.lacrymans* is thought to initiate cellulose depolymerization in the cell wall by the production of a non-enzymatic H_2O_2/Fe^{2+} system (Koenigs, 1974; Murmanis *et al.*, 1988b). However, *Trichoderma* spp. have been shown to produce siderophores that sequester iron (Srinivasan *et al.*, 1992b; Anke *et al.*, 1991) and Srinivasan *et al.* (1995) showed that siderophore

production by *Trichoderma* isolates was significant in the inhibition of wood decay fungi. If these siderophores can remove iron from the wood before the *S.lacrymans* can obtain it then the decay process could be halted. Certain siderophores have also been shown to be potent antibiotics (Leong, 1986) and can inhibit the growth of certain fungi and bacteria (Anke *et al.*, 1991), and this may also account for some of the inhibition of the *S.lacrymans*.

The results from the percentage decay estimation (Table 4.2) of the medium-scale stake segments do not seem to show an obvious trend. Three of the stakes (1, 4, 16) treated with the *T.harzianum* 25 bran mixture seem to show a sudden reduction in decay close to the application point of the control agent. The other stake (19) shows no decay at the application point or beyond. The stakes treated with the uninoculated bran generally only show a continuation of decay up to, including and beyond the application point. This would suggest that the *T.harzianum* 25 was providing some antagonism towards the *S.lacrymans*. Whether this antagonism was due to the spread of the *T.harzianum* into the stakes (evidence for which may be shown by certain of the PCR results for stake 19) or merely due to its presence within the bran is conjecture at this time. It is also possible that antagonistic substances released by the *T.harzianum* 25 during its growth within the bran may have leached out into the surrounding stake, although this is only likely to have an effect within the immediate area of the application point.

The moisture content readings of the stakes indicate that the humidity maintained the moisture content at around 20-30%. Although no decay measurements were taken at the same time as the moisture contents, visual observations were made which recorded that stakes 1, 4, 6, 13 and 15-19 all had visual signs of decay at brick level (sections 6-7 of the stakes). Although half of the stakes had high moisture contents in the segments with high decay levels, the others had moisture contents near to the 20-30% level, and no pattern

could be determined. *S.lacrymans* has an optimum moisture content range of 30-40%, although it has been reported that *S.lacrymans* can decay wood that has a moisture content of 100% (Schmidt and Moreth-Kebernik, 1990).

The apparent dying back of the *S.lacrymans* was unexpected although may have been caused by air currents produced by the opening and closing of the cellar door. The *S.lacrymans* growing on the stake immediately behind the door grew as normal. It is known that *S.lacrymans* requires limited air-movement (Low *et al*, 1997) and it is likely that the air currents adversely affected the growth of the fungus on the stake surface but as the decay of the stakes did not show any obvious interruptions then the adverse effect was minimal.

The PCR results although not conclusive do seem to indicate that the *T.harzianum* 25 did spread from the bran mixture to the stakes even if only in the immediate area of the application point. If this is the case then the effects of the *Trichoderma* presence upon the *S.lacrymans* does seem to have been substantial with the rapid reduction in decay even some distance from the application point. *Trichoderma* spp. have been shown to act via a number of mechanisms, for example, production of antifungal metabolites (e.g. harzianolide (Avent *et al.* 1992)), volatiles (e.g. sesquiterpenes (Claydon *et al.* 1987)), hydrolytic enzymes (e.g. chitinase and laminarinase (Srinivasan *et al.* 1992b)) and siderophores (Anke *et al.* 1991; Srinivasan *et al.* 1995). The application of the *T.harzianum* 25 by way of a spore suspension does not appear to have been successful even though one of the stakes showed (stake number 20) very little decay. This lack of decay seems to have been linked to the inability of the *S.lacrymans* to decay the wood (since no decay was detected in those segments beneath brick level and prior to *Trichoderma* application) rather than the presence of the *Trichoderma.* The PCR results also seem to indicate the presence of *Trichoderma* in stake 17, which was not treated with

the *Trichoderma* control agent. Either the control agent spread via spores to the stake or another *T.harzianum* species was detected by the PCR system. Arisan-Atac *et al* (1995) reported that *Trichoderma* isolates identified as the same species, for example, *T.harzianum*, although given a different classification number often gave identical RAPD-PCR fingerprints.

Overall, the results indicate that the *Trichoderma* treatment will only offer protection if the fungus completely colonises the wood prior to contact with *S.lacrymans*. If colonisation is incomplete then the *S.lacrymans* can grow around the treated area without the formation of specialised exploration organs. If the colonisation is complete then the *S.lacrymans* can still counteract the presence of the *Trichoderma* by producing cords which then advance over the treated area. If the treated area is large enough then it may be possible that the cords will eventually succumb to the *Trichoderma*. More research will be required to determine if a more suitable application method can be devised for the *Trichoderma* to allow it to more successfully colonise the wood to be protected.

Chapter 5

The Biological Control of *S.lacrymans* by *Trichoderma* spp. in a Field Trial

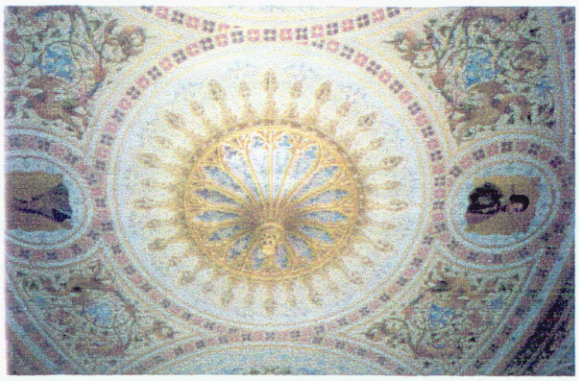
5.1 Introduction

Studies reported in this thesis have shown that whereas *S.lacrymans* can be effectively controlled by certain *Trichoderma* species on agar-based systems the control on wood substrates was less effective. Whilst the *Trichoderma* species could not prevent the spread of the *S.lacrymans* their presence did interrupt the decay of the wood by the basidiomycete. However none of the systems used could be said to be truly reflective of the situation in which the control agent would be used. Therefore, a field site was chosen to test the effectiveness of the *Trichoderma* control agent against *S.lacrymans* growing within a building.

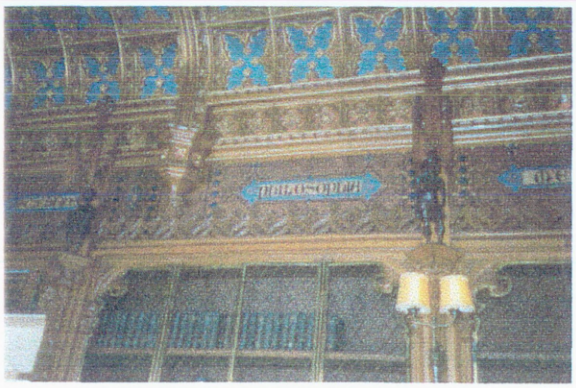
Previous studies have shown that the transfer of biocontrol agents from the laboratory to the field has been less than satisfactory. Bruce and King (1986b) showed that although the control of *N.lepideus* by *Trichoderma* sp. in laboratory-based systems was successful this control was limited in the field situation due to incomplete colonisation of the wood by the *Trichoderma*. Benko (1989) observed a similar result where the effective control of blue stain fungi by *P.cepacia* decreased over time in the field.

The field trial was based at Taymouth Castle, situated at Kenmore, which is located on the Eastern end of Loch Tay. Figure 5.1 (a, b and c) shows the artwork and woodwork that is threatened by moisture ingress and dry rot attack. Dry rot is noticeable in many parts of the castle but the east wing is arguably the most visibly damaged. The test site was situated in room G114 that was located on the ground floor of the east wing. Dry rot has

entered the room from the adjacent corridor (which had collapsed) and a site of active growth was evident along the west wall. The field trial was designed to determine whether the dry rot infection of the wooden floor of room G114 could be successfully treated using a *Trichoderma* sp..



(a)



(b)

(c)

Figure 5.1 :Taymouth Castle field site. (a) Ceiling of the library; (b)Woodwork and paint work of the library; (c) Paint and gilt work on the ceiling in one of the reception rooms.

5.2 Methods

Figure 5.2 represents a plan of room G114. The floor was originally covered by linoleum, however, this was removed to reveal the surface of the wooden planks. Using the positions of the joists under the floor, the floor was divided into ten lanes (A-J) with lane A nearest the window and lane J next to the door. Lines were then drawn between the north and south walls, after dividing these walls into equal divisions (ten in all), which resulted in the formation of a grid (10 by 10) of squares of dimensions approximately 47 cm by 36 m. These squares were further sub-divided into 12 squares (segments) and each was given a number between 1 and 24. Lane J was ultimately not used in the experiment as the lane was half the size of the others, therefore, the experimental area was a 10 by 9 grid. The walls were also subdivided to a height of two metres, depending on the division of the floor.

The positions of the treatment and control lanes were determined by the position of the *S.lacrymans* colony on the surface of the floor. The colony was effectively split into two, such that half the underside of the floor covered by the colony would be treated and half was not. The two lanes nearest the window (lanes A and B) were not colonised by *S.lacrymans*, so lane A was treated and Lane B was left as a control. Therefore, lanes A, E, F and G were treatment lanes, and lanes B, C, D, H and I were control lanes. To maintain the humidity at floor level, plastic sheeting was laid on the floor and secured.

The first treatment to be tested was a spore suspension of *T.harzianum* 25 (1.25 x 10^6 spores per ml) that was prepared in the same manner as that prepared for the medium-scale stake experiment. Two holes were drilled in each lane equidistant from each

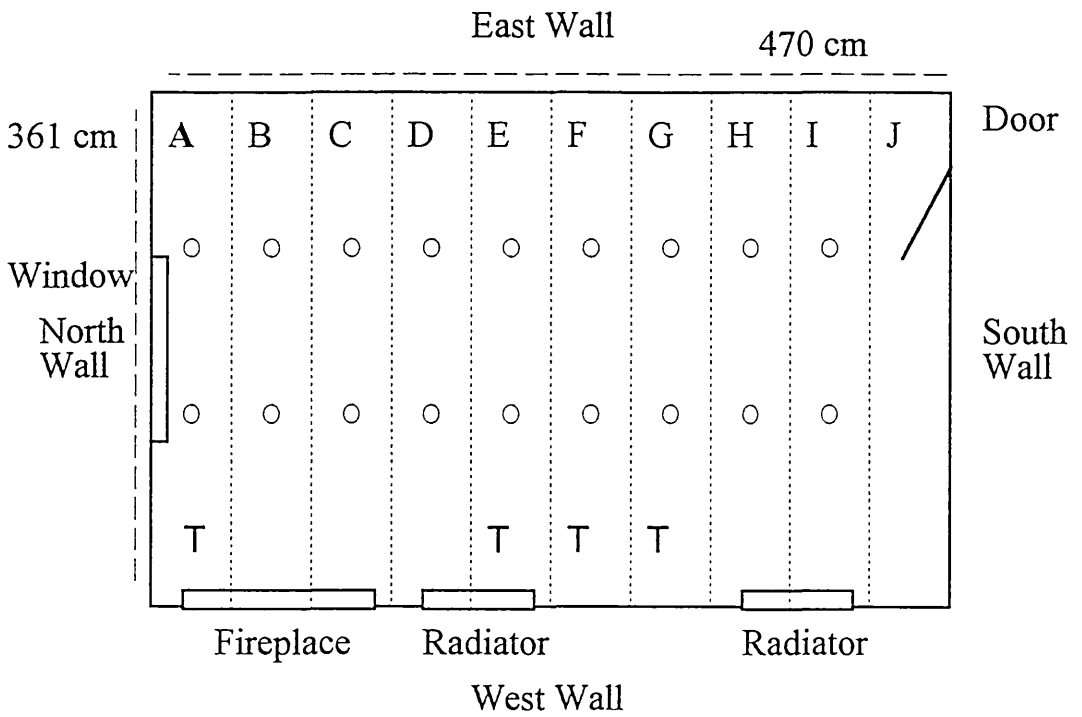


Figure 5.2 : Diagram of the room at Taymouth Castle where the field trial took place. Key : Letters A-J refer to floor lane divisions; T refers to lanes treated with *T.harzianum* 25; the small circles refer to the approximate positions of the treatment holes cut in the floor.

other and the east/west walls. By using a pressurised garden sprayer the suspension was sprayed onto the underside of the floor around the holes drilled in the floor in the treatment lanes.

After 5 months it became clear that the *T.harzianum* 25 spores had not germinated and colonised the floor and another treatment method was carried out. Four litres of treatment fluid were prepared by incubating *T.harzianum* 25 in flasks containing 3% malt extract broth for 10 days, after which time the contents of the flasks were macerated in a blender for 15 seconds. Around the treatment holes, 800 mls of sterile distilled water was applied to the underside of the floor to raise the moisture content. Then 800 mls of the treatment fluid was applied to the underside of each lane by using the pressurised garden sprayer, with the vapouriser removed from the spray head. The control lanes were treated with water and sterile malt extract broth. By plating out an appropriate dilution of the treatment fluid it was determined that the fluid contained 1.26×10^8 colony forming units per ml at the first treatment and 1.3×10^8 at the second. This treatment was applied twice, the first application on 24/6/94 and the second on 14/12/94.

Over the period of the experiment, the moisture content of the floor and walls (from each division), and the room temperature and air humidity were taken at every visit to the field site (every 4-8 weeks over a 68 week period). Notes were taken on the extent of spread of the dry rot colony, both on the surface of the floor and its underside (with the aid of a boroscope). To determine the spread of both fungi through the floor over the experiment two samples were taken from each floor segment (randomly selected) and plated onto selective media (malt extract agar plus 4 p.p.m. benomyl for isolation of *S.lacrymans* and malt extract agar plus methyl orange for *T.harzianum* 25). Unfortunately,

it soon became clear that the isolation media would not suppress the growth of *Mucor* spp. that covered the agar before the *S.lacrymans* or *T.harzianum* 25 had a chance to grow. Sampling of the floor was abandoned and it was decided to use the polymerase chain reaction to map the spread of the fungi through the floor by taking samples at the end of the experiment. The holes left in the floor after sampling were plugged with pine sapwood dowels that had been sterilised by autoclaving at 121°C ± 1°C for 30 minutes. In time the *S.lacrymans* grew up through the dowels onto the surface of the floor (Figure 5.3). Table 5.1 shows the timing of the field trial from January 1994 to May 1995.

At the termination of the experiment, each lane was individually removed from the floor and inspected. Samples (width of the lane by the width of the plank) were taken by removing planks from each of the lane divisions, giving a maximum of ten samples per lane. A 5 mm strip of wood was removed from the centre of each sample and ground into sawdust. The fungal DNA from these samples were removed and used in the Polymerase Chain Reaction to determine the presence of *S.lacrymans* and *T.harzianum* 25 in each of the sections and therefore determine their relative spread through the floor.

1994												1995				
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
1,S	S	S	S	S	2						3					U

Table 5.1 : Timing of the field trial over the 17 month period from January 1994 to May 1995. Key : 1 - Application of *T.harzianum* 25 spore suspension; 2 - First application of *T.harzianum* 25 treatment fluid ; 3 - Second application of *T.harzianum* 25 treatment fluid; S - Core samples removed from floor; U - Termination of experiment and uplift of floor.

Figure 5.3 : Growth of *S.lacrymans* through the dowels from the underside of the floor.

5.3 Results

5.3.1 Visual Observations

Figures 5.4 (a, b) indicate the extent of the *S.lacrymans* colony and visual decay of the floor prior to the start of the experiment. Note the *S.lacrymans* fruit-bodies evident at the skirting-board and spore dust lying on top of the skirting board and electrical socket in Figure 5.4 (a). Figure 5.5 (a, b) show the extent of the colony and visual decay at the termination of the experiment. Note the spore dust that is evident over the floor, radiator and pipes in Figure 5.5 (b). Six months after the start of the experiment the *S.lacrymans* colony began to show obvious signs of growth on the surface of the floor. Decay of the floor also continued with collapse of some of the floorboards evident (Figure 5.6). Observations of the growth indicated that on the surface of the floor the fungus continued to advance over the floor despite the changes in temperature, the periodical removal of the plastic sheeting and the accidental flooding of the room. Figure 5.7 shows the formation of moisture on the plastic sheeting, which shows that the humidity was maintained to a reasonably high level which maintained the growth of the *S.lacrymans*.

Changes in mycelial pigmentation were observed, for example, lilac and yellow pigmentation associated with normal *S.lacrymans* growth. Brown pigmentation was also observed at certain points of the colony edge where the fungus had come into contact with other fungal organisms, such as *Penicillium* and *Aspergillus* species that have been isolated from the floor. Figure 5.8 shows the formation of cords and brown pigmentation (note the brown liquid droplets). It also shows the presence of a sporulating fungal organism that may be responsible for causing the brown colouration in the *S.lacrymans* colony.

(a)

(b)

Figure 5.4 : Room at the beginning of the experiment. (a) Initial extent of *S.lacrymans* colony on floor surface. (b) Initial extent of damage to floor.

(a)

(b)

Figure 5.5 : Room at the end of the experiment. (a) Final extent of *S.lacrymans* colony. (b) Final extent of damage to the floor

Figure 5.6 : Colony extent and floor damage six months from start of experiment.

Figure 5.7 : Growth of *S.lacrymans* under the plastic sheeting. Note formation of moisture on the underside of the sheet.

Figure 5.9 shows the interaction between *S.lacrymans* and another fungus present within the floor. The colony periphery is showing pigmentation changes indicative of an antagonistic reaction. The hole shown in the picture is one of the holes drilled for treatment application and shows the dispersal of *S.lacrymans* spores released by fruiting bodies formed beneath the floor. It should also be noted that the floor at the colony periphery is moist. Figure 5.10 shows the colonisation of the *S.lacrymans* fruiting bodies, growing at the skirting- board, by another fungus possibly *Penicillium* spp.. Towards the end of the trial (during 1995) the colony appeared to stop advancing in the treatment lanes but continued to grow in the control lanes. Figure 5.11 is a representation of the visually assessed growth of the *S.lacrymans* colony on the floor surface during the period of the field trial.

On the underside of the floor, there tended to be more visible changes in the morphology of the *S.lacrymans* colony. In the control lanes, there was a tendency for more fluffy mycelia to be present during the summer months compared to the winter months. The spore treatment produced no obvious effects upon the *S.lacrymans* growth, although there was some yellowing of the *S.lacrymans* mycelia around the treatment holes. In the treatment lanes, after the first macerated hyphae treatment, there was an increase in the number and size of fruiting bodies formed by the mycelia (Lanes C, D, E, F and G). A green coloured fungus was observed growing on the underside of the floor around the treatment holes in Lanes A and F. Pigmentation changes associated with antagonism were also noted at the colony periphery in Lane G. It was only after the second macerated hyphae treatment that the *S.lacrymans* stopped advancing in the treatment lanes and the colony edge collapsed and changed colour (yellow/brown). Four months after the

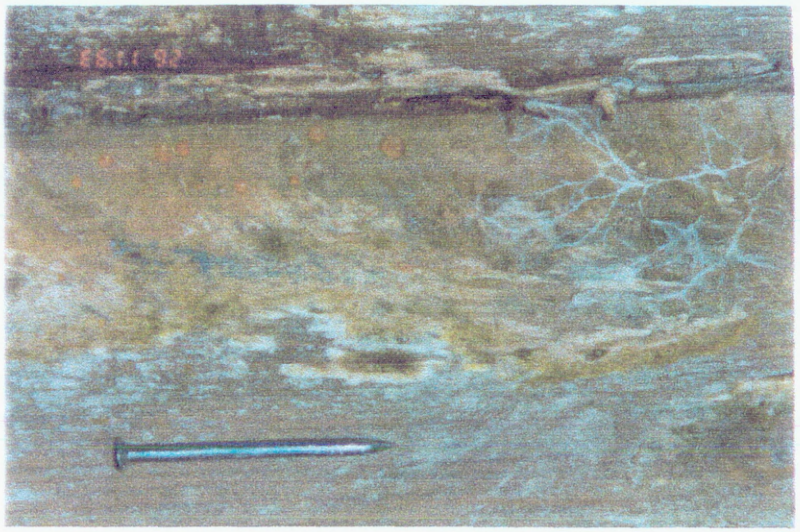


Figure 5.8 : Cord formation on floor surface. Note brown pigmentation of hyphae, brown droplets and a blue-coloured fungus growing on the dead *S.lacrymans* mycelia.

Figure 5.9 : Interactions of *S.lacrymans* periphery with another fungus growing in the floor. Note the moist areas of floor around the periphery and the deposition of spore around the area of the viewing hole.

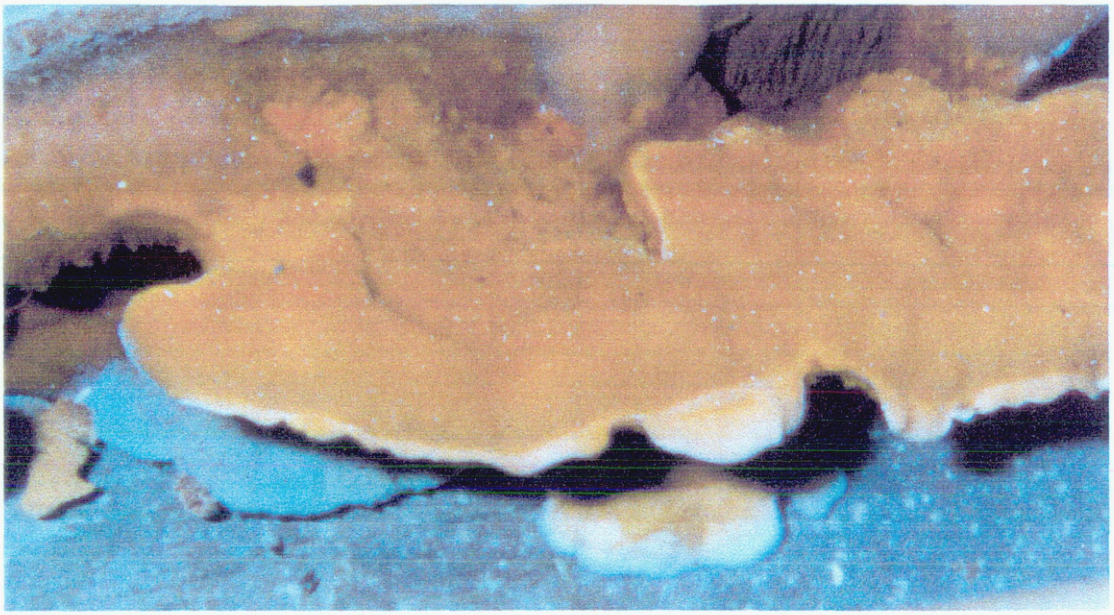


Figure 5.10 : *S.lacrymans* fruiting bodies partially colonised by another fungal organism(s) as shown by the blue colour.

East Wall

Divisions	Treatment Lanes								
	A	B	C	D	E	F	G	H	I
1									
2									
3				11	8	8			
4				6	5	5	6		
5			6	5	4	4	6		
6		12	5	4	3	4	5		
7		10	5	3	1	4	5		
8		6	4	4	1	2	5		
9	13	#	#	#	1	1	5		
10		#	#	#	1	1	#	#	#

North Wall

South Wall

West Wall

Key :

26/01/94	1
15/03/94	2
19/05/94	3
21/07/94	4
07/09/94	5
25/10/94	6
25/11/94	7

14/12/94	8
18/01/95	9
15/02/95	10
14/03/95	11
12/04/95	12
25/05/95	13

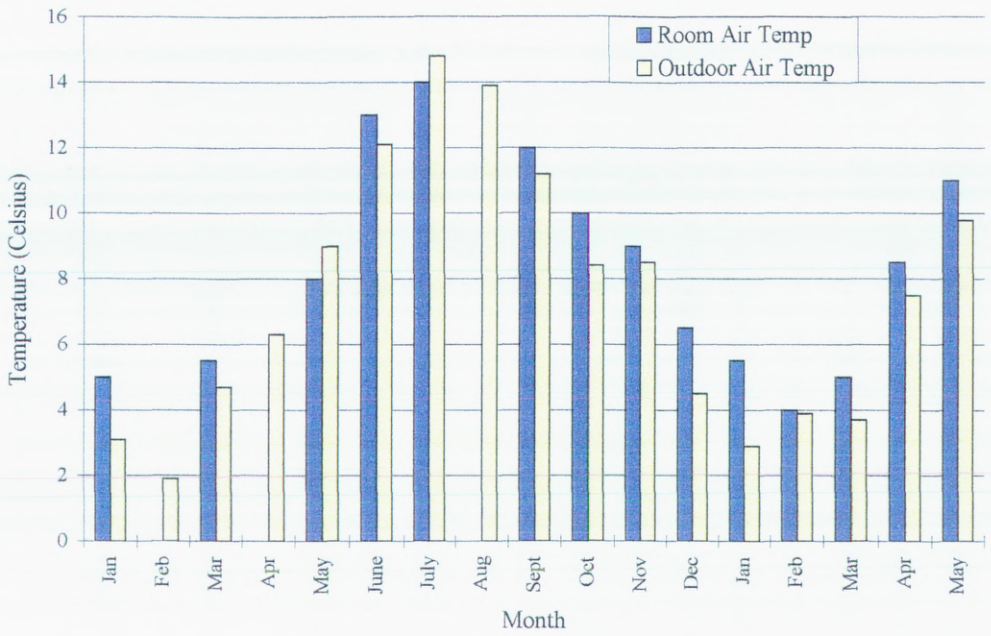
Figure 5.11 : Spread of *S.lacrymans* over the period of the field trial. N.B. : # signifies that no growth was evident due to the presence of the fire-place or collapse of the floorboards; green shading represents the position of the *Trichoderma* treatment.

second treatment the *S.lacrymans* began to recover and show signs of regrowth in the treatment lanes at the colony edge, however, this growth could not advance past the original colony margin. During July of 1994, large numbers of liquid droplets were observed on the *S.lacrymans* colony growing on the underside of the floor. Those droplets produced by the mycelia in the control lanes were clear, however, those produced in the treatment lanes were generally yellow although brown droplets were produced at the colony periphery. Large numbers of spores were also released at this time by the fruiting bodies formed on the underside of the floor.

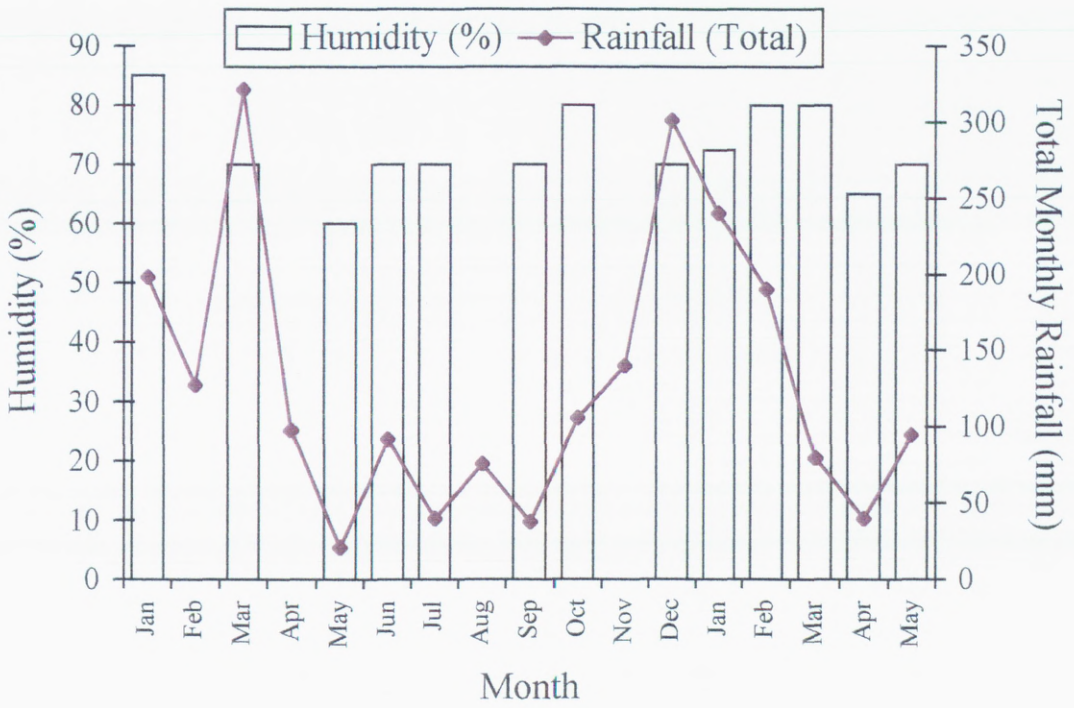
Upon lifting the floor at the end of the trial it was seen that the uninfected wood ahead of the *S.lacrymans* colony (in the treatment lanes) was dotted with clumps of green spores. Figure 5.12 shows the *S.lacrymans* colony periphery in Lane G. The mycelia had collapsed and a yellow/brown colouration developed along the periphery indicative of fungal antagonism. Another example of antagonism was found in Lane H where the *S.lacrymans* colony edge had been colonised by another resident fungus producing an antagonistic reaction that was evident by pigmentation changes.

Figure 5.13 (a, b) displays the changes in air temperature (indoor and outdoor), rainfall and room humidity over the period of the experiment. The graph shows that the air humidity did not change dramatically over the period of the experiment and did not appear to be influenced by the external temperature. The air temperature of the room followed the normal seasonal variations. Figures 5.14 (a-g) show the changes in the percentage moisture content of the floor and walls. These indicate that the major ingress of water was from the north and west walls, which contained the window and fireplace/chimney respectively. Figure 5.15 shows the wall above the fireplace where the plaster has been damaged by

Figure 5.12 : *S.lacrymans* colony periphery in Lane G at the termination of the experiment.



(a)



(b)

Figure 5.13 : Comparison of the internal and external air temperature at Taymouth castle (a) and the external rainfall and room humidity (b). The x-axis range is from January 1994 to May 1995. The outside air temperature and external rainfall data were recorded by the Ardtalnaig weather station situated on the southern shores of Loch Tay and kindly provided by the Meteorological Office at Glasgow.

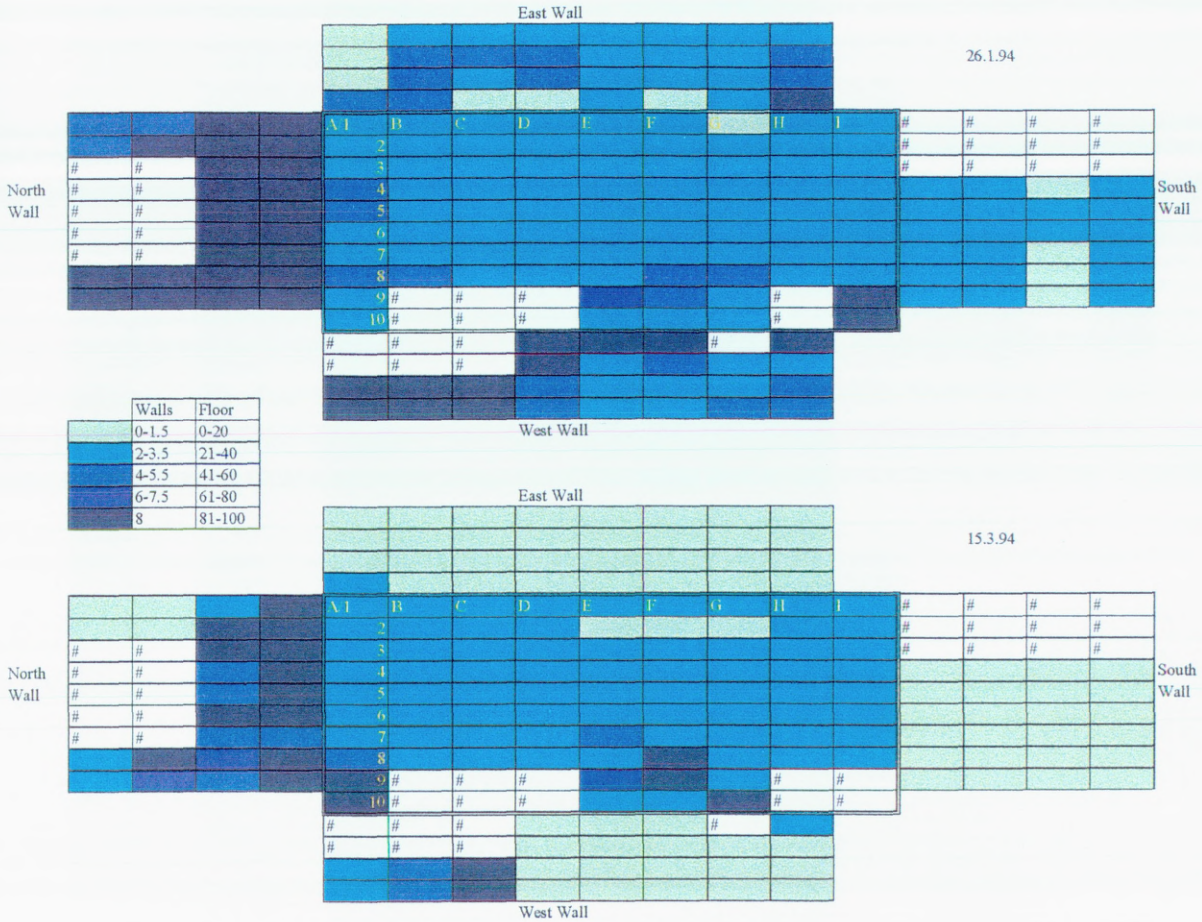


Figure 5.14 a : Maps of the percentage moisture content of the walls (four outer rectangles) and floor (inner, double-lined rectangle) over the period of the field trial (N.B. : The key refers to the scales on the two moisture meters used to read the moisture contents of the walls and floor. The two scales have been approximated to each other so that one colour scale could be used for the walls and the floor. The # symbol indicates that no reading could be taken from this lane division).

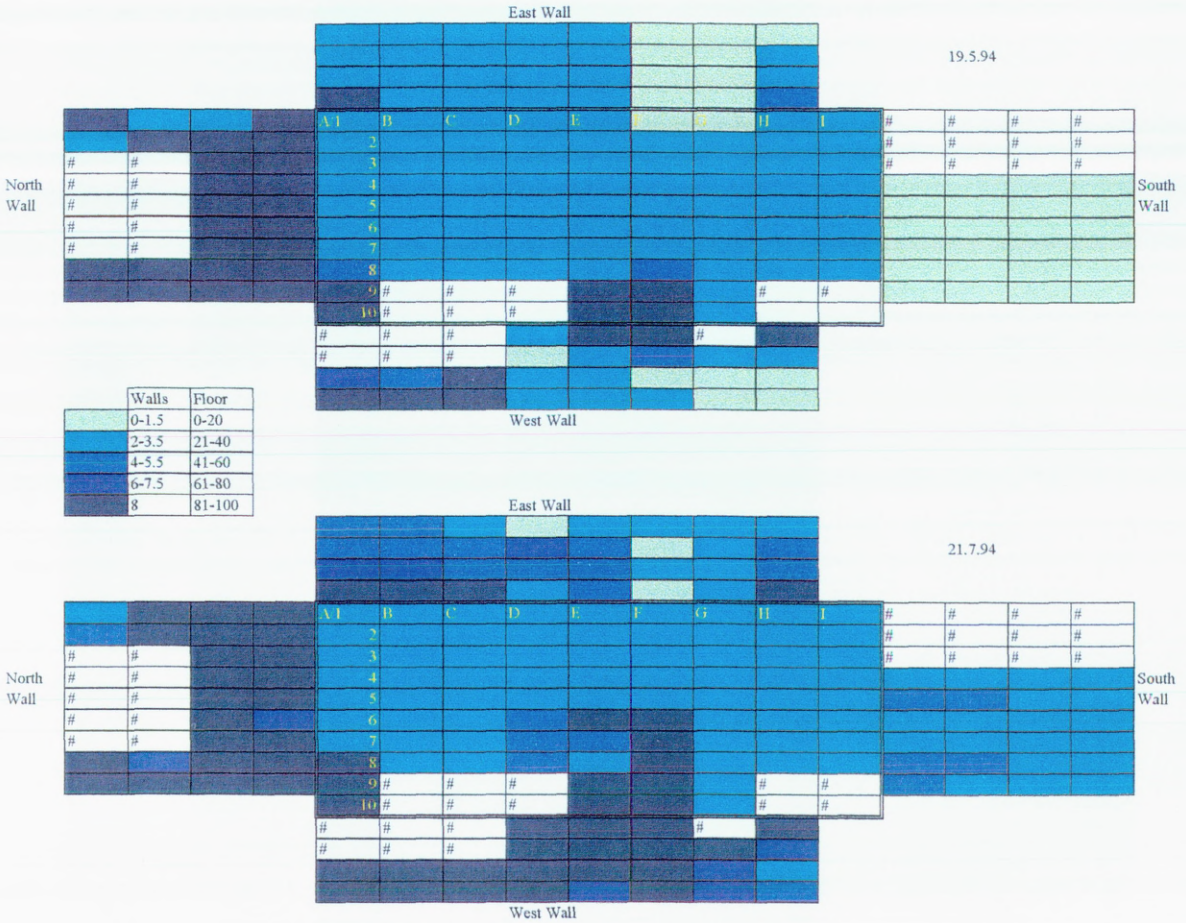


Figure 5.14 b : Continuation of moisture maps.

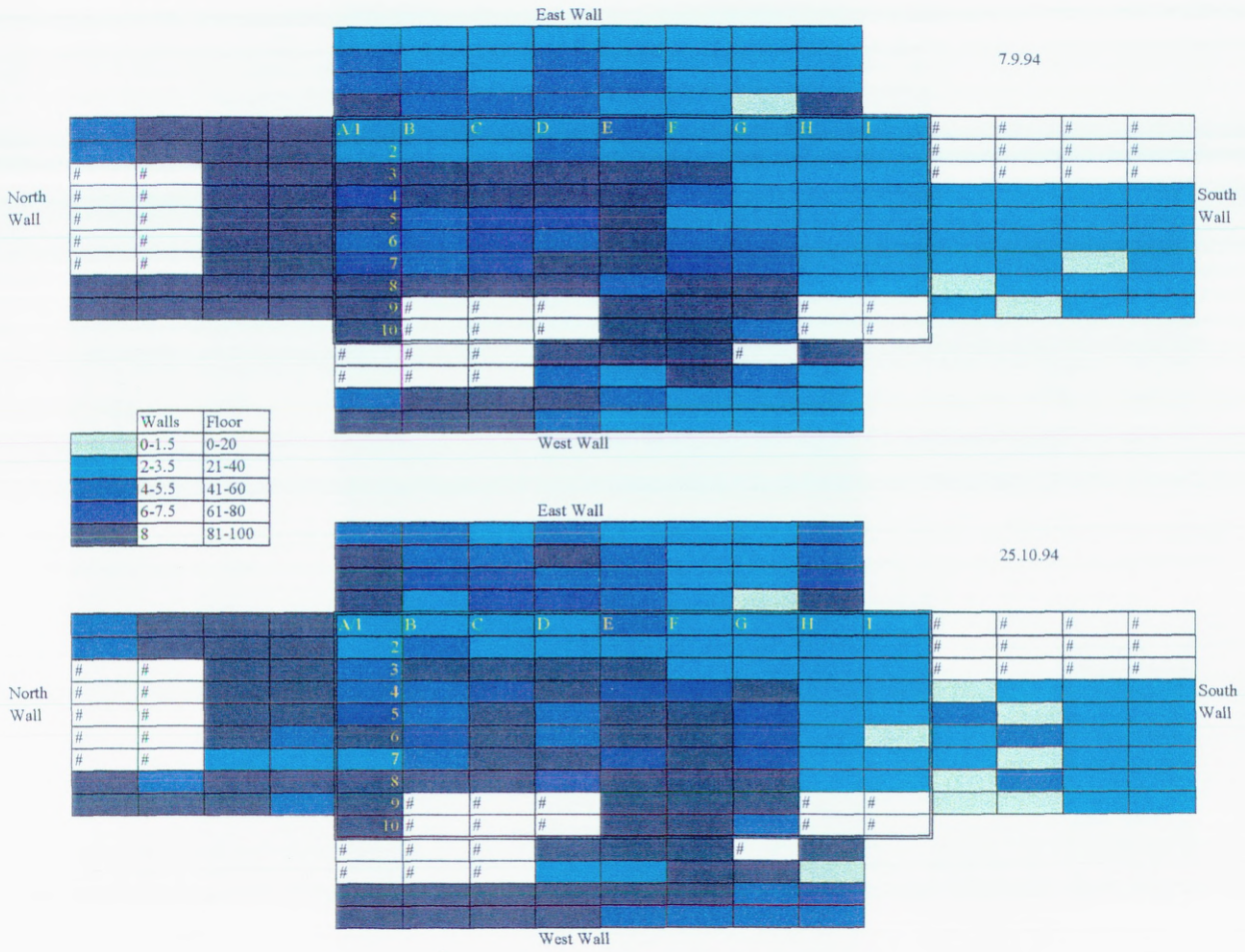


Figure 5.14 c : Continuation of moisture maps

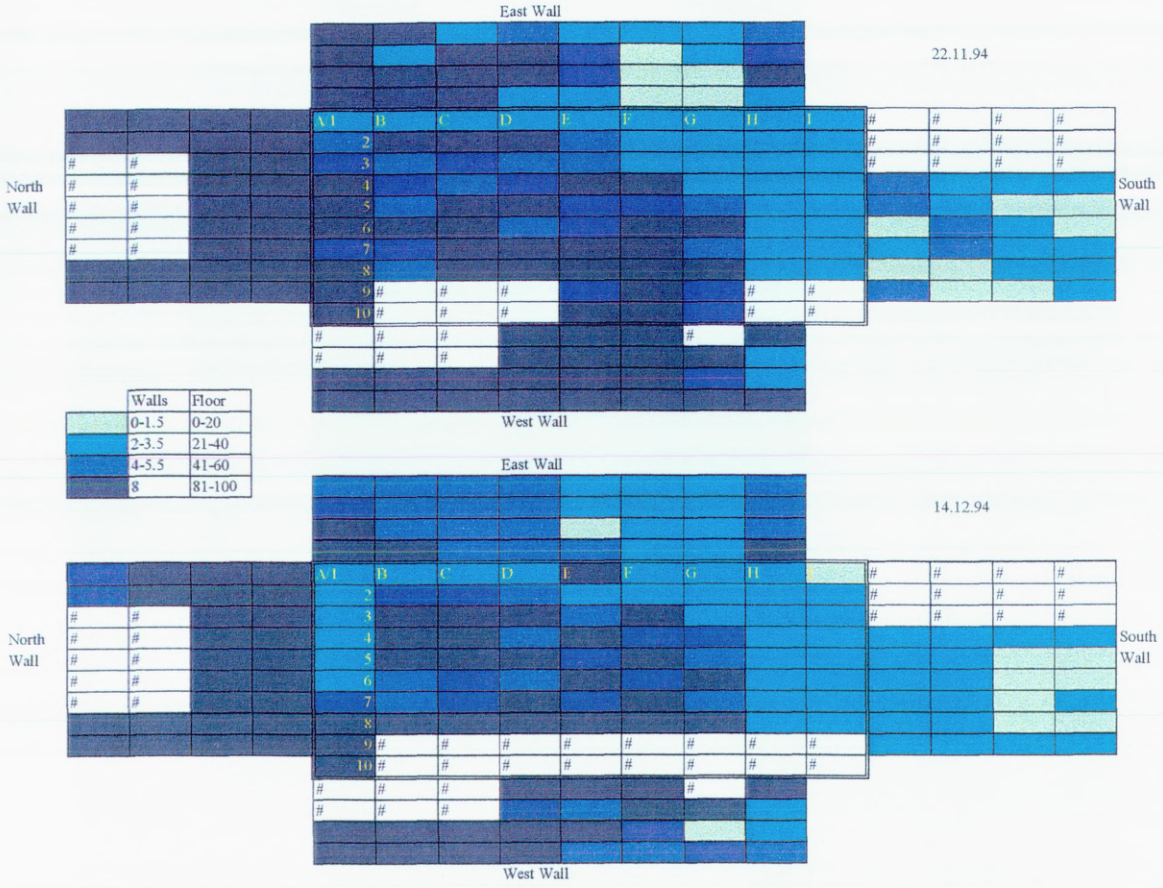


Figure 5.14 d : Continuation of moisture maps

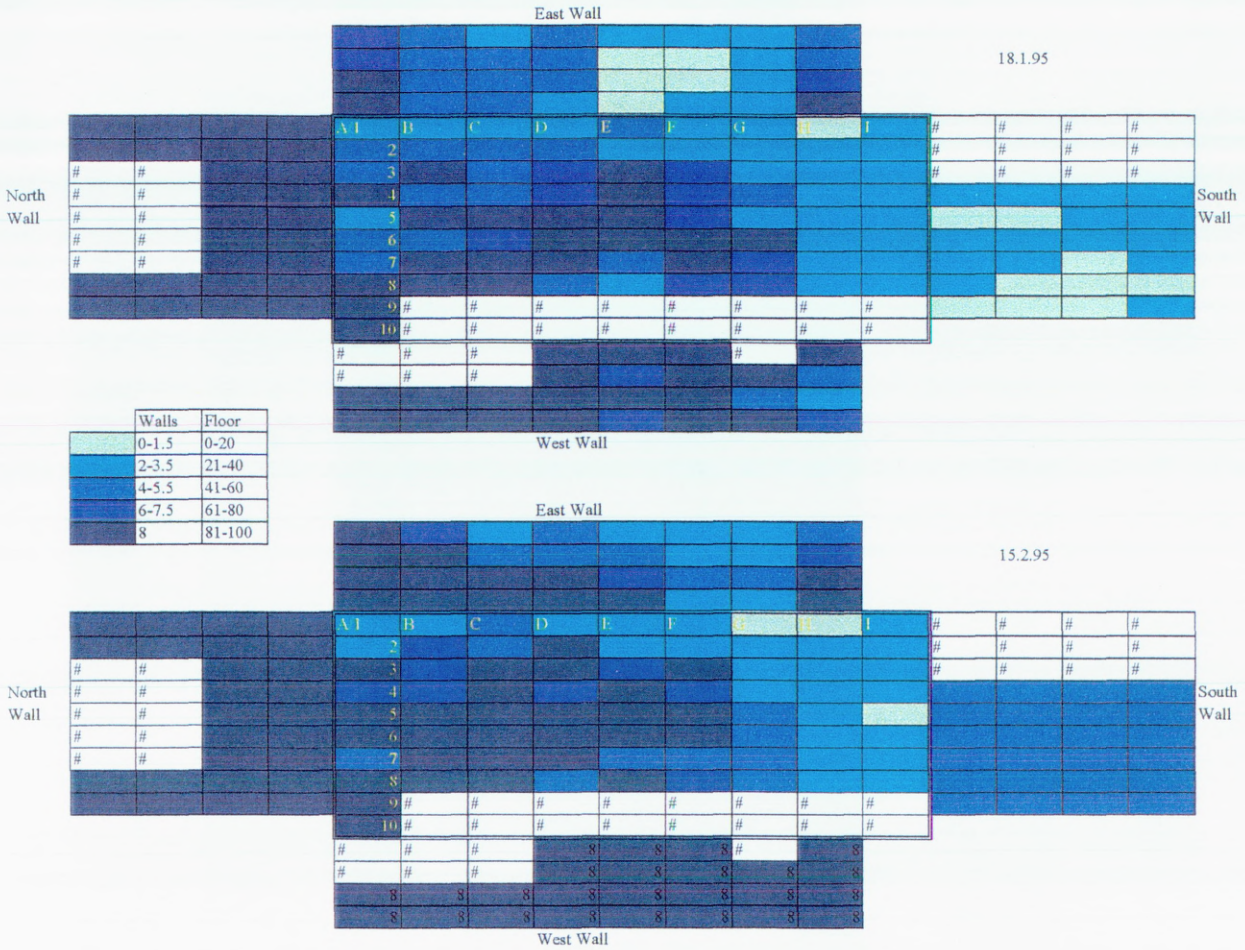


Figure 5.14 e : Continuation of moisture maps

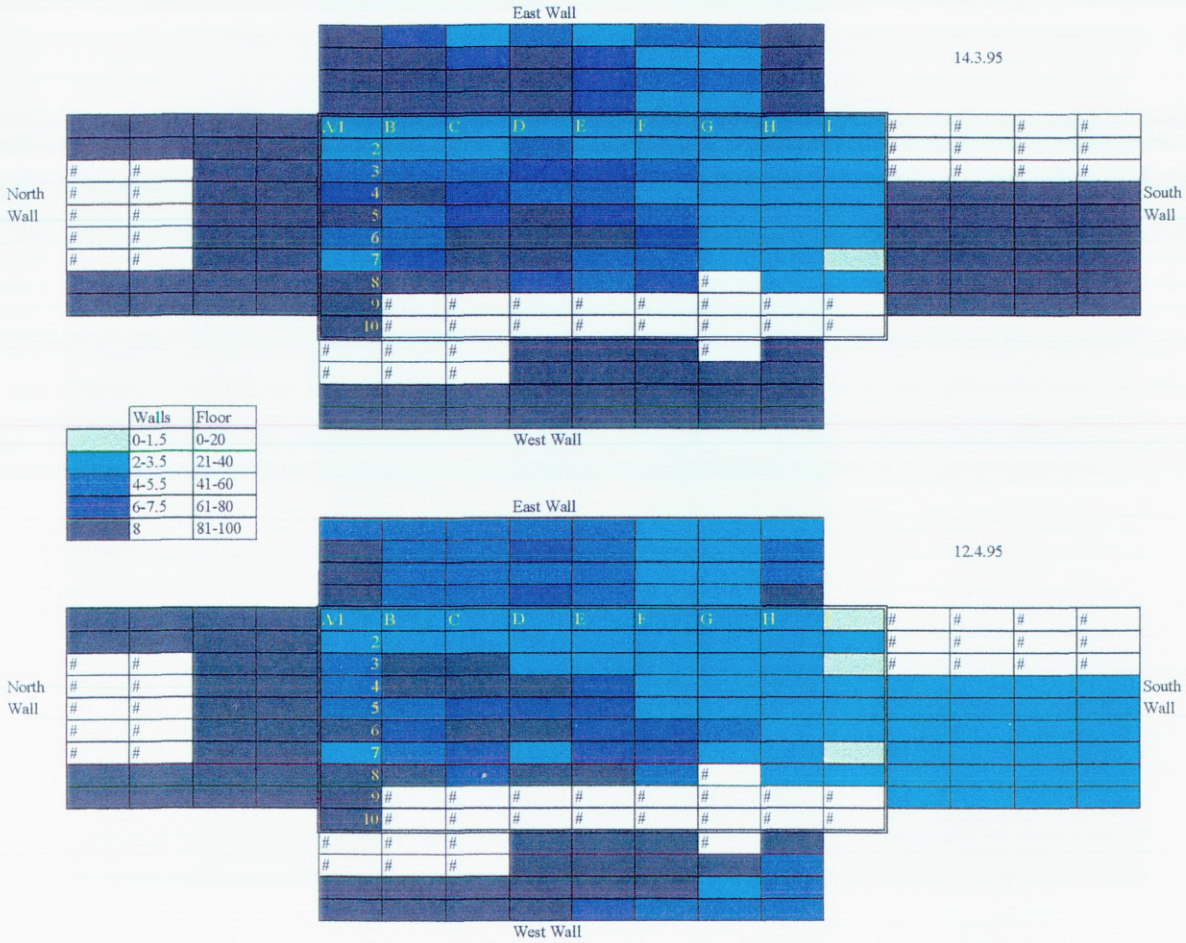


Figure 5.14 f : Continuation of moisture maps

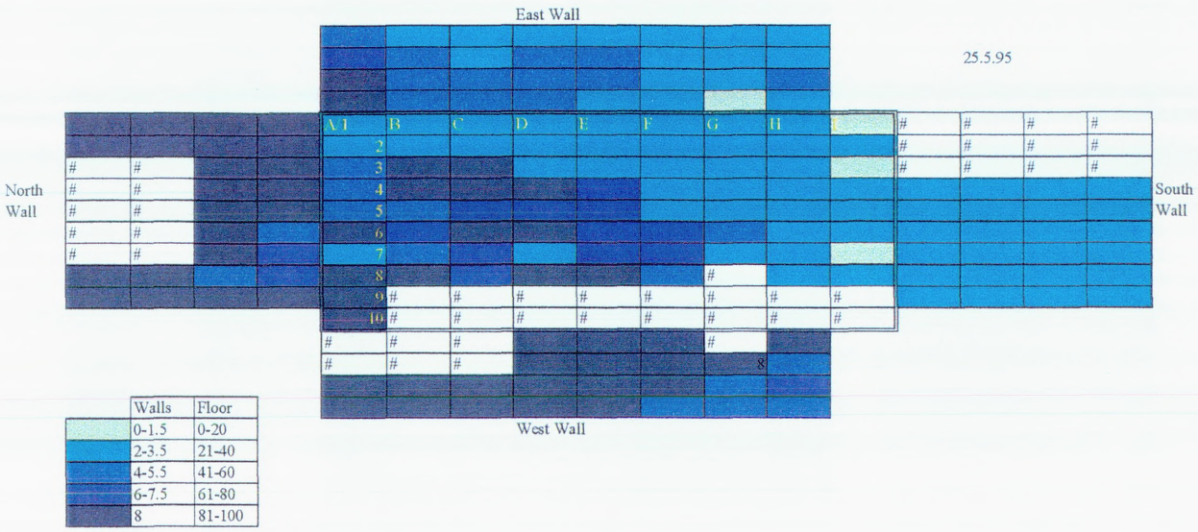


Figure 5.14 g : Continuation of moisture maps

Figure 5.15 : Water damage to the plaster above the fireplace. Note the presence of moulds at the leading edge of the water damage.

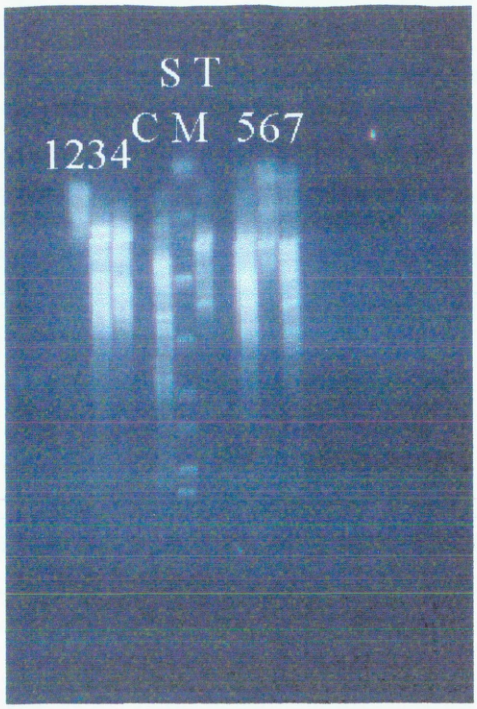
water ingress and mould growth is also evident. There were, however, other sources of moisture, particularly in the east and west walls adjacent to the south wall. Although the moisture content of the walls reached very high levels, the moisture content of the floor remained relatively constant. This remained the case until the 7/9/94 (Figure 5.14 c) when it was found that the room had been flooded a few weeks previous because of blocked drains on the outside wall of the floor above the room. From this point the moisture content of the floor remained high, although there were indications that the floor was drying out by the end of the experiment.

5.3.2 Microbial isolation

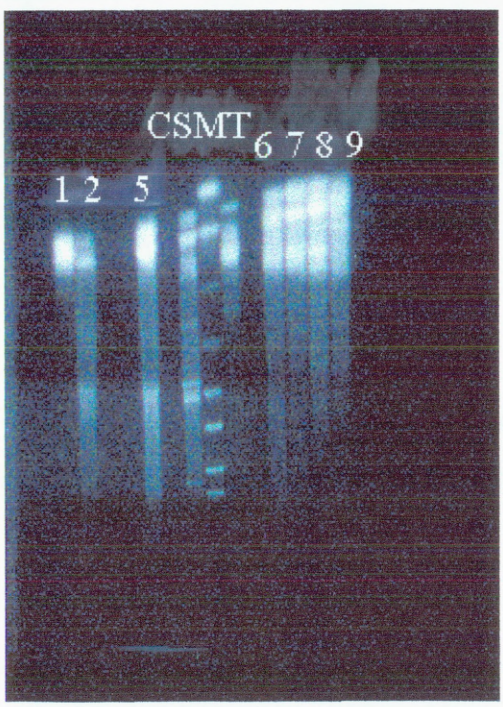
Despite the use of specialised isolation media, neither *S.lacrymans* or *T.harzianum* 25 could be isolated from the wood samples. The inhibitory substances used in the media did not stop the growth and spread of *Mucor* spp. that swamped the plates before the *S.lacrymans* or *T.harzianum* 25 had a chance to grow. It was noted, however, that *Penicillium*, *Aspergillus* and at least one other *Trichoderma* species (tentatively identified as a *T.viride* spp. due to the shape of its conidia) were present in the samples.

5.3.3 Polymerase Chain Reaction

The PCR results showed the presence of *S.lacrymans* in Lanes D-G (Figures 5.17-5.18) although the results were not consistent, even within lanes. Only the Lane F results (Figure 5.18 a) showed the presence of *S.lacrymans* throughout the lane, including parts of the lane not visually infected with *S.lacrymans*. Evidence of *T.harzianum* 25 could not be found in any of the treated lanes.

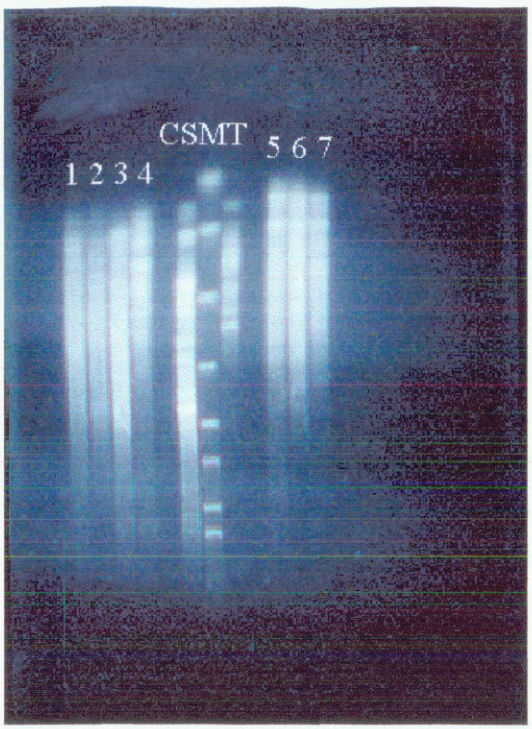


(a)

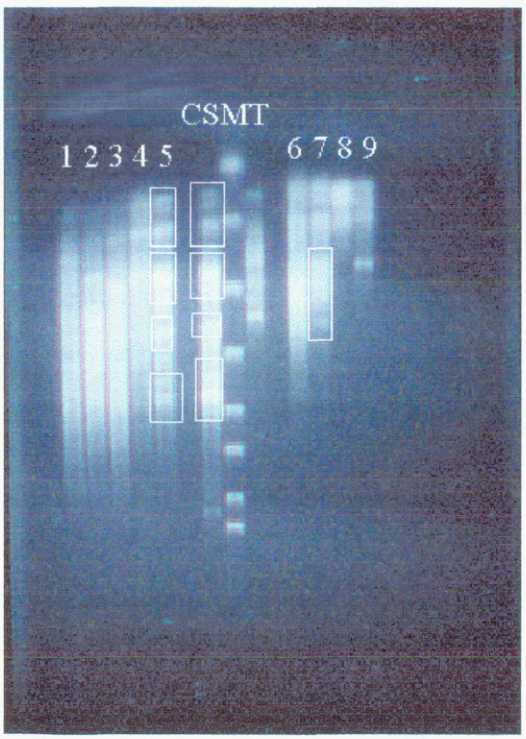


(b)

Figure 5.16 : PCR analysis of Lane B (a) and Lane C (b). Key : C - no DNA control; S - *S.lacrymans* FPRL 12 C DNA; T - *T.harzianum* 25 DNA; M - molecular weight markers.

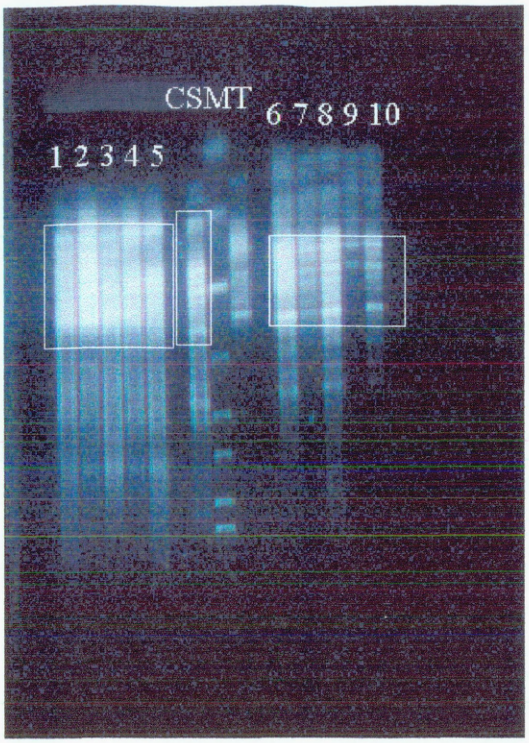


(a)

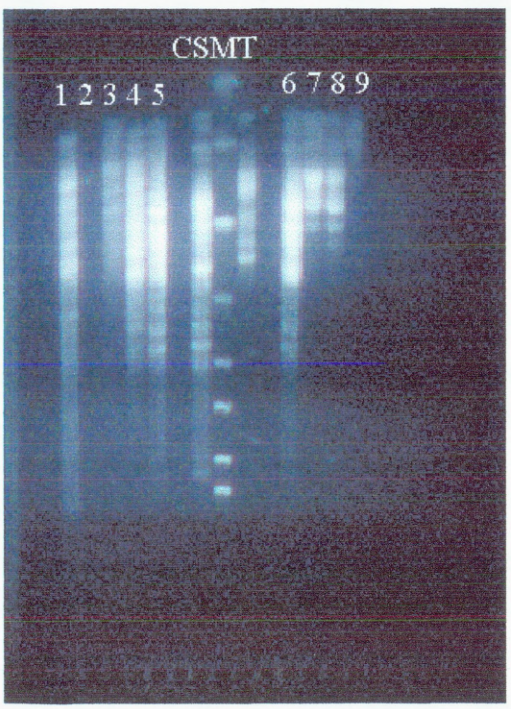


(b)

Figure 5.17 : PCR analysis of Lane D (a) and Lane E (b). Key as for Figure 5.16. Boxes indicate matching bands found in the sample and *S.lacrymans* lanes.

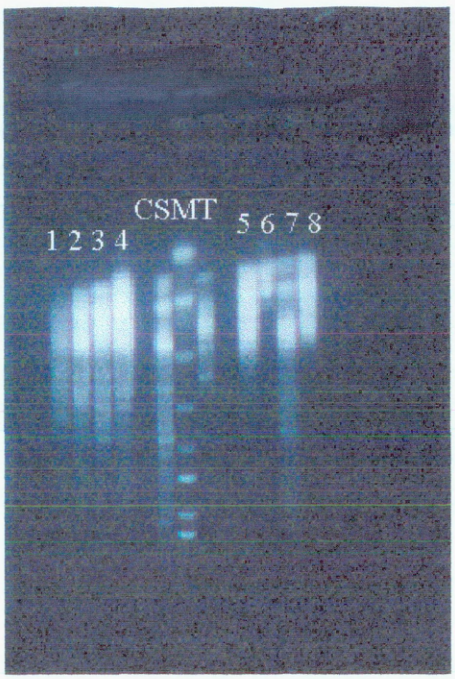


(a)

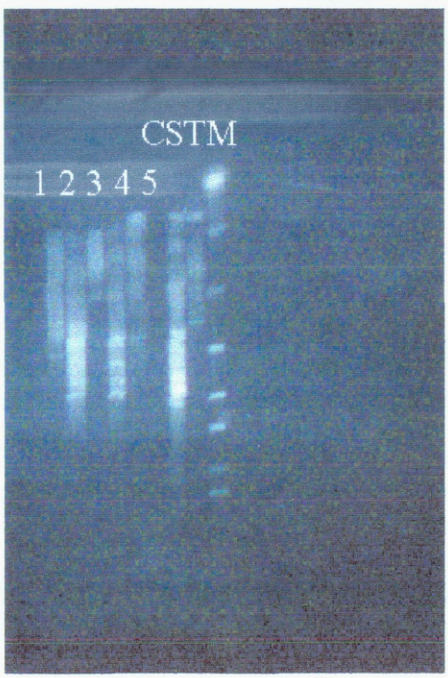


(b)

Figure 5.18 : PCR analysis of Lane F (a) and Lane G (b). Key as for Figure 5.16.



(a)



(b)

Figure 5.19 : PCR analysis of Lane H (a) and Lane I (b). Key as for Figure 5.16.

5.4 Discussion

The potential use of *T.harzianum* 25 in the biological control of *S.lacrymans* has been shown in the laboratory, however, as yet it had not been tested in a field situation. The work described in this chapter is the first known field test of a biological control agent against *S.lacrymans*. Two methods of application were tested and the effects upon the *S.lacrymans* were noted, and by using the Polymerase Chain Reaction the spread of both fungal species through the floor was mapped.

In general, the environmental conditions within the room were conducive to the growth of *S.lacrymans*. Since the room was unheated the temperature did not vary to any great extent from the outside temperature, although the room did tend to be slightly warmer especially through the winter months. Despite the wide fluctuations in temperature, the *S.lacrymans* showed viable growth throughout the period of the trial. This despite the temperature falling close to 0°C in January 1995 when new growth was seen on the upper surface of the floor. It has been reported that *S.lacrymans* can grow at -2°C (Bech-Andersen, 1994) and survive -5°C (Doi, 1991).

The air humidity within the room varied between 60 and 85% and this is somewhat lower than 93.5% which was reported to be the minimum required for growth of *S.lacrymans*. However, it is possible that the humidity was much higher beneath the plastic sheeting, as indicated by the collection of moisture on the underside of the sheeting (Figure 5.7). If the linoleum had not been removed then this would have maintained the high humidity level required for *S.lacrymans* growth.

The growth of *S.lacrymans* on the upper surface of the floor was affected by the flooding of the floor during the summer of 1994. This was demonstrated by the fact that

most growth was achieve during the summer months prior to the flooding, with little growth after it (Figure 5.11). The flooding resulted in the majority of the surface colony (with the exception of the colony periphery) being severely affected, although this recovered within one month. The flooding raised the moisture content of the floorboards but this did not appear to have had any serious effect upon the spread of the fungus on the underside of the floor, although the effect upon decay could not be determined. However, Thornton (1989) reported that *S.lacrymans* was able to decay wood blocks with moisture contents as high as 100%. The results also indicate that the area of floor that was infected with *S.lacrymans* showed a high moisture content even before the flooding of the room. The area of floor surrounding the colony periphery tended to be damp (Figure 5.9), and may simply be the result of the condensation build-up under the plastic sheeting. However, it has been reported that *S.lacrymans* can release water from the hyphal tips (Coggins *et al*, 1980) which may be due to either a control of the hyphal internal hydrostatic pressure (used to move solutes though the hyphae; Coggins *et al*, 1980) or through the production of metabolic water produced by the breakdown of cellulose (Schmidt and Moreth-Kebernik, 1990; Bech-Andersen, 1991). Ultimately the wood ahead of the colony periphery would be moistened although this moisture may not be necessary for growth *per se* but necessary to reduce water loss from the mycelium (Clarke, 1976).

Although the *S.lacrymans* did produce cords on the surface of the floor, it did not produce cords in order to bypass the antagonistic organisms that was present on the underside of the floor. *S.lacrymans* produces cords in order to advance over areas that are nutritionally deficient in search of new food sources (Rizzo *et al.*, 1992) or in the conservation of nitrogen in the mycelium (Jennings and Watkinson, 1982; Coggins, 1976). Cord production noticed on the surface of the floor was largely observed behind fresh

growth at the *S.lacrymans* colony periphery. However, in at least one case the production of cords was an attempt by the *S.lacrymans* to advance over parts of the colony killed by the presence of an antagonistic fungus (Figure 5.8). The production of cords to bypass an antagonistic organism was shown by Score *et al* (1998) (see also Chapter 4). It is open to speculation why the *S.lacrymans* did not produce cords at the colony peripheries in contact with the antagonistic organism on the underside of the floor. It may be that since growth was still possible at other parts of the colony, the energy expenditure required to produce the cords was redirected elsewhere within the colony. If this was the case, however, why was the cord produced to bypass the antagonist on the surface of the floor?. It may be possible that the presence of the antagonist on the floors' upper surface was coincidental with the production of the cords, which may have been produced for another reason, e.g. nitrogen conservation.

Despite the lack of confirmation in the molecular studies, visual observations seemed to indicate that the *T.harzianum* 25 had colonised the underside of the floor ahead of the *S.lacrymans* colony periphery. The periphery in the treatment lanes did show changes indicative of the presence of an antagonistic organism, for example, pigmentation changes and cessation of growth (Figure 5.12). Since the PCR system did not detect the *Trichoderma* applied to the floor it is possible that the control of the *S.lacrymans* was due to the presence of another antagonistic organism. The presence of organisms capable of inducing pigmentation changes in the *S.lacrymans* hyphae was noted (Figure 5.9) although the antagonistic effect seems to have been localised. If the observed control was due to the presence of antagonistic organisms already present within the floor then the control should have been recorded in the untreated lanes also.

The failure of the spore suspension to colonise the floor-boards may have been due to a number of factors. It is possible that the presence of other fungal species within the wood inhibited the germination of the spores. Since the spores were suspended in water with no added nutrients there was not enough nutrients to initiate and support germination. Other possible reasons include an insufficient inoculum potential within the suspension; the wood may have been too dry to allow colonisation by the *T.harzianum* 25 (King (1981) reported that to allow colonisation by mould and stain fungi wood must have a minimum moisture content of approximately 60%. However, in those areas of the floor near the moisture sources, e.g., next to the north wall, there was moisture at levels high enough to allow spore germination); the temperature during the first months of the year may have been too low to allow the *Trichoderma* to germinate since the temperature did not rise above 6°C.

The first macerated *T.harzianum* 25 treatment did produce some changes in the growth characteristics of the *S.lacrymans* and it is possible that the *Trichoderma* treatment induced the formation of *S.lacrymans* fruit-bodies. *Trichoderma* species have been reported as producing a number of volatile and non-volatile metabolites that are inhibitory towards other fungal species (Dennis and Webster, 1971 a, b, c; Claydon *et al*, 1987). In addition, it was reported that certain of these metabolites can act as paramorphogens, i.e., they alter the distribution of an organism's biomass but not its rate of production (Claydon *et al*, 1987). These metabolites may have inhibited the growth of *S.lacrymans*, at least initially, but in doing so induced the formation of the fruit-bodies. This may have occurred through the direct action of the *T.harzianum* 25 metabolites or through the redirection of energy by the *S.lacrymans* from the colony periphery (that had ceased growth) to fruit-body formation. The induction of spore production may, at first, seem detrimental but if

the conditions are not right for spore germination then the treatment has caused a redirection of valuable energy to a non-productive process.

Bruce and King (1986b) did show that although *Trichoderma* treated wood was resistant to decay by brown-rot fungi this protection was limited by the incomplete colonisation of the wood by the *Trichoderma* species. It is possible that the failure of the *T.harzianum* 25 to spread through the floorboards was due to the presence of other non-decay organisms as reported by Bruce *et al* (1990), such as those seen growing on the fruit-bodies and mycelia of the *S.lacrymans,* and even of the *S.lacrymans* itself. This would tend to agree with the results from the small- and medium-scale experiments (see Chapter 4) where the *Trichoderma* species could not spread into wood already infected with *S.lacrymans.*

The visual observations of the growth of the *S.lacrymans* did show the overall growth pattern of the colony throughout the period of the experiment (Figure 5.11). However, the observations were qualitative in nature and tended to be general. These observations were to complement the PCR results that were meant to show the full spread of the *S.lacrymans* and the *T.harzianum* 25. The PCR did show that the *S.lacrymans* had spread to a greater degree than the visual observations indicated, for example, *S.lacrymans* was detected in segments 3-10 of Lane F. This could indicate that although the growth of the *S.lacrymans* was inhibited on the surface of the floorboards its growth was not hindered within the floorboards. This may concur with the results obtained from the medium-scale stake experiment that may have also showed advance of the *S.lacrymans* colony through the central portion of the stakes (see Chapter 4). The failure of the PCR to detect the *T.harzianum* 25 may simply indicate that the system was not optimised to detect this fungus. Score *et al* (1998) reported that the amount of visible *Trichoderma* mycelium

was considerably reduced when grown on sawdust agar compared to malt extract agar, thus the amount of *Trichoderma* in the floor samples may have been too small to detect.

Field trials, such as the one described in this chapter, are used to investigate a particular hypothesis, for example, can *Trichoderma* spp. control the spread of *S.lacrymans* within a housing environment. As such the hypothesis should be relatively non-specific in the sense that a field trial set-up to investigate a specific hypothesis has a greater chance of failure because of unanticipated factors occurring. These factors could include, for example, unseasonable weather conditions, unforeseen actions by the organisms involved and physical disruption to the test site. The greater the number of factors being measured ensures that, if unexpected events do occur, at least some results are achieved at the termination of the trial.

The results reported from this field trial indicate that *Trichoderma* species could have a role to play in the prevention of decay of housing timbers. Despite the fact that the presence of *Trichoderma* could not be confirmed the biocontrol treatment did have an effect upon the *S.lacrymans*. At the very least it caused the *S.lacrymans* to re-route valuable resources and energy into reproduction and probably stopped the advance of the *S.lacrymans* on the underside of the floor. The optimum inoculation protocol will have to establish if *Trichoderma* species could be used to prevent wood decay by *S.lacrymans*. Factors such as inoculum size, use of more than one *Trichoderma* isolate and addition of specific nutrients and/or fungicides should be investigated.

Overall, the results of the field trial tend to indicate that the biological control of *S.lacrymans* with *Trichoderma* species may not be a viable prospect as a sole remedial system. However, another field trial should be undertaken to confirm or repudiate these

findings. The spread of the *Trichoderma* has to be confirmed within the wood to determine whether it is the presence of the added *Trichoderma* that is achieving the control or whether it is simply combining with the organisms already present within the flooring to inhibit the *S.lacrymans* growth.

Chapter 6

Production of Fungal Melanins in Response to Offensive or Defensive Strategies

6.1 Introduction

During the mycelial confrontations of higher fungal species (i.e. basidiomycetes and ascomycetes) a number of morphogenic changes are observed including pigment accumulation and the production of non-substrate hyphae, i.e., hyphae not in immediate contact with the substrate (Rayner *et al*, 1994). Confrontations involving *S.lacrymans* and *Trichoderma* species usually result in some pigmentation changes associated with the *S.lacrymans*. Generally, these pigment changes are the result of the *S.lacrymans* colony being invaded by antagonistic mycelia or subject to a deadlock reaction (see Chapter 2; Score and Palfreyman, 1994; Score *et al*, 1998). However, it was noted that *S.lacrymans* strands rarely went through the same pigment changes as ordinary colony hyphae (Score *et al*, 1998), and, hyphae emerging from a deadlocked *S.lacrymans* colony was white in colour even if the colony zone from which this hyphae emerged were pigmented (e.g. yellow). This seemed to suggest that *S.lacrymans* strands and replacement hyphae were protected in some way against antagonistic fungi.

Fungal strands are specialised structures designed for the exploration of uncolonised substrates. Rhizomorphs, although more complex, perform basically the same function as strands. The rhizomorphs of *Armillaria* species, which are particularly well studied, consist of a core of differentiated hyphae surrounded by the cortex the cells of which are melanised, and this is thought to be a major factor in the survival of these rhizomorphs (Rizzo *et al*, 1992). A number of fungal structures (hyphal cell walls, spores, sclerotia and pseudosclerotial plates (Bell and Wheeler, 1986)) contain melanin which protects them from U.V. irradiation, moisture stress and lysis by hydrolytic enzymes such

as chitinases and β-glucanases (Bloomfield and Alexander, 1967; Bull, 1970; Bell and Wheeler, 1986). Other possible functions of cellular melanin include enhancing structural integrity or as a physical barrier against microbial attack (Chet and Henis, 1969; Bell and Wheeler, 1986).

There are a number of substances which are known to be either the substrate or precursor to melanin production, for example, tyrosine, γ-glutaminyl-3,4-dihydroxy-benzene (GDHB), catechol and 1,8-dihydroxynaphthalene (DHN) (Bell and Wheeler, 1986). However, melanin production has not yet been demonstrated in *S.lacrymans* although catechol has been shown to be the immediate precursor to melanin synthesis in the basidiomycete *Ustilago maydis* (Piatelli *et al,* 1963; Bell and Wheeler, 1986). The shikimate pathway in fungi is responsible for the production of chorismate which is the common precursor for the three aromatic amino acids (Trp, Tyr and Phe) (Hawkins *et al,* 1993). Tyrosine can be oxidised by tyrosinase to form 3,4-dihydroxy-phenylalanine melanin (Bell and Wheeler, 1986).

A number of substances have also been shown to inhibit melanin synthesis, for example, tricyclazole (Bell and Wheeler, 1986), hexachloroacetone (Durrell, 1964) and ethylenediaminetetraacetic acid (EDTA) (Bell and Wheeler, 1986). The possible involvement of catechol or shikimate in melanin production by *S.lacrymans* was tested as a prelude to an investigation of the possible role of melanin in the stress responses of *S.lacrymans.*

6.2 Methods

6.2.1 Determination of the presence of melanin in field samples of *S.lacrymans*.

Samples of *S.lacrymans* fruiting bodies, cords and mycelia were collected from Taymouth Castle, and 10g (wet weight) of each were freeze-dried. 1g amounts were then taken and a modified version of the extraction procedure of Gadd (1980) was used to extract melanin. Each sample was placed into an universal bottle containing 1 M NaOH (6 mls) and then autoclaved at 121°C ± 1°C for 20 minutes. After cooling the samples were centrifuged at 4000 g for 5 minutes, after which the supernatant was decanted into a fresh universal bottle and borate buffer (6 mls; pH 8) was added. The optical density (OD) of the solution was then read at 540 nm.

Following the above procedure, the cord sample gave a reduced amount of supernatant compared to the other samples. To ensure that a dilution factor would not become a factor in the results equal quantities of supernatant and borate buffer were mixed for all subsequent extractions.

A standard curve was constructed using a synthetic melanin made from tyrosine in the concentrations 1 $mgml^{-1}$, 0.5 $mgml^{-1}$, 0.1 $mgml^{-1}$, 25 μgml^{-1}, 12.5 μgml^{-1}, 6.25 μgml^{-1}, 3.125 μgml^{-1} and 1.5625 μgml^{-1} (Figure 6.1).

6.2.2 Induction and inhibition of melanin synthesis in *S.lacrymans*

To induce melanin synthesis, catechol (10%) and shikimate (100 p.p.m.) were prepared, with hexachloroacetone (100 p.p.m.) and EDTA (10^{-3} M) prepared as inhibitors. Distilled water was used as a control and all solutions were sterilised by filtration (through a 0.2 μm filter). Fifteen 250 ml Duran bottles, containing 3% malt extract broth, were inoculated with *S.lacrymans*. Once fungal growth had started, 1 ml of inducer, inhibitor or control was added to three of the bottles every four days. After 3 weeks incubation (at 22°

C ± 1°C) the fungal colonies and broth were harvested from each bottle and subjected to the melanin extraction procedure. The OD of the extracts were then read at 540 nm with the blank being 3% malt extract broth that had also been through the extraction procedure.

To determine whether excess inducer/inhibitor in the media would give a positive reading, media was prepared with the total amount of inducer/inhibitor added and an extraction carried out.

6.2.3 Determination of the concentration of catechol required to induce melanin formation in *S.lacrymans*.

Five concentrations of catechol solution (0.1, 0.5, 1.0, 5.0 and 10.0%) and a solution of cetyltrimethylammonium bromide (CTAB) were prepared and sterilised by filtration through a 0.2 μm filter. Plates containing minimal media broth (see Chapter 2) were inoculated with 5 mm cores of *S.lacrymans* and incubated until the mycelia had started to spread into the broth. Using five replicates per catechol treatment, 1 ml of each solution (catechol, CTAB or sterile distilled water) was added to the relevant plates. The following treatments were prepared:

1. *S.lacrymans* + catechol solution + water (5 replicates)
2. *S.lacrymans* + catechol solution + CTAB (5 replicates)
3. Control (no *S.lacrymans*) + catechol + water (5 replicates)
4. *S.lacrymans* + CTAB (5 replicates)
5. *S.lacrymans* + water (5 replicates)

The first addition of treatment solution was carried out four days after the start of the experiment and continued every four days after that, up to three weeks incubation. Prior to each addition of treatment solution, 1 ml of media was removed from each replicate and pooled. This was then subjected to the melanin extraction procedure and the absorbance between 200 and 550 nm read using a scanning spectrophotometer. At the end

of the experiment, the mycelia was also collected from each replicate, pooled and extracted for melanin.

6.3 Results

6.3.1 Determination of the presence of melanin in field samples of *S.lacrymans.*

The level of melanin detected varied with the different fungal structures examined (mean of three extractions) : Fruit body - 428.26 μg/ml; Mycelia - 382.09 μg/ml; Cords - 80.48 μg/ml. The cord sample had been diluted approximately by a factor of three, therefore, the cord results can be increased to 241.44 μg/ml.

6.3.2 Induction and inhibition of melanin synthesis in *S.lacrymans*

Melanin was only detected in one of the three *S.lacrymans* control plates, giving a mean with a large standard deviation, i.e. 0.235 ± 0.406. The effects of the inducers/inhibitors on melanin synthesis are shown in Figure 6.2, with significance testing of the test values shown in Table 6.2. The results indicate that the inducers/inhibitors used did not affect melanin synthesis within the fungal colony. The high results obtained with catechol give a false perception as the results in the next section show that catechol either self-polymerises or reacts with a medium component to form a dark coloured pigment.

6.3.3 Determination of the concentration of catechol required to induce melanin formation in *S.lacrymans.*

From the scanning spectroscopy results it was clear that there were no peaks at 540 nm the presence of which would indicate the formation of melanin within *S.lacrymans.* However, there were two peaks, one at 250 nm and the other at 272 nm, which seemed to increase both with the level of catechol added and the incubation period of the plates. Tables 6.3 - 6.6 give a representation of the intensities of these two peaks over the period of the experiment. Table 6.3 indicates that an absorbance increase was shown by the catechol only controls and the liquid media in these plates did darken over time (with the exception of the 0.1% catechol plates) with evidence of a dark precipitate forming in the media. This may indicate that the catechol was polymerising to form this

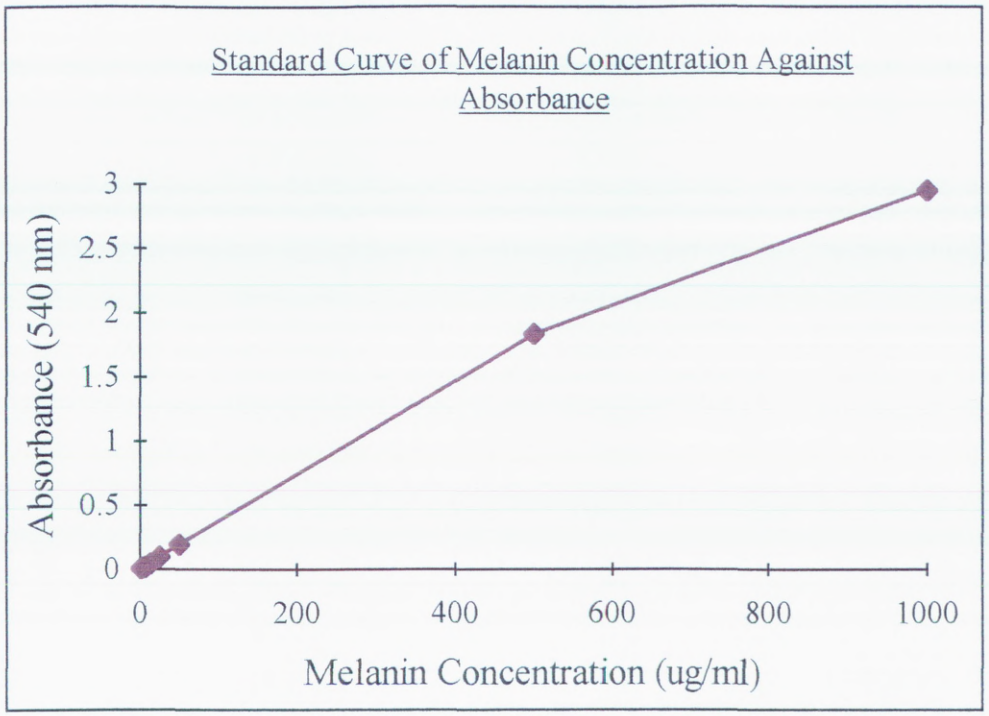


Figure 6.1 : Standard curve of melanin concentration against absorbance at 540 nm.

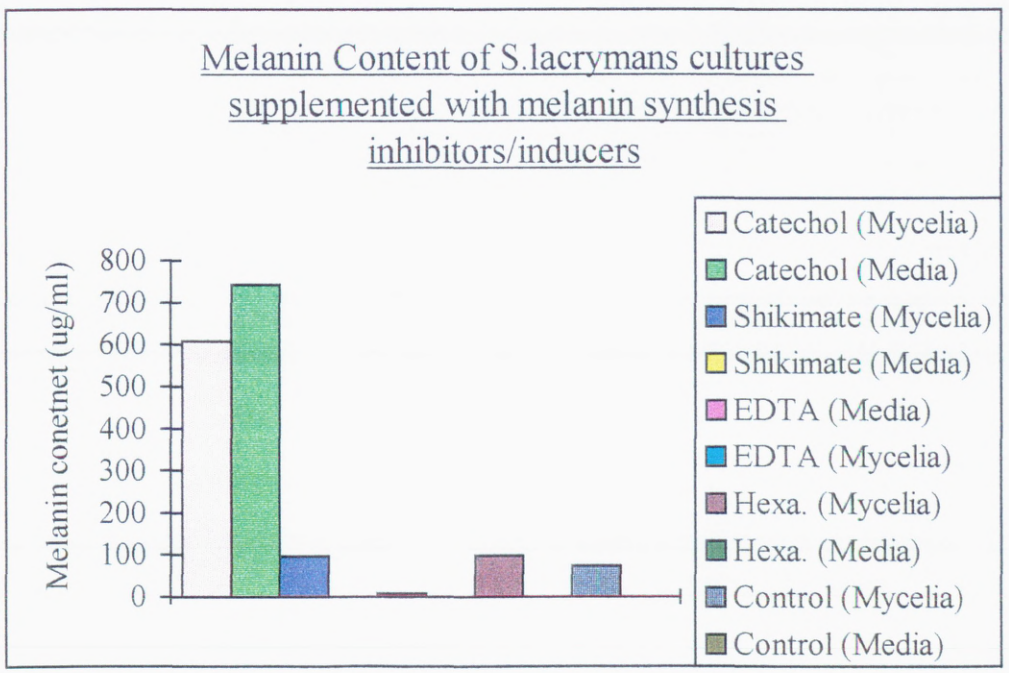


Figure 6.2 : Melanin content of *S.lacrymans* mycelia and culture medium after addition of melanin synthesis inducers or inhibitors. (N.B. : Hexa. - Hexachloroacetone)

	Melanin Inducer		Melanin Inhibitor		
	Catechol	Shikimate	EDTA	Hexa.	Control
Catechol	-				
Shikimate		-			
EDTA			-		
Hexa.				-	
Control					-

Table 6.1 : Significance testing (1% level) of the effects of the melanin synthesis inducers/inhibitors on *S.lacrymans*. Solid boxes indicate a significant difference, clear boxes indicate no significant difference and boxes with a '-' indicate no result.

black precipitate or precipitate formation was caused by the catechol attaching to an unknown component of the medium. Since the precipitate was apparently not formed in the 0.1% catechol control plates the absorbance increase at 272 nm was caused by some other factor.

When 0.1% and 0.5% catechol was added to the *S.lacrymans* plates (Table 6.4), there was a greater increase in the 250/272 absorbencies compared to the catechol only control plates. However, there does not appear to be any pattern to the absorbance readings of the plates to which the higher concentrations of catechol were added. The addition of CTAB to the media did reduce the absorbencies to some degree but the readings were still higher than those for the catechol only plates. However, upon analysing the *S.lacrymans* mycelia the level of melanin was reduced compared to the *S.lacrymans* control (Table 6.7).

	Catechol Solution Added											
	0.0%		0.1%		0.5%		1.0%		5.0%		10.0%	
	250	272	250	272	250	272	250	272	250	272	250	272
S	++	++	+	++	++	++++	++	+++++	++++	+++++	+++++	+++++
1	++	++	+	++	++	++++	++	+++++	++++	+++++	+++++	+++++
2	++	++	+	+++	++	++++	+++	+++++	++++	+++++	+++++	+++++
3	++	++	+	++++	+++	++++	++++	++++	+++++	++++	+++++	+++++
4	++	++	+	++++	++++	++++	++++	++++	+++++	++++	+++++	+++++
5	++	++	++	+++++	++++	++++	++++	++++	+++++	++++	+++++	+++++

Table 6.2 : Relative intensities of absorbencies at 250 and 272 nm for the Control + Catechol plates. Key : S - start of experiment; 1 - day 4 sample; 2 day 8 sample; 3 - day 12 sample; 4 - day 16 sample; 5 - day 20 sample; + - absorbance value 0-0.99; ++ - absorbance value 1.0-1.99; +++ - absorbance value 2.0-2.99; ++++ - absorbance value 3.00-3.99; +++++ - absorbance value 4.0-4.99.

	Catechol Solution Added											
	0.0%		0.1%		0.5%		1.0%		5.0%		10.0%	
	250	272	250	272	250	272	250	272	250	272	250	272
S	++	++	++	+++	+++	+++++	-	-	+++++	+++++	-	-
1	++	++	+++	+++	+++	+++++	++++	++++	+++++	+++++	++++	++++
2	++	++	++++	+++++	++++	++++	+++++	+++++	+++++	+++++	+++++	++++
3	++	++	+++++	+++++	++++	++++	+++++	+++++	+++++	+++++	+++++	++++
4	++	++	++++	++++	++++	++++	++++	++++	+++++	+++++	++++	++++
5	++	++	++++	++++	++++	++++	++++	++++	+++++	+++++	+++++	+++++

Table 6.3 : Relative intensities of absorbencies at 250 nm and 272 nm for the *S.lacrymans* + Catechol plates. Key as for Table 6.3, with '-' indicating no result.

	Catechol Solution Added											
	0.0%		0.1%		0.5%		1.0%		5.0%		10.0%	
	250	272	250	272	250	272	250	272	250	272	250	272
S	++	++	++	+++	++	++++	+++	++++	++++	++++	++++	++++
1	++	++	++	+++	+++	+++++	++++	++++	++++	++++	+++++	+++++
2	++	++	++	+++	+++	++++	++++	++++	+++++	++++	++++	++++
3	++	++	+++	+++++	++++	++++	++++	++++	++++	++++	++++	++++
4	++	++	+++	+++++	++++	++++	++++	++++	++++	++++	++++	++++
5	++	++	+++	+++++	++++	++++	+++++	++++	+++++	+++++	++++	++++

Table 6.4 : Relative intensities of absorbencies at 250 nm and 272 nm for the *S.lacrymans* + Catechol + CTAB plates. Key as for Table 6.3

	Control + Catechol				*S.lacrymans* + Catechol				*S.lacrymans* + Catechol + CTAB			
	Media		Mycelia		Media		Mycelia		Media		Mycelia	
Catechol	250	272	250	272	250	272	250	272	250	272	250	272
0.0 %	++++	+++	++++	++++	++++	++++	+++++	++++	++++	++++	+++++	++++
0.1 %	++++	++++	++++	++++	++++	++++	+++++	++++	++++	++++	+++++	++++
0.5 %	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
1.0 %	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++	++++	++++
5.0 %	++++	++++	+++	++++	++++	++++	++++	+++	+++	+++	++++	+++
10.0 %	++++	++++	+++	+++	++++	+++	++++	+++	+++	+++	++++	+++

Table 6.5 : Relative intensities of absorbencies at 250 nm and 272 nm for the final melanin extractions. Key as for Table 6.3.

	S.lacrymans + Water		*S.lacrymans* + CTAB	
	250	272	250	272
Media	++++	++++	++++	++++
Mycelia	++++	++++	++	++

Table 6.6 : Relative intensity of absorbencies at 250 nm and 272 nm for the *S.lacrymans* cultures supplemented with water and CTAB. Key as for Table 6.3

6.4 Discussion

The role of fungal melanins in enhancing the survival of fungal structures has been known for many years (Bell and Wheeler, 1986). The importance of some fungal melanins became clear when it was discovered that certain plant pathogens (for example, *Pyricularia oryzae*, *Colletotrichum lagenarium* and *C.lindemuthianium* which are pathogens of rice, cucumber and bean respectively) require melanin for penetration of plant tissues (Bell and Wheeler, 1986). A number of fungicides (for example, tricyclazole, pyroquilon, chlobenthiazone and fthalide) have been developed to control fungal plant pathogens by inhibiting melanin synthesis (Bell and Wheeler, 1986). In other fungi melanin production is in response to unfavourable conditions, for example, environmental factors outwith normal growth parameters or the presence of antagonistic organisms (Li, 1981; Bell and Wheeler, 1986). Although melanin production has not yet been fully investigated in wood decay fungi, the presence of phenoloxidase enzymes (such as laccase and tyrosinase) has been shown in certain basidiomycetes, for example, *Hypholoma fasciculare* and *Phlebia radiata* (Griffith *et al*, 1994b). It has been suggested that one of the roles of these enzymes is in the production of melanin or melanin-like compounds (Bell and Wheeler, 1986; Griffith *et al*, 1994a).

The extractions of the field samples determined that melanin was present in all the samples, although the highest levels were found in the fruit-bodies and mycelial specimens. Melanin has been reported in chlamydospores (Bell and Wheeler, 1986), sclerotia (Bloomfield and Alexander, 1967) and spores (Durrell, 1964; Mendoza *et al*, 1979). The melanin present in these structures protects the propagules from adverse environmental conditions and from lytic attack by other microbial organisms. Similarly, melanin has been found in fungal mycelia that may protect it from unfavourable conditions and antagonistic attack both of which the *S.lacrymans* colony at Taymouth Castle had been

exposed to (see Chapter 5). The low level of melanin found in the cord sample was unexpected since this structure would have to endure harsher conditions than those normally experienced by the colony as cords are organs of exploration. It may be possible that the fungal cords had not encountered the trigger required to initiate melanin synthesis, i.e. an unfavourable environment. If this is the case then it is clear that previous exposure to unfavourable conditions does not necessarily result in melanin production in structures developed a period of time after exposure to the trigger. This is not unexpected as this would mean that the fungal colony was not wasting precious resources in protecting fungal structures when it was not necessary.

The inducer/inhibitor experiment indicated that none of the substances tested had any effect upon melanin synthesis by *S.lacrymans*. Both catechol and shikimic acid have been precursors in the production of melanin in fungal species. Piatelli *et al* (1963) showed that catechol was the precursor for the dark pigment, most likely a catechol melanin, found in the spores of *Ustilago maydis*. Percentages of nitrogen, carbon, hydrogen and carboxyl groups in *Ustilago* melanin are nearly identical with those in catechol melanin formed by oxidation of catechol with mushroom tyrosinase (Bell and Wheeler, 1986). Catechol melanin synthesis may proceed through free radicals or quinone-catechol adducts, and although the biosynthetic origin of catechol is unknown, the shikimic acid pathway is suspected (Bell and Wheeler, 1986).

The skikimic acid pathway leads to the formation of an extensive number of end products in addition to the aromatic amino acids (including tyrosine) such as metal chelators, vitamins E and K, folic acid, ubiquinone and plastoquinone (Hawkins *et al*, 1993). The oxidation of tyrosine by tyrosinase leads to the formation of DOPA melanin, which forms the black pigments in animals (Bell and Wheeler, 1986). However, although tyrosinase had been shown to be produced by fungi (see also Chapter 7) the presence of

DOPA melanin has yet to be confirmed and furthermore there is evidence that the phenoloxidase enzyme laccase is the more likely enzyme associated with melanin synthesis (Bell and Wheeler, 1986). The addition of shikimate to the media of *S.lacrymans* seems to indicate that the end products of the shikimate pathway do not have a role in melanin synthesis. This would also seem to cast doubt on the shikimate pathway being the biosynthetic origin of catechol postulated by Bell and Wheeler (1986). However, it is also possible that *S.lacrymans* was unable to take up the shikimate, which therefore meant that it played no role in the biochemical pathways of the fungus.

There is the possibility that the extraction procedure was not leading to the isolation of melanin despite the fact that the procedure was successful with *Armillaria* species (Rizzo *et al*, 1992). However there is also the possibility that melanin (or a melanin-like precursor) is only synthesised when the *S.lacrymans* comes under some form of stress. Laccase has been reported as being involved with melanin synthesis in fungi (Bell and Wheeler, 1986) and looking for the presence of this enzyme could indicate the presence of a melanin synthesis pathway in *S.lacrymans*.

Despite the inability of either EDTA or hexachloroacetone to inhibit melanin synthesis in *S.lacrymans*, the inhibition of melanin synthesis in *S.lacrymans* could lead to an increased susceptibility of the fungus to antagonistic attack. *Trichoderma* species release hydrolytic enzymes such as chitinase and β-1,3-glucanase (Srinivasan *et al*, 1992b). The ability of these enzymes to digest fungal cell walls is inversely related to the melanin content of the walls (Bloomfield and Alexander, 1967; Bull, 1970; Kuo and Alexander, 1967; Bell and Wheeler, 1986). Therefore, if melanin synthesis in *S.lacrymans* could be reduced or inhibited then the presence of antagonistic organisms such as *Trichoderma* spp. would be more effective.

Over the period of the catechol experiment no melanin was released into the culture media. Peaks at 250/272 nm did change over time but this was probably down to the formation of a black pigment produced by the self-polymerisation of the catechol. Although this pigment formation was not due to the presence of the *S.lacrymans*, the cultures did have some effect on the black pigment production in the 0.1% catechol plates (with and without CTAB). This could indicate that the cultures were releasing some unknown compound into the media that could enhance the self-polymerisation of the catechol. *S.lacrymans* has been shown to release phenoloxidase enzymes such as tyrosinase and laccase (Score *et al*, 1998; see also Chapter 7) which can act to produce melanin or melanin-like compounds. This could indicate that *S.lacrymans* can produce melanin and that the substrate is catechol. The presence of the CTAB reduced the production of the pigment and since CTAB is a melanin synthesis inhibitor this could also indicate the ability of *S.lacrymans* to produce melanin.

The ability of *S.lacrymans* to survive abnormal environmental conditions and to adapt to the presence of antagonistic organisms could be explained by its capacity to produce melanin. The presence of melanin has been shown to enhance the survival of fungal structures which makes the production of a biocontrol agent all the more difficult. However, if a biocontrol agent could be found or designed via genetic engineering to produce enzymes that could degrade the melanin then the treatment of dry rot by biocontrol could still be possible. The combination of melanin synthesis inhibitor (for example, tricyclazole) and biocontrol agent could make a more effective treatment. The production of melanin could also be disrupted if the enzymes or biochemical pathway responsible could be found. The production of melanin in some fungi has been shown to be controlled by phenoloxidase enzymes. Prior to the start of this experiment the production of phenoloxidase enzymes by *S.lacrymans* was not shown. Therefore, an

investigation into the possible release of enzymes by *S.lacrymans* was started and this is discussed in Chapter 7.

Chapter 7

Extracellular Phenoloxidase and Peroxidase Enzyme Production During Interspecific Fungal Interactions

7.1 Introduction

During interspecific and intraspecific interactions fungal colonies release metabolites that enhance their ability to capture previously colonised substrates or to defend their own substrate base. Most of these metabolites are non-enzymatic in nature, however, some enzymes are secreted in response to the presence of another fungus. For example, certain *Trichoderma* species release chitinase and laminarinase in order to parasitise susceptible fungal hyphae (Srinivasan *et al.*, 1992a). Phenoloxidase enzymes (such as laccase and tyrosinase) have been implicated in a range of roles, one of which is in mycelial morphogenesis where the fungal mycelium undergoes changes in response to the presence of another fungus (Rayner *et al*, 1994; White and Boddy, 1992). Peroxidase enzymes have been implicated in the production of highly toxic compounds that are antifungal in nature, in the production of melanin and melanin-like pigments (Li, 1981) and in the formation of different mycelial formations (Rayner *et al*, 1994).

Laccase (ρ-diphenol oxidase, E.C. 1.10.3.2) is a member of the family of blue copper oxidases, which also includes the plant ascorbate oxidases and the mammalian protein ceruloplasmin (Thurston, 1994). Laccases are widespread in nature and are produced by plants, fungi and bacteria. Laccases oxidise phenolic compounds with the resulting reduction of oxygen to water, and they can also demethylate, polymerise and depolymerise phenolic compounds (Coll *et al*, 1993). They are relatively non-specific as to their reducing substrate, and the range of substrates oxidised varies from one laccase to another (Thurston, 1994). Laccases can oxidise monophenols, o- and ρ-diphenols, aminophenols and diaminoaromatic compounds (DeVries *et al*, 1986). These enzymes

have been implicated in lignin degradation and/or detoxification of lignin degradation products, pigmentation accumulation, sporulation (DeVries *et al*, 1986), detoxification of toxic compounds (Dean and Eriksson, 1994), plant pathogenesis (Thurston, 1994) and mycelial morphogenesis (Rayner *et al.*, 1994). Laccase isoenzymes can vary between and within species, and can also have different functions within these species and under different environmental conditions (Assavanig *et al*, 1992).

Tyrosinase (monophenol monooxygenase, E.C. 1.14.18.1) is a copper-containing monooxygenase which catalyses the o-hydroxylation of monophenols (cresolase or monophenolase activity) and the oxidation of o-diphenols to o-quinones (catecholase or diphenolase activity) (Lerch, 1995). Tyrosinase is widely distributed in bacteria, fungi, plants and animals, where it is involved in the biosynthesis of melanins and other polyphenolic compounds (Lerch, 1995). Tyrosinase oxidises L-DOPA and enkephalins (*in vitro*) to form melanin and melanin-like pigments (Choi and Sapers, 1994; Rosei *et al*, 1992).

Peroxidase enzymes (such as lignin peroxidase and manganese peroxidase) are capable of oxidising a variety of compounds producing radicals that spontaneously degrade via non-enzymatic reactions to yield a variety of aliphatic and aromatic end products. The peroxidase enzyme from groundnut is known to catalyse the oxidation of mono- and diphenols and aromatic amines to the highly toxic quinones in the presence of hydrogen oxides. Groundnut peroxidase is also toxic to the growth of the wheat rust pathogen *Puccinia graminis* f. sp. *triciti* (Velashahan and Vidhyasekaran, 1994).

All of these enzymes may have some role to play in the offence and defence of fungi during interactions. Interactions involving *S.lacrymans* have shown that this fungus undergoes certain pigmentation changes and phenoloxidase/peroxidase enzymes may

control these. This study was designed to determine whether phenoloxidase and peroxidase enzymes were involved in the interaction of *S.lacrymans* with *Trichoderma* species.

7.2 Methods

7.2.1 Fungal organisms used in the experiment

Basidiomycetes - *Serpula lacrymans* (FPRL 12C), *Coniophora puteana* FPRL 11E, *Neolentinus lepideus* and *Gloeophyllum trabeum* (obtained from the Building Research Establishment, Garston, UK).

Deuteromycetes - *Trichoderma harzianum* SIWT 25, *T.viride* SIWT 60, *T.viride* SIWT 110 [for origins of the *Trichoderma* spp. see Srinivasan *et al.* (1992a)] and *Scytalidium* FY (for origin of this isolate see Bruce and King, 1983).

All fungi were maintained on malt extract agar (5% w/v malt extract and 2% w/v agar in distilled water) and stored at 4°C ± 1°C.

7.2.2 Enzyme Assays

Enzyme tests were carried out as described by Poppe and Welvaert (1983). The following enzyme substrates were prepared for qualitative assaying of extracellular enzymes: for the laccase assay, 1 x 10^{-3}M α-naphthol in 96% ethanol, for the tyrosinase assay, 1 x 10^{-3}M ρ-cresol in 96% ethanol and for the peroxidase assay, 0.79 x 10^{-3}M pyragallol in distilled water (the pyragallol mixture was added to an equal volume of 0.4% hydrogen peroxide prepared in distilled water immediately prior to the assay). The culture plates were flooded with 5 mls of the relevant substrate, which was then immediately poured off. The plates were left at room temperature for 24-48 hours to allow any colour reaction to develop.

7.2.2.1 The effect of fungal interaction on enzyme release

Enzyme assays were carried out on pure and mixed fungal cultures (each isolate was paired against the others isolates in succession), grown both on malt extract agar and a minimal medium designed to reflect the carbon:nitrogen ratio in wood (Score and Palfreyman, 1994). All plates were inoculated using 5 mm diameter cores taken from stock culture plates and interaction studies were carried out as described in Score and Palfreyman (1994). Pure cultures were incubated until the colony periphery was almost at the edge of the petri-dishes. Two identical sets of confrontations were carried out with one set being incubated until the colony peripheries had just begun to intermingle (termed 'incomplete' interaction) and the second set incubated until either a deadlock or replacement result had been achieved (termed 'complete' interaction). Four duplicate plates were prepared for each enzyme assay.

7.2.2.2 The effect of different amino acids upon the release of the enzymes

S.lacrymans and *T.harzianum* 25 were grown on malt extract agar and minimal media supplemented with different amino acids. Each of the amino acids contain varying amounts of nitrogen, therefore, the amount of amino acid added was calculated to maintain the carbon:nitrogen level at that achieved by adding asparagine. The following amino acids were added to the different batches of minimal medium (with the amounts added in brackets) : asparagine (1.3×10^{-2} gl^{-1}; control); tyrosine (3.6×10^{-2} gl^{-1}), tryptophan (2.0×10^{-2} gl^{-1}), phenylalanine (3.2×10^{-2} gl^{-1}), threonine (02.3×10^{-2} gl^{-1}) and asparagine/tyrosine mixture (6.5×10^{-3} gl^{-1}/1.8×10^{-2} gl^{-1}). Interactions and assays were carried out as in 7.2.2.1.

7.2.2.3 The effect of temperature stress upon production of laccase by *S.lacrymans*

Twelve plates were inoculated with *S.lacrymans* and incubated at 22°C ± 1°C until a minimum colony diameter of 20 mm had been achieved. Four plates were then removed and incubated at 4°C ± 1°C, with another set of four removed and incubated at 28°C ± 1° C, and the final four plates left at 22°C ± 1°C. All plates were then incubated for a further 72 hours at which time the *S.lacrymans* incubated at 22°C ± 1°C had covered the plates and underwent enzyme testing. The 4°C and 28°C plates were returned to 22°C ± 1°C and incubated for another 48 hours. The cultures were then tested for enzyme production as in 7.2.2.1.

7.3 Results

7.3.1 Growth Observations

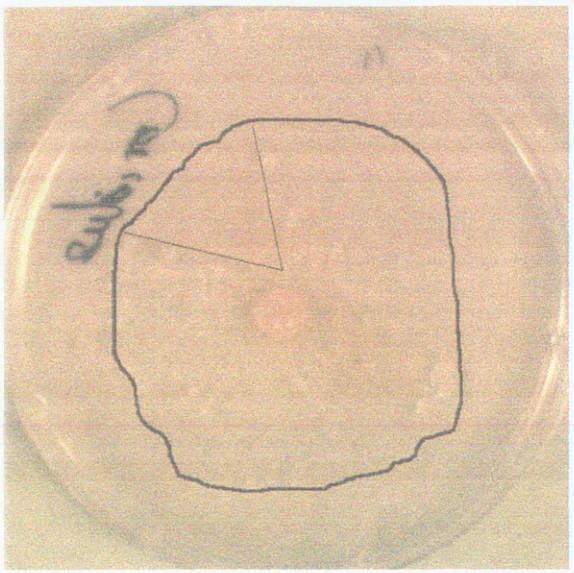
Trichoderma harzianum 25 began to replace both *S.lacrymans* (causing a yellowing and browning of the mycelia) and *C.puteana*. However, *T.harzianum* 25 produced deadlock reactions against the other *Trichoderma* isolates and *Scytalidium* FY indicating somatic incompatibility. All the other fungi produced deadlock reactions against each other, although *S.lacrymans* did start to grow over *Scytalidium* FY.

Fungal growth on the two media types was generally similar, although hyphal extension rates on the minimal media were slower. However, *C.puteana* did show two different mycelial morphs on the minimal medium (Figure 7.1). The initial morph was similar to that seen growing on the malt extract agar, although there was a reduction in the amount of aerial mycelia. The subsequent morph showed zonation where part of the colony appeared similar to that of the first morph, but the remainder of the colony showed appressed mycelium and the medium beneath these areas appeared semi-liquid in nature.

7.3.2 Qualitative enzyme activities

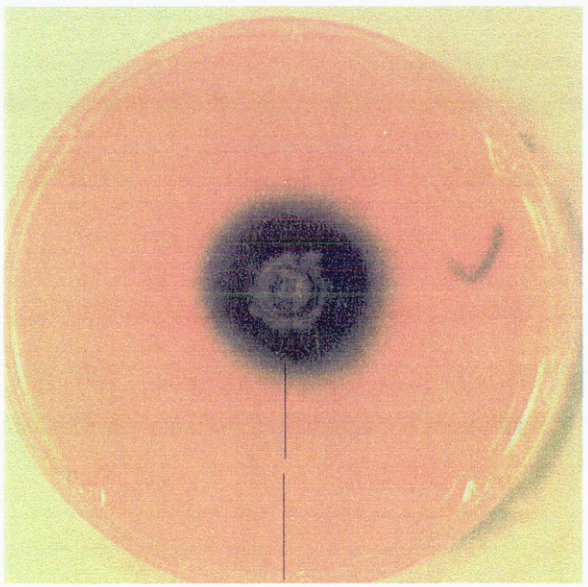
7.3.2.1 Pure cultures

Tables 7.1 and 7.2 give the results for the enzyme analyses carried out on the pure fungal cultures. *C.puteana* (Figure 7.2) and *Scytalidium* FY were the only isolates to produce laccase in pure culture. Laccase activity was evident under the entire *Scytalidium* FY colony. Only a small area around the *C.puteana* inoculum showed activity that coincided with the location of aerial mycelium around the inoculum. The zonation produced by *C.puteana* on minimal medium influenced the results as no activity (for either laccase and tyrosinase) was observed within the appressed colony areas above the semi-liquid portions of the medium. Tyrosinase activity was detected in pure cultures of



Colony periphery

Figure 7.1 : Zonation produced by *C.puteana* when grown on minimal media.



Laccase staining

Figure 7.2 : *C.puteana* grown on malt extract agar and stained for laccase release.

S.lacrymans, *N.lepideus*, *C.puteana*, *T.harzianum* 25 and *T.viride* 60 when grown on malt extract agar. However, when transferred to minimal medium *S.lacrymans* and *T.harzianum* 25 colonies stopped releasing the enzyme and *T.viride* 110 produced it. *S.lacrymans*, *C.puteana* and *Scytalidium* FY released peroxidase when grown on malt extract agar although the three *Trichoderma* isolates did release peroxidase when grown on minimal medium.

7.3.2.2 Incomplete interaction studies

In the incomplete interactions between *S.lacrymans* and the *Trichoderma* isolates on malt extract agar (Figure 7.3), laccase staining was observed at those points in the interaction zone where the two fungi had been in contact for the longest period of time, which coincided with areas of browning of the *S.lacrymans* mycelium. Laccase was also detected in pairings of *T.harzianum* 25 and *G.trabeum*/*C.puteana* and *T.viride* 110 and *C.puteana*. On minimal medium, laccase was detected in the pairings of *G.trabeum*/*N.lepideus* with the *Trichoderma* isolates, and in the pairings of the *Trichoderma* isolates with *Scytalidium* FY.

Tyrosinase activity appeared throughout the entire fungal colony (as for *T.harzianum* SIWT 25 paired against itself, Figure 7.4) or was located away from the interaction zone (as for *T.harzianum* SIWT 25 paired with *T.viride* SIWT 110, Figure 7.5). Staining was not localised within the interaction zone only. Tyrosinase production by *T.harzianum* SIWT 25 was not inhibited during the interactions, whereas enzyme production by *S.lacrymans*, *T.viride* SIWT 60 and *C.puteana* was affected by the confrontation of other fungal isolates. On minimal media, tyrosinase production was reduced although pure cultures of *T.viride* SIWT 110 did release tyrosinase on this medium.

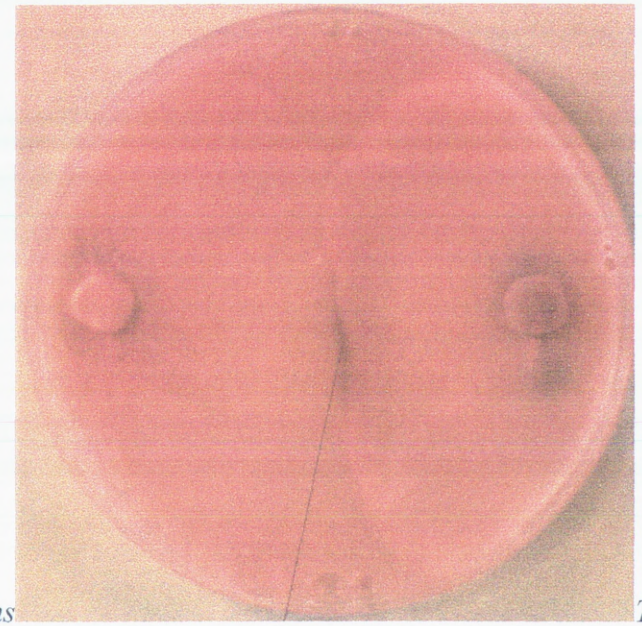


Figure 7.3 : Pairing between *S.lacrymans* and *T.viride* on malt extract agar stained for laccase release.

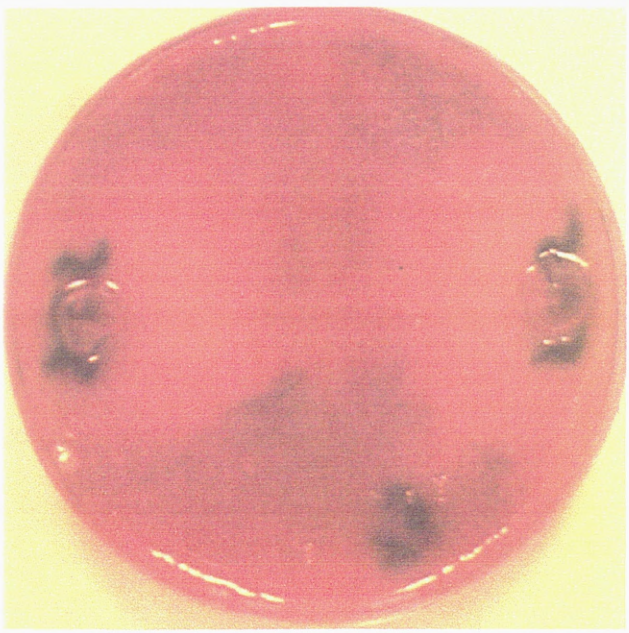


Figure 7.4 : Self-pairing of *T.harzianum* on malt extract agar stained for tyrosinase release.

All fungi tested released peroxidase with *S.lacrymans*, *Scytalidium* FY and *C.puteana* releasing it in pure cultures grown on malt extract agar. The three *Trichoderma* isolates released peroxidase when paired against other fungi, but peroxidase release was inhibited during certain *Trichoderma*/*Trichoderma* pairings. Peroxidase release by *T.viride* SIWT 110 was only detected when paired against *Scytalidium* FY and *C.puteana* on malt extract agar. On minimal media, all three *Trichoderma* isolates released peroxidase with the production of peroxidase by *T.viride* SIWT 60 being inhibited by *S.lacrymans* and *T.viride* SIWT 110. Peroxidase release by *T.viride* SIWT 110 was affected when it was paired against itself and the other *Trichoderma* isolates. Peroxidase activity could be detected in all areas of the fungal colony, however, the strongest staining was in the interaction zones of pairings involving *S.lacrymans* on malt extract agar (Figure 7.6). In the pairing of *S.lacrymans* and *T.viride* SIWT 110, mycelial fans were produced by the *S.lacrymans* that proceeded to advance over the periphery of the *T.viride* SIWT 110 colony (Figure 7.7).

7.3.2.3 Completed interactions

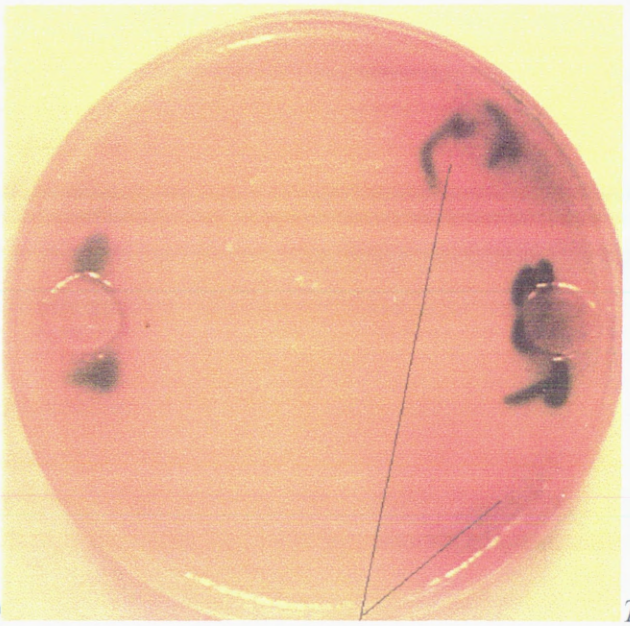
Tables 7.3 and 7.4 show the results from the analyses carried out on the completed interactions. The results of the completed interactions, i.e., overgrowth or deadlock are also indicated. The *Trichoderma* isolates tended to overgrow the basidiomycetes and the *Scytalidium* FY, with *T.harzianum* 25 invading the *T.viride* 60 colony. All other interactions resulted in deadlock reactions, with the exceptions of *S.lacrymans* overgrowing *C.puteana*, *Scytalidium* FY and *T.viride* 60. This was reflected in the minimal medium results although the number of overgrowths by the *Trichoderma* isolates was reduced. Overgrowths of *N.lepideus* by *Scytalidium* FY and *C.puteana* by *G.trabeum* were also noted.

Fungus	Laccase	Tyrosinase	Peroxidase	Competitor	Laccase	Tyrosinase	Peroxidase
S12		T	P	-	-	-	-
S12		T	P	S12		T	P
S12		T	P	NL		T	P
S12			P	GT			
S12			P	CP	L	T	P
S12	L?	T	P	T25	L?	T	P
S12	L?	T	P	T60	L?	T	P
S12	L?		P	T110	L?		
S12		T	P	SCY	L		P
NL		T		-	-	-	-
NL				NL			
NL				GT			
NL		T	P	CP	L	T	P
NL			P	T25		T	
NL		T		T60			P
NL		T		T110			P
NL		T		SCY	L		P
GT				-	-	-	-
GT				GT			
GT		T	P	CP	L	T	
GT				T25	L	T	P
GT				T60		T	P
GT				T110	L		P
GT				SCY	L		P
CP	L	T	P	-	-	-	-
CP	L		P	CP	L		P
CP	L	T	P	T25	L	T	P
CP	L		P	T60			P
CP	L		P	T110			P
CP	L		P	SCY	L		P
T25		T		-	-	-	-
T25		T	P	T25		T	P
T25		T	P	T60		T	
T25		T	P	T110			
T25		T	P	SCY	L		P
T60		T		-	-	-	-
T60			P	T60			P
T60			P	T110			
T60		T	P	SCY	L		P
T110				-	-	-	-
T110				T110			
T110			P	SCY	L		P
SCY	L		P	-	-	-	-
SCY	L		P	SCY	L		P

Table 7.1 : Enzymes detected in pure cultures and incomplete interactions carried out on malt extract agar. A blank space indicates that no enzyme release was detected. Key : S12 - *S.lacrymans* 12C; NL - *N.lepideus*; GT - *G.trabeum*; CP - *C.puteana*; T25 - *T.harzianum* SIWT 25; T60 - *T.viride* SIWT 60; T110 - *T.viride* SIWT 110; SCY - *Scytalidium* FY; '-' no result; '?' indicates that the source of the enzyme was not determined, with no '?' indicating that the enzyme was identified as being produced by a specific colony. Bold letters indicate enzyme release detected during pairings that was not detected in pure cultures.

Fungus	Laccase	Tyrosinase	Peroxidase	Competitor	Laccase	Tyrosinase	Peroxidase
S12			P	-	-	-	-
S12			P	S12			P
S12				NL			
S12				GT			
S12			P	CP			
S12	L?			T25	L?		P
S12				T60			
S12			P	T110			P
S12		T	P	SCY	L		
NL	L	T		-	-	-	-
NL			P	NL			P
NL				GT			
NL		T	P	CP	L	T	P
NL	L	T	P	T25	L	T	P
NL	L	T	P	T60	L	T	P
NL	L	T	P	T110	L	T	P
NL		T		SCY	L		
GT				-	-	-	-
GT				GT			
GT		T		CP		T	P
GT	L		P	T25	L		P
GT	L			T60			P
GT	L	T	P	T110			P
GT		T	P	SCY	L		P
CP	L	T	P	-	-	-	-
CP	L	T	P	CP	L	T	P
CP	L		P	T25			P
CP	L		P	T60			P
CP	L		P	T110			P
CP	L		P	SCY	L		P
T25			P	-	-	-	-
T25			P	T25		T	P
T25		T	P	T60		T	P
T25		T	P	T110			
T25	L		P	SCY	L		P
T60		T	P	-	-	-	-
T60			P	T60			P
T60			P	T110			
T60	L		P	SCY	L		P
T110		T	P	-	-	-	-
T110				T110			
T110	L		P	SCY	L		P
SCY	L		P	-	-	-	-
SCY	L		P	SCY	L		P

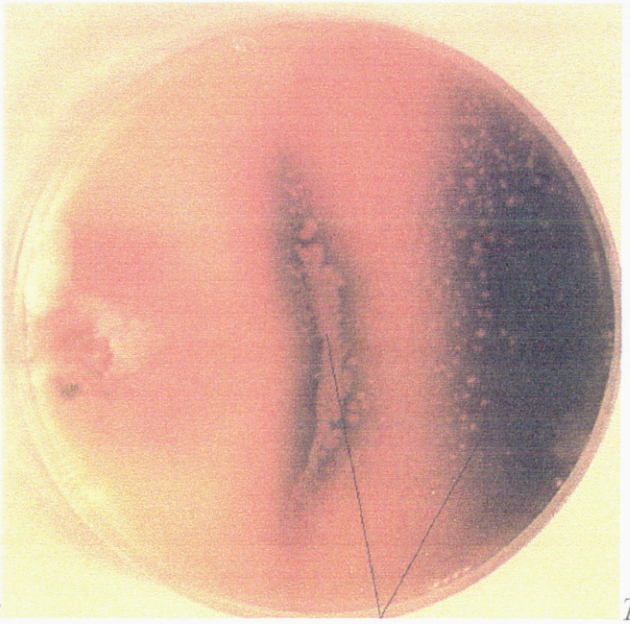
Table 7.2 : Enzymes detected in pure cultures and incomplete interactions carried out on minimal medium. A blank space indicates that no enzyme release was detected.



T.viride 110 *T.harzianum* 25

Tyrosinase staining

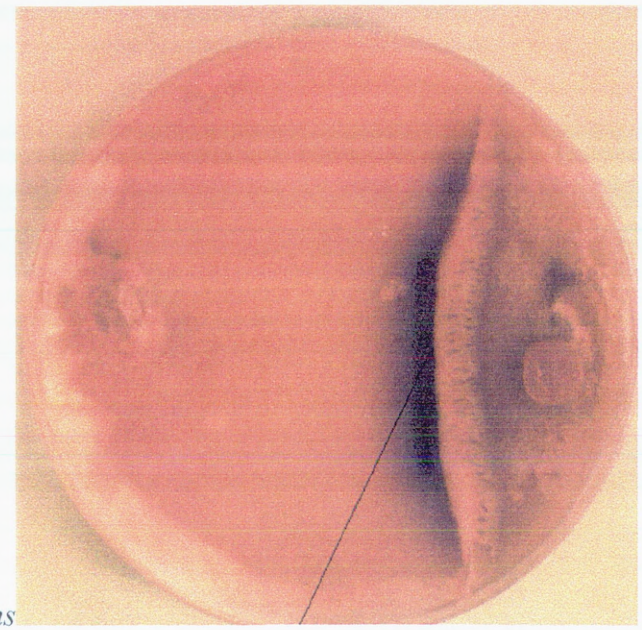
Figure 7.5 : Pairing of *T.harzianum* and *T.viride* 110 grown on malt extract agar and stained for tyrosinase release.



S.lacrymans *T.harzianum* 25

Peroxidase staining

Figure 7.6 : Pairing between *S.lacrymans* and *T.harzianum* 25 grown on malt extract agar stained for peroxidase release.



S.lacrymans *T.viride* 110

Peroxidase staining

Figure 7.7 : Pairing between *S.lacrymans* and *T.viride* 110 grown on malt extract agar and stained for peroxidase release. Note the overgrowth of the *T.viride* 110 colony periphery by *S.lacrymans*.

Fungus	Laccase	Tyrosinase	Peroxidase	Competitor	Laccase	Tyrosinase	Peroxidase
S12		T	P	-	-	-	-
S12	L	T	P	S12[D]	L	T	P
S12	L	T	P	NL[D]		T	P
S12		T	P	GT[D]	L	T	P
S12	L	T	P	CP[O(S)]	L	T	
S12	L		P	T25[O(T)]		T	
S12			P	T60[O(S)]			
S12	L	T	P	T110[D]		T	P
S12	L	T	P	SCY[O(S)]	L		P
NL		T		-	-	-	-
NL		T	P	NL[D]		T	P
NL	L	T	P	GT[D]			
NL		T	P	CP[D]	L	T	P
NL		T	P	T25[O(T)]			P?
NL		T	P	T60[O(T)]			
NL		T	P	T110[O(T)]	L		P
NL		T	P	SCY[D]	L		P
GT				-	-	-	-
GT				GT[D]			
GT			P	CP[D]	L	T	P
GT	L	T	P	T25[O(T)]		T	P
GT	L	T	P	T60[O(T)]			P
GT			P	T110[O(T)]			P
GT		T	P	SCY[D]	L		P
CP	L	T	P	-	-	-	-
CP	L	T	P	CP[D]	L	T	P
CP	L	T	P	T25[O(T)]		T	
CP	L	T	P	T60[O(T)]			
CP	L	T	P	T110[D]			P
CP	L	T	P	SCY[D]	L		P
T25		T		-	-	-	-
T25		T		T25[D]		T	
T25		T	P	T60[O(25)]		T	P
T25		T	P	T110[D]		T	P
T25	L	T	P	SCY[O(T)]	L		P
T60		T		-	-	-	-
T60		T	P	T60[D]		T	P
T60			P	T110[D]		T	P
T60		T	P	SCY[O(T)]	L		P
T110				-	-	-	-
T110		T	P	T110[D]		T	P
T110		T	P	SCY[D]	L		P
SCY	L		P	-	-	-	-
SCY	L			SCY[D]	L		

Table 7.3 : Enzymes detected in pure cultures and complete interactions carried out on malt extract agar. A blank space indicates that no enzyme release was detected. Key as for Table 7.1. The interaction results are given in the 'Competitor' column. Interaction results key : O - overgrowth; D - deadlock; S - *S.lacrymans*, T - *Trichoderma*, 25 - *T.harzianum* 25

Fungus	Laccase	Tyrosinase	Peroxidase	Competitor	Laccase	Tyrosinase	Peroxidase
S12			P	-	-	-	-
S12			P	S12[D]			P
S12			P	NL[D]	L	T	P
S12	L?	T		GT[D]	L?	T	P
S12			P	CP[O(S)]		T	
S12	L		P	T25[O(T)]			P
S12		T	P	T60[D]		T	
S12	L	T	P	T110[D]		T	P
S12	L	T	P	SCY[D]	L		P
NL	L	T	P	-	-	-	-
NL	L	T	P	NL[D]	L	T	P
NL	L	T	P	GT[D]	L	T	P
NL	L	T	P	CP[D]	L	T	P
NL	L	T	P	T25[O(T)]			P
NL	L	T	P	T60[O(T)]		T	P
NL	L	T	P	T110[O(T)]			P
NL	L	T		SCY[O(F)]	L		
GT				-	-	-	-
GT			P	GT[D]			
GT		T		CP[O(G)]		T	P
GT	L		P	T25[O(T)]			P
GT			P	T60[D]			
GT	L		P	T110[D]			P
GT	L	T	P	SCY[D]	L	T	P
CP	L	T	P	-	-	-	-
CP	L	T	P	CP[D]	L	T	P
CP	L		P	T25[O(T)]			P
CP	L		P	T60[O(T)]			P
CP	L		P	T110[D]			P
CP	L		P	SCY[D]	L		P
T25			P	-	-	-	-
T25		T	P	T25[D]		T	P
T25		T	P	T60[D]		T	P
T25		T	P	T110[D]	L		P
T25	L		P	SCY[D]	L		P
T60		T	P	-	-	-	-
T60			P	T60[D]			P
T60		T	P	T110[D]			P
T60	L		P	SCY[D]	L		P
T110		T	P	-	-	-	-
T110			P	T110[D]			P
T110	L		P	SCY[D]	L		P
SCY	L		P	-	-	-	-
SCY	L		P	SCY[D]	L		P

Table 7.4 : Enzymes detected in pure cultures and complete interactions carried out on minimal media. A blank space indicates that no enzyme release was detected. Key as for Table 7.2, including in the interaction results key : G - *G.trabeum*; F - *Scytalidium FY.*

There are a number of differences between the incomplete and complete interaction staining results. Generally, enzyme release was detected in more of the completed interactions. However, in certain pairings enzymes that were detected at the beginning of the interactions were not detected at their completion.

In the malt extract agar completed pairings laccase production was produced by *S.lacrymans* in the majority of its pairings. However, in those pairings of *S.lacrymans* overgrowth by *T.harzianum* 25, laccase staining was limited to those brown pigmented areas of the *S.lacrymans* colony that were produced upon initial contact with the antagonist. Laccase was also detected in selected pairings of *N.lepideus* and *G.trabeum.* With the exception of *T.viride* 110 against *N.lepideus*, laccase was not detected in those *Trichoderma* pairings where the *Trichoderma* overgrew the competitor fungus. In contrast, in at least some of these pairings, laccase was produced by the colony that was being invaded by the *Trichoderma* isolates. On minimal media the results were generally similar although laccase production was detected in all of the *N.lepideus* pairings irrespective of interaction result. Again, laccase was produced by those basidiomycetes that were overgrown by the *Trichoderma* isolates, with no laccase produced by the invading colonies.

Tyrosinase release was detected in more of the completed interactions rather then the incomplete interactions. There were, however, examples of tyrosinase being released at the being of the interaction and not being detected in the completed pairings, e.g., *G.trabeum* against *C.puteana* and *T.viride* 60 against *S.lacrymans* and *G.trabeum.* On minimal medium, tyrosinase release was reduced, with *S.lacrymans*, *C.puteana* and *T.harzianum* 25 being the most affected. Peroxidase release was induced in *N.lepideus*, *G.trabeum* and in the *Trichoderma* isolates in the majority of the pairings on malt extract

agar involving these isolates. With few exceptions, most pairings showed peroxidase release and no obvious pattern could be determined.

7.3.2.4 Effect of amino acid content upon enzyme production

In general, the pure cultures and competitive pairings were visually similar to those described in 7.3.2.1 (Table 7.5). However, the one notable difference concerned *S.lacrymans* growing on minimal medium containing tyrosine. The colony advanced onto the agar but after 2-3 days the *S.lacrymans* stopped growing and the colony mycelium appeared dense and compact in nature. In pure culture the only amino acid that had any effect upon enzyme release was tyrosine which induced the production of laccase from *S.lacrymans*. In the pairings, the results on malt extract agar and minimal medium (plus asparagine) mirrored those recorded for the interactions described previously. Laccase release by *S.lacrymans* was also recorded on minimal media supplemented with tyrosine, tryptophan and the asparagine/tyrosine mixture. Despite producing peroxidase in pure culture when grown on minimal medium plus phenylalanine, enzyme release by *S.lacrymans* was not detected in the interaction pairing on this medium. Enzyme release by the *T.harzianum* 25 was not influenced by the amino acid present within the minimal medium.

7.3.2.5 Effect of temperature upon laccase release by *S.lacrymans*

When switched from the normal incubation of 22°C ± 1°C, the *S.lacrymans* stopped growing at 4°C and 28°C (Figure 7.8). Growth did resume, although at a slower rate, when the plates at 4°C were returned to 22°C. During the 48 hours incubation at 22°C after 28°C the colonies showed minimal signs of growth with minor increases in the colony diameters. After adding the laccase substrate, staining was observed on the *S.lacrymans* cores on the 22°C and 4°C plates. However, actual colony staining was only observed on the colonies incubated at 28°C (Figure 7.9).

Organism	Media Type						
	MEA	MM+Asp	MM+Try	MM+Thr	MM+Tyr	MM+Phe	MM+Asp/Tyr
S12	P	P	P	P	L	P	L
T25	P	P	P	P	P	P	P
S12 / T25	LTP/TP	L/P	LT/P	T/P	L/P	-/P	L/P

Table 7.5 : Extracellular enzyme production by pure and mixed cultures of *S.lacrymans* and *T.harzianum* 25. Key : S12 - *S.lacrymans*; T25 - *T.harzianum* 25; MEA - malt extract agar; MM - minimal medium; Asp - asparagine; Try - tryptophan; Thr - threonine; Tyr - tyrosine; Phe - phenylalanine; L - laccase detected; T - tyrosinase detected; P - peroxidase detected. - indicates no enzymes detected.

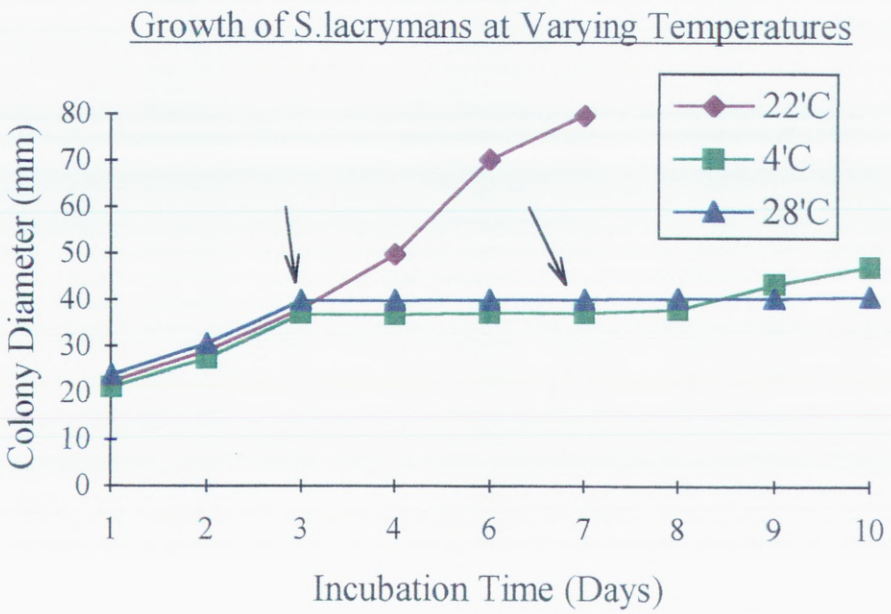


Figure 7.8 : Growth of *S.lacrymans* at 4°C, 22°C and 28°C. Arrows indicate changes in incubation temperature at days 3 and 7. The colony growing at 22°C reached the plate edge at day 7 and incubation was stopped.

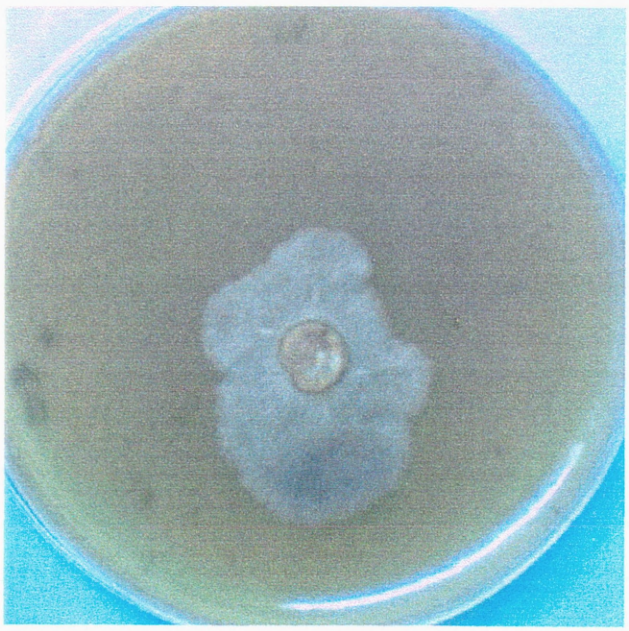


Figure 7.9 : Laccase staining produced on a *S.lacrymans* colony incubated at 28°C.

7.4 Discussion

Confrontation experiments carried out on agar media are often used for screening fungal species that could be potentially used as biocontrol organisms. Previous studies have indicated that nutrient composition is an important factor in the success of an antagonistic fungus (Srinivasan *et al*, 1992a; Score and Palfreyman, 1994). Most studies have centred upon improving the antagonistic fungus' production of compounds of an antibiotic nature. One possible way of increasing the effectiveness of biocontrol would be to enhance the ability of a fungus to invade previously colonised substrates without relying on the production of antifungal metabolites.

Laccase has been hypothesised to have a number of different functions and it has been suggested that the family of laccase isoenzymes can carry out different reactions (Dean and Eriksson, 1994). Many organisms are known to produce laccases, but no brown-rot Basidiomycetes have been reported to produce laccase. However, Dsouza *et al*, (1996) reported that *G.trabeum* did have a nucleotide sequence that coded for the N-terminal domains of known basidiomycete laccases. In this study, one brown-rot fungus, *C.puteana*, was shown to produce laccase in pure culture, and *S.lacrymans*, *N.lepideus* and *G.trabeum* released extracellular laccase in response to the presence of an antagonistic organism. Similarly, there has been no report of *Scytalidium* FY releasing laccase in pure or mixed culture, but the release of laccase by *Trichoderma* spp. has been reported previously (Assavanig *et al*, 1992).

The purpose of these experiments was to determine if extracellular phenoloxidase and peroxidase enzymes were involved during the initial contact of antagonistic fungi. From the results, it can be seen that there are two main inducers to the production of laccase. The first is shown by *C.puteana* and *Scytalidium* FY in pure culture where the enzyme activity is probably induced by the presence of certain components in the medium.

The second is shown in confrontations involving the *Trichoderma* isolates where laccase is released in response to competition for the available substrate.

During interactions involving the *Trichoderma* and *S.lacrymans* isolates, laccase release was detected in the interaction zone suggesting that it is likely to be involved in the offensive strategy of the *Trichoderma* isolate or the defensive strategy of the *S.lacrymans*. Although in the incomplete interactions the pattern of staining did not indicate which of the two fungal isolates released the laccase, microscopic examination indicated that the staining was located on the *S.lacrymans* hypha. In the completed interactions the staining was limited to the area of the *S.lacrymans* colony that had been invaded by the *Trichoderma* isolate after 1-2 days of contact. Evidence suggests that extracellular enzymes secreted by Basidiomycete fungi remain closely associated with the hyphae, possibly within a hyphal sheath (Wood, 1985). Studies have shown that by acting on phenolic compounds, laccase can produce reactive quinone compounds that may be involved in oxidative polymerisations with cell surface components, such as carbohydrates or proteins, resulting in a change of hydrophobicity of the hyphal walls. These reactions might serve to chemically cross-link adjacent hyphae thus leading to the construction of aggregated tissue (for example, hyphal strands) from spatially divergent hyphae (Wood, 1985). The data reported here suggest that *S.lacrymans* is either attempting to produce invasive hyphae (such as hyphal strands) or hyphal barrages designed to stop invasion (Rayner *et al*, 1994; White and Boddy, 1992; Li, 1981). The supposition that the laccase is being released in a defensive response is supported by the fact that all the brown-rot fungi produced laccase in response to invasion of the colony by the *Trichoderma* isolates.

Changes in hyphal hydrophobicity could also reduce or negate the effectiveness of the hydrolytic enzymes released by *Trichoderma* isolates when parasitising susceptible hyphae. Laccase is also involved in the formation of pigments (Thurston, 1994) and

melanin deposition (Dean and Eriksson, 1994). Interaction between *S.lacrymans* and *Trichoderma* isolates invariably result in the production of yellow and brown pigmentation in *S.lacrymans* (Score and Palfreyman, 1994). Production of intra- and extracellular pigments may implicate phenoloxidase enzymes as a factor in interaction responses. The formation of pigments may indicate the formation of melanin or melanin-like compounds that could protect hyphal structures from hydrolytic enzymes and antagonistic organisms (Rizzo *et al*, 1992).

Since laccase release by *Trichoderma* isolates has been reported previously (Assavanig *et al*, 1992) and was seen during the interaction with *C.puteana* a hypothesis may be made that the *Trichoderma* isolates release laccase to appropriate the media already occupied by the *S.lacrymans*. The *Trichoderma* isolates may also produce laccase to detoxify compounds released by the *S.lacrymans* as part of a chemically orientated defence system. With the possible formation of melanin-like pigments by *S.lacrymans*, the *Trichoderma* isolates may be releasing laccase to degrade these compounds. Laccase release by the *Trichoderma* isolates was detected at the beginning of the interaction but not at its completion, indicating that the laccase is important in the initial invasion of the competitor fungal colony.

Tyrosinase activity was apparently affected by the presence of a confronting isolate and on which medium the interaction was carried out on. For example, on malt extract agar *S.lacrymans* only released tyrosinase during self-pairings and in interaction with *Scytalidium* FY, although on minimal medium tyrosinase production was inhibited in self-pairings and in interactions with the *Trichoderma* species or *C.puteana*. One fungal isolate, *Scytalidium* FY only released tyrosinase on one occasion (a completed interaction against *G.trabeum* on minimal medium) despite a deadlock reaction being produced on both media types used. Tyrosinase was detected more in the completed interactions and

production of this enzyme is likely to be time-dependent, for example, *T.viride* 110 released tyrosinase in a number of confrontations that were completed but never in those that were incomplete. It is also possible that minimal media contains an inducer to tyrosinase release by *T.viride* 110 since only pure cultures grown on minimal media showed tyrosinase release.

Tyrosinase is known for its ability to form melanin and melanin-like pigments from phenolic compounds such as L-tyrosine, L-DOPA and GHB (Choi and Sapers, 1994). Therefore, it is possible that if the production of insulating pigments was linked to the defensive strategy of fungi, then tyrosinase would be detected in the interaction zone as would laccase. Since tyrosinase was not detected in the interaction zones of any of the fungal pairings, then either the formation of pigments does not play a role in fungal defence with these fungi, or tyrosinase production can be inhibited by the presence of other fungi. It is also possible that it does not play a role in pigment production. However, since pigments are produced in certain interactions, notably between *S.lacrymans* and the *Trichoderma* isolates, then another pathway for pigment production must be in operation within the interaction zone.

On malt extract agar, S.lacrymans and Scytalidium FY produced peroxidase in pure culture. The three *Trichoderma* isolates only released peroxidase during interactions that may support the substrate acquisition strategy. Peroxidase release was enhanced on minimal media indicating that some component of this medium stimulated the release of peroxidase or, conversely, malt extract agar contained a repressor. Peroxidase staining was predominant in the interaction zones of certain pairings indicating that peroxidase enzymes are being released as part of an offensive/defensive strategy operated by the *S.lacrymans* and the *Trichoderma* isolates. This strategy could be linked to the release of laccase that was also detected in the interaction zone. Peroxidase activity has been linked to the

development of certain structures such as fruit bodies, sclerotia and rhizomorphs (Nia White, personal communication) and to the formation of melanin-like pigments (Li, 1981). S.lacrymans produced Mycelial fans when paired with *T.viride* SIWT 110, and although the fans themselves did not release peroxidase, the area of the colony periphery immediately behind the fans did. This could indicate that peroxidase activity is linked to the production of mycelial formations designed to invade opposing fungal colonies. It may also be possible that the peroxidase is being released in an attempt to produce pigments to resist invasion by other fungi.

The effects of the amino acid content in minimal media did influence the release of laccase, tyrosinase and peroxidase. Minimal media containing tyrosine resulted in poor growth of the *S.lacrymans* colony with corresponding dark pigmentation at the colony edges and also induced the release of laccase, both in pure and mixed culture with *T.harzianum* 25. This could indicate that either the tyrosinase was an inducer of laccase production or the amino acid was being used to construct a polyphenolic compound via the actions of laccase. The other amino acids did influence enzyme release during the interactions with tyrosinase release induced by tryptophan and threonine, but threonine and phenylalanine inhibited laccase release.

S.lacrymans has a maximum growth temperature of 28°C when growth stops (Thornton, 1989; Sienkiewicz *et al*, 1997) and this is shown in Figure 7.8. Even when the colony is returned to 22°C no growth is detected, which conflicts with the 4°C incubated colonies that did regrow when returned to 22°C. Laccase was detected in those colonies incubated at 28°C and this may indicate that the laccase was being used in some way to protect the colony from the high temperature. *S.lacrymans* has a relatively low temperature maximum (26°C, White *et al*, 1995) and temperatures above this result in heat stress and eventual death of the fungus. It has been shown that incubation of *S.lacrymans* at 28°C

induces the production of a heat shock protein providing thermotolerance, for at least a limited period of time. This may indicate that laccase is either a heat shock protein or is simply a general shock protein that is produced under different types of stress (for example, antagonistic, nutrient or heat stress).

In summary clearly there is a link between phenoloxidase/peroxidase activity and fungal antagonism. Both laccase and peroxidase appear to be important in the interaction zone with a connection between mycelial invasion and peroxidase release in *S.lacrymans*. Brown-rots appear to use laccase as part of a defensive response against invasion by antagonistic fungi, although the exact role of the enzyme has not yet been determined. It is also apparent that laccase activity by *C.puteana* is linked to pigmentation of the colony and to the formation of invasive mycelial fans. Results also seem to indicate that the incidence of laccase activity is increased by the presence of aromatic amino acids in the medium, in particular tyrosine, and temperature stress also induces laccase release. Since laccase has been detected under three different types of stress (antagonistic, nutrient and temperature) it is possible that laccase is a general shock protein released under conditions of stress to protect vital cellular components. It may be possible to develop a biological control strategy for wood decay basidiomycetes whereby the release of phenoloxidase enzymes is either enhanced or inhibited, depending upon which fungus is releasing them, thus leading to a more effective control system. The number of fungi reported to release phenoloxidase enzymes is increasing suggesting that these enzymes are more widespread than first thought. Therefore these enzymes may have a more general application than attributed previously, for example, in the adaptation of fungi to stress due to the environment or the presence of antagonistic fungi. It is also possible that since there is a large number of instances where these enzymes are involved, particularly for laccase, the definition of a

phenoloxidase enzyme needs to be redefined and made more specific with regards to its function.

Chapter 8

8.0 General Discussion

S.lacrymans causes upwards of £400 million pounds of wood decay each year in the United Kingdom. Although there are treatment regimes than can effectively eradicate a *S.lacrymans* infection from timber, these regimes are necessarily harsh. They result in the removal of infected timber and involve the use of powerful fungicides. Where historically important or ornate timbers are involved, removal of the timber to treat the *S.lacrymans* infection is not a desirable option. The fungicides used are powerful and can only be employed by trained personnel, and with the present attitudes towards environmental compatibility the use of these chemicals is questioned. This project was designed to determine whether *S.lacrymans* could be effectively controlled within timber by using an antagonistic fungus from the *Trichoderma* genus.

The first aim of the project was to screen a number of *Trichoderma* isolates for their antagonistic potential against *S.lacrymans* The media screening experiments indicated that only some of the *Trichoderma* isolates were antagonistic towards *S.lacrymans*, in particular certain *T.harzianum* and *T.viride* isolates. The experiments also indicated that media composition played an important role in the level of antagonism against *S.lacrymans*. Low nitrogen levels decreased the effectiveness of the *Trichoderma* isolates and this will be important since timber contains very little reserves of nitrogen. The screening experiments on wood generally reflected the media results with *T.harzianum* and *T.viride* isolates proving to be the better antagonists. However, one *T.viride* isolate (T110) proved to be the most effective antagonist on wood but not on media designed to reflect the carbon:nitrogen ratio in wood. This result indicates the problem associated with results obtained from screening experiments carried out in the

laboratory which are later extrapolated to the field situation. Screening experiments cannot fully reflect the nutritional and environmental status of the field situation although if the field substrate (in this case wood with no further nutrients added) can be used within the laboratory then a close approximation could be achieved. However, a compromise must be made as the closer to the field situation the screening experiment gets the longer the experiment will take to perform. Overall, the screening experiments provided one *T.harzianum* isolate (T25) that could be taken to the next stage of the project.

The next aim was to design a medium-scale experiment to determine the effectiveness of *T.harzianum* 25 in controlling *S.lacrymans* growing on timber stakes in a semi-controlled environment. The results obtained indicated that the *T.harzianum* 25 did not spread effectively through the timber stakes. However, in those areas of the plank that were colonised by the *T.harzianum* 25 no decay by the *S.lacrymans* was observed. To increase the effectiveness of the *Trichoderma* isolate a method must be found to increase its colonisation of wood. This may be achieved by using a more appropriate application procedure, for example, one that provides all of the nutrients necessary for the *Trichoderma* until it can gain all of its nutrient from the wood and/or maximise the area that is inoculated with the *Trichoderma* isolate.

The third aim was to develop a field site to test the chosen *Trichoderma* isolate in a non-controlled environment and attempt to control the spread of *S.lacrymans* within a building. Observations indicated that the *Trichoderma* did spread through the floor and halted the spread of the *S.lacrymans* colony into new areas of the floor. However, biomass accumulation by the *S.lacrymans* continued despite the presence of the *Trichoderma* and fruiting by the *S.lacrymans* was induced when the control agent was applied directly onto the colony.

These experiments indicate that there is potential in using *Trichoderma* species to control *S.lacrymans* within housing timbers. Although the *Trichoderma* control agent cannot kill the existing *S.lacrymans* colony the spread of the colony can be inhibited. Therefore, the *Trichoderma* could be used to enclose the *S.lacrymans* in a certain area and, by using other control measures, the *S.lacrymans* colony could then be eradicated. Since the *S.lacrymans* colony would be contained the measures used to exterminate the colony could be less intensive, for example, less powerful fungicides or environmental measures (i.e. heat or a moving airflow) could be used. However, further work is required to determine the effectiveness of using a *Trichoderma* control agent in conjunction with other control measures. Investigations should also be carried out into increasing the antagonistic effectiveness of the *Trichoderma* isolates used as control agents. If their effectiveness could be increased then it may be possible for the agent to kill the existing colony and therefore not rely upon another control measure to eradicate the *S.lacrymans*.

The final project aim was to investigate certain biochemical changes that occur during interactions, namely pigmentation changes. The interaction between *S.lacrymans* and certain *Trichoderma* species results in the production of pigments by the *S.lacrymans*. An investigation of the importance of these pigmentation changes has not previously been carried out. At first, the pigmentation changes were thought to be linked to the production of melanin within the *S.lacrymans* hyphae. However, the extraction procedure employed seemed to indicate that although melanin was present within the hyphae, melanin synthesis could not be induced or inhibited. Therefore, it was decided to investigate whether the phenoloxidase enzymes that have been reported as being involved in melanin synthesis were present in *S.lacrymans*. The investigation showed that the phenoloxidase enzymes laccase and tyrosinase, and peroxidase were released during interactions between

basidiomycete fungi and fungal antagonists. With the exception of *C.puteana* (which released laccase in pure culture) the basidiomycete fungi tested released laccase when antagonised by *Trichoderma* species, and that the *Trichoderma* species released laccase when invasion of the opposing colony was initiated. Therefore, it appears that laccase plays an important role in the offensive and defensive strategies of certain fungal isolates. It also appears that laccase is a possible stress enzyme employed by *S.lacrymans* to protect cellular components when the fungus comes under stress (nutrient, environmental and temperature). The roles of tyrosinase and peroxidase are less clear although they were induced on specific media types and during certain fungal interactions.

Overall the project results indicate that control of *S.lacrymans* within housing timbers is possible by using *Trichoderma* isolates, although it may have to be used in conjunction with another control measure. A detailed investigation into the biochemical aspects of the interaction process also needs to be carried out to determine the strategies that *S.lacrymans* employs to succeed in wood in the housing environment.

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THE INTERNATIONAL RESEARCH GROUP ON WOOD PRESERVATION

Section 1 Biology

Biological control of *Serpula lacrymans* using *Trichoderma* spp

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Introduction

The use of biocontrol systems for the protection of ground line timbers, e.g. distribution poles, has been subject of a large amount of research in recent years. However less emphasis has been given to the remedial treatmetn of timber in other situations, for example in buildings, despite the fact that there is a recognised need for new products and processes in this area to overcome the perceived problems with some of the technologies currently in use, particularly for the treatment of the dry rot caused by the brown rot organism *Serpula lacrymans*. Whilst some initial observations have been reported by Doi and Yamada (1991,1992) there has been no reported work in this area from western Europe where decay in buildins caused by dry rot is a major economic problem. (The bill for dry rot repairs in the UK alone is estimated at over £200 milliom per annum (Singh, 1994).

The problems with current treatment methods for dry rot centre around two areas, first an increased resistance to the use of perceived toxic chemicals in buildings and second, the rather destructive nature of the generally recommended treatment regimes. As part of a large international programme designed to investigate and compare a range of alternative technologies we have studied the possible use of *Trichoderma* spp. in the biocontrol of *Serpula lacrymans* starting from agar based experiments as undertaken for other systems (Bruce, 1983) and moving, more recently, to small and large scale wood systems as well as a field trial. In this paper we will describe some of the results of the laboratory experiments undertaken so far. Further information on the field trial experiment may be available in the next few months.

Materials and Methods

Organisms

The following organisms were used in this study, *S.lacrymans* FPRL 12C, *S.lacrymans* CMI 152233 and thirty *Trichoderma* spp. The origin, and some information about each of these organisms, is given in Table 1.

Cross plating

Standard cross plating experiments were undertaken using systems described by Rayner and Todd (1977) using a range of different media as described by Srinivasan *et al* (1992) and further detailed by Score and Palfreyman (1994). Basically media contained either 5% malt extract, 5% malt extract + 5% sawdust (pine sapwood) or minimal essential components plus various concentrations of ferric chloride and L-asparagine (Srinivasan *et al*, 1992). The exact ionic conditions of the media, as regards iron and nitrogen, is given in the next section. Experiemnts were undertaken on a range of different organisms from the list given in Table 1.

Small wood blocks

Experiments were undertaken on small (1x1x1cm) pine sapwood blocks to investigate the effects of pretreatment with *Trichoderma* spp. followed by incubation with *S.lacrymans.* Details of the system used can be found in Palfreyman *et al* (1991). Basically blocks were weighed, dried, reweighed, steam sterilised, incubated on plastic meshes on top of cultures of *Trichoderma* spp. for various times then transferred to cultures of *S.lacrymans.* After incubation for an appropriate time wood samples were dried, reweighed and weight loss determined by normal methods.

Experiments were also undertaken to determine if *Trichoderma* spp. could halt, or slow down decay being caused by *S.lacrymans.* In this case samples were partially decayed with *S.lacrymans* then sterilised and transferred on to *Trichoderma* cultures for various time periods. As with the pretreatment experiments weight loss in blocks was determined.

Stick experiment

In order to more fully analyse the interaction of *S.lacrymans* and *Trichoderma* spp. in a wood based system the apparatus shown in Figure 1 was set up. The details of the system will be published elsewhere but essentially the system allowed the infection of one end of a stick with an organism, in this case *S.lacrymans* and, after suitable colonisation the stick could then be moved to a separate jar in which it could be incubated with a second organism, in this case one of a number of *Trichoderma* spp. Following incubation for an appropriate time, sticks were divided into two and weight losses for the two halves of the stick determined. In addition observational information about the sticks was made at all times during the interaction process and data from these observations will be reported elsewhere.

Results

The results of the interaction of a range of different *Trichoderma* spp. with *S.lacrymans* FPRL 12C are shown in Table 2. Results included deadlock, overgrowth and probable killing by *S.lacrymans* and overgrowth and probable killing by *Trichoderma* spp. Results were performed using 6 replicates and in the great majority of cases similar results were obtained with all replicates. As well as the interactions, data was also produced regarding growth rates of organisms before, during and after interactions. Of particular interest was the observation that some species of *Trichoderma* could reduce the rate of growth of *S.lacrymans* even before interaction occurred.

These initial interaction experiments were undertaken on a malt extract based medium which is very rich in nutrients. It has been shown by Srinivasan *et al* (1992) that medium constituents can have an important effect on the interaction process and to investigate if this was true for the interaction between *Trichoderma* spp. and *S.lacrymans* the experiment, whose results are shown in Figure 2, was undertaken. The interactions were carried out on four types of medium which contained respectively, normal iron/ low nitrogen (LNM-NFe), normal iron/ high nitrogen (HNM-NFe), low iron/ low nitrogen (LNM-LFe) and low iron/ high nitrogen (HNM-LFe). All ion concentrations were made up in minimal essential medium as described by Srinivasan *et al* (1992). For the puposes of this paper low nitrogen is defined as 0.4mM, high nitrogen as 7.9mM, low iron as 0.01mM and normal iron as 0.1mM.

Isolate	Coding	Location	Code
S.lacrymans (Schumacher ex Fr)	CMI 152233	CMI, 1959	SC
S.lacrymans (Schumacher ex Fr)	FPRL 12C	CMI, 1970	S12
FY Trichoderma	-	-	TF
J.W. Trichoderma	-	-	TJ
T.aureoviride	IMI 91968	*Fagus sylvaticus*, Scotland, 1962	TA
T.harzianum	IMI 206040	*Picea excelsa*, Sweden, 1976	TH
T.koningii	IMI 54693	Soil, Nigeria, 1953	TK
T.longibrachiatum	IMI 53608	*Coffea* sp., Tanzania, 1955	TL
T.polysporium	IMI 206039	*Picea excelsa*, Sweden, 1970	TP
T.reesei	IMI 192656ii	Derived from IMI 192655ii	TR
T.saturnisporum	IMI 14685	Forest soil, USA, 1970	TS
T.viride	IMI 24039	Soil, Jealott's Hill, 1970	TV1
T.viride	IMI 49791	Soil, Dacca, 1952	TV2
Trichoderma Isolate (Cellulose Tape)	-	-	TIC
Trichoderma Isolate (Softwoods, CCA)	-	-	TIS
T.viride +	-	(Chloropicrin)	
T.viride +	-	(Vorlex)	
T.pseudokoningii +	-	(Methylisothiocyanate)	
T.viride +	-	(Chloropicrin)	
T.viride +	-	(Chloropicrin)	
T.viride +	-	(Chloropicrin)	
T.pseudokoningii +	-	(Chloropicrin)	
T.viride +	-	(Chloropicrin)	
T.viride +	-	(Chloropicrin)	
T.pseudokoningii +	-	(None)	
T.pseudokoningii +	-	(Vorlex)	
T.viride +	-	(Chloropicrin)	
T.pseudokoningii +	-	(Chloropicrin)	
T.viride +	-	(Chloropicrin)	
T.viride +	-	(Chloropicrin)	
T.viride +	-	(Chloropicrin)	
Unknown *Trichoderma* Isolate +	-	-	

Table 1 : Isolates used during the interaction study. Isolates labelled '+' were isolated from Douglas-fir roots following fumigation with the fumigant shown in brackets. They were obtained from Mr. E. Nelson, United States Department of Agriculture, Forest Service, Pacific Northwest Research Station, 3200 SW Jefferson Way, Corvallis, OR 97331.

Trichoderma Isolate	Agar Type	
	2% Purified Agar\5% Malt Extract Agar	2% Purified Agar\5% Malt Extract\5% Pine Sapwood Sawdust agar
T.harzianum IMI 206040	Target Replacement	Target Replacement
T.aureoviride	Target Replacement	Target Replacement
FY Trichoderma	Target Replacement	Target Replacement
J.W.Trichoderma	Target Replacement	Target Replacement
T.polysporium	Target Replacement	Target Replacement
Trichoderma Isolate (Cellulose Tape)	Target Replacement	Target Replacement
T.koningii	Target Replacement	Target Replacement
T.viride 49791	Target Replacement	Target Replacement
T.saturnisporum	Deadlock	Antagonist Replacement
Trichoderma Isolate Softwoods (CCA)	Antagonist Replacement	Target Replacement
T.viride 24039	Antagonist Replacement	Target Replacement
T.reesei	Antagonist Replacement	Antagonist Replacement
T.longibrachiatum	Antagonist Replacement	Antagonist Replacement
T.viride (T1)	Target Replacement	-
T.viride (T14)	Target Replacement	-
T.pseudokoningii (T22)	Target Replacement	-
T.viride (T24)	Target Replacement	-
T.viride (T28)	Target Replacement	-
T.viride (T30)	Target Replacement	-
T.pseudokoningii (T33)	Target Replacement	-
T.viride (T40)	Target Replacement	-
T.viride (T43)	Target Replacement	-
T.pseuokoningii (T51)	Target Replacement	-
T.pseudokoningii (T55)	Target Replacement	-
T.viride (T60)	Target Replacement	-
T.pseudokoningii (T64)	Deadlock	-
T.viride (T70)	Target Replacement	-
T.viride (T90)	Target Replacement	-
T.viride (T100)	Target Replacement	-
Unknown Trichoderma Isolate (T140)	Target Replacement	-

Table 2 : Results of the interaction study. 'Target' refers to the *S.lacrymans* species and 'Antagonist' refers to the *Trichoderma* species.

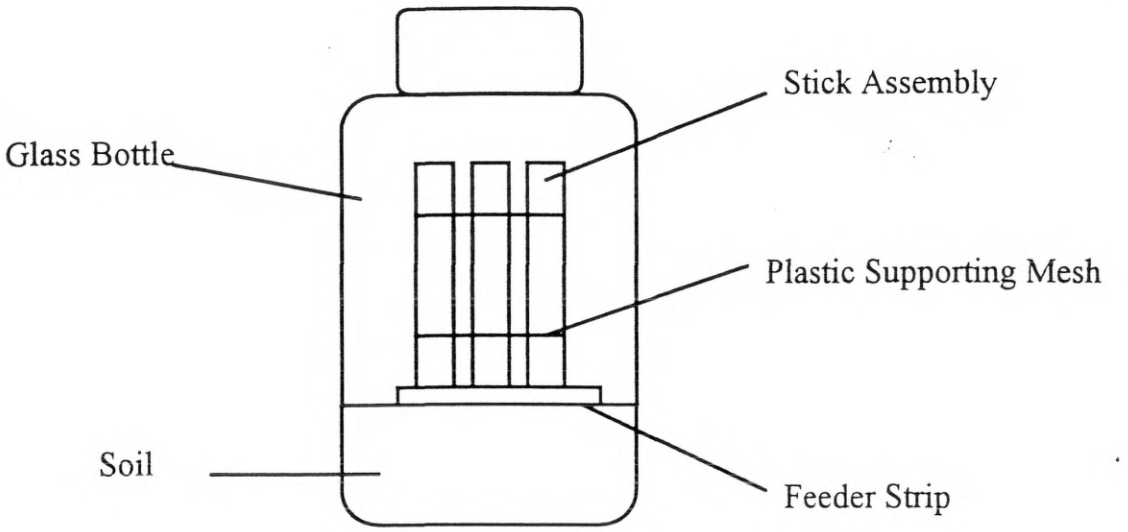

Figure 1 : Diagrammatic representation of the stick experiment.

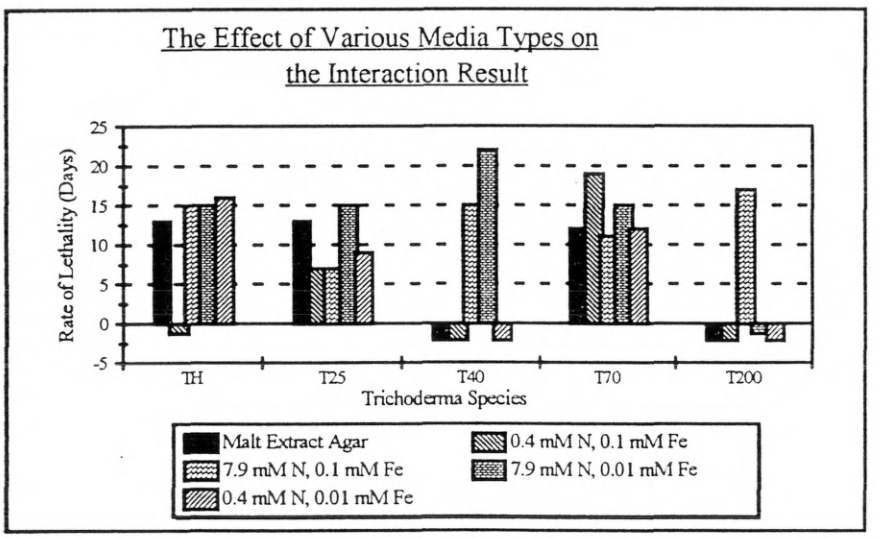

Figure 2 : The effect of various media on the interaction results. Large blocks below the line indicate situations where the *S.lacrymans* overgrew the *Trichoderma*, and the smaller blocks indicate a deadlock reaction.

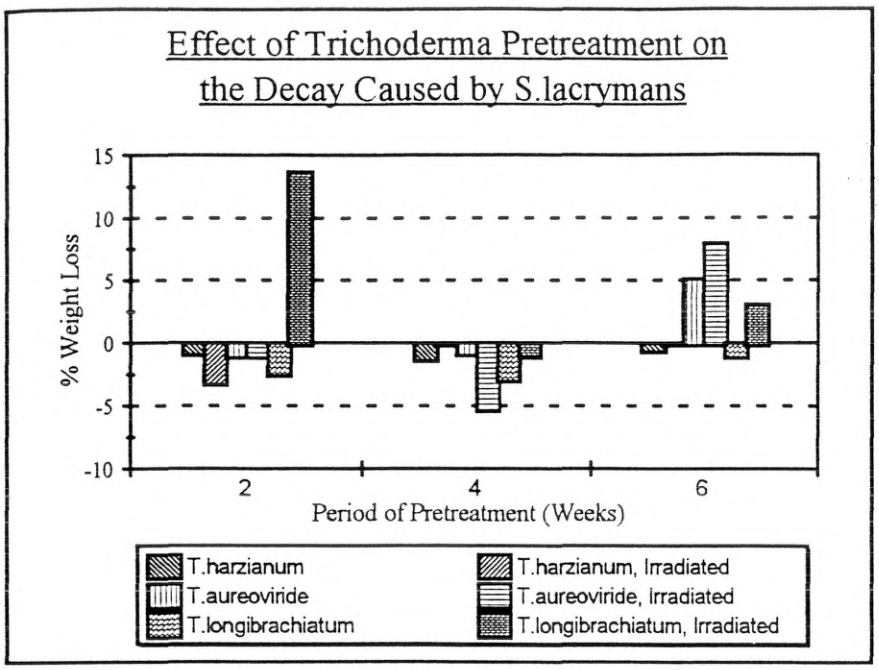

Figure 3: The effect of the pretreatment of wood blocks with *Trichoderma* species prior to incubation with *S.lacrymans* for three weeks. Weight losses of ± 5% are generally considered to be within the accuracy limits of the experiment.

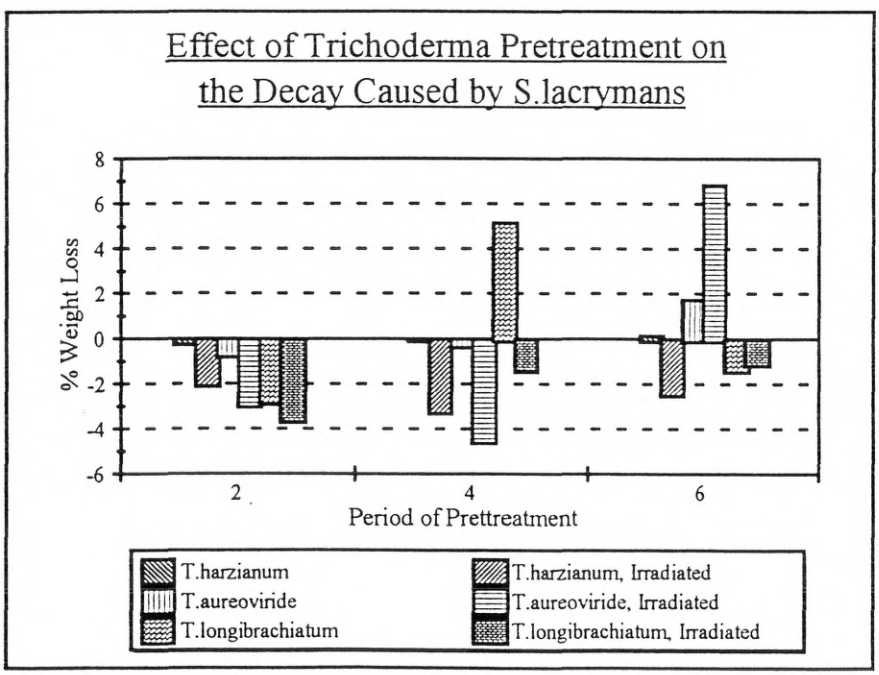

Figure 4 : The effect of the pretreatment of wood blocks with *Trichoderma* species prior to incubation with *S.lacrymans* for six weeks.

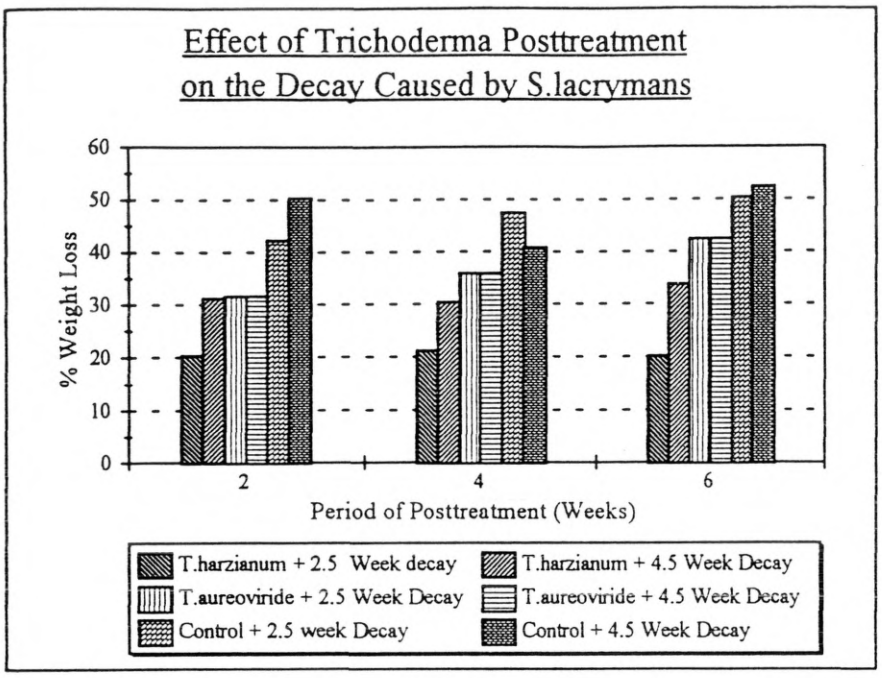

Figure 5 : The effect of the posttreatment of wood blocks infected with *S.lacrymans* for different periods of time.

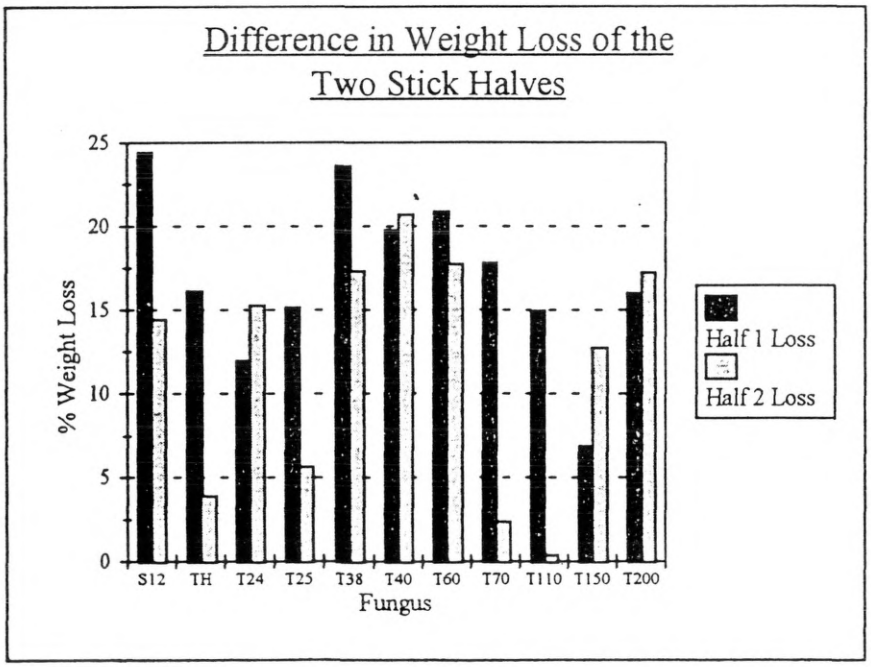

Figure 6 : Comparison of the weight losses of the two stick halves for each *Trichoderma* species.

The results shown in Figure 2 indicate that indeed medium concentrations did have an effect on the nature of the interactions between *S.lacrymans* and *Trichoderma* spp. and that some of the later species, most notably T25 and T70 were effective at killing in all the media tested. Generally *Trichoderma* organisms were most effective in the HNM-NFE medium and, perhaps surprisingly, least effective in the malt extract agar.

Pretreatment of small wood blocks with *Trichoderma* did, as expected, allow the the protection of blocks from subsequent decay. The results shown in Figures 3 and 4 are relatively short term but do indicate that it is possible to prevent decay caused by *S.lacrymans* by preinfections with *Trichoderma* spp.. Pretreatment, whilst of interest, is not crucial to the treatment of dry rot where the objective is to kill an organism which is already in possession of the nutrient substrate. When small wood blocks were partially decayed by *S.lacrymans* then treated with *Trichoderma* the results were more ambiguous (Figure 5). Though some small reduction in rate of decay was found weight loss continued after blocks were placed on to *Trichoderma* and the eventual final weight loss was similar in the experiment whether or not the blocks had been placed on to *Trichoderma*. However all attempts to isolate live *S.lacrymans* from such blocks were unsuccessful indicating that the organsim had in fact been killed.

To try to resolve the dificulties in the analysis of the remedial treatment experiment the apparatus shown in Figure 1 was constructed and a set of sticks infected at one end with *S.lacrymans*. After infections were well established the sticks were removed from their original jars, inverted and placed on mats of a variety of *Trichoderma* isolates. After a second incubation decay was assessed as described in the materials and methods and the results are displayed in Figure 6. Overall it can be seen that whilst decay continued at the same rate as for controls in some instances, some of the *Trichoderma* isolates (notably &25 and T70) were indeed successful at preventing further decay and prevented further progress of the growth of the decay organisms.

Discussion

The data presented in this paper indicate that it is possile, at least in small experimental systems, to control the growth of the dry rot fungus, *S.lacrymans*, with the potential biocontrol agent *Trichoderma* spp.. The data also show some of the difficulties of implementing such systems. For example the nature of the various interactions found between the competing organisms was very much dependant upon the nature of teh experimental conditions used. So an organsim wthat was particularly effective in the malt agar system (e.g. T40) might be ineffective in either the minimal media based system or the stick system. However there is no doubt that some organisms, in particular *Trichoderma* sp. T25, were effective at controlling the basidiomycete in a range of the systems tested.

On malt extract agar most *Trichoderma* spp. tested could efectively control *S.lacrymans*. On the ion supplemented meida a more limited number of organisms were successful. There are a number of reasons why the nature of the interactions may vary with a change of substrate. In the first instance it was obvious, data not shown, that the growth rate of the *S.lacrymans* was significantly affected by the growth medium and, for example all MEM based media, supported slower growth rates for this organism than the malt extract medium. Considering the MEM based media neither iron nor nitrogen had any effect on the extension rates of *S.lacrymans* as previously reported for nitrogen by Thornton and McConalogue (1990). Though Paajanen (1993) indicated that high levels of iron could affect growth the level used by this worker was 50 times those used in these studies.

Of the organisms tested on the MEM the most effective was certainly *Trichoderma* T25. Indeed the effectiveness of this organism seemed to improve when the interactions were carried out on the low nitrogen media despite the report by Doi and Yamada (1992) that a reduction in nutrients affects the ability of *Trichoderma* to act as an effective antagonist. Of course the low nitrogen may not only affect the *Trichoderma* and it possible that the *S.lacrymans* is being stressed by the MEM/ low nitrogen medium and becoming more susceptible to specific antagonists. The actual mechanisms of how such media affect antagonistic reactions is the basis of another paper at this meeting by Srinivasan and Bruce (1994).

The potential role of iron in wood decay, and hence biocontrol, has been discussed elsewhere (Koenigs, 1974, Murmanis *et al*, 1988, Srinivasan *et al*, 1992). In the experiments reported here it was found that most of the *Trichoderma* spp. were more effective in the low iron media than in than in the normal iron media. Now both *Trichoderma* and *.S. lacrymans* produce iron scavenging compounds called siderophores (Anke *et al.,* 1991, Srinivasan *et al.,* 1992, and Score (unpublished observations). The results reported in this paper may therefore indicate that the siderophores produced by the T*richoderma* spp., notably strains T25, T70, T110 and 206040 were more effective at scavenging iron than those produced by the basidiomycete antagonist.

The objective of utilising different media for analysing interactions experiments is to try to nearly represent the nutrient conditions found in wood. However no system will ever accurately mimic wood and it is therefore necessary to undertake laboratory scale experiments on wood blocks before moving into the field. In this paper we have reported on experiments undertaken on small wood blocks and on a larger, stick based system. The protection of small wood blocks by preincubation with *Trichoderma* spp. represents the simplest wood based system with which to work and, as might be expected, protection of wood samples was easily demonstrated in this system. By contract, the more realistic system, where wood blocks were partially decayed with *S. lacrymans* prior to incubation with *Trichoderma* represented a more difficult situation for the latter organism and the results are ambiguous.

Continued weight loss was found after incubation with *Trichoderma* even though it was not possible to isolate live *S. lacrymans*. There are a number of possible explanations for this observation. First, the presence of *Trichoderma* on wood samples may be sufficient to prevent isolation. However, samples were taken from the interior of wood blocks, plated out on to benomyl containing agar which should have prevented growth of *Trichoderma* and there should have been every opportunity for *S. lacrymans* to have been isolated had viable mycelia been present. Alternatively it is possible that the residual enzymes from the *S. lacrymans* present in the wood blocks were continuing the degradation of cellulose even in the absence of viable basidiomycete. A third, and most likely, explanation is that once the *S. lacrymans* has depolymerised the cellulose it becomes available for metabolism and the *Trichoderma* cellulases can then utilise this cellulose. Initial observations (Phillips, unpublished data) indicate that this may well be the correct explanation.

In order to determine if the positive interactions demonstrated on the agar plates could be mimicked in a simple wood system the apparatus shown in Figure 1 was established. This apparatus allowed the partial decay of a piece of pine sap wood in a spatially controlled manner, an objective which could not be achieved in the block system. A number of types of apparatus were tried before the one illustrated was developed. However, the advantage of the current system is that it allows the easy removal of samples after initial colonisation and transfer of these samples to a new apparatus. Control of decay was well shown in this

system with some of the *Trichoderma* spp. eg., T25, T70 tested indicating that the remedial treatment of timber is potentially possible.

The next stage of the project is to try remedial treatment of full size timbers, currently underway, and the treatment of a field site, also underway at Taymouth Castle in Perthshire, Scotland. It is hoped that the results of these experiments will be presented at future conferences.

To date the data is consistent with the use of *Trichoderma* spp. in the remedial treatment of timber. However there are many situations where *Trichoderma* treatment of timber would be contra-indicated and it is not proposed that *Trichoderma* treatment could ever act as a universal treatment for dry rot but that in certain specialised situations it might be useful. For example in situations where property is not expected to be renovated immediately but where a holding operation is necessary. Alternatively in areas which are difficult or slow to dry out after a repair process treatment with *Trichoderma* might represent an appropriate measure to prevent further decay before environmental conditions supress growth completely.

It is hoped that at some stage in the future a range of appropriate treatment methods for dry rot will have been validated and that the use of biocontrol will be an element within the battery of methods.

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