AN ABSTRACT OF THE THESIS OF

Michael Freitag for the degree of Master of Science in Forest Products presented on December 18, 1989.

Title: Measuring Extracellular Enzymes in Pure and Mixed Cultures of Trametes versicolor (L.:Fr.) Pilát and Trichoderma harzianum Rifai.

Signature redacted for privacy.

Abstract approved: _____
Jeffrey J. Morrell

The biocontrol potential of Trichoderma harzianum Rifai, Trichoderma polysporum (Link.:Pers.) Rifai, Scytalidium aurantiacum Klingstr. et Beyer or a Penicillium sp. against Trametes versicolor (L.:Fr.) Pilát, Neolentinus lepideus (Fr.) Redh. et Ginns, Postia placenta (Fr.) M.Lars. et Lomb. and Irpex lacteus Fr. was evaluated using agar plate, soil bottle and small size wood wafer tests. Although T. harzianum arrested growth of several wood decay fungi, it never killed them on wood samples. Of the tested microfungi, S. aurantiacum performed best in the wood-based assays.

At the same time, the utility of small size wood assays for evaluation of biocontrol potential was demonstrated. Such assays are more rapid and versatile than full-scale soil bottle or sawdust tube tests, and provide more information than agar plate screenings.

Microscopic examination of hyphal interactions between T. versicolor and T. harzianum on microscope slide cultures yielded little additional information, partly due to the experimental system chosen. No hyphal interference or lysis of cell walls was observed, possibly due to the high nutrient levels in the slide cultures.

The activities of enzymes related to wood decay were studied in pure and mixed liquid cultures of T. versicolor and T. harzianum at low (0.4 mM) and high (4 mM) nitrogen concentrations. In high nitrogen medium, total filter paper cellulase, specific and total cellobiase, and specific and total laccase increased when compared to activities obtained in low nitrogen concentrations. Conversely, specific filter

paper cellulase and peroxidase activities were enhanced under nitrogen limiting conditions.

Influences of T. harzianum on extracellular enzyme production of T. versicolor were observed. Total and specific laccase activities were induced in media containing both low and high nitrogen levels, whilst filter paper cellulase activities decreased. Peroxidase and cellobiase activities remained at approximately the same level or were decreased in mixed cultures.

The experimental system chosen allowed no separation of mycelia or culture liquids of the two fungi incubated in mixed culture. Therefore, few conclusions with respect to induction, inhibition or regulation of the monitored enzymes could be reached. None of the enzyme activities was correlated with biomass production. Laccases and Poly R-478 peroxidase activity indicated survival of the T. versicolor, since T. harzianum did not produce these two enzymes.

Measuring Extracellular Enzymes in Pure and Mixed Cultures of *Trametes versicolor* (L.:Fr.) Pilát and *Trichoderma harzianum* Rifai

by

Michael Freitag

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed December 18, 1989

Commencement June 1990

APPROVED:

Signature redacted for privacy.

Professor of Forest Products in charge of major

Signature redacted for privacy.

Head of the Department of Forest Products

Signature redacted for privacy.

Dean of Graduate School

Date thesis is presented December 18, 1989

Typed by Michael Freitag

Acknowledgement

Foremost, I wish to thank Dr. Jeff Morrell for his interest, patience and assistance during the preparation of this thesis. In many helpful discussions he pointed out important aspects or shortcomings of this project. At all times I appreciated his ability for letting me determine my own research topics. I also wish to thank Drs. Peter Bottomley and Everett Hansen, who gave me insights into microbial ecology, thus planting ideas which led to this study.

Thanks are also due to Dr. Paul Przybylowicz for providing almost inaccessible literature, and especially to Dr. Chris Biermann for sharing his equipment, lab space and knowledge of analytical techniques with me.

I am deeply indebted to my parents for their encouragement and continued support throughout my education.

Last, but not least, I wish to thank Camille, Nathan and Colin, without whom I would remain a visitor.

Table of Contents

List of Figures

List of Tables

"My love was science - more specifically, when placed in a common jar, which of two organisms would devour the other."

Gary Larson

Preface

The process of wood decay plays an indispensable role in the nutrient cycling of forest ecosystems. At the same time, decay leads to substantial economic losses by reducing the quality of trees or the service life of wood products. The microbial community present and the prevailing environmental factors, including the condition of the wood as a host or substrate are the factors which determine decay progression. Wood in service that is maintained under conditions adverse to microbial growth, i.e. dry or completely submerged under water, withstands biodegradation for long periods of time.

Many different preservation methods have been developed to extend the service life of timber used under moist conditions. These include the early, rather crude external applications of natural substances, as well as the development of modern standard methods, especially pressure treatment of timber with chemicals prior to installation (Knigge & Schulz, 1966; Hunt & Garratt, 1967). A comparatively new approach to decay control involves volatile compounds, collectively called fumigants. A large number of fumigants are able to eradicate or inhibit further growth of wood decay fungi through remedial treatment (Morrell & Corden, 1987).

Whilst preservative treatment extends wood service life and reduces the need to harvest more timber, the use of toxic chemicals is hazardous. Before chemicals are actually used as preservatives, they create hazards during the manufacturing process, the handling and the transport to the treatment sites (Greaves, 1987). During treatments, spills and local accumulation of preservatives in soils may occur (Thompson, 1973; Richardson, 1988; Stroo *et al.*, 1989). While in service, active toxic ingredients may leach into the environment (Green

et al., 1987; Evans, 1987; Nijman, 1987; Duguet & Dartigues, 1988). Residual chemicals in wood that is eventually taken out of service creates a toxic waste problem (Talarek, 1988). To minimize these dangers, extensive research is underway to identify less environmentally hazardous chemicals or treating processes (Willeitner & Peek, 1988; Hill & Killmeyer, 1988), evaluate application of lower concentrations of active chemicals (Pizzi et al., 1987), apply combinations of chemicals with synergistic effects (Landsiedel, 1987; Straetmans, 1987), identify environmental factors affecting fungitoxicity (Zahora & Morrell, 1987) and search for natural fungistatic or fungitoxic substances (Bultman et al., 1987).

A quite different, and more recent approach to minimize environmental impacts and reduce dependency on chemicals for wood protection is the use of biological control methods, also referred to as biocontrol (Baker & Cook, 1982). Compared to the difficulties encountered in the application of specific biocontrol organisms against insect pests or in soil environments, the potential for biocontrol of wood decay should be high: the wood environment is relatively stable and supports a less diverse microbial community than the surrounding soil (Greaves, 1972), especially if the wood has been treated with preservatives prior to exposure (Bruce & King, 1983; 1986a+b). Moreover, biocontrol organisms will act in a manner closely resembling natural processes and may avoid the abrupt changes in ecosystems which are normally associated with chemical treatments. The failure to completely eradicate pathogens or decay organisms has often been perceived as a weakness of biocontrol: short-term eradication of decay organisms solely by biocontrol strategies is deemed impossible. Studies on biocontrol of root pathogens suggest that sufficient control will eventually occur over an extended period of time in these systems (Cook & Baker, 1983). "Sufficient" control implies a pre-determined and acceptable level of, mostly, financial loss associated with disease. It should be stressed that this philosophy of control is not applicable to wood preservation. Many agricultural crops and the associated diseases are annual, whereas wood decay fungi create a perennial problem. Failure to stop the decay process completely and for long periods of time, may lead to structural failure of inadequately treated timber and fatal accidents.

Unfortunately, wood decay organisms have rarely been completely eradicated solely by means of biocontrol (Morris & Calver, 1986; Bruce et al., 1989).

Based on research on biocontrol in forest pathology (Rishbeth, 1963), the general feasibility of biological control systems on wood products was investigated (Ricard & Bollen, 1968; Ricard et al., 1969). These early studies produced conflicting conclusions (Ricard, 1970; Graham, 1973), but encouraged research in Europe (Klingström & Johansson, 1973; Dubos & Ricard, 1974; Lundborg & Unestam, 1980). Recent studies have revived the search for efficient biocontrol organisms to control wood decay in fumigated stumps (Nelson & Thies, 1985; 1986; Nelson et al., 1987; Goldfarb et al., 1989 a+b), post-logging treatments (Benko, 1987; 1988; 1989) and processed timber (Morris & Dickinson, 1981; Bruce & King, 1983, 1986 a+b; Bruce et al., 1984; 1987; 1989; Morris et al., 1984; 1986; Highley & Ricard, 1988; Morrell & Sexton, 1988; Highley, 1989). Previous studies were largely empirical and concentrated on two genera, Scytalidium spp. and Trichoderma spp., but the recent investigations have screened a multitude of organisms and explored different mechanisms by which biocontrol may be effected.

Still, most biocontrol studies have focussed on the characteristics of the microfungi, their potentially toxic metabolites or physical reactions detrimental to decay fungi, whilst effects of the interaction on the target decay fungus were only measured on the basis of growth rate, wood weight loss or survival. The responses of enzyme systems of the target decay fungus to the presence of a potential biocontrol organism have generally been ignored or have been evaluated only in relation to synergistic cellulose degradation (Wood, 1969; Hulme & Stranks, 1970; Hulme & Shields, 1975). However, enzymatic responses of a decay fungus to the presence of a biocontrol organism may provide important clues to biocontrol mechanisms which, in turn, may be used to identify more effective control methods.

In this study effects of selected biocontrol organisms on decay fungi were investigated by:

- characterizing the effects of one proposed biocontrol organism, Trichoderma harzianum Rifai, on several white and brown rot fungi using a series of laboratory tests on agar plates and in soil bottles;

- determining the extent of hyphal interactions between T. harzianum and Trametes versicolor (L.:Fr.) Pilát on slide cultures at the light microscopic level;

- and, most importantly, measuring the levels of selected enzymes present in pure and mixed culture filtrates of T. harzianum and T. versicolor to determine effects of a potential biocontrol organism on extracellular wood degrading enzyme complexes of a decay fungus.

MEASURING EXTRACELLULAR ENZYMES IN PURE AND MIXED CULTURES OF TRAMETES VERSICOLOR (L:Fr.) Pilát AND TRICHODERMA HARZIANUM Rifai

1. General Introduction and Literature Review - Biological Control and Fungal Interactions

1.1. The Concept of Biological Control

Outbreaks of plant diseases and insect epidemics in human-managed ecosystems often indicate a biological imbalance which has permitted one organism to rise above endemic levels. Conversely, epidemics are rare events in most unmanaged natural ecosystems (DeBach, 1964). Late last century, entomologists realized the potential of influencing one population in a disease system to control another population. This strategy was called "parasitic control", "biological method", "biological control" or "biocontrol" (DeBach, 1964; Baker & Cook, 1982). The latter two terms are widely and interchangeably used by entomologists and plant pathologists.

There are numerous definitions of biocontrol. Whilst entomologists generally agree on a biocontrol strategy emphasizing pest-enemy population dynamics dominated by interactions between predators and parasites (Beddington et al., 1978, Murdoch et al., 1985), biocontrol methods in plant pathology most often involve the concepts and mechanisms of competition and antibiosis (Hornby, 1978, Cook & Baker, 1983). Biocontrol was broadly defined by Baker & Cook (1982) as:

> "...the reduction of inoculum density or disease- producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists."

The conscious modification of the environment or direct predation by humans were excluded as potential sources of antagonism (Baker, 1987).

The objective of biocontrol is the development of a disease inhibiting biological balance of crops, pathogens, antagonists and physical environment by the:

> "1. Reduction of inoculum of the pathogen through decreased survival between crops, decreased production or release of viable propagules, or decreased spread by mycelial growth;
> 2. Reduction of infection of the host...;
> 3. Reduction of severity of attack..."
> (Baker & Cook, 1982).

Thus, biocontrol organisms will rarely eradicate plant pathogens completely, but will reduce the frequency of disease or keep it below a pre-determined economic threshold. The concept of biocontrol as just one strategy within integrated control programs has been widely accepted (Papavizas & Lumsden, 1980; Papavizas, 1985; Baker, 1987; Papavizas & Lewis, 1988). The ideal biocontrol fungus against soil-borne pathogens should possess the ability to grow rapidly, sporulate profusely, efficiently disperse its propagules and subsequently colonize new substrates rapidly, thus exploiting favourable habitats by a mixture of rapid growth, migration and dormancy (Faull, 1988).

The importance of how and where to detect and characterize potential biocontrol systems is obvious. Often agonizing attempts to completely understand plant-pathogen-antagonist systems prior to developing control strategies, coupled with the dogmatic use of pure culture techniques, have slowed the identification and application of biocontrol systems in plant pathology (Linderman et al., 1983). Generally, the most efficient biocontrol systems are likely to occur where no disease is present, but conditions are conducive for its development (Cook & Baker, 1983; Whipps et al., 1988). Moreover, most biocontrol systems appear to be very specific, requiring experimental designs which resemble natural conditions very closely (Linderman et al., 1983; Faull, 1988). The concepts of biocontrol applied in plant pathology are largely based on the pioneering work of soil microbiologists (Sanford, 1926; Millard & Taylor, 1927; Weindling, 1932; 1941;

Brian, 1951; Wood & Tveit, 1955; Garrett, 1956), and the reviews of commercially successful biocontrol systems mirror this preoccupation with soil-borne plant diseases (Snyder, 1960; Menzies, 1963; Baker & Snyder, 1965; Papavizas, 1966; 1981; 1985; Baker, 1968; Bruehl, 1969; 1975; Garrett, 1970; Mitchell, 1973; Wilhelm, 1973; Snyder et al., 1976; Cook, 1977; Lumsden, 1980 a+b; Papavizas & Lumsden, 1980; Parker et al., 1985; Mukerji & Garg, 1986; Burge, 1988). Only during the last decade have research efforts been directed towards developing a better understanding of microbial interactions on the phylloplane (Dickinson & Preece, 1976; Dubos & Bulit, 1981; Blakeman & Fokkema, 1982; Windels & Lindow, 1985; Blakeman, 1988).

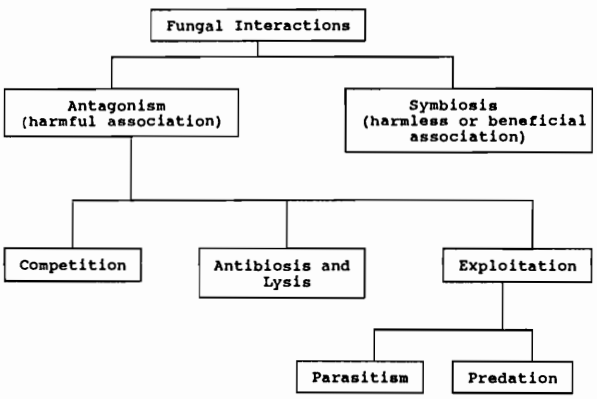
1.2. Interspecific fungal interactions

Dynamic interspecific interactions form the basis for all biocontrol systems. Organisms are said to interact when the presence of one affects the performance of another (Rayner & Webber, 1984), both on an intra- and interspecific level (Rayner & Todd, 1979). Whilst specialization into separate niches can minimize interspecific interactions for organisms with determinate body form, described by the competitive exclusion principle (Frederickson & Stephanopoulos, 1981), the distribution of mycelial fungi with their indeterminate feeding and reproductive structures is more likely to be influenced by interspecific interactions (Rayner & Webber, 1984). Studies of fungal interactions, therefore, are not only useful for controlling disease, they improve our understanding of the general development of fungal communities. This part of the review is an attempt to compare and combine several classifications of possible fungal interactions. It will follow closely the approach chosen by Rayner & Webber (1984).

(a)

Type of Interaction	Effect on Population A	B
Neutralism	0	0
Commensalism	0	+
Synergism	+	+
Mutualism (Symbiosis)	+	+
Competition	-	-
Amensalism (Antibiosis, "Antagonism")	0/+	-
Exploitation (Predation, Parasitism)	+	-

(b)



(c)

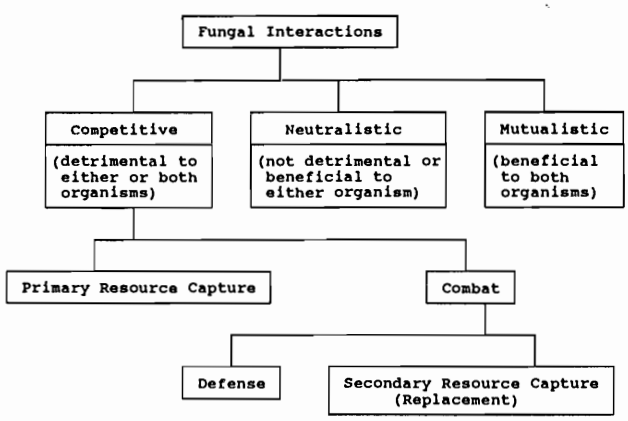


Figure 1.1.: Proposed interactions between diverse microbial populations: (a) possible interactions between two populations (0 = no effect, + = beneficial and - = detrimental effect for the respective population (after Atlas & Bartha, 1987), (b) previous classification of fungal interactions (after Park, 1960; 1965; Clark, 1965; Baker & Cook, 1982), (c) classification of fungal interactions proposed by Rayner & Webber (1984).

1.2.1. The Traditional Classification of Fungal Interactions

It is difficult, if not impossible, to unequivocally classify all possible types of interspecific interactions occurring between populations of mycelial fungi (Odum, 1971; Park, 1968; Meers, 1978; Culver, 1982). Traditionally, interactions have been classified as "positive" or "negative" for either both or only one organism. There is also a possibility of no interaction ("neutralism"). This "non-interaction" is difficult to demonstrate since it involves a negative concept and may occur among populations with extremely different metabolic capabilities (Atlas & Bartha, 1987).

Positive interactions include commensalism, synergism and mutualism (Fig. 1.1.a.). In commensalism, one organism benefits through the production of growth factors (Bell et al., 1974), increase in substrate availability (Beam & Perry, 1974; Cappenberg, 1975) and detoxification or removal of chemical compounds (Jeffries, 1982) by a second organism which remains unaffected. In synergism, both organisms benefit but are still able to survive separately, whereas strict mutualism, or symbiosis, implies an obligatory association of two organisms, typically requiring both high specificity and close physical proximity (Atlas & Bartha, 1987).

These definitions for positive interactions are widely accepted. The classification of negative fungal interactions, however, has recently been reviewed (Rayner & Webber, 1984). Traditionally, interactions detrimental to at least one organism were termed "antagonism" and included competition, exploitation (= parasitism and predation) and antibiosis (Fig. 1.1.b.). The boundaries between these interactions are indistinct. The term competition was applied in its narrow sense, subservient to antagonism, including the utilization or removal of resources, but omitting space, water and oxygen as factors in competition (Clark, 1965; Veldkamp et al., 1984). Garrett's (1956; 1970) concept of competitive saprophytic ability, which can be based on various modes of action, including antibiosis and competition for nutrients, was completely ignored. Also, some "antagonistic" mechanisms

did not fit in the old scheme: hyphal interference or the action of cell-wall lytic enzymes cannot be classified as competition, parasitism or antibiosis in the classic sense. The old scheme mirrors the early dependence on interaction studies in soil environments, where spore-producing fungi dominate, and the difficulties associated with the inspection of fungal hyphae in a rather complex system (Rayner & Webber, 1984). Perhaps the most important disadvantage of the traditional system is that interactions cannot be adequately classified before their mechanisms are completely understood: descriptions of interaction outcome (harmless, beneficial or harmful) were mixed with terminology describing their mechanisms of interaction (competition, antibiosis or exploitation).

1.2.2. The Proposed Classification of Fungal Interactions

The praxis of mixing descriptions of interaction outcome with the respective mechanisms of interaction is avoided when the classification is based solely on the outcome of interactions (Rayner & Webber, 1984; Fig. 1.1.c.). The new scheme incorporates the concept of selection along a r-K gradient, reflecting the fact that fungal mycelia are heterogenous, complex and dynamic entities with overlapping phases of establishment, exploration and exploitation of resources as well as reproduction, during which a variety of interactions can occur simultaneously (Cooke & Rayner, 1984; Lockwood, 1988). R-selected species or populations depend on high reproductive rates for continued survival of a population, but lack most other adaptive advantages. Conversely, K-selected species or populations rely on physiological adaptations to the environment and competing populations (Waksman, 1953; Harper & Ogden, 1970; Andrews & Rouse, 1982; Andrews & Harris, 1986). In ephemeral fungi, rapid germination and hyphal extension are associated with explorative mycelia that are able to utilize readily available substrates and to reproduce at an early stage (r-strategy). Maintenance of

a sustained feeding structure over extended periods of time, the ability to utilize refractory substances or the need to withstand interspecific or environmental pressure characterize fungal K-strategists. On a scale from pure r- to K-strategy, plant pathogens and soil fungi tend to lie near the r-extreme, whereas wood decay fungi come closer to the typical K-strategist (Rayner & Webber, 1984; Lockwood, 1988). Based on this general ecological concept, three major survival strategies have been proposed for fungi: ruderal, competitive and stress-tolerant (Pugh, 1980).

Fungal interactions are still classified as neutralistic, mutualistic and competitive (Fig. 1.1.c.), but competition is now broadly defined as active demand by two or more organisms for the same resource (Rayner & Webber, 1984). During initial colonization of a vacant resource, either primary resource capture or, gradually, combative mechanisms will result. The former strategy is solely dependent on early arrival and sequestering of resources and does not involve the direct challenges between organisms found in the latter strategy (Cooke & Rayner, 1984). Success in primary resource capture is determined by effective dispersal mechanisms, spore germination, mycelial extension rates, possession of suitable enzymes to utilize easily available substrates and tolerance to adverse conditions associated with the resource (Rayner & Webber, 1984). Thus, ruderal and stress-tolerant fungi are favoured. The advantage of a given fungus will depend on its relative combative abilities as it comes in contact with mycelia of its own or invading competitive fungal species. Combative competition may lead to secondary resource capture, also called replacement (Cooke & Rayner, 1984), or to successful defense of the resource.

If combative competition results from direct physiological challenges of organisms and not purely nutritional factors, two main mechanisms may be recognized: interactions mediated at a distance by volatile or diffusible antibiotics or interactions depending on contact, such as hyphal interference or hyphal coiling. Stress-tolerant fungi adapted to a K-strategy of survival are generally favoured, as are fungi producing cell-wall lytic enzymes, antibiotics or hyphal interference

(competitive fungi).

The proposed scheme overemphasizes the combative mechanisms in fungal interactions which occur in challenges between K-strategists. Whilst the scheme is extremely helpful when dealing with complex mycelial interactions, it seems most appropriate for the classification of interactions among Basidiomycotina. Interactions occurring between soil fungi with high capacity for primary resource capture are not further classified, again illustrating the focus on Basidiomycotina.

1.2.3. Mechanisms of Combative Competition

Lysis of fungal mycelium is a common phenomenon in soils (Cook & Snyder, 1965) and was defined as "the loss of protoplasms in fungal structures and the dissolution of the cell walls" (Lloyd & Lockwood, 1966). "Autolysis", due to starvation and self-digestion (Sequeira, 1962) and "heterolysis", due to antibiosis (Carter & Lockwood, 1957; Garrett, 1965; Lloyd & Lockwood, 1966) or extracellular enzymes of another organism (Skujins et al., 1965; Hüttermann & Cwielong, 1982; Mercer; 1986) are, in praxis, difficult to distinguish.

When a microbial population produces substances which are inhibitory or lethal to another population, the resulting interaction is called "antibiosis" and the substances produced "antibiotics" (Jackson, 1965). The first population may gain a positive effect by this mechanism or may remain unaffected. This broad definition of antibiosis makes it difficult to separate and distinguish from other mechanisms, since it includes all fungal secondary and sometimes even primary metabolites as potential antibiotics. The significance of antibiotics in natural environments is a controversial topic and has been the subject of many reviews (Weindling et al., 1950; Stallings, 1954; Garrett, 1956; Brian, 1957; 1960; Burgess, 1958; Goldberg, 1959; Krasil'nikov, 1960; Park, 1960; 1967; Bilai, 1963; Dekker, 1963; Jackson, 1965; Baker, 1968; Bruehl et al., 1969; Gottlieb & Shaw, 1970;

Gottlieb, 1976; Moore, 1978; Sebek, 1980; Williams, 1982; Leong, 1986; Bruehl, 1987; Fravel, 1988).

The difficulties in understanding antibiosis and its role in natural environments are essentially based on the: (a) organisms' need for excess substrate (especially carbon sources) which may be absent in natural systems to produce antibiotics; (b) difficulty of detection and extraction of antibiotics from natural environments; (c) production of antibiotics under artificial conditions, but failure to produce the same compounds in nature; (d) inactivation of antibiotics; (e) dispersion of antibiotics in soil, although locally high concentrations may occur; (f) resistance of organisms to antibiotics; (g) lack of studies showing the mutual inhibition associated with antibiotic production of one of two organisms (Baker & Cook, 1982; Cooke & Rayner, 1984; Rayner & Webber, 1984; Atlas & Bartha, 1987). Since antibiotic producing organisms are not spectacularly more successful in colonizing or utilizing substrates (Gottlieb, 1976), many of the above-cited authors believe that antibiosis plays a marginal role in the outcome of interspecific interactions. However, the outcome of the interaction may not always mirror the mechanisms of interaction between species, and multi-species competition in an interactional network may counterbalance specific advantages of an antibiotic producer. Bruehl et al. (1969) suggested that antibiotic producers gained easy access to a substratum and at least temporarily colonized it, whilst antibiotic negative strains were overrun by competitiors.

Parasitism and predation were collectively called "exploitation" (Boosalis & Mankau, 1965; Baker & Cook, 1982). The boundaries between these two mechanisms are indistinct with fungi, since the morphology and physiology of fungi prevents predation as defined in, for example, mammals or arthropods. Mycoparasites benefit by deriving their nutritional requirements partly or completely from living cells of their susceptible host. The relationship is typically of long duration, depending on the degree of specialization and aggressiveness of the parasite (Atlas & Bartha, 1987). Mycoparasitism, often interchangeably used with the term "hyperparasitism", has been reviewed extensively

(Butler, 1957; Barnett, 1963; Boosalis, 1964; Boosalis & Mankau, 1965; Griffith & Barnett, 1967; Hashioka & Fukita, 1969; Barnett & Binder, 1973; Traquair & McKeen, 1973; Cerrato et al., 1976; Rayner, 1977; 1978; Tzean & Estey, 1978; Rayner & Todd, 1979; Lumsden, 1980 a+b; Boddy & Rayner, 1983; Sundheim & Tronsmo, 1986; Whipps et al., 1988).

Mycoparasitism is often sub-classified as biotrophic or necrotrophic mycoparasitism based upon the pattern of attack and the duration of the interaction (Barnett, 1963). Necrotrophic parasitism leads to lysis of host hyphae and loss of intercellular components on which the parasite feeds (Moore-Landecker, 1982). No specific nutrients are required and the interaction somewhat resembles predation. Necrotrophic parasitism is often associated with what has been called "hyphal coiling" (Butler, 1957; Tzean & Estey, 1978; Elad et al., 1980, 1983b), which leads to secondary resource capture or "self-parasitism" (Nguyen & Niederpruem, 1984). Biotrophic parasitism is characterized by its long duration and the presence of a delicate physiological balance between host and parasite (Hoch, 1978), forming the link to a true symbiosis, and involves the whole range of combative mechanisms: lysis, antibiotic production and resource capture (Rayner & Webber, 1984).

Hyphal interactions form one important mechanism of mycoparasitism which appears to be especially common among Basidiomycotina (Ikediugwu et al., 1970; Traquair & McKeen, 1977). These interactions require close contact between hyphae of different organisms, involving changes in membrane permeability, increased refractivity, vacuolation and lysis, eventually leading to mutual or unilateral death of hyphae (Rayner & Webber, 1984). Hyphal interactions seem to be triggered by directed growth towards a nutrient source (Whipps et al., 1988), followed by surface recognition of hyphae by fungal lectins (Elad et al., 1983a; Sharon & Lis, 1989) and are species-specific (Boddy & Rayner, 1983; Rayner & Turton, 1982). Rayner & Webber (1984) postulated a close relationship between hyphal fusion and interspecific hyphal interactions leading to both necro- and biotrophic parasitic relationships.

1.3. Successful Biocontrol of Wood Decay Fungi

After defining possible fungal interactions and describing major mechanism of competition, we can now take a closer look at biocontrol of wood decay fungi. Although successions and interactions of fungal communities on both hardwoods and softwoods have been studied extensively (Harder, 1911; D'Aeth, 1939; Griffith & Barnett, 1967; Shigo, 1967; 1970; Driver & Ginns, 1969; Eslyn, 1970; 1986; Toole, 1971; Greaves, 1972; Smith, 1973; Käärik, 1975; Rayner, 1976; 1977 a+b; 1978; Rayner & Todd, 1979; Shigo & Marx, 1977; Merrill & Shigo, 1979; Zabel et al., 1982; 1985; Przybylowicz et al., 1987; Chapela et al., 1988), few reliable biocontrol systems have been generated from this research. To illustrate the practical exploitation of fungal interactions, three biocontrol systems will be reviewed.

The best known and commercially most successful example of biocontrol of a wood decay fungus is the inoculation of Scots pine (Pinus sylvestris L.) stumps with Phanerochaete (= Peniophora) gigantea (Fr.:Fr.) Rattan, to prevent the invasion of Heterobasidion annosum (Fr.) Bref., the major cause of root and butt rot in conifers in Europe. Rishbeth (1963) developed a strategy of biocontrol based on the high competitive saprophytic ability of P. gigantea. This concept, also termed "possession principle" (Barton, 1961), was first advocated by Leach (1939). When P. gigantea was inoculated on stump surfaces immediately after cutting, this fungus was able to colonize the stumps and root systems well in advance of H. annosum. If H. annosum was already established, P. gigantea could only partially replace it and the short range spread of H. annosum by root contacts and grafts was not controlled (Ikediugwu et al., 1970; Holdenrieder, 1982). However, this biocontrol method has become standard practice to prevent medium and long range spread of H. annosum into first-rotation pine plantations in Britain (Greig, 1984).

Another example for successful biocontrol is the inhibition of root rot caused by Armillaria mellea (Vahl:Fr.) Karst. in orchards. Soil fumigation with sublethal doses of carbon disulfide (Bliss, 1951)

or methyl bromide (Ohr et al., 1973), weakens the mycelia of A. mellea, which are subsequently parasitized and killed by Trichoderma species. Differences in the resident soil population led to varied degrees of control (Mughogho, 1968). The interaction is likely to involve a combination of primary resource capture and combative competition via necrotrophic or biotrophic parasitism.

The colonization of unprotected pruning wound tissue on plum trees by Trichoderma species inhibited the invasion by Chondrostereum purpureum (Pers.:Fr.) Pouz. due to altered nutrient conditions (Brooks & Storey, 1922; Grosclaude, 1970; Mercer & Kirk, 1984a+b). Both preventive and curative effects of Trichoderma viride Pers.:Fr. were noted on plum (Dubos & Ricard; 1974), apricot (Carter & Price, 1975) and pear trees (Corke, 1974), as well as on European beech (Mercer & Kirk, 1984a+b). Saprophytic fungi like Trichoderma spp. may exhaust the easily available substrates in the wound tissue and probably defend their resource by a combination of parasitism and antibiosis, preventing invasion by spores of C. purpureum, but the mechanism of control has not been sufficiently investigated (Mercer & Kirk, 1984b).

The three examples illustrate the importance of both primary resource capture and combative competition in interactions between mycelial fungi. It should be stressed that there are important differences in the survival strategies of the saprophytic Trichoderma spp. and the facultative parasitic H. annosum or A. mellea. Trichoderma spp. can be classified as ruderal fungi, although they can be competitive under favourable environmental conditions (Domsch et al., 1980) and command, species by species, a variety of combative mechanisms. Wood decay fungi are mostly stress-tolerant or competitive (Rayner & Webber, 1984). It is difficult to envision how predominantly ruderal fungi may be able to inhibit or eradicate physiologically well adapted wood decay fungi. However, this biocontrol strategy has been employed to inhibit A. mellea and C. purpureum in orchard trees and is also the central strategy in all attempts to establish biocontrol organisms on wood products. These methods are feasible when the environment is either conducive to Trichoderma spp. or other organisms with high competitive

saprophytic ability, or when these organisms are favoured by modifications of environmental conditions by humans. How stable this advantage will be is doubtful, but should be of concern when long term control is required since "populations are a reflection of habitat [and] any change due to introduction without change in habitat will be a transient one" (Odum, 1971).

Each of the following chapters will concentrate on a different approach to quantify biocontrol potential. First, the potential of biocontrol organisms was tested in experimental designs most likely measuring the combined effect of primary resource capture and combative competition. Second, only hyphal interaction as one important mechanism of mycoparasitism was investigated. Finally, and most importantly, enzymatic responses of the target decay fungus should enable us to better understand how the nutritional capabilities of decay fungi may be affected by the presence of potential biocontrol organisms.

2. Potential of *Trichoderma harzianum* Rifai as a Biological Control Fungus

2.1. Literature Review - Biological Control of Decay Fungi on Wood Products

Compared to living trees, dead timber offers only limited resistance to microbial degradation (Scheffer, 1973; Merrill & Shigo, 1979). The change in status from tree to finished product, from living host to readily available substrate, makes it more difficult to achieve adequate control of wood decay. Also, the philosophy of decay control changes: a loss of trees is accepted as inevitable in forest disease control, whereas complete decay control is the ultimate goal of wood preservation. Most studies on biocontrol of decay fungi on wood products attempted to prevent decay in utility poles and concentrated on two genera, *Scytalidium* spp. and *Trichoderma* spp. (Bruce & Highley, 1989). These two taxa exemplify the evolving concepts of biocontrol on wood products since *Scytalidium* spp. were investigated due to their capability of producing diffusible antibiotics, whereas *Trichoderma* spp. were employed due to their combined competitive saprophytic, antibiotic and mycoparasitic capabilities.

Ricard (1966) isolated a previously undescribed *Scytalidium* strain, termed "FY", from undecayed Douglas-fir poles. This strain inhibited the growth and decay associated with *Antrodia carbonica* (Overh.) Ryv. *et* Gilbn. in laboratory and field studies (Ricard *et al.*, 1968; 1969). Similar strains were isolated and tested in Europe (Björkman, 1947; Klingström & Beyer, 1965; Bergman & Nilsson, 1971) and Canada (Shields & Shih, 1967). The "FY" strain produced an antibiotic, designated scytalidin (Strunz *et al.*, 1972; Stillwell *et al.*, 1973; Stranks, 1976) or scytalidic acid (Overeem & Mackor, 1973). The most thorough screening of the genus *Scytalidium* for biocontrol purposes was performed by Klingström & Johansson (1973). They compared growth rates, influence of nutrient conditions, cellulolytic activities, production of resting structures, pigments, and crystals of 38 European and North

American strains of Scytalidium aurantiacum Klingström et Beyer, Scytalidium album Beyer et Klingström, and Scytalidium lignicolum Pesante, and measured their antagonistic action against four decay fungi (H. annosum, T. versicolor, Coniophora puteana [Fr.] Karst., and Neolentinus lepideus [Fr.:Fr.] Redh. et Ginns) and a blue-stain fungus (Leptographium lundbergii Lagerb. et Melin) in cross-plating tests, wood blocks, and tree stems. The 38 strains differed markedly in antagonism against the wood-decay fungi but this variation was not due to geographical distribution of the strains or the hosts. All tested strains caused less then two percent wood weight loss, results comparable with those obtained by Ricard et al. (1968; 1969). The interaction between inhibited decay fungi, Scytalidium spp. and wood led to the development of the "immunizing commensal" (IC) concept (Ricard et al., 1968). The term was subsequently used to describe antagonists which grow on or in a substrate without damaging its important properties and which, by their antagonistic capabilities, protect it against other invading microorganisms (Ricard, 1970; 1976). "Immunizing commensalism" describes the relationship between wood products and potential biocontrol organisms rather poorly, since a dead substrate can hardly be described as a "commensal". Although Ricard (1977) suggested "immunizing commensalism" as a means to achieve complete bio-control, many problems regarding the inoculation, control and overall efficacy of his proposed methods remained unsolved.

Studies on wood block systems showed that both S. lignicola and T. viride were able to control decay by N. lepideus (Oxley, 1976), but not by Postia placenta (Fr.) M.Lars. et Lomb. or Gloeophyllum trabeum (Pers.:Fr.) Murr (Highley, 1989). Morris & Dickinson (1981) developed a "sawdust tube" method to quantitatively evaluate the antagonism between decay fungi and Scytalidium strains. They found promising results for both tested strains against N. lepideus and A. carbonica, whereas P. placenta was resistant. More recently Bruce & King (1983) evaluated the antagonistic properties of Scytalidium strain "FY" against N. lepideus on Scots pine and lime (Tilia vulgaris L.) blocks and creosoted pine poles, using the commercially available Binab FYT™ preparation. This

product contained spores and mycelium of Scytalidium strain "FY", Trichoderma polysporum (Link.:Pers.) Rifai, and T. harzianum. All three fungi independently inhibited the growth of N. lepideus. Since a residual antagonistic effect of all three tested species was detected after the wood blocks had been thoroughly leached with hot water prior to exposure to N. lepideus, Bruce & King (1983) concluded that this residual effect could not be due to water-soluble antibiotics, such as scytalidin. If this residual effect would take place under natural conditions there would be no need for survival of the biocontrol organism for effecting wood preservation. However, other authors found no residual effect of Scytalidium FY or S. lignicolum against N. lepideus, C. puteana (Morris et al., 1986) or P. placenta and G. trabeum (Highley, 1989), possibly due to modified test conditions. No inhibition of N. lepideus by Scytalidium strain "FY" due to volatile antibiotics or mycoparasitism was shown (Bruce et al., 1984). On birch blocks, no permanent inhibition of selected white- or brown-rot fungi by Scytalidium spp. was found: white-rot fungi occluded xylem cells with hyphae and formed zone lines, also called pseudosclerotial plates (Cease et al., 1989).

Trichoderma spp. have been considered of value for biocontrol purposes in agriculture and horticulture (Hadar et al., 1979; Elad et al., 1980; Baker & Cook, 1982; Papavizas, 1985; Lifshitz et al., 1985; Windham et al., 1986; Vannacci & Harman, 1987), but the use of Trichoderma spp. in forestry has seldom been successful (Nelson & Thies, 1985; 1986; Nelson et al., 1987; Goldfarb et al.; 1989 a+b).

Trichoderma spp. were long believed to produce antibiotics. Brian & Hemming (1945) showed the production of gliotoxin by a fungus they thought to be a strain of T. viride. In subsequent studies Brian et al. (1946) isolated viridin from a closely related strain. Webster & Lomas (1964) detected neither gliotoxin nor viridin in cultures of Trichoderma viride or its perfect stage Hypocrea rufa (Pers.:Fr.) Fr. and showed that Brian's (1945, 1946) strains were not Trichoderma spp., but Gliocladium virens Miller, Gidens et Foster. However, Godtfredsen & Vangedal (1965) showed the production of a sesquiterpene antibiotic,

trichodermin, by T. viride. The genus Trichoderma was subsequently revised taxonomically and divided into nine species aggregates (Rifai, 1969), which reduced the widespread confusion with regard to species-specific antibiotic production. Dennis & Webster (1971a) tested Trichoderma isolates from different species aggregates for their ability to inhibit the growth of H. annosum, Rhizoctonia solani Kühn, Pyronema domesticum (Sow.:Fr.) Sacc., Fusarium oxysporum Schlecht., Pythium ultimum Trow and Mucor hiemalis Wehmer. They found production of trichodermin, dermatin and peptide antibiotics (alamethicin and suzukacillin), but no gliotoxin or viridin production. Large variations between and within species groups were observed, showing that strains may differ physiologically, although they are morphologically very similar. Trichoderma polysporum caused growth inhibition of all fungi tested, T. viride, Trichoderma longibrachiatum Rifai and Trichoderma pseudo-koningii Rifai were active against most of the test fungi, and T. harzianum showed only limited antagonistic action due to the production of non-volatile antibiotics. Brewer et al. (1987) investigated the production of alamethicins by strains of T. viride, and Donnelly & Sheridan (1986) isolated anthraquinones from cultures of T. polysporum and T. viride. The antraquinones inhibited H. annosum on agar plates and led to the production of yellow crystals by the decay fungus (Donnelly et al., 1982; Sonnenbichler et al., 1983), which may play a role in the resistance mechanism of H. annosum.

Some Trichoderma isolates tested by Dennis & Webster (1971b), using the cellophane-agar plate technique of Dick & Hutchinson (1966), produced volatile antibiotics active against H. annosum and R. solani and supported earlier reports (Bilai, 1963). Subsequently, several alkyl pyrones, as well as acetone, ethanol, ethylene and acetaldehyde were isolated from cultures of T. viride (Collins & Halim, 1972; Kikuchi et al., 1974; Moss et al., 1975) and T. harzianum (Tamimi and Hutchinson, 1975; Taylor, 1986; Claydon et al., 1987); Hutchinson & Cowan (1972) claimed that ethanol and carbon dioxide production by T. harzianum may have been responsible for the inhibition of Aspergillus niger v.Tiegh. and Pestalotia rhododendri Guba. Bruce et al. (1984; 1987)

showed control of N. lepideus in agar plates by volatile compounds produced by Trichoderma spp. The extent of inhibition was a function of the distance between antagonist and test fungi, the age of Trichoderma cultures and the amount of volatile antibiotics produced (Bruce et al., 1987). The combination of two Trichoderma spp., as in Binab FYT™, produced greater inhibition than either of the two species alone (T. polysporum and T. harzianum).

Hüttermann & Cwielong (1982) suggested control of H. annosum by an extracellular enzyme complex from T. harzianum based on studies of DeVries & Wessels (1973). This enzyme complex not only specifically degraded existing cell walls of H. annosum, but also prevented cell wall synthesis. The enzyme complex is commercially available for the preparation of fungal protoplasts ("Novozym™234"; Sigma; Eveleigh, 1985; Collings et al., 1988; Kitamoto et al., 1988). The mode of action is based on the recognition of chitin as one of the major cell wall components in H. annosum, whilst chitin is rare in cell walls of T. harzianum (Hüttermann & Cwielong, 1982). Similar mechanisms were postulated earlier, since Trichoderma spp. were known to produce a variety of cell wall lytic enzymes (Dennis & Webster, 1971c). Enhanced cellulase and chitinase activities during parasitism by T. harzianum on Sclerotium rolfsii Sacc. was observed (Elad et al., 1983).

Mycoparasitism seems to be a common feature of Trichoderma spp. (Chi, 1960; Durrell, 1966). Dennis & Webster (1971c) found that hyphae of most tested Trichoderma spp. coiled around host hyphae, but they could rarely show penetration. Most antibiotic producing strains showed inhibition of test fungi before hyphal contact occurred. Vacuolation and coagulation of cytoplasma in hyphae of H. annosum, R. solani, and Lentinula edodes (Berk.) Pegl. as a result of combined antibiotic production and hyphal interference were shown by several authors (Rishbeth, 1950; Komatsu, 1968; Holdenrieder, 1982; Wazny et al., 1987). Often, coagulation was followed by hyphal bursting, possibly providing Trichoderma spp. with additional nutrients (Dennis & Webster, 1971c). One of the polypeptide antibiotics, alamethicine, could induce such leakage of hyphal contents.

Field studies with Trichoderma spp. were not nearly as successful as laboratory tests. Based on observations of Falck (1931) and Björkman (1947), Shields & Atwell (1963) tested the ability of a T. viride strain to prevent decay of birch wood blocks and outside stored birch bolts. Trametes versicolor, Trametes hirsuta (Wulf.:Fr.) Quél., and C. purpureum were significantly inhibited on agar plates, wood blocks and during the field test, even if inoculated at the same time as the antagonist. Similar studies by Lindgren (1952) and DeFreitas & Erickson (1969) tried to establish the use of Trichoderma spp. to alter permeability and enhance treatability of softwoods and hardwoods, but their work did not lead to any feasible processes. Trichoderma spp. are often considered to be a nuisance since they affect surface characteristics of seasoning or stored wood (Smith & Cserjesi, 1983). Toole (1971) examined the antagonistic action of T. viride against P. placenta in lab studies using soil-block tests and measured weight loss, change of modulus of elasticity, loss in stress at proportional limit, and loss in stress at 5% compression strain. T. viride caused no significant change, whilst P. placenta caused significant reduction in strength properties and wood weight. In field studies Bruce & King (1986b; 1989) examined T. polysporum and T. harzianum strains isolated from Binab FYT™. Although the biocontrol fungi were found in about 90% of the poles, they concluded that the efficacy of the preparation was limited, because of an inability to spread rapidly through the poles due to the resident microbial communities in poles.

These results show quite drastically the contrast between results obtained on agar plates or wood blocks and results from experiments under natural conditions (Lundborg & Unestam, 1980; Morris & Calver, 1986; Bruce et al., 1989b). Although application of biocontrol preparations as prophylactic treatments on "microbiologically clean" poles directly or shortly after pressure treatments has been suggested (Bruce & King, 1986b), the short-term chances for feasible bio-control on utility poles remain rather poor.

In this part of the study, the effects of a Penicillium sp., S. aurantiacum, T. harzianum and T. polysporum on several white- and brown-rot fungi was investigated in laboratory tests on agar plates, wood blocks and wood wafers. One objective was a general screening for biocontrol potential of these fungi, whilst another goal was the development of more appropriate screening methods.

2.2 Materials and Methods

2.2.1. Fungi and Wood Species Tested

Four Basidiomycotina were employed to test and compare bio-control properties of four microfungi. In this study, the term "microfungus" describes members of the subdivision Ascomycotina and the form-subdivision Deuteromycotina, as proposed by Ainsworth (1966). The current names and most recent synonyms, strain numbers and sources for all test organisms are given in Table 2.1.

Two wood species were used in soil bottle and wood wafer tests. Douglas-fir heartwood (Pseudotsuga menziesii [Mirb.] Franco) and ponderosa pine sapwood (Pinus ponderosa Laws.) were cut into squares (10 x 10 x 3 mm), subsequently referred to as feeders or wafers, and blocks (10 x 10 x 10 mm). The wafers and blocks were oven dried at 60°C, cooled in a dessicator and weighed.

2.2.2. Agar Plate Test

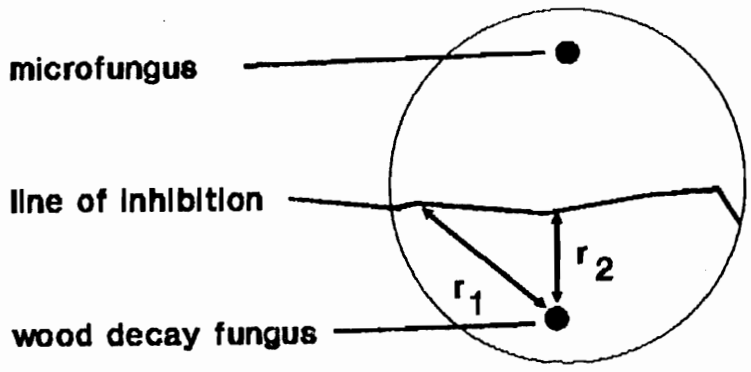
Agar plate tests were performed using three different growth media. Medium 1 contained 1.5 % (w/v) malt extract (Difco) and 1 % (w/v) agar (Sigma). Medium 2 was prepared by aseptically adding 10 ppm (active ingredient) benomyl to molten Medium 1. Benomyl inhibits the growth of many microfungi, but has minimal effects on most Basidiomycotina. Medium 3 (cf. Medium A, 4.2.1.) contained 0.5 % (w/v) glucose and 0.182 % (w/v) cellulose powder in a basal salt mixture (Hüttermann & Volger, 1973) and was solidified with 1 % (w/v) agar (Sigma).

The potential bio-control fungi were inoculated simultaneously on opposite edges of plastic Petri plates, following a slightly modified procedure described by Rayner & Todd (1979) (Fig. 2.1.a). Five repli-

Table 2.1.: Sources of fungi evaluated in agar plate, soil bottle and wafer sandwich studies.

Fungal Species	Strain Number	Source	Experiments
Basidiomycotina			
Irpex lacteus (Fr.:Fr.) Fr.	FP-105915-SP	Forest Products Laboratory (FPL), Madison, WI	Soil Bottle Test Wafer Sandwich Test
Neolentinus lepideus (Fr.:Fr.) Redhead et Ginns (Lentinus lepideus [Fr.:Fr.] Fr.)	44-C	NRCCC, Ottawa, Canada	Soil Bottle Test
Postia placenta (Fr.) M. Larsen et Lombard (Poria placenta [Fr.] Cooke)	FP-94267-R	FPL, Madison, WI	Agar Plate Test Soil Bottle Test Wafer Sandwich Test
Trametes versicolor (L.:Fr.) Pilát (Coriolus versicolor [L.:Fr.] Quél.)	R-105	FPL, Madison, WI	Agar Plate Test Soil Bottle Test
Microfungi			
Penicillium sp.	PEN-1	Oregon State Univ., Forest Research Lab (FRL), Corvallis, OR	Soil Bottle Test Wafer Sandwich Test
Scytalidium aurantiacum Klingström et Beyer	SCY-3	FRL, Corvallis, OR	Soil Bottle Test
Trichoderma harzianum Rifai	ATCC #26799	E. Nelson, USDA Forestry Sciences Laboratory (FSL), Corvallis, OR	Agar Plate Test Soil Bottle Test Wafer Sandwich Test
Trichoderma polysporum (Link.:Pers.) Rifai	ATCC #20475	E. Nelson, FSL, Corvallis, OR	Soil Bottle Test

(a)



Percent inhibition = $([r_1 - r_2]/ r_2) \times 100$ [%]

(b)

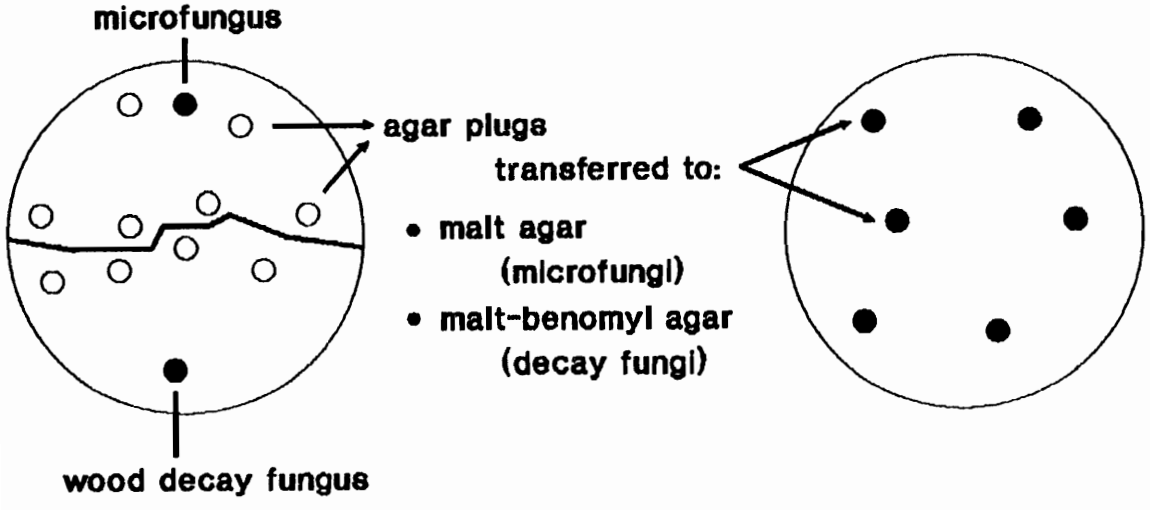


Figure 2.1.: Arrangement of inoculum and measurement of growth radii (r_1 = maximal radial growth of decay fungus in mm; r_2 = growth of decay fungus to inhibition zone in mm) (a) and reisolation pattern to determine survival or death of test fungi (b) following cross-plating experiments with T. versicolor, P. placenta and T. harzianum on agar-plates.

cates for each combination were prepared. The plates were sealed with Parafilm^R and incubated in the dark at 19° - 23°C (room temperature) for up to 15 weeks. At the end of the incubation period, reisolation of both the respective decay fungus and the potential bio-control fungus was attempted by plating mycelium aseptically removed from the test plates on Medium 1 and Medium 2 (Fig. 2.1.b.). Death was defined as the inability of test fungi to grow or germinate.

Inhibition of hyphal extension, reflected by the maximal radial hyphal growth (mm) on plates measured at 2 day intervals over period of two weeks, was used as the measure of bio-control potential (Fig. 2.1.a.). After this point, measurements were only made every 7 days. Percent inhibition was calculated following the proposal of Benko (1988):

$$\text{percent inhibition } (\%) = [(r_1 - r_2) / r_2] \times 100$$

where: r_1 = maximum radial growth (mm) of decay fungus,

r_2 = radial growth (mm) of decay fungus to inhibition zone

2.2.3. Soil Bottle Tests

A modified soil-block test (ASTM, 1989) was used to determine the biocontrol potential of microfungi on wood substrates. Inoculum for soil bottle tests was prepared by adding 25 cm^3 of garden soil to each of 64 deep Petri dishes (radius = 45 mm, height = 25 mm), 32 dishes for each wood species. Forty feeder squares were placed on the soil surface of each dish and 10 ml of distilled water was added to raise the soil moisture content (MC) to 50-60 % (Fig. 2.2.a.). The dishes were autoclaved at 121°C for 45 min. After cooling, each feeder square in a dish was inoculated with a mycelial plug, cut from the actively growing edge of a 7 day old malt agar culture of the appropriate test fungus.

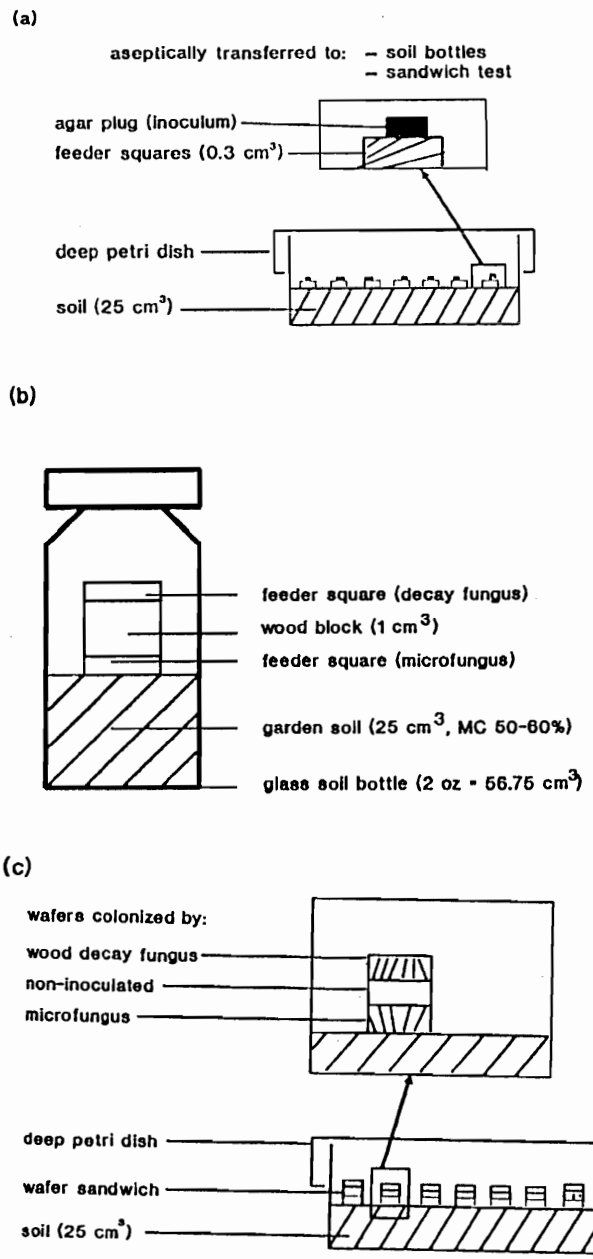


Figure 2.2.: Arrangement of (a) feeder wafers in deep Petri dishes on garden soil, (b) of 1 cm^3 ponderosa pine or Douglas-fir blocks and feeder wafers inoculated with a biocontrol fungus (bottom) and decay fungus (top) in 2 oz (56.75 ml) soil bottles and (c) of wafer sandwich consisting of a feeder inoculated with a biocontrol fungus, a non-inoculated wafer and a feeder inoculated with a decay fungus (from bottom to top).

Two dishes were inoculated for each combination of fungus and wood species. The Petri dishes were incubated in the dark for 30 days at 20°-23°C (room temperature).

Soil bottles (2 oz., 56.75 ml; n = 545) were filled with 25 cm^3 of garden soil whose initial MC ranged from 15 - 20 %. Five ml of distilled water were added to raise the MC to approximately 35 - 40 %. It was not possible to reach the desired 50 - 60 % MC in one single step because of the high sand fraction in the test soil. Therefore, 5 ml of distilled water was aseptically added to each soil bottle two weeks after inoculation, raising the MC to 50 - 55 %.

The filled bottles were autoclaved (121°C, 45 min), cooled overnight and autoclaved a second time. A feeder square colonized by the respective potential bio-control fungus was placed on the soil surface in the bottles (n = 320), a steam-sterilized block (100°C, 40, min) of one wood species was placed on the feeder square, and a second feeder square colonized by a decay fungus was placed on top (Fig. 2.2.b.). Ten replicates for each combination were prepared. Control bottles contained either one test fungus alone (n = 160) or noninoculated feeder squares (n = 20). The assembled bottles were incubated in the dark at 19° - 23°C (room temperature). Soil MC was monitored by harvesting control bottles containing noninoculated wood (n = 45) at approximately 3 day intervals to ensure that the MC remained at levels conducive to fungal survival and decay.

Control bottles containing P. placenta and the noninoculated blocks were harvested after 9 weeks of incubation. The remaining bottles were harvested after 12 weeks. The blocks were removed from the bottles with sterile forceps, carefully scraped clean of adhering mycelium and weighed to determine MC. Collected mycelium was plated on 1.5 % (w/v) malt extract agar to determine survival of the micro- and decay fungi. The blocks were oven-dried for 48 hrs at 60°C and weighed to determine wood weight loss, which served as a measure of wood decay and, when compared to controls, as a measure of inhibition of decay by the microfungi.

Statistical analyses were performed with the GLM-ANOVA test of the NumberCruncher statistical program using a Newman-Keul post-hoc test at $\alpha = 0.05$ (Steel & Torrie, 1980; Hintze, 1987).

2.2.4. Sandwich Wafer Tests

The inoculated feeder squares (Fig. 2.2.a.) were also used to evaluate a more rapid method for assessing bio-control potential of Penicillium sp. or T. harzianum against P. placenta and Irpex lacteus Fr.

Ten feeder squares, either colonized by a given microfungus or noninoculated, were placed on the surface of 25 cm^3 garden soil in each of 10 deep Petri dishes, topped with a weighed, noninoculated feeder square ("wafer") and finally with a feeder square either colonized by a decay fungus or noninoculated for controls (Fig. 2.2.c.). Each combination of decay vs microfungus was represented by 10 "sandwiches"; twenty sandwiches were prepared as noninoculated controls and for each test fungus alone (n = 100). The sandwiches were incubated in the dark for 6 weeks at 19° - 23°C (room temperature) before being harvested and evaluated as described for the blocks (cf. 2.2.2.).

2.3. Results and Discussion

2.3.1. Agar Plate Tests

The maximal radial growth of the test fungi was different on the three chosen media (Fig. 2.3.). As expected, benomyl inhibited the growth of T. harzianum almost completely, but P. placenta and T. versicolor were only slightly affected by the chemical. Trichoderma

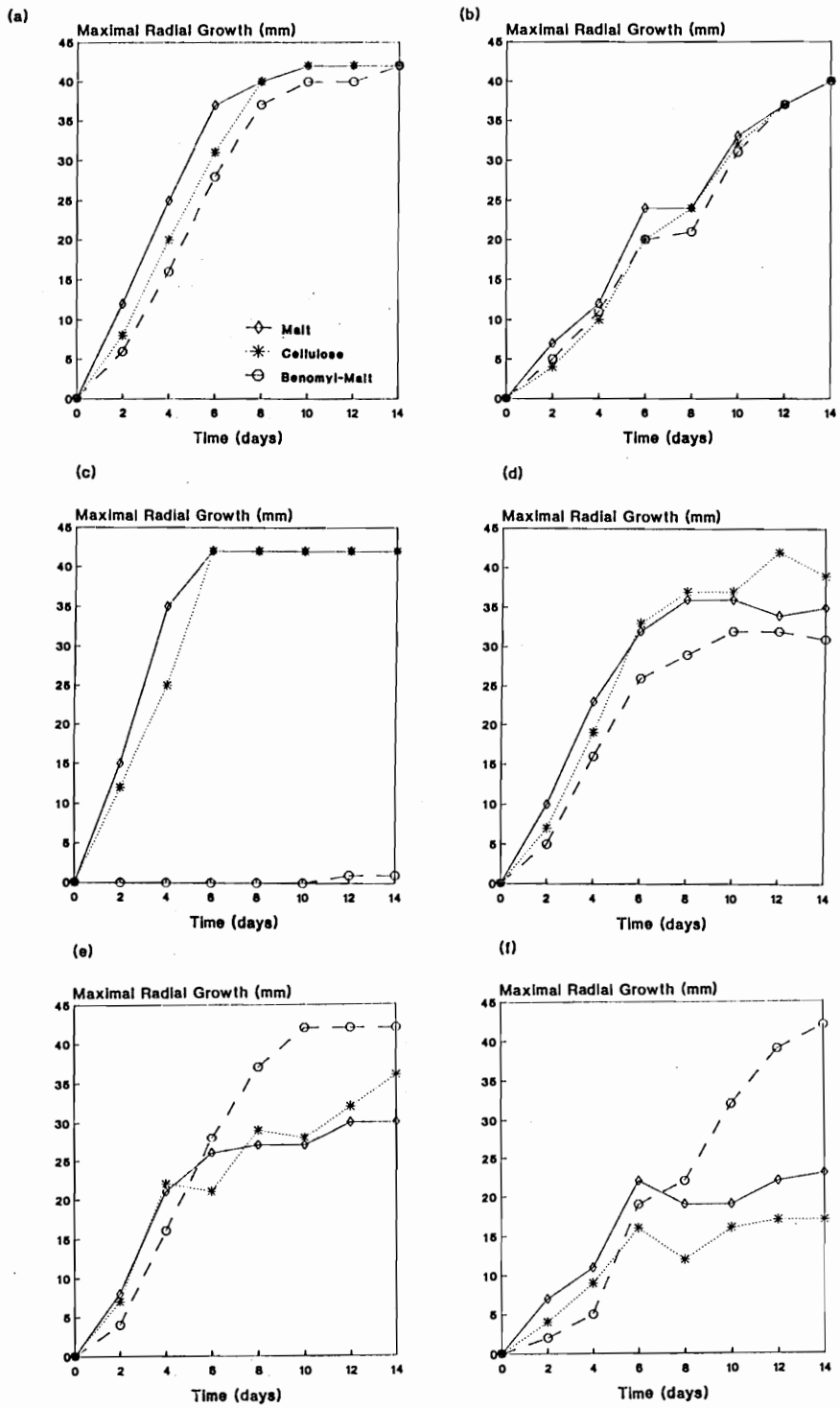


Figure 2.3.: Maximal radial growth (mm) of (a) T. versicolor, (b) P. placenta, (c) T. harzianum, (d) T. versicolor vs. P. placenta, (e) T. versicolor vs. T. harzianum or (f) P. placenta vs. T. harzianum on malt agar, cellulose-glucose agar or malt-benomyl agar.

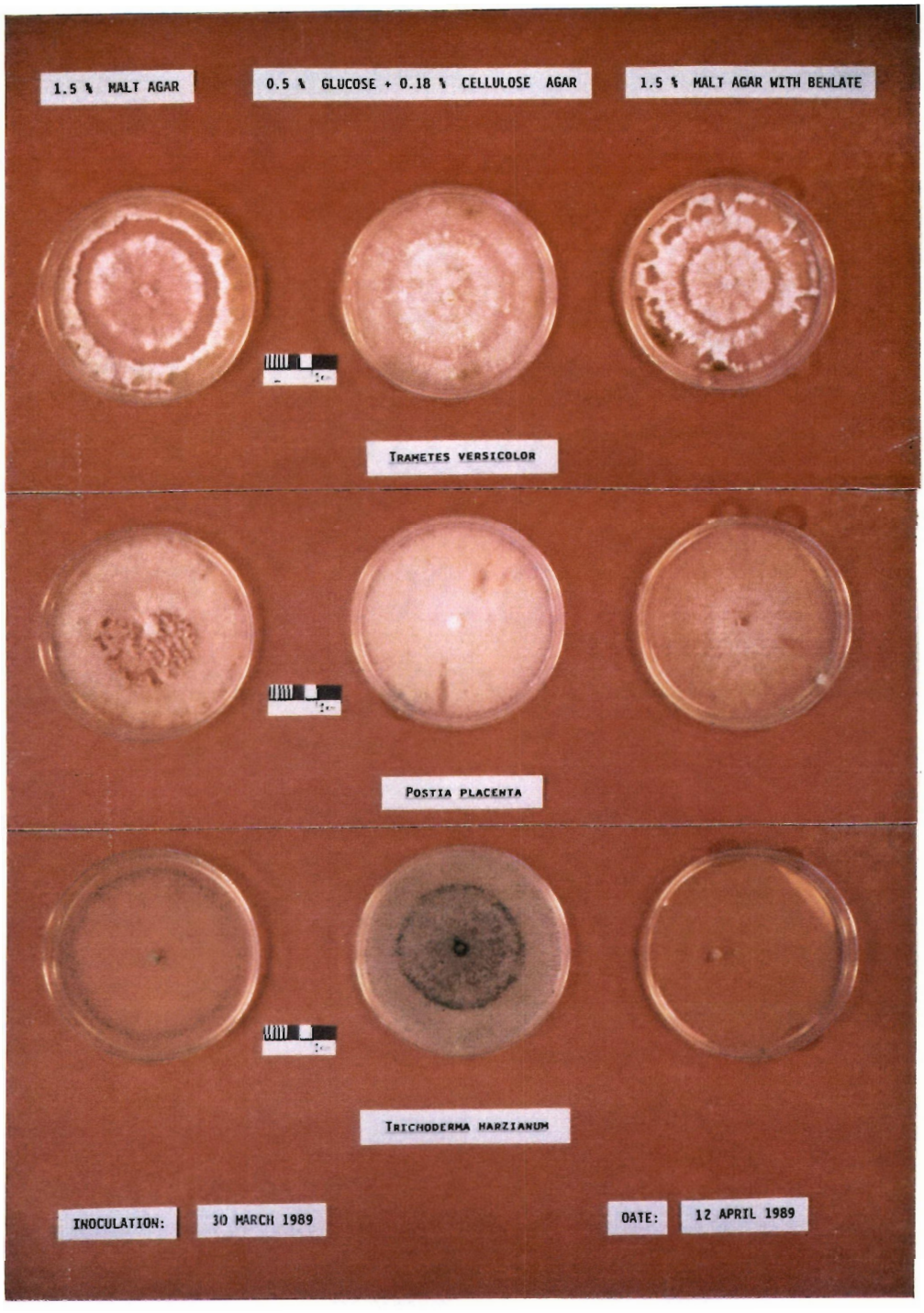
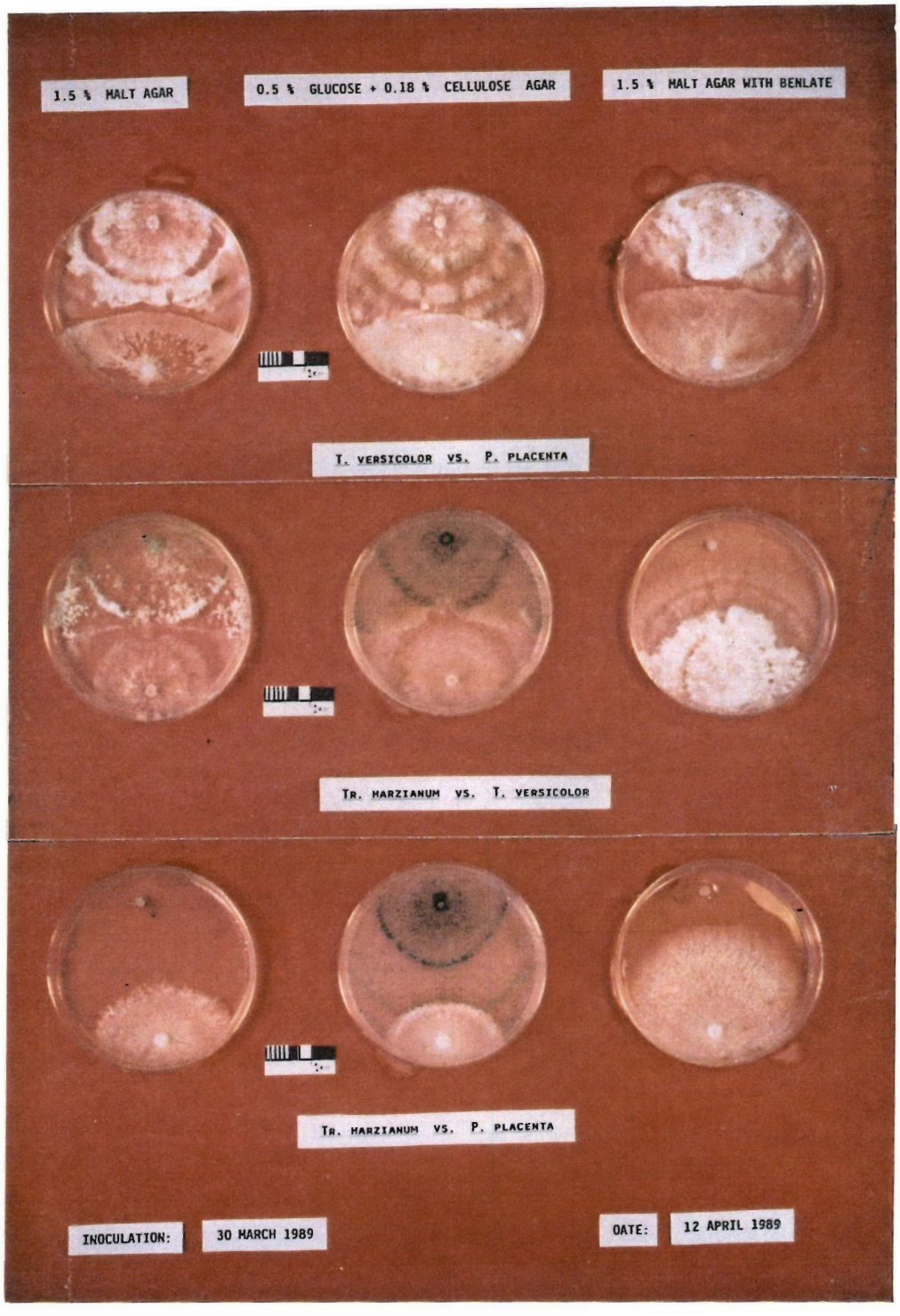


Figure 2.4.: Appearance of pure and mixed cultures of (a) T. versicolor, (b) P. placenta, (c) T. harzianum, (d) T. versicolor vs. P. placenta, (e) T. versicolor vs. T. harzianum, or (f) P. placenta vs. T. harzianum on malt agar, cellulose-glucose agar or malt benomyl agar [(d) - (f) = following page).

(d)



(e)

(f)

(Figure 2.4.: continued)

harzianum was the fastest growing fungus.on malt and cellulose-glucose agar, however, followed by T. versicolor and P. placenta.

In combinations of T. harzianum with either decay fungus, inhibition of growth occurred as soon as the mycelia met, but no inhibition zone was formed (Fig. 2.4.e.+f.). Instead, the mycelia overgrew each other or formed more dense mycelium at the line of contact. Trichoderma harzianum formed conidia rapidly on this line, after only 3 or 4 days after inoculation (Fig. 2.4.). Moreover, T. harzianum appeared to grow through the agar at the bottom of plates, thus at least initially avoiding the mycelium of T. versicolor or P. placenta.

Mycelia of T. versicolor and P. placenta grew into each other throughout the depth of the agar, forming a dense line or zone of contact. No barrages ("zone lines") or inhibition zones occurred, however. Sometimes, but not regularly, T. versicolor was able to arrest the growth of P. placenta for about one week, resulting in complete overgrowth of the plates with T. versicolor. After approximately three weeks, P. placenta was able to recover and the interaction resulted in complete deadlock in all plates inspected (Fig. 2.4.). Some plates showed hyphal cord formation of T. versicolor in attempts to overgrow the line of contact with P. placenta, a feature of interspecific mycelial interactions described by Cooke & Rayner (1984).

A quantification of these interactions as percent inhibition showed that T. harzianum could not sufficiently control P. placenta and T. versicolor on malt agar (Tab. 2.2.). Benko (1988) considered a value of 85 - 90 % percent inhibition as a measure of high bio-control potential for bacteria. Trichoderma harzianum never reached that level during the first two weeks of the test and its mycelium was in fact regularly killed on malt agar. On cellulose-glucose agar, T. harzianum inhibited both decay fungi and was eventually able to kill them. Postia placenta and T. versicolor inhibited each other's mycelial development on all three media to approximately the same extent. Due to its faster growth, T. versicolor generally covered a larger plate area, but the interactions nevertheless always ended in deadlock.

Table 2.2.: Percent inhibition and survival of cross-plated pairings of *T. versicolor*, *P. placenta* and *T. harzianum* after 1, 2 or 12 weeks of incubation.

Test Fungus	Antagonist	Malt[1]				Glucose-Cellulose				Benomyl			
		1 wk	2 wk	12 wk	Surv.[2]	1 wk	2 wk	12 wk	Surv.	1 wk	2 wk	12 wk	Surv.
T. versicolor	*T. harzianum*	48[3]	63	50	+/-	60	14	100	-/+	0	0	75	+/+
	P. placenta	23	68	60	+/+	48	54	45	+/+	21	52	55	+/+
P. placenta	*T. harzianum*	13	50	0	+/-	40	43	96	-/+	0	0	0	+/-
	T. versicolor	2	60	70	+/+	12	50	60	+/+	30	45	35	+/+

[1] Malt = 1% (w/v) malt-agar; Glucose-cellulose = same as liquid medium, solidified with 1% (w/v) agar; Benomyl = 1% Malt agar with 1% (v/v) benomyl.
[2] Surv. = Survival of test fungus/antagonist after 14 wks, where (+) denotes survival and (-) denotes death of fungi.
[3] Percent inhibition (%) = $[(r_1 - r_2)/r_2]100$, where r_1 = maximum radial growth of test fungus, r_2 = radial growth of test fungus to inhibition zone.

The results presented are comparable with those obtained with the same strain of T. harzianum in earlier studies (Bettucci et al., 1988; Highley & Ricard, 1988; Bruce & Ricard, 1989). Only slight inhibition of P. placenta and T. versicolor was reported, with brown rot fungi generally being controlled more readily by cell-free filtrates of T. harzianum, incorporated into agar plates (Bruce & Highley, 1989). Culture conditions and malt agar composition were not exactly the same, but approximated each other. The variable responses on different media tested in this study, however, should caution against only screening on agar plates.

2.3.2. Soil Bottle Tests

Weight losses of ponderosa pine blocks obtained in soil bottle tests were higher than those in Douglas-fir blocks, except for blocks exposed to P. placenta (Tab. 2.3.). Postia placenta is frequently isolated from dead Douglas-fir (Eslyn, 1970; Graham & Corden, 1980; Zabel et al., 1980; Przybylowicz et al., 1987; Meyer et al., 1988). It was, however, reported to occur on dead southern pine (Zabel et al., 1982) and on both hardwood and softwood structural timbers (Gilbertson, 1981; Ryvarden, 1978). Irpex lacteus, T. versicolor and N. lepideus showed almost no weight loss (0.8 - 1.1 %) and therefore no decay of Douglas-fir, even after 12 weeks of incubation. This was somewhat unexpected, since all three fungi have been reported to colonize Douglas-fir wood (Davidson et al., 1942; Nobles, 1965; Bega, 1978), although it is by no means a preferred substrate (Farr et al., 1989).

On ponderosa pine blocks, S. aurantiacum was associated with the lowest weight losses. When compared to the single fungus controls, weight losses were decreased by 83 - 84 % (P. placenta, I. lacteus and T. versicolor) and 75 % (N. lepideus) in the respective combinations with S. aurantiacum (Tab. 2.3.). The other three potential bio-control fungi exerted less clear influences on wood weight losses. Trichoderma

Table 2.3.: Weight losses of Douglas-fir heartwood and ponderosa pine sapwood blocks (1 cm^3) exposed to decay fungi, biocontrol fungi or combinations of both in a modified soil bottle test.

Decay fungus	Microfungus	Wood weight loss[1] (%) Ponderosa pine	Douglas-fir
Control[2]		-0.7 a[3]	-1.1 a
Control[4]	*Penicillium* sp.	0.5 a	-0.3 a
	Scytalidium aurantiacum	0.2 a	-0.4 a
	Trichoderma harzianum	-0.3 a	-0.7 a
	Trichoderma polysporum	0.4 a	-0.4 a
Postia placenta	Control[5]	41.9 i	44.6 g
	Penicillium sp.	31.5 h	25.3 f
	Scytalidium aurantiacum	6.7 c	13.9 d
	Trichoderma harzianum	28.2 h	21.4 e
	Trichoderma polysporum	17.1 g	19.8 e
Irpex lacteus	Control	20.1 g	1.1 b
	Penicillium sp.	13.0 f	2.1 c
	Scytalidium aurantiacum	3.2 b	2.6 c
	Trichoderma harzianum	12.4 e	1.9 c
	Trichoderma polysporum	14.9 f	1.9 c
Trametes versicolor	Control	17.3 g	0.8 b
	Penicillium sp.	12.4 e	2.4 c
	Scytalidium aurantiacum	3.0 b	2.2 c
	Trichoderma harzianum	11.1 d	2.2 c
	Trichoderma polysporum	12.3 e	2.4 c
Lentinus lepideus	Control	6.1 c	0.8 b
	Penicillium sp.	1.6 a	2.3 c
	Scytalidium aurantiacum	1.5 a	2.7 c
	Trichoderma harzianum	4.0 b	2.2 c
	Trichoderma polysporum	3.6 b	3.0 c

1 = values represent means of 10 replicates.
2 = non-inoculated feeders and blocks.
3 = values for a given wood species followed by different letters are significantly different according to Newman-Keul Test ($\alpha = 0.05$).
4 = non-inoculated top feeder.
5 = non-inoculated bottom feeder.

harzianum reduced the weight losses associated with P. placenta and N. lepideus by 34 %. In combination with T. versicolor and I. lacteus, the reduction was 36 % and 38 %, respectively. Trichoderma polysporum led to reductions in weight loss of 59 % for P. placenta, 41 % for N. lepideus, 26 % for I. lacteus and 29 % for T. versicolor, respectively. The Penicillium sp. was quite successful as an inhibitor for N. lepideus (75 %), but performed poorly against the three other fungi: 25 % in P. placenta, 28 % in T. versicolor and 35 % in I. lacteus.

However, all four microfungi significantly reduced the weight losses associated with the respective decay fungus on ponderosa pine. The results are comparable to those obtained by Oxley (1976), who evaluated the ability of six microfungi, among them T. viride and S. lignicola, to arrest decay of N. lepideus in Scots pine (Pinus sylvestris L.) wood blocks. The T. viride strain decreased decay development by 60 %, whilst the S. lignicola strain almost completely inhibited the decay fungus during a 12 week tests.

The effects of the potential bio-control fungi on weight losses in Douglas-fir blocks were quite different: all microfungi had little or no inhibitory effect on weight losses associated with I. lacteus, T. versicolor or N. lepideus. In most cases, decay appeared to be stimulated by the presence of microfungi (Tab. 2.3.), a phenomenon also reported by Dennis & Webster (1971a). Lindgren (1952) found that colonization with Trichoderma spp. prior to pressure treatments enhanced wood penetrability and thus treatability of southern pine timber. It is possible that microfungi such as Penicillium spp. or Trichoderma spp., although themselves not capable of actively decaying solid wood, are nevertheless able to condition the wood for colonization by decay fungi. This may be especially true if the wood in question is comparatively decay-resistant towards a given decay fungus and thus not a regular host or substrate. "Conditioning" of wood may promote attack by decay fungi during natural successions (Hulme & Shields, 1970; 1972a+b; 1975; Merrill & Shigo, 1979; Bjurman, 1988).

Although conditioning of wood by microfungi or bacteria can occur, the rather perplexing results for Douglas-fir may be entirely due to the

effect of wood species on decay development. Trametes versicolor is a cosmopolitan decay fungus capable of colonizing many substrates, but prefers hardwood or pine species to Douglas-fir (Ryvarden, 1978; Zabel et al., 1985). Similarly, I. lacteus and N. lepideus are more commonly isolated from various pine species than from Douglas-fir (Bruce & King, 1983; Zabel et al., 1982; Eslyn, 1986). The weight losses show clearly that decay of Douglas-fir blocks by these three species was, at best, marginal (Tab. 2.3.). In fact, weight losses of 1 - 3 % are within the error margin of the soil bottle experiment itself so that definitive conclusions concerning the inhibitory or conducive effects of the microfungi on fungal decay of Douglas-fir cannot be drawn. The results, therefore, illustrate the importance of selecting the appropriate wood material for soil bottle tests: in addition to the two softwood species, a hardwood species should have been selected to allow evaluation of the effects of microfungi on decay by T. versicolor and I. lacteus. In earlier studies, complete decay resistence of Scots pine wood against N. lepidues and A. carbonica and reduction of wood weight losses due to T. versicolor were achieved (Bruce & Highley, 1989a; Bruce et al., 1989a). The same was true for Japanese beech (Fagus crenata Bl.) or cedar (Cryptomerica japonica D.Don.) blocks precolonized by Trichoderma spp. (Tanaka et al., 1988).

The only fungus controlled by the four microfungi on Douglas-fir was P. placenta, which is most often associated with this wood species (Graham & Corden, 1980). Scytalidium aurantiacum was again the best inhibitor (69 %), followed by T. polysporum (56 %), T. harzianum (52 %) and the Penicillium sp. (43 %).

The soil bottle experiment showed that the bio-control potential of microorganisms on wood can be evaluated using a small block test, but the time required to obtain substantial weight losses was considered too long to fulfill the requirements for screening tests, particularly in moderately decay-resistant wood species such as Douglas-fir. Smaller samples may prove more useful for a rapid evaluation of bio-control organisms.

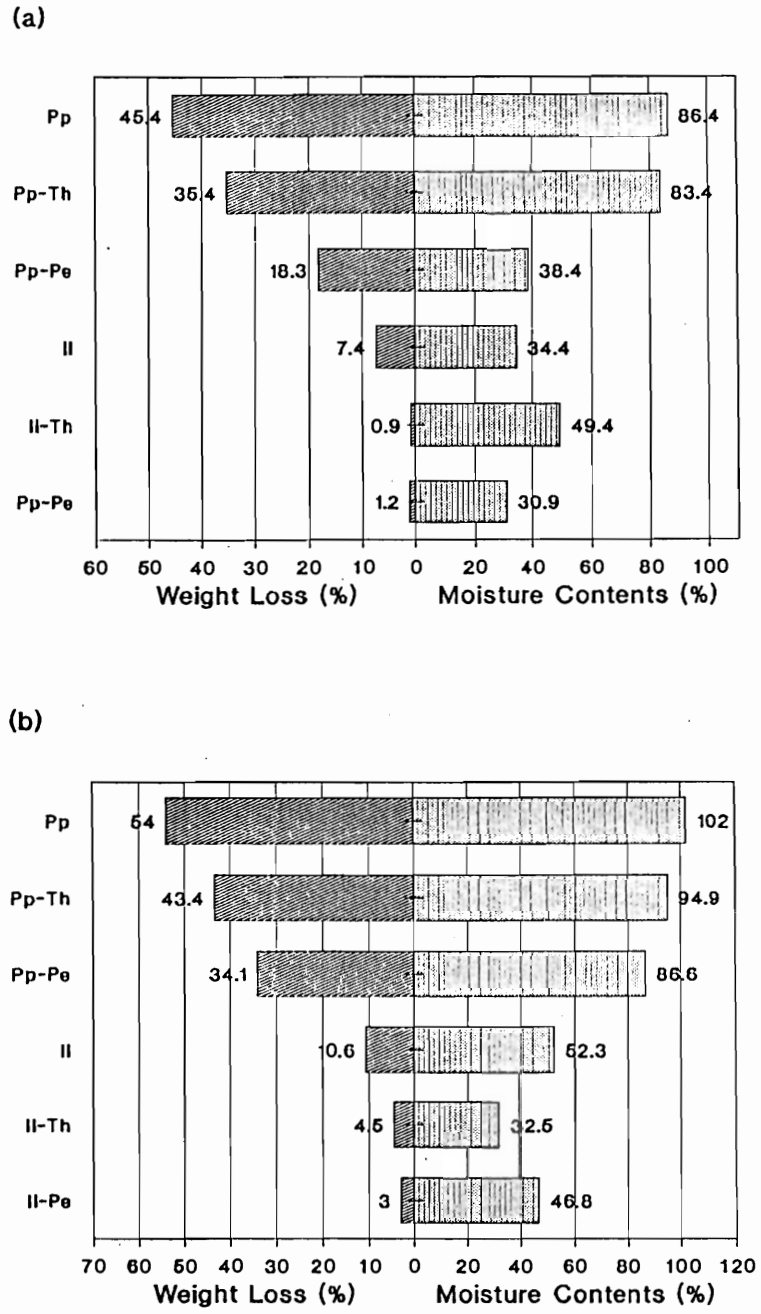


Figure 2.5.: Wood weight loss and moisture contents of wafers after 6 weeks of exposure to combinations of P. placenta (Pp), T. harzianum (Th), Penicillium sp. (Pe) or I. lacteus (Il) in a wafer sandwich test on (a) Douglas-fir wafers or (b) Ponderosa pine wafers.

2.3.3. Sandwich Wafer Tests

Only two Basidiomycotina and two microfungi were evaluated using the sandwich wafer method (Fig. 2.5.). Wood weight losses brought about by P. placenta were comparable to those found in the soil bottle test (on ponderosa pine: 54 % in the wafer, 42 % in the blocks; on Douglas-fir: both 45 %). Weight losses associated with I. lacteus increased for Douglas-fir wafers (7.4 % compared to 1.1 % for blocks), but decreased for ponderosa pine wafers (10.6 % compared to 20.1 % for blocks).

Both potential bio-control fungi inhibited the growth of both decay fungi (Fig. 2.6.). Weight losses caused by I. lacteus were decreased by 58 % and 88 % in the presence of T. harzianum and 72 % and 84 % in the presence of Penicillium sp. on ponderosa pine and Douglas-fir, respectively. For P. placenta the values were 20 % and 22 % in the presence of T. harzianum and 37 % and 60 % in the presence of Penicillium sp. for ponderosa pine and Douglas-fir, respectively. The results were thus almost the reverse of those obtained for the respective combinations on blocks: Penicillium sp. showed a higher bio-control potential than T. harzianum on wafers. The weight losses were comparable to those obtained in the soil bottle test if the length of incubation and wood surface to wood volume ratio were taken into account: the smaller sample size and shape allowed fungal colonization to progress faster and significant weight losses were recorded after only 3 weeks for P. placenta.

The small dimensions of the wafers produced more variation in MC when compared to the small blocks. The MC of decayed blocks ranged from 57 % to over 300 %, therefore being well over the minimal MC range for decay, generally considered to be about 30 % depending on the specific fiber saturation point of each sample. Trichoderma harzianum was able to grow well at a sawdust MC from 32 - 72 % (Komatsu, 1976). It is apparent that in the sandwich wafer test, high weight losses were strongly associated with high MC (Fig. 2.5.), which was expected since MC and progression of decay are generally directly correlated (Ammer, 1964). Low weight losses of specific wafers may have been due to

desiccation, particularly of the wafers inoculated with decay fungi which were placed on top of the sandwich and not in contact with the wet soil (40 - 55 % soil MC). Careful monitoring of MC during every test is one way to mitigate this problem. An even more convenient solution may be to rotate the sandwiches by 90 degrees, thus exposing all three wafers equally to the soil surface (Dawson-Andoh, 1989, pers. commun.).

2.3.4. Comparison of the Test Methods

Assessing the bio-control potential of microorganisms by laboratory techniques will remain a pre-requisite for successful field tests (Bruce & Highley, 1989). It is therefore necessary to develop adequate screening assays like those used in this study. Both, the soil bottle test and the sandwich wafer test gave results which are comparable to those obtained by either agar plate methods, full-scale wood block tests or the sawdust tube assay (Oxley, 1976; Morris et al., 1984; Highley & Ricard, 1988; Morrell & Sexton, 1988; Seifert et al., 1988; Bruce & Highley, 1989). This suggests the usefulness of agar plate tests for initial screenings, since predictions with regard to bio-control potential were confirmed. Moreover, no efficient methods of antibiotic isolation from wood samples are available. If the presences of antibiotics is suspected, these chemicals must be purified and evaluated from liquid or agar cultures (Stillwell et al., 1969; Klingström & Johansson, 1973; Drews, 1983). Mechanisms and metabolism of antibiotics, such as gliotoxin and viridin from G. virens, are now well understood (Jones & Hancock, 1987; 1988), and research on toxins from Trichoderma continues (Claydon et al., 1987), almost entirely carried out on artificial media. At the same time, the utility of small wood sample tests for detecting modes of interactions, possibly completely overlooked in agar tests, is obvious. Whilst agar tests can predict the presence of diffusible toxic metabolites and allow the evaluation of morphological changes due to interactions (Cooke & Rayner, 1984), it is

not possible to unequivocally detect the presence of volatile compounds, hyphal interactions or toxic metabolites as they would exist and react in wood (Dowding, 1978; Andrews, 1985). Therefore, any screening employing agar plates should be followed by small size wood sample tests of the potential bio-control organisms.

Overall, the wafer test appears to be a simple, rapid method for determining the relative ability of microfungi to decay wood or inhibit decay fungi when compared to a full-scale soil-block test. Although the incubation period is somewhat longer than for agar plate tests, the assay provides more information than agar or even sawdust tube assays (Morris et al., 1984), since the fungi are forced to interact on or within solid wood under conditions which more closely resemble a natural environment. Many modifications of this methods are possible, allowing either an assessment of the influence of various decay determining parameters such as soil and wood MC, incubation temperature or surface nutrients, or the investigation of specific modes of antagonism. Moreover, organic and inorganic, liquid and solid toxicants - alone or in combination with biocontrol organisms - may be screened for their efficacy using this method. Fumigant resistant strains of Trichoderma may be used to simultaneously evaluate biocontrol potential and the influence of fumigants on decay fungi (Papavizas et al., 1982; Nelson et al., 1987; Ahmad & Baker, 1987).

Some disadvantages or uncertainties with regard to the soil bottle and sandwich wafer test remain to be rectified, however. Moisture relationships in blocks or wafers must be more tightly controlled than during this study to reach adequate wood weight losses within a reasonable time period. Wood samples may be equilibrated at a MC commonly found for high hazard wood decay conditions to stimulate rapid colonization by decay fungi.

Equally problematic is the determination of an adequate incubation period for assays using small wood samples. In standard soil bottle tests, developed to determine effects of chemicals on decay fungi, wood weight loss is usually determined after a defined incubation time known to be sufficient for significant decay. The influence of sample size

has been the object of many investigations, which mostly caution against samples smaller than 4 cm^3 or 8 cm^3 (5 x 20 x 40 mm for 30 days, or 10 x 20 x 40 mm for 60 days of incubation; Ueyama, 1966). Below these dimensions, decay rate and wood weight loss are not strictly correlated if defined incubation periods are chosen (Ueyama, 1966). Carey (1988) carried out successful soil block studies with sample sizes of 5 x 10 x 30 mm and incubation periods of 3 weeks. Alternatively, specified minimal weight losses reached in a certain percentage of sample bottles may be employed to indicate sufficient incubation. Either way, microbial interactions will not occur according to incubation schedules and survival of both organisms, referred to before as deadlock, is probably the most common microbial interaction over an extended period of time, with each fungus occupying a portion of the substratum (Rayner & Todd, 1979; Rayner & Webber, 1984). The parameters of interaction measured can only reflect an interaction pattern at a very specific time during the course of the interaction. Therefore, when using small scale wood-based or agar plate tests, it is always important to realize that the results will reflect changing testing conditions during the incubation or testing period.

The mode of colonization of wafers by microfungi and decay fungi, connected to their survival strategies and the pre-defined length of an incubation period presents a third problem. Both blocks and wafers were most often rapidly surface-colonized by Penicillium sp. and the two Trichoderma spp., whereas S. aurantiacum seemed to require a longer colonization period. The microfungi formed conidia rapidly, mostly on the wood surface. In the soil, chlamydospores of Penicillium sp. and the Trichoderma spp. were commonly seen around wafers. Whilst these survival structures were not present during the first two or even three weeks after inoculation with S. aurantiacum, they were later most frequently found on wood which became overgrown by the decay fungus.

The respective decay fungus, on the other hand, was initially inhibited by the microfungus, mostly due to a higher inoculum potential of the latter. To become established, survive or actively decay the wood, the decay fungus had to grow into the wood and become established

before the whole wafer or block surface was covered by mycelium of the microfungus. Once it colonized the wood, the decay fungus gained wood surface area back and could eventually colonize the interior of the blocks and wafers.

It was impossible to follow colonization patterns or the eventual outcome of interactions, even in a simple system like the wafer sandwich; however, these assays were proposed for screening purposes and not for in-depth ecological studies. Although Garrett's (1951; 1963) concept of stages during wood decomposition (primary saprophytic fungi → cellulose decomposers → secondary saprophytic fungi → lignin decomposers) is widely cited in the literature, Käärik (1975) cautions against the undifferentiated application of this over-simplified model to a specific wood decay situation. It would be useful to intensively study colonization patterns and interactions of fungi on wood products in a natural environment without time constraints. The methods of Rayner & Todd (1979) may be particularly useful to develop appropriate models of fungal interactions. Although first steps in that direction have been made, the colonization of wood by decay fungi has always been emphasized (Bruce et al., 1984b; Zabel et al., 1985; Sexton et al., 1990; Smith et al., 1990). Any decay development study should also include work on microfungi or bacteria present. An example is a study by Gramss (1987), who investigated the competitive saprophytic ability (also called "kratovirulence") and combative competition ("pathovirulence") of 41 wood decay fungi with various other xylophilous fungi, leading to the development of a classification system for ecological capabilities of wood decay fungi.

2.3.5. Conclusions

Although highly artificial when compared to natural conditions of wood decay, a combination of agar plate and small size wood sample assays may be sufficient to detect organisms with high bio-control

potential. Generally, wood assays are to be preferred over agar tests, since they better approximate natural wood decay, but agar plate assays have their place during initial screening periods when high numbers of organisms and strains need to be sorted out, or when antibiotics need to be purified. Agar plate assays will normally detect extraordinarily potent bio-control organisms, but strains or species which do not produce diffusible toxic metabolites or grow poorly on agar plates may be overlooked. To mitigate problems encountered on artificial media, studies may be conducted in greenhouse soil beds using accelerated stake tests or outside on small size wood samples. Although "contamination" with resident or invading organisms is likely, these studies may provide insights into possible durable habitat changes associated with the tested potential biocontrol organisms.

Trichoderma harzianum would not have been chosen for the subsequent experiments of this study if a decision would have been based on agar plate tests only. Likewise, T. polysporum alone seems to be a rather weak bio-control fungus. The commercial bio-control formulation Binab FYT™ is composed of both T. harzianum and T. polysporum and a strain of Scytalidium sp., and together these fungi seem to exert at least some degree of control. Scytalidium aurantiacum seems to be a rather promising bio-control fungus, although difficulties establishing this organism in stumps or poles have not been solved. It was dropped from this study because it grows very poorly on synthetic liquid media.

3. Microscopic Characteristics of Test Fungi

3.1. Literature Review - Methods for Microscopic Inspection of Growing Hyphae and Microscopic Evidence for Biological Control Mechanisms

Examining hyphal interactions microscopically was, until recently, primarily a domain of fungal taxonomists and geneticists (Raper, 1966; Rayner & Todd, 1977; 1978; Nguyen & Niederpruem, 1984; Aylmore & Todd, 1984; Rayner & Webber, 1984; Todd & Aylmore, 1985; Rayner & Coates, 1987). As it became evident that many specific fungal biocontrol systems were based on various forms of mycoparasitism, the biocontrol potential of fungi was studied using light or electron microscopy (Chi, 1960; Durrell, 1966; Dennis & Webster, 1971c; Traquair & McKeen, 1977; 1978; Tzean & Estey, 1978; Hadar et al., 1979; Elad et al., 1980; Burdsall et al., 1980; Barron, 1982; Nordbring-Hertz, 1984; Bowers et al., 1986; Murmanis et al., 1988).

Many techniques have been developed for studying fungal growth and morphology using phase contrast (light) and electron microscopy. The fungi are most often examined using microscope slide cultures in various types of growth chambers on agar (Riddell, 1950; Bowers et al., 1986) or on cellophane membranes (Chet et al., 1981; Aylmore & Todd, 1984; Ainsworth & Rayner, 1986). These techniques permit undisturbed examination of actively growing hyphae at various developmental stages. The method of Aylmore & Todd (1984) is perhaps the most sophisticated, but also laborious method: specially prepared and ventilated aluminium microscope slides with custom-made cellophane membranes holding an electron microscope object carrier are inoculated with fungal mycelial fragments or conidia and incubated in a moist chamber. These slides may be examined using first phase contrast and then electron microscopes.

Mycoparasitism appears to be a frequent phenomenon (Moore-Landecker, 1982). Commercially grown basidiomycetes such as Agaricus brunnescens Peck (= A. bisporus [Lange] Imbach; button mushroom) or L. edodes (shii-take) are frequently attacked by lower fungi, especially Tricho-

derma spp. (Barnett, 1963; Komatsu, 1976; Tokimoto, 1985). These fungi were most often classified as mycoparasites, since hyphal coiling, hyphal interference and subsequent cell wall lysis were associated with their presence. There appear to be two distinct types of mycoparasitism, biotrophic and necrotrophic (cf. 1.2.2.3.). Biotrophic mycoparasitism depends on long-term hyphal interactions, leading to a relationship which resembles typical plant-pathogen systems based on parasitism (Whipps et al., 1988); however, other combative mechanisms such as antibiosis and competitive abilities are important for fungi which form long-lasting parasitic interactions.

Necrotrophic parasitism is more commonly found in fungi. Hyphal interference accompanied by cell wall lysis are characteristics of this interaction which resembles predation by mammals or arthropods: hyphal association is followed by penetration and sometimes growth of parasitic hyphae within host hyphae or the leakage of cell contents (Whipps et al., 1988). The term hyphal interference was coined to describe extensive invagination and the presence of extraplasmalemma structures through which cell contents were lost, which were found on hyphal tips of H. annosum when grown in the presence of P. gigantea (Ikediugwu et al., 1970; Ikediugwu, 1976a+b). Similar observations were made by Holdenrieder (1982). Host hyphae or propagules may be dead before penetration or die due to the action of the parasite, the latter mode of action being more common (Whipps et al., 1988). Tzean & Estey (1978) reported necrotrophic parasitism by a strain of Schizophyllum commune Fr., adding to early reports of parasitism of T. viride (Weindling, 1932) and Coprinus heptemerus Lange et Smith (Ikediugwu, 1976a). Coiling around host mycelium or fructifications and penetration via hyphae or penetration pegs and subsequent intrahyphal growth were observed after the host cell wall was enzymatically digested. As in early studies (Weindling, 1932; Ikediugwu, 1976a), no diffusible antibiotics were found in this strain of S. commune (Tzean & Estey, 1978). Necrotrophic mycoparasitism generally requires intimate contact betwen the host and parasite mycelia, excretion of a specific toxic substance (Traquair & McKeen, 1978; Bowers et al., 1986) and destruction

of the host cell wall by digestive enzymes or the toxin (Barnett & Binder, 1973).

It should be stressed that the classification of mycoparasitism as either biotrophic or necrotrophic (Barnett, 1963) is highly artificial. It is difficult to determine the duration of hyphal interference, even in laboratory studies, let alone field experiments. Moreover, it is not always possible to determine whether host hyphae or, more importantly, whether a whole mycelium, i.e. fungal individuum, is living or dead. Rather, the two "types" of parasitism describe two more or less extreme phenomenal expressions of parasitism on a gradient from a para-saprophytic, short-lived, predation-like parasitism to a highly advanced, long-term parasitism.

The above discussion illustrates the limitations for using mycoparasites in biocontrol: (a) the pathogen or decay fungus has to be in an active stage with a well established population; (b) close contact is required for hyphal interactions; (c) parasitic action may seldom lead to complete eradication, since a successful, well-adapted parasite does not normally kill its host outright. Mycoparasites should be most effective against survival stages or secondary spread of pathogens (Baker, 1968). Their action will be slow, but mycoparasites may provide long-term protection if their population stabilizes over several years.

This part of the study served to link soil block and enzyme studies by investigating possible biocontrol mechanisms employed by *T. harzianum* to inhibit the growth of *T. versicolor* at the microscopic level. Since *T. harzianum* is a known mycoparasite (Dennis & Webster, 1971c; Murmanis *et al.*, 1988), hyphal coiling or other forms of hyphal interaction between the two fungi as indications of mycoparasitism were expected. Another goal of this experiment was to develop a less elaborate slide culture technique to replace the laborious and complicated cellophane membrane methods used in earlier studies (Chet *et al.*, 1981; Aylmore & Todd, 1984). The basic microscopic methods described here were also used routinely for identifying the test fungi reisolated from mixed wood or liquid cultures.

3.2. Materials and Methods

3.2.1. Test Fungi

Only T. versicolor and T. harzianum (cf. 2.2.1. and Tab. 2.1.) and their combinations on malt-agar plates (1 % [w/v] malt extract, Difco; 1.5 % [w/v] agar-agar, Sigma) and microscope slides were examined microscopically, to help relate subsequent enzyme studies with possible hyphal interactions.

3.2.2. Cultures on Agar Plates

Needlepoint agar pieces containing hyphae or aerial mycelium of the respective fungus were taken from growing edges of test fungi and from the zone of interaction in cross-plated fungal combinations, squash-mounted in 1 % phloxin or Cotton Blue-lactophenol and examined at 100 - 1000x magnification under a Leitz microscope (Nr.538170; Leitz, Wetzlar, FRG). Surface and submerged hyphae were examined. This technique was used for general identification purposes during all stages of this study, but proved insufficient for detecting hyphal interactions of fungi, since the original orientation of hyphae in agar was altered during squash-mounting.

3.2.3. Slide Cultures

Fungal cultures on microscope slides were prepared using a modification of a method described by Riddell (1950). Agar medium (1 % [w/v] malt extract, Difco; 1.5 % [w/v] agar-agar, Sigma) and all small equipment, microscope slides and cover slips were autoclaved (121°C, 25

min). The medium was aseptically poured into a Petri dish to form a 1 - 2 mm thick layer. After the medium had solidified, 5 x 5 mm squares were cut and four pieces were transferred to each microscope slide (Fig. 3.1.a.). This arrangement was chosen after fungal mycelia failed to deeply penetrate the agar and instead grew along the edge of the cover slips when one 10 x 10 mm square had been used.

The microscope slides were inoculated with a needlepoint inoculum from 4 day old cultures of the test fungi. Mycelial fragments or conidia of either only T. versicolor or T. harzianum or T. versicolor (right side) and T. harzianum (left side) were placed between two agar pieces on the microscope slide (Fig. 3.1.a.). Each slide was placed on sterilized glass rods over a saturated NaCl solution (relative humidity: 76 %; Winston & Bates, 1960) in a sterile deep Petri dish. The dish was sealed with Parafilm^{R} and incubated for up to 30 days at 19° - 22°C (room temperature). Five replicates per combination were prepared in three separate experiments. The slides were examined after 1, 3, 7, 14 and 21 days at 100 - 400x magnification using the Leitz microscope.

At each sampling point, one slide from each combination (T. versicolor and T. harzianum alone and combined) was stained with phloxin and Cotton Blue-lactophenol. The cover slip was carefully lifted from the agar and was placed aside, fungal growth facing upwards. The colonized cover slip and microscope slides were treated in essentially the same way: adhering mycelium was removed carefully and after the addition of a drop ethanol and lactophenol or phloxin, the cover slip was placed on a clean microscope slide and a clean cover slip on the original microscope slide. The dye was distributed by gently pressing down on the cover slips, yielding two new stained preparations for each originally inoculated slide. The removed agar pieces and most of the fungal growth were either squash-mounted or discarded. All that usually remained on the original microscope slide and the cover slip were lines of growth, partially connected by narrow mycelial strands (Fig. 3.1.b.). The drop of ethanol was added to wet the remaining mycelium and Cotton Blue-lactophenol or phloxin were added for staining purposes to enhance contrast of mycelial structures. The prepared slides were examined at

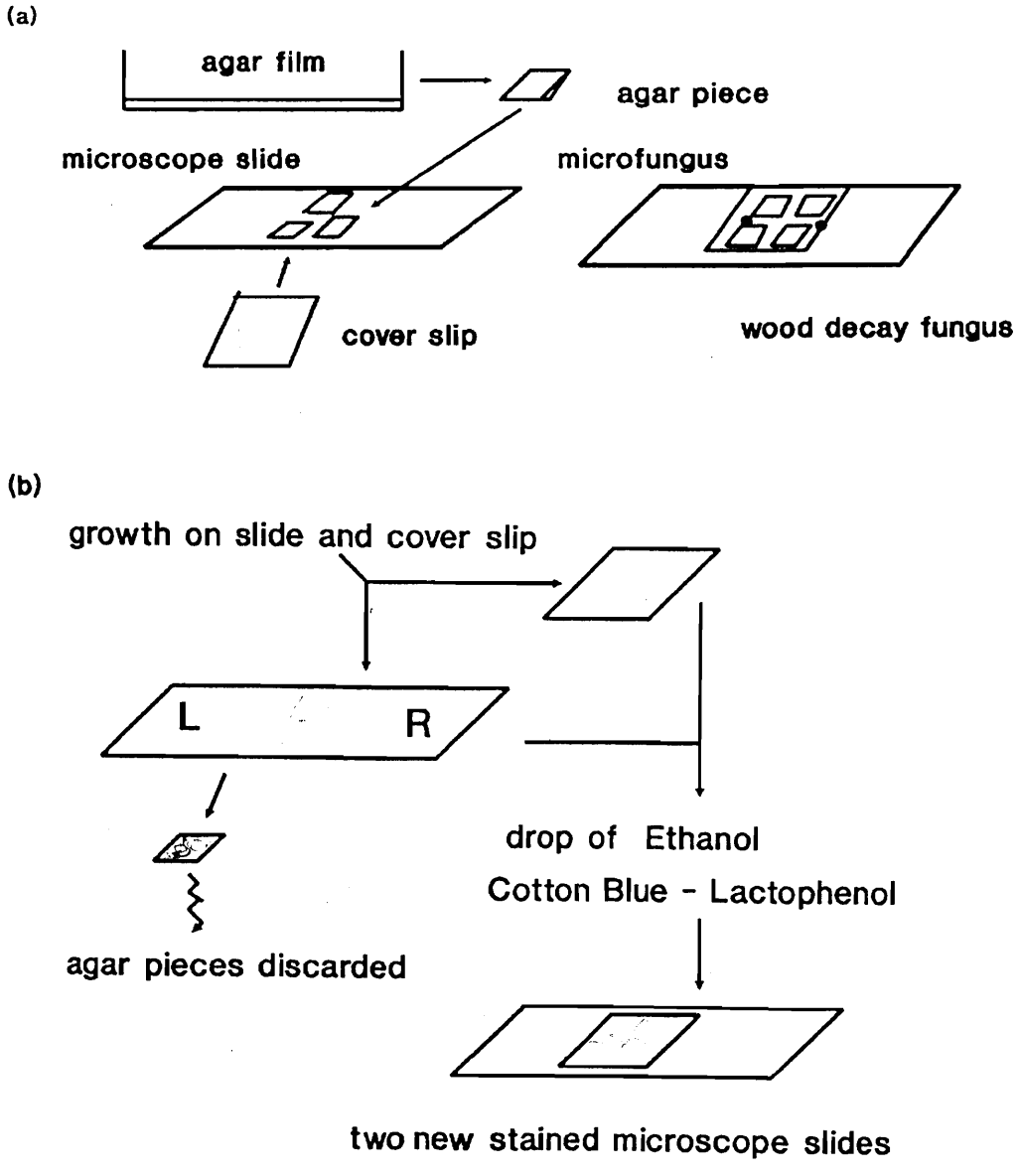


Figure 3.1.: Arrangement of agar pieces and inoculum (labelled slide on the right was used for inspections in unstained condition) (a) and procedure employed to stain adhering mycelium on cover slips and slides (b).

100 - 1000x magnification using the Leitz microscope. Selected slides were preserved for later observation by sealing the edges of the cover slips with nail-polish.

Photographs were taken with a Zeiss C35 camera back attached to a Zeiss microscope adapter and Ikophot M lightmeter (Zeiss Ikon, Stuttgart, FRG).

3.3. Results and Discussion

3.3.1. Cultural Characteristics and Identification of Test Fungi

No sexual reproductive structures of either fungi were observed in culture, which was expected. The identification of the fungi from cross-plated combinations was based on asexual spore production of T. harzianum and hyphal characteristics of both T. versicolor and T. harzianum based on published keys (Davidson et al., 1942; Nobles, 1948; 1965; Rifai, 1969; Komatsu, 1976; Domsch et al., 1980; Stalpers, 1978; Lombard & Chamuris, 1988; Wang, 1988).

Distinctive cultural features of the T. harzianum species aggregates are: no spore ornamentation, short, crowded and regular phialides, regular branching of conidiophores and overall pale green coloration of the colonies (Rifai, 1969). To be sure of conidial smoothness, conidia of 10 - 14 day old cultures were examined with an immersion lens (1000x; Domsch et al., 1980). The coconut odor (Claydon et al., 1987) is not necessarily a feature unique to T. harzianum (Rifai, 1969).

Colonies of T. harzianum grew very rapidly on malt agar, covering Petri plates within 3 - 4 days. First, a white smooth mycelium was produced, which soon turned aerial and produced bright green-yellow to dull green conidial areas which formed distinct rings of tufts on the agar surface. The hyphae were septate, much branched, hyaline, smooth-

walled and 1.2 - 4 (12) µm wide. Chlamydospores were globose, smooth-walled and appeared hyaline and varied from 6 - 12 µm in diameter. The conidiophores were numerous and much-branched, the branches arising in right angles. The 3 - 9 phialides arose terminally at a wide angle from the branches, producing successively single phialospores, which were smooth-walled and appeared dark greenish, measuring 2.8-3.2 x 2.5-2.8 µm.

T. versicolor covered agar plates within 7 - 10 days with a cottony-wooly or felty mycelial mat which formed crusty, brown edges after 10 - 14 days. Chlamydospores were rare, subglobose or rectangular and 10-15 x 5-8 µm large ("arthroconidia-like"; Riddell, 1950; Stalpers, 1978). No other conidia were found. The hyphae were generally wider (2 - 7 µm) than those produced by T. harzianum, thin-walled, short-branched, hyaline, xanthochroic and with clamp connections at septa (nodose-septate). Thick-walled fiber hyphae, branched or unbranched, were also common.

Thus, hyphae of T. versicolor were easily distinguished from T. harzianum by the average hyphal diameter, the presence of clamp connections and thick-walled fiber hyphae, and the absence of phialospores and other conidial structures.

3.3.2. Hyphal Interactions on Malt Agar Plates and Slide Cultures

As previously noted (cf. 3.2.2.), mounts of agar pieces and hyphae from agar plates yielded no useful information with regard to hyphal interactions when examined microscopically. Even when hyphae were removed with utmost care, the relative orientation of hyphae and their connections to the main mycelial body was undoubtedly disturbed by the rather crude squash-mounting. No evidence for intra- or interspecific hyphal coiling was found in the slides examined.

Slide cultures provided a better approach for examining hyphal and conidial structures of fungi. Although four agar pieces were arranged

on the slides (Fig. 3.1.a.), the agar itself was only sporadically penetrated by hyphae and most hyphae grew aerially. However, this technique permitted examination of unstained hyphal and asexual structures from both T. versicolor and T. harzianum (Fig. 3.2. - 3.4.).

Hyphal interference combined with lysis of hyphal walls has been reported to be a common capability of T. harzianum strains (Dennis & Webster, 1971c; Komatsu, 1976; Hadar et al., 1979; Elad et al., 1980; Hüttermann & Cwielong, 1982; Murmanis et al., 1988). However, neither evidence for hyphal coiling by T. harzianum around hyphae of T. versicolor, nor lysis or the presence of "appressorium-like" structures such as those found in T. polysporum were noted in this study (Murmanis et al., 1988). Longitudinal association was observed among hyphae of the same species on pure culture or combination slides (Fig. 3.4.), but never ocurred between species. This intraspecific hyphal coiling was more frequent after 14 - 21 days of incubation and may represent a form of necrotrophic "self-parasitism" for recycling of nutrients under starvation conditions (Nguyen & Niederpruem, 1984).

After 21 days of incubation, it became exceedingly difficult to differentiate between, for example, fiber hyphae of T. versicolor and thick hyphae of T. harzianum, especially in unstained slides. Slide cultures may actually not allow the detection of hyphal interference by light microscopic examination of intertwining hyphae. It would have been useful to examine some areas of dense mycelial growth by electron microscopy or to develop a staining procedure specific for chitinase to identify localized areas of cell wall lysis. The approach chosen proved to be, overall, cumbersome and time consuming and therefore not a better method than the one developed by Aylmore & Todd (1984).

A remarkable modification of normal growth was found on one microscope slide inoculated with both T. versicolor and T. harzianum (Fig. 3.4.c.). Hyphae of T. versicolor grew normally for about 2 days, then turned and grew back in a u-turn pattern. Growth in this particular area of the slide ceased after about 16 days. The pattern was stable, but only found once in 15 replicates. This growth aberration may either reflect inorganic factors, such as salt toxicity due to creep

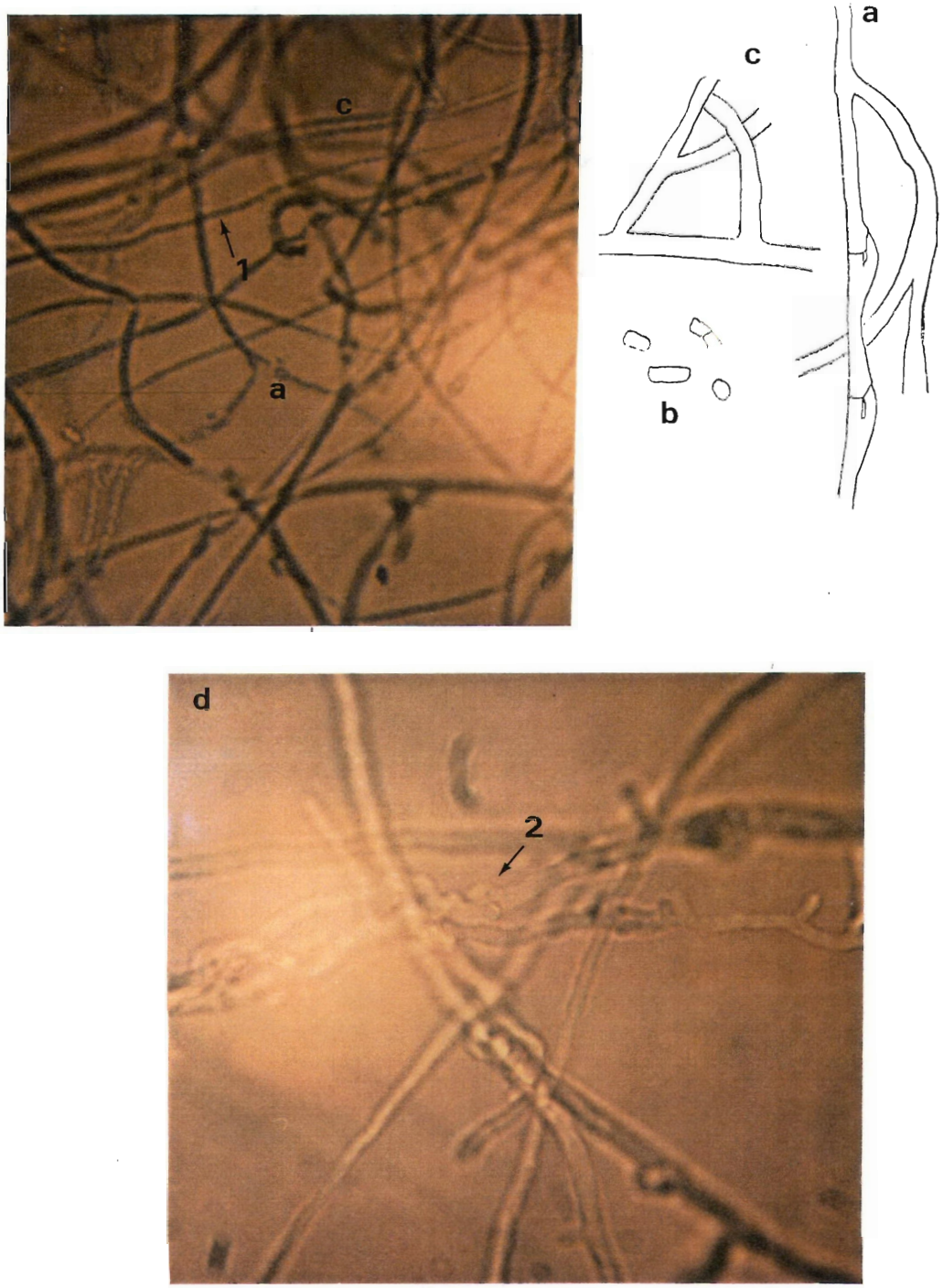


Figure 3.2.: Cultural characteristics of T. versicolor: (a) vegetative and fiber hyphae with clamp connections, (b) chlamydospores, (c) fiber hypha (1) and clamp connection on vegetative hypha (2), and (d) coiling hyphae (all 400x).

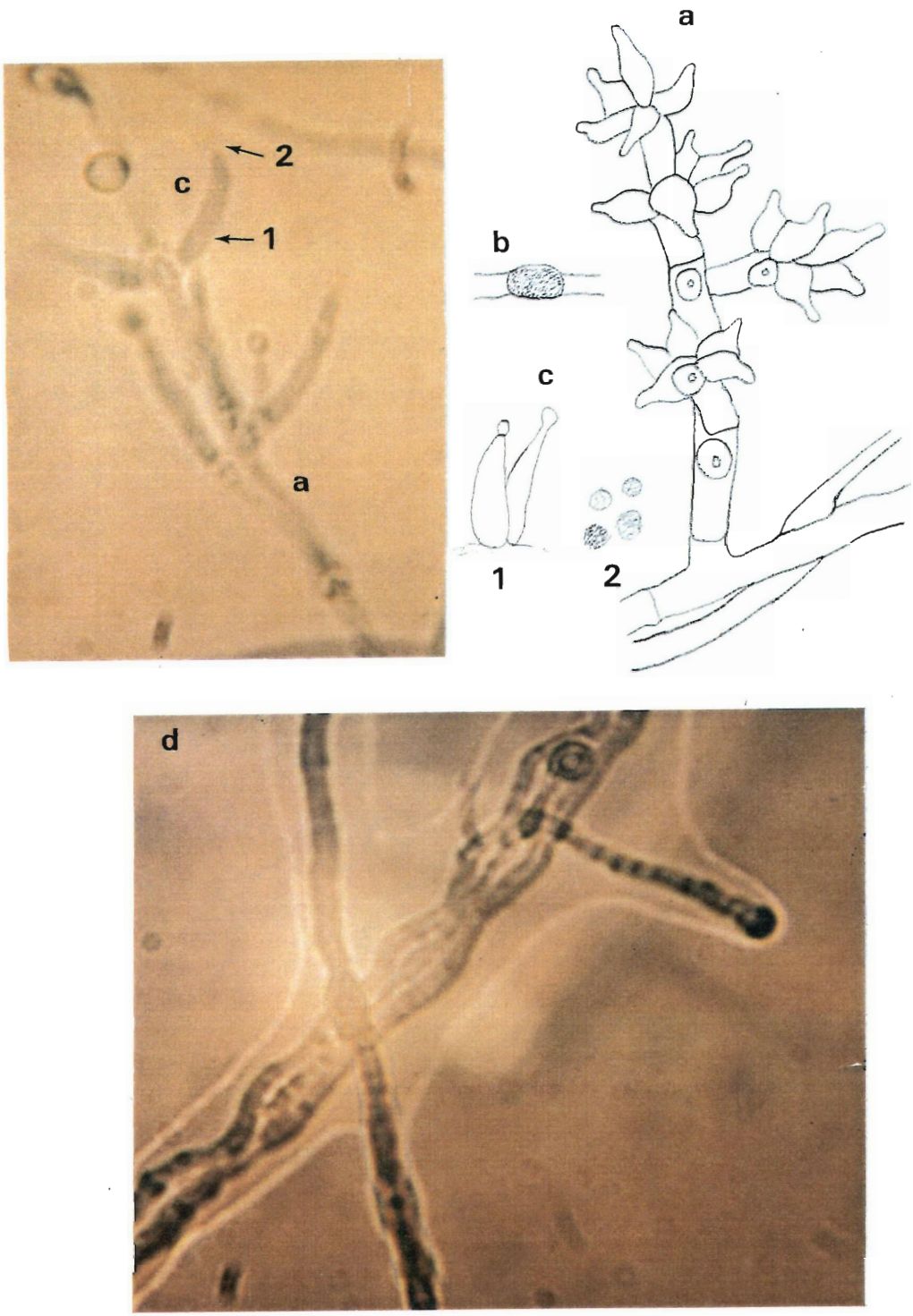


Figure 3.3.: Cultural characteristics of T. harzianum: (a) hyphae with pin-shaped phialides on conidiophores, conidia, (b) chlamydospores, (c) phialides (1) and conidia (2), and (d) coiling hyphae (all 400x).

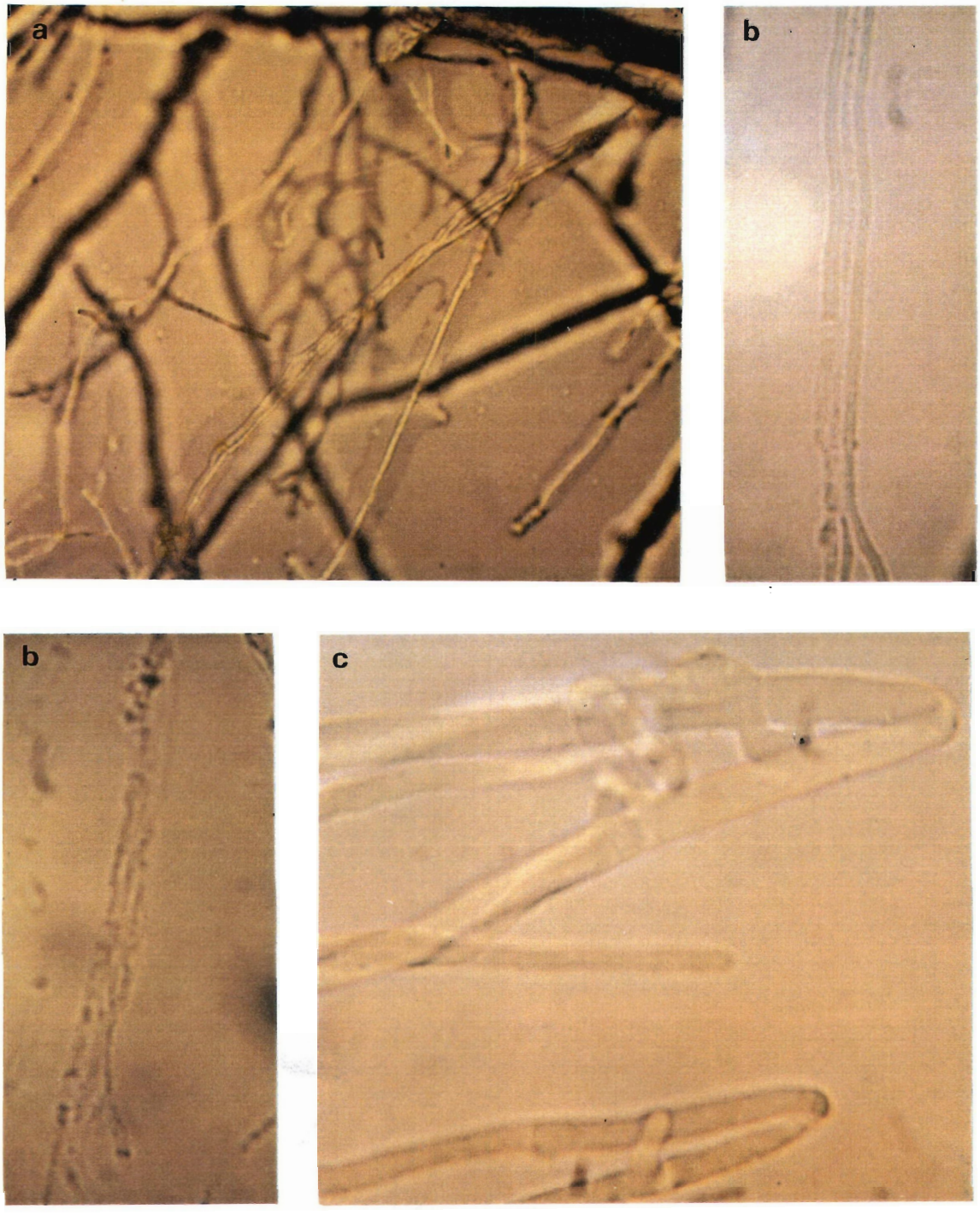


Figure 3.4.: Hyphal system of T. versicolor and T. harzianum showing (a) strands of hyphae from T. harzianum, (b) senescent, partially lysed hyphae of T. versicolor and T. harzianum and (c) growth aberration of T. versicolor in mixed slide cultures.

from the NaCl solution onto the microscope slide, localized unfavourable changes in atmospheric oxygen or carbon dioxide levels, or the influence of T. harzianum. Whilst the growth pattern of T. versicolor was altered, no changes were observed in the growth of T. harzianum. The production of volatile metabolites, diffusing towards T. versicolor along the agar may explain this phenomenon; however, the true nature of this effect could not be elucidated.

The absence of definitive interspecific hyphal interactions on malt agar plates or slide cultures may have been due to a departure from the methods used in previous studies, employing either phase contrast or electron microscopy (Aylmore & Todd, 1984; Murmanis et al., 1988). To ascertain the presence or absence of hyphal interaction in the strain of T. harzianum, the more elaborate cellophane membrane method should be employed (Chet et al., 1981; Murmanis et al., 1988). Murmanis et al. (1988) showed penetration and lysis of cell walls of several decay fungi due to the action of the same strain of T. harzianum, obtained from the same source as in this study. The cellophane membrane method is still quite artificial, since it does not involve growth on wood as a substrate. However, the fungi are grown under severely nutrient-limited conditions, leading more readily to hyphal interactions and various forms of parasitism (Moore-Landecker, 1982). Nutrient conditions in this study were not limited: even after 14 - 21 days, fast extending, exploitative hyphae of T. versicolor and T. harzianum were still present. The agar was never entirely colonized and a large food reserve remained basically untouched. Generally, the growth of potentially parasitic hyphae towards host hyphae is induced by low nutrient conditions (Tokimoto & Komatsu, 1979; Murmanis et al., 1988; Whipps et al., 1988). Similar effects may be exerted by the incubation temperature (Tronsmo & Dennis, 1978). Intraspecific hyphal coiling was a very common phenomenon, especially among senescent hyphae and hyphal cordons, as shown in studies with Laetisaria arvalis Burds. (Fig. 1-3 in: Burdsall et al., 1980) and is characterized by a mixture of hyphae with different widths which may initially be confused with interspecific interactions.

3.3.3. Conclusions

The slide culture method allowed the examination of hyphal interactions between two inocula, but gave no advantages when compared to the earlier methods. Although the method may be adapted, disturbance-free observation at the electron microscopic level is not possible. The absence of hyphal interactions between T. versicolor and T. harzianum may have been associated with the nutrient-rich agar employed.

The results of this study yielded no additional information concerning the secretion of cell wall lytic enzymes was obtained. Selective immunoassays or other labelling techniques would be more useful for locating secreted enzymes.

4. Extracellular Enzyme Activities of Trametes versicolor (L.:Fr.) Pilát and Trichoderma harzianum Rifai in Pure and Mixed Liquid Cultures

4.1. Literature Review - Mechanisms and Role of Fungal Enzymes in Wood Decay and Effects of Microorganisms on Fungal Enzymes

The preceeding chapters emphasized interactions of organisms with their microenvironment, especially interspecific fungal interactions. Wood decay and its prevention may be considered on two levels: interactions between wood and microorganisms on a macroscopic or microscopic scale and the effects of these interactions on the enzyme catalysed chemical reactions which bring about the degradation of wood (Jeffries, 1987). The structure of wood polymers and the enzymes catalyzing their depolymerization, the proposed pathways of wood degradation for white-, brown- and soft-rot fungi, and enzyme interactions between different species, expressed as synergism or inactivation, will be addressed in this chapter.

4.1.1. Biochemical Aspects of Wood Decay

Relationships between wood structure and chemical or biological decomposition have been the topic of many reviews and specialized texts (Cowling, 1958; 1961; Nilsson, 1973; Eriksson et al., 1975; Kirk, 1975; Reese, 1975; Adler, 1977; Ander & Eriksson, 1977; 1978; Brown & Jurasek, 1979; Kirk et al., 1978b; 1980; Eriksson, 1981; Montgomery, 1982; Kirk & Fenn, 1982; Higuchi, 1985; Higuchi et al., 1983; Paterson et al., 1984; Eriksson & Wood, 1985; Kirk & Shimada, 1985; Harvey et al., 1986; Highley, 1987a; Jeffries, 1987; Enoki et al., 1988; Wood & Kellogg, 1988a+b; Gold et al., 1989; Green et al., 1989). Three principal classes of components are found in varying quantities in cell walls of wood. Cellulose, organized into laminar crystallites with amorphous and

crystalline sections, is cross-linked by hydrogen bonds and bundled into microfibrils (Jeffries, 1987). Hemicelluloses are bound to the amorphous sections of cellulose and at the same time to parts of the third class of polymeric component, the lignins (Whitmore, 1982). As those covalent bonds between polysaccharides and lignins are only broken by altering the properties of either, the term lignocellulose is often used (Jeffries, 1987). Lignins encrust both classes of polysaccharides and give the wall rigidity and possibly decay resistance towards most microorganisms (Vance et al., 1980).

Whilst cellulose is a homopolymer of ß-[1→4]-linked glucose units, both hemicelluloses and lignins are heteropolymers (Hori & Elbein, 1985). Hemicelluloses contain glucose, but predominantly mannose, xylose, arabinose, galactose and various derivatives of these sugars, ß-[1→4], ß-[1→6] or ß-[1→3]-linked, leading to a mixture of straight chain and branched molecules. This polymerization is not completely random, however, which distinguishes it from the reactions leading to lignins. Three derivatives of p-hydroxycinnamic acid have been found to be the building blocks of these amorphous, cross-linked, optically inactive, and high molecular weight polymers: p-coumaryl, coniferyl and sinapyl alcohol (Freudenberg, 1965). Lignins have no repeating units like cellulose or most hemicelluloses. Phenylpropane radicals are randomly copolymerized under formation of various C-C and C-O-C bonds, a reaction catalyzed by peroxidases and phenoloxidases. The dominant linkage is the arylglycerol-ß-aryl ether (ß-O-4) bond, the ß here designating the second carbon atom in the propane side chain of a phenyl group (Higuchi, 1985).

Many microorganisms can aerobically degrade intact plant cell walls and therefore polysaccharides and lignins. When plant material has been ground, polysaccharides become available for anaerobic decomposition, due to the separation of cellulose and hemicelluloses from the protective lignin barrier (Buswell & Odier, 1987). Three types of wood decay fungi are distinguished: white, brown, and soft rot fungi (Nilsson, 1988). White-rot fungi, which are able to completely mineralize both lignins and polysaccharides, leave cellulosic residues behind,

resulting in white colored areas in wood. Brown-rot fungi, on the other hand, preferentially degrade polysaccharides. Their group name stems from the brownish colored wood residue, mainly comprised of altered lignins. Decay caused by soft-rot fungi is characterized either by the occurrence of rhombus- and spindle-shaped cavities or an erosion pattern in the wood cell wall (Nilsson, 1988). Early studies described and compared the biochemistry of white- and brown-rot decay, describing the major chemical and physical differences between types of decay, trying to link them to the enzymes produced by the respective fungi. Evidence indicating the nature and rates of enzyme production was generated from experiments comparing particle-size distribution, solubility, composition, degree of polymerisation, hygroscopicity, X-ray diffraction, and histological analyses of wood in progressive stages of white- and brown-rot decay (Cowling, 1961).

Screenings of white- and brown-rot fungi and cellulase-less mutants have been undertaken (Nilsson, 1974; Eriksson et al., 1980). However, only a few, mostly very active degraders or enzyme producers have been used in wood decay research. Early studies about enzymatic cellulose hydrolysis centered on the genus Trichoderma, due to its high potential for cellulase production (Mandels & Reese, 1960; Brown & Jurasek, 1979). The same holds true for lignin biodegradation research: although research on several aggressive white-rot fungi was conducted, Phanerochaete chrysosporium Burds. was studied more in depth than all other organisms combined (Kirk et al., 1980; Kern, 1981; Leisola et al., 1983; Janshekar & Fiechter, 1983; Leisola & Fiechter, 1985; Kirk & Farrell, 1987). This approach harbors both advantages and dangers. One advantage is associated with the adaptibility of the respective organisms to the laboratory environment and therefore ease of study. The danger lies in the potential generalization of mechanisms and pathways which may be unique to the studied organism. In recent years, however, more bacteria and fungi have been screened and evaluated for their cellulolytic and ligninolytic capabilities (Wood & Kellogg, 1988a+b).

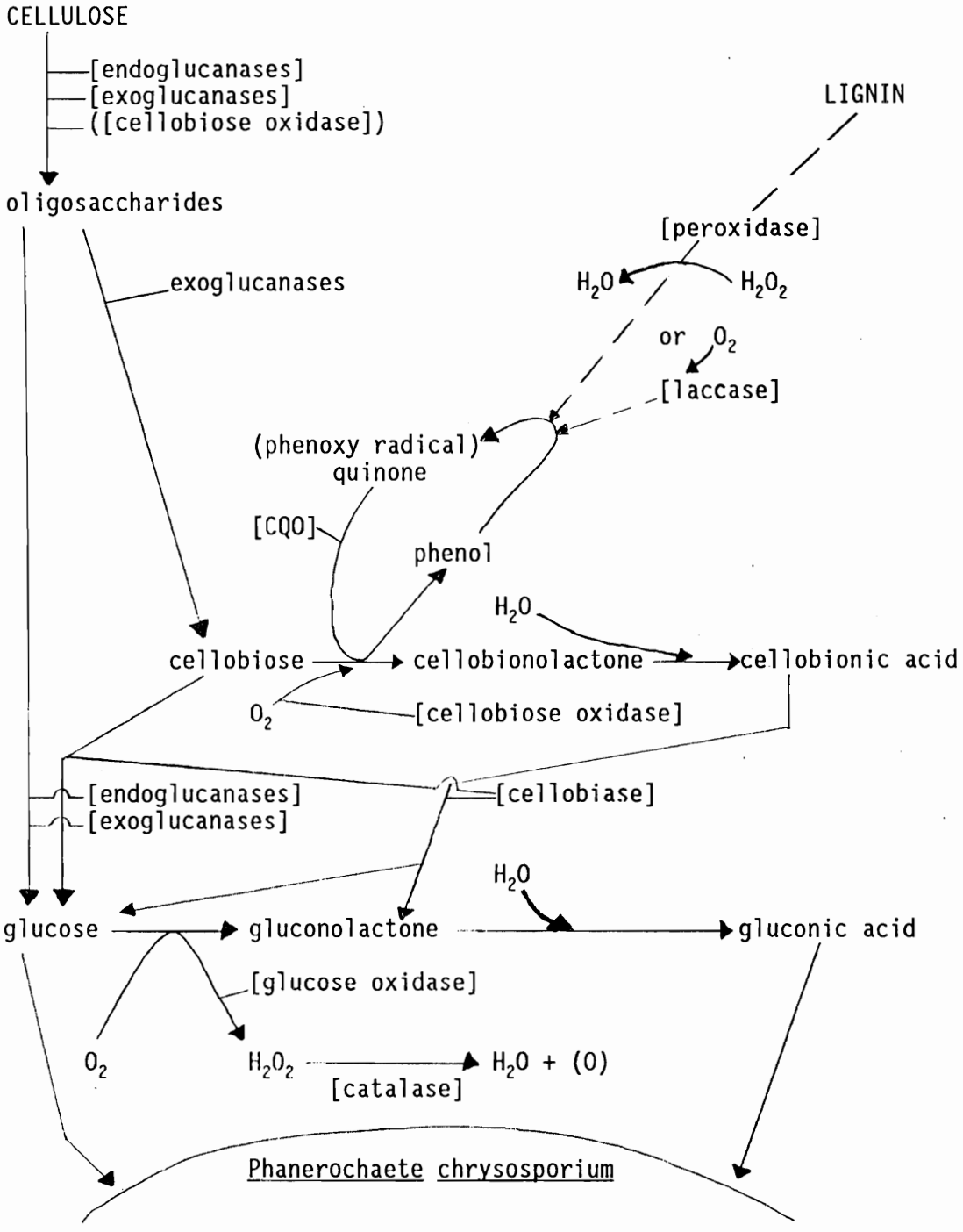


Figure 4.1.: Proposed pathway of cellulose degradation by the white rot fungus Phanerochaete chrysosporium Burds. (after Eriksson, 1978 [in Eriksson & Wood, 1985]); CQO = cellobiose:quinone oxidoreductase.

The degradation of cell wall polysaccharides and lignins clearly requires enzymes with different capabilities. Cellulose and hemicelluloses, when liberated from the lignocellulosic matrix are comparatively easily hydrolysed by a complex of enzymes often called collectively "cellulases". These hydrolases are produced by plants during differentiation processes, but most importantly are also used by pathogenic or saprophytic insects, mollusks or microorganisms to invade or degrade plant material (Finch & Roberts, 1985). Three types of hydrolases act upon cellulose: (a) "endoglucanases", cleaving erratically along the cellulose chain; (b) "exoglucanases", cleaving cellobiose units terminally from the non-reducing end; and (c) "cellobiase", cleaving the ß-[1→4] linkage in cellobiose (Fig. 4.1.; Wood & McCrae, 1979; Eriksson, 1981). Cellulose degradation by white-rot fungi may also include oxidative enzymes, such as cellobiose oxidase and glucose oxidase (Fig. 4.1.). These two hemoprotein enzymes are responsible for larger reaction rates under high oxygen tension and result in the formation of the respective aldonic acids using molecular oxygen (Eriksson & Wood, 1985). Another pathway to oxidize cellobiose in P. chrysosporium involves cellobiose:quinone oxidoreductase (Westermark & Eriksson, 1974a+b). This enzyme reduces quinones and phenoxy radicals in the presence of cellobiose and may be involved in lignin biodegradation. Whilst it is specific for cellobiose, the requirements for the quinones reduced are not nearly as high: both para- and ortho-substituted compounds may serve as substrate (Westermark & Eriksson, 1974b). Acidic proteases present in the culture liquid of P. chrysosporium can increase cellulase activity (Eriksson & Wood, 1985).

The three groups of enzymes, collectively called cellulases, act together in a synergistic manner (Wood & McCrae, 1979) and only under such conditions is complete degradation of native crystalline cellulose by white-rot fungi achieved. However, direct evidence for this action on wood is not available, since there are no methods with which cellulases are measured directly in wood. Enzyme assays on artificial substrates are, however, accompanied by ultrastructural and, most recently, immunolabelling studies (Ruel & Barnoud, 1985; Goodell et al.,

1988; Daniel et al., 1988; 1989a+b; Blanchette et al., 1989). The "synergism" of cellulose hydrolysis by white-rot fungi mirrors the complexity of intertwined pathways, which are still not completely understood. Brown-rot fungi do not follow the pathways exhibited by most white-rot or other xylophilous fungi, since they lack exoglucanases (Highley, 1987a). The exact manner in which brown-rot fungi depolymerize crystalline cellulose is not known, but the most recent hypothesis postulates the diffusion of small radicals and activated oxygen species or an hydrogen peroxide/Fe^{2+} system into the lignocellulosic matrix (Halliwell, 1965; Koenigs, 1974; Eriksson & Wood, 1985; Highley, 1987a; Kirk et al., 1989).

The complexity of substrates and enzymes able to catalyze their decomposition has led to the development of numerous assays for cellulase activity (Wood, 1985; Wood & Bhat, 1988). Overall cellulolytic activity is often determined by the release of reducing sugars from crystalline cellulose, most often filter paper (Goksøyr & Eriksen, 1980; Mullings, 1985). Many assay procedures have been developed to determine the activity of endocellulases, based either on the release of reducing sugars from cellulose derivatives, such as carboxymethylcellulose, or viscosimetric determinations of chain length (Demeester et al., 1979; Wood & Bhat, 1988). Cellobiase is measured by the release of glucose from cellobiose, determined by the enzymatic conversion of glucose to gluconic acid by glucose oxidase (Wood & Bhat, 1988).

Factors controlling the induction of cellulases have been the object of numerous studies (Hulme & Stranks, 1970; Enari & Markkanen, 1977; Ghose, 1977; Goksøyr & Eriksen, 1980; Lee et al., 1980a+b; Merivuori et al., 1985). Cellulases are inducible in most fungi, but single components of the complex may be produced constitutively (Enari & Markkanen, 1977). Cellobiose induces overall cellulase activity (Mandels & Reese, 1960), whilst glucose acts as catabolite or end-product repressor. Cellulase production only starts after available free sugars have been depleted (Mandels & Reese, 1965; Goksøyr & Eriksen, 1980).

Whilst the action of the cellulase complex is based mostly on hydrolysis of long chain cellulose molecules, lignin degrading enzymes must be of an oxidative nature (Kirk et al., 1980; Kirk, 1987; 1988). Most of the current insights regarding lignin degradation have been obtained in experiments with P. chrysosporium. The optimum conditions for ligninolytic activity by wild-type and mutant strains of this fungus have been determined. Presence of an easily degradable carbon source, high oxygen tension, growth as mycelial mats, correct buffer, correct levels of minerals and trace elements, correct temperature and growth-limiting amounts of nitrogen are required to obtain optimum "ligninase" activity (Kirk et al., 1976; 1978a; Keyser et al., 1978; Reid, 1979; Fenn & Kirk, 1981; Jeffries et al., 1981; Shimada et al., 1981; Kern, 1983; Ulmer et al., 1983a; Leisola et al., 1984; Agosin & Odier, 1985; Leatham, 1986), although there are mutant strains with differing requirements (Kuwahara et al., 1987).

Four types of oxidative enzymes have been implicated in lignin biodegradation: laccases (Ander & Eriksson, 1976; 1978; Ishihara, 1980; Noguchi et al., 1980; Ishihara & Nishida, 1983; Morohoshi et al., 1987a-d); lignin peroxidase (Kirk & Tien, 1983; Tien & Kirk, 1984; Gold et al., 1983; 1984; Glenn et al., 1983); manganese peroxidase (Kuwahara et al., 1984; Glenn & Gold, 1985); and hydrogen peroxide producing enzymes (Kirk, 1988).

Purified fungal laccases (p-diphenol:oxygen oxidoreductase, EC 1.10.3.2; Sheldon & Kochi, 1981) are copper-containing proteins capable of oxidizing paraphenols and aromatic amines by abstraction of an electron and hydrogen ion from an hydroxyl group, thus forming an aryloxy free radical, which converts into quinone and water (Ander & Eriksson, 1978). Laccases are closely related to tyrosinases (o-diphenol:oxygen oxidoreductase, EC 1.14.18.1; Sheldon & Kochi, 1981) which catalyze, among other reactions, the oxidation of tyrosine to melanin and the dehydrogenation of catechols (Bouchilloux et al., 1963; Jolley & Mason, 1965; Robb, 1984). Recently, the elucidation of the nature of the catalytic site(s) and the state of the copper ions during catalysis have been emphasized (Reinhammar & Malmström, 1981).

The oxidative demethylation of several dimeric lignin model compounds using purified laccases from various Basidiomycotina has been studied (Blaich & Esser, 1975; Ishihara, 1980; Bollag & Leonowicz, 1984). Common assays use syringaldazine or 2,6-dimethoxyphenol as laccase substrates (Hurst, 1963; Mayer, 1987). Ander & Eriksson (1976; 1978) suggested potential roles of laccase in lignin biodegradation including: (a) detoxification of low-molecular weight phenolic compounds (Ishihara & Nishida, 1983; Hoff et al., 1985; Bollag et al., 1988), (b) initiation of lignin depolymerization by chemical transformations due to laccase action (Kaplan, 1979; Ishihara, 1980; Kawai et al., 1987), (c) regulation of lignin-degrading and cellulose-degrading enzyme complexes and fruiting (Turner, 1974; Turner et al., 1975; Wood & Goodenough, 1977), and (d) functioning in an enzymatic cycle together with cellobiose:quinone oxidoreductase (Westermark & Eriksson, 1974a+b). The role of phenoloxidases in the re-polymerization of lignin precursors in cultures of H. annosum is well documented (Haars & Hüttermann, 1980). Attempts to prove one or more of the proposed functions of laccase are numerous, however, no definitive conclusions with respect to mechanisms, regulation and roles of laccase have been reached.

In 1983, two research groups independently announced the discovery of lignin depolymerizing peroxidases from P. chrysosporium (Gold et al., 1983; Kirk & Tien, 1983). Gold et al. (1984) referred to these enzymes more appropriately as "diarylpropane oxygenases", because they were found in studies with dimeric model compounds, but they are now generally known as "lignin peroxidases" (Tien & Kirk, 1984). Since the first isolation, these enzymes have also been found in other white-rot fungi, including T. versicolor (Evans & Plamer, 1983; Dodson et al., 1987), but conclusive proof of lignin degrading capabilities in vivo is still lacking (Kirk, 1988).

Peroxidases (donor:hydrogen peroxide oxidoreductase, EC 1.11.1.7) are hemiproteins which are widely distributed in microorganisms and normally not highly substrate specific (Sheldon & Kochi, 1981). Peroxidases oxidize hydrogen donor substrates under concurrent reduction of hydrogen peroxide to water. "Ligninases" are true peroxidases,

containing one mole protoporphyrin IX per mole enzyme (Tien & Kirk, 1984). Crude preparations consist of multiple ligninases, and at least four lignin peroxidase isoenzymes with slightly different specificities have been characterized (Kirk et al., 1986). In addition to the originally described C_{α}-C_{β} cleavage, lignin peroxidases catalyse the oxidation of a variety of mono- and dimeric lignin-model compounds; detailed pathways of enzymatic oxidation are discussed by Kirk (1987). The underlying mechanism is based on radical chemistry, as elucidated from electron-spin resonance spectra (Kersten et al., 1985). Based on kinetic studies of the oxidation of veratryl alcohol by the lignin peroxidase-H_2O_2 system (Tien et al., 1986), a catalytic cycle for lignin peroxidases has been proposed (Kirk, 1987).

Lignin peroxidases alone are not able to degrade lignin and other enzymes, such as manganese peroxidase and the hydrogen peroxide producing enzymes, might participate in lignin degradation pathways (Haemmerli et al., 1986). Manganese peroxidases may be involved in some reactions during lignin degradation, many of which are also catalyzed by laccases (Glenn & Gold, 1985; Kawai et al., 1987). In the catalytic cycle of manganese peroxidase, a Mn^{3+}-lactate complex is the true oxidant (cf. Fig. 4.20.; Gold et al., 1989). The same kind of catalytic cycle with veratryl alcohol as mediator has been proposed for lignin peroxidases (Harvey et al., 1986). Both peroxidases have been sequenced (Tien & Ti, 1987; Pribnow et al., 1989) and research towards cloning of these enzymes into industrial microorganisms continues.

4.1.2. Influence of Microorganisms on Extracellular Enzymes

Primary metabolism involves basically the same reactions in all organisms. For initial growth, energy production and structural purposes, organisms need to produce RNA, proteins, simple and complex carbohydrates, lipids and DNA. During those principal metabolic reactions, few intermediates accumulate and the turnover rate of simple

compounds is high (Rawn, 1989). When all easily available substrates are depleted, a shift from primary to secondary metabolism is observed and specific metabolites are produced which have no direct function in primary metabolism. This model has been derived largely from laboratory batch culture experiments and it should be stressed that growth and secondary metabolism are really competitive modes for key metabolic intermediates and not mutually exclusive phenomena (Moss, 1984).

Secondary metabolites, encompassing various classes of enzymes, toxins, antibiotics and other, ill-defined components, may play an important role in survival by their solubilizing, detoxifying or antibiotic action (Yoder, 1980; Faull, 1988). For example, under nitrogen or carbon starvation conditions the synthesis of hydrolases is induced or derepressed (Demain, 1972). This has led to the idea of controlling the mycelium or germination of pathogenic soil fungi by hydrolase hyper-secreting strains of potential biocontrol organisms such as T. harzianum (Kelly et al., 1981; Hüttermann & Cwielong, 1982; Faull, 1988). There have been few successful selection programs and it is feared that selected strains will rapidly lose their ability for hyper-secretion upon prolonged culturing or changed environmental conditions (Neijssel & Tempest, 1979; Vanek & Hostalek, 1986). Moreover, controlling the biocontrol system in the heterogenous soil system has proven difficult, and successful control may only be achieved in combination with high competitive saprophytic abilities of the respective biocontrol organism (Faull, 1988).

The direct application of hydrolytic enzymes to achieve control is only one possibility for biochemical interference of a biocontrol organism with a pathogen. Another alternative is the inactivation of enzyme capabilities of pathogens by the controlling organism. Enzyme inactivation in vivo represents one mechanism of enzyme regulation, next to the regulation of enzyme synthesis rate and control of enzyme activity by effects of non-covalent binding of ligands (Switzer, 1977; Rawn, 1989). "Inactivation" means the irreversible loss of catalytic activity in a physiologically significant reaction of the respective enzyme (Switzer, 1977), and is thereby distinguished from inhibition,

which may be associated with a temporary reversible loss of catalytic ability. Inactivation is most often associated with regulation in the organism itself, brought about by shifts in carbon or nitrogen metabolism, differentiation processes and turnover of proteins (Switzer, 1977). However, it might be exogenously induced by the presence of other organisms, either directly by chemical compounds in close physical proximity to the first organism, or indirectly by microenvironmental changes associated with the second organism. Although the detrimental inactivation of degradative enzymes is postulated to be the dominant process, there are reports which show that specific enzymes may thrive under "stress" conditions, enhancing their catalytic capabilities and the competitiveness of the affected organism (Neidleman, 1989).

Although it is apparent that many low and high molecular weight compounds may interfere with extracellular enzyme production by fungi (Merivuori et al., 1987), few studies have examined the relations between the presence of wood degradative enzymes released by wood decay fungi and potential biocontrol organisms. The scope of this study only allowed a descriptive approach to this problem, encompassing the overall measurement of degradative activities of a wood decomposer, T. versicolor, as influenced by the presence of the potential biocontrol organism T. harzianum under low and high nitrogen regimes. It was hypothesized that: (a) T. versicolor would produce extracellular enzymes or enzyme complexes required for wood degradation in pure culture. In mixed culture the enzymatic activity should be reduced or enhanced due to the interactions with T. harzianum; (b) T. harzianum would vary in its capability to produce the enzyme complexes chosen. No extracellular laccase and ligninase activity was expected to be found with this fungus; (c) the laccase and peroxidase activities may be found to be growth-linked if adequate culture conditions are chosen; and (d) it should be possible to estimate some of the antagonistic abilities of the examined microfungi by means of biomass and extracellular enzyme production.

Objectives of this part of the study were:

- to assess the qualitative and quantitative production of extracellular enzymes or enzyme complexes important in biodegradation of wood by the selected test fungi;

- to determine whether the enzymatic methods chosen are suitable for estimating growth rates and biomass production in pure and mixed cultures of the test fungi, with the final goal to develop a simple screening method for detecting fungal antagonists;

- to examine changes in extracellular enzyme activity of the test fungi in pure and mixed cultures, possibly leading to a better understanding of antagonistic mechanisms involved in biological control against wood decay fungi.

4.2. Materials and Methods

4.2.1. Growth Media

The basal growth medium (Medium A) was based on a nutrient solution by Hüttermann & Volger (1973):

5.000 g	glucose
0.500 g	yeast extract
0.520 g	L-asparagine (anhydrous)
1.000 g	KH_2PO_4
0.300 g	$MgSO_4$ (anhydrous)
0.500 g	KCl
0.010 g	$FeSO_4$
0.008 g	$Mn(CH_3COO)_2 \times 4\ H_2O$
0.002 g	$Zn(NO_3)_2 \times 6\ H_2O$
0.050 g	$Ca(NO_3)_2 \times 4\ H_2O$
0.002 g	$CuSO_4$ (anhydrous)
	in 1000 ml distilled H_2O

Two alternative growth media (Media B + C) containing varied amounts of carbon and nitrogen were used for enzyme studies, based upon the basal medium (Tab. 4.1.). These media contained the same salt solution as the basal medium, but no yeast extract or asparagine as complex nitrogen sources. They were used for measuring extracellular enzyme activities. All media described were autoclaved at 121°C for 20 min. The pH was adjusted to 5.5 after autoclaving.

Table 4.1.: Composition of growth media used in enzyme studies.

Medium	Carbon Source (C)	Amount added (g/l)	Total Carbon (mM)	Nitrogen[1] Source (N)	Amount added (g/l)	Total Nitrogen (mM)	C/N-Ratio[2]
A (Basal Medium)	D-glucose	5.000	166.7	L-asparagine yeast extract	0.520 0.500	8.00[3]	10:1
B (Low Nitrogen)	D-glucose cellulose	5.000 1.822	233.3	L-asparagine NH_4NO_3	0.013 0.008	0.40	500:1
C (High Nitrogen)	D-glucose cellulose[4]	5.000 1.822	233.3	L-asparagine NH_4NO_3	0.130 0.080	4.00	50:1

1 = Nitrogen sources in salts were kept constant, and their overall contribution to the C/N-ratio was negligible.

2 = C/N-ratio based on g/l carbon and nitrogen in growth media.

3 = Approximate only, since the nitrogen content of yeast extract varies widely between batches.

4 = Cellulose powder for chromatography (Standard Grade, Whatman).

4.2.2. Inoculation of Test Cultures

4.2.2.1. Stock Cultures of Test Fungi

Stock cultures of T. versicolor and T. harzianum (cf. 3.2.1. and Tab. 2.1. for strain numbers and sources) were grown in 250 ml Erlenmeyer flasks containing 50 ml of Medium A. The flasks were inoculated with approximately 5 mm x 5 mm mycelial plugs cut from actively growing edges of 7 day old malt agar cultures of the test fungi. The flasks were incubated at 29°C in the dark for two days, then at 20° - 22°C (room temperature) on a Labline rotary shaker (Labline Instr. Inc., Melrose Park, IL) at 80 rpm for an additional two days.

4.2.2.2. Test Cultures

Inoculum for test cultures was prepared by aseptically decanting the culture filtrate of 4 stock cultures, transferring the residual mycelium of each flask into a Waring blender containing 200 ml fresh medium and homogenizing the combined mycelium at low speed for 15 sec. Erlenmeyer flasks (250 ml) containing 50 ml of the selected growth media were aseptically inoculated with 5 ml of the homogenized mycelial suspension. Five ml of suspension corresponded to 0.26×10^6 - 0.3×10^6 and 0.25×10^6 - 0.66×10^6 colony forming, viable mycelial fragments for T. versicolor and T. harzianum, respectively. These numbers were determined using dilution plating methods (Drews, 1983; Atlas & Bartha, 1987).

Mixed cultures were prepared by pipetting 5 ml of mycelial suspension of each fungus into the flask as described above; however, since the inoculum potential and initial growth rates of the test fungi were quite different under the chosen conditions, T. harzianum was added

to flasks 4 days after inoculation with T. versicolor to permit the latter fungus to overcome its comparatively longer lag-phase.

4.2.3. Culture Conditions

Stationary cultures were incubated in the dark at 29°C. Cultures containing 0.2 % (w/v) of the polymeric dye Poly R-478 were grown on a rotary shaker (cf. 4.2.2.1.) at 80 rpm and 20° -22°C (room temperature).

4.2.4. Harvest of Test Cultures

Both culture filtrates and wet mycelia were sampled at about 4 day intervals after inoculation. At each time point, the contents of 6 flasks per test fungus or combination of fungi were harvested by filtration through Whatman # 40 filter paper using a Büchner funnel. Mycelial pads were washed twice with distilled H_2O and either immediately used for dry weight determinations or frozen (-20°C) for subsequent protein measurements.

The crude culture filtrate was made cell-free by sterile filtration through membrane filters (Millipore AAWP, 0.8 μm; Millipore, Bedford, MA). The cell-free filtrate was immmediately cooled in an ice box and used within 4 - 6 hrs for the various enzyme assays. Figure 4.2. shows a flowchart of assays performed at each sampling point for the regular test cultures and those with added Poly R-478.

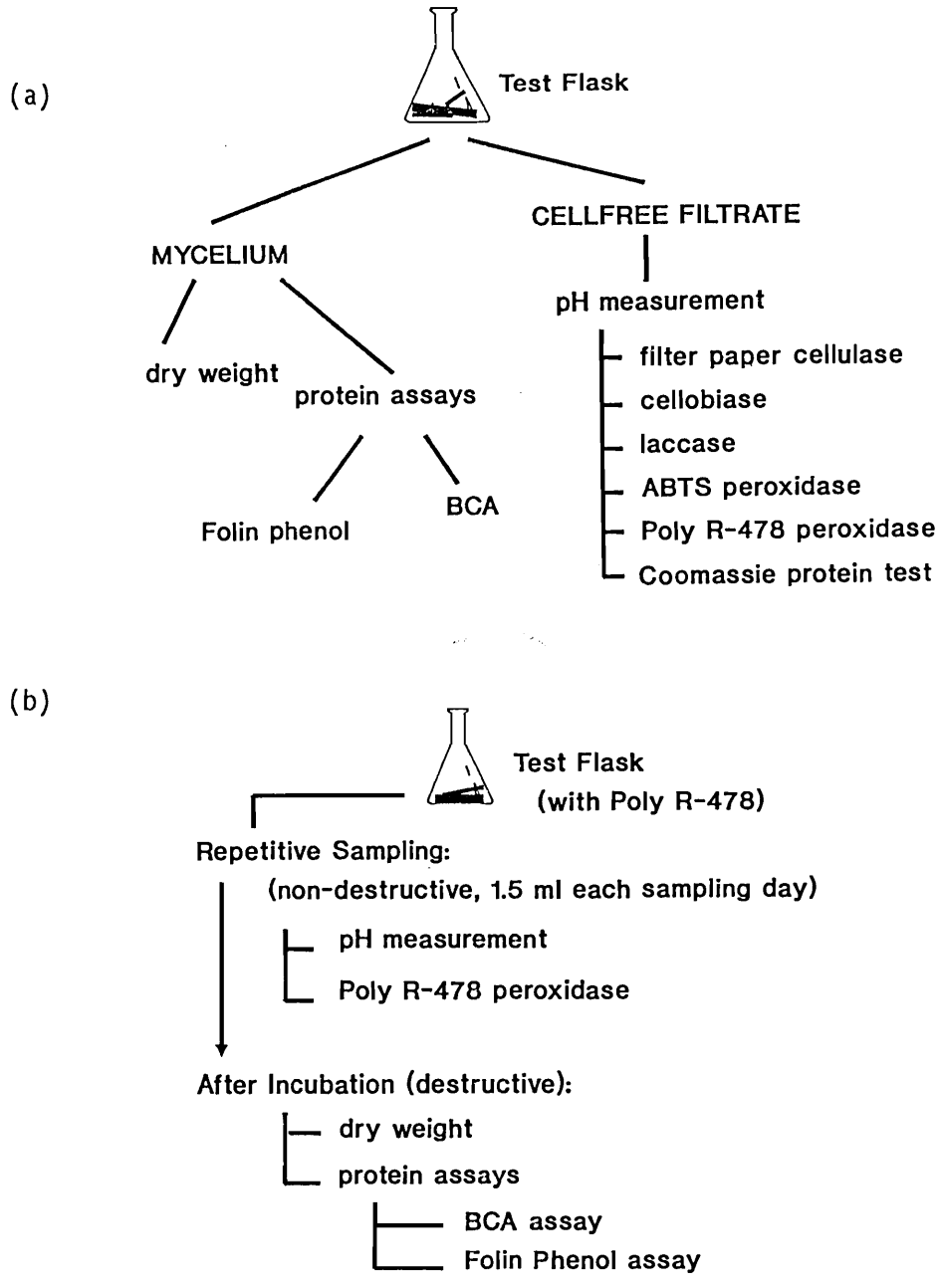


Figure 4.2.: Flowcharts of assays performed to characterize physiologic differences between mixed and pure cultures of T. versicolor and T. harzianum on (a) normal growth media and (b) media amended with the polymeric dye Poly Red-478.

4.2.5. Measurement of Fungal Growth Characteristics

4.2.5.1. Glucose Determination

The depletion of glucose as major carbon source in the growth media was measured by injecting 25 µl supernatant of centrifuged (5 min at 10,000 rpm; Eppendorf Microcentrifuge 5415, Brinkmann Instr. Co., Westbury, NY) cellfree culture filtrate into an YSI 23A Glucose Analyzer (YSI Inc., Yellow Springs, OH).

The determination of glucose concentration in this instrument is based on the glucose oxidase catalysed reaction of glucose with oxygen to form gluconic acid and hydrogen peroxide, followed by oxidation of hydrogen peroxide to protons, electrons and molecular oxygen at a platinum anode, and the reduction of oxygen to water by a silver cathode, all at 37°C. The current created by the oxidation of a constant portion of hydrogen peroxide is directly proportional to the glucose concentration in the sample (YSI Inc., 1988). Glucose concentration was read as mg/dl, but was converted to mM.

4.2.5.2. Biomass Determination

Determination of mycelial dry weight is a simple, comparatively accurate method for estimating fungal biomass production and growth rate (Matcham et al., 1984). Mycelial pads remaining after filtration were oven-dried to constant weight at 70°C, cooled in a desiccator and weighed.

4.2.5.3. Protein Determination

Fungal biomass and growth rates in the exponential growth phase may be estimated from protein determinations (Drews, 1983; Kresze, 1984). In addition, determining the ratio of filtrate to mycelial protein gives an indication of overall extracellular enzyme production by the test fungi.

Standard procedures to measure total protein only provide a relative measure of protein production by a test organism, since the results of assays will vary with the type of predominant protein and its amino acid sequence (Peterson, 1983). Two different methods for determination of total protein were used in this study: the Folin phenol assay and the bicinchoninic acid assay.

4.2.5.3.1. Folin Phenol Assay

The most widely used protein test is based on Folin & Ciocalteu's phenol reagent (Lowry et al., 1951, Peterson, 1977; 1979; 1983). The reduction of phospho-molybdic-tungstic mixed acid chromagen by the aromatic amino acid residues tyrosine and tryptophan, as well as copper chelates of peptide chains and polar side chains in alkali result in a blue colorization of the test mixture (Lowry et al., 1951; Peterson, 1983). Color development is related to total protein, but the relation is not strictly linear over a wide range of protein concentrations (Peterson, 1979).

The mycelial pads were rapidly thawed in a stirred water bath at 50°C and transferred into test tubes which were brought to 10 ml with distilled H_2O and sonified for 120 sec at 55 W (50 % output, control at 5.5; Branson Sonifier 250 with a tapered 1/18'' microtip, Branson Ultrasonics Co., Danbury, CT). After addition of 5 ml 1 M trichloroacetic acid (TCA), the contents of test tubes were emptied into centrifuge tubes (Nalge #3117; VWR, Seattle, WA). The test tubes were then rinsed

with 10 ml H_2O which was added to the centrifuge tubes. Proteins were precipitated (final concentration of TCA: 0.2 M) by storage for 2 or 4 hrs at 4°C followed by centrifugation for 20 min at 10,000 rpm (Sorvall centrifuge SERVALL RC-2 with rotor SS 34, giving 12,100 xg at 10,000 rpm). The resulting pellets were dissolved in 20 ml 1 M NaOH and kept in a stirred water bath at 60°C for 1 hr. After cooling, mixing, and centrifugation (5 min at 13,000 rpm; Eppendorf microcentrifuge 5415), 500 µl of the supernatant containing dissolved proteins was used for the colorimetric protein assay.

The dissolved protein (500 µl) was diluted with 950 µl distilled H_2O and 3.75 ml of a freshly prepared reaction mixture, containing 1.33 ml 1% (w/v) $CuSO_4$ x 5 H_2O, 1.33 ml 2% (w/v) K-Na-tartrate and 2.67 g Na_2CO_3 in 100 ml distilled H_2O, was added. After 30 min, 0.5 ml Folin & Ciocalteau's reagent (diluted 1:2 with distilled H_2O) was added and immediately and thoroughly mixed. After 60 min, this solution was measured at 750 nm in a glass cuvette (1 cm path length; #58016-425, VWR, Seattle, WA), using a Milton Roy Spectronic 301 spectrophotometer (Milton Roy, Rochester, NY). Blanks contained 950 µl distilled H_2O, 500 µl 1 M NaOH, and 3.75 ml reaction mixture.

Absorbance readings were converted to protein concentrations using a calibration curve based on bovine serum albumin (BSA; Sigma #B-2518) in 1 M NaOH as dissolved protein. Protein levels found in mycelial pads and cell-free filtrates in this study were within the linear range of the Folin phenol assay. Therefore, a standard linear regression procedure (sum of least squares) was used to calculate total protein concentrations. Usually double logarithmic plots, reciprocal power functions or polynomial equations as graphical transforms of the calibration protein concentrations vs absorbance at 750 nm have been employed (Bates & McAllister, 1974; Stauffer, 1975; Coakley & James, 1978).

4.2.5.3.2. Bicinchoninic Acid (BCA) Assay

The bicinchoninic acid assay is based on the reduction of cupric Cu^{2+} by proteins in an alkaline medium to cuprous Cu^{+} (= biuret reaction), coupled with the quantitative formation of a purple product due to the association of two molecules of bicinchoninic acid with one cuprous Cu^{+} (Fig. 4.3.; Smith et al., 1985). The assay was used in the form of a commercially available reaction kit (#23225; Pierce Chem. Co., Rockford, IL).

Dissolved protein (0.1 ml) was pipetted into small test tubes and 2 ml protein reagent, consisting of 50 parts BCA reagent and 1 part 4% (w/v) $CuSO_4$, was added. The test tubes were incubated for 30 min at 60°C (enhanced protocol; Pierce Chem Co., 1988), cooled to room temperature and absorbance of the solution was measured at 562 nm in a glass cuvette (1 cm path length; #58016-425, VWR, Seattle, WA) using the Milton Roy spectrophotometer. Calibration curves were prepared using dissolved BSA (Sigma, #B-2518) as the standard, following the same procedure as for the Folin phenol assay. Distilled H_2O and 1 M NaOH, mixed with the protein reagent and incubated as described above, were used as blanks.

4.2.5.4. pH Measurements

The pH of the respective cell-free filtrates was determined at each sampling point using a Corning pH/ion meter 150 with a Combination X-EL electrode (Corning Sci. Prod., Medfield, MA), standardized at 22° C with Brinkmann pH 4 and pH 7 solutions.

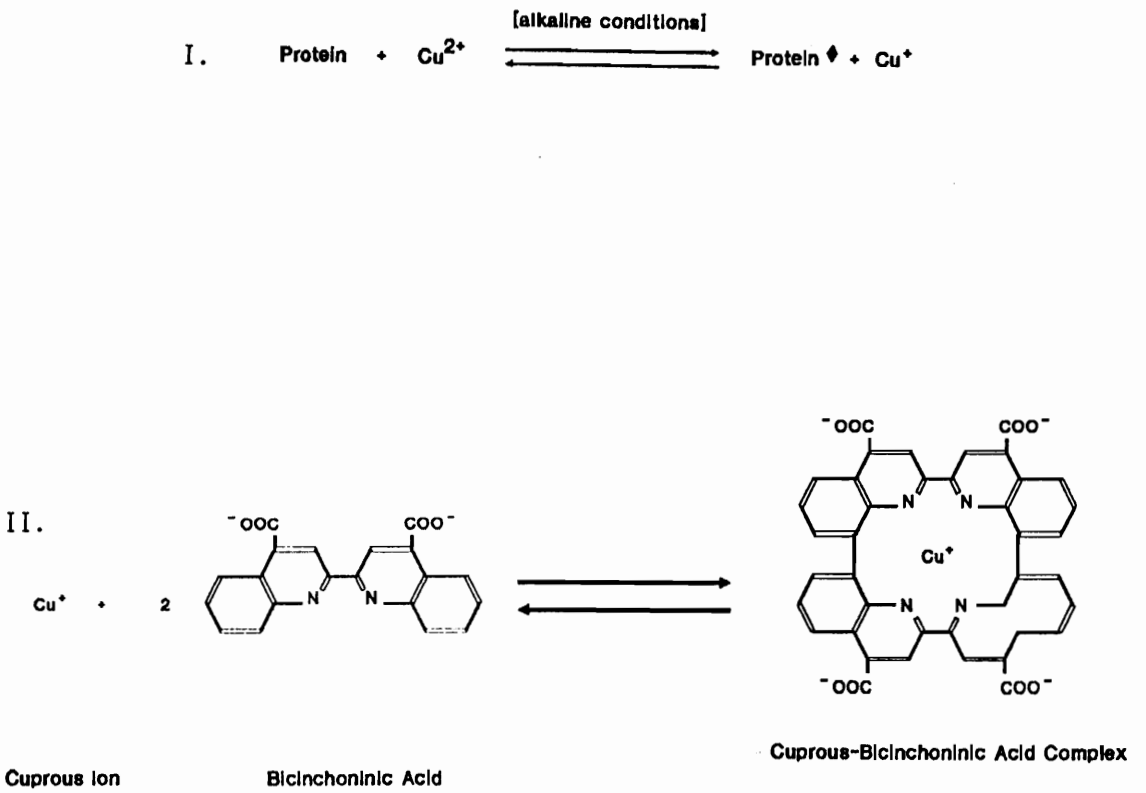


Figure 4.3.: Reaction scheme of the bicinchoninic acid (BCA) protein assay.

4.2.6. Determination of Extracellular Enzyme Activities

4.2.6.1. Filter Paper Cellulase

Filter paper cellulase activity was measured using a modification of earlier methods (Warzywoda et al., 1983; Ishihara, 1986; Wood & Bhat, 1988), based on the release of glucose from filter paper as a result of the hydrolytic activity of the complete cellulase complex. Whatman # 1 filter paper (50 mg) was placed in test tubes with 1 ml of 0.05 M Na-citrate buffer (pH 4.5) and 3 ml cellfree filtrate (cf. 4.2.4.). The initial glucose concentration and the release of glucose after 6 and 24 hrs of incubation at 50°C was determined by injecting 25 µl of the reaction mixture into the YSI Glucose Analyzer. Glucose concentration was read as mg/dl and was converted to total filter paper cellulase activity (µM glucose / min) and specific filter paper cellulase activity (mM glucose / min x mg filtrate protein) by subtracting the initial glucose concentration from the concentrations measured after 6 and 24 hrs. The activity of commercial cellulase derived from Trichoderma viride (EC 3.2.1.4; Sigma, #C-2274) was determined to test the validity of the modified assay (Fig. 4.4.a.).

4.2.6.2. Cellobiase

Cellobiase (ß-[1→4]-glucosidase) is the last in the chain of proposed hydrolytic enzymes which break down crystalline cellulose (Fig. 4.1.). Numerous assays have been devised to measure cellobiase activity (Wood & Bhat, 1988). In this study, cellobiase activity in the cellfree filtrate was measured using a modification of the IUPAC Biotechnology Commission Recommended Method (Wood & Bhat, 1988), where the glucose reagent kit was substituted by measuring glucose concentration in the YSI Glucose Analyzer.

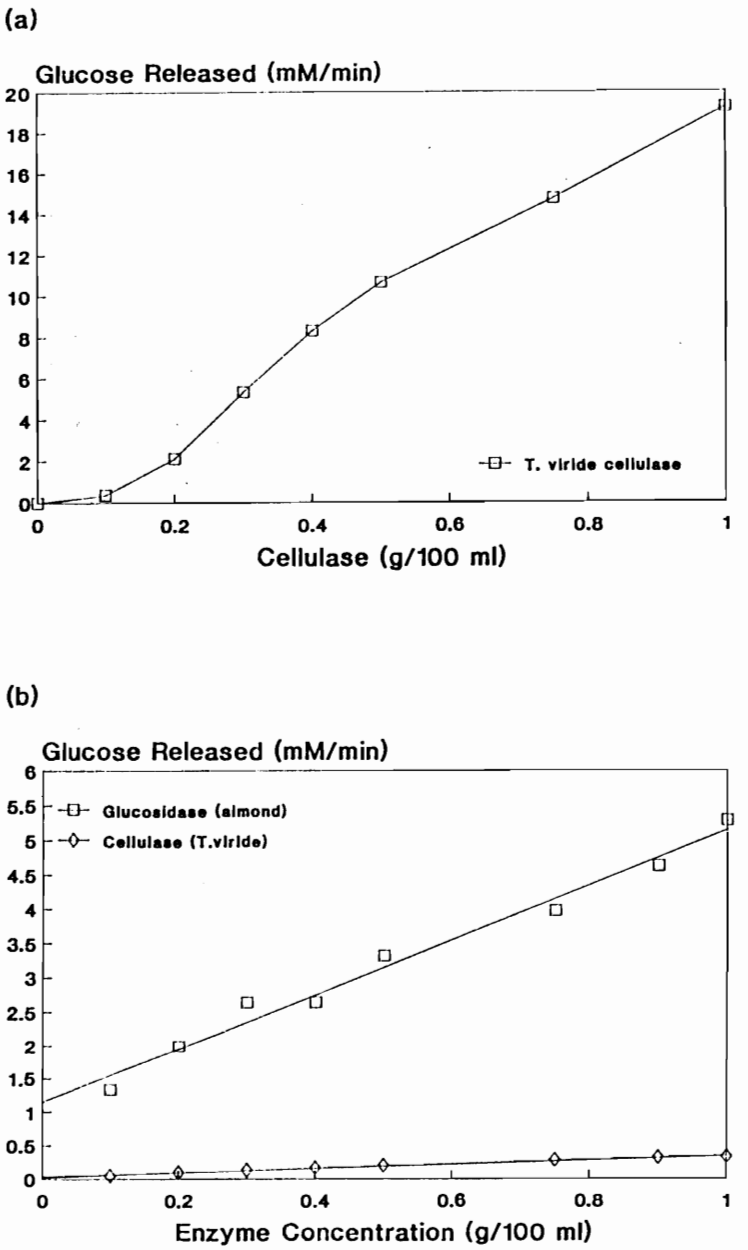


Figure 4.4.: Activities of (a) T. viride cellulase acting upon filter paper or (b) T. viride cellulase and ß-(1→4)-glucosidase from almonds acting upon cellobiose, measured as glucose release.

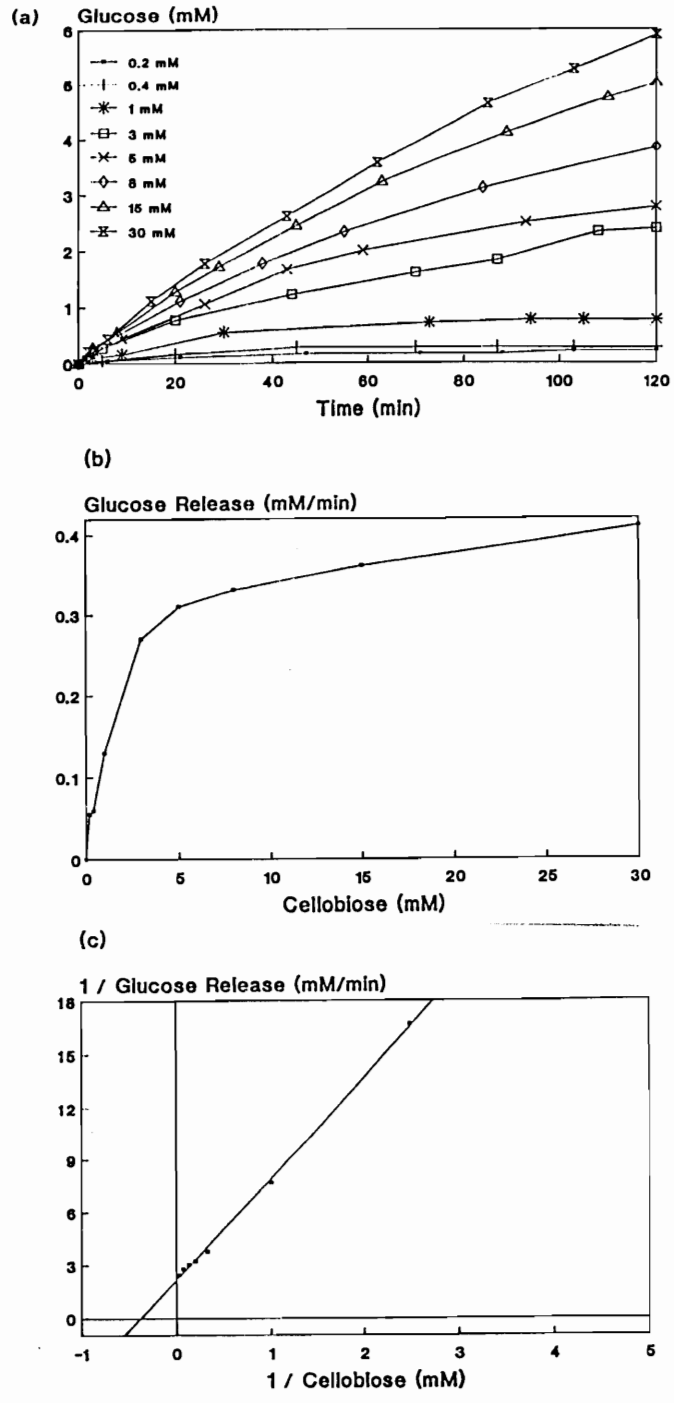


Figure 4.5.: Effect of cellobiose concentration on activity of T. viride cellulase as measured by (a) glucose release over time, (b) initial reaction velocities vs substrate concentration (0.2 - 30 mM) and (c) double reciprocal Lineweaver-Burk plot of (b): 1/glucose release = 2.1 and v_{max} = 0.47 mM/min; $(-1/K_{M\ app})$ = -0.5 and $K_{M\ app}$ = 2.0 mM.

Cell-free filtrate (1 ml) was pipetted into test tubes which were equilibrated in a stirred water bath at 50°C. One ml of 15 mM cellobiose (0.2567 g / 50 ml buffer) in 0.05 M Na-citrate buffer (pH 4.5) was added to the test tubes which were incubated for 30 min at 50°C and then boiled for 5 min at 90°C to stop the reaction. After cooling to room temperature, 25 µl of the reaction mixture was injected into the YSI Glucose Analyzer. Distilled H_2O and 1 ml filtrate mixed with 1 ml 0.05 M Na-citrate buffer served as blanks. Glucose concentrations were read as mg/dl and were converted to total cellobiase activity (µM glucose / min) and specific cellobiase activity (mM glucose / min x mg filtrate protein) by subtracting the blank readings from the glucose concentrations measured in mixtures where cellobiose had been added.

The expression of filter paper cellulase and cellobiase activities as µM glucose released was chosen to indicate which property of the assay system was measured. Quite commonly, arbitrary "units" are derived which are difficult to accurately define, even in the case of "katal" (= the catalytic amount of any catalyst that catalyses a reaction rate of one mole/sec in an assay system; Demeester et al., 1979). Wood & Bhat (1988), for example, listed three cellobiase assays, all of which have differently defined units, depending on the assay system. It is important to realize that these units are relative and only pertain to the respective assay. The measured release of glucose represents a more general way to express cellulolytic activity.

The activities of commercial T. viride cellulase (EC 3.2.1.4; Sigma #C-2274) and ß-D-glucoside glucohydrolase from almonds (EC 3.2.1.2; Sigma #G-0395) were determined to provide additional reference (Fig. 4.4.b). Kinetic parameters for the T. viride cellulase were determined using various concentrations of cellobiose and enzyme to determine initial velocities (Fig. 4.5.a.). Initial velocities were plotted vs. substrate concentration and the apparent Michaelis constant ($K_{M\ app}$) and maximal velocity (v_{max}) were determined from a double reciprocal Lineweaver-Burk plot (Fig. 4.5.c.). The kinetics of the T. viride cellulase showed a non-competitive pattern with cellobiose as a substrate which corresponded to earlier reports (Lee et al., 1980).

4.2.6.3. Laccase

One colorimetric assay for laccase, based on yellow colorization due to the oxidation of 2,6-dimethoxyphenol to 2,2'-bis-[2,6-dimethoxyquinone] (Fig. 4.6.), was developed by Hurst (1963) and refined by Prillinger (1976). It was used in this study according to Haars et al. (1981).

A reagent stock solution was prepared by dissolving 0.3 g 2,6-dimethoxyphenol in 0.5 ml dimethylsulfoxide (DMSO) and making up to 100 ml with distilled H_2O. A substrate solution was prepared by mixing 1 ml of the reagent stock solution with 99 ml of McIlvaine's buffer (500 ml of 0.1 M citric acid, slow addition of 0.2 M K_2HPO_4 until pH 4.5 is reached). The final concentration of 2,6-dimethoxyphenol was 20 μM. The substrate solution was freshly prepared for every test, since the concentration of 2,6-dimethoxyphenol in the buffer changes over time, even at low temperatures.

For each test, 1 ml of the substrate solution was added to test tubes with 20, 60, 100 and 150 μl cell-free filtrate, respectively. The test tubes were incubated for 5 to 15 minutes at 37°C in a heating block (VWR, Seattle, WA). The reaction was stopped by adding 1 ml DMSO. After thorough mixing, the solution was pipetted into a reduced plastic cuvette (1 cm path length; #58017-847; VWR, Seattle, WA) and absorbance at 468 nm was measured using the Milton Roy spectrophotometer. The blank solution contained 1 ml DMSO, 1 ml substrate solution and the respective amount of cell-free filtrate, the latter being added after incubation and stopping of the reaction. Units of enzyme activity in the cell-free filtrate were determined using a formula derived by Haars et al. (1981), where:

$$1\ U = A_{468\ nm} \times 0.2 \times min^{-1} \times ml^{-1}\ \text{cellfree filtrate}$$

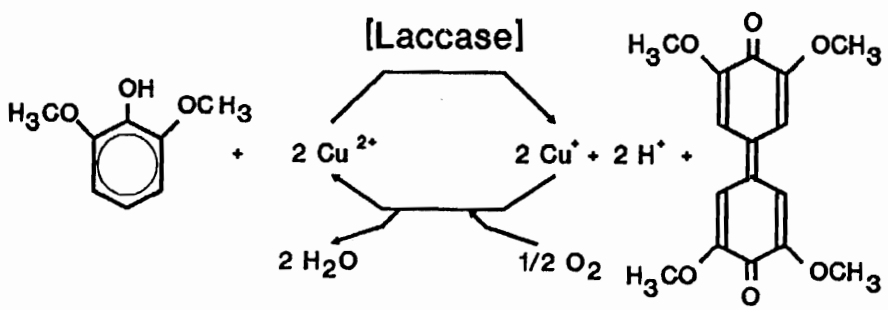
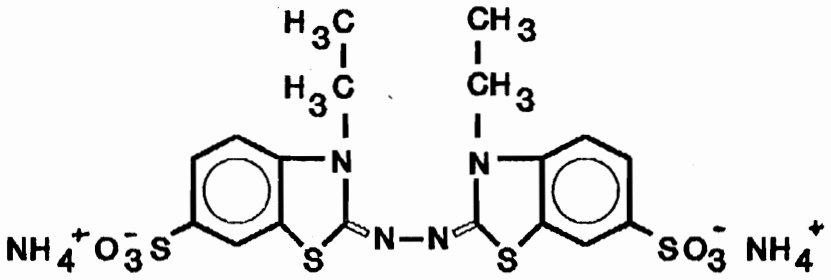


Figure 4.6.: Reaction pathway for the oxidation of 2,6-dimethoxyphenol by laccase in the presence of cupric ions to form 4,4′-bis-[2,6-dimethoxy-quinone].

(a)



2,2'-Azino-bis-[3-ethylbenzothiazole-6-sulfonic acid]

[ABTS]

(b)

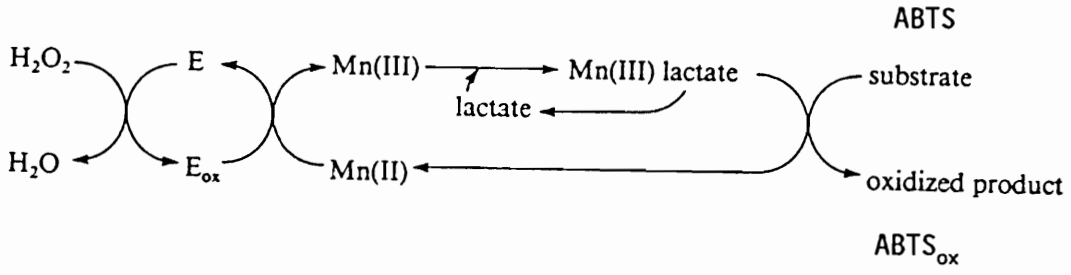


Figure 4.7.: Structure (a) and proposed reaction scheme of 2,2'-azino-bis-[3-ethylbenzothiazole-6-sulfonic acid] with manganese lactate, catalyzed by peroxidases (b; altered from Gold & Glenn, 1988).

4.2.6.4. ABTS-Peroxidase

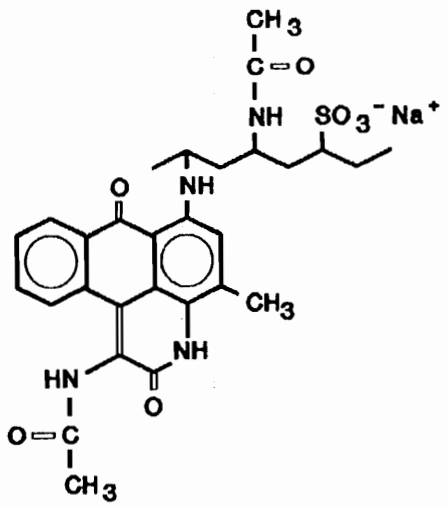
The assay used in this study was a modification of a method used by Mustranta (1987). It employed a reaction mixture containing 0.7 ml 20 mM 2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) as the substrate (Fig. 4.7.), 2.2 ml 0.05 M glycine-HCl buffer (pH 3) and 30 µl cellfree filtrate, mixed thoroughly in a plastic cuvette (#58017-825; VWR, Seattle, WA). The reaction was initiated by fast addition of 0.1 ml of 0.03 % (v/v) H_2O_2 at 22°C (room temperature). The absorbance of this reaction mixture was followed at 436 nm for up to 30 min using the Milton Roy spectrophotometer. Enzyme activity was calculated by determining the mean rate of increase in absorbance at 436 nm over a period of 5 - 10 min and calculating initial rates (= slope of time vs absorbance curve at time 0).

4.2.6.5. Poly Red-478 Peroxidase

The decolorization of the polymeric dyes Poly Blue-411, Poly Yellow-606 and Poly Red-481 by P. chrysosporium appeared to be catalyzed at least partly by a Mn^{2+}-peroxidase (Glenn & Gold, 1985). In this study, the polymeric dye Poly Red-478 was used since the dyes used in earlier studies were no longer commercially available. Poly Red-478 differs from Poly Red-481 by the presence of an acetyl group, substituting the phenyl ring on the pyrimidone ring (Fig. 4.8.; Dawson, 1981). Absorption spectra of Poly R-478 in various solvents and buffers are comparable to spectra for Poly R-481 (Fig. 4.9.; Glenn & Gold, 1983; Gold et al., 1988).

Two measures of dye decolorization as an indicator for the presence of peroxidases were employed. In the first, a 1 % (w/v) solution of the dye was filter sterilized (Acrodisc, 0.2 µm; #4192, Gelman Sciences, Ann Arbor, MI) and 1 ml aliquots were added to 250 ml Erlenmeyer flasks with 50 ml Media B and C, which had been freshly

(a)



(b)

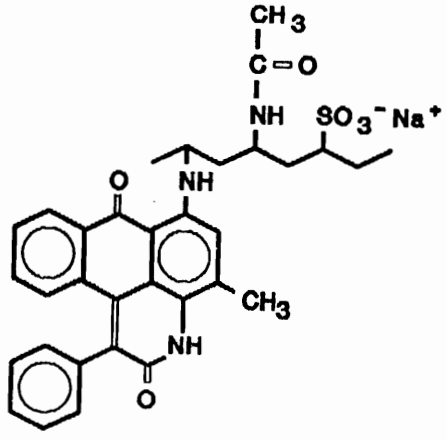


Figure 4.8.: Molecular structures of the monomeric units of two polymeric anthraquinone sulfonate dyes (a) Poly R-478, the dye employed in this study and (b) Poly R-481, used in earlier studies. The latter dye differs only in the substitution of a phenyl group for an acetyl group on the pyrimidone ring.

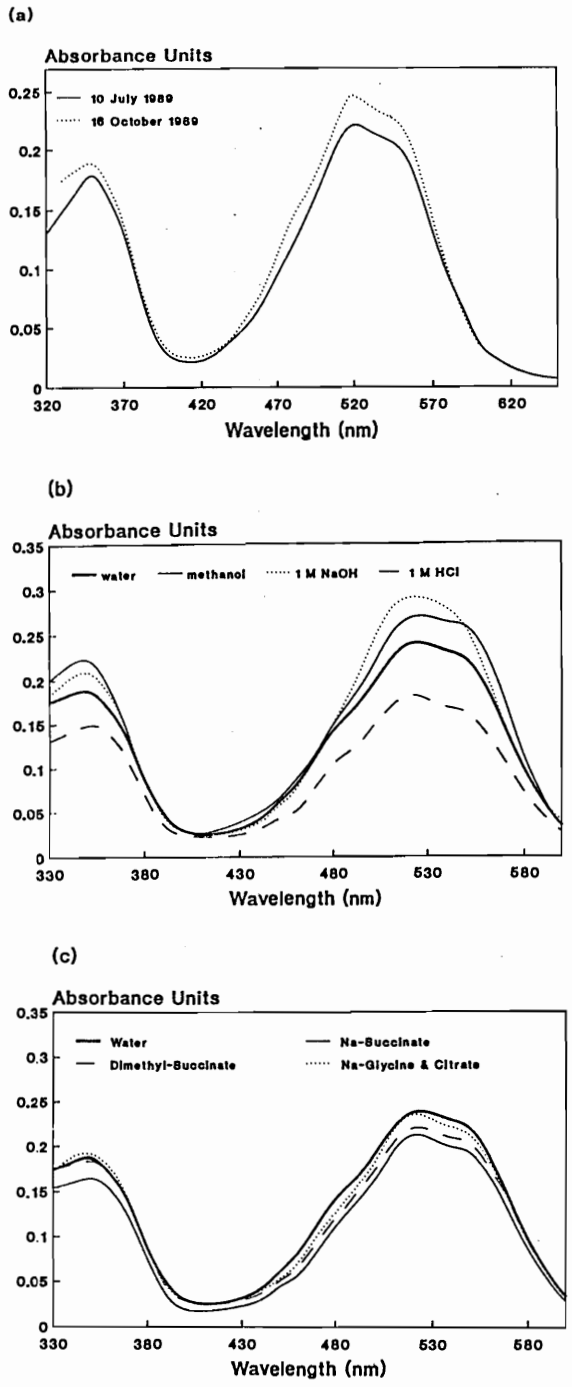


Figure 4.9.: Absorbance spectra of the polymeric dye Poly Red-478 (a) when stored at 4°C for 3 months in water (higher October values are due to evaporation), (b) when dissolved in water, methanol, sodium hydroxide and hydrogen chloride or (c) in common buffers.

inoculated with the test fungi (final concentration of Poly R-478: 0.2 % [w/v]). Three flasks containing either only the growth media or growth media with dye were used as controls. The experiment was repeated using Medium B and incubating on a rotary shaker (cf. 4.2.2.2.) at 80 rpm.

In 2 day intervals, 1 ml of culture liquid was aseptically removed from each flask and centrifuged (5 min at 10,000 rpm; Eppendorf microcentrifuge 5415). Supernatant (100 μl) was diluted with 900 μl H_2O. The absorbance at 520 and 350 nm was determined against a water blank using the Milton Roy spectrophotometer. Decolorization was quantified by determining the decrease in absorbance ratio at 520 nm and 350 nm ($A_{520\ nm}$ / $A_{350\ nm}$). While maximal absorbance of the dye at 520 nm decreases due to dye adsorption to mycelium in culture, the calculated ratio remains stable over prolonged time periods if organisms are unable to decolorize the dye (Glenn & Gold, 1983).

This method provided a measure of cumulative decolorization over time, but did not show the time of highest enzyme activity. To measure peak activity, 1.5 ml culture liquid was aseptically removed from Erlenmeyer flasks without dye at 2 day intervals, centrifuged (5 min at 10,000 rpm; Eppendorf microcentrifuge 5415) and 1 ml of the supernatant incubated with 20 μl of 1 % (w/v) filter sterilized Poly R-478 (final concentration: 0.02 % [w/v]) for various time intervals at 37°C. Absorbance at 520 nm and 350 nm was determined as above. Enzyme activity was calculated as change in $A_{520\ nm}$ / $A_{350\ nm}$ per min.

A second peroxidase assay, based on the oxidation of the dye Phenol Red by H_2O_2, catalyzed by peroxidase was employed (Kuwahara et al., 1984). One ml of a 0.01 % (w/v) Phenol Red solution in 20 mM Na-succinate buffer (pH 4.5) was mixed with 10 μl H_2O_2 solution (from 102 μl 30 % [w/v] H_2O_2 brought to 100 ml with distilled water). The reaction was initiated by adding 100 μl cell-free filtrate. Non-inoculated, centrifuged medium and distilled H_2O served as blanks. Initial rates of peroxidase activity were calculated by monitoring the reaction at 610 nm over 10 to 15 min in a glass cuvette (1 cm path length; #58016-425, VWR, Seattle, WA) using the Milton Roy spectrophotometer.

4.2.6.6. Coomassie Blue Protein Assay

Filtrate protein concentrations were routinely determined to calculate specific enzyme activities using the Coomassie Blue protein assay (Bradford, 1976). This rapid method, based on the binding of the dye Coomassie Brilliant Blue G-250 to proteins, is about four times more sensitive than the unaltered Lowry assay (Bradford, 1976). A protein determination kit (#23200; Pierce Chem. Co., Rockford, IL) which incorporates changes to the original assay suggested by Read & Northcote (1981) was used.

The reaction mixture (1 ml), containing Coomassie Brilliant Blue G-250, H_3PO_4, methanol, water and solubilizers (Pierce Chem. Co., 1988) was added to 1 ml protein standard (BSA in the respective centrifuged medium) or cellfree filtrate. The test tubes were immediately vortexed and absorbance read at 595 nm in reduced plastic cuvettes (1 cm path length; #58017-847, VWR, Seattle, WA) using the Milton Roy spectrophotometer. Protein concentrations were read from a calibration curve fitted by linear regression, using BSA (Sigma #B-2518) as the standard protein.

4.2.7. Chemicals

Malt and yeast extract were from Difco, Detroit, MI; H_2O_2 from Fisher Scientific Corp., Fair Lawn, NJ; dimethylsulfoxide (DMSO) from Crown Zellerbach, Camas, WA; glucose and citric acid from J.T. Baker, Phillipsburg, NJ; 2,6-dimethoxyphenol from Aldrich Chemicals, Milwaukee, WI;: Cellobiose, Agar, L-asparagine, Folin & Ciocaltaeu's Reagent, bovine serum albumin, horseradish peroxidase, Phenol Red, Poly Red-478 and 2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) from Sigma, St. Louis, MO; and the Coomassie Protein Kit and BCA Protein Kit from Pierce Chem. Co., Rockford, Il.

All other chemicals were obtained from Van Waters and Rogers (VWR), Seattle, WA and were analytical or HPLC grade.

4.3. Results and Discussion

4.3.1. Growth Characteristics of Test Fungi

Growth of T. versicolor and T. harzianum in pure and mixed cultures was evaluated using four parameters: (a) residual glucose concentration, (b) pH, (c) mycelial dry weight and (d) filtrate and mycelial protein contents (Fig. 4.10. - 4.12.). These parameters allowed an evaluation of the influence of the two variables, pure vs. mixed cultures and high vs. low nitrogen level, on biomass production by the test fungi.

4.3.1.1. Mycelial Dry Weights

Mycelial dry weights did not differ substantially between pure and mixed and low and high nitrogen cultures of T. versicolor (Fig. 4.10. a.). Mycelial yields on low nitrogen (low N) medium were highest after 8 - 10 days and at the end of the 30 day incubation period, whilst stagnation and subsequently autolysis began after only 10 - 12 days in cultures on high nitrogen (high N) medium. The same general growth pattern was obtained for T. harzianum (Fig. 4.10.b.), but the difference between low and high N cultures was more pronounced. Mixed cultures of T. harzianum and T. versicolor (Fig. 4.10.c.) produced slightly, but hardly significantly more dry mycelial mass on low than on high N medium.

The previous emphasis placed on carbon to nitrogen (C/N) ratios led to the use of two distinct ratios in this study. Low N medium had a C/N ratio of 500:1, which adequately describes the overall C/N ratio in many wood species and wood tissues (Merrill & Cowling, 1966b; King et al., 1976). High N medium had a C/N ratio of 50:1, comparable to ratios found in mycelia when grown in artificial culture or on wood substrates

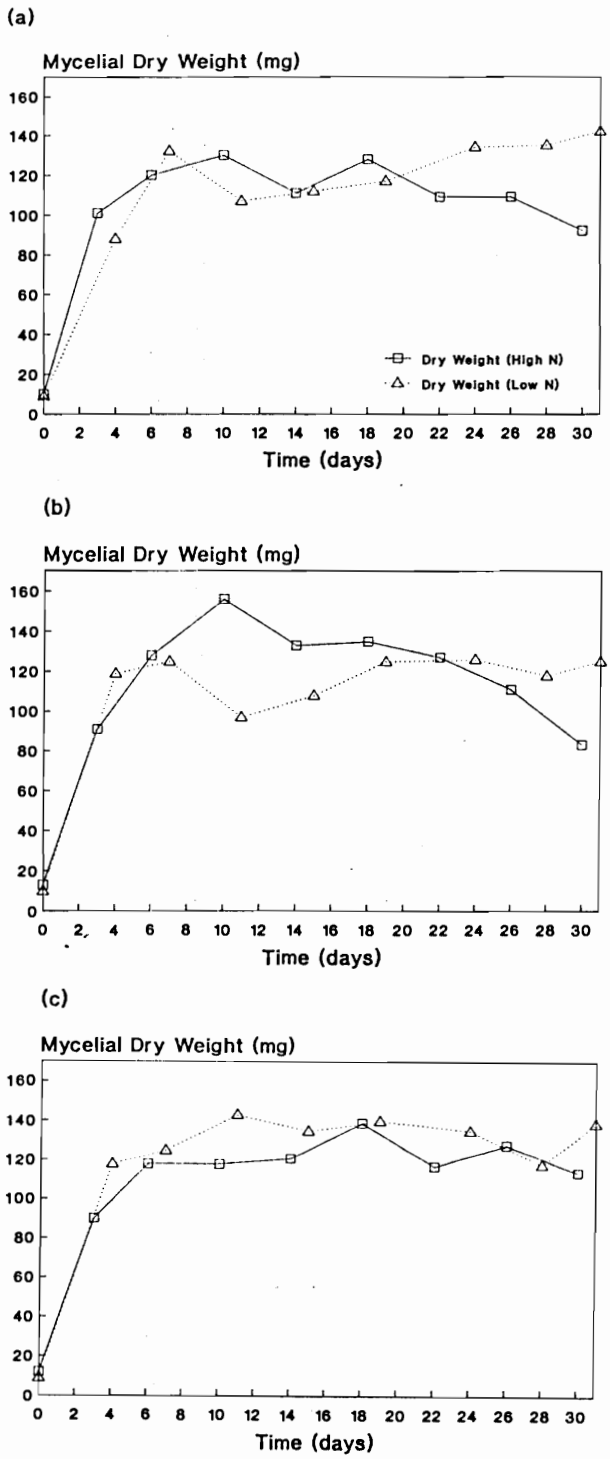


Figure 4.10.: Mycelial dry weights (mg/flask) of (a) pure cultures of T. versicolor, (b) pure cultures of T. harzianum or (c) mixed cultures of T. versicolor and T. harzianum grown in liquid media containing low (0.4 mM) and high (4 mM) nitrogen concentrations.

under non-nitrogen limiting conditions (Merrill & Cowling, 1966a; Levi et al., 1968). Absolute conversion and decay rate of wood were shown to be higher when wood was amended with exogenous nitrogen (Duncan, 1960; Merrill & Cowling, 1965; 1966c). The same held true, however, when the absolute amount of easily accessible carbon was varied: cultures grown on media with C/N ratios of 4:1 - 400:1 were found to be carbon-limited and, above 400:1, nitrogen-limited (Levi & Cowling, 1969). Completely different results were obtained when a fixed C/N ratio of 16:1 was chosen, but the amounts and composition of the carbon and nitrogen sources were varied (Levi & Cowling, 1969). Thus, the absolute amounts of carbon and nitrogen available are independent parameters governing mycelial growth and the C/N ratio is "merely an arbiter of which factor will limit growth" (Rayner & Boddy, 1988) and, by itself, cannot give a clear indication of overall growth or biomass conversion by fungi, neither on synthetic media nor on wood. However, the rates of growth or biomass conversion and turnover due to respiration and mycelial autolysis seem to be affected by the relative amounts of carbon and nitrogen present (Park, 1976; Dowding, 1981).

In this study, the differences between dry weights obtained from low and high N artificial media were smaller than those of earlier studies (Levi & Cowling, 1968; Danielson & Davey, 1973b+c; Sierota, 1976; Park, 1976; Keyser et al., 1978; Kirk et al., 1978; Reid, 1979; Dowding, 1981). The low level (0.4 mM) represents severe nitrogen starvation conditions. The results therefore suggest that both test fungi either command efficient nitrogen cycling mechanisms which allow further growth even when the total amount of nitrogen is limited or have the ability to "adapt" to very low nitrogen levels by preferential synthesis of essential compounds (Cowling & Merrill, 1966; Levi & Cowling, 1968; 1969; Ulmer et al., 1983b; Rayner & Boddy, 1988). These characteristics are reflected by the roughly bimodal curves for mycelial dry weight production on low N medium by both T. versicolor and T. harzianum (Fig. 4.10.a.+b.). Under high N conditions (4 mM) the curves were not bimodal, although this nitrogen level is still low compared to earlier studies (Danielson & Davey, 1973a+b; Sierota, 1976). Lowering

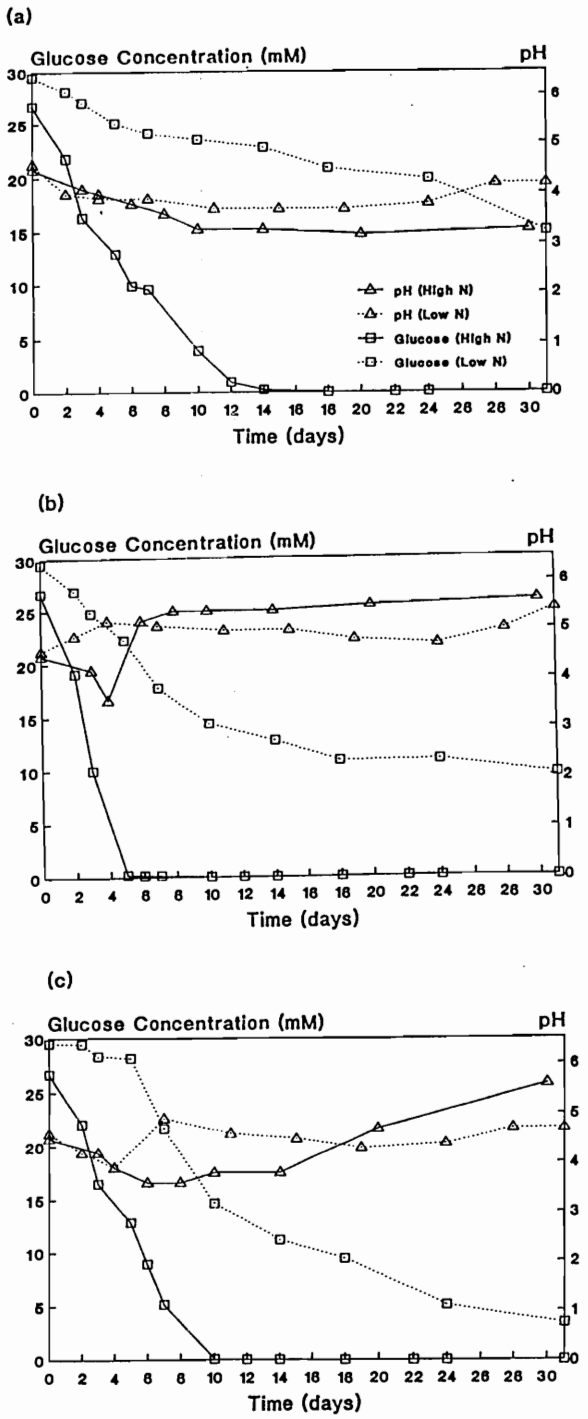


Figure 4.11.: Glucose concentration (mM) and pH in (a) pure cultures of T. versicolor, (b) pure cultures of T. harzianum or (c) mixed cultures of T. versicolor and T. harzianum grown in liquid media containing low (0.4 mM) and high (4mM) nitrogen concentrations.

the nitrogen level to 0.1 mM or beyond might result in a more distinct bimodal pattern. The fact that both T. versicolor and T. harzianum exhibited bimodal growth curves supports the argument that soil fungi may also have nitrogen cycling mechanisms (Rayner & Boddy, 1988); however, the total protein levels did not exhibit bimodal patterns (Fig. 4.12.), indicating that the synthesis of protein in mycelia was more or less constant after completion of the logarithmic growth phase.

4.3.1.2. Glucose Concentration

Glucose concentrations in both pure and mixed high N cultures of T. versicolor and T. harzianum fell rapidly during the incubation period: T. harzianum depleted the glucose completely within only 5 days of incubation (Fig. 4.11.b.), while it took T. versicolor 14 - 16 days to deplete glucose (Fig. 4.11.a.). Mixed cultures of T. versicolor and T. harzianum were intermediate, requiring 9 - 12 days for glucose depletion (Fig. 4.11.c.). In low N cultures, T. harzianum used glucose at a faster rate than T. versicolor, but the mixed cultures were more efficient in glucose depletion (Fig. 4.11.a.-c.). Glucose was never completely removed from the low N media over the 31 day incubation period.

Thus, glucose utilization during primary metabolism is strongly dependent on nitrogen level. There may even be "synergistic" depletion as T. versicolor and T. harzianum compete for the same substrate. This competition was reflected in the overall biomass production, measured as mycelial dry weight, where mixed low N cultures were associated with slightly higher dry weights than those obtained from mixed high N cultures or pure cultures of T. versicolor and T. harzianum (Fig. 4.10.). Similar results were reported for the growth of T. versicolor (Levi et al., 1968; Levi & Cowling, 1969) and Phanerochaete chrysosporium Burds. (Keyser et al., 1978; Reid, 1979), where the mycelial yield and efficiency of glucose usage were reduced when cultures were grown in low N media.

4.3.1.3. Acidity of the Culture Liquid

The pH of the media was adjusted to 4.5 before inoculation and pH changes associated with the growth of T. versicolor and T. harzianum in pure and mixed cultures were studied. In all cases, the pH was altered in the presence of fungi, with T. versicolor changing the pH to 3.3 and 3.6, and T. harzianum altering it to 5.6 and 5.4 in high and low N medium, respectively (Fig. 4.11.). The pH in high N cultures of T. harzianum dropped first and rose sharply after 5 days. The same patttern was found in low N mixed cultures, whilst the pH in high N mixed cultures first dropped steadily and later rose to values comparable to those obtained from pure cultures of T. harzianum. Similar patterns have been reported, but the magnitude of pH alterations was larger in those studies (Levi et al., 1968; Levi & Cowling, 1969; Danielson & Davey, 1973b+c; Sierota, 1976; Shepherd & Carels, 1983).

Secretion of secondary metabolites or protons may affect acidity of the culture liquid, but the observed changes in pH were most likely due to selective utilization of nitrogen compounds (Shepherd & Carels, 1983). Carbon compounds have less effect on culture pH (Sierota, 1976). When L-asparagine or nitrate are used preferentially, the culture liquid may become more basic due to the accumulation of ammonia, whilst it becomes more acidic when ammonium is preferred to the other major nitrogen compounds. The latter effect seemed more pronounced when NH_4NO_3 is offered as the sole nitrogen source (Sierota, 1976). Trametes versicolor generally grows better on protein precursors and ammonium compounds and grows poorly on nitrate as nitrogen source (Levi et al., 1968). However, the media became more acidic, possibly due to the release of protons during ammonium metabolism. During secondary metabolism, endogenous, nitrogen-rich carbon sources are recycled and ammonia excreted, leading eventually to increased pH in the culture liquid (Grabbe et al., 1968). Trichoderma harzianum also metabolizes preferentially L-asparagine and ammonium (Danielson & Davey, 1973; Sierota, 1976), but accumulation of ammonia from amino acid decomposition or during secondary metabolism may increase the pH.

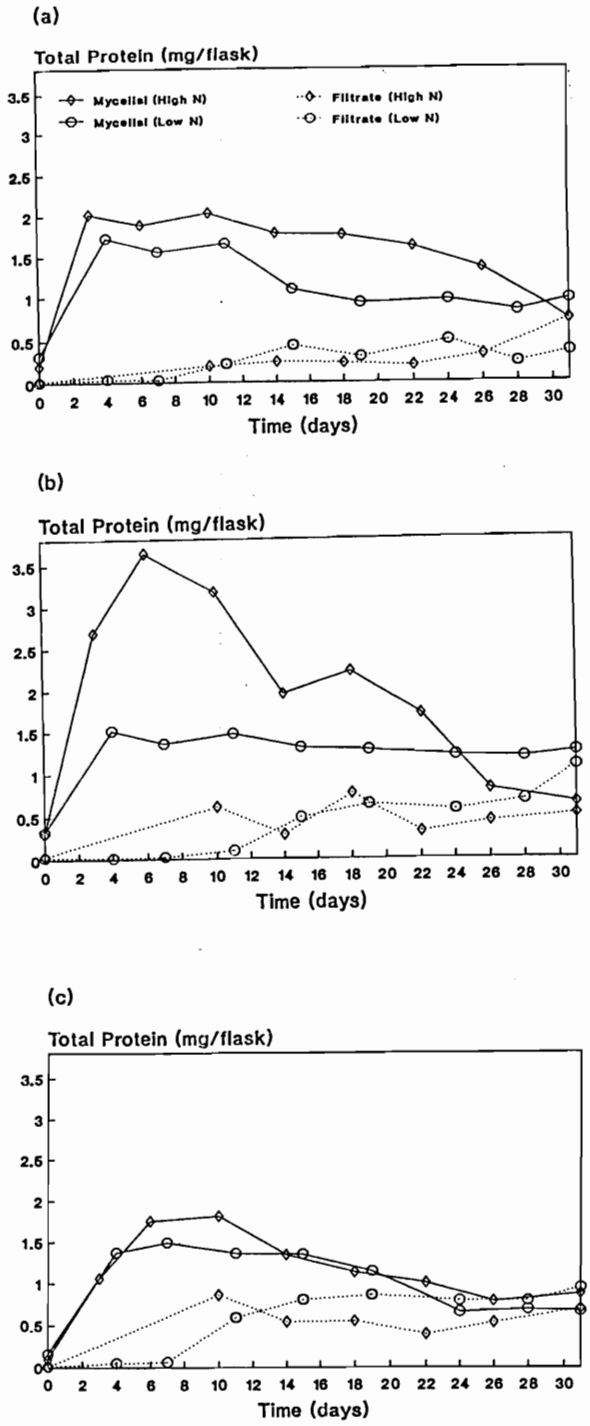


Figure 4.12.: Total mycelial and filtrate protein (mg/flasks) of (a) pure cultures of T. versicolor, (b) pure cultures of T. harzianum or (c) mixed cultures of T. versicolor and T. harzianum grown in liquid media containing low (0.4 mM) and high (4mM) nitrogen concentrations.

Alterations of pH with time in mixed cultures appeared to be affected by which fungus was growing more actively. Fungal growth was, in turn, related to the different pH in cultures of T. versicolor and T. harzianum, which were only marginally influenced by the overall nitrogen level. T. harzianum altered the media pH after two weeks of incubation in high N media, whilst pH was intermediate between pure cultures of T. versicolor and T. harzianum in low N media.

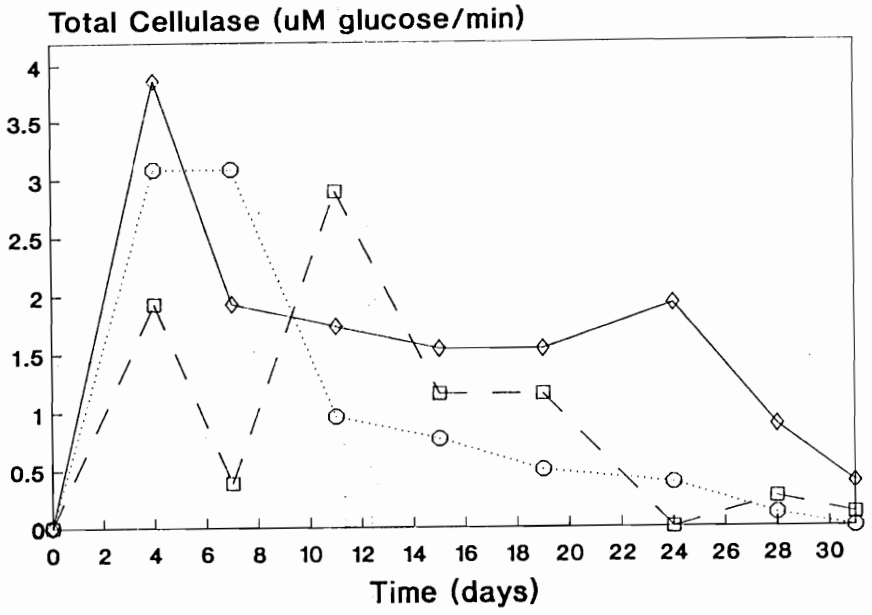
4.3.1.4. Levels of Mycelial and Filtrate Proteins

Rayner & Boddy (1988) explained variations in nitrogen content of mycelia grown at different C/N ratios (Levi & Cowling, 1969) by suggesting different fungal allocation strategies for either carbon or nitrogen. If carbon is in excess and nitrogen limits growth, nitrogen will be incorporated into compounds required to sustain primary metabolism and further growth, whilst carbon compounds will be transformed into storage products. When carbon is limiting, however, nitrogen will be incorporated into secondary metabolites or released into the culture liquid (Rayner & Boddy, 1988). This hypothesis would explain earlier results without the need for a specific "adaptation" mechanism suggested by Levi & Cowling (1969).

Significantly more protein was found in the mycelium in high N cultures of T. harzianum than in mycelium obtained from low N cultures. These curves followed those for the development of mycelial dry weight (Fig. 4.12.b). Nitrogen allocation towards filtrate protein appeared to be independent of total nitrogen level, although the highest level of filtrate protein appeared later in low N cultures. Whilst the latter trend was also noted with T. versicolor, the difference in mycelial protein production in high and low N media was far less pronounced with this fungus (Fig. 4.12.a.).

Quite surprisingly, no significant differences were found between mycelial and filtrate protein production on high and low N media in

(a)



(b)

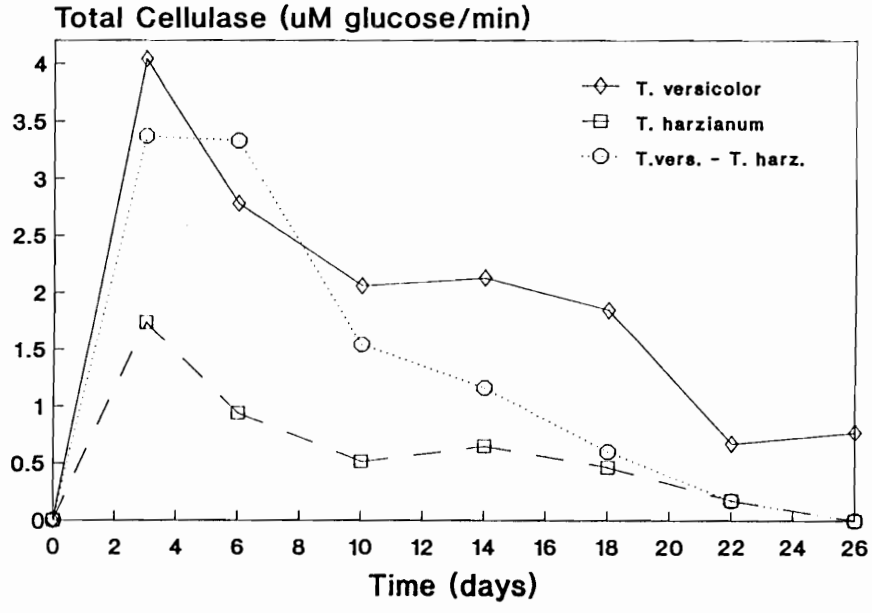


Figure 4.13.: Total filter paper cellulase activity (μM glucose released/min) of pure and mixed cultures of T. versicolor and T. harzianum in liquid media at (a) low (0.4mM) and (b) high (4mM) nitrogen concentrations.

mixed cultures of T. versicolor and T. harzianum (Fig. 4.12.c.). However, the absolute amounts of mycelial protein decreased in mixed cultures, whilst the absolute amounts of filtrate proteins were slightly increased, possibly due to an increased release of extracellular enzymes.

4.3.2. Extracellular Enzyme Activities in Pure and Mixed Cultures

4.3.2.1. Filter Paper Cellulase

Filter paper cellulase activities in low N cultures of T. versicolor peaked after 4 days of incubation, whilst activity was highest after 12 days in T. harzianum cultures (Fig. 4.13.a.). In mixed cultures, the peak was extended from 4 - 8 days after inoculation. Activities were highest for T. versicolor and mixed cultures of T. versicolor and T. harzianum and did not appear to be influenced by nitrogen level. Peaks of cellulase activity early in the growth cycle correspond to those found in earlier studies on synthetic or solid media (Mandels & Reese, 1960; 1965; Wood & McCrae, 1979; Warzywoda et al., 1980; Gritzali & Brown, 1979; Gong et al., 1979; Montgomery, 1982; Willick et al., 1984; Finch & Roberts, 1985; Breuil & Saddler, 1985), although they conflict with those found using Agaricus brunnescens Peck (= A. bisporus [Lange] Imbach) on compost medium (Wood & Goodenough, 1977; Claydon et al., 1988; Smith et al., 1989). Trichoderma harzianum produced higher filter paper cellulase activities on low N medium. The overall low cellulase activity of T. harzianum was unexpected and somewhat surprising, since other members of this genus and even other strains of the same species aggregate are thought to be among the most active cellulase producers (Eveleigh, 1985; Finch & Roberts, 1985; Saddler et al., 1985). The results illustrate the importance of consider-

ing species and strain variation in studies of wood or cellulose utilization.

"Specific enzyme activity" is usually defined as enzyme activity per unit weight of enzyme (Rawn, 1989). Since the determination of enzyme mass was not feasible in this study, "specific" cellulase activities were calculated as total cellulase per mg total filtrate protein. These specific activities allow comparisons of protein allocation within a species and the culture fraction investigated. High specific enzyme activities reflect high relative amounts of the respective enzyme in the investigated protein fraction, whilst low specific enzyme activities suggest that either low amounts of enzyme or large amounts of other extracellular proteins are present.

Specific cellulase activities were low in high N medium for both pure and mixed cultures of T. versicolor and T. harzianum (Fig. 4.14.). Since the total activities on high N medium were among the highest values obtained for both pure cultures of T. versicolor and mixed cultures, cellulases accounts for only a small fraction of the whole range of extracellular enzymes produced under high N regime. The contribution of cellulase to the total extracellular protein present was larger on low N medium, especially in T. harzianum cultures (Fig. 4.14.b.). These results are comparable to those obtained by Levi & Cowling (1968; 1969) and suggest that cellulase production is enhanced when carbon and nitrogen levels are limiting (Rayner & Boddy, 1988). These results conflict with those obtained by Butcher & Drysdale (1974) in studies on soft-rot fungi, which showed maximal cellulase production at a C/N ratio of 50:1 and 100:1. However, Butcher & Drysdale (1974) used two times more carbon or nitrogen, again showing the importance of absolute amounts of nitrogen and carbon and the difficulties of correctly interpreting C/N ratios.

Peak specific cellulase activities were always observed after 4 - 5 days, due to the relatively small amounts of proteins present in the extracellular liquid. This was expected, since cellulose-degrading hydrolases are secreted during primary metabolism of Basidiomycotina

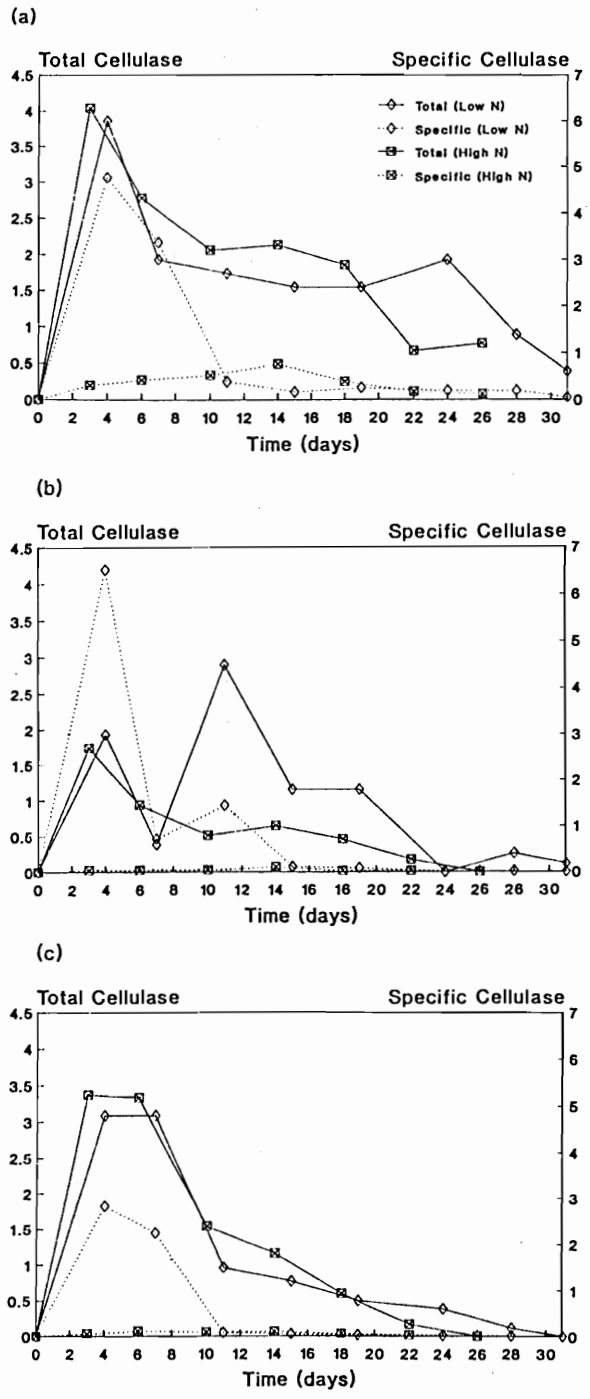


Figure 4.14.: Comparison of total (μM glucose released/min) and specific (mM glucose released/min) filter paper celulase activities of (a) T. versicolor, (b) T. harzianum and (c) mixed cultures of T. versicolor and T. harzianum grown in liquid media containing low (0.4 mM) and high (4 mM) nitrogen concentrations.

(Eriksson & Wood, 1985). Sequential secretion of carboxymethyl cellulase followed by cellobiase associated with varied carbon sources have been noted in labelling and immunoprecipitation studies with Schizophyllum commune Fr. (Willick et al., 1984). These results are consistent with the pattern of cellulase and cellobiase release noted in this study.

The lower levels of cellulase activity in mixed cultures conflicts with reports of synergistic action of cellulase systems of wood decay fungi with secondary sugar fungi like Trichoderma spp. or Scytalidium spp. (Hulme & Stranks, 1970; Hulme & Shields, 1975; Rayner & Webber, 1984; Morris & Calver, 1986). These earlier studies discussed the possibility that the feedback inhibition or repression of cellulases by glucose or cellobiose in wood decay fungi would be overridden by the action of sugar-capturing Trichoderma spp., thus leading to either a constant or even an enhanced production of cellulases. Results from this study do not support this hypothesis. The activities of filter paper cellulase on both low and high N media was lower in mixed cultures than in cultures of T. versicolor alone. However, cellulases may have been inactivated by the action of proteases produced by either of the two fungi. Even in a simplified interactional system using only two fungi, total amounts of enzymes produced are, of course, not additive. Thus, the total and specific enzyme activities measured reflect only the outcome of an interaction between T. versicolor and T. harzianum, and not the true enzyme activities produced by either of the two fungi or both combined.

Recent studies of synergistic cellulase production by pairings of fungi on crystalline cellulose yielded variable results (Lundborg, 1988a+b). Whilst the growth of Heterobasidion annosum (Fr.) Bref. on cellulose agar plates and wood weight losses in sawdust tubes associated with this white-rot fungus were greatly reduced by the presence of S. album, cellulase activities were only moderately affected and increased when other decay fungi were co-inoculated (Lundborg, 1988b). The decay process was slowed or stopped when Norway spruce wood was amended with various nitrogen and carbon sources and incubated with H. annosum in the

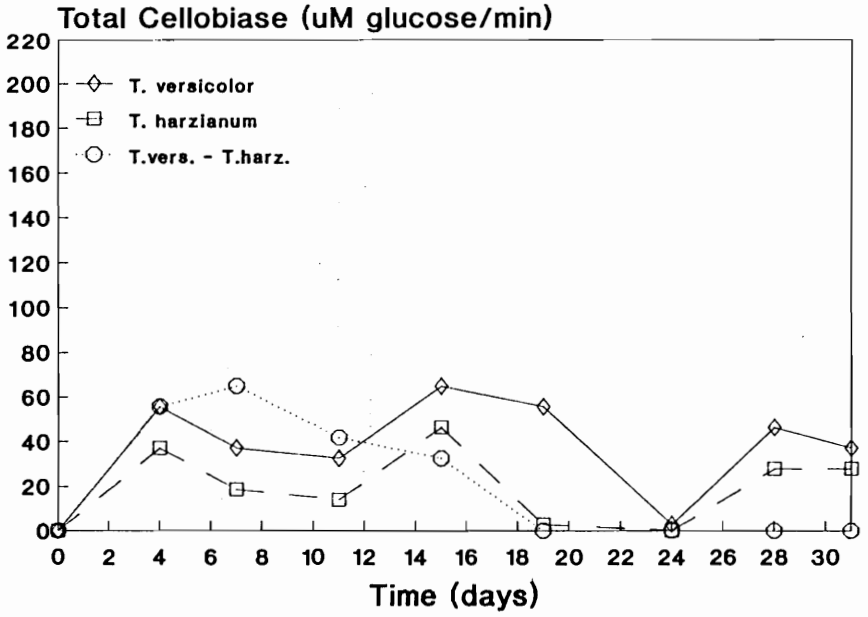
presence of two potential biocontrol fungi (Lundborg, 1988a). Feedback-inhibition of cellulases of H. annosum by the added glucose, overruling the induced ligninolytic effect due to low nitrogen concentrations, was postulated (Lundborg, 1988a).

4.3.2.2. Cellobiase

Cellobiase activities in pure cultures of T. harzianum were low in both low and high N medium, whilst the activities for both pure cultures of T. versicolor and mixed cultures of T. versicolor and T. harzianum were about two times higher in high than in low N medium (Fig. 4.15.). Highest activities were measured in high N mixed cultures, but they were still substantially lower than activities of the purified commercial T. viride cellulase (Fig. 4.5). Very pronounced peaks of cellulase activities were found in high N medium with T. versicolor and mixed cultures after 18 days of incubation, whilst T. harzianum cultures showed highest activities after only 14 days. The peaks in cellobiase activities on high N medium were correlated with the disappearence of glucose from the culture filtrate (Fig. 4.11.). As the concentration of the feedback inhibitor glucose in culture liquid decreased, the production of inducible cellobiase started or was derepressed, and peaked close to the time of complete glucose depletion.

A completely different pattern of cellobiase activity was associated with low nitrogen levels (Fig. 4.15.a.): mixed cultures produced comparatively high amounts of cellobiase for up to 18 days, but then became completely inactive. Pure cultures of T. versicolor produced more cellobiase than T. harzianum. The pattern suggests cycling of nitrogen and repeated switching from primary to secondary metabolism. The reason for cessation of cellobiase production in mixed cultures after three weeks is unknown. Cellobiase activities in pure cultures of T. versicolor continued to cycle for up to 45 days, with a steadily declining amplitude.

(a)



(b)

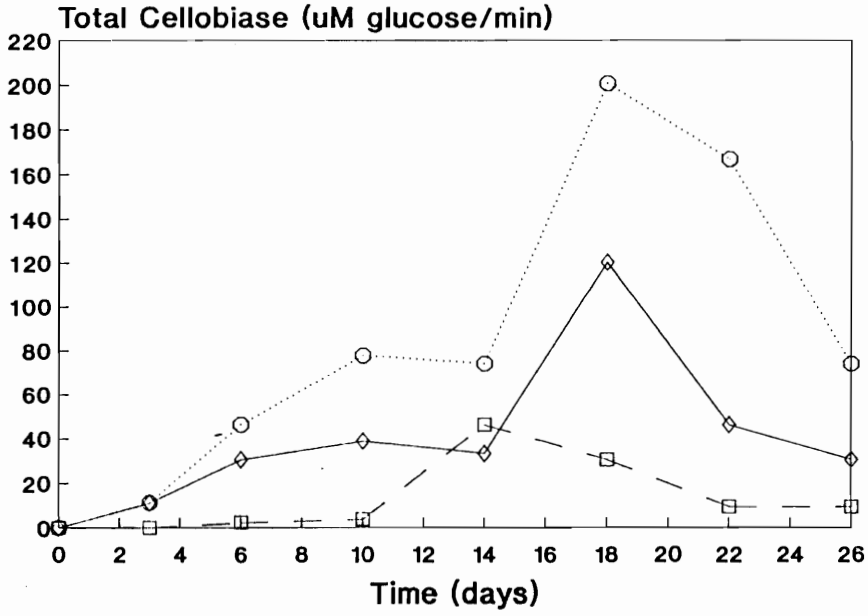


Figure 4.15.: Total cellobiase activity (μM glucose released/min) of pure and mixed cultures of T. versicolor and T. harzianum in liquid media at (a) low (0.4mM) and (b) high (4mM) nitrogen concentrations.

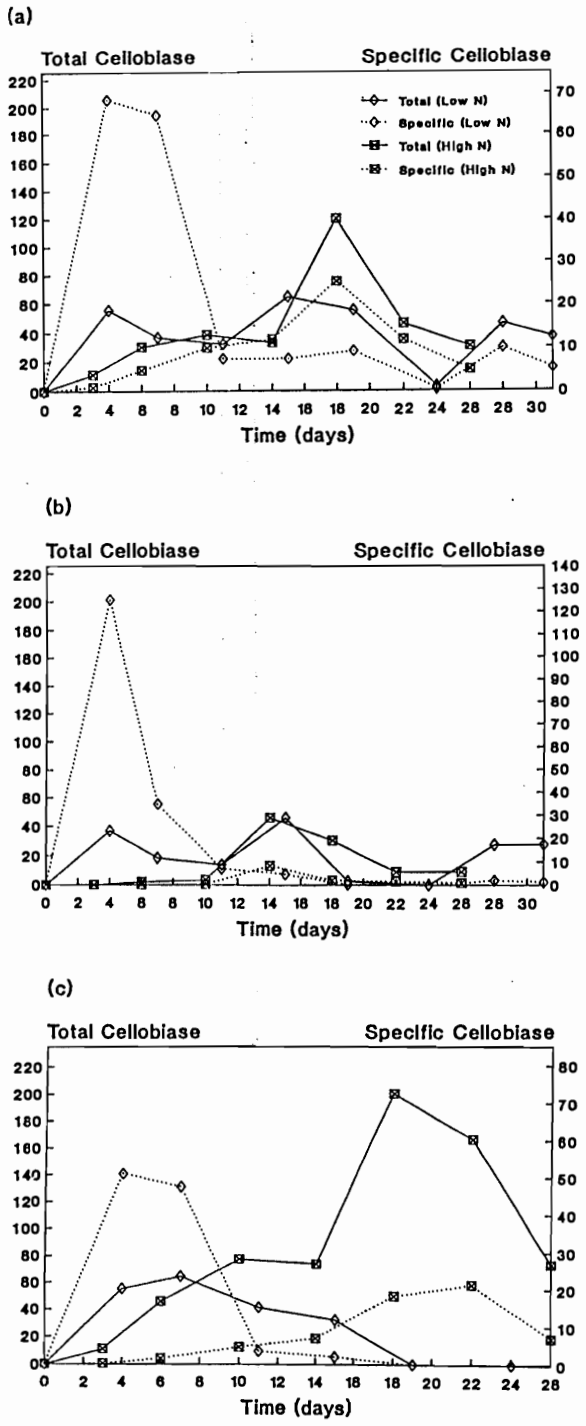


Figure 4.16.: Comparison of total (μM glucose released/min) and specific (mM glucose released/min) cellobiase activities of (a) T. versicolor, (b) T. harzianum and (c) mixed cultures of T. versicolor and T. harzianum grown in liquid media containing low (0.4 mM) and high (4 mM) nitrogen concentrations.

Specific cellobiase activities were also much higher in low N cultures and peaked after 4 - 5 days during primary growth (Fig. 4.16.). The highest specific cellobiase activities on high N media were calculated after 18, 14 and 22 days for cultures of T. versicolor, T. harzianum and their combination, respectively, indicating that the relative contribution of cellobiase to the sum of extracellular proteins in the culture liquid was small on high N medium, but much larger during the primary growth phase in low N cultures. The peak of specific cellobiase activity in mixed high N cultures occurred in the third week of incubation, indicating that cycling from primary to secondary and later, after 16 days, back to primary metabolism occurred (Figs. 4.15.b. and 4.16.a+c.). Cellobiase accounted for a large percentage of the protein in filtrates of pure cultures of T. versicolor and mixed cultures of T. versicolor and T. harzianum after glucose was depleted.

Total cellobiase activities were enhanced in mixed cultures (Fig. 4.15. and 4.16.), possibly due to competition for small chain polyglucosides, paralleling earlier studies. For example, Trichoderma reesei Simm. is a potent producer of endo- and exoglucanases, but its cellobiase production is rather low (Panda et al., 1989). One way to enhance the saccharification rate is to supplement reactors containing T. reesei with industrially prepared cellobiases (Dekker, 1986). Cellobiase activities of T. reesei have also been enhanced by directly mixing cultures with Aspergillus wentii Wehm. in batch stirred-tank reactors (Panda et al., 1989).

4.3.2.3. Laccase

Laccase activity was not detected in regular cultures of T. harzianum, nor was it inducible by the addition of catechol and ferulic acid or the presence of another non-laccase producing fungus, P. placenta, (unpublished results). Thus, T. harzianum does not produce laccases under the chosen conditions and may therefore serve as an

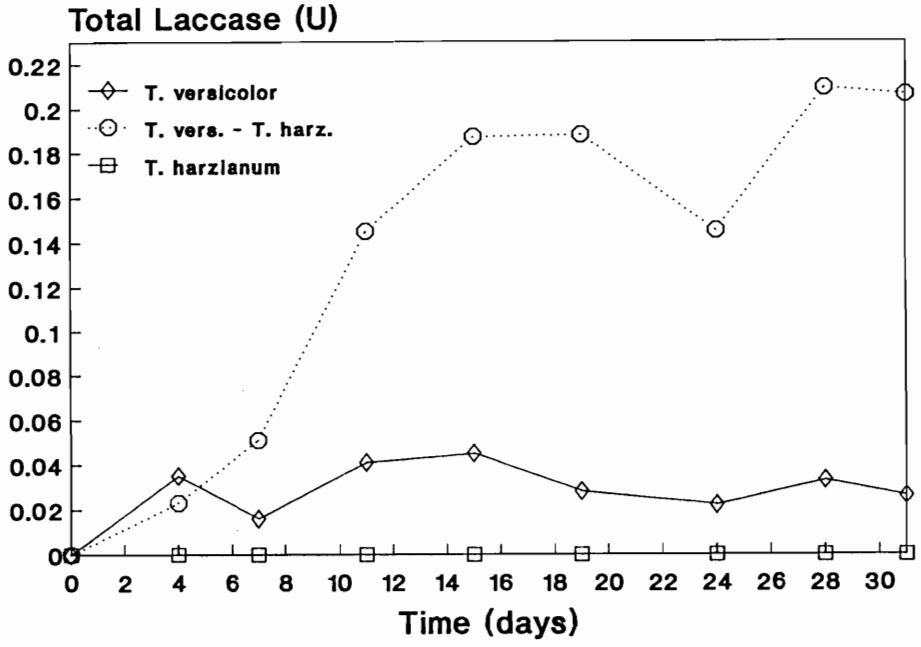
enzyme indicating the viability of a laccase producing wood decay fungus, such as T. versicolor, in a mixed inoculum with a potential biocontrol organisms. In this study, however, no strict correlation between laccase production and mycelial dry weights or total mycelial protein production was found (Figs. 4.10., 4.12. and 4.17.), contrary to earlier studies on solid media (Turner, 1974; Wood & Goodenough, 1977; Wood, 1980a). Laccase activities in A. brunnescens, peak just before sporulation, in conjunction with the appearance of primordia and fruiting bodies (Turner, 1974; Wood & Goodenough, 1977; Smith et al., 1989). Once sporulation starts, laccase production decreases 10 to 20 fold (Wood & Goodenough, 1977). No causal relationship between these two events has been confirmed, but both laccase inactivation and enhanced proteolysis are responsible for the decline in laccase activity (Wood, 1980b; 1985). Turner et al. (1975) found high levels of ethylene, which is thought to inhibit laccase activities (Haars et al., 1981), between 2 and 3 weeks of incubation, and again after sporulation of the mature crop, coinciding with very low cellulase and medium high laccase levels. Similar patterns of laccase release were found in S. commune: laccase activity diminished when mature fruiting bodies were formed, but continued to rise if fruiting body formation was exogenously inhibited (Leonard, 1971; Phillips & Leonard, 1976). Laccase activities in both A. brunnescens and S. commune were correlated with biomass production expressed as mycelial dry weight. In a mixed culturing system containing laccase-producing and non-producing fungal species, laccase measurements may thus replace dry weight determinations as one reliable method for estimating biomass if laccase production and regulation are not affected by the presence of the non-producing species. In this study, however, no direct correlation between laccase activity and biomass production was observed, possibly due to culture conditions and a different, species-specific regulation of laccase in T. versicolor.

Under nitrogen limiting conditions (0.4 mM), laccase activities were only 10 or 20 percent of those measured in pure T. versicolor or mixed high (4 mM) N cultures, respectively. However, laccase induction

studies using ferulic acid and veratryl alcohol showed that raising the nitrogen level to 14 mM nitrogen inhibited laccase production in pure cultures of T. versicolor (unpublished results). Darbyshire et al. (1969) also found higher laccase activities in very low N media, whilst Grabbe et al. (1968) found that high (2.5 g/l) levels of L-asparagine coupled with high levels of glucose (10 g/l) led to faster ammonia production and reduced laccase activities in T. versicolor.

Laccase activity curves are frequently double-peaked (Fåhraeus, 1952; Haars & Hüttermann, 1980; Kharazipour, 1983). This bimodal pattern, explained by a shift from primary to secondary metabolism or by the presence of inducers (Taylor et al., 1986a+b), occurred in both low and high N cultures (Fig. 4.17.) and was even more pronounced when specific activities were calculated (Fig. 4.18.). The first peak of laccase activity occurred after 12 days and 16 - 18 days in high and low N mixed cultures, respectively, compared to a peak in high N pure cultures of T. versicolor after 18 days. Laccase activities in low N pure cultures was relatively stable and very low over the incubation period. These results correspond with previous reports of laccase production before or after induction by phenolic compounds in Neurospora crassa Shear et Dodge (Froehner & Eriksson, 1974), H. annosum (Haars & Hüttermann, 1980; 1983; Haars et al., 1981; Hüttermann et al., 1980), Pleurotus ostreatus (Jacq.:F.) Kumm. (Hiroi & Eriksson, 1976; Kharazipour, 1983) and L. edodes (Leatham and Stahmann, 1981; Leatham, 1985a+b; Schmidt & Kebernik, 1987), but differs from the single peak pattern obtained in S. commune (Leonard, 1971; Leonhard & Phillips, 1973; Phillips & Leonhard, 1976a+b), and A. brunnescens (Dijkstra et al., 1972; Turner, 1974; Turner et al., 1975; Wood & Goodenough, 1977; Wood, 1980 a+b; Smith et al., 1989). Laccase activity in liquid cultures of P. ostreatus and H. annosum is usually greatest between 16 - 40 days, depending on conditions (Kharazipour, 1983). The highest laccase activity with both S. commune and A. brunnescens was recorded as a single peak late in the incubation period (30+ days), both on solid and liquid medium (Leonard, 1971; Dijkstra et al., 1972; Wood & Goodenough, 1977; Wood, 1985). These differences among the studied fungi may

(a)



(b)

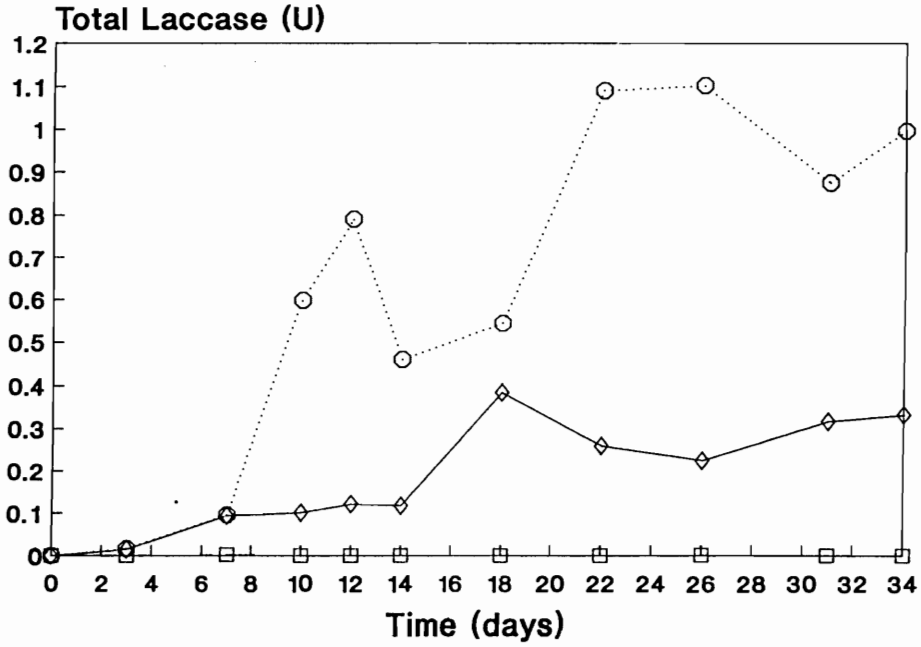


Figure 4.17.: Total laccase activity in pure and mixed cultures of T. versicolor and T. harzianum grown in liquid media containing (a) low (0.4mM) and (b) high (4mM) nitrogen concentrations.

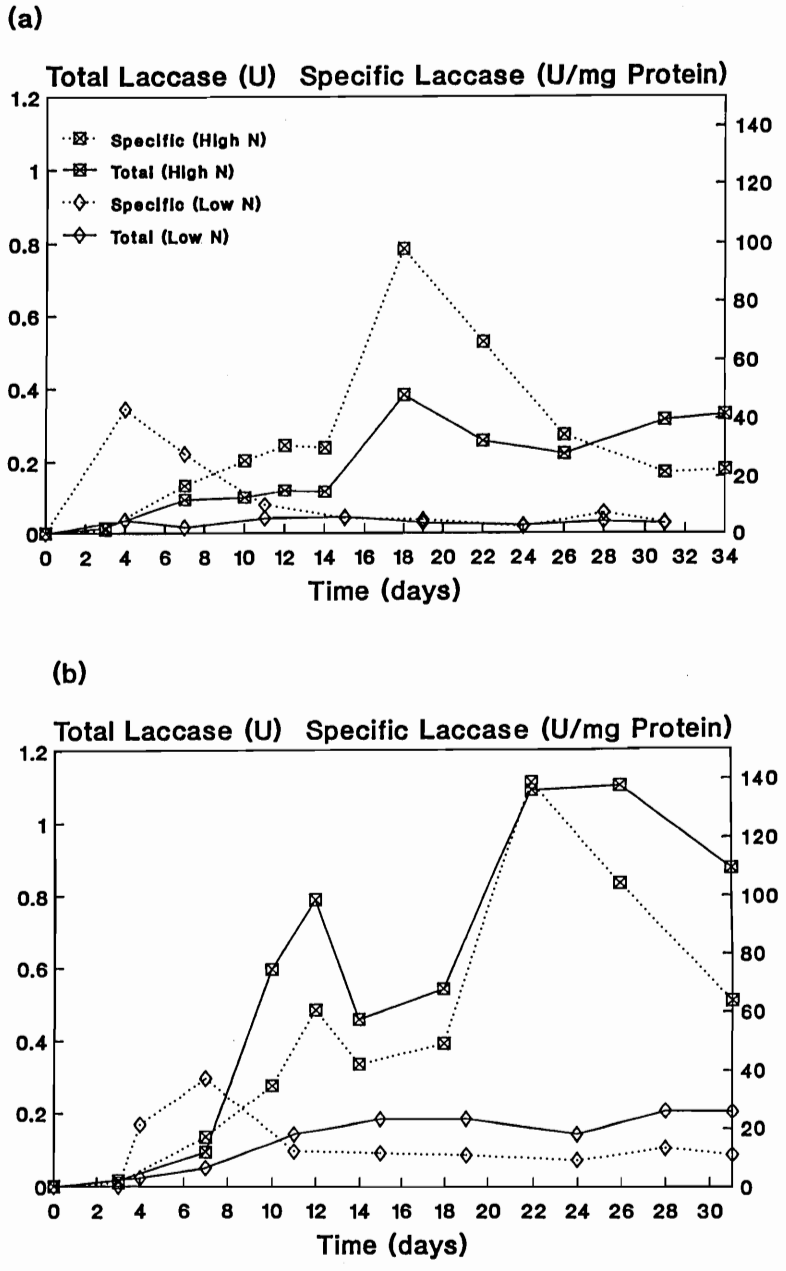


Figure 4.18.: Comparison of total and specific laccase activities of (a) T. versicolor and (b) mixed cultures of T. versicolor and T. harzianum grown in liquid media containing low (0.4 mM) and high (4 mM) nitrogen concentrations.

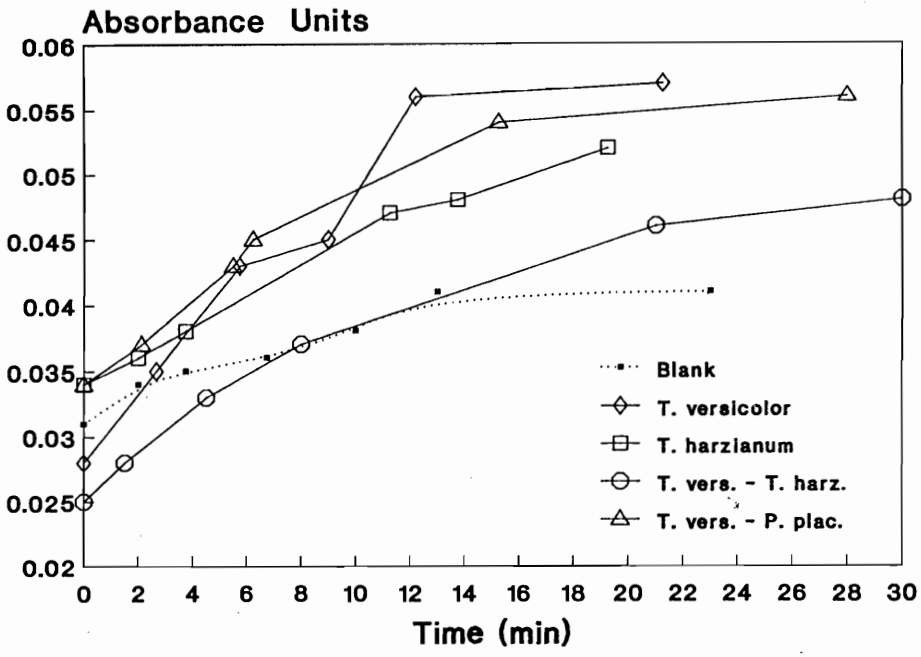
reflect differences in laccase regulation or culture conditions. The reason for laccase inactivation during sporulation of A. brunnescens is unknown (Wood, 1985).

Both specific and total extracellular laccase activity were 1.5- to 2-times higher in mixed cultures of T. versicolor and T. harzianum when compared to pure cultures of T. versicolor (Fig. 4.17. and 4.18.). Similar influences on the phenoloxidase activity of H. annosum and L. edodes have been reported (Ishikawa et al., 1980; Lundborg, 1988b), but only a marginal combined influence of resident soil fungi on laccase activities was observed in non-sterile commercial mushroom beds infested with various soil fungi (Wood, 1985). The induction pattern associated with the presence of T. harzianum in cultures of T. versicolor parallels direct laccase induction in fungi in the presence of aromatic compounds and lignins (Hüttermann et al., 1980; Ishikawa et al., 1980; Haars et al., 1981; Taylor et al., 1986a+b), or indirect induction via limited inhibition of protein synthesis (Froehner & Eriksson, 1974).

Since decreased protein production was observed, and thus protein synthesis in T. versicolor was possibly inhibited, laccase might have been derepressed, associated with a switch from primary to secondary metabolism. This pattern is typical of N. crassa in which laccases are inducer regulated and are not produced during rapid growth (Froehner & Eriksson, 1974), but differs from constitutive laccase production found in most white-rot fungi (Wood, 1980a).

Specific laccase activity exhibited almost the same pattern as total laccase activtiy, particularly in low N media (Fig. 4.18.), indicating that, regardless of the presence of another organism, approximately the same fraction of protein is secreted as laccase. Since filtrate protein levels increased in mixed cultures at levels which could not be completely accounted for with the activities of the enzymes monitored, the release of other enzymes or protein- or glycoprotein-containing secondary metabolites by T. versicolor may have occurred. Whilst total mycelial protein levels were somewhat lower in mixed cultures, the increase in filtrate protein levels would suggest that partial inhibition of protein synthesis, as shown in N. crassa, was not

(a)



(b)

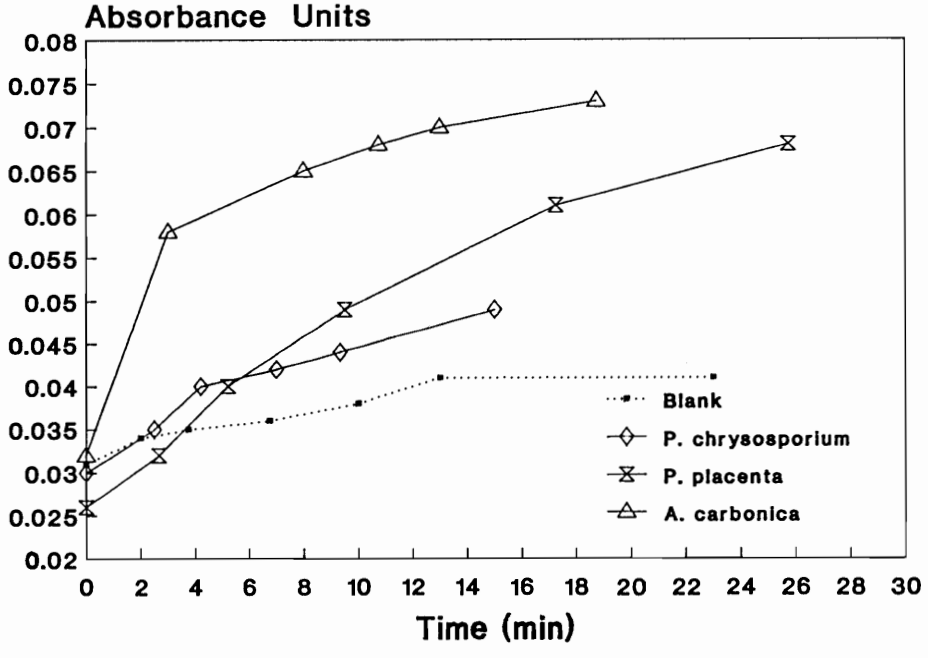


Figure 4.19.: Change in absorbance (at 436 nm) measured during the oxidation of ABTS by peroxidases of (a) pure and mixed cultures of T. versicolor and T. harzianum or T. versicolor and P. placenta and (b) pure cultures of P. placenta, A. carbonica or P. chrysosporium.

Table 4.2.: ABTS peroxidase activity (mU)[1] of pure and mixed cultures of *T. versicolor*, *T. harzianum* or *P. placenta* after 3, 6, and 9 days grown in liquid media containing low (0.4 mM) and high (4 mM) nitrogen concentrations.

Fungi tested	Nitrogen Level (mM)	Incubation Period (days)		
		3	6	9
T. versicolor	0.4	4.90[1]	3.40	1.00
	4.0	1.40	4.80	3.80
T. harzianum	0.4	1.35	1.07	0.46
	4.0	1.90	2.30	2.00
P. placenta	0.4	1.11	1.30	1.16
	4.0	0.87	1.50	1.10
T. versicolor vs. *T. harzianum*	0.4	0.13	1.80	1.30
	4.0	-[2]	0.93	0.86
T. versicolor vs. *P. placenta*	0.4	0.93	1.80	1.23
	4.0	-	1.90	1.10
P. placenta vs. *T. placenta*	0.4	0.83	0.95	0.71
	4.0	-	0.88	0.65

1 = mU = (change in Absorbance / min)*1000
2 = - = not determined

responsible for laccase induction. Aromatic metabolites, more specifically phenols produced by either fungus, may have induced laccase activity. Laccase reportedly has a detoxifying role in phenol metabolism of white-rot fungi (Grabbe et al., 1968; Haars et al., 1981), and might provide a possible defense mechanism against other fungi present in the same culture.

4.3.2.4. ABTS Peroxidase

The ability of cell-free culture filtrates of T. versicolor, T. harzianum, mixed cultures of T. versicolor and T. harzianum or T. versicolor and P. placenta, as well as P. chrysopsorium, P. placenta and Antrodia carbonica Gilbn. & Ryv., to oxidize 2,2′-azino-bis-[3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) was tested. More fungal species were added for comparative purposes. Oxidation of ABTS by P. chrysosporium was comparable to the capability of T. versicolor and T. harzianum to oxidize this compound (Fig. 4.19.a.). The activity of the peroxidase from P. chrysosporium was substantially lower than found under optimized conditions and after partial purification for the same original strain used elsewhere (Gold & Glenn, 1988). Differences in results may reflect the influence of the specific test conditions (Glenn & Gold, 1985; Mustranta, 1987; Gold & Glenn, 1988).

Oxidation of ABTS by brown rot fungi has not been reported previously. Postia placenta was as active as the white-rot fungi, whilst A. carbonica was the most active fungus tested (Fig. 4.19.b.). That ABTS was oxidized by both brown rot fungi and T. harzianum under the chosen conditions suggests that the assay has limited utility for detection of specific peroxidases, the peroxidases which are thought to mediate lignin decomposition are non-specific or the same peroxidases are produced by white- and brown-rot fungi. Reaction schemes for the catalytic cycle of lignin and manganese peroxidase have been proposed (Fig. 4.20.; Gold & Glenn, 1988; 1989). The importance of adding

exogenous Mn^{2+}, α-hydroxy acids, such as lactate, and H_2O_2 for optimum conditions of catalysis has been repeatedly stressed (Kuwahara et al., 1984; Glenn & Gold, 1985; Pasczynski et al., 1986; Gold et al., 1989). The Mn^{2+}/Mn^{3+} pair may act as a freely diffusible redox couple, able to oxidize lignin within the lignocellulose matrix. This model is analogous to the Fe^{2+}/H_2O_2 system possibly involved in cellulose degradation by brown-rot fungi (Highley, 1987a). Lactate can form stabilized redox complexes by chelating Mn^{3+} (Gold et al., 1989). In this study, however, no Mn^{2+} or lactate was added and all test fungi showed at least some activity towards ABTS. The results suggest that ABTS is either a broad-spectrum substrate for other enzymes present or that endogenous amounts of Mn^{2+}, added with the culture medium, and α-hydroxy acids were sufficient to mediate some oxidation. Another possibility is the reduction of enzyme intermediate I (Fig. 4.20.b.) by phenolic compounds. Phenolics are known substrates for manganese peroxidase but are oxidized at a substantially lower rate (Gold et al., 1989). Moreover, manganese peroxidase cannot complete its catalytic cycle if reduced by phenolics from compound I to compound II, since phenolics are not able to reduce compound II back to the native enzyme state (Fig. 4.20.b.; Gold et al., 1989). More detailed studies, particularly with brown rot fungi are needed to elucidate the specificity of the proposed lignin-degrading peroxidases.

Recently, Enoki et al. (1989) reported the production of H_2O_2-producing or one-electron oxidases by brown rot fungi and suggested a preliminary scheme for cellulose and lignin degradation by brown rot fungi. These activities were measured using ethylene development from 2-keto-4-thiomethylbutyric acid (KTBA), an assay considered relatively non-specific for lignin degradation and dependent on the metabolism of the organisms studied (Gold et al., 1983; Kelley, 1988). Earlier reports of peroxidases in brown-rot fungi relate mostly to intracellular enzymes (Koenigs, 1970). Non-specific intra- and extracellular peroxidases were reported from I. versicolor (Schánel et al., 1971) and P. weirii (Koenigs, 1972), but recently Dodson et al. (1987) found extracellular peroxidases of I. versicolor which cleaved C_α-C_β linked

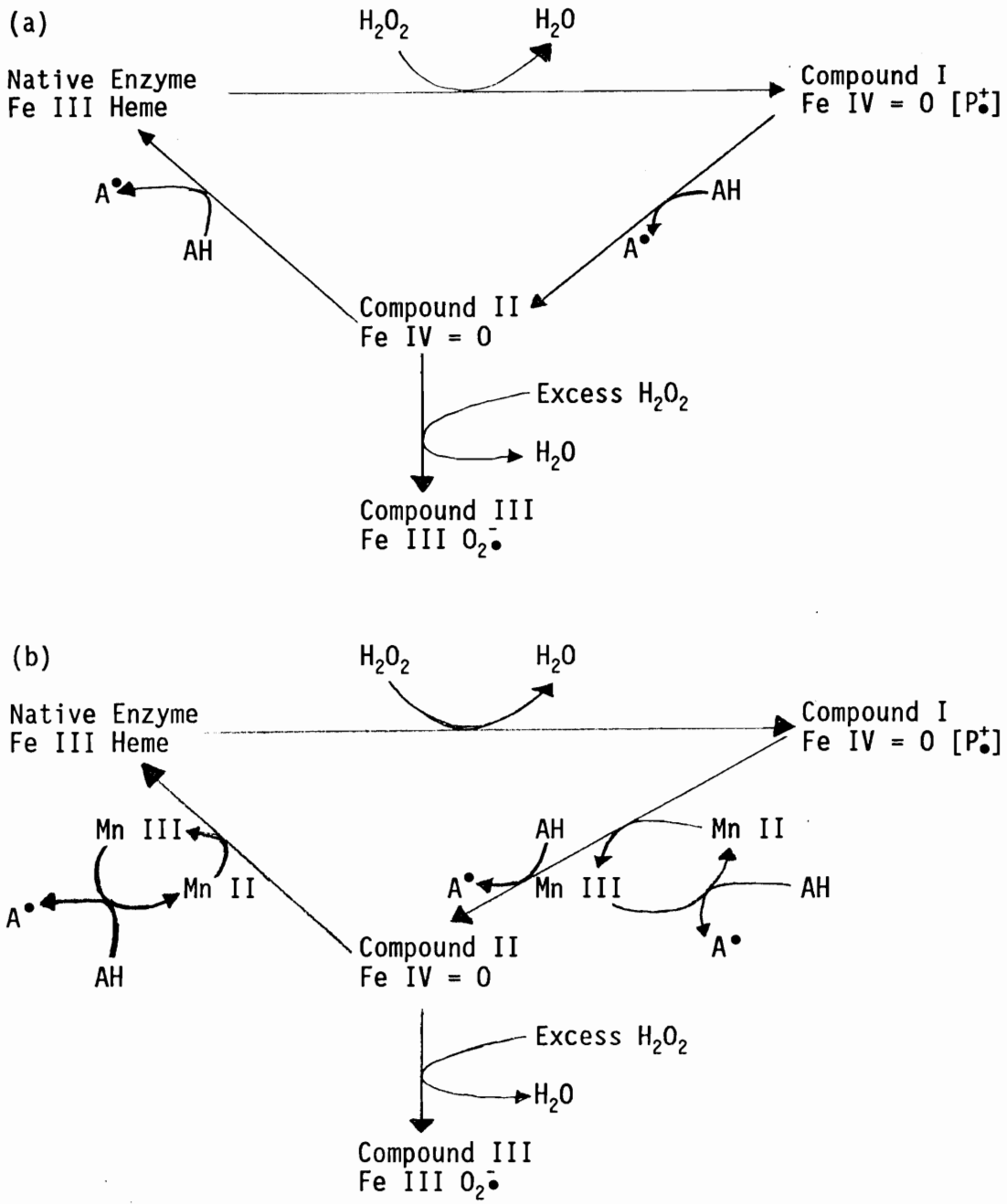


Figure 4.20.: Catalytic cycle of (a) lignin and (b) manganese peroxidase (after Gold et al., 1989) involving two single electron steps: manganese peroxidase heme is oxidized by H_2O_2 forming intermediate compound I. This is reduced by one equivalent Mn^{2+} to compound II, whilst the porphyrin radical [$P_\bullet$] is reduced back to a normal porphyrin. A second equivalent of Mn^{2+} reduces compound II back to the native heme.

lignin model compounds. ABTS oxidation by horseradish peroxidase was used to detect bacterial (Müller, 1984) and fungal H_2O_2 production (Highley, 1987b) by alcohol oxidase (Farmer et al., 1960; Highley & Murmanis, 1985) or glucose oxidase (Kuwahara et al., 1984), but neither study investigated the oxidation of ABTS by fungal peroxidases. Mustranta (1987) found peroxidases in Phellinus weirii (Murr.) Gilbn. which acted similarly to Mn^{2+}-peroxidases from P. chrysosporium (Leisola et al., 1985; 1987; Gold et al., 1989), but did not require Mn^{2+} or lactate. These results suggest that ABTS is indeed not an highly specific substrate or that specific requirements of fungal peroxidases with respect to metal coenzymes or presence of short-chain carboxylic acid may vary widely.

The activity of ABTS peroxidase in mixed cultures was lower for all tested fungal combinations when compared to values obtained with pure cultures of T. versicolor and P. placenta, regardless of the nitrogen concentration (Tab. 4.2.). The initial high activity measured in T. versicolor after only 3 days was surprising and remains unexplained. In studies with P. chrysosporium under optimized conditions, peaks were obtained after 7 - 9 days of incubation and activities reached maxima in P. weirii after only 12 days (Glenn & Gold, 1985; Gold & Glenn, 1988; Mustranta, 1987).

4.3.2.5. Poly R-478 Peroxidase

Oxidation of selected dyes, such as Phenol Red or polymeric dyes, may provide information about the oxidative ligninolytic capabilities of fungal culture liquids and their cell-free filtrates (Gold et al., 1983; Glenn & Gold, 1983). Dye decolorization and degradation assays have served as inexpensive and rapid methods for assaying enzyme or lignin-degrading activities (Glenn & Gold, 1983; Chet et al., 1985; Platt et al., 1985; Glenn & Gold, 1985; Gold et al., 1988; Nishida et al., 1988).

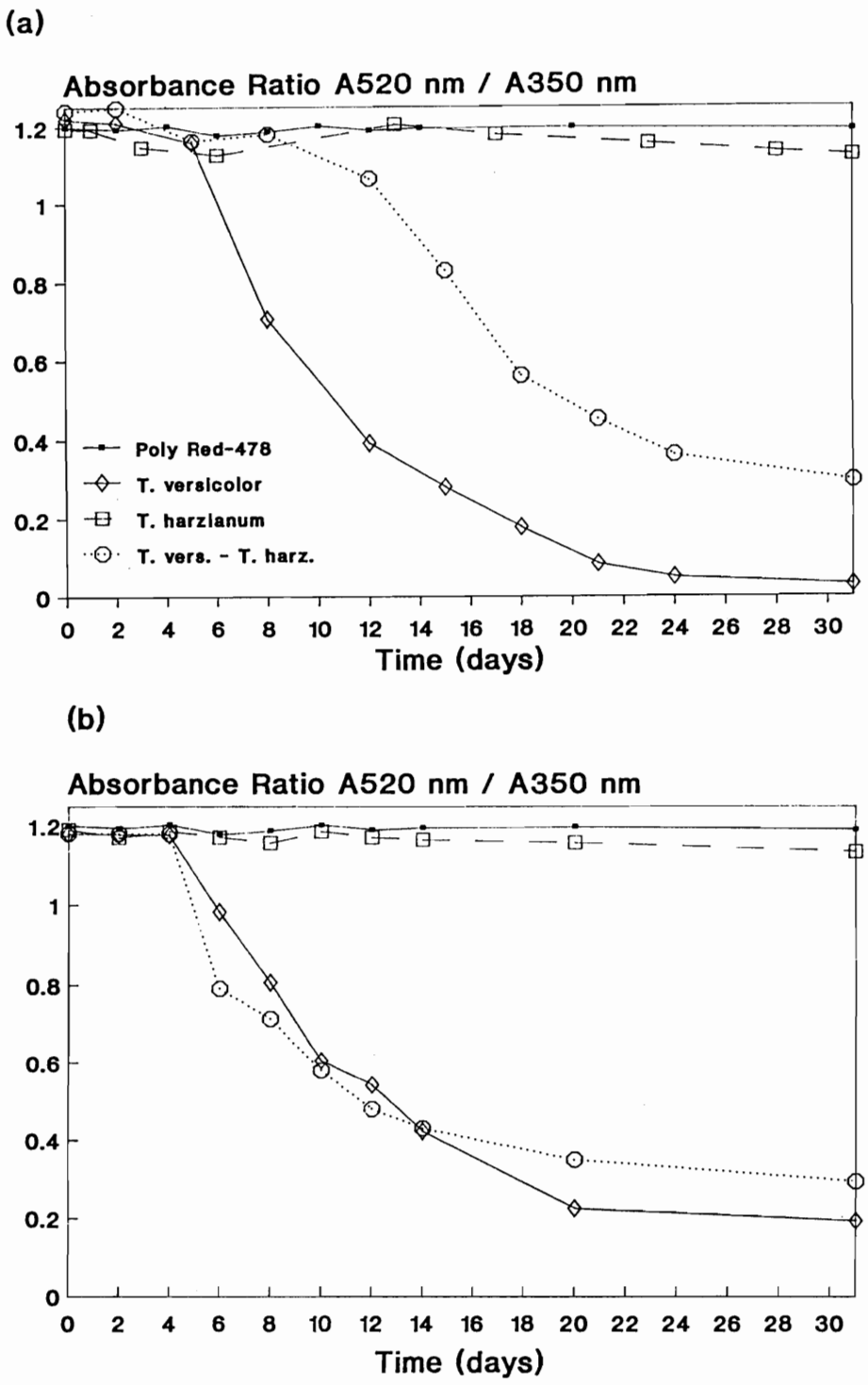


Figure 4.21.: Changes in the absorbance ratio A520 nm / A 350 nm of pure and mixed cultures of T. versicolor and T. harzianum grown in liquid media containing (a) low (0.4 mM) and (b) high (4 mM) nitrogen concentrations.

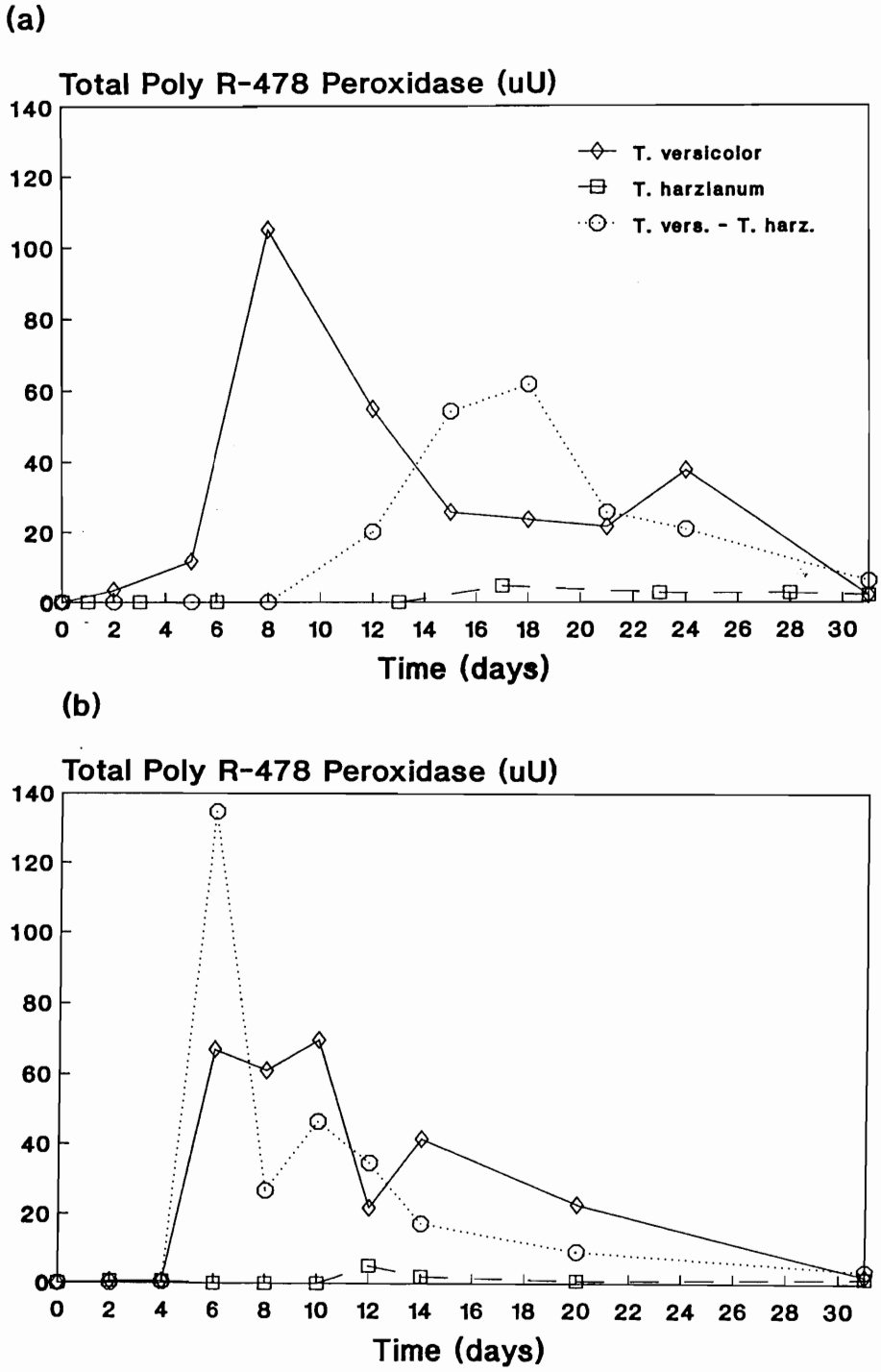


Figure 4.22.: Total Poly R-478 peroxidase activities of pure and mixed cultures of T. versicolor and T. harzianum grown in liquid media containing (a) low (0.4 mM) and (b) high (4 mM) nitrogen concentrations.

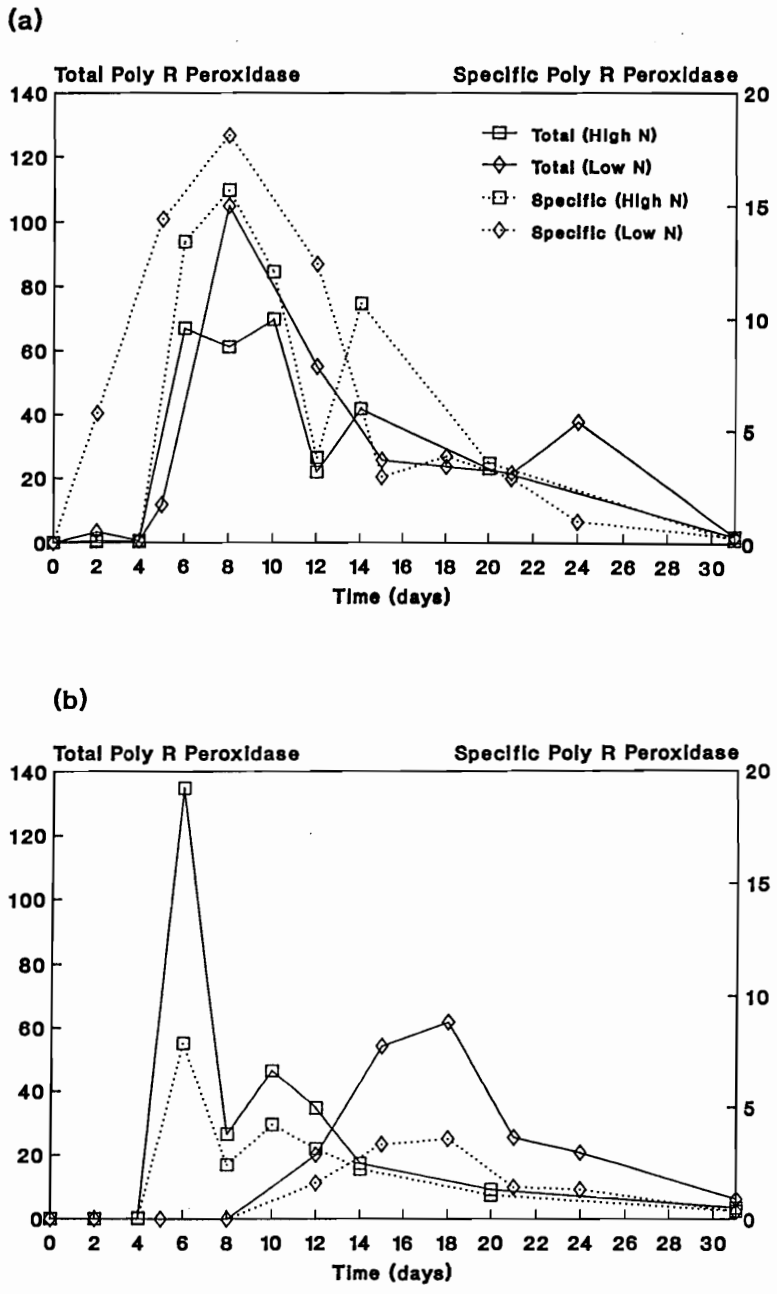


Figure 4.23.: Comparison of total (mU) and specific Poly R-478 peroxidase activities (mU/mg protein) of (a) T. versicolor and (b) mixed cultures of T. versicolor and T. harzianum grown in liquid media containing low (0.4 mM) and high (4 mM) nitrogen concentrations.

The decolorization of culture medium by pure and mixed cultures of T. versicolor and T. harzianum was used as an additive measure of peroxidase activity which paralleled extracellular laccase activities (Fig. 4.21.). In low N media, T. versicolor decolorized the culture liquid after a lag-period of 5 days. Whilst T. harzianum showed no activity on either medium, mixed cultures were intermediate in their activity on low N medium and as efficient as pure cultures of T. versicolor on high N medium.

A subsequent screening of 42 white, brown and soft rot fungi on agar plates containing 0.2 % (w/v) Poly R-478 showed that some brown-rot fungi, especially members of the genus Antrodia and Coniophora puteana (Fr.) Karst., decolorized the dyed agar. Conversely, some strongly ligninolytic white-rot fungi, such as Phanerochaete sordida (Karst.) J.Eriks. et Ryv. and S. commune, failed to decolorize the agar (unpublished results). Platt et al. (1985) found that Rhizoctonia solani Kühn was able to decolorize the dye Poly Blue-411, but did not release ^{14}C from lignin model compounds. These results parallel results from our screening, indicating that decolorization of polymeric dyes may indicate the presence of peroxidases, but can not always be used as a direct measure of ligninolytic activity. There are, however, reports of direct correlation between dye decolorization and ligninolytic activity, measured either by degradation of Klason lignin (Nishida et al., 1988) or release of $^{14}CO_2$ from ^{14}C-labelled dehydroconiferyl alcohol (Chet et al., 1985; Trojanowski, 1989, pers. commun.).

Total Poly R-478 peroxidase activity (cf. 4.2.6.5.) peaked after 8 days in low and high N cultures of T. versicolor (Fig. 4.22.). Peaks in mixed cultures of T. versicolor and T. harzianum were obtained after 18 days and 6 days in low and high N medium, respectively. The high activity in mixed cultures on high N medium is quite puzzling, since earlier studies (Gold & Glenn, 1988; Gold et al., 1989) and results from pure cultures of T. versicolor (Fig. 4.22.a.) showed increased decolorization in low N medium. Very low activities were measured for T. harzianum, possibly due to experimental errors, since no decolorization was observed after adding dye to stationary cultures of this fungus. Small

differences were found between the two decolorization assays employed: values for the decolorization of Phenol Red were generally higher than those for the decolorization of Poly R-478, possibly due to the different structures. Also, differences in decolorization of the originally used dye Poly R-481 and Poly R-478 were small, allowing Poly R-478 to replace Poly R-481 in further studies (Glenn & Gold, 1983). Shaking of cultures with added dye resulted in initially faster decolorization, but did not affect total Poly R-478 peroxidase activities after 4 days of inoculation.

Specific Poly R-478 peroxidase activities generally paralled the total activities (Fig. 4.23.). Specific activity was higher in cultures of T. versicolor with low N medium, although the overall difference with regard to the nitrogen concentration was smaller than would have been expected from earlier reports (Glenn & Gold, 1983; Gold et al., 1983). Mixed cultures showed different patterns of activities when the nitrogen levels where varied: in low N medium the peak of activity was delayed by 10 - 12 days when compared to high nitrogen cultures (Fig. 4.23.b.). Both ABTS-peroxidase and Poly R-478 peroxidase exhibited this delayed or reduced activity in mixed low N medium, whereas laccase activity was enhanced. The need for enhanced nitrogen uptake for de novo synthesis of proteins, such as the two peroxidases, or nucleic acids (Leonowicz et al., 1972) may be inhibited by the presence of T. harzianum, whilst decreased nitrogen uptake might inhibit protein synthesis and indirectly stimulate laccase production (Froehner & Eriksson, 1974). More detailed studies are needed to elucidate the true nature of this effect.

4.3.4. Summary

Mycelial dry weight production, glucose depletion and pH in mixed cultures of T. versicolor and T. harzianum were all intermediate between the extremes exhibited by either fungus in pure cultures, whilst protein levels were slightly lower than in pure cultures. Overall secretion of

(a) Influence of Nitrogen on Enzyme Activity

- higher activity in high N medium:

↑ total cellulase
total cellobiase
specific and total laccase

- higher activity in low N medium:

↑ specific cellulase
specific cellobiase
peroxidases

(b) influence of T. harzianum on extracellular enzymes of T. versicolor

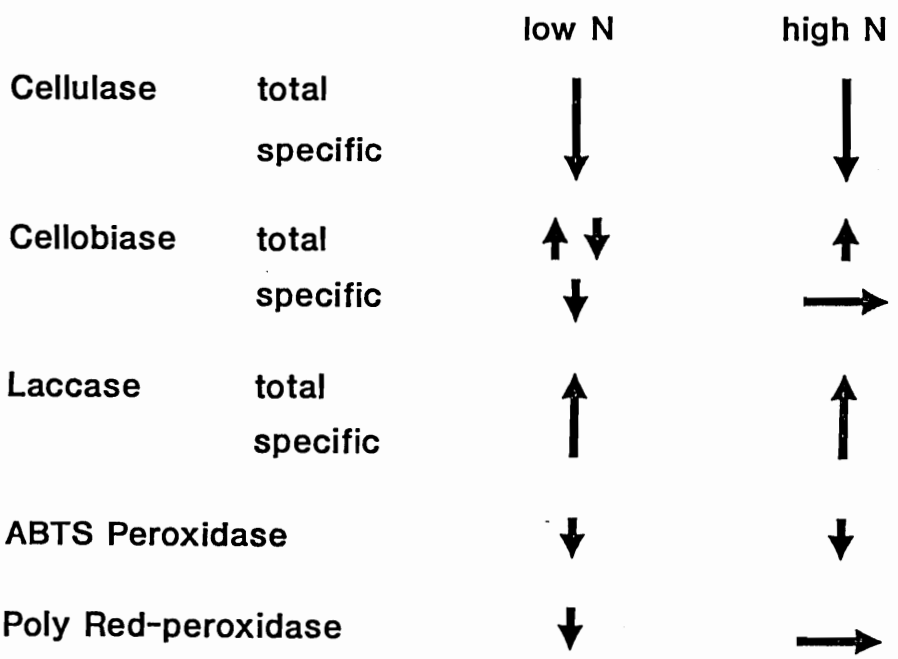


Figure 4.24.: Comparison of total and specific filter paper cellulase, laccase, ABTS peroxidase and Poly R-478 peroxidase activities in pure and mixed cultures of T. versicolor and T. harzianum containing low (0.4 mM) and high (4 mM) nitrogen concentrations.

proteins into the filtrate was enhanced in mixed cultures and was stable over a three week period on high N medium, whilst total protein production was lowered by the presence of a second fungus. This may have been due to increased protein synthesis and active release into the media or to increased cell wall lysis.

The system used in the experiments prevented separation of mycelia or cell-free filtrates of the two fungi in mixed cultures, making it impossible to determine which fungus was favored under the test conditions. Mycelial dry weight or total protein production in mixed cultures do not provide reliable indicators of the physiological state of either of the two fungi, whilst comparing glucose depletion and pH in mixed and pure cultures of T. harzianum suggested that this fungus was physiologically dominant. However, reisolation of both fungi from 34 day old liquid cultures onto agar plates (cf. 2.2.2.) indicated that T. harzianum was unable to kill T. versicolor.

One limitation of this study is that the sources of nitrogen used do not reflect availability, sources or distribution of nitrogen in wood. Since nitrogen is predominantly bound as proteins and glycoproteins in wood (Whitmore, 1982; Dill et al., 1984), the use of inorganic nitrogen sources, such as NH_4NO_3, and organic precursors, such as L-asparagine, in experiments on artificial media do not accurately reflect the actual usage of nitrogen compounds by wood decay fungi (Rayner & Boddy, 1988). The widespread use of C/N ratios to describe nitrogen supply may be especially misleading. C/N ratios not only disguise qualitative differences in nitrogen and carbon sources (i.e. ammonium vs. lignocellulose-bound glycoprotein or glucose vs. crystalline cellulose), they also provide no information concerning the spatial distribution of nitrogen and carbon in heterogenous media such as wood.

None of the enzyme activities monitored were directly correlated with biomass production under test conditions. Therefore, neither cellulases nor enzymes involved in lignin biodegradation can serve as an indicator for the interactive mechanisms in mixed liquid cultures of T. versicolor and T. harzianum. However, laccase and Poly R-478 peroxidase activities indicated survival of T. versicolor. Laccase and cellulase

activities can be monitored in compost soils (Smith et al., 1989) and it seems likely that methods will be developed to measure the activities of these enzymes in wood. Negative correlations between enzyme activities and biomass production must then be reevaluated.

Changes in extracellular enzyme activities in pure and mixed cultures of T. versicolor and T. harzianum containing low and high nitrogen concentration are summarized in Figure 4.24. No definite explanation of laccase induction in T. versicolor by T. harzianum can be given, due to the experimental system chosen in this study. Culturing of two organisms separated by thin semi-permeable membranes allowing mixing of culture liquid but separation of mycelia may serve as one method to more precisely control culture conditions. Labelling studies, involving carbon, nitrogen and oxygen sources are needed to elucidate the regulation of laccase induction in T. versicolor but these methods were beyond the scope of this investigation. Based on activity patterns obtained in this study, laccase induction associated with phenolic compounds in the culture medium is postulated.

The determination of ABTS- and manganese peroxidase yielded some unexpected results. The high levels of peroxidases measured in A. carbonica and P. placenta were surprising, but may have been due to the culture conditions. Another explanation would be a lack of substrate specificity of the peroxidases present. It is also possible that brown-rot fungi secrete the same peroxidases as white-rot fungi. Research on this particular aspect of the study will be continued.

The decolorization of Poly R-478 can provide a measure of fungal viability and peroxidase activity, but may not necessarily correlated with lignin degradation. Poly R-478 peroxidase activity in preliminary screenings of 42 white- and brown-rot was correlated with fungal genera and might become a useful taxonomic tool, paralleling the well-known Bavendamm reaction of polyphenol oxidase producing fungi on agar supplemented with lactic or tannic acid.

5. General Conclusions and Outlook

The assessment of biological control potential of T. harzianum in this study yielded new information with respect to small size wood block tests. The wood-based screening methods proposed in this study are currently used to identify biocontrol organisms and test their chemical resistance and adaptibility to an integrated control strategy involving the prophylactic treatment of poles or possibly remedial treatments in concert with fumigants (Dawson-Andoh, 1989; pers. commun.).

Tests on agar plates have their place during the very early screening stages, but should be viewed cautiously as preliminary indicators for biocontrol potential. Large scale ecological decay development studies should be undertaken if biocontrol strategies for decay prevention on wood products are to rely on an understanding of successions and interspecific interactions between xylophilous organisms. Although this very basic knowledge is not necessary for identifying potential biocontrol organisms, it would possibly validate the currently employed concept of control, namely the attempt to control well adapted, physiologically versatile and potentially long-living wood decay fungi by more or less ruderal and opportunistic microfungi. Levels of control obtained by application of microfungi on wood products are sometimes astounding, but mostly short-lived. Results from long-term studies (Bruce et al., 1983-1989) support models which postulate a steady-state of "interference competition" (Wicklow, 1981), rather than the presence of efficient control mechanisms.

One of the objectives of this study was to assess the potential of wood decay enzymes to serve as indicators of nutritional states of wood decay fungi. Laccases and peroxidases may have potential in that role; at present, insufficient information about regulation and nutritional requirements of fungi for production of laccases and peroxidases in pure culture make it difficult to assess the influence exerted by other organisms. In mixed batch culture studies with T. reesei and A. wentii, Panda et al., (1989) found that the activities of cellulases and xylanases were dependent on the biomass concentrations of the respective

fungi and they developed a method to estimate biomass production by monitoring a species-specific pigment produced by A. wentii. This approach may also prove useful for Trichoderma spp., since some of the most commonly employed strains for biocontrol purposes produce green conidia. Correlating the production of pigments in conidia, growth rates, biomass production and physiological state of these strains may present one way to monitor these strains in mixed cultures. The study of the biochemistry of microbial interactions and regulation of enzyme activities of one organism by another will benefit from the development of new labelling methods. Since it is possible to specifically label, for example, primary metabolites, enzymes or lectins, more information concerning growth factors, inhibition and regulation during secondary metabolism can be obtained.

The experimental system chosen to evaluate influences of mixed culturing on extracellular enzyme production of T. versicolor was sufficient to describe overall changes in activities, but it was not possible to determine which factors or mechanisms were responsible for the observed changes. Culturing of fungi separated by semipermeable membranes or the use of mutant strains, unable to synthesize particular enzymes, may be approaches to mitigate these problems. Other shortcomings of the study are related to the concentration on only one decay fungus - biocontrol fungus system. More systems, however, may be evaluated and correlations between changes in enzyme activities and macroscopic or microscopic may be found. Particularly useful would be an evaluation of a pattern of change, using electrophoresis, rather than the description of changes in some enzyme activities. The localization of enzymes using immunolabelling or lectin-dye systems could, at the same time, provide insights into transfer mechanisms of enzymes and substrates from membranes and hyphae into extracellular spaces, which may be affected by the presence of a second organism.

6. Bibliography

Adler, E. 1977. Lignin chemistry - past, present and future. Wood Sci. Technol. **11**:169-218.

Agosin, E. and E. Odier. 1985. Solid-state fermentation, lignin degradation and resulting digestibility of wheat straw fermented by selected white-rot fungi. Appl. Microbiol. Biotechnol. **21**:397-403.

Ahmad, J.S. and R. Baker. 1987. Rhizosphere competence of benomyl-tolerant mutants of Trichoderma spp. Can. J. Microbiol. **34**:694-696.

Ainsworth, G.C. 1966. A general purpose classification for fungi. Bibl. Syst. Mycol. **1**:1-4.

Ainsworth, A.M. and A.D.M. Rayner. 1986. Responses of living hyphae associated with self and non-self fusions in the basidiomycete Phanerochaete velutina. J. Gen. Microbiol. **132**:191-201.

American Society for Testing and Materials (ASTM). 1989. Standard method of test for accelerated laboratory test of natural decay resistance of wood. ASTM Des. D 2017. Ann. ASTM Standards. Philadelphia. PA.

Ammer, U. 1964. Über den Zusammenhang zwischen Holzfeuchtigkeit und Holzzerstörung durch Pilze. Holz als Roh- und Werkstoff. **22(2)**:47-52.

Ander, P. and K.-E. Eriksson. 1976. The importance of phenol oxidase activity in lignin degradation by the white-rot fungus Sporotrichum pulverulentum. Arch. Microbiol. **109**:1-8.

Ander, P. and K.-E. Eriksson. 1977. Selective degradation of wood components by white-rot fungi. Physiol. Plant. **41**:239-248.

Ander, P. and K.-E. Eriksson. 1978. Lignin degradation and utilization by micro-organisms. Prog. Ind. Microbiol. **14**:1-58.

Andrews, J.H. 1985. Strategies for selecting antagonistic microorganisms from the phylloplane. p 31-44. In: Windels, C.E. and S.E. Lindow (eds). Biological Control on the Phylloplane. American Phytopathological Society. St.Paul, MN.

Andrews, J.H. and R.F. Harris. 1986. r- and K-selection and microbial ecology. Adv. Microb. Ecol. **9**:99-148.

Andrews, J.H. and D.I. Rouse. 1982. Plant pathogens and the theory of r- and K-selection. American Naturalist. **120**:283-296.

Atlas, R.M. and R. Bartha. 1987. Microbial Ecology. Benjamin-Cummings. Menlo Park, CA.

Aylmore, R.C. and N.K. Todd. 1984. A microculture chamber and improved method for combined light and electron microscopy of filamentous fungi. J. Microbiol. Meth. **2**:317-322.

Baker, K.F. 1987. Evolving concepts of biological control of plant pathogens. Ann. Rev. Phytopath. **25**:67-85.

Baker, K.F. and R.J. Cook. 1982. Biological Control of Plant Pathogens. The American Phytopathological Society. St.Paul. MN.

Baker, K.F. and W.C. Snyder (eds). 1965. Ecology of Soil-Borne Plant Pathogens. Prelude to Biological Control. Univ. Calif. Press. Berkeley, CA.

Baker, R. 1968. Mechanisms of Biological Control of Soil-Borne Pathogens. Ann. Rev. Phytopath. **6**:263-294.

Barnett, H.L. 1963. The nature of mycoparasitism by fungi. Ann. Rev. Microbiol. **17**:1-14.

Barnett, H.L. and F.L. Binder. 1973. The fungal host-parasite relationship. Ann. Rev. Phytopath. **11**:273-292.

Barron, G.L. 1982. Nematode-destroying fungi. p 533-552. In: Burns, R.G. and J.H. Slater (eds). Experimental Microbial Ecology. Blackwell Scientific Publ. London, UK.

Barton, R. 1961. Saprophytic activity of Phythium mamillatum in soils. II. Factors restricting P. mamillatum to pioneer colonization of substrates. Trans. Brit. Mycol. Soc. **44**:105-118.

Bates, W.K. and D.F. McAllister. 1974. Some methods for nonlinear regression using desk-top calculators with an application to the Lowry protein method. Anal. Biochem. **59**:190-199.

Beam, H.W. and J.J. Perry. 1974. Microbial degradation of cycloparaffinic hydrocarbons via cometabolism and commensalism. J. Gen. Microbiol. **82**:163-169.

Beddington, J.R., C.A. Free and J.H. Lawton. 1976. Concepts of stability and resilience in predator-prey models. J. Anim. Ecol. **45**:791-816.

Bega, R.V. 1978. Diseases of Pacific Coast Conifers. USDA Forest Service. Agric. Handb. 521.

Bell, W.H., J.M. Lang and R. Mitchell. 1974. Selective stimulation of marine bacteria by algal extracellular compounds. Limn. Oceanogr. **19**:833-839.

Benko, R. 1987. Antagonistic effect of some mycorrhizal fungi as biological control of blue-stain. Int. Res. Group Wood Preserv. IRG/WP/1314.

Benko, R. 1988. Bacteria as possible organisms for biological control of blue-stain. Int. Res. Group Wood Preserv. IRG/WP/1339.

Benko, R. 1989. Biological control of blue-stain on wood with Pseudomonas cepacia 6253. Laboratory and field test. Int. Res. Group Wood Preserv. IRG/WP/1380.

Bergman, O. and T. Nilsson. 1971. Studies on the outside storage of sawmill chips. Res. Notes R 71. Dept. Forest Prod. Royal Coll. Forest. Stockholm, S.

Bettucci, L., S. Lupo and S. Silva. 1988. Control of growth of wood-rotting fungi by non-volatile metabolites from Trichoderma spp. and Gliocladium virens. Cryptogamie, Mycol. **9**:157-165.

Bilai, V.I. 1963. Antibiotic producing microscopic fungi. Elsevier. Amsterdam, NL.

Björkman, E. 1947. On the development of decay in building timber injured by blue stain. Pappers Tidn. **11(B)**:49.

Bjurman, J. 1988. The importance of blue-stain attack for the colonization by wood-rotting fungi of wood not in contact with the ground. Int. Res. Group Wood Preserv. IRG/WP/1349.

Blaich, R. and K. Esser. 1975. Functions of enzymes in wood destroying fungi. II. Multiple forms of laccase in white rot fungi. Arch. Microbiol. **103**:271-277.

Blakeman, J.P. and N.J. Fokkema. 1982. Potential for biological control of plant diseases on the phylloplane. Ann. Rev. Phytopath. **20**:167-192.

Blanchette, R.A., A.R. Abad, R.L. Farrell and T.D. Leathers. 1989. Detection of lignin peroxidase and xylanase by immunocytochemical labeling in wood decayed by basidiomycetes. Appl. Env. Microbiol. **55**:1457-1465.

Bliss, D.E. 1951. The destruction of Armillaria mellea in citrus soils. Phytopath. **36**:302-318.

Boddy, L. and A.D.M. Rayner. 1983. Mycelial interactions, morphogenesis and ecology of Phlebia radiata and Phlebia rufa from oak. Trans. Brit. Mycol. Soc. **80**:437-448.

Bollag, J.-M. and A. Leonowicz. 1984. Comparative studies of extracellular fungal laccase. Appl. Env. Microbiol. **48**:849-854.

Bollag, J.-M., K.L. Shuttleworth and D.H. Anderson. 1988. Laccase-mediated detoxification of phenolic compounds. Appl. Env. Microbiol. **54**:3086-3091.

Boosalis, M.B. 1964. Hyperparasitism. Ann. Rev. Phytopath. **2**:363-376.

Boosalis, M.G. and R. Mankau. 1965. Parasitism and predation of microorganisms. p 374-389. In: Baker, K.F. and W.C. Snyder (eds). Ecology of Soil-Borne Plant Pathogens. Univ . Calif. Press. Berkeley, CA.

Bouchilloux, S., P. McMahill and H.S. Mason. 1963. The multiple forms of mushroom tyrosinase. Purification and molecular properties of the enzyme. J. Biol. Chem. **238**:1699-1707.

Bowers, W.S., H.C. Hoch, P.H. Evans and M. Katayama. 1986. Thallophytic allelopathy: isolation and identification of laetisaric acid. Science. **232**:105-106.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgramm quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248-254.

Breuil, C. and J.N. Saddler. 1985. Comparison of the 3,5-dinitrosalicylic acid and Nelson-Somogyi methods of assaying for reducing sugars and determining cellulase activity. Enzyme Microb. Technol. **7**:327-332.

Brewer, D., F.G. Mason and A. Taylor. 1987. The production of alamethicins by Trichoderma spp. Can. J. Microbiol. **33**:619-625.

Brian, P.W. 1951. Antibiotics produced by fungi. Bot. Rev. **17**:357-430.

Brian, P.W. 1957. The ecological significance of antibiotic production. p 168-188. In: Williams, R.E.O. and C.C. Spicer (eds). Microbial Ecology. Cambridge Univ. Press. London, UK.

Brian, P.W. 1960. Antagonistic and competitive mechanisms limiting survival and activity of fungi in soil. p 115-129. In: Parkinson, D. and J.S. Waid (eds). The Ecology of Soil Fungi. Liverpool Univ. Press. Liverpool, UK.

Brian, P.W. and H.G. Hemming. 1945. Gliotoxin, a fungistatic metabolic product of Trichoderma viride. Ann. Appl. Biol. **32**:214-220.

Brian, P.W., P.J. Curtis, H.G. Hemming and J.C. McGowan. 1946. The production of viridin by pigment-forming strains of Trichoderma viride. Ann. Appl. Biol. **33**:190-200.

Brooks, F.T. and H.A. Storey. 1922. Silver-leaf disease. IV. J. Pomology. **3**:117-141.

Brown, R.D., Jr. and L. Jurasek (eds). 1979. Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Hydrolysis. Advances in Chemistry. Vol. 181. American Chemical Society. Washington, DC.

Bruce, A. and B. King. 1983. Biological control of wood decay by Lentinus lepideus (Fr.) produced by Scytalidium and Trichoderma residues. Mat. Org. **18**:171-181.

Bruce, A. and B. King. 1986a. Biological control of decay in creosote treated distribution poles. I. Establishment of immunizing commensal fungi in poles. Mat. Org. **21**:1-13.

Bruce, A. and B. King. 1986b. Biological control of decay in creosote treated distribution poles. II. Control of decay in poles by immunizing commensal fungi. Mat. Org. **21**:165-169.

Bruce, A., W.J. Austin and B. King. 1984. Control of growth of Lentinus lepideus by volatiles from Trichoderma. Trans. Brit. Mycol. Soc. **82**:423-428.

Bruce, A., B. King, C. Bruce and G.M. Smith. 1984. Three dimensional computer representations of growth of microbial populations in wood. Int. Res. Group Wood Preserv. IRG/WP/1243.

Bruce, A., C. Johnstone and J.A.P. McVey. 1987. Susceptibility of Lentinus lepideus (Fr.:Fr.) Fr. to volatiles produced by Trichoderma spp. Int. Res. Group Wood Preserv. IRG/WP/1316.

Bruce, A. and T.L. Highley. 1989a. The effect of precolonization of blocks with common pole resident fungi on subsequent biological control of L. lepideus by Trichoderma spp. Int Res. Group Wood Preserv. IRG/WP/1387.

Bruce, A. and T.L. Highley. 1989b. Control of growth of wood decay basidiomycetes by soluble metabolites from Trichoderma spp. and other potentially antagonistic fungi. (in preparation)

Bruce, A, B. King and T.L. Highley. 1989a. Decay resistance of wood removed from poles treated with Trichoderma. Int. Res. Group Wood Preserv. IRG/WP/1386.

Bruce, A., A. Fairnington and B. King. 1989b. Biological control of decay in creosote treated distribution poles. III. Control of decay in poles by immunizing commensal fungi after extended incubation period. Mat. Org. (in press).

Bruehl, G.W. 1969. Factors affecting the persistence of fungi in soil." In: Cook, R.J. and R.D. Watson (eds). Nature of the Influence of Crop Residues on Fungus-Induced Root Diseases", Wash. Agric. Exp. Stn. Bull. No. 716.

Bruehl, G.W. (ed). 1975. Biology and Control of Soil-Borne Plant Pathogens. American Phytopathological Society. St. Paul, MN.

Bruehl, G.W. 1987. Antibiotics. p 135-143. In: Bruehl, G.W. (ed). Soilborne Plant Pathogens. MacMillan. New York, NY.

Bruehl, G.W., R.L. Millar and B. Cunfer. 1969. Significance of antibiotic production by Cephalosporium gramineum to its saprophytic survival. Can. J. Plant Sci. **49:**235-246.

Bultman, J.D., R.L. Gilbertson, T.L. Amburgey, J.E. Adaskaveg and C.A. Baisley. 1987. The anti-fungal efficacy of guayule resin. Int. Res. Group Wood Preserv. IRG/WP/3429.

Burdsall, H.H., H.C. Hoch, M.G. Boosalis and E.C. Setliff. 1980. Laetisaria arvalis (Aphyllophorales, Corticiaceae): a possible biological control agent for Rhizoctonia solani and Pythium species. Mycologia. **72:**729-736.

Burge, M.N. (ed). 1988. Fungi in Biological Control Systems. Manchester Univ. Press. Manchester, UK.

Burges, A. 1958. Microorganisms in the Soil. Hutchinson and Co. Ltd. London, UK.

Buswell, J. and E. Odier. 1987. Lignin biodegradation. CRC Crit. Rev. Biotechnol. **6:**1-60.

Butcher, J.A. and J. Drysdale. 1974. Effect of nitrogen source and carbon:nitrogen ratio on cellulase activity and decay capability of certain soft-rot fungi. Mat. Org. **9:**255-268.

Butler, E.E. 1957. Rhizoctonia solani as a parasite of fungi. Mycologia. **49:**354-373.

Cappenberg, T.E. 1975. A study of mixed continuous cultures of sulfate reducing and methane producing bacteria. Micob. Ecol. **2:**60-72.

Carey, J.K. 1988. Some observations on miniaturized soil/block tests. Int. Res. Group Wood Preserv. IRG/WP/2317.

Carter, H.P. and J.L. Lockwood. 1957. Lysis of fungi by soil microorganisms and fungicides including antibiotics. Phytopath. **47:**154-158.

Carter, M.V. and T.V. Price. 1975. Biological control of Eutypa armeniacae. III. A comparison of chemical, biological, and integrated control. Australian J. Agric. Res. **26:**537-543.

Cease, K.R., R.A. Blanchette and T.L. Highley. 1989. Interactions between scytalidium species and brown- or white-rot basidiomycetes in birch wood. Wood Sci. Technol. **23:**151-161.

Cerrato, R.P., R.E. DeLaCruz and D.H. Hubbel. 1976. Further studies on a mycoparasitic basidiomycete species. Appl. Env. Microbiol. **31:**60-62.

Chapela, I.H., L. Boddy and A.D.M. Rayner. 1988. Structure and development of fungal communities in beech logs four and a half years after felling. FEMS Microbiol. Ecol. **53:**59-70.

Chet, I., G.E. Harman and R. Baker. 1981. Trichoderma hamatum: Its hyphal interactions with Rhizoctonia solani and Pythium spp. Microbiol. Ecol. **7:**29-38.

Chet, I., J. Trojanowski and A. Hüttermann. 1985. Decolorization of the dye Poly B-411 and its correlation with lignin degradation by fungi. Microbiol. Letters. **29:**37-43.

Chi, C.C. 1960. Effects of Streptomyces and Trichoderma on Fusarium. Phytopath. **50:**631.

Clark, F.E. 1965. The concept of competition in microbial ecology. p 339-345. In: Baker, K.F. and W.C. Snyder (eds). Ecology of Soil-Borne Plant Pathogens. Univ. Calif. Press. Berkeley, CA.

Claydon, N., M. Allan, J.R. Hanson and A.G. Avent. 1987. Antifungal alkyl pyrones of Trichoderma harzianum. Trans. Brit. Mycol. Soc. **88:**503-513.

Claydon, N., M. Allan and D.A. Wood. 1988. Fruit body mass regulated production of extracellular endocellulase during periodic fruiting by Agaricus bisporus. Trans. Brit. Mycol. Soc. **90:**85-90.

Coakley, W.T. and C.J. James. 1978. A simple linear transform for the Folin-Lowry protein calibration curve to 1.0 mg/ml. Anal. Biochem. **85:**90-97.

Collings, A., B. Davis and J. Mills. 1988. Factors affecting protoplast release from some mesophilic, thermophilic and thermotolerant species of filamentous fungi using Novozym 234. Microbios. **53:**197-210.

Collins, R.P. and A.F. Halim. 1972. Characterization of the major aroma constituent of the fungus Trichoderma viride (Pers.). J. Agr. Food Sci. **20:**437-438.

Cook, R.J. and K.F. Baker. 1983. The Nature and Practice of Biological Control of Plant Pathogens. American Phytopathological Society. St.Paul. MN.

Cook, R.J. and W.C. Snyder. 1965. Influence of host exudates on growth and survival of germlings of Fusarium solani f. phaseoli in soil. Phytopath. **55:**1021-1025.

Cooke, R.C. and A.D.M. Rayner. 1984. The Ecology of Saprophytic Fungi. Longman. London, UK.

Corke, A.T.K. 1974. The prospect for biotherapy in trees infected by silver leaf. J. Horticult. Sci. **49:**391-394.

Cowling, E.B. 1958. A review of literature on the enzymatic degradation of cellulose and wood. USDA For. Serv. For. Prod Lab. Rep. 2116.

Cowling, E.B. 1961. Comparative biochemistry of the decay of sweetgum sapwood by white-rot and brown-rot fungi. USDA For. Serv. Tech. Bull. 1258.

Cowling, E.B. and W. Merrill. 1966. Nitrogen in wood and its role in wood deterioration. Can. J. Bot. **44:**1539-1554.

Crawford, R.L. 1981. Lignin Biodegradation and Transformation. John Wiley & Sons. New York, NY.

Culver, D.C. 1981. Introduction to the theory of species interactions. p 281-294. In: Wicklow, D.T. and G.C. Carroll (eds). The Fungal Community. Dekker. New York, NY.

D'Aeth, H.R.X. 1939. A survey of interactions between fungi. Biol. Rev. 14:105-131.

Daniel, G.F., T. Nilsson and B. Petterson. 1988. Immunolabelling studies on the detection of enzymes during the degradation of wood by Phanerochaete chrysosporium. Int. Res. Group Wood Preserv. IRG/WP/1364.

Daniel, G.F., T. Nilsson and B. Pettersson. 1989a. Intra- and extracellular localization of lignin peroxidase during the degradation of solid wood and wood fragments by Phanerochaete chrysosporium by using transmission electron microscopy and immuno-gold labeling. Appl. Env. Microbiol. **55:**871-881.

Daniel, G., B. Pettersson, T. Nilsson and J. Volc. 1989b. Cytoplasmic and extracellular localization of manganese II dependent peroxidase(s) in white-rot fungi during degradation of woody materials. Int. Res. Group Wood Preserv. IRG/WP/1416.

Danielson, R.M. and C.B. Davey. 1973a. The abundance of Trichoderma propagules and the distribution of species in forest soils. Soil Biol. Biochem. **5:**485-494.

Danielson, R.M. and C.B. Davey. 1973b. Non nutritional factors affecting the growth of Trichoderma in culture. Soil Biol. Biochem. **5:**495-504.

Danielson, R.M. and C.B. Davey. 1973c. Carbon and nitrogen nutrition of Trichoderma. Soil Biol. Biochem. **5:**505-515.

Darbyshire, B., G.C. Wade and K.C. Marshall. 1969. In vitro studies of the role of nitrogen and sugars on the susceptibility of apple wood to decay by Trametes versicolor. Phytopath. **59:**98-102.

Davidson, R.W., W.A. Campbell and D.B. Vaughn. 1942. Fungi causing decay of living oaks in the eastern United States and their cultural identification. USDA Tech. Bull. 785.

Dawson, D.J. 1981. Polymeric dyes. Aldrichim. Acta. **14(2):**23-29.

Dawson-Andoh, B. 1989. personal communication.

DeBach, P. (ed). 1964. Biological Control of Insect Pests and Weeds. Reinhold. New York, NY.

DeFreitas, A.R. and H.D. Erickson. 1969. Propagation of molds on red alder roundwood - their effect on oil soak treatments. For. Prod. J. 19(10):45-52.

Dekker, J. 1963. Antibiotics in the control of plant disease. Ann. Rev. Microbiol. **17:**243-262.

Dekker, R.F.H. 1986. Kinetic, inhibition, and stability properties of a commercial ß-D-glucosidase (cellobiase) preparation from Aspergillus niger and its suitability in the hydrolysis of lignocellulose. Biotechnol. Bioeng. **28:**1438-1442.

Demain, A. 1972. Cellular and environmental factors affecting the synthesis and excretion of metabolites. J. Appl. Chem. Biotechnol. **22:**345-362.

Demeester, J.M. Bracke, and A. Lauwers. 1979. Absolute viscosimetric method for the determination of endocellulase (C_x) activities based upon light-scattering interpretations of gel chromatographic fractionation data. p 91-125. In: Brown, R.D. and L. Jurasek (ed). Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis. Advances in Chemistry. Vol. 181. American Chemical Society. Washington, DC.

Dennis, C. and J. Webster. 1971a. Antagonistic properties of species-groups of Trichoderma. I. Production of non-volatile antibiotics. Trans. Brit. Mycol. Soc. **57:**25-39.

Dennis, C. and J. Webster. 1971b. Antagonistic properties of species--groups of Trichoderma. II. Production of volatile antibiotics. Trans. Brit. Mycol. Soc. **57:**41-48.

Dennis, C. and J. Webster. 1971c. Antagonistic properties of species-groups of Trichoderma. III. Hyphal interaction. Trans. Brit. Mycol. Soc. **57:**363-369.

DeVries, O. and J. Wessels. 1973. Effectiveness of a lytic enzyme preparation from Trichoderma viride in releasing spheroplasts from fungi, particularly basidiomycetes. Ant. v. Leeuwenhoeck. **39:**397-400.

Dick, C.M. and S.A. Hutchinson. 1966. Biological activity of volatile fungal metabolites. Nature. **211:**868.

Dickinson, C.H. and T.F. Preece. 1976. Microbiology of Aerial Plant Surfaces. Academic Press. London, UK.

Dijkstra, F.L., W.A. Scheffers, and T.O. Wiken. 1972. Submerged growth of the cultivated mushroom, Agaricus bisporus. Antonie van Leeuwenhoeck. **38**:329-340.

Dill, I., J. Salnikow and G. Kraepelin. 1984. Hydroxyproline-rich protein material in wood and lignin of Fagus sylvatica. Appl. Env. Microbiol. **48**:1259-1261.

Dodson, P.J., C.S. Evans, P.J. Harvey and J.M. Palmer. 1987. Production and properties of an extracellular peroxidase from Coriolus versicolor which catalyzes C_{α}-C_{β} cleavage in a lignin model compound. FEMS Microbiol. Lett. **42**:17-22.

Domsch, K.H., W. Gams and T.-H. Anderson. 1980. Compendium of Soil Fungi. Vol. 1. Academic Press. London, UK.

Donnelly, D.M.X. and M.H. Sheridan. 1986. Anthraquinones from Trichoderma polysporum. Phytochem. **25**:2303-2304.

Donnelly, D.M.X., J. O'Reilly, J. Polonsky and G.W. Van Eijk. 1982. Fomajorin S and D from Fomes annosus (Fr.) Cooke. Tetrahedron Lett. **23**:5451-5452.

Dowding, P. 1978. Concluding remarks: methods for studying microbial interactions. Ann. Appl. Biol. **89**:167-171.

Dowding, P. 1981. Nitrient uptake and allocation during substrate exploitation by fungi. p 621-635. In: Wicklow, D.T. and G.C. Carroll (eds). The Fungal Community. Marcel Dekker. New York, NY.

Drews, G. 1983. Mikrobiologisches Praktikum. Springer Verlag. Berlin, FRG.

Driver, C.H. and J.H. Ginns. 1969. Ecology of slash pine stumps: fungal colonization and infection by Fomes annosus. For. Sci. **15**:2-10.

Dubos, B. and J. Bulit. 1981. Filamentous fungi as biocontrol agents on aerial plant surfaces. p 353-367. In: Blakeman, J.P. (ed). Microbial ecology of the phylloplane. Academic Press. London, UK.

Dubos, B. and J. Ricard. 1974. Curative treatment of peach trees against silver leaf disease (Stereum purpureum) with Trichoderma viride preparations. Plant Dis. Rep. **58**:147-150.

Duguet, J.S. and V. Dartigues. 1988. Evaluating possibilities of leaching of deltamethrin and TCMTB by rain water or soaking in water. Int. Res. Group Wood Preserv. IRG/WP/3464.

Duncan, C.G. 1960. Wood-attacking capacities and physiology of soft-rot fungi. For. Prod. Res. Lab. Rep. 2173. p 1-28. Madison, WI.

Durrell, L.W. 1966. Hyphal invasion by Trichoderma viride. Mycopath. Mycol. Appl. **35**:138-144.

Elad, Y., I. Chet and J. Katan. 1980. Trichoderma harzianum: a biological agent effective against Sclerotium rolfsii and Rhizoctonia solani. Phytopath. **70**:119-121.

Elad, Y., R. Barak, and I. Chet. 1983a. Role of lectins in mycoparasitism. J. Bacteriol. **154**:1431-1451.

Elad, Y., I. Chet, P. Boyle and Y. Henis. 1983b. Parasitism of Trichoderma spp. on Rhizoctonia solani and Sclerotium rolfsii - scanning electron microscopy and fluorescence microscopy. Phytopath. **73**:85-88.

Enari, T.-M. and P. Markkanen. 1977. Production of cellulolytic enzymes by fungi. Adv. Biochem. Eng. **5**:1-24.

Enoki, A., H. Tanaka and G. Fuse. 1988. Degradation of lignin-related compounds, pure cellulose, and wood components by white-rot and brown-rot fungi. Holzforschung. **42**:85-93.

Enoki, A., H. Tanaka and G. Fuse. 1989. Relationship between degradation of wood and production of H_2O_2-producing or one-electron oxidases by brown-rot fungi. Wood Sci. Technol. **23**:1-12.

Eriksson, K.-E. 1981. Fungal degradation of wood components. Pure Appl. Chem. **53**:33-43.

Eriksson, K.-E. and T.M. Wood. 1985. Biodegradation of cellulose. p 469-503. In: Higuchi, T. (ed). Biosynthesis and Biodegradation of Wood Components. Academic Press. Orlando, FA.

Eriksson, K.-E., B. Petterson and W. Westermark. 1975. Enzymic mechanisms of cellulose degradation caused by the rot fungus Sporotrichum pulverulentum. p 143-152. In: Liese, W. (ed). Biological Transformation of Wood by Microorganisms. Springer Verlag, Berlin, FRG.

Eriksson, K.-E., A. Grünewald, and L. Vallander. 1980. Studies of growth conditions in wood for three white-rot fungi and their cellulaseless mutants. Biotechnol. Bioeng. **22**:363-376.

Eslyn, W.E. 1970. Utility pole decay. Part 2. Basidiomycetes associated with decay in poles. Wood Sci. Technol. **4**:97-103.

Eslyn, W.E. 1986. Utility pole decay. Part 4. Growth-temperature relations and decay capabilities of eleven major utility pole decay fungi. Holzforschung. **40**:69-77.

Evans, C.S. and J.M. Palmer. 1983. Ligninolytic activity of Coriolus versicolor. J. Gen. Microbiol. **129:**2103-2108.

Evans, F.G. 1987. Leaching from CCA-impregnated wood to food, drinking water, and silo-fodder. Int. Res. Group Wood Preserv. IRG/WP/3433.

Eveleigh, D.E. 1985. Trichoderma. p 487-509. In: Demain, A.L. and N.A. Solomon (eds). Biology of Industrial Microorganisms. Benjamin/Cummings Publ. Menlo Park, CA.

Fåhraeus, G. 1952. Formation of laccase by Polyporus versicolor in different culture media. Physiol. Plant. **5:**284-292.

Falck, R. 1931. Über den Einfluss des Flossens auf die Widerstandsfähigkeit gegen Trockenfäule und über den Holzschutz durch Schimmelbefall und Diffussionstränkung. Mitt. Forstwirt. Forstwiss. **2:**480-485.

Farmer, V.C., M.E.K. Henderson and J.D. Russell. 1960. Aromatic-alcohol oxidase activity in the growth medium of Polystictus versicolor. Biochem. J. **74:**257-262.

Farr, D.F., G.F. Bills, G.P. Chamuris and A.Y. Rossman. 1989. Fungi on Plants and Plant Products in the United States. American Phytopathological Society. St.Paul. MN.

Faull, J.L. 1988. Competitive antagonism of soil-borne plant pathogens. p 125-140. In: Burge, M.N. (ed). Fungi in Biological Control Systems. Manchester Univ. Press. Manchester, UK.

Fenn, P. and Kirk, T.K. 1981. Relationship of nitrogen to the onset and suppression of lignolytic activity and secondary metabolism in Phanerochaete chrysosporium. Arch. Microbiol. **130:**59-65.

Finch, P. and J.C. Roberts. 1985. Enzymatic degradation of cellulose. p 312-343. In: Nevell, T.P and S.H. Zeronian (eds). Cellulose Chemistry and Its Applications. Ellis Horwood Ltd. Chichester, UK.

Fravel, D.R. 1988. Role of antibiosis in the biocontrol of plant diseases. Ann. Rev. Phytopath. **26:**75-91.

Frederickson, A.G. and G. Stephanopoulos. 1981. Microbial competition. Science. **213:**972-979.

Freudenberg, K. 1965. Lignin: Its constitution and formation from p-hydroxycinnamyl alcohols. Science. **148:**595.

Froehner, S.C. and K.-E. Eriksson. 1974. Induction of Neurospora crassa laccase with protein synthesis inhibitor. J. Bacteriol. **120:**450-457.

Garrett, S.D. 1951. Ecological groups of soil fungi: a survey of substrate relationships. New Phytologist. **50:**159-176.

Garrett, S.D. 1956. Biology of Root-Infecting Fungi. Cambridge Univ. Press. London, UK.

Garrett, S.D. 1963. Soil Fungi and Soil Fertility. Macmillan Co. New York, NY.

Garrett, S.D. 1965. Toward biological control of soil-borne plant pathogens. p 4-17. In: Baker, K.F. and W.C. Snyder (eds). Ecology of Soil-Borne Plant Pathogens. Univ. Calif. Press. Berkeley, CA.

Garrett, S.D. 1970. Pathogenic Root-Infecting Fungi. Cambridge Univ. Press. Cambridge, UK.

Ghose, T.K. 1977. Cellulase biosynthesis and hydrolysis of cellulosic substances. Adv. Biochem. Eng. **5**:39-75.

Gilbertson, R.L. 1981. North American wood-rotting fungi that cause brown rots. Mycotaxon. **12**:372-416.

Glenn, J.K. and M.H. Gold. 1983. Decolorization of several polymeric dyes by the lignin-degrading Basidiomycete Phanerochaete chrysosporium. Appl. Env. Microbiol. **45**:1741.

Glenn, J.K. and M.H. Gold. 1985. Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading Basidiomycete Phanerochaete chrysosporium. Arch. Biochem. Biophys. **242**:329-341.

Glenn, J.K, M.A. Morgan, M.B. Mayfield, M. Kuwahara and M.H. Gold. 1983. An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white-rot Basidiomycete Phanerochaete chrysosporium. Biochem. Biophys. Res. Commun. **114**:1077-1083.

Godtfredsen, W.O. and S. Vangedal. 1965. Trichodermin, a new sesquiterpene antibiotic. Acta Chem. Scand. **19**:1088-1102.

Goksoyr, J. and J. Eriksen. 1980. Cellulases. p 283-330. In: Rose, A.H. (ed). Microbial Enzymes and Bioconversions. Economic Microbiology. Vol. 5. Academic Press. London, UK.

Gold, M.H. and J.K. Glenn. 1988. Manganese peroxidase from Phanerochaete chrysosporium. Methods in Enzymology. **161**:258-264.

Gold, M.H., J.K. Glenn, and M. Alic. 1988. Use of polymeric dyes in lignin biodegradation assays. Methods in Enzymology. **161**:74-78.

Gold, M.H., H. Wariishi and K. Valli. 1989. Extracellular peroxidases involved in lignin degradation by the white rot basidiomycete Phanerochaete chrysosporium. p 127-140. In: Whitaker, J.R. and P.E. Sonnet (eds). Biocatalysis in Agricultural Biotechnology. American Chemical Society. Washington, DC.

Gold, M.H., M. Kuwahara, A.A. Chiu and J.K. Glenn. 1984. Purification and characterization of an extracellular H2O2-requiring diarylpropane oxygenase from the white rot basidiomycete Phanerochaete chrysosporium. Arch. Biochem. Biophys. **234**:353-362.

Gold, M.H., J.K. Glenn, M.B. Mayfield, M.A. Morgan and H. Kutsuki. 1983. Biochemical and genetic studies on lignin degradation by Phanerochaete chrysosporium. p 219-232. In: Higuchi, T., H.-m. Chang and T.K. Kirk (eds). Recent Advances in Lignin Biodegradation Research. Uni Pub. Co. Tokyo, JP.

Goldberg, H.S. (ed). 1959. Antibiotics, Their Chemistry and Non-Medical Uses. D. van Nostrand. Princeton, NJ.

Goldfarb, B., E.E. Nelson and E.M. Hansen. 1989a. Trichoderma species from Douglas-fir stumps and roots infested with Phellinus weirii in the Western Cascade mountains of Oregon. Mycologia. **81**:134-138.

Goldfarb, B., E.E. Nelson and E.M. Hansen. 1989b. Trichoderma spp.: Growth rates and antagonism to Phellinus weirii in vitro. Mycologia. **81**:375-381.

Gong, C.S., M.R. Ladisch and G.T. Tsao. 1979. Biosynthesis, purification, and mode of action of cellulases of Trichoderma reesei. p 261-288. In: Brown, R.D. and L. Jurasek (ed). Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis. Advances in Chemistry. Vol. 181. American Chemical Society. Washington, DC.

Goodell, B., G. Daniel, J. Jellison and T. Nilsson. 1988. Immunolocalization of extracellular metabolites from Poria placenta. Int. Res. Group Wood Preserv. IRG/WP/1361.

Gottlieb, D. 1976. The production and role of antibiotics in soil. J. Antibiot. **29**:987-1000.

Gottlieb, D. and P.D. Shaw. 1970. Mechanism of action of antifungal antibiotics. Ann. Rev. Phytopath. **8**:371-402.

Grabbe, K., R. Koenig and K. Haider. 1968. Die Bildung der Phenoloxydase und die Stoffwechselbeeinflussung durch Phenole bei Polystictus versicolor. Arch. Microbiol. **63**:133-153.

Graham, R.D. 1973. Preventing and stopping internal decay of Douglas-fir poles. Holzforschung. **27**:168-173.

Graham, R.D. and M.E. Corden. 1980. Controlling biological deterioration of wood with volatile chemicals. Final Report EPRI EL-1480. Electrical Power Res. Inst. Palo Alto. CA.

Gramss, G. 1987. The colonization of timber by wood-decay fungi as a dynamic interaction with microbial wood substrate contaminants. Mat. Org. **22**:271-287.

Greaves, H. 1972. Microbial ecology of untreated and copper- chrome--arsenic treated stakes exposed in a tropical soil. 1. The initial invaders. Can. J. Micorbiol. **18**:1923-1931.

Greaves, H. 1987. Environmental aspects of wood preservatives. Int. Res. Group Wood Preserv. IRG/WP/3406.

Green, C.A., G.M. Smith and B. King. 1987. The effect of aquaeous leaching on moisture uptake by CCA treated blocks during soil burial. Int. Res. Group Wood Preserv. IRG/WP/2283.

Green, F., M.J. Larsen, L.L. Murmanis and T.L. Highley. 1989. Proposed model for the penetration and decay of wood by the hyphal sheath of the brown-rot fungus Postia placenta. Int. Res. Group Wood Preserv. IRG/WP/1391.

Greig, B.J.W. 1984. Management of East England pine plantations affected by Heterobasidion annosum root rot. Eur. J. For. Path. **14**:392-397.

Griffith, N.T. and H.L. Barnett. 1967. Mycoparasitism by basidiomycetes in culture. Mycologia. **59**:149-154.

Gritzali, M. and R.D. Brown, Jr. 1979. The cellulase system of Trichoderma: relationships between purified extracellular enzymes from induced or cellulose-grown cells. p 237-260. In: Brown, R.D. and L. Jurasek (ed). Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis. Advances in Chemistry. Vol. 181. American Chemical Society. Washington, DC.

Grosclaude, C. 1970. Premiers essais de protection biologique des blessures de taille vis-a-vis du Stereum purpureum Pers. Ann. Phytopath. (Paris). **2**:507-516.

Haars, A.; A. Hüttermann. 1980. Function of laccase in the white-rot fungus Fomes annosus. Arch. Microbiol. **125**:233-237.

Haars, A. and A. Hüttermann. 1983. Laccase induction in the white-rot fungus Heterobasidion annosum (Fr.) Bref. (Fomes annosus Fr. Cooke). Arch. Microbiol. **134**:309-313.

Haars, A, I. Chet and A. Hüttermann. 1981 Effect of phenolic compounds and tannin on growth and laccase activity of Fomes annosus. Eur. J. For. Path. **11**:67-76.

Hadar, Y., I. Chet and Y. Henis. 1979. Biological control of Rhizoctonia solani damping-off with wheat bran culture of Trichoderma harzianum. Phytopath. **69**:64-68.

Haemmerli, S.D., M. Leisola and A. Fiechter. 1986. Oxidation of organic pollutants by lignin peroxidases of Phanerochaete chrysosporium. Proc. 3rd Int. Conf. Biotechnol. Pulp Paper Ind., Stockholm, S. p 130-132.

Halliwell, G. 1965. Catalytic decomposition of cellulose under biological conditions. Biochem. J. **95**:35-40.

Harder, R. 1911. Über das Verhalten von Basidiomyceten und Ascomyceten in Mischkulturen. Naturw. Zeitschr. Forst- und Landwirtsch. **9**:129-160.

Harper, J.L. and J. Ogden. 1970. The reproductive strategy of higher plants. I. The concept of strategy with special reference to Senecio vulgaris L. J. Ecol. **58**:681-689.

Harvey, P.J., H.E. Schoemaker and J.M. Palmer. 1986. Lignin degradation by wood-degrading fungi. Int. Res. Group Wood Preserv. IRG/WP/1310.

Hashioka, Y. and T. Fukita. 1969. Ultrastructural observations on mycoparasitism of Trichoderma, Gliocladium, and Acremonium to phytopathogenic fungi. Rep. Tottori Mycol. Inst. (Japan). **7**:8-18.

Highley, T.L. 1987a. Biochemical aspects of white-rot and brown-rot decay. Int. Res. Group Wood Preserv. IRG/WP/1319.

Highley, T.L. 1987b. Effect of carbohydrate and nitrogen on hydrogen peroxide formation by wood decay fungi in solid medium. FEMS Microbiol. Lett. **48**:373-377.

Highley, T.L. 1989. Antagonism of Scytalidium lignicola against wood decay fungi. Int. Res. Group Wood Preserv. IRG/WP/1392.

Highley, T.L. and L.L. Murmanis. 1985. Determination of hydrogen peroxide production in Coriolus versicolor and Poria placenta during wood degradation. Mat. Org. **20**:241-252.

Highley, T.L. and J. Ricard. 1988. Antagonism of Trichoderma spp. and Gliocladium virens against wood decay fungi. Mat. Org. **23**:157-169.

Higuchi, T. 1985. Biosynthesis of Lignin. p 141-160. In: Higuchi, T. (ed). Biosynthesis and Biodegradation of Wood Components. Academic Press. Orlando, FA.

Higuchi, T., H.-m. Chang and T.K. Kirk (eds). 1983. Recent Advances in Lignin Biodegradation Research. Uni Publ. Tokyo, JP.

Hill, R. and A.J. Killmeyer. 1988. Chemical and biological investigations of organotin compounds as wood preservatives. Preprint Am. Wood Preserv. Assoc. Ann. Meet. 1988. Minneapolis, MN.

Hintze, J.L. 1987. Number Cruncher Statistical System. Version 5.01. Reference Manual. Kaysville, UT.

Hiroi, T. and K.-E. Eriksson. 1976. Microbiological degradation of lignin. Part 1. Influence of cellulose on the degradation of lignins by

the white-rot fungus Pleurotus ostreatus. Svensk Pappperstidn. **79:**157-161.

Hoch, H.C. 1978. Mycoparasitic relationships. IV. Stephanoma phaeospora parasitc on a species of Fusarium. Mycologia. **70:**370-379.

Hoff, T., S-Y. Liu and J.-M. Bollag. 1985. Transformation of halogen-, alkyl-, and alkoxy-substituted anilines by a laccase of Trametes versicolor, Appl. Env. Microbiol., **49:**1040-1045

Holdenrieder, O. 1982. Untersuchungen zur biologischen Bekämpfung von Heterobasidion annosum (Fr.) Bref. (Fomes annosus [Fr.] Karst.) an Fichte (Picea abies P.Karst.) mit antagonistischen Pilzen. PhD. Thesis. University Munich, FRG.

Hori, H. and A.D. Elbein. 1985. Biosynthesis of plant cell wall polysaccharides. p 109-140. In: Higuchi, T. (ed). Biosynthesis and Biodegradation of Wood Components. Academic Press. Orlando, FA.

Hornby, D. 1978. Microbial antagonism in the rhizosphere. Ann. Appl. Biol. **89:**97-100.

Hulme, M.A. and J.K. Shields. 1970. Biological control of decay fungi in wood by competition for non-structural carbohydrates. Nature. **227:**300-301.

Hulme, M.A. and J.K. Shields. 1972a. Interaction between fungi in wood blocks. Can. J. Bot. **50:**1421-1427.

Hulme, M.A. and J.K. Shields. 1972b. Effect of a primary fungal infection upon secondary colonization of birch bolts. Mat. Org. **7:**177-188.

Hulme, M.A. and J.K. Shields. 1975. Antagonistic and synergistic effects for biological control of decay. p 52-63. In: W. Liese (ed.). Biological Transformation of Wood by Microorganisms. Springer Verlag. Berlin. FRG.

Hulme, M.A. and D.W. Stranks. 1970. Induction and the regulation of production of cellulase by fungi. Nature. **226:**469-470.

Hunt, G.M. and G.A. Garratt. 1967. Wood Preservation. McGraw-Hill Book Company, New York.

Hurst, H.M. 1963. Aromatic acid-reducing systems in fungi. p 121-138. In: Proc. Plant Phenolics Group Symp., 1962. Liverpool, UK.

Hutchinson, S.A. and M.E. Cowan. 1972. Identification and biological effects of volatile metabolites from cultures of Trichoderma harzianum. Trans. Brit. Mycol. Soc. **59:**71-77.

Hüttermann, A. and C. Volger. 1973. Induction of aryl-ß-glucosidase in Fomes annosus by cellobiose. Arch Microbiol. **93:**195-204.

Hüttermann, A. and P. Cwielong. 1982. The cell wall of Fomes annosus (Heterobasidion annosum) as target for biochemical control. I. Direct lysis of the cell wall by lytic enzymes from Trichoderma harzianum. Eur. J. For. Path. **12:**238-245.

Hüttermann, A., C. Herche and A. Haars. 1980. Polymerization of water-insoluble lignins by Fomes annosus. Holzforschung. **34:**64-66.

Ikediugwu, F.E.O. 1976a. Ultrastructure of hyphal interference between Coprinus heptemerus and Ascobulus crenulatus. Trans. Brit. Mycol. Soc. **66:**281-290.

Ikediugwu, F.E.O. 1976b. The interface in hyphal interference by Peniophora gigantea against Heterobasidion annosum. Trans. Brit Mycol. Soc. **66:**291-296.

Ikediugwu, F.E.O., C. Dennis and J. Wenster. 1970. Hyphal interference by Peniophora gigantea against Heterobasidion annosum. Trans. Brit. Mycol. Soc. **54:**307-309.

Ishihara, M. 1986. An approach to biomass utilization. V. Screening of cellulolytic Trichoderma and Hypocrea species. Mokuzai Gakkaishi. **32:**59-66.

Ishihara, T. 1980. The role of laccase in lignin biodegradation. p 17-31. In: Kirk, T.K., T. Higuchi and H-m Chang (eds.). Lignin Biodegradation: Microbiology, Chemistry, and Potential Applications. Vol II. CRC Press. Boca Raton, FA.

Ishihara, T. and A. Nishida. 1983. The role of oxidation and reduction enzymes in lignin biodegradation. p 134-142. In: Higuchi, T., H.-m. Chang and T.K. Kirk (eds). Recent Advances in Lignin Biodegradation Research. Uni Publ. Tokyo, JP.

Ishikawa, H., M. Nagao, T. Oki and K. Kawabe. 1980. Physiological changes in Lentinus edodes (Berk.) Sing. mycelia induced by Trichoderma metabolites. (Japanese with English abstract and figures). Rep. Tottori Mycol. Inst. (Japan). **18:**197-204.

Jackson, R.M. 1965. Antibiosis and fungistasis of soil microorganisms. p 363-369. In: Baker, K.F. and W.C. Snyder (eds). "Ecology of Soil-Borne Plant Pathogens. Univ. Calif. Press. Berkeley, CA.

Janshekar, H. and A. Fiechter. 1983. Lignin: Biosynthesis, applications, and biodegradation. Adv. Biochem. Eng. Biotechnol. **27:**119-178.

Jeffries, T.W. 1982. The microbiology of mercury. Progr. Ind. Microbiol. **16:**23-75.

Jeffries, T.W. 1987. Physical, chemical and biochemical considerations in the biological degradation of wood. p 213-230. In: Kennedy, J.F., G.O. Phillips and P.A. Williams (eds). Wood and Cellulosics: Industrial

Utilization, Biotechnology, Structure and Properties. Ellis Horwood Ltd. Chichester, UK.

Jeffries, T.W., S. Choi and T.K. Kirk. 1981. Nutritional regulation of lignin degradation by Phanerochaete chrysosporium. Appl. Env. Microbiol. **42:**290-296.

Jolley, R.L., Jr. and H.S. Mason. 1965. The multiple forms of mushroom tyrosinase. J. Biol. Chem. **240:**1489-1491.

Jones, R.W. and J.G. Hancock. 1987. Conversion of viridin to viridol by viridin-producing fungi. Can. J. Microbiol. **33:**963-966.

Jones, R.W. and J.G. Hancock. 1988. Mechanism of gliotoxin action and factors mediating gliotoxin sensitivity. J. Gen. Microbiol. **134:**2067-2075.

Käärik, A. 1975. Successions of microorganisms during wood decay. p 39-51. In: Liese, W. (ed.). Biological Transformation of Wood by Microorganisms. Springer Verlag. Berlin, FRG.

Kaplan, D.L. 1979. Reactivity of different oxidases with lignins and lignin model compounds. Phytochem. **18:**1917-1919.

Kawai, S.; T. Umezawa; M. Shimada; T. Higuchi; K. Koide; T. Nishida; N. Morohoshi; T. Haraguchi. 1987. C_{α}-C_{β} cleavage of phenolic ß-1 lignin substructure model compound by laccase of Coriolus versicolor. Mokuzai Gakkaishi. **33:**792-797.

Kelley, R.L. 1988. Ligninolytic activity of Phanerochaete chrysosporium measured as ethylene production from α-keto-γ-methylthiolbutyric acid. Meth. Enzymol. **161:**79-82.

Kelly, D.M.T., L.H.G. Morton and M. Edmunds. 1981. The effect of extracellular fungal metabolites on the germination of wood-inhabiting microfungal spores. Mat. Org. **16:**133-140.

Kern, H.W. 1981. Microbial degradation of lignosulfonates. FEMS Symposium. **12:**299-324.

Kern, H.W. 1983. Increased biooxidation of lignosulfonates by Sporotrichum pulverulentum in the presence of polyacrylic acid. Appl. Microbiol. Biotechnol. **17:**182-186.

Kersten, P.J., M. Tien, B. Kalyanaraman and T.K. Kirk. 1985. The ligninase of Phanerochaete chrysosporium generates cation radicals from methoxybenzenes. J. Biol. Chem. **260:**2609-2612.

Keyser, P., T.K. Kirk and J.G. Zeikus. 1978. Ligninolytic enzyme system of Phanerochaete chrysosporium: synthesized in the absence of lignin in response to nitrogen starvation. J. Bacteriol. **135:**790-797.

Kharazipour, R. 1983. Optimierung eines Verfahrens zur Herstellung von Bindemitteln für Holzwerkstoffe auf der Basis von Ligninsulfonat und Laccase. Ph.D. Thesis. University of Göttingen.

Kikuchi, T., T. Mimura, K. Harimaya, H. Yano, T. Arimoto, Y. Masada and T. Inoue. 1974. Volatile metabolite of aquatic fungi. Identification of 6-pentyl-α-pyrone from Trichoderma and Aspergillus species. Chem. Pharm. Bull. **22:**1946-1948.

King, B., T.A. Oxley and K.D. Long. 1976. Some biological effects of redistribution of soluble nutrients during drying of wood. In: Becker, G. and W. Liese (eds). Organismen und Holz. Mat. Org. Beiheft 3. p 263-276.

Kirk, T.K. 1975. Chemistry of lignin degradation by wood-destroying fungi. p 153-164. In: Liese, W. (ed). Biological Transformation of Wood by Microorganisms. Springer Verlag, Berlin, FRG.

Kirk, T.K. 1987. Lignin-degrading enzymes. Phil. Trans. Roy. Soc. London. **A321:**461-474.

Kirk, T.K. 1988. Biochemistry of lignin degradation by Phanerochaete chrysosporium. p 315-332. In: Aubert, J.-P., P. Beguin and J. Millet (eds). Biochemistry and Genetics of Cellulose Degradation. Academic Press. San Diego, CA.

Kirk, T.K. and R.L. Farrell. 1987. Enzymatic "combustion": the microbial degradation of lignin. Ann. Rev. Microbiol. **41:**465-505.

Kirk, T.K. and P. Fenn. 1982. Formation and action of the ligninolytic system in basidiomycetes. p 67-90. In: Frankland, J.C., J.N. Hedger and M.J. Swift (eds). Decomposer Basidiomycetes: Their Biology and Ecology. Cambridge Univ. Press. Cambridge, UK.

Kirk, T.K. and M. Shimada. 1985. Lignin biodegradation: the microorganisms involved, and the physiology and biochemistry of degradation by white-rot fungi. p 579-605. In: Higuchi, T. (ed). Biosynthesis and Biodegradation of Wood Components. Academic Press. Orlando, FA.

Kirk, T.K. and M. Tien. 1983. Biochemistry of lignin degradation by Phanerochaete chrysosporium: investigations with non-phenolic model compounds. p 233-245. In: Higuchi, T., H.-m. Chang and T.K. Kirk (eds). Recent Advances in Lignin Biodegradation Research. Uni Publ. Tokyo, JP.

Kirk, T.K., W.J. Connors and J.G. Zeikus. 1976. Requirement for a growth substrate during lignin decomposition by two wood-rotting fungi. Appl. Env. Microbiol. **32:**192-194.

Kirk, T.K., T. Higuchi and H.-m. Chang (eds). 1980. Lignin Biodegradation: Microbiology, Chemistry, and Potential Applications. Vol. I + II. CRC Press. Boca Raton, FA.

Kirk, T.K., H.H. Yang and P. Keyser. 1978b. The chemistry and physiology of the fungal degradation of lignin. Dev. Ind. Microbiol. **19:**51-61.

Kirk, T.K., T.L. Highley, R. Ibach and M.D. Mozuch. 1989. Identification of terminal structures in cellulose degraded by the brown-rot fungus Postia placenta. Int. Res. Group Wood Preserv. IRG/WP/1389.

Kirk, T.K., S. Croan, M. Tien, K.E. Murtagh and R.L. Farrell. 1986. Production of multiple ligninases by Phanerochaete chrysosporium: effect of selected growth conditions and use of a mutant strain. Enz. Microb. Technol. **8:**27-32.

Kirk, T.K., E. Schultz, W.J. Connors, L.F. Lorenz and J.G. Zeikus. 1978a. Influence of culture parameters on lignin metabolism by Phanerochaete chrysosporium. Arch. Microbiol. **117:**277-285.

Kitamoto, Y., N. Mori, M. Yamamoto, T. Ohiwa and Y. Ichikawa. 1988. A simple method for protoplast formation and improvement of protoplast regeneration from various fungi using an enzyme from Trichoderma harzianum. Appl. Microbiol. Biotechnol. **28:**445-450.

Klingström, A.E. and L. Beyer. 1965. Two new species of Scytalidium with antagonistic properties to Fomes annosus (Fr.) Cke. Svensk Bot. Tidskr. **59:**30-36.

Klingström, A.E. and S.M. Johansson. 1973. Antagonism of Scytalidium isolates against decay fungi. Phytopath. **63:**473-479.

Knigge, W. and H. Schulz. 1966. Grundriss der Forstbenutzung. Paul Parey, Hamburg.

Koenigs, J.W. 1970. Peroxidase Activity in Brown-Rot Basidiomycetes. Arch. Microbiol. **73:**121-124.

Koenigs, J.W. 1972. Poria weirii as a possible commercial source of peroxidase. Appl. Microbiol. **23:**835-836.

Koenigs, J.W. 1974a. Production of hydrogen peroxide by wood-rotting fungi in wood and its correlation with weight loss, depolymerization, and pH changes. Arch. Microbiol. **99:**129-145.

Koenigs, J.W. 1974b. Hydrogen peroxide and iron: a proposed system for decomposition of wood by brown-rot basidiomycetes. Wood Fiber. **6:**66-80.

Komatsu, M. 1968. Trichoderma viride as an antagonist of the wood-inhabiting Hymenomycetes. VIII. The antibiotic activity against the mycelial growth of Lentinus edodes (Berk.) Sing. of three genera, Trichoderma, Pachybasium, Gliocladium, and other sterile forms. Rep. Tottori Mycol. Inst. (Japan). **8:**29-42.

Komatsu, M. 1976. Studies on Hypocrea, Trichoderma and allied fungi antagonistic to shiitake, Lentinus edodes (Berk.) Sing. [in Japanese

with English figures and abstract]. Rep. Tottori Mycol. Inst. (Japan). **13**:1-113.

Krasil'nikov, N.A. 1960. The biological role of microbes-antagonists, producers of antibiotic substances. Soil Plant Food. **5**:184-193.

Kresze, G.B. 1984. Methods for protein determination. p 84-89. In: Bergmeyer, H.U. (ed.). Methods of Enzyme Analysis. Volume 3. Verlag Chemie. New York, NY.

Kuwahara, M., Y. Asada, Y. Kimura and M. Aokage. 1987. Isolation of mutants of a lignin-degrading basidiomycete, Phanerochaete chrysosporium, that produce lignin peroxidase in high nitrogen cultures. Mokuzai Gakkaishi. **3**:821-823.

Kuwahara, M., J.K. Glenn, M.A. Morgan and M.H. Gold. 1984. Separation and characterization of two extracellular H_2O_2-dependent oxidases from lignolytic cultures of Phanerochaete chrysosporium. FEBS Lett. **169**:247-250.

Landsiedel, H.A.B. 1987. Fungicidal Combination Products. Int. Res. Group Wood Preserv. IRG/WP/3426.

Leach, R. 1939. Biological control and ecology of Armillaria mellea (Vahl.) Fr. Trans. Brit. Mycol. Soc. **23**:320-329.

Leatham, G.F. 1985a. Extracellular enzymes produced by the cultivated mushroom Lentinus edodes during degradation of a lignocellulosic medium. Appl. Env. Microbiol. **50**:859-867.

Leatham, G.F. 1985b. Growth and development of Lentinus edodes on a chemically defined medium. p 403-427. In: Moore, D. L.A. Casselton, D.A. Wood and J.C. Frankland (eds). Developmental Biology of Higher Fungi. Cambridge Univ. Press. Cambridge, UK.

Leatham, G.F. 1986. The lignolytic activities of Lentinus edodes and Phanerochaete chrysosporum. Appl. Microbiol. Biotechnol. **24**:51-58.

Lee, Y.-H. and L.T. Fan. 1980. Properties and mode of action of cellulase. Adv. Biochem. Eng. **17**:101-129.

Lee, Y.-H., L.T. Fan and L.-S. Fan. 1980. Kinetics of hydrolysis of insoluble cellulose by cellulase. Adv. Biochem. Eng. **17**:131-168.

Leisola, M.S. and A. Fiechter. 1985. New Trends in Lignin Biodegradation. Adv. Biotech. Processes. **5**:59-89.

Leisola, M., U. Thaney-Wyss and A. Fiechter. 1985. Strategies for production of high ligninase activities by Phanerochaete chrysosporium. J. Biotechnol. **3**:97-107.

Leisola, M.S., D.C. Ulmer and A. Fiechter. 1983. Problem of oxygen transfer during degradation of lignin by Phanerochaete chrysosporium. Appl. Microbiol. Biotechnol. **17:**113-116.

Leisola, M.S., D.C. Ulmer and A. Fiechter. 1984. Factors affecting lignin degradation in lignocellulose by Phanerochaete chrysosporium. Arch. Microbiol. **137:**171-175.

Leisola, M., B. Kozulic, F. Meussdoerffer and A. Fiechter. 1987. Homology among multiple peroxidases from Phanerochaete chrysosporium. J. Biol. Chem. **262:**419-424.

Leonard, T.J. 1971. Phenoloxidase activity and fruiting body formation in Schyzophyllum commune. J. Bacteriol. **106:**162-167.

Leonard, T.J. and L.E. Phillips. 1973. Study of phenoloxidase activity during the reproductive cycle of Schizophyllum commune. J. Bacteriol. **114:**7-10.

Leong, J. 1986. Siderophores: their biochemistry and possible role in the biocontrol of plant pathogens. Ann. Rev. Phytopath. **24:**187-209.

Leonowicz, A. and J. Trojanowski. (1975), Induction of a new laccase form in the fungus Pleurotus ostreatus by ferulic acid, Microbios, **13:**167-174

Leonowicz, A., J. Trojanowski and G. Nowak. 1972. Ferulic acid as the inductor of messenger RNA synthesis related to laccase formation in the wood rotting fungus Pleurotus ostreatus, Microbios, **6:**23-28.

Levi, M.P. and E.B. Cowling. 1968. The effect of carbon to nitrogen ratio of substrate on the growth, composition, cellulase production, and wood-destroying capacity of Polystictus versicolor. p 575-583. In: Walters, A.H. and J.J. Elphick (eds). Biodeterioration of Materials. Vol. 1. Elsevier. London, UK.

Levi, M.P. and E.B. Cowling. 1969. Role of nitrogen in wood deterioration. VII. Physiological adaptation of wood-destroying and other fungi to substrates deficient in nitrogen. Phytopath. **59:**460-468.

Levi, M.P., W. Merrill and E.B. Cowling. 1968. Role of nitrogen in wood deterioration. VI. Mycelial fractions and model nitrogen compounds as substrates for growth of Polyporus versicolor and other wood-destroying and wood-inhabiting fungi. Phytopath. **58:**626-634.

Lifshitz, R., M.T. Windham and R. Baker. 1986. Mechanism of biological control of preemergence damping-off of pea by seed treatment with Trichoderma spp. Phytopath. **76:**720-725.

Linderman, R.G., L.W. Moore, K.F. Baker and D.A. Cooksey. 1983. Strategies for detecting and characterizing systems for biological control of soilborne plant pathogens. Plant Dis. **67:**1058-1064.

Lindgren, R.M. 1952. Permeability of southern pine as affected by mold and other fungus infection. Proc. Am. Wood Preserv. Assoc. **48**:158-168.

Lloyd, A.B. and J.L. Lockwood. 1966. Lysis of fungal hyphae in soil and its possible relation to autolysis. Phytopath. **56**:595-602.

Lockwood, J.L. 1988. Evolution of concepts associated with soilborne plant pathogens. Ann. Rev. Phytopath. **26**:93-121.

Lombard, F.F. and G.P. Chamuris. 1988. Basidiomycetes. p C13-C88. In: Meyer, R.W., R.A. Zabel, C.J.K. Wang and F.C. Terracina (eds) Wood pole decay characterization. Vol.2: Soft rot characteristics and an identification manual for the decay fungi in utility poles in New York. Final Report EP 84-5. Empire State Electric Energy Research Corporation. New York.

Lowry, O.H., H.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **93**:265-275.

Lumsden, R.D. 1980a. Hyperparasitism for the control of plant pathogens. In: Handbook of Agriculture, Vol 1. Sect. B. Pest Management. CRC Press. West Palm Beach, FA.

Lumsden, R.D. 1980b. Ecology of mycoparasitism. p 295-318. In: Wicklow, D.T. and G.W. Carroll (eds). The Fungal Community. Dekker. New York, NY.

Lundborg, A. 1988a. Long time effects of nutrient addition to Norway spruce wood on wood decay caused by Heterobasidion annosum. Mat. Org. **23**:125-131.

Lundborg, A. 1988b. Interactions between fungi affect their ability to degrade crystalline cellulose. Mat. Org. **23**:171-189.

Lundborg, A. and T. Unestam. 1980. Antagonism against Fomes annosum. Comparison between different test methods in vitro and in vivo. Mycopathologia. **70**:107-115.

Mandels, M. and E.T. Reese. 1960. Induction of cellulase in fungi by cellobiose. J. Bacteriol. **79**:816-826.

Mandels, M. and E.T. Reese. 1965. Inhibition of Cellulases. Ann. Rev. Phytopath. **3**:85-102.

Matcham, S.E., B.R. Jordan and D.A. Wood. 1984. Methods for assessment of fungal growth on solid substrates. p 5-18. In: Grainger, J.M. and J.M. Lynch (ed.). Microbiological Methods for Environmental Biotechnology. Society for Applied Bacteriology. Tech. Series No. 19. Academic Press. London, UK.

Mayer, A.M. 1987. Polyphenol oxidases in plants - recent progress. Phytochem. **26**:11-20.

Meers, J.L. 1978. Growth of bacteria in mixed cultures. Ann. Appl. Biol. **89:**143-145.

Menzies, J.D. 1963. Survival of microbial plant pathogens in soil. Bot. Rev. **29:**79-122.

Mercer, P.C. 1986. Biological control of decay fungi in wood. p 177-198. In: Mukerji, K.G. and K.L. Garg (eds). 1986. Biocontrol of Plant Diseases. Vol. 1. CRC Press. Boca Raton, FA.

Mercer, P.C. and S.A. Kirk. 1984a. Biological treatments for the control of decay in tree wounds. I. Laboratory tests. Ann. Appl. Biol. **104:**211-219.

Mercer, P.C. and S.A. Kirk. 1984b. Biological treatments for the control of decay in tree wounds. II. Field tests. Ann. Appl. Biol. **104:**221-229.

Merivuori, H., B.S. Montenecourt and J.A. Sands. 1987. Ethanol perturbs glycosylation and inhibits hypersecretion in Trichoderma reesei. Appl. Env. Microbiol. **53:**463-465.

Merivuori, H., K.M. Siegler, J.A. Sands and B.S. Montenecourt. 1985. Regulation of cellulase biosynthesis and secretion in fungi. Biochem. Soc. Trans. (UK). **13:**411-414.

Merrill, W. and E.B. Cowling. 1965. Effect of variation in nitrogen content of wood on rate of decay. Phytopath. **55:**1067-1068.

Merrill, W. and E.B. Cowling. 1966a. Role of nitrogen in wood deterioration: amount and distributionof nitrogen in fungi. Phytopath. **56:**1085-1090.

Merrill, W. and E.B. Cowling. 1966b. Role of nitrogen in wood deterioration: amounts and distribution of nitrogen in tree stems. Can. J. Bot. **44:**1555-1580.

Merrill, W. and E.B. Cowling. 1966c. Role of nitrogen in wood deterioration. IV. Relationship of natural variation in nitrogen content of wood to its susceptibility to decay. Phytopath. **56:**1324-1325.

Merrill, W. and A.L. Shigo. 1979. An expanded concept of tree decay. Phytopath. **69:**1158-1160.

Meyer, R.W., R.A. Zabel, C.J.K. Wang and F.C. Terracina. 1988. Wood pole decay characterization. Vol.2: Soft rot characteristics and an identification manual for the decay fungi in utility poles in New York. Final Report EP 84-5. Empire State Electric Energy Research Corporation. New York.

Millard, W.A. and C.B. Taylor. 1927. Antagonism of microorganisms as the controlling factor in the inhibition of scab by green manuring. Ann. Appl. Biol. **14:**202-215.

Mitchell, J.E. 1973. The mechanisms of biological control of plant diseases. Soil Biol. Biochem. **5:**721-728.

Montgomery, R.A.P. 1982. The role of polysaccharidase enzymes in the decay of wood by basidiomycetes. p 51-65. In: Frankland, J.C., J.N. Hedger and M.J. Swift (eds). Decomposer Basidiomycetes: Their Biology and Ecology. Cambridge Univ. Press. Cambridge, UK.

Moore, L.W. 1978. Practical use and success of Agrobacterium radiobacter strain 84 for crown gall control. p 553-568. In: Schippers, B. and W. Gams (eds). Soil-Borne Plant Pathogens. Academic Press. New York, NY.

Moore-Landecker, E. 1982. Fundamentals of the Fungi. 2nd Edition. Prentice-Hall Inc. Englewood Cliffs, NJ.

Morohoshi, N. and T. Haraguchi. 1987. Degradation of lignin by the extracellular enzymes of Coriolus versicolor. III. Degradation of variuos substrates by the phenol oxidase fraction. Mokuzai Gakkaishi. **33:**143-150.

Morohoshi, N., K. Fujita, H. Wariishi and T. Haraguchi. 1987. Degradation of lignin by the extracellular enzymes of Coriolus versicolor. V. Degradation of isolated lignin fractions by laccase I, II, and III. Mokuzai Gakkaishi. **33:**310-315.

Morohoshi, N., M. Nakamura, A. Yoshitake, Y. Katayama and T. Haraguchi. 1987. Isolation of a ligninolytic laccase and its messenger ribonucleic acid from Coriolus versicolor. Mokuzai Gakkaishi. **33:**743-746.

Morohoshi, N., H. Wariishi, C. Muraiso, T. Nagai and T. Haraguchi. 1987. Degradation of lignin by the extracellular enzymes of Coriolus versicolor. IV. Properties of three laccase fractions fractionated from the extracellular enzymes. Mokuzai Gakkaishi. **33:**218-225.

Morrell, J.J. and M.E. Corden. 1987. Controlling wood deterioration with fumigants: a review. For. Prod. J. **36(10):**26-34.

Morrell, J.J. and C.M. Sexton. 1988. Feasibility of using biological control agents to arrest and prevent colonization of Douglas-fir and southern pine by decay fungi. Int. Res. Group Wood Preserv. IRG/WP/1345.

Morris, P.I. and B. Calver. 1986. Wood Pole Decay - Mythology and Magic! 2. Magic. Distribution Developments. March 1986. p 3-10.

Morris, P.I. and D.J. Dickinson. 1981. Laboratory studies on the antagonistic properties of Scytalidium spp. to basidiomycetes with regard to biological control. Int. Res. Group Wood Preserv. IRG/WP/1130.

Morris, P.I., D.J. Dickinson and J.F. Levi. 1984. The nature and control of decay in creosoted electricity poles. Rec. Brit. Wood Preserv. Assoc. p 42-53.

Morris, P.I., N.A. Summers and D.J. Dickinson. 1986. The leachability and specificity of the biological protection of timber using Scytalidium sp. and Trichoderma spp. Int. Res. Group Wood Preserv. IRG/WP/1302.

Moss, M.O. 1984. The mycelial habit and secondary metabolite production. p 127-142. In: Jennings, D.H. and A.D.M. Rayner (eds). The Ecology and Physiology of the Fungal Mycelium. Cambridge Univ. Press. Cambridge, UK.

Moss, M. O., R.M. Jackson and D. Rogers. 1975. The characterization of 6-(pent-1-enyl)-α-pyrone from Trichoderma viride. Phytochem. **14:**2706-2708.

Mughogho, L.K. 1968. The fungus flora of fumigated soils. Trans. Brit. Mycol. Soc. **51:**441-459.

Mukerji, K.G. and K.L. Garg (eds). 1986. Biocontrol of Plant Diseases. Vol. 1 + 2. CRC Press. Boca Raton, FA.

Müller, H.E. 1984. ABTS peroxidase medium as a highly sensitive plate assay for detection of hydrogen peroxide production in bacteria. J. Microbiol. Meth. **2:**101-102.

Mullings, R. 1985. Measurement of saccharification by cellulases. Enz. Microb. Technol. **7:**586-591.

Murdoch, W.W., J. Chesson and P.L. Chesson. 1985. Biological Control in Theory and Practice. American Naturalist. **125:**344-366.

Murmanis, L.L., T.L. Highley and J. Ricard. 1988. Hyphal interaction of Trichoderma harzianum and Trichoderma polysporum with wood decay fungi. Mat. Org. **23:**271-279.

Mustranta, A. 1987. Production of peroxidase by Inonotus weirii. Appl. Microbiol. Biotechnol. **27:**21-26.

Neidleman, S.L. 1989. Enzymes under stress. ASM News. **55:**67-70.

Neijssel, O.M. and D.W. Tempest. 1979. The physiology of metabolite overproduction. p 53-82. In: Bull, A.T., D.C. Ellwood and C. Ratledge (eds). Microbial Technology, Current State, Future Prospects. Cambridge Univ. Press. Cambridge, UK.

Nelson, E.E. and W.G. Thies. 1985. Colonization of Phellinus weirii infested stumps by Trichoderma viride. 1. Effect of isolate and inoculum base. Eur. J. For. Path. **15:**425-431.

Nelson, E.E. and W.G. Thies. 1986. Colonization of Phellinus weirii infested stumps by Trichoderma viride. 2. Effects of season of inoculation and stage of wood decay. Eur. J. For. Path. **16**:56-60

Nelson, E.E., B. Goldfarb and W.G. Thies. 1987. Trichoderma species from fumigated Douglas-fir roots decayed by Phellinus weirii. Mycologia. **79**:370-374.

Nguyen, T.T. and D.J. Niederpruem. 1984. Hyphal interactions in Schizophyllum commune: the di-mon mating. p 73-102. In: Jennings, D.H. and A.D.M. Rayner (eds). The Ecology and Physiology of the Fungal Mycelium. Cambridge Univ. Press. Cambridge, UK.

Nijman, H.F.M. 1987. Risk of pulmonary damage as a result of an evaporation from wood treated by difluorides. Int. Res. Group Wood Preserv. IRG/WP/3401.

Nilsson, T. 1973. Studies on wood degradation and cellulolytic activity of microfungi. Stud. For. Sue. No. 104.

Nilsson, T. 1974. Comparative study on the cellulolytic activity of white-rot and brown-rot fungi. Mat. Org. **9**:173-198.

Nilsson, T. 1988. Defining fungal decay types - final proposal. Int. Res. Group Wood Preserv. IRG/WP/1355.

Nishida, T., Y. Kashino, A. Mimura and Y. Takahara. 1988. Lignin biodegradation by wood-rotting fungi. I. Screening of lignin-degrading fungi. Mokuzai Gakkaishi. **34**:530-536.

Nobles, M.K. 1948. Studies in forest pathology. VI. Identification of cultures of wood-rotting fungi. Can. J. Res. **C26**:281-431.

Nobles, M.K. 1965. Identification of cultures of wood-inhabiting Hymenomycetes. Can. J. Bot. **43**:1097-1139.

Noguchi, A., M. Shimada and T. Higuchi. 1980. Studies on lignin biodegradation. I. Possible role of non-specific oxidation of lignin by laccase. Holzforschung. **34**:86-89.

Nordbring-Hertz, B. 1984. Mycelial development and lectin-carbohydrate interactions in nematode-trapping fungi. p 419-432. In: Jennings, D.H. and A.D.M. Rayner (eds.). The Ecology and Physiology of the Fungal Mycelium. Cambridge Univ. Press. Cambridge, UK.

Odum, E.P. 1971. Fundamentals of Ecology. 3rd Edition. W.B. Saunders Co. Philadelphia, PA.

Ohr, H.D., D.E. Munnecke and J.L. Bricjer. 1973. The interaction of Armillaria mellea and Trichoderma spp. as modified by methyl bromide. Phytopath. **63**:965-973.

Overeem, J.C. and A. Mackor. 1973. Scytalidic acid, a novel compound from Scytalidium species. Recueil. **92:**349-359.

Owen, C.A., Jr. 1982. Biochemical Aspects of Copper. Copper Proteins, Ceruloplasmin, and Copper Protein Binding. Noyes Publications. Park Ridge, NY.

Oxley, T.A. 1976. Report on biological control of decay in poles. Int. Res. Group Wood Preserv. IRG/WP/149

Panda, T., V.S. Bisaria and T.K. Ghose. 1989. Method to estimate growth of Trichoderma reesei and Aspergillus wentii in mixed culture on cellulosic substrates. Appl. Env. Microbiol. **55:**1044-1046.

Papavizas, G.C. 1966. Biological methods for the control of plant diseases and nematodes. p 82-94. In: Pest Control by Chemical, Biological, Genetic, and Physical Means - A Symposium. USDA ARS 33-110. Washington, DC.

Papavizas, G.C. (ed). 1981. Biological Control in Crop Production. Allanheld Osmun Co. London, UK.

Papavizas, G.C. 1985. Trichoderma and Gliocladium: biology, ecology, and potential for biocontrol. Ann. Rev. Phytopath. **23:**23-54.

Papavizas, G.C. and J.A. Lewis. 1988. The use of fungi in integrated control of plant diseases. p 235-253. In: Burge, M.N. (ed). Fungi in Biological Control Systems. Manchester Univ. Press. Manchester, UK.

Papavizas, G.C. and R.D. Lumsden. 1980. Biological control of soilborne fungal propagules. Ann. Rev. Phytopath. **18:**389-413.

Papavizas, G.C., J.A. Lewis and T.H. Abd-El Moity. 1982. Evaluation of new biotypes of Tricoderma harzianum for tolerance to benomyl and enhanced biocontrol capabilities. Phytopath. **72:**126-132.

Park, D. 1960. Antagonism - the background of soil fungi. p 148-159. In: Parkinson, D. and J.S. Waid (eds). The Ecology of Soil Fungi. Liverpool Univ. Press. Liverpool, UK.

Park, D. 1967. The importance of antibiotics and inhibiting substances. p 435-447. In: Burges, A. and F. Row (eds). Soil Biology. Academic Press. New York, NY.

Park, D. 1968. The ecology of terrestrial fungi. p 1-39. In: Ainsworth, G.C. and A.S. Sussman (eds). The Fungi. Vol.3. Academic Press, London, UK.

Park, D. 1976. Carbon and nitrogen levels as factors influencing fungal decomposers. p 41-59. In: Andersen, J.M. and A. Macfadyen (eds). The Role of Terrestrial and Aquatic Organisms in Decomposition Processes. Blackwell Scientific Publ. Oxford, UK.

Parker, C.A., A.D. Rovira, K.J. Moore and P.T.W. Wong (eds). 1985. Ecology and Management of Soilborne Plant Pathogens. American Phytopathological Society. St. Paul, MN.

Paszcynski, A., V.-B. Huynh and R. Crawford. 1986. Comparison of ligninase-1 and peroxidase-M2 from the white-rot fungus Phanerochaete chrysosporium. Arch. Biochem. Biophys. **244:**750-765.

Paterson, A., A.J. McCarthy and P. Broda. 1984. The application of molecular biology to lignin degradation. p 33-68. In: Grainger, J.M. and J.M. Lynch (ed.). Microbiological Methods for Environmental Biotechnology. Society for Applied Bacteriology. Tech. Series No. 19. Academic Press. London, UK.

Peterson, G.L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. **83:**346-356.

Peterson, G.L. 1979. Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Anal. Biochem. **100:**201-220.

Peterson, G.L. 1983. Determination of total protein. Methods in Enzymology. **91:**95-119.

Phillips, L.E. and T.J. Leonard. 1976a. Extracellular and intracellular phenoloxidase activity during growth and development in Schizophyllum. Mycologia. **68:**268-276.

Phillips, L.E. and T.J. Leonard. 1976b. Benzidine as a substrate for measuring phenoloxidase activity in crude cell-free extracts of Schizophyllum commune. Mycologia. **68:**277-285.

Pierce Chem. Co. 1988a. Coomassie protein asssay reagent. Info #23200. Pierce Chemical Co. Rockford, IL.

Pierce Chem. Co. 1988b. BCA Protein asssay reagent. Info #23220/23225. Pierce Chemical Co. Rockford, IL.

Pizzi, A., W.E. Conradie, A. Jansen and A.R. Vosloo. 1987. A series of low toxicity wood preservatives. Int. Res. Group Wood Preserv. IRG/WP/3408.

Platt, M., Y. Hadar and I. Chet. 1985. The decolorization of the polymeric dye Poly Blue by lignin degrading fungi. Appl. Microbiol. Biotechnol. **21:**394-396.

Pribnow, D., M.B. Mayfield, V.J. Nipper, J.A. Brown and M.H. Gold. 1989. Characterization of a cDNA encoding a manganese peroxidase, from the lignin-degrading basidiomycete Phanerochaete chrysosporium. J. Biol. Chem. **264:**5036-5040.

Prillinger, H. 1976. Genetische Kontrolle der Phenoloxidase "Laccase" des Ascomyceten Podospora anserina. Bibliotheca Mycologica. Band 51. Vaduz, FL.

Przybylowicz, P.R., B.R. Kropp, M.E. Corden and R.D. Graham. 1987. Colonization of Douglas-fir poles by decay fungi during air-seasoning. For. Prod. J. **37(4):**17-23.

Pugh, G.J.F. 1980. Strategies in fungal ecology. Trans. Brit. Mycol. Soc. **75:**1-14.

Raper, J.R. 1966. Genetics of Sexuality in Higher Fungi. Ronald Press Co. New York, N.Y.

Rawn, J.D. 1989. Biochemistry. Patterson Publ. Burlington, NC.

Rayner, A.D.M. 1976. Dematiaceous hyphomycetes and narrow dark zones in decaying wood. Trans. Brit. Mycol. Soc. **67:**546-549.

Rayner, A.D.M. 1977a. Fungal colonization of hardwood stumps from natural sources. I. Non-Basidiomycetes. Trans. Brit. Mycol. Soc. **69:**291-302.

Rayner, A.D.M. 1977b. Fungal colonization of hardwood stumps from natural sources. II. Basidiomycetes. Trans. Brit. Mycol. Soc. **69:**303-312.

Rayner, A.D.M. 1978. Interactions betwen fungi colonizing hardwood stumps and their possible role in determining patterns of colonization and succession. Ann. Appl. Biol. **89:**131-134.

Rayner, A.D.M. and L. Boddy. 1988. Fungal Decomposition of Wood. Its Biology and Ecology. John Wiley and Sons. Chichester, UK.

Rayner, A.D.M. and D. Coates. 1987. Regulation of mycelial organisation and responses. p 115-136. In: Rayner, A.D.M., C.M. Brasier and D. Moore (eds). Evolutionary Biology of the Fungi. Cambridge Univ. Press. Cambridge, UK.

Rayner, A.D.M. and N.K. Todd. 1977. Intraspecific antagonism in natural populations of wood-decaying basidiomycetes. J. Gen. Microbiol. **103:**85-90.

Rayner, A.D.M. and N.K. Todd. 1978. Polymorphism in Coriolus versicolor and its relation to interfertility and intraspecific antagonism. Trans. Brit. Mycol. Soc. **71:**99-106.

Rayner, A.D.M. and N.K. Todd. 1979. Population and community structure and dynamics of fungi in decaying wood. Adv. Bot. Res. **7:**333-420.

Rayner, A.D.M. and M.N. Turton. 1982. Mycelial interactions and population structure in the genus Stereum: S. rugosum, S. sanguinolentum and S. rameale. Trans. Brit. Mycol. Soc. **78**:483-494.

Rayner, A.D.M. and J.F. Webber. 1984. Interspecific mycelial interactions - an overview. p 382-417. In: Jennings, D.H. and A.D.M. Rayner (eds.). The Ecology and Physiology of the Fungal Mycelium. Cambridge Univ. Press. Cambridge, UK.

Read, S.M. and D.H. Northcote. 1981. Minimization of variation in the response to different proteins of the Coomassie Blue G dye-binding assay for protein. Anal. Biochem. **116**:53-64.

Reese, E.T. 1975. Polysaccharases and the hydrolysis of insoluble substrates. p 165-181. In: Liese, W. (ed). Biological Transformation of Wood by Microorganisms. Springer Verlag, Berlin, FRG.

Reid. I.D. 1979. The influence of nutrient balance on lignin degradation by the white-rot fungus Phanerochaete chrysosporium. Can. J. Bot. **57**:2050-2058.

Reinhammar, B. and B.G. Malmström. 1981. "Blue" copper-containing oxidases. p 109-149. In: Spiro, T.G. (ed.). Copper Proteins. John Wiley & Sons. New York, NY.

Ricard, J. 1966. Detection, identification, and control of Poria carbonica and other fungi in Douglas-fir poles. Ph.D. Thesis. OSU. Corvallis, OR.

Ricard, J. 1970. Biological control of Fomes annosus in Norway spruce (Picea abies) with immunizing commensals. Stud. For. Sue. Vol. 84.

Ricard, J. 1976. Biological control of decay in standing creosote-treated poles. J. Inst. Wood Sci. **7(4)**:6-9.

Ricard, J. 1977. Experience with immunizing commensals. Neth. J. Plant Path. **83(Suppl.1)**:443-448.

Ricard, J. and W.B. Bollen. 1968. Inhibition of Poria carbonica by Scytalidium sp. an imperfect fungus isolated from Douglas-fir poles. Can. J. Bot. **46**:643-647.

Ricard, J., M.M. Wilson and W.B. Bollen. 1969. Biological control of decay in Douglas-fir poles. For. Prod. J. **19(8)**:41-45.

Richardson, B. A. 1988. Organotin wood preservatives. Activity and safety in relation to structure. Preprints Am. Wood Preserv. Assoc. Ann. Meet. 1988. Minneapolis, MN.

Riddell , R.W. 1950. Permanent stained mycological preparations obtained by slide culture. Mycologia. **42**:265-270.

Rifai, M.A. 1969. A revision of the genus Trichoderma. Mycol. Papers. Vol. 116.

Rishbeth, J. 1950. Observations on the biology of Fomes annosus, with particular reference to East Anglian pine plantations. I. The outbreaks of disease and ecological status of the fungus", Ann. Bot. (N.S.). **14**:3-65-384.

Rishbeth, J. 1963. Stump protection against Fomes annosus. III. Inoculation with Peniophora gigantea. Ann. Appl. Biol. **52**:63-77.

Robb, D.A. 1984. Tyrosinase. p 207-240. In: Lontie, R. (ed.). Copper Proteins and Copper Enzymes. Vol. 2. CRC Press. Boca Raton, FA.

Ruel, K. and F. Barnoud. 1985. Degradation of wood by microorganisms. p 441-468. In: Higuchi, T. (ed). Biosynthesis and Biodegradation of Wood Components. Academic Press. Orlando, FA.

Ryvarden, L. 1978. Polyporaceae of North Europe. Vol. 1 & 2. Fungiflora. Oslo. Norway.

Saddler, J.N., C.M. Hogan and G. Louis-Seize. 1985. A comparison between the cellulase systems of Trichoderma harzianum E58 and Trichoderma reesei C30. Appl. Microbiol. Biotechnol. **22**:139-145.

Sanford, G.B. 1926. Some factors affecting the pathogenicity of Actinomyces scabies. Phytopath. **16**:525-547.

Schánel, L., R. Blaich and K. Esser. 1971. Functions of enzymes in wood decaying fungi. I. Comparative studies of intracellular and extracellular enzymes in Trametes versicolor and Trametes hirsuta. Arch. Microbiol. **77**:140-150.

Scheffer, T.C. 1973. Microbiological degradation and the causal organisms. p 31-106. In: Nicholas, D.D. (ed). Wood Deterioration and its Prevention by Treatments. Vol. I. Syracuse Univ. Press. Syracuse, NY.

Schmidt, O. and U. Kebernik. 1987. Wuchsansprüche, Enzyme und Holzabbau des auf Holz wachsenden Speisepilzes "Shii-take" (Lentinus edodes) sowie einiger seiner Homo- and Dikaryonten. Mat. Org. **22**:237-255.

Sebek, O.K. 1980. Microbial transformations of antibiotics. p 575-612. In: Rose, A.H. (ed). Microbial Enzymes and Bioconversions. Economic Microbiology Vol. 5. Academic Press. London, UK.

Seifert, K.A., C. Breuil, L. Rossignol, M. Best and J.N. Saddler. 1988. Screening for microorganisms with the potential for biological control of sapstain on unseasoned lumber. Mat. Org. **23**:81-95.

Sequeira, L. 1962. Influence of organic amendments on survival of Fusarium oxysporum f. cubense in the soil. Phytopath. **52**:976-982.

Sexton, C.M., J.J. Morrell, B.R. Kropp, M.E. Corden and R.D. Graham. 1990. Colonization of Douglas-fir poles during air seasoning: I. Identity of Basidiomycotina after 1, 2, or 3 years of exposure. Mycol. Res. (in review)

Sharon, N. and H. Lis. 1989. Lectins as recognition molecules. Science. **246:**227-234.

Sheldon, R.A. and J.K. Kochi. 1981. Metal-Catalyzed Oxidations of Organic Compounds. Academic Press, New York, NY.

Shepherd, D. and M. Carels. 1983. Product formation and differentiation in fungi. p 515-535. In: Smith, J.E. (ed). Fungal Differentiation. Marcel Dekker. New York, NY.

Shields, J.K. and E.A. Atwell. 1963. Effect of a mold, Trichoderma viride, on decay of birch by four storage-rot fungi. For. Prod. J. **13(7):**262-265.

Shields, J.K. and M. Shih. 1967. Reference culture collection of wood-inhabiting microorganisms. Can. For. Prod. Lab. Inform. Rep. No. OP-X-9.

Shigo, A.L. 1967. Successions of organisms in discolorization and decay of wood. Int. Rev. For. Res. **2:**237-299.

Shigo, A.L. 1970. An expanded concept of decay in living trees. Bull. No. 13. Fonds de recherche forestrieres de l'Universite Laval, Quebec, CND.

Shigo, A.L. and H.G. Marx. 1977. Compartmentalization of decay in trees. USDA For. Serv. Agric. Inf. Bull. No. 405.

Shimada, M., F. Nakatsubo, T.K. Kirk and T. Higuchi. 1981. Biosynthesis of the secondary metabolite veratryl alcohol in relation to lignin degradation in Phanerochaete chrysosporium. Arch. Microbiol. **129:**321-324.

Sierota, Z.H. 1976. Yield of dry mycelium of Trichoderma viride Pers. ex Fr. on some carbon and nitrogen sources. Eur. J. For. Path. **7:**65-76.

Skujins, J.J., H.J. Potgieter and M. Alexander. 1965. Dissolution of fungal cell walls by a streptomycete chitinase and ß(1→3)-glucanase. Arch. Biochem. Biophys. **111:**358-364.

Smith, J.F., N. Claydon, M.E. Love, M. Allan and D.A. Wood. 1989. Effect of substrate depth on extracellular endocellulase and laccase production of Agaricus bisporus. Mycol. Res. **93:**292-296.

Smith, P.K., R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson and D.C. Klenk. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150:**76-85.

Smith, R.S. 1973. Colonization and degradation of outside stored softwood chips by fungi. XVI:1-15. In: Proc. IUFRO Symp. Protection of Wood in Storage, Norway & Sweden, July 1972.

Smith, R.S. and A.J. Cserjesi. 1983. Protection from biodeterioration of unseasoned Canadian softwood lumber during storage and shipment. Biodeterioration. **5**:97-105.

Smith, S.M., J.J. Morrell and C.M. Sexton. 1990. Colonization of Douglas-fir poles during air seasoning: II. Distribution of selected fungi over a 3-year period. Mycol. Res. (in review)

Snyder, W.C. 1960. Antagonism as a plant disease control principle. p 127-136. In: Reitz, L.P. (ed). Biological and Chemical Control of Plant and Animal Pests. Am. Assoc. Adv. Sci. Washington, DC.

Snyder, W.C., G.W. Wallis and S.N. Smith. 1976. Biological control of plant pathogens. p 521-539. In: Huffaker, C.B. and P.S. Messenger (eds). Theory and Practice of Biological Control. Academic Press. New York, NY.

Sonnenbichler, J., V. Lamm, A. Gieren, O. Holdenrieder and H. Lotter. 1983. A cyclopentabenzopyranone produced by the fungus Heterobasidion annosum in dual cultures. Phytochem. **22**:1489-1491.

Stallings, J.H. 1954. Soil produced antibiotics - Plant disease and insect control. Bacteriol. Rev. **18**:131-146.

Stalpers, J.A. 1978. Identification of wood-inhabiting Aphyllophorales in pure culture. Stud. Mycol. No.16.

Stauffer, C.E. 1975. A linear standard curve for the Folin Lowry determination of protein. Anal. Biochem. **69**:646-648.

Steel, R.D. and J.H. Torrie. 1980. Principles and Procedures of Statistics: A Biometrical Approach. McGraw-Hill Book Co. New York. NY.

Stillwell, M.A., R.E. Wall and G.M. Strunz. 1973. Production, isolation, and antifungal activity of scytalidin, a metabolite of Scytalidium species. Can. J. Microbiol. **19**:597-602.

Straetmans, U. 1987. Some tests on ES-AS 11, a novel anti- sapstain formulation, and its properties. Int. Res. Group Wood Preserv. IRG/WP/3399.

Stranks, D.W.. 1976. Scytalidin, hyalodendrin, cryptosporiopsin - antibiotics for prevention of blue stain in white pine sapwood. Wood Sci. **9**:110-112.

Stroo, H.F., M.A. Jurek, J.A. Bumpus, M.F. Torpy and S.D. Aust. 1989. Bioremediation of wood preserving wastes using the white rot fungus

Phanerochaete chrysosporium. Preprints Am. Wood Preserv. Assoc. Ann. Meet. 1989. San Francisco, CA.

Strunz, G.M., M. Kakushima and M.A. Stillwell. 1972. Scytalidin: a new fungitoxic metabolite produced by a Scytalidium species. J. Chem. Soc. Perkin Trans. I. 18:2280-2283.

Sundheim, L. and A. Tronsmo. 1986. Hyperparasites in biological control. p 53-70. In: Mukerji, K.G. and K.L. Garg (eds). 1986. Biocontrol of Plant Diseases. Vol. 1. CRC Press. Boca Raton, FA.

Switzer, R.L. 1977. The inactivation of microbial enzymes in vivo. Ann. Rev. Microbiol. 31:135-157.

Talarek, W.G. 1988. The regulation of wood preserving industry wastes under the Resource Conservation and Recovery Act. Preprints Am. Wood Preserv. Assoc. Ann. Meet. 1988. Minneapolis, MN.

Tamimi, K.M. and S.A. Hutchinson. 1975. Differences between the biological effects of culture gases from several species of Trichoderma. Trans. Brit. Mycol. Soc. 64:455-463.

Tanaka, H., A. Enoki, G. Fuse and K. Nishimoto. 1988. Interactions in successive exposure of wood to varying wood-inhabiting fungi. Holzforschung. 42:29-35.

Taylor, A. 1986. Some aspects of the chemistry and biology of the genus Hypocrea and its anamorphs Trichoderma and Gliocladium. Proc. N.S. Inst. Sci. 36:27-58.

Taylor, R., G.C. Llewellyn, J.E. Mayfield, W.C. Shortle and W.V. Dashek. 1986b. Time-dependent appearance of extracellular polyphenol oxidase in relation to catechol-induced bimodal growth response of Coriolus versicolor. p 63-74. In: Llewellyn, G.C. and C.E. O'Rear (eds). Biodeterioration Research 1. Plenum Press. New York, NY.

Taylor, R., J.E. Mayfield, W.C. Shortle, G.C. Llewellyn and W.V. Dashek. 1986a. Attempts to determine whether the products of extracellular polyphenol oxidase modulate the catechol-induced bimodal growth response of Coriolus versicolor. p 43-62. In: Llewellyn, G.C. and C.E. O'Rear (eds). Biodeterioration Research 1. Plenum Press. New York, NY.

Thompson, W.S. 1973. Pollution Control. p 345-395. In: Nicholas, D.D. (ed). Wood Deterioration and Its Prevention by Preservative Treatments. Vol. II. Syracuse Univ. Press. Syracuse, NY.

Tien, M. and T.K. Kirk. 1984. Lignin-degrading enzyme from Phanerochaete chrysosporium: purification, characterization, and catalytic properties of a unique H2O2-requiring oxygenase. Proc. Natl. Acad. Sci. USA. 81:2280-2284.

Tien, M. and C.-P.D. Tu. 1987. Cloning and sequencing of a cDNA for a ligninase from Phanerochaete chrysosporium. Nature. **326:**520-523.

Tien, M., T.K. Kirk, C. Bull and J.A. Fee. 1986. Steady-state and transient-state kinetic studies on the oxidation of 3,4-dimethoxylbenzyl alcohol catalyzed by the ligninase of Phanerochaete chrysosporium. J. Biol. Chem. **261:**1687-1693.

Todd, N.K. and R.C. Aylmore. 1985. Cytology of hyphal interactions and reactions in Schizophyllum commune. p 231-248. In: Moore, D., L.A. Casselton, D.A. Wood and J.C. Frankland (eds). Developmental Biology of Higher Fungi. Cambridge Univ. Press. Cambridge, UK.

Tokimoto, K. 1985. Physiological studies on antagonism between Lentinus edodes and Trichoderma spp. in bedlogs of the former. [in Japanese with English figures and abstract]. Rep. Tottori Mycol Inst. (Japan). **23:**1-54.

Tokimoto, K. and M. Komatsu. 1979. Effect of carbon and nitrogen sources in media on the hyphal interference between Lentinus edodes and some species of Trichoderma. Ann. Phytopath. Soc. Japan. **45:**261-264.

Toole, E.R. 1971. Interaction of mold and decay fungi on wood in laboratory tests. Phytopath. **61:**124-125.

Traquair, J.A. and W.E. McKeen. 1977. Hyphal interference by Trametes hispida. Can. J. Microbiol. **23:**1675-1682.

Traquair, J.A. and W.E. McKeen. 1978. Necrotrophic mycoparasitism of Ceratocystis fimbriata by Hirschioporus pargamenus (Polyporaceae). Can. J. Microbiol. **24:**869-874.

Trojanowski, J. 1989. personal communication.

Tronsmo, A. and C. Dennis. 1979. Effect of temperature on antagonistic properties of Trichoderma species. Trans. Brit. Mycol. Soc. **71:**469-474.

Turner, E.M. 1974. Phenoloxidase activity in relation to substrate and development stage in the mushroom, Agaricus bisporus. Trans. Br. Mycol. Soc. **63:**541-547.

Turner, E.M., M. Wright, T. Ward, D.J. Osborne and R. Self. 1975. Production of ethylene and other volatiles and changes in cellulase and laccase activities during the life-cycle of the cultivated mushroom, Agaricus bisporus. J. Gen. Microbiol. **91:**167-176.

Tzean, S.S. and R.H. Estey. 1978. Schizophyllum commune Fr. as a destructive mycoparasite. Can. J. Microbiol. **24:**780-784.

Ueyama, A. 1966. The size effect of sample blocks on decay rate of beech wood (Fagus crenata) in laboratory tests. In: Becker, G. and W. Liese (eds). Holz und Organismen. Mat. Org. (Beiheft 1). p 301-307.

Ulmer, D.C., M.S. Leisola, B. Schmidt and A. Fiechter. 1983a. Rapid degradation of isolated lignins by Phanerochaete chrysosporium. Appl. Env. Microbiol. **45**:1795-1801.

Ulmer, D.C., M.S. Leisola, J. Puhakka and A. Fiechter. 1983b. Phanerochaete chrysosporium and lignin degradation: growth pattern and lignin degradation. Eur. J. Appl. Microbiol. Biotechnol. **18**:153-157.

Vance, C.P., T.K. Kirk and R.T. Sherwood. 1980. Lignification as a mechanism of disease resistance. Ann. Rev. Phytopath. **18**:259-288.

Vanek, Z. and Z. Hostalek (eds). 1986. Overproduction of Microbial Metabolites. Strain Improvement and Process Control Strategies. Butterworth Publ. Boston, MA.

Vannacci, G. and G.E. Harman. 1987. Biocontrol of sedd-borne Alternaria raphani and A. brassicicola. Can. J. Microbiol. **33**:850-856.

Veldkamp, H., H. van Gemerden, W. Harder and H.J. Laanbroeck. 1984. Competition among bacteria: an overview. p 279-280. In: Klug, A.J. and C.A. Reddy (eds). Current Perspectives in Microbial Ecology. American Society of Microbiology. Washington, DC.

Waksman, S.A. 1953. Sergei N. Winogradsky - His Life and Work. Rutgers Univ. Press. New Brunswick, N.J.

Wang, C.J.K. 1988. Microfungi. p C89-C250. In: Meyer, R.W., R.A. Zabel, C.J.K. Wang and F.C. Terracina (eds) Wood pole decay characterization. Vol.2: Soft rot characteristics and an identification manual for the decay fungi in utility poles in New York. Final Report EP 84-5. Empire State Electric Energy Research Corporation. New York.

Warzywoda, M., V. Ferre and J. Pourquie. 1983. Development of a culture medium for large-scale production of cellulolytic enzymes by Trichoderma reesei. Biotechnol. Bioeng. **25**:3005-3010.

Wazny, J., L. Brodziak and A. Kundzewicz. 1987. Biotic influence of some fungi on Lentinus edodes (Berk.) Sing. in laboratory experiments. Mat. Org. **22**:103-110.

Webster, J. and N. Lomas. 1964. Does Trichoderma viride produce gliotoxin and viridin?. Trans. Brit. Mycol. Soc. **47**:535-540.

Weindling, R. 1932. Trichoderma lignorum as a parasite of other soil fungi. Phytopath. **22**:837-845.

Weindling, R. 1941. Experimental consideration of the mold toxins of Gliocladium and Trichoderma. Phytopath. **31**:991-1003.

Weindling, R., H. Katznelson and H.P. Beale. 1950. Antibiosis in relation to plant diseases. Ann. Rev. Microbiol. **4**:247-260.

Westermark, U. and K.-E. Eriksson. 1974a. Carbohydrate-dependent enzymic quinone reduction during lignin degradation. Acta Chem Scand. **B28:**204-208.

Westermark, U. and K.-E. Eriksson. 1974b. Cellobiose:quinone oxidoreductase, a new wood-degrading enzyme from white-rot fungi. Acta Chem. Scand. **B28:**209-214.

Whipps, J.M., K. Lewis and R.C. Cooke. 1988. Mycoparasitism and plant disease control. p 161-187. In: Burge, M.N. (ed). Fungi in Biological Control Systems. Manchester Univ. Press. Manchester, UK.

Whitmore, F.W. 1982. Lignin-protein complex in cell walls of Pinus elliottii: amino acid constituents. Phytochem. **21:**315-318.

Wicklow, D.T. 1981. Interference competition and the organization of fungal communities. p 351-375. In: Wicklow, D.T. and G.C. Carroll (eds). The Fungal Community. Marcel Dekker, New York, NY.

Wilhelm, S. 1973. Principles of biological control of soil-borne plant diseases. Soil Biol. Biochem. **5:**729-737.

Willeitner, H. and R.D. Peek. 1988. Less pollution due to technical approaches on accelerated steam fixation of chromated wood preservatives. Int. Res. Group Wood Preserv. IRG/WP/3487.

Williams, S.T. 1982. Are antibiotics produced in soil? Pedobiologia. **23:**427-435.

Willick, G.E., R. Morosolo, V.L. Seligy, M. Yaguchi and M. Desrochers. 1984. Extracellular proteins secreted by the basidiomycete Schizophyllum commune in response to carbon source. J. Bacteriol. **159:**294-299.

Windels, C.E. and S.E. Lindow (eds). 1985. Biological Control on the Phylloplane. American Phytopathological Society. St. Paul, MN.

Windham, M.T., Y. Elad and R. Baker. 1986. A mechanism for increased plant growth induced by Trichoderma spp. Phytopath. **76:**518-521.

Winston, P.W. and D.H. Bates. 1960. Saturated solutions for the control of humidity in biological research. Ecology. **41:**232-237.

Wood, A.W. and S.T. Kellogg (eds). 1988a. Biomass. Part A Cellulose and Hemicellulose. Meth. Enzymology. Vol. 160.

Wood, A.W. and S.T. Kellogg (eds). 1988b. Biomass. Part B Lignin, Pectin and Chitin. Meth. Enzymology. Vol. 161.

Wood, D.A. 1980a. Production, purification, and properties of extracellular laccase of Agaricus bisporus. J. Gen. Microbiol. **117:**327-338.

Wood, D.A. 1980b. Inactivation of extracellular laccase during fruiting of Agaricus bisporus. J. Gen. Microbiol. **117:**339-345.

Wood, D.A. 1985. Production and roles of extracellular enzymes during morphogenesis of basidiomycete fungi. p 375-387. In: Moore, D. L.A. Casselton, D.A. Wood and J.C. Frankland (eds). Developmental Biology of Higher Fungi. Cambridge Univ. Press. Cambridge, UK.

Wood, D.A. and P.W. Goodenough. 1977. Fruiting of Agaricus bisporus. Changes in extracellular enzyme activities during growth and fruiting. Arch. Microbiol. **114:**161-165.

Wood, R.K.S. and M. Tveit. 1955. Control of plant diseases by use of antagonistic organisms. Bot. Rev. **21:**441-492.

Wood, T.M. 1969. Relationship between cellulolytic and pseudo-cellulolytic microorganisms. Biochim. Biophys. Acta. **192:**531-534.

Wood, T.M. 1985. Properties of cellulolytic enzyme systems. Biochem. Soc. Trans. (UK). **13:**407-410.

Wood, T.M. and K.M. Bhat. 1988. Methods for measuring cellulase activities. Methods in Enzymology. **160:**87-112.

Wood, T.M. and S.I. McCrae. 1979. Synergism between enzymes involved in the solubilization of native cellulose. p 181-210. In: Brown, R.D. and L. Jurasek (ed). Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis. Advances in Chemistry. Vol. 181. American Chemical Society. Washington, DC.

Yoder, O.C. 1980. Toxins in pathogenesis. Ann. Rev. Phytopath. **18:**103-129.

YSI Inc. 1988. YSI Model 23A and 23AM Glucose Analyzer Instruction Manual. Yellow Springs Instrument Co. Yellow Springs, OH.

Zabel, R.A., F.F. Lombard and A.M. Kenderes. 1980. Fungi associated with decay in treated Douglas-fir transmission poles in the northeastern United States. For. Prod. J. **30(4):**51-56.

Zabel, R.A., C.J.K. Wang and F.C. Terracina. 1982. The fungal associates, detection, and fumigant control of decay in treated southern pine poles. Final Report EPRI EL-2768. Electric Power Res. Ins. Palo Alto. CA.

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

Zahora, A.R. and J.J. Morrell. 1987. Influence of wood moisture content on the fungitoxicity of methylisothiocyanate in Douglas- fir heartwood. Int. Res. Group Wood Preserv. IRG/WP/3430.