STUDIES ON MICROFUNGI FROM LITTER AND SOIL IN RELATION TO THE ECOLOGY OF LIGNIN DECOMPOSITION

A thesis presented for the degree

of

Doctor of Philosophy

in the University of Stirling

by

R. L. B. Black B.A. (Oxon.)

Department of Biology, University of Stirling.

October, 1974.

ABSTRACT

The studies reported here on microfungi from litter and soil involved:

1) The behaviour of microfungi in pure culture towards lignin related-phenolic acids.
2) The chemistry and microbiology of Scots Pine litter in relation to lignin decomposition.
3) The decomposition of ferulic acid by mixed populations of microorganisms.

Ferulic acid incorporated into agar could be inhibitory or stimulatory to the spore germination and germ hyphal growth of microfungi depending upon its concentration and the fungal species. Within a certain concentration range, the total growth potential of some Angiosperm leaf saprophytes was depressed relative to control whereas there was an increase in the case of conifer needle litter saprophytes and some common soil fungi. Other phenolic acids produced similar patterns of results but the differential effects were generally less marked.

The ability of litter and soil microfungi to utilise ferulic acid as a sole carbon source was defined from the decomposition of the substrate after inoculating culture solution with spores. The pattern in the ability to decompose ferulic acid was not related to the effects of this compound on germination on agar or in the liquid medium.

Dothichiza pityophila and Fusicoccum bacillare were isolated as the principal primary saprophytes of Scots Pine needle litter while the widespread primary saprophytes of

Angiosperm leaf litters, including Aureobasidium pullulans were absent, confirming and reinforcing the results of previous European work. The use of Infra-red spectroscopy of finely divided, but unextracted, Pine needles and humus to follow the course of the decomposition of the litter permitted an evaluation of the role of soil-inhabiting fungi in this process.

Burial of litter needles in a deciduous woodland soil or in a field soil led to colonisation by several soil fungi not characteristic of Pine litter, notably Gliocladium roseum and an Actinomycete of genus Nocardia. These two species were able to colonise the needle interior in the presence of needle saprophytes surviving in buried needles whereas Trichoderma viride remained restricted to the needle surface by prior-colonisation.

Finally, experiments are described in which Pine needle and soil material from the burial experiments and samples of two other soils of contrasting ligninolytic characteristics were used as inoculum for the decomposition of ferulic acid, the results of these experiments relating to known microbiological and chemical properties of the soils.

The importance of phenolic acids released during lignin decomposition in the fungal colonisation of substrates in litter and soil is discussed, and some conclusions are made regarding the validity of phenolic acids as 'lignin model compounds'.

ACKNOWLEDGEMENTS

My special thanks go to my supervisor, Dr. Neville Dix, for his unfailing enthusiasm and encouragement. I should also like to thank all my colleagues at Stirling with whom I have had much helpful discussion, particularly Dr. Peter Bannister for advice on numerical techniques.

This study was undertaken during the tenure of a Science Research Council research studentship.

I am grateful to Miss Margaret Keilt for typing this thesis.

The work presented in this thesis is the result of my own investigations and has neither been accepted nor is being submitted for any other degrees.

R. Black ..... Candidate

Neville Dix ..... Supervisor

Oct 28 1974 ..... Date

Presque

À Fontainebleau
Devant l'hôtel de l'Aigle Noir
Il y a un taureau sculpté par Rosa Bonheur
Un peu plus loin tout autour
Il y a la forêt
Et un peu plus loin encore
Joli corps
Il y a encore la forêt
Et le malheur
Et tout à côté le bonheur
Le bonheur avec les yeux cernés
Le bonheur avec des aiguilles de pin dans le dos
Le bonheur qui ne pense à rien
Le bonheur comme le taureau
Sculpté par Rosa Bonheur
Et puis le malheur
Le malheur avec une montre en or
Avec un train à prendre
Le malheur qui pense à tout...
À tout
À tout....à tout....à tout....
Et à Tout
Et qui gagne 'presque' à tous les coups
Presque.

Jaques Prévert

CONTENTS

GENERAL INTRODUCTION

Lignin has a type of chemical structure that is unique among biological polymers conferring a remarkable degree of resistance to chemical and biological attack. A consequence of this is the attention that has been given to the microbiological decomposition of lignin by workers in various fields:- the pathology of woody plants, timber decay, disposal of waste products of paper manufacture and the decomposition of plant remains on and in the soil. In litter/soil systems in particular the existence of slowly decomposing lignin is of considerable ecological significance and the work reported here relates to this aspect.

Chemically lignin is best visualised as "a three-dimensional, branched polymer formed from the oxidative polymerisation of three substituted cinnamyl alcohols:- p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol" (Kirk, 1971). Another consequence of this structure, and the inter-linking of lignin and cell-wall carbohydrate, is that it is not possible at present to obtain chemically pure and unaltered lignin in a representative yield. 'Björkman lignins' (Björkman 1956) have been widely used in recent years for decomposition studies as they are thought to be relatively unchanged but this fraction is only a small part of the native lignin(Harkin, 1967). Biosynthetic lignins are thought to resemble 'Björkman' lignins so this same criticism applies to these materials. An alternative is to use native material, as favoured by Mangenot & Kiffer (1972) for example, but here the assay of the lignin may

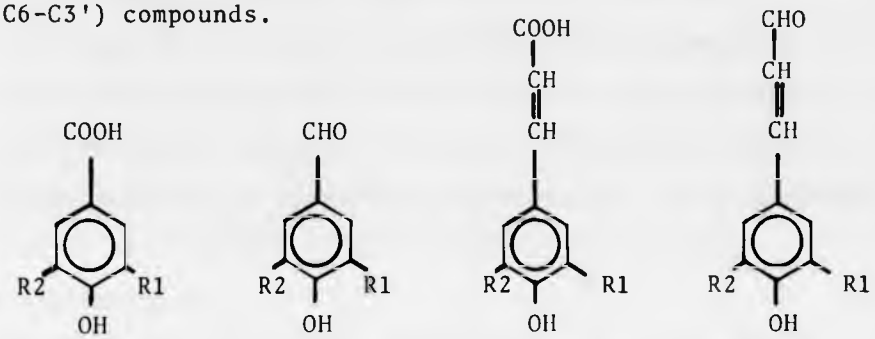
present problems and also the activity of organisms decomposing other fractions of the substrate. Gulyas (1957) showed in fact that a chemically extracted lignin was decomposed less readily than the native lignin in wheat straw. This material was a lot milder than the phenol extracted lignin used by Fischer (1953) or sulphuric acid lignin of Smith & Brown (1935).

Much of the available data on lignin decomposition concerns the activity of white rot fungi - various members of the Homobasidiomycetes and also certain Ascomycete species. Falck & Haag (1927) showed that the significance of brown and white rotting processes of wood lay in the preferential removal from the substrate of cellulose by brown rot fungi and lignin by white rot fungi although there is never exclusive decomposition of either cellulose or lignin. It is important to note here that white rot fungi can oxidise lignin completely to carbon dioxide and water; they carry out a true 'decomposition' as defined by Kirk (1971). Brown rot fungi on the other hand can only carry out a limited 'degradation' i.e. they do not destroy the lignin polymer as such, but cause some structural changes, possibly related to the bonding between lignin and polysaccharide.

Biochemical studies on the decomposition of lignin by white rot fungi (e.g. Ishikawa et al., 1963a) have shown that various phenolic compounds are liberated during this process; so presumably microorganisms attacking lignin would be subjected to their presence, and organisms using lignin as a carbon source would have to metabolise them. In addition, the alkaline nitrobenzene oxidation of lignin yields several derivatives of benzoic acid.

There has been much discussion as to how important is

this release during decomposition of single ring compounds, particularly in relation to the significance of extracellular Laccase enzymes. However, phenolic decomposition products of lignin have been used extensively as Lignin model compounds in order to circumvent the problem of lignin extraction. A series of substituted Benzoic and Cinnamic acids (and the corresponding aldehydes) are particularly appropriate, among a range of benzyl ('C6-C1') and phenylpropyl ('C6-C3') compounds.



R1 = R2 = H

p-hydroxybenzoic acid | p-hydroxy benzaldehyde | p-coumaric acid | P-coumaraldehyde

R1 = OCH_3 R2 = H

vanillic acid | vanillin | ferulic acid | coniferaldehyde

R1 = R2 = $-OCH_3$

syringic acid | syringaldhyde | sinapic acid | sinapaldehyde

The decomposition of lignin in leaf litter was first investigated systematically by Lindeberg (1946) who showed that a wide variety of soil inhabiting Hymenomycetes were ligninolytic. Much later Sundman et al. (1964) used populations of bacteria and fungi from soil to study the decomposition of lignin in brown rotted wood. They found that the soil inocula decomposed lignin more vigorously

than the white rotting fungus Coriolus versicolor, which could not in fact compete with the soil microbial populations. The importance of these two pioneer studies was the implication that lignin decomposition in the soil, being spread over a wide range of microorganisms, cannot be resolved into a division of function between white rot and brown rot activities, comparable to that in wood decay.

Meanwhile, several groups of workers had been investigating the details of the metabolic pathways involved in the complete assimilation of the C6-C3 and C6-C1 breakdown products of lignin; Henderson (1956, 1961b) and Cain et al. (1968) on microfungi; Flaig & Haider (1961a) and Ishikawa et al. (1963b, c) on white rot Basidiomycetes.

But most importantly, Henderson and her co-workers had screened species of Fungi Imperfecti from soil for the ability to utilise p-hydroxy-benzaldehyde, vanillin, syringaldehyde and ferulic acid (Henderson 1960, 1961a, Henderson & Farmer, 1955); and they had also used a soil enrichment technique for isolating fungi that could metabolise these compounds (Henderson, 1966, Jones & Farmer 1967). As with lignin, the ability to grow on these simple substrates seemed to be widespread among soil fungi, although some fungi could utilise particular compounds more readily than others and not all species could metabolise every compound completely.

The ligninolytic activity of micro-fungi in pure culture has been studied by Haider & Domsch (1969) using ultra-thin foils of maple wood, Gulyas (1967) with isolated lignin from wheat straw, Jones & Farmer (1967) with lignin from

Phragmites and barley straw, and Fischer (1953) using a phenol lignin. Due to the sparcity of this section of the literature and the difficulties attendant upon the use of native or isolated lignin rather few microfungi from soil have been shown convincingly to decompose lignin.

In spite of the lack of information concerning which fungi are responsible for lignin decomposition in the soil, it is clear, however, that there is another important difference between lignin decomposition in this situation and that in wood. In soil the process is characteristically incomplete, with the residues being important intermediates in the formation of humic substances.

The analysis of lignins and humic acids by infra-red spectroscopy has provided evidence for the gradual transformation of lignin to substances resembling humic acids, during the rotting of wheat straw (Flaig et al., 1959) and the degradation of a Björkman lignin by soil microorganisms. (Wojtas-Wasilewska et al. 1973). That the fulvic acid fraction of soil organic matter may contain partially degraded lignins and tannins in the process of humification has been suggested by Sinha (1972).

Various models have been suggested for the formation of humic substances from simple phenolic compounds released during lignin decomposition which generally involve enzymic oxidation of the phenols to quinones and autoxidation of the latter with nitrogen compounds to form co-polymers, (Flaig 1964, Whitehead & Tinsley 1964) although the situation is complicated by phenol synthesis and humic acid formation by fungi from non-aromatic substrates. (Martin & Haider 1969, Martin et al. 1972). Grabbe & Haider (1971a,b) and

Martin & Haider (1969) reported the incorporation of phenolic decomposition products of lignin into the humic acids produced during the growth of micro-fungi and Basidiomycetes on plant residues.

Perhaps one of the most significant discoveries in this field was that the proportions of p-hydroxyphenyl, guaiacyl (vanillyl) and syringyl residues in humic acids from different soils bore a relation to the proportions of these types of aromatic nuclei in the lignin from the vegetation overlying the soils (Burges et al. 1964).

Another important aspect of the incompleteness of lignin breakdown in soil is the release of phenolic acids related to lignin into the environment. Whitehead (1964) found p-hydroxybenzoic, vanillic, p-Coumaric and Ferulic acids in soil; and lignin related phenolic acids have been extracted with cold water from cereal residues by Börner (1960) and from conifer and deciduous leaf litters by Bruckert and colleagues (Bruckert & Jaquin 1966, Jaquin & Bruckert 1965, Vedy & Bruckert 1970).

Free phenolic acids have been implicated in the complexing of soil cations (Bloomfield, 1963) and therefore in podzolisation (Bruckert's group above); in the toxicity of crop residues to plants (Patrick & Tousson, 1965, Guenzi & McCalla 1966, Winter & Schönbeck 1957); and in the phenomenon of soil fungistasis (Lingappa & Lockwood 1962, Maurer & Baker 1964, Lewis & Papavizas 1968). Phenolic acids have been specifically suggested as causes of phytotoxicity.

The various characteristics of the breakdown of lignin in soil and its effects have been summarised very elegantly by Mangenot & Kiffer (1972) who considered these processes

in the light of a postulated interaction between brown and white rot - like processes in the soil:-

"In the first case glucans are degraded preferentially in such a way that the structure of plant matter is gradually destroyed. At the same time the lignin is isolated and undergoes alterations which can include splitting-off of side chains and the opening of some benzene rings; the lignin becomes richer in nitrogen and would seem to be transformed into dark substances partially soluble in alkali. This picture corresponds to brown and soft rotting, the work of complex populations in which cellulolytic organisms abound.

In a second process lignin is fragmented at the same time as the glucan framework - but not necessarily at the same rate - with the result that the materials attacked retain initially their integrity during decolourisation and loss of mechanical strength. At the same time, soluble compounds are formed, including phenolic acids and aldehydes split off from the lignin molecule. The latter are relatively unstable and can be metabolised, or oxidised to form brown substances, somewhat condensed. This second kind corresponds to white rotting, of which the main agents are higher Basidiomycetes and a few Ascomycetes.

There is very little information on the fate of lignin in soils and yet it is possible that, following the schemes above, the degradation of lignified materials could influence, through opposing factors, soil development.

In the case of brown rotting, lignin could be gradually transformed into substances analogous to humus. In the case of "white rotting ('la corrosion') the soluble substances produced could have many effects; for example

in the light of a postulated interaction between brown and white rot - like processes in the soil:-

"In the first case glucans are degraded preferentially in such a way that the structure of plant matter is gradually destroyed. At the same time the lignin is isolated and undergoes alterations which can include splitting-off of side chains and the opening of some benzene rings; the lignin becomes richer in nitrogen and would seem to be transformed into dark substances partially soluble in alkali. This picture corresponds to brown and soft rotting, the work of complex populations in which cellulolytic organisms abound.

In a second process lignin is fragmented at the same time as the glucan framework - but not necessarily at the same rate - with the result that the materials attacked retain initially their integrity during decolourisation and loss of mechanical strength. At the same time, soluble compounds are formed, including phenolic acids and aldehydes split off from the lignin molecule. The latter are relatively unstable and can be metabolised, or oxidised to form brown substances, somewhat condensed. This second kind corresponds to white rotting, of which the main agents are higher Basidiomycetes and a few Ascomycetes.

There is very little information on the fate of lignin in soils and yet it is possible that, following the schemes above, the degradation of lignified materials could influence, through opposing factors, soil development.

In the case of brown rotting, lignin could be gradually transformed into substances analogous to humus. In the case of "white rotting ('la corrosion') the soluble substances produced could have many effects; for example

react with cations, contribute to fungistasis, exert an antagonistic effect on the micro-fauna or influence the physiology of higher plants". (Translated).

Current theories of the mechanism of soil fungistasis are not based on the action of preformed chemical inhibitors; they take account of the fact that the active growth of microorganisms is necessary, because, for example, fungistasis is completely eliminated from soil by sterilisation by gamma-radiation (Kusakari & Takagi, 1973). Lingappa & Lockwood (1962) concluded that lignin decomposition products may be important in fungistasis, after demonstrating their inhibitory effect on soil fungi. They were at that time interested in the inhibitor theory of fungistasis; but they were aware that lignin decomposition products could not be a source of widespread fungistasis because fungistasis is a feature of soils of low organic matter content as well as more normal soils.

Maurer & Baker (1964) obtained decreased severity of Bean root-rot through lignin amendments to soil but this was regarded as being due to competition for nutrients provided by the decomposition of lignin leading to a suppression of the pathogen, rather than to a direct effect from decomposition products. Lewis & Papavizas (1968) found that lignin amendments were ineffective against Fusarium solani in soil and that the severity of bean root rot was increased, possibly because there were phytotoxic effects causing increased susceptibility to the disease.

However, phenolic compounds have been implicated widely as providing defence mechanisms in plants against fungal pathogens because of their occurrence in the living plant and the activity of such compounds in vitro against the growth of pathogens. Specific reference to phenolic acids

which are also decomposition products of lignin is given in the reports of Li et al. (1969, 1972), Srinivasan & Naransimhan (1971), Christie (1965) and Molot (1970) and there is the very interesting work of van Sumere et al. (1957) and Macko et al. (1972) on endogenous inhibitors of germination of rust spores. As with the study of soil components with anti-fungal properties, there are great difficulties in interpreting results in the light of the situation in vivo, let alone in directly experimenting with the latter. Most of the studies done in vivo, in fact, have not shown the direct effect of preformed phenolics on a pathogen but effects resultant upon an interaction of host and pathogen. Thus there is the inhibition of fungal pectinases by oxidation products of pre-formed phenolics (Deverall & Wood 1961, Wilson et al. 1973, Metliski et al. 1973) or the synthesis of structural substances from such phenolics (Friend et al. 1973, Sridhar & Ou 1974).

It can be concluded from these considerations that from the viewpoint of fungal ecology the effects of decomposition products of lignin deserve more attention. On the one hand there is the apparently widespread ability of fungi from soil to metabolise them, and also their involvement in humification (i.e. these compounds are probably valid substitutes for lignin in decomposition studies); while on the other hand there seems to be the general principle that phenolic compounds, and certain phenolic acids in particular, are inhibitory to fungi.

However in previous work concerning phenolic acids and microfungi from soil there has been little attention paid to the ecology of the particular fungi being tested, in relation

to the fairly well defined groups of fungi that do occur on different substrates and to a certain extent in different soils. And so consideration has been given to this aspect in a study of the interactions between phenolic acids and micro-fungi.

In his review of the ecology of fungi colonising decaying plant material above the soil Hudson (1968) put forward a generalised pattern of succession on this type of substrate consisting of a series of waves of fungi belonging to different ecological groups. The essential features of this pattern (for non-lignicolous substrates) are given below.

1. PARASITES	Ascomycetes & Fungi Imperfecti	Host specific or restricted
2. COMMON PRIMARY SAPROPHYTES)	Ascomycetes Fungi Imperfecti	May be weak parasites
3. RESTRICTED SECONDARY SAPROPHYTES)	Ascomycetes, Basidiomycetes Fungi Imperfecti	
4. SOIL FUNGI	All groups	On some substrates in contact with mineral soil.

The scheme presented here is very generalised and the only group to be represented in practically every substrate are the common primary saprophytes. In temperate situations the characteristic fungi in this group are Cladosporium herbarum, Alternaria tenuis, Epicoccum nigrum, Aureobasidium pullulans and Botrytis cinerea. Hudson has considered in detail suggestions put forward to explain the widespread occurrence of these fungi, and, since they appear to be very successful colonisers, the reasons for their later disappearance from the substrate.

The leaf litter of Scots Pine (Pinus sylvestris) is interesting in this context because the common primary saprophytes are apparently absent from this material with the possible exception of Aureobasidium pullulans (which has been confused with Dothichiza (=Sclerophoma) pityophila) as the very comprehensive work of Kendrick & Burges (1962) indicated. This may be a feature of the litter of other species of Pinus in Britain at least, (Parkinson & Balasooriya, 1967, 1969, Balasooriya & Parkinson 1967) although Widden & Parkinson (1973) found that only Botrytis cinerea was absent from the litter of Pinus spp. in Canada.

Even disregarding this question, the pine leaf litter system has many features of interest. In addition to the studies on it mentioned above there have been those of Gremmen (1957) and Hayes (1965a, b) all of which have led to the characterisation of several host specific or restricted parasites, disappearing after different periods after leaf fall (e.g. Lophodermium pinastri, Dothichiza pityophila,) specific saprophytes (e.g. Desmazierella acicola, Helicoma monospora, Sympodiella acicola); and some very common soil fungi, particularly Trichoderma viride, Penicillium spp. and members of the Mucorales. The occurrence of this latter group of fungi on the fermentation layers of the litter (F1 and F2) seems particularly significant in view of the apparent absence of the common primary saprophytes.

Of importance also is the superficial colonisation of these soil fungi on F layer needles, as revealed by the use of washing and surface sterilisation techniques. The Mucorales may be sugar fungi growing in association with

cellulolytic and lignin decomposing Basidiomycetes, while Kendrick & Burges regarded T. viride and the Penicillia as being present as a high spore potential only. Certainly the F2 layer is decomposed very slowly, only finally being reduced to amorphous humus with the agency of the microfauna.

In addition to mycological studies there have been a number of important comparative studies on the rate and nature of the decomposition of litter of coniferous and deciduous trees, those of Bruckert and co-workers mentioned above, Beck et al .(1969 and Nykvist (1959, 1963). This subject has attracted the attention of French workers particularly because the long decomposition period of conifer litter is associated with mor humus formation and podzolisation. This research has yielded important data on the production of phenolic acids in litter, showing that although the rate of release of these compounds is slower in conifer leaf litter, they are less readily broken down than in deciduous litter. Hence there is more possibility of phenolic acids produced from lignin exerting effects in conifer leaf litter than in deciduous litter.

It thus seemed worthwhile to study the behaviour of fungi from different ecological groups, particularly microfungi colonising pine litter, towards phenolic acid decomposition products of litter; and to investigate the ecology of these fungi in relation to lignin decomposition.

The investigations reported here involve studies on microfungi in pure culture, experiments with mixed populations on pine needles and soil particles and chemical investigations of pine leaf litter.

The effect of phenolic acids on the germination of

fungi in pure culture was studied, and the decomposition of these compounds by fungi in pure culture and in mixed populations.

MATERIALS AND METHODS

FUNGI

Isolates used in pure culture work with phenolic acids

The following isolates of microfungi were used in this study:
Mortierella ramanniana (Moller) Linnemann (2 isolates), Mucor hiemalis Wehmeyer (2 isolates), M. racemosus Fresenius, Desmazierella acicola Lib., Aureobasidium pullulans (de Bary) Arnaud (Syn. Guignardia fulvida Sanderson), Botrytis cinerea Pers. ex Fr., Cladosporium herbarum Link ex S.F. Gray, Dreschlera rostrata (Drescler) Richardson et Fraser, Fusarium oxysporum Schlecten., Gilmaniella humicola Barron, Gliocladium roseum Bainier, Penicillium expansum Link ex S.F. Gray, P. funiculosum Thom, P. janthinellum Biourge, P. spinulosum Thom, Stemphylium dendriticum Sousa da Camara, Thysanophora penicilloides (Roum) Kendrick, Trichoderma viride Pers. ex S.F. Gray (7 isolates) Coniella sp., Dothichiza pityophila (Corda) Petrak (2 isolates), Phoma eupyrena Sacc.; full details of the sources of these isolates and the substrates from which they were obtained are given in Appendix 1.

Maintenance of cultures

Cultures of the fungi were maintained on Potato Dextrose Agar (Oxoid) or on Malt Extract Agar (Oxoid) in

Petri dishes when spore suspensions were required, and stock cultures were kept on slopes.

ACTINOMYCETE

One isolate of the genus Nocardia was used. Cultures were maintained on Nutrient Agar (Oxoid).

PHENOLIC ACIDS

Ferulic acid and p-hydroxybenzoic acid were obtained from Kodak, vanillic acid from K & K Laboratories and sinapic, p-coumaric, protocatechuic and syringic acids from Koch-Light Laboratories. All compounds gave only a single trace on Thin Layer Chromatography except syringic acid which showed a very slight trace of an impurity. Ferulic acid was re-crystallised from hot aqueous methanol.

PREPARATION OF MEDIA

Agar

Tap water agar for spore germination tests was prepared with 1.5% Difco Bacto Agar and tap water. Malt agar for Pine needle isolations was made up from 2 % Malt Extract (Oxoid), 1.5% Oxoid Agar No. 1 and distilled water.

After mixing the media were sterilised by autoclaving at 121^{o}C for 15 minutes.

Liquid culture solutions

The two basal media used in the study of the decomposition of phenolic acids and in the studies on spore germination in liquid medium had the following compositions:

Czapek salts at pH 4.5		Ammonium nitrate medium at pH 5.5	
$NaNO_3$	2.0 g	NH_4NO_3	5.0 g
KH_2PO_4	2.0 g	KH_2PO_4	1.5 g
KCl	0.5 g	$MgSO_4.7H_2O$	0.5 g
$MgSO_4.7H_2O$	0.5 g	$CaCO_3$	0.2 g
$FeSO_4.7H_2O$	0.01 g	$FeSO_4.7H_2O$	0.01 g
Distilled water	1000 cm^3	Distilled water	1000 cm^3

The solutions were autoclaved at 121°C for 15 minutes and after correcting the volume of the cooled solutions the phenolic acids were added to give the required concentrations (the solutions being re-warmed slightly to dissolve ferulic acid), and finally the media were re-sterilised by membrane filtration.

EXPERIMENTAL TECHNIQUES

pH determinations

A Corning (Model 7) pH meter was used.

Preparation of spore suspensions

Spore suspensions were prepared by washing off spores from cultures 14-28 days old with sterile distilled water, centrifuging and resuspending the spores at the appropriate concentration; for the agar block tests and the decomposition experiments the concentration of spores

(and cells of Nocardia) was 2-6 X 10^5 cm^{-3}; for studies of spore germination in liquid medium the concentration was 1-3 X 10^6 cm^{-3}.

Assay of phenolic acids on spore germination and germ hyphal growth on agar

Water agar blocks were formed in metal bottle caps, 2 cm in diameter, from which the rubber seals had been removed. After the application of phenolic acids the blocks were manipulated according to the method of assay. For the preliminary tests with six phenolic acids, a central well was made in the block by removing a core with a Number 2 cork borer. A drop of spore suspension pipetted into the well was absorbed into the agar, depositing the spores over the walls of the well, and from which hyphae grew out over the surface of the block. The growth of the hyphae in this manner was facilitated by hanging the bottle caps upside down from the lid of the damp chamber by surface tension. For measurement of the effects of ferulic acid and sinapic acid on the spore-germination and hyphal growth, an outer ring of agar was removed from the block, leaving a central disc on which the spore suspension was deposited.

The preparations were incubated in glass Petri dishes kept damp with a moist seed test pad. After incubation the agar blocks were transferred to microscope slides, stained with Lactophenol/Cotton Blue and examined microscopically.

Spore germination tests in liquid culture

25 cm^3 aliquots of sterile culture solution (Czapek medium ± ferulic acid) were measured into 150 cm^3 sterile conical flasks, and inoculated with 0.5 cm^3 of spore suspension (to give a spore concentration of about 5×10^4 cm^{-3}). Aeration of the cultures was achieved by magnetic stirring using autoclavable polypropylene-covered 'fleas'. The flasks were incubated at 25°C in a constant temperature room, and to protect the cultures from the heat given off from the magnetic stirrers the flasks were supported above the stirrers.

Percentage germination and germ tube lengths were examined in drops of the cultures pipetted onto cavity slides and stained with 1% aqueous Rose Bengal.

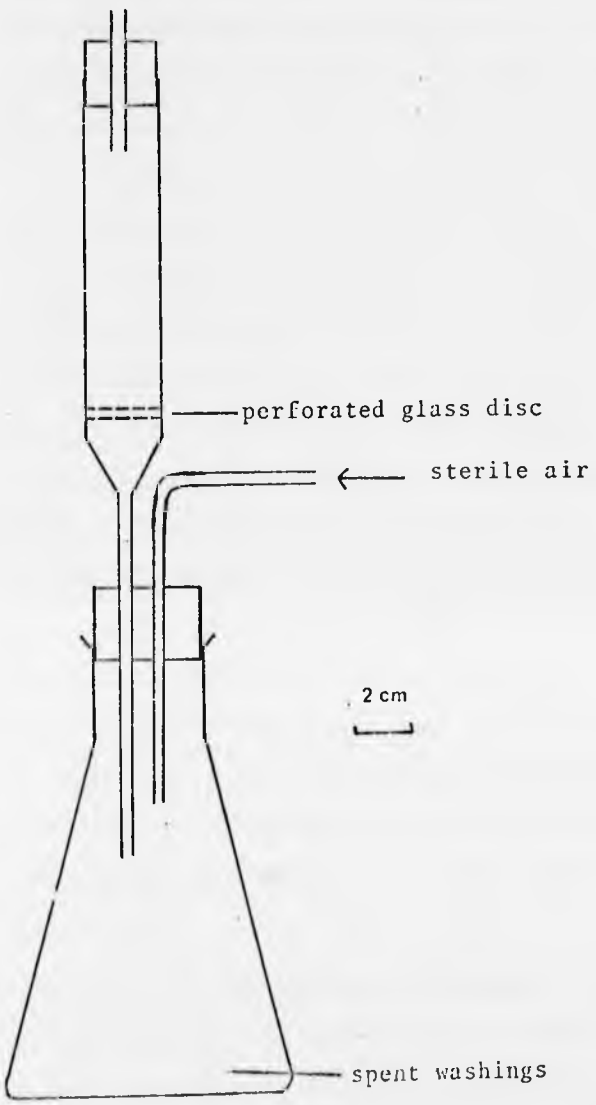
Surface sterilisation of Pine needles

The simple and readily autoclavable apparatus shown in Figure 1 was used. Needles introduced into the washing chamber are treated in a water column supported by air pressure delivered through a filter from a small rotary pump with a variable drive. After each sterilisation or washing period the pressure is released, allowing the spent water to drain into the conical flask, and then fresh Mercuric chloride solution or sterile water is introduced through the top of the apparatus with the pressure re-applied. A still water column (for surface sterilisation) can be supported by the pump working at low speeds, while higher speeds produce a stream of bubbles through the perforations in the glass disc, which facilitates vigorous agitation of the needles.

Soil washing

The technique of Gams & Domsch (1967) was used,

Fig. 1. Apparatus used for surface sterilisation of Pine needles.


perforated glass disc
sterile air
2 cm
spent washings

whereby soil particles are washed in a column of flowing water. The apparatus was a modification of the original designs, being constructed of glass rather than plastic or brass, and is shown in Figure 2. The three sections form an air-tight cylinder when the ground glass surfaces on the flanges are held together in steel clamps. The central washing chamber is partitioned by two grids of fine stainless steel mesh held in place by bevels in the flanges. After autoclaving, the top two sections are removed from the bottom part under sterile conditions, a soil sample is placed on the lower grid and the apparatus is reassembled. The inlet tube is connected to a reservoir of sterile water. When the flow of water is commenced, the apparatus is inverted until it is nearly full and then turned right-way up. In this manner water flows through the apparatus but the soil is fully immersed under a constant head of water, achieving efficient washing. During the washing the apparatus is shaken up and down to remove air from the soil particles and ensure thorough soaking of the soil.

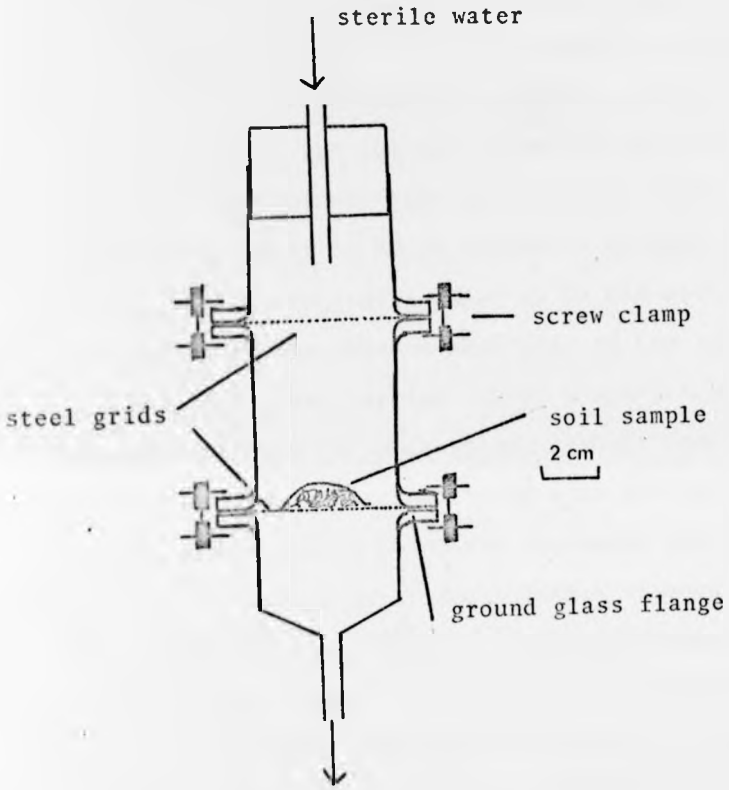
Chromatographic analysis of phenolic compounds

Culture filtrates and water extracts of Pine litter were evaporated to dryness on a rotary vacuum evaporator, desiccated *in vacuo* and the residues stored at -20°C until required. The residues were then dissolved in the appropriate solvent (chloroform or pyridine) and analysed without further extraction.

Two-dimensional thin layer chromatography was carried out on 20 X 20 cm plates of Silica Gel 60 (Merck), 0.25 mm thick. The substances to be analysed were spotted on to the plate in chloroform and the chromatograms were run in

steel

Fig. 2. Soil Washing Apparatus


sterile water
screw clamp
steel grids
soil sample
2 cm
ground glass flange

chloroform/glacial acetic acid (8:2) followed by n-butyl ether/glacial acetic acid (10:1). Phenolic acids and aldehydes and other compounds were visualised by absorption or fluorescence in UV light (254 nm and 336 nm).

Gas liquid chromatography of phenolic acids and aldehydes was carried out after formation of trimethylsilyl derivatives on a Pye 104 Gas Chromatograph with twin columns. The columns were of stainless steel, 1.5 m long and packed with 3% SE 30 on Diatomite CW DMCS 100-120 mesh. Loading of the stationary phase on to the solid support and packing of the columns were done in the laboratory.

Silylation was carried out on a micro-scale in approximately 1.0 cm^3 pear-shaped vessels made by drawing out 8 dram glass vials and closed with the original plastic screw caps in which the cardboard seals had been replaced by washers of silicone rubber wrapped in Teflon. The residue was transferred to the silylation vessels in solvent and silylated with NO-Bis-(trimethylsilyl)-acetamide (BSA). Originally silylation was done in pyridine (100 µl pyridine, 10 µl BSA, at 50°C for 10 mins.) but later chloroform was employed as the solvent (100 µl chloroform, 40 ul BSA, at room temp.) to reduce solvent tailing.

Separation of the silyl derivatives was achieved by a temperature programmed run (8 C min^{-1}) from 150° to 270°C with a nitrogen flow rate of 20 cm^3 min^{-1}.

Spectrophotometry

For ultra-violet spectrophotometry a Pye Unicam SP 1800 Recording Spectrophotometer was used, with 1 cm path length quartz cells. The culture filtrates were diluted as appropriate and analysed against a distilled water blank.

The concentrations of protocatechuic acid and p-coumaric acids were calculated from extinction coefficients available in the literature (see 'Organic Electronic Spectral Data' Wiley - Interscience). The extinction coefficient of ferulic acid in aqueous solution had to be determined from standard solutions. Spectra of substances eluted from T.L.C. plates with ethanol were run against pure ethanol.

Infra-red spectra were obtained on a Perkin-Elmer 457 Grating IR-Spectrophotometer. The reference for the KBr discs was air (plus attenuator).

FIELD EXPERIMENTS

Source of Pine litter for fungal isolations and IR analysis

Needle litter was obtained from a stand of *Pinus sylvestris* var. *scotica* in the Blackwood of Rannoch (Grid reference 551 545, sheet 48).

Sites for burial of Pine needles on the University Estate

Hermitage Wood is part of the wooded slopes on base-rich lavas leading up to the Ochils. The Experimental Gardens are situated right at the foot of these hills.

The pH and the exchangeable cation content of the soils at the two sites are given below:

	pH (soil paste)	Exchangeable Cations (m.equivalents per 100g of air-dried soil)			
		Ca	Mg	K	Na
Hermitage Wood	4.1	1.50	0.10	0.05	0.13
Experimental Gardens	6.2	0.17	0.37	0.06	0.30

Texturally, both soils are silty loams with abundant stones.

The seed trays in which the Pine needles were buried could only be placed at a depth of about 15 cm in Hermitage Wood due to the appearance of the parent material below this level. A much deeper hole could be dug in the soil in the Gardens and the trays were buried at a depth of about 40 cm because the top layer of this soil freezes solid in a heavy frost whereas the soil in Hermitage Wood is insulated by leaf litter.

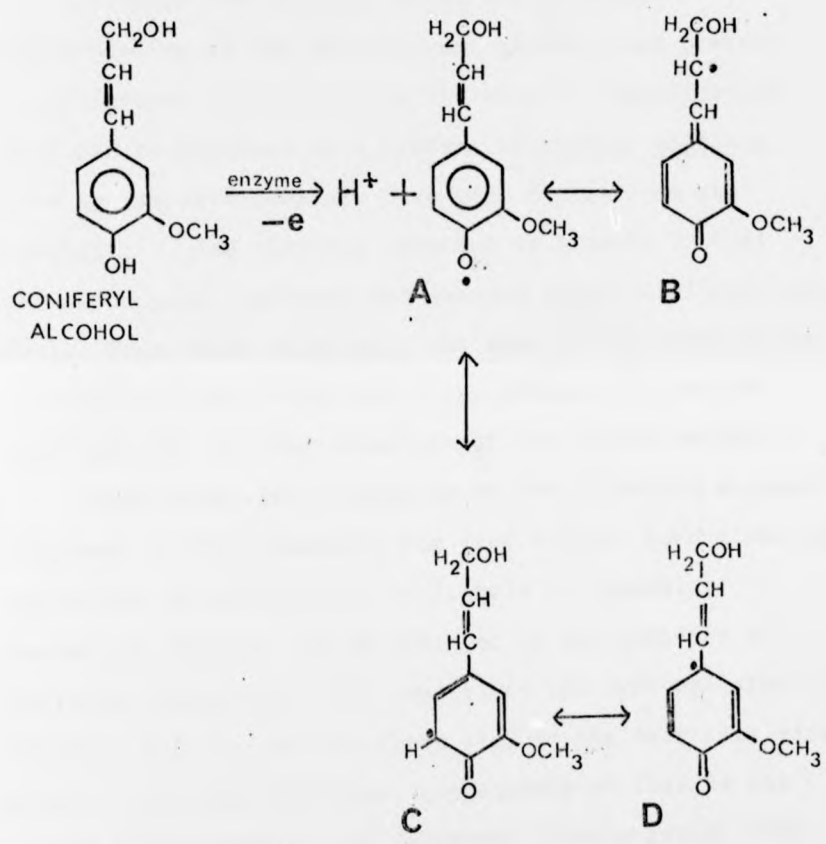
CHAPTER ONE

THE BEHAVIOUR OF MICROFUNGI IN PURE CULTURE TOWARDS LIGNIN RELATED PHENOLIC ACIDS

Phenolic compounds have been used to replace lignin in decomposition studies for two reasons; firstly, because certain phenolics are considered to be structurally related to lignin and so can be used as 'model compounds'; and secondly, because some are known products of the decomposition of lignin. However, physiological studies on microfungi from litter and soil involving phenolic acids have generally not taken into account the ecology of the organisms concerned, even though much information is available concerning the detailed distribution of species within this general habitat.

The development of the theory of lignification has been reviewed by Brown (1964), Harkin (1967) and Schubert (1968). The process of the formation of lignin by oxidative polymerisation of cinnamyl alcohols is best considered in terms of the effects of phenol oxidising enzymes on 'Lignols'. These are C6-C3 compounds with a free para-hydroxl group and an unsaturated side chain conjugated with the benzene ring, which permits the formation of a stable, mesomeric free radical system. Removal of an electron from coniferyl alcohol gives rise to the system shown in Figure 1.1. Coupling of coniferyl alcohol radicals of different sorts could alone give rise to the majority of bonds thought to be present in lignin. The three main types of dilignols formed from coniferyl

Fig. 1.1. Formation of mesomeric radical system on oxidation of coniferyl alcohol (Adapted from Harkin, 1967).

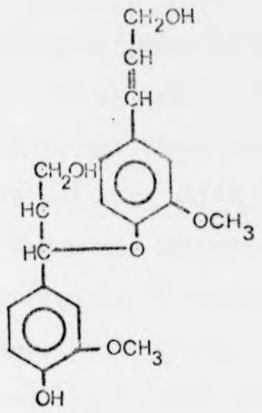

CONIFERYL
ALCOHOL
enzyme
−e
A
B
C
D

alcohol are shown in Figure 1.2. Further coupling and condensation reactions can take place between these and higher lignols to produce many more bonds. The guaiacylglycerol-β-coniferylether structure may comprise one quarter to one third of the bonds present in lignin.

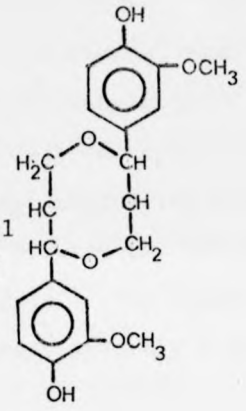
Although the cinnamic acids and aldehydes corresponding to the alcohols are probably not present as precursors transported to the site of lignification, they can be produced by a process of radical exchange, once appropriate radicals have been formed from the alcohols. And they can function as lignols so that the carboxylic, carbonyl and lactone groups of lignin may derive from their coupling. As long as the products of coupling contain p-hydroxyls, the process can proceed indefinitely with the formation of the lignin polymer.

Peroxidases are thought to be the principle enzymes involved in lignification, but free radical formation, and indeed the polymerisation of lignols to something resembling lignin, can be achieved in the presence of inorganic catalysts - the removal of the hydrogen atom from the free hydroxyl at the first step is the only catalysed phase. The most important consequence of this is the random arrangement of the different linkage groups with respect to one another; although lignin from a particular source can be characterised with the proportion of each type of bond, each sample of lignin is in fact unique. And thus the possibilities of enzymatic degradation of lignin must be viewed in a completely different perspective from that of other biological polymers.

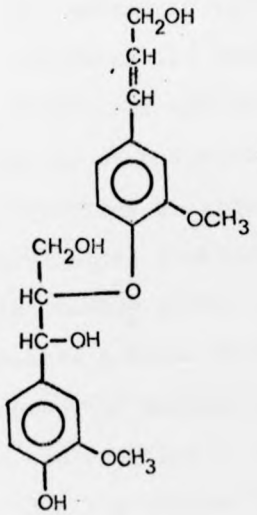
Fig. 1.2 The three most important dilignols involved in lignin biosynthesis. The letters refer to radicals in fig. 1.1.



Dehydrodiconiferyl alcohol
(B + C)



D, L-pinoresinol
(B + B)



Guaiacylglycerol-β-coniferyl ether
(A + B and addition of
water)

All three types of aromatic nuclei - p-hydroxyphenyl, guaiacyl and syringyl - are found in the lignin of all seed plants but their relative proportions vary. In conifers guaiacyl units predominate with some p-hydroxyphenyl and minor amounts of syringyl units; in woody angiosperms lignin contains approximately equal amounts of guaiacyl and syringyl residues with very little of the other type; while grass lignin has major contributions from all three types of nuclei (Kirk, 1971). Fairly recently Higuchi et al. (1967a, b) indicated that in grass lignin the majority of the p-hydroxyphenylpropane component is merely esterified with lignin as p-coumaric acid and not polymerised by lignol coupling, suggesting that there is little fundamental difference between the polymeric systems of hardwood and grass lignin. The lignin of Sphagnum has been found to be almost entirely a polymer of p-Coumaryl alcohol.

In the mesomeric radical system derived from sinapyl alcohol the formation radicals of type C in fig. 1.1 will be suppressed and this leads to an augmentation of the frequency of benzyl-aryl ethers in lignin containing a high proportion of sinapyl groups (Harkin, 1967). As benzyl-aryl ether linkages are among the easiest degraded in lignin, Harkin (1966) has suggested that the evolutionary trend towards greater methoxylation has come about through the advantage of having lignin that can be more readily degraded and recycled - the incorporation of more sinapyl alcohol into angiosperm lignin being accompanied by the evolution of xylem vessels to replace the weaker tracheids.

Thorough chemical degradation of lignin can only be achieved by mild oxidation with permanganate (eg Larsson

& Mikische, 1967, 1969, 1971) or with alkaline nitrobenzene (e.g. Bicho et al. 1966) as hydrolysis leads to further condensation. Some of the structures thought to be present in lignin have been deduced from dimeric and trimeric products of these oxidations, and one also obtains a variety of benzoic acid and benzaldehyde derivatives.

Very little is known of the biochemistry of lignin breakdown by micro-organisms, particularly as regards the intermediates in the process, and most of the available data are concerned with the activity of white rot fungi. And although the catabolic pathways for the phenolic compounds postulated as primary products have been established in macro- and microfungi and bacteria, how they are produced from the lignin polymer in the first place is not clear, even for white-rot fungi. These problems have been reviewed by Kirk (1971) as well as by Harkin (1967) and Schubert (1968).

A topic that has been central to this issue is the role of extracellular phenol oxidising enzymes that are secreted by fungi, in particular enzymes requiring molecular oxygen with the 'Laccase' (oxygen : p-diphenol oxidoreductase, E.C. 1.10.3.2) and 'Tyrosinase' (oxygen : o-diphenol oxidorductase, E.C. 1.11.3.1) types of activity. The secretion of these enzymes is stimulated in the presence of phenols (and also aromatic amines) and this is the basis of the Bavendamm test for detecting their production by fungi growing on agar using tannic acid. The implication of these enzymes in lignin decomposition derives from early observations that among

wood rotting fungi only white rot species and not brown rot fungi gave a positive result for the Bavendamm test. However, Higuchi & Kitmara (1953) subsequently showed that only white rot species produce Laccase, but that both white rot fungi and some brown rot species secreted Tyrosinase. They suggested that the oxidation of tannic acid in the Bavendamm test may be caused by either enzyme, although Kirk & Kelman (1965) showed that the reaction of Tyrosinase secreting brown rot fungi was very much weaker than the reaction of white rot fungi to various phenolic compounds.

A mechanism is available for the oxidative depolymerisation of lignin under the action of Laccase (or Tyrosinase or Peroxidase). In this scheme (Harkin, 1967) oxidation of benzyl carbons leads to side chain cleavage and the formation of quinones. However, Ishikawa & Myazaki (1972) found that phenol oxidising enzymes could only cause limited degradation on the periphery of the polymer while further polymerising the core. In vitro studies with peroxidase and phenolic model compounds have indicated the likelihood of this happening, particularly with softwood lignin (Young & Steelink, 1973).

Kirk & Kelman (1965) found that certain wood decomposing Basidiomycetes that could decompose lignin did not give a positive Bavendamm test and Sundman et al. (1964) failed to find Laccase production in any of the fungi isolated during their studies of lignin decomposition by soil micro-organisms.

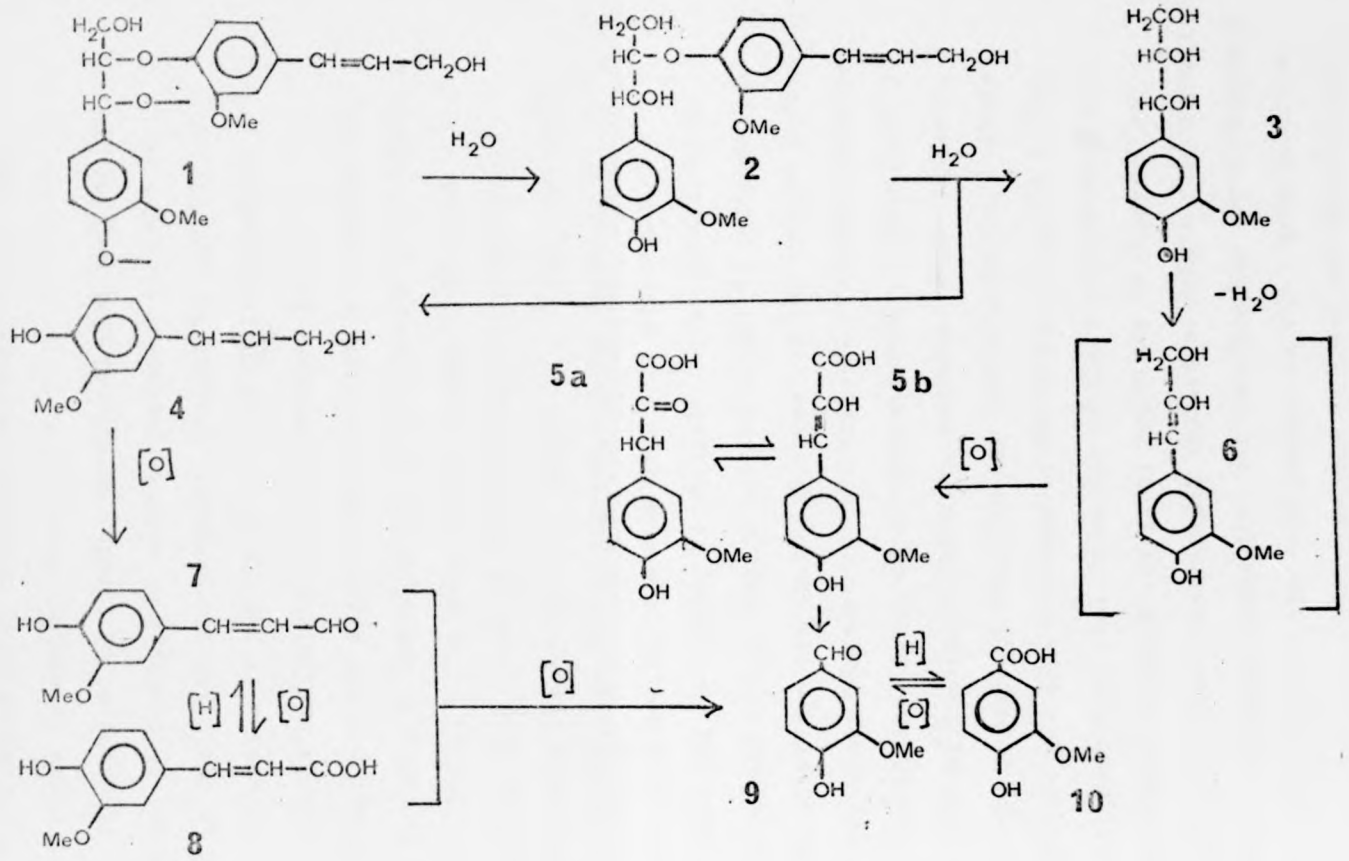
Current opinion would seem to favour an auxilliary role for phenol oxidases in lignin decomposition in wood, in the detoxification of the decomposition products themselves and of heartwood compounds. Other enzymatic models for the breakdown of lignin include the demethylation of aromatic nuclei and ring cleavage within the polymer, suggested by Harkin (1967) and the rupture of the important glycerol-β-aryl ether bonds by an 'Etherase'. However there is very little experimental evidence to support these models.

Much attention has been given, however, to the guaiacylglycerol-β-aryl ether structures in lignin because of their numerical importance. Ishikawa et al. (1963a) obtained guaiacylglycerol-β-coniferyl ether (Fig. 1.2) and guaiacylglycerol from lignin attacked by white rot organisms and subsequently these workers (1963b,c) proposed pathways for the guaiacylglycerol and coniferyl alcohol moieties of the above ether into vanillic acid (Fig. 1.3). The interpretation of their results and this model is very complicated, and unfortunately the reviews only seem to add to the confusion. Kirk (1971) has pointed out some of the problems in accepting this scheme, but in view of the fact that the metabolic conversion of ferulate to vanillate had already been established in microfungi (Henderson & Farmer, 1955) the main difficulties would seem to arise from the need for enzymatic cleavage of the ether bond. Attempts at demonstrating 'Etherase' activity, let alone the isolation of an enzyme system, have been hampered by experimental difficulties. And

Fig. 1.3 Pathways proposed by Ishikawa et al. (1963b) for the metabolism of guaiacylglycerol- -coniferyl ether by white rot fungi.

1. Guaiacylglycerol-β-coniferyl ether units.
2. Guaiacylglycerol-β-coniferyl ether.
3. Guaiacylglycerol.
4. Coniferyl alcohol.
5. 4-hydroxy-3-methoxy-phenylpyruvic acid.
   a) Keto form b) Enol form
6. B-hydroxyconiferyl alcohol.
7. Coniferaldehyde.
8. Ferulic acid.
9. Vanillin.
10. Vanillic acid.

Me = CH_3


1
2
3
4
5a
5b
6
7
8
9
10
H_2O
H_2O
$-H_2O$
[O]
[O]
[O]
[H]
[O]
[H]
[O]

how the dimeric ether molecules are split off in the first place is another question.

Ishikawa et al. (1963c) postulated that polyphenol oxidases (i.e. diphenol oxido-reductases) played a key role in the metabolic pathways outlined in fig. 1.3 in the conversion of the C6-C3 units to C6-C1 compounds, but this is unlikely as all the evidence points to coupling, and not degradation, of phenolic compounds in the presence of these enzymes or peroxidase (e.g. Gierer & Opara, 1973). In this context Harkin (1967) has indicated that "some of the intermediates postulated hardly seem likely to occur freely in a system containing phenol oxidases". In the first place, however, a complicated metabolic system like that proposed by Ishikawa and co-workers is unlikely to be extracellular. It is a possibility that a certain amount of intermediates produced within the cytoplasm appear in the culture medium and are there polymerised by extracellular phenol oxidases.

Refluxing lignin in dioxane-water-HCl ('Acidolysis') was used by Lundquist (1970) to hydrolyse ether linkages, with the release of vanillin and coniferaldehyde. These compounds are only released in low yield, from which Kirk (1971) concluded that "cleavage of the arylglycerol-β-aryl ether bonds by white rot fungi could not be expected in itself to release substantial amounts of single ring products". However Lundquist found that the vanillin and coniferaldehyde were released from the 'end groups' in the lignin molecule. Also on the basis of comparisons of sound and white-rotted lignins, Hata (1966) found evidence of the side chain

oxidation of coniferyl alcohol end groups; and he went on to suggest a mechanism for progressive and extensive depolymerisation of lignin by enzymatic cleavage of ether bonds, with the release of vanillic acid.

In summary, the low yields of aromatic compounds that are encountered in the decomposition of lignin or in the metabolism of primary products must thus be viewed in the context of a) the low availability of ether linkages that can be split at any one time, and b) the prompt removal of decomposition products from the substrate or culture medium by assimilation by the fungus or polymerisation by extracellular enzymes.

In addition to the existence of C6-C3 and C6-C1 structures as end groups attached by ether bonds, there also appear to be benzoic and cinnamic acid residues esterified to lignin. The work of Higuchi et al. (1967a, b) has already been referred to, but much earlier Smith (1955 a,b) had shown that esters of vanillic, p-hydroxybenzoic, ferulic and p-coumaric acids occurred in various lignins. And recently, Morrison (1974) has suggested that ferulic and p-coumaric acids may be important in cross linking in the lignin-carbohydrate complex in grass lignin.

Henderson (1960) pointed out that the apparently widespread ability of soil fungi to metabolise and utilise lignin model compounds "could be of importance in the decomposition of lignin in the soil only if these units can exist there in the free state." This condition has been satisfied and many lignin decomposition products can be released from crop residues by hot water treatment or mild hydrolysis (e.g. Guenzi & McCalla, 1966) so one can

conclude that phenolic acids and related compounds are important environmental factors associated with the attack of lignin in and on soil. However, as has been discussed above, the place of these compounds in the overall conversion of lignin to low molecular weight substances is unclear.

To complement the work of Henderson's group on the utilisation of phenolic acids and aldehydes by soil fungi and yeasts, attention in this study was first directed to the effects of phenolic acids on the spore germination of microfungi. Phenolic compounds, including phenolic acids, have been implicted widely in the interaction of higher plants and fungal pathogens, in mechanisms of preformed resistance to attack or through involvement in the interaction after the host has been attacked. There is little really positive evidence for these roles and it is possible that in many studies the free phenolic acids found in plants have arisen by hydrolysis of esters or glycosides during extraction (Li et al. 1972, Niemann, 1969 a, b, 1972). But it is pertinent to consider here the methods used in the study of the direct effects of these compounds on fungal pathogens in vitro. A variety of assay methods have been used:- growth on agar for sporulation assessment (Naranyanarao et al. 1972), percentage germination and appressorial formation in drops of aqueous solutions of the phenolic compounds on glass slides (Srinivasan & Naransimhan, 1971), mycelial growth rate in liquid culture (Christie, 1965; Li et al. 1969). Three of these works have used more or less the same group of twenty or more compounds, but few conclusions can be made about chemical structure and biological activity as

insufficient fungi have been tested by any one assay method. Generally, cinnamic acids are more inhibitory than benzoic acids, but the marked effects of substituents to the benzene ring distorts this pattern.

Some useful data have been produced from studies of endogenous inhibitors of the uredospores of Rust fungi. Van Sumere et al. (1957) extracted eleven coumarins and phenolic acids from uredospores of Puccinia graminis var. tritici and tested their effects on the germination of uredospores floating on aqueous solutions. Some compounds were stimulatory while ferulic, vanillic and p-hydroxybenzoic acids were markedly inhibitory. These workers considered that the stimulation of germination was due to the counteraction of the self-inhibition produced by compounds already present in the spore, and so were unable to identify the endogenous inhibitor(s). More recently, however, Macko et al. (1972) established that the self-inhibitors of the uredospores of certain rusts were methyl ferulate or methyl-3:4-dimethoxycinnamate and furthermore that it was only the cis-isomers of these compounds that were inhibitory.

The effects of lignin decomposition products themselves on soil fungi have been investigated by Lingappa & Lockwood (1962). They incorporated a wide variety of phenolic substances, lignin, tannins and lipid fractions from soil into water agar for the measurement of their effects on the spore germination of species of Glomerella, Fusarium and Penicillium. Lignin preparations were variably inhibitory

but these data are difficult to interpret in view of the vigorous chemical methods of extraction of these fractions. However, ethanol extractions from lignin amended soil and pure phenolic decomposition products depressed percentage germination and germ tube growth.

Several methods are available for testing the effects of certain substances on the spore germination of fungi. Spores can be placed in drops of aqueous solutions containing the test compounds, or on thin films on glass surfaces on which the substances have been deposited by evaporation of the solvent, or on agar containing the test substances. The suitability of these methods was investigated in preliminary work.

It was found early on that germination of the spores of fungi under consideration was unsatisfactory in or on water droplets. Miller (1949) reviewed the use of dried deposits on glass slides, to be rewetted by the spore suspension. The main problem here relates to obtaining a uniform water droplet and therefore a uniform deposit, and Miller describes the use of cellulose nitrate sheets for this purpose. It was necessary, however, to prepare deposits with solutions of the phenolic acids in absolute methanol, as some of them are not very soluble in cold water, particularly ferulic and sinapic acids. Methanol and ethanol do not form drops on glass but spread, and so the problem of uniformity is increased. Some standardisation was achieved by pipetting the methanolic solutions into the wells of cavity slides, and on to various absorbent surfaces such as cellulose or fine sand. However agar was found to be the best medium for the deposition of phenolic acids.

Previously, Lingappa & Lockwood (1962) mixed test substances into cooled molten agar in petri dishes, or occasionally prepared a deposit on the bottom of the dish. They also showed that it was advisable not to autoclave the compounds in the agar, although some compounds were apparently not affected. Pero & Owens (1971) describe a method for the assay of a fungicide in which a pyridine solution of the fungicide is evaporated off the surface of a microscope slide and then molten agar is poured over to form the germination surface.

A modification of these methods was found to be the most satisfactory method of impregnating agar with phenolic acids. Agar blocks of standard size are prepared by pipetting 1 cm^3 aliquots of tap water agar into metal bottle caps, autoclaving and allowing the agar to set. Methanol solutions applied to the surface of the agar can be seen to be immediately absorbed into the agar and to replace water which is left as a surface film. Unlike pyridine or ethanol, methanol is not irreversibly adsorbed by agar and this method of preparation has no effect on the control germination. Because the methanol is contained by the walls of the bottle cap, run off is prevented and the test substance is distributed uniformly throughout the agar. Control blocks are prepared using 0.1 cm^3 of absolute methanol, test blocks using 0.1 cm^3 of a methanol solution of the phenolic acid, pipetted by an Eppendorf Micropipette.

During the development of this method Stemphylium dendriticum was the prinicipal fungus used to standardise conditions with a range of phenolic acids. Ferulic acid

was found to be the most inhibitory acid during the trial of various materials to provide a surface for the evaporation of the methanol. But, up to a certain level, concentrations highly inhibitory to the percentage germination and germ tube growth of Stemphylium were stimulatory to Dothichiza pityophila, irrespective of the method of assay.

In preliminary tests with p-hydroxybenzoic, vanillic, syringic, p-coumaric, ferulic and sinapic acids the method of assay involved the growth of hyphae over the surface of the agar discs formed in bottle caps from a central well inoculated with a spore suspension. Details of the manipulation of the agar discs, the sources of the phenolic acids and the preparation of the spore suspensions are given in the Materials and Methods section. Each compound was applied at the level of 400 µg per disc to give a concentration of approximately 400 mg l^{-1} since each disc had a volume of about 1 cm^3. There were three replicates of each treatment and control.

The species of fungi tested were:- four primary saprophytes of Angiosperm leaves, Dreschlera rostrata, Stemphylium dendriticum, Botrytis cinerea, Cladosporium herbarum; two saprophytes of conifer needle litter Thysanophora penicilloides and Dothichiza pityophila; three soil fungi also common on certain types of litter, including that of conifers, Trichoderma viride, Mucor hiemalis and Penicillium spinulosum; and Penicillium expansum, a pathogen of apples for comparison with P. spinulosum. Details of the origins of the isolates used are given in the Materials and Methods section.

After incubation at 24°C in petri dish damp chambers the effect of the phenolic acids on the germination and growth of the fungi was measured by counting the number of hyphae growing from the well over the disc. Within treatments variability of this parameter is high, particularly when the mean number of hyphae is large. To partly correct this mean/variance association statistical analysis of the data was performed by t-tests between control and treatment on the logarithms of the hyphal numbers. For comparison, the Mann-Whitney test was also used (Siegel, 1956), a non-parametric analogue of the t-test.

The full results and details of the statistical analyses are given in Appendix 2 while mean hyphal numbers and a summary of the analysis are given in Table 1.1.

On the basis of these results it would appear that the primary leaf saprophytes and P. expansum behave differently from the soil fungi and conifer needle saprophytes, (which will be referred to as groups A and B respectively. Every species in group A has hyphal production suppressed by at least one phenolic acid and ferulic acid is inhibitory to all the leaf saprophytes. There are no significant inhibitions in group B and on the basis of the t-test every acid is stimulatory to at least one species and every species shows at least one positive response. Only in one case does a phenolic acid (vanillic) stimulate the hyphal numbers of a species in group A (D. rostrata).

It should be noted that group A species produced more hyphae in control than group B fungi, which is a consequence

TABLE 1.1 Effect of phenolic acids on the production of hyphae by microfungi on agar blocks

	Mean number of hyphae to nearest whole						
	'Control'	FER	VAN	P-COU	P-HYD	SIN	SYR
Dreschlera rostrata	20	1*-	37*+	17	24	27	26
Stemphylium dendriticum	40	7*-	11 -	12	9	21	33
Botrytis cinerea	13	0*-	6	10	13	11	17
Cladosporium herbarum	39	5*-	11*-	15*-	35	18 -	31
Penicillium expansum	11	4 -	16 -	2*-	27	4 -	18
Penicillium spinulosum	2	12	16 +	11 +	3	43*+	14*+
Trichoderma viride 'B'	3	128*+	173*+	61*+	181*+	91*+	115*+
Mucor hiemalis 'A'	0	2*+	2	11*+	6	50*+	32
Mucor hiemalis 'B'	0	7	0	0	14	27*+	11
Dothichita pityophila 'A'	8	52 +	45	36 +	50*+	40	14
Thysanophora penicilloides	0	21*+	21*+	7*+	22 +	6 +	10 +

* Significant effect at $p < 0.05$ in t test (log transformation)

+ Significant stimulation in Mann-Whitney u-test

- Significant inhibiton in Mann-Whitney u-test

of the slower intrinsic rate of germ hyphal growth in the latter. The effects of this on the interpretation of the results will be discussed later.

Sinapic acid seems to be the most stimulatory compound to group B but this compound, syringic acid and p-hydroxybenzoic acid are not particularly inhibitory to group A at the concentration used. Ferulic acid on the other hand is the most inhibitory to the leaf saprophytes and is also quite stimulatory to the needle saprophytes and soil fungi.

The effect of ferulic and sinapic acids on the spore germination and germ hyphal growth of microfungi was then examined. In this assay a spore suspension is pipetted on to the surface of a disc left in the centre of the water agar block formed in the bottle caps. After incubation at 24^{o}C in damp chambers percentage germination and germ tube lengths are determined in each of the three replicates of each treatment, and the means calculated. Germ hyphal lengths were measured by means of a projection microscope, the mean value for a treatment being obtained from the 15 individual hyphal lengths measured in each replicate. Statistical analysis of the effects of a phenolic acid was carried out using the t-test to compare treatments with control. The values for percentage germination were transformed by the arc-sin transformation before analysis. Fuller details of the techniques are to be found in the Materials and Methods section.

The response of the spore germination and germhyphal growth of Stemphylium dendriticum, Dothichiza pityophila

isolate 'C' and Trichoderma viride isolate 'A' to different concentrations of ferulic and sinapic acids was first investigated. The concentrations of each compound were 0 (Control), 200, 400, 600 and 800 µg per block. The results for T. viride and S. dendriticum after 16 hours incubation are given in Figure 1.4 (% germination) and Figure 1.5 (germ hyphal lengths). The percentage germination of S. dendriticum is appreciably, but not significantly, depressed by 200 µg ferulic acid and is zero at higher levels. The percentage germination of T. viride is not affected significantly by 200 µg ferulic acids per disc but is significantly inhibited at higher concentrations. Sinapic acid does not affect the percentage germination of either species significantly, but it would appear that there is a tendency for inhibition in S. dendriticum and for stimulation in T. viride. The germ hyphal length of S. dendriticum is significantly suppressed by 200 µg ferulic acid where percentage germination exceeds zero and at all concentrations of sinapic acid. T. viride is slower growing than S. dendriticum but its germ hyphal length is significantly stimulated by both acids at 200 µg per block but at higher concentrations only ferulic has a significant effect (inhibitory).

It is not possible to estimate the percentage germination of Dothichiza pityophila reliably because of the proliferation of secondary blastospores, which are detached from the hyphae when the agar discs are prepared for examination. The secondary blastospores do not germinate during the experimental period, however,

Fig. 1.4 Effect of a) Ferulic acid and b) Sinapic acid on the percentage germination of conidia of Trichoderma viride 'B' and Stemphylium dendriticum. 16 hours incubation.

□ T. viride 'B'

○ S. dendriticum

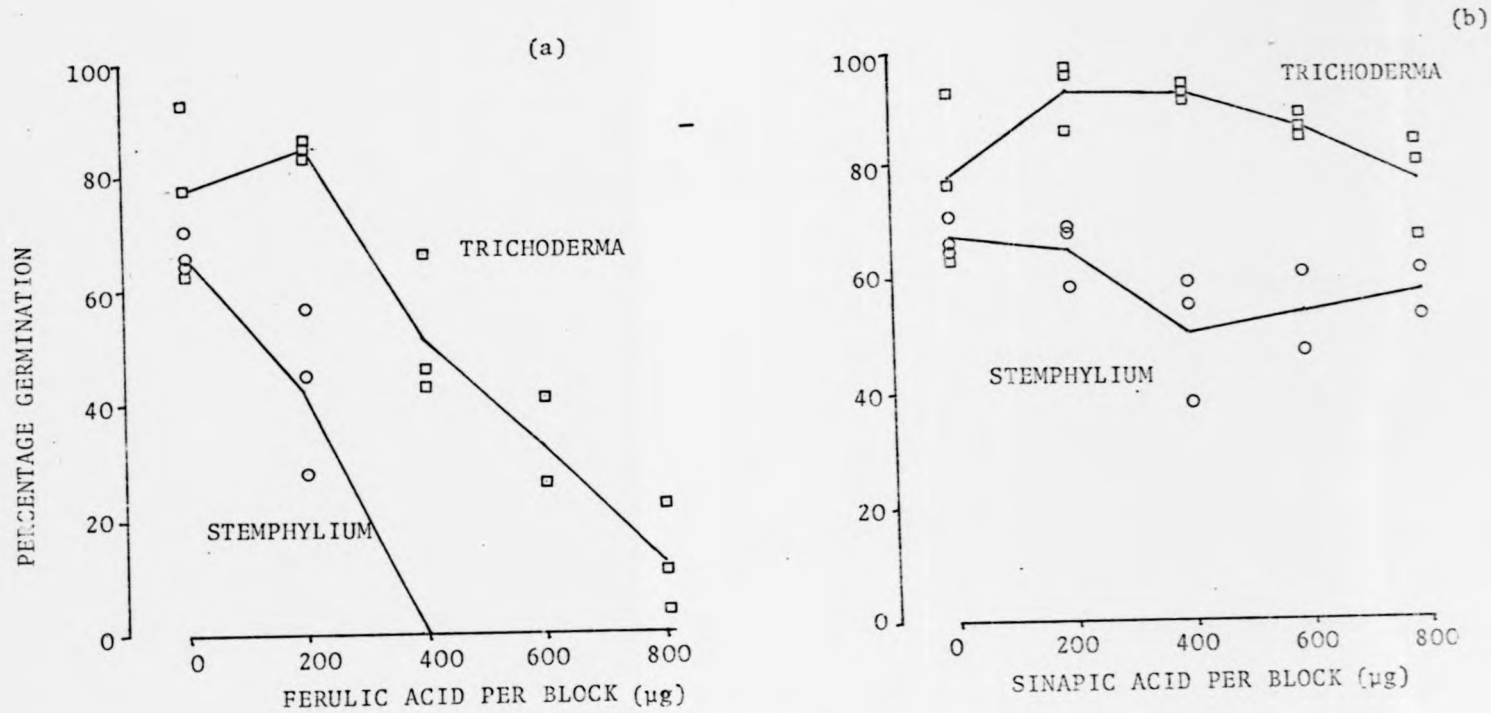
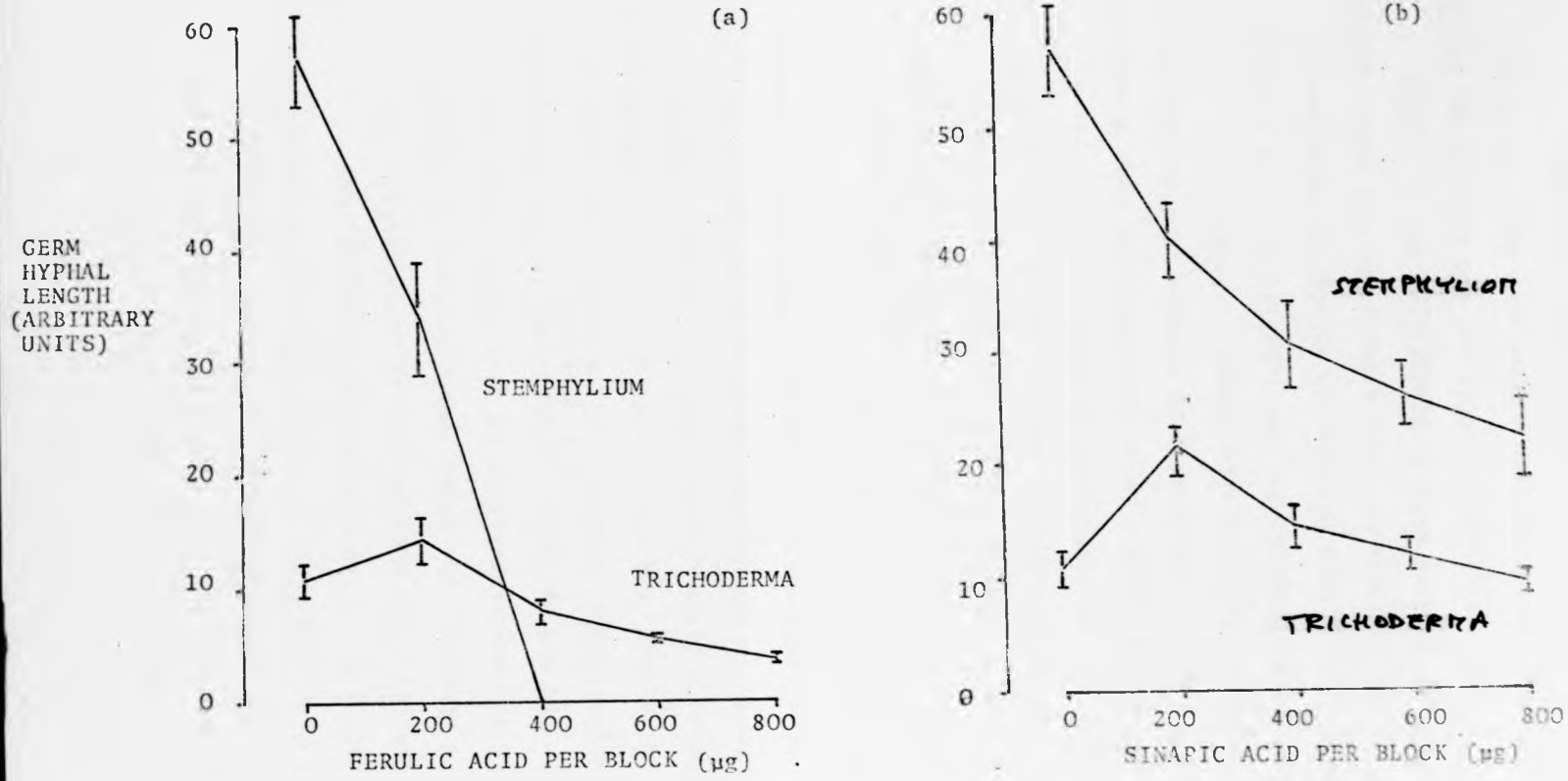

(a)
PERCENTAGE GERMINATION
100
80
60
40
20
0
TRICHODERMA
STEMPHYLIUM
0
200
400
600
800
FERULIC ACID PER BLOCK (µg)
(b)
TRICHODERMA
STEMPHYLIUM
SINAPIC ACID PER BLOCK (µg)

Fig. 1.5 Effect of a) Ferulic acid and b) Sinapic acid on the germ hyphal lengths of Trichoderma viride 'B' and Stemphylium dendriticum. 16 hours incubation.

I Least significant difference at p = 0.05


(a)
GERM HYPHAL LENGTH (ARBITRARY UNITS)
60
50
40
30
20
10
0
STEMPHYLIUM
TRICHODERMA
0
200
400
600
800
FERULIC ACID PER BLOCK (µg)
(b)
STEMPHYLIUM
TRICHODERMA
SINAPIC ACID PER BLOCK (µg)

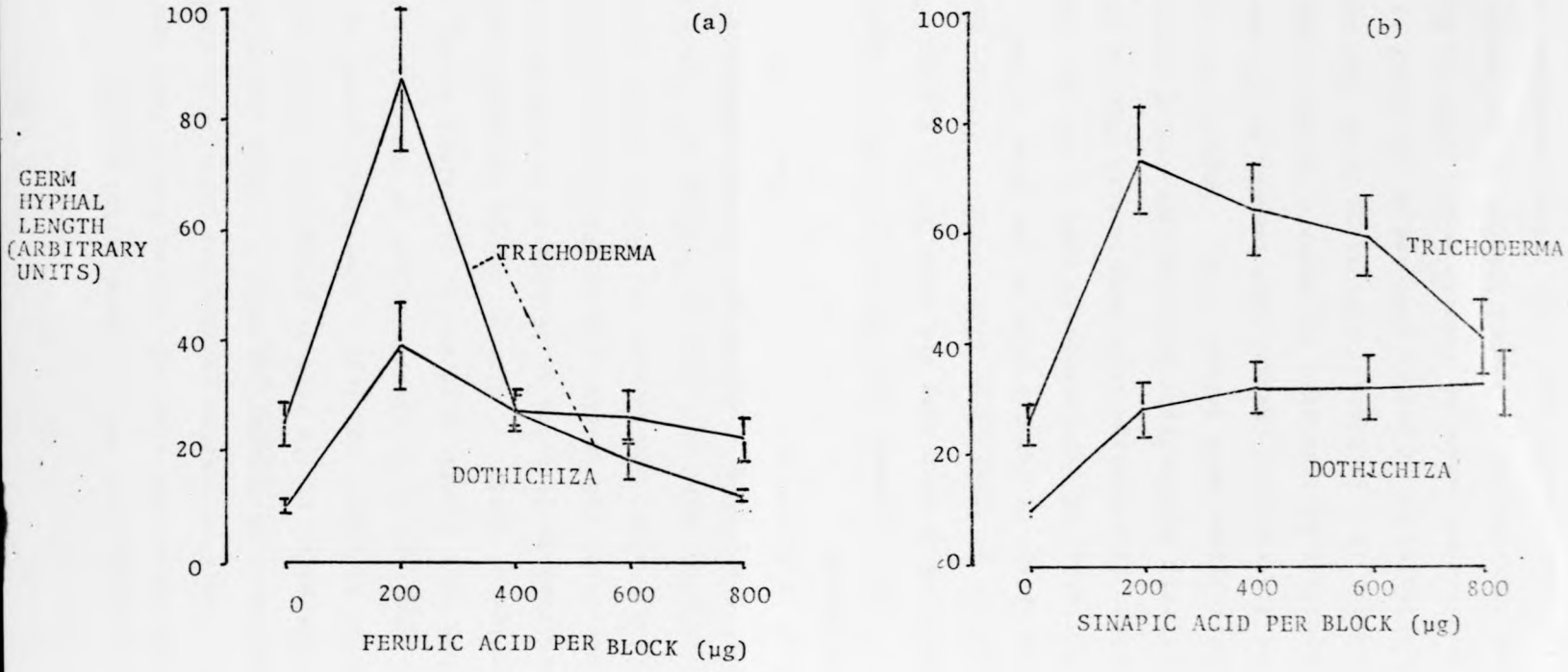
and so do not present a problem in the measurement of hyphal growth. The effect of ferulic and sinapic acids on the germ hyphal lengths of D. pityophila and T. viride after 26 hours incubation is shown in Figure 1.6. The results for T. viride show essentially the same pattern as after 16 hours but more clearly; the higher concentrations of sinapic acid are now stimulatory to germ hyphal length and only 600 and 800 µg of ferulic acid are now inhibitory. The germ hyphal length of D. pityophila is stimulated by all levels of ferulic acid, but the effects of increasing concentration produce a pattern very similar on the whole to that shown by T. viride, but there is no decline in the stimulated level of germ hyphal length with increasing concentration of sinapic acid.

Both sinapic and ferulic acids have produced stimulation and inhibition of percentage germination and hyphal length, the effects depending on the concentration and the fungus. On the basis of the occurrence of statistically significant effects, germ tube length appears to be more sensitive than percentage germination. Qualitatively sinapic acid appears to have the same effects on each fungus as ferulic acid, but the former compound is less inhibitory. As a result only ferulic acid was used in subsequent experiments.

The progress of spore germination and hyphal growth with time was studied with S. dendriticum and T. viride isolate 'B' in control conditions and with 200 µg ferulic acid per block. 30 germ hyphae were measured in each treatment. The results are shown in figures 1.7

Fig. 1.6. Effect of a) Ferulic acid and b) Sinapic acid on the germ hyphal lengths of Dothichiza pityophila 'C' and Trichoderma viride 'B'. 26 hours incubation.

⊢⊣ Least significant difference at p = 0.05


(a)
GERM HYPHAL LENGTH (ARBITRARY UNITS)
100
80
60
40
20
0
TRICHODERMA
DOTHICHIZA
0
200
400
600
800
FERULIC ACID PER BLOCK (µg)
(b)
100
80
60
40
20
0
TRICHODERMA
DOTHICHIZA
0
200
400
600
800
SINAPIC ACID PER BLOCK (µg)

(% germination) and 1.8 (germ hyphal growth). Ferulic acid delays the start of germination in both species but the final percentage germination relative to control is depressed in Stemphylium only. There are no obvious differences in the rate of germination in the exponential phase in either species. Germ hyphal growth of S. dendriticum is clearly faster in the control treatment than with ferulic acid and is maintained during the period of study. With T. viride the mean hyphal length achieved in 24 hours is stimulated by ferulic acid. Because of the initial lag in germination caused by ferulic acid, and because the hyphal growth in neither treatment shows any signs of falling off this must mean that the growth of the fungus in the exponential period must be faster under ferulic acid than in the control treatment.

The frequency distribution of hyphal lengths measured at each time are shown for T. viride (fig. 1.9) and for S. dendriticum (fig. 1.10). With T. viride the effect of ferulic acid on the delay in the initiation of germination is seen in the more negatively skewed distribution with ferulic acid than in the control, indicating a higher proportion of relatively short hyphae with ferulic acid. However the shape of the distribution changes more rapidly in the case of the ferulic acid treatment (12 hours - 15 hours) than in the control (9 - 15 hours), indicating again that there is a stimulation of growth obscured by the delay in germination caused by ferulic acid. With S. dendriticum the distribution of hyphal lengths in the ferulic acid treatment remains negatively skewed throughout the

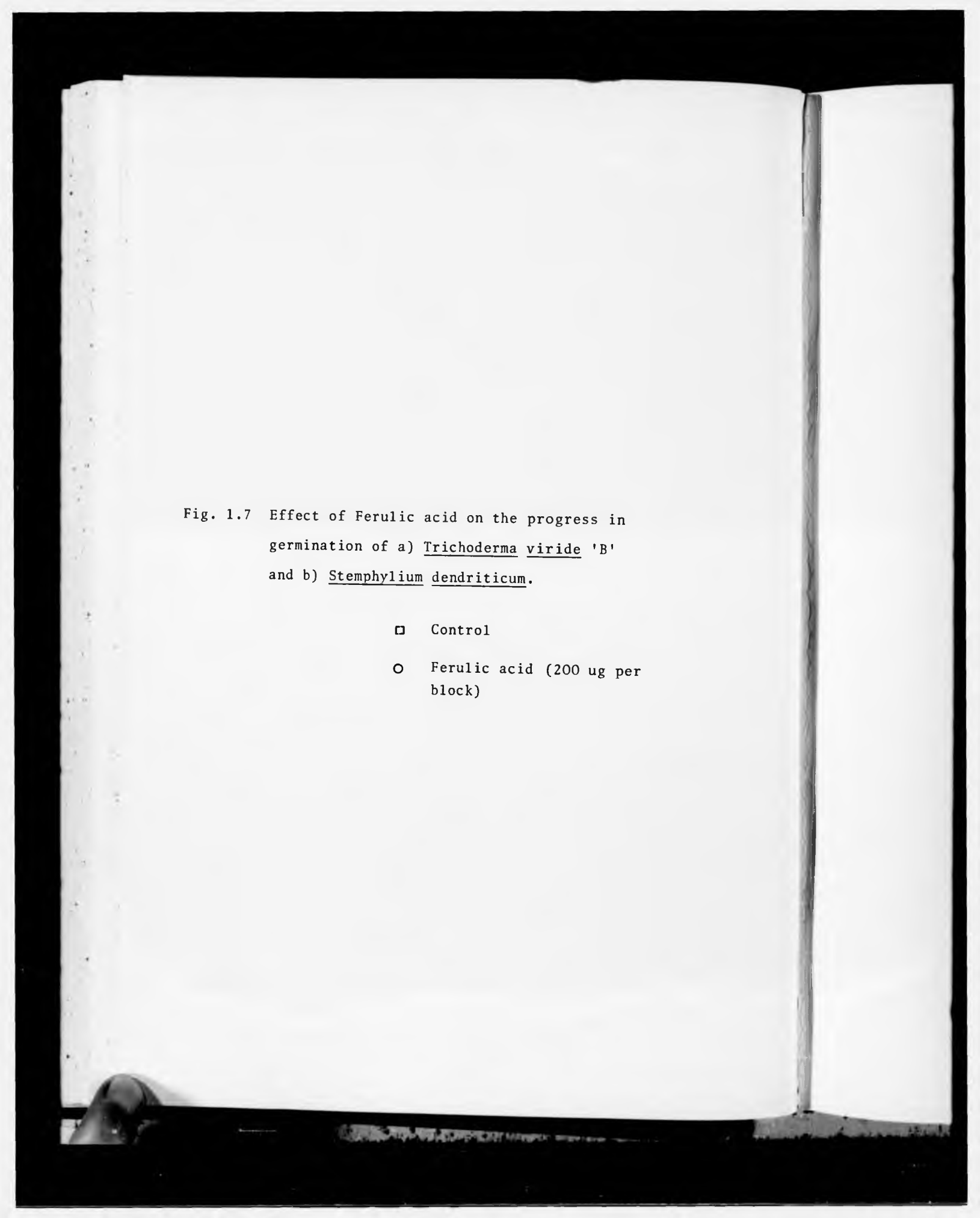
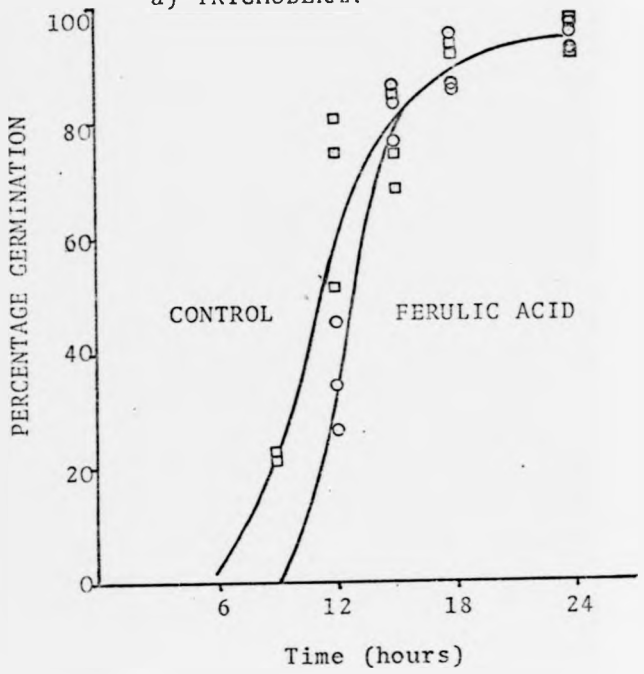


Fig. 1.7 Effect of Ferulic acid on the progress in germination of a) Trichoderma viride 'B' and b) Stemphylium dendriticum.

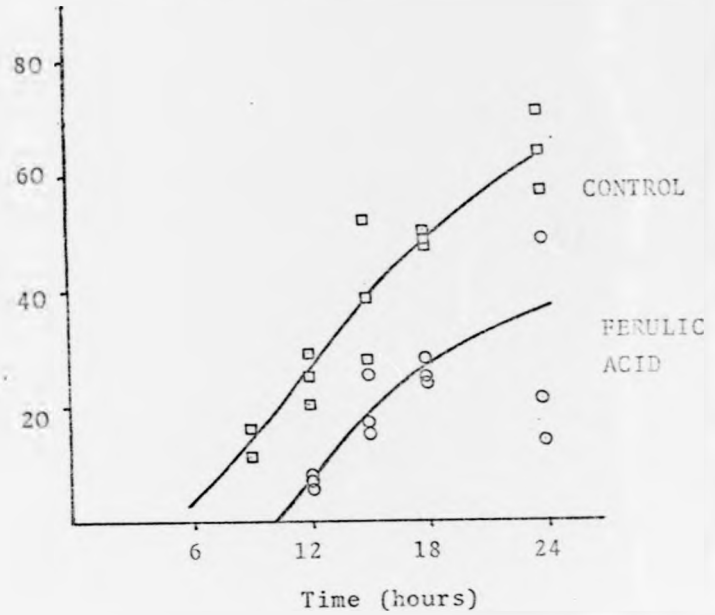
□ Control

○ Ferulic acid (200 ug per block)

a) TRICHODERMA



b) STEMPHYLIUM



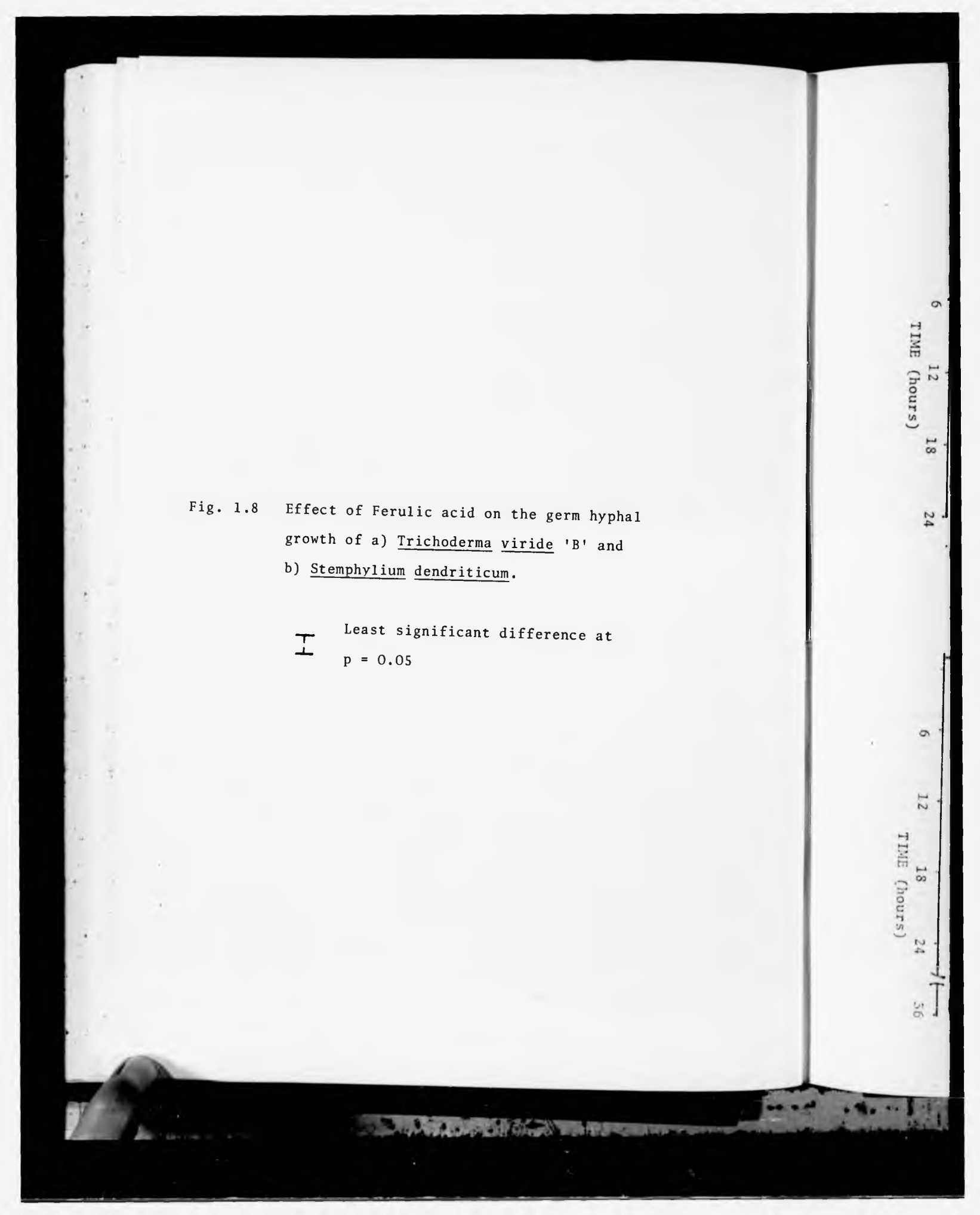


Fig. 1.8 Effect of Ferulic acid on the germ hyphal growth of a) Trichoderma viride 'B' and b) Stemphylium dendriticum.

⊣⊢ Least significant difference at p = 0.05

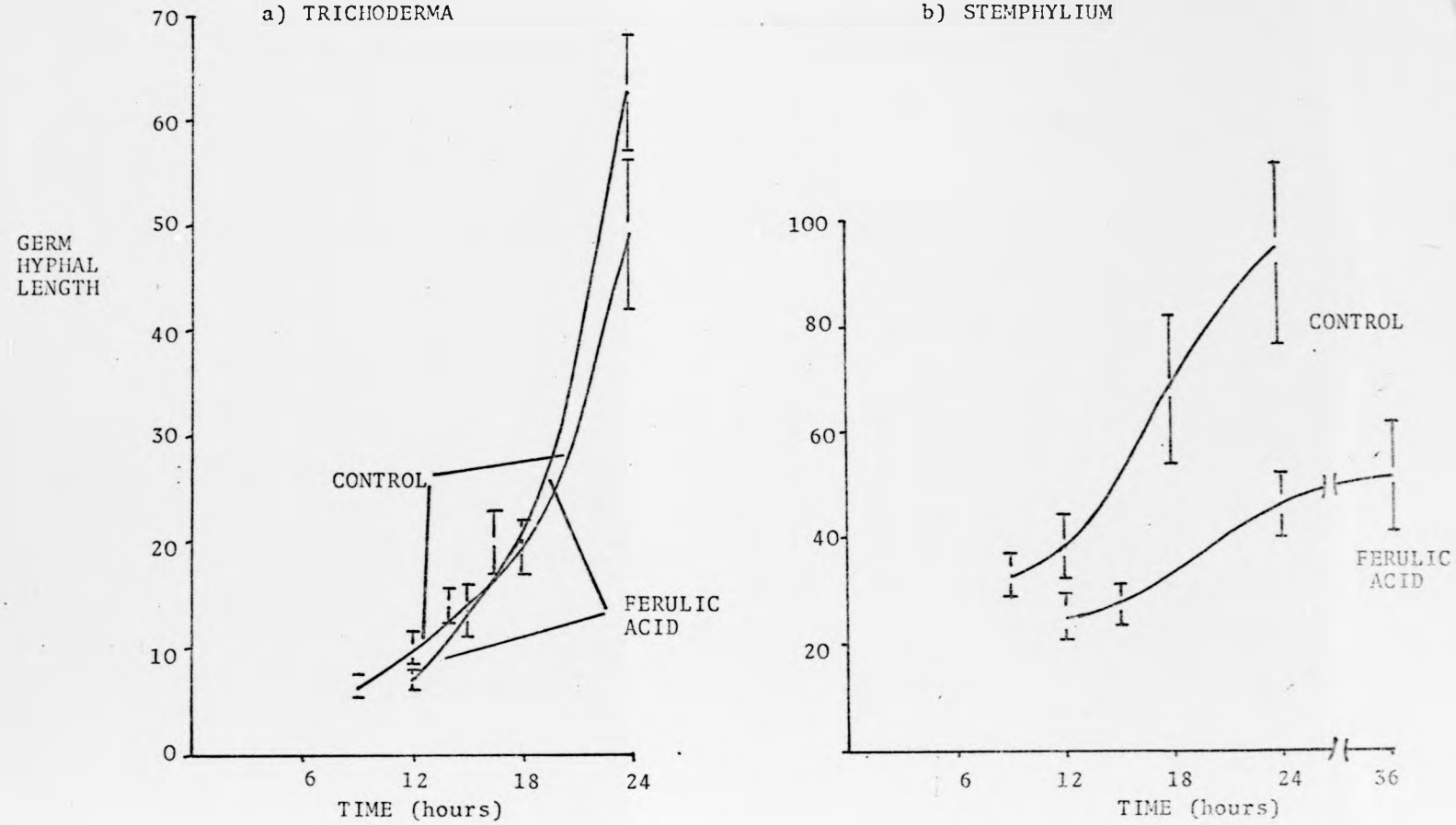

a) TRICHODERMA
b) STEMPHYLIUM
GERM HYPHAL LENGTH
70
60
50
40
30
20
10
0
CONTROL
FERULIC ACID
6
12
18
24
TIME (hours)
100
80
60
40
20
CONTROL
FERULIC ACID
6
12
18
24
36
TIME (hours)

Fig. 1.9 Frequency distributions of hyphal lengths of Trichoderma viride 'B' during the course of germination. (see figs. 1.7a and 1.8a).

Time (h)	Mean hyphal lengths: Control	Ferulic acid
9	6.6	-
12	9.9	6.5
15	13.5	13.9
18	19.5	19.8

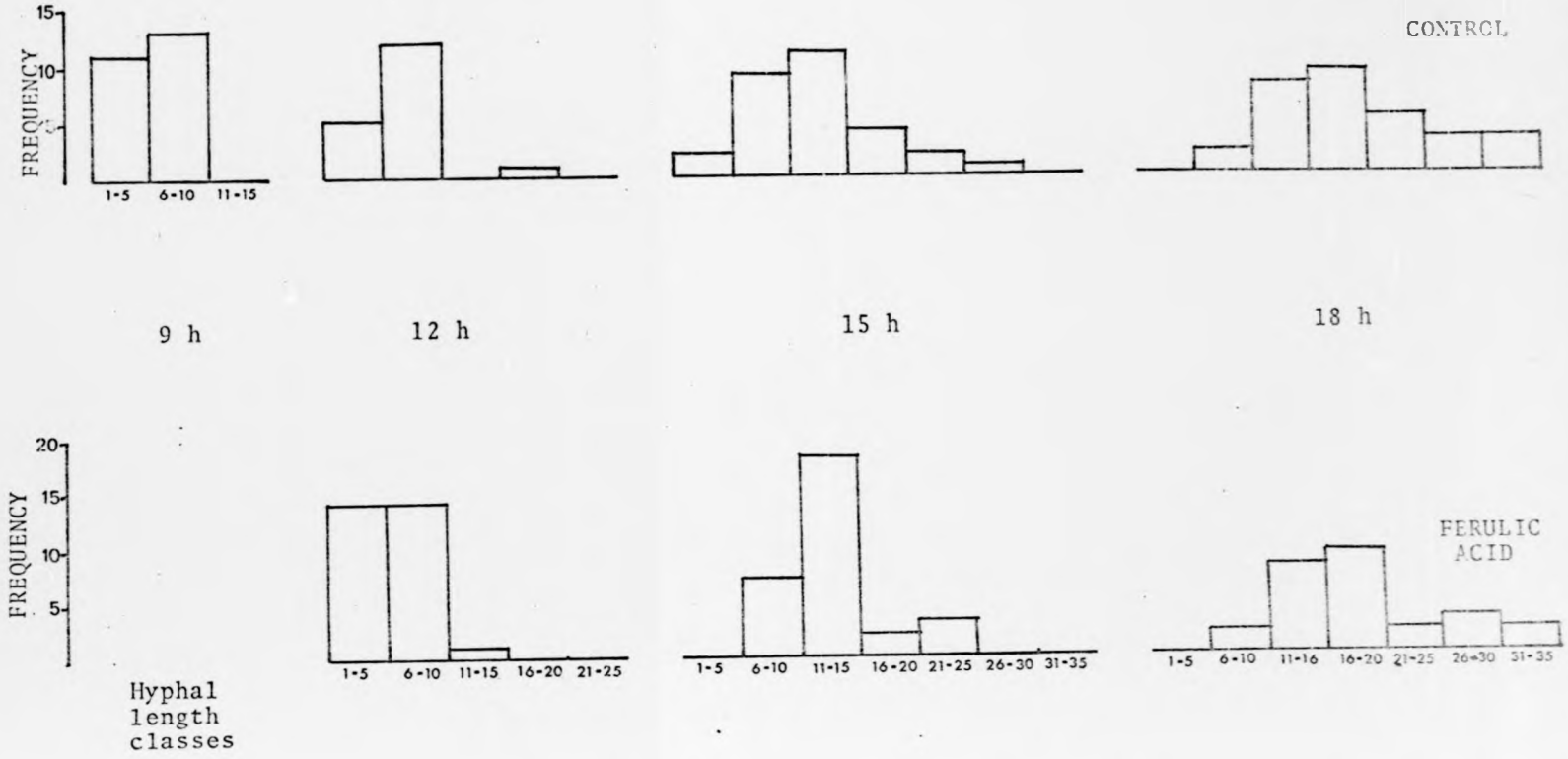
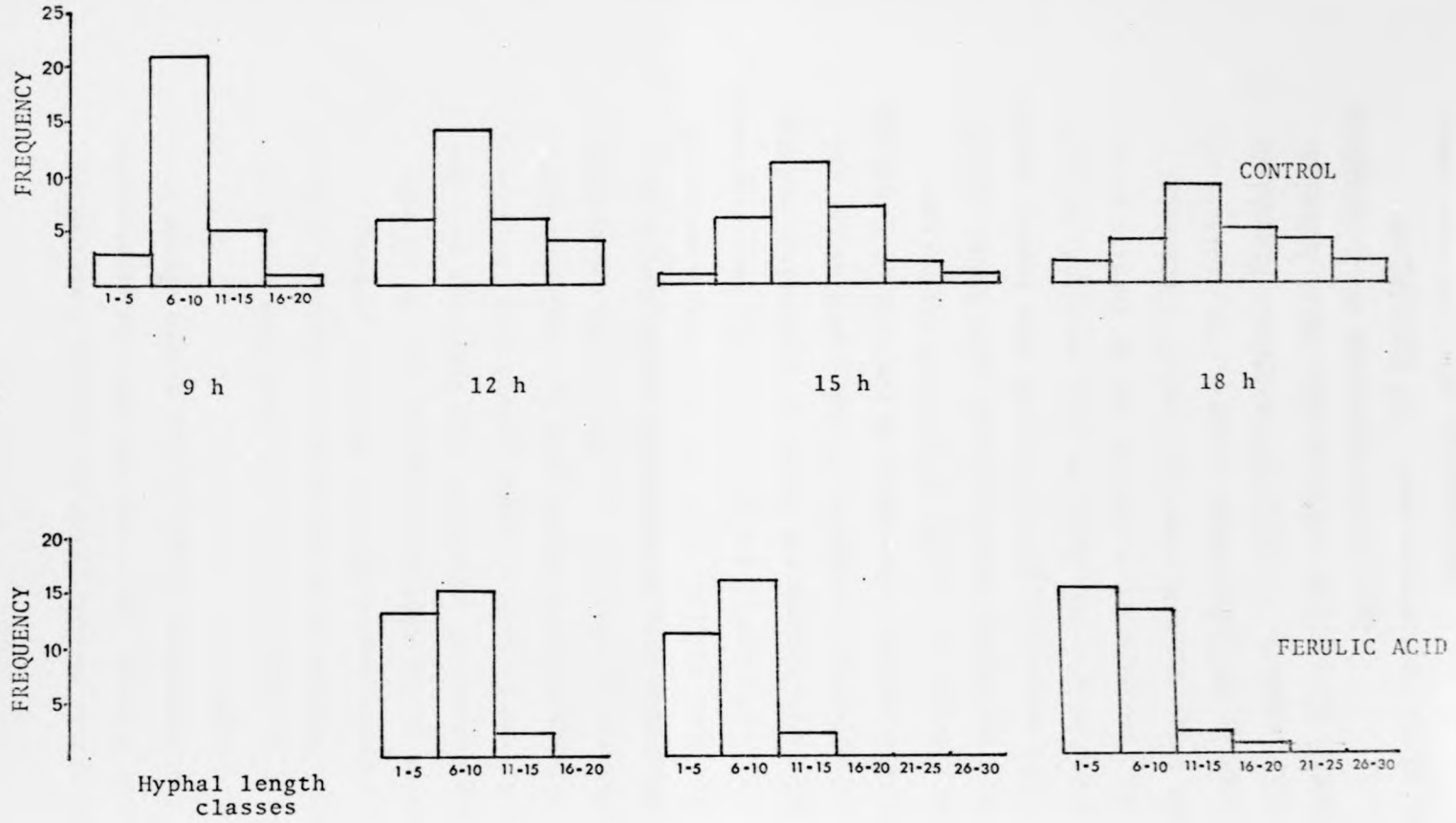

FREQUENCY
15
10
5
1-5
6-10
11-15
CONTROL
9 h
12 h
15 h
18 h
FREQUENCY
20
15
10
5
1-5
6-10
11-15
16-20
21-25
26-30
31-35
FERULIC ACID
Hyphal length classes

Fig. 1.10 Frequency distribtuions of hyphal lengths of Stemphylium dendriticum during the course of germination (See figs.1.7b and 1.8b).

Time (h)	Mean hyphal lengths: Control	Ferulic acid
9	8.3	-
12	9.5	6.2
15	16.5	6.8
18	17.1	10.5


FREQUENCY
25
20
15
10
5
1-5
6-10
11-15
16-20
CONTROL
9 h
12 h
15 h
18 h
FREQUENCY
20
15
10
5
1-5
6-10
11-15
16-20
21-25
26-30
FERULIC ACID
Hyphal length classes

experimental period, while the control distribution becomes diffuse, indicating the delay in germination and the depression of germ hyphal growth caused by ferulic acid.

It is suggested that when conditions cause an initial depression of percentage germination followed by a stimulation of the rate of hyphal growth, relative to the control situation, the use of mean hyphal lengths at successive time intervals will not give a true picture of hyphal growth rate, because of the disproportionate amount of short hyphae caused by the delayed germination. Ideally the measurement of the growth rate of individual hyphae should be carried out. (Casida, 1972, has described a method for this). Dix (1972) and Mitchell (1973) have considered correlations between the levels of fungistatic effects on the different parameters of spore germination and germ tube growth. But here, in the case of T. viride, is an example of a purely statistical interaction between percentage germination and germ hyphal growth.

The percentage germination and germ hyphal lengths of a number of other fungi was also measured, on agar blocks incorporating 0, 200 and 600 µg ferulic acid. These fungi included other isolates of Dothichiza pityophila and Trichoderma viride; the isolates of Botrytis cinerea, Cladosporium herbarum, Dreschlera rostrata, Thysanohora penicilloides, Mucor hiemalis, Penicillium spinulosum and P. expansum used in the preliminary work; and Penicillium funiculosum and P. janthinellum, which are very common

soil species. Aureobasidium pullulans, a ubiquitous primary leaf saprophyte, was also studied. On water agar this fungus does not produce hyphae but grows by forming colonies of blastospores, a form of growth it shows on leaves (Skidmore & Dickinson, 1973; Pugh & Buckley, 1971). The growth of A. pullulans in this manner is markedly inhibited by ferulic acid at 200 µg ferulic acid per block). The origins of the new isolates will be found in the Materials and Methods section.

In these experiments percentage germination and germ hyphal lengths were only measured at a single time (usually 16-18 hours after incubation) except in the case of Penicillium expansum (17 and 26 hours). The results are given in Table 1.2 (% germination) and Table 1.3 (germ hyphal length). Although different objectives had to be used in the projection microscope for different fungi, depending on the intrinsic rates of hyphal growth, the values in Table 1.3 have been given as if all were measured at the same magnification (400x).

With 200 µg ferulic acid per block there is significant inhibition of percentage germination of four isolates, while an equal number are significantly stimulated. Because the species which are stimulated are those showing low percentage germination on the control discs, it is likely that the effect of ferulic acid is to increase the potential for germination. Germ tube lengths are significantly affected in both directions, with five isolates showing significant inhibition and seven being significantly stimulated, by 200 µg ferulic acid.

TABLE 1.2 Effect of ferulic acid on the germination of spores of fungi from litter and soil.

	Percentage germination [a] Ferulic acid per block (µg)		
	0 (Control)	200	600
Dreschlera rostrata	96.2	96.6	16.5 ***
Stemphylium dendriticum	67.1	43.0	0
Botrytis cinerea	86.9	78.2*	0
Cladosporium herbarum	54.2	31.3**	0
Penicillium expansum 17h	61.3	48.8**	0
26h	82.7	72.3	45.9 ***
P. janthinellum	90.7	87.3	89.6
P. spinulosum	53.7	78.3*	4.4 ***
P. Funiculosum	35.3	75.3***	3.1 ***
Trichoderma viride 'B'	77.6	84.9	32.8 *
'C'	77.3	59.1**	0
Mucor hiemalis 'A'	90.1	88.0	0
'B'	26.0	57.5***	0
Thysanophora penicilloides	6.4	29.1**	44.7**

a) Measured after 16-18 h. incubation except for

M. hiemalis 'B'	20 h	* Significant	$p < 0.05$
C. herbarum	24 h	** "	$p < 0.01$
T. penicilloides	29 h	*** "	$p < 0.001$

TABLE 1.3. Effect of ferulic acid on the germ hyphal growth of microfungi from litter and soil

	Germ hyphal length [a] (Arbitrary units) Ferulic acid per block (µg)		
	0 (Control)	200	600
Dreschlera rostrata	405.1	308.9 *	54.5 ***
Stemphylium dendriticum	56.8	34.0 ***	0
Botrytis cinerea	26.7	19.7 ***	0
Cladosporum herbarum	24.4	21.0	0
Penicillium expansum 17 h	8.6	6.7 *	0
26 h	14.3	12.2	7.1 ***
P. janthinellum	7.2	11.1 ***	8.8
P. spinulosum	7.1	8.8 *	3.9 ***
P. funiculosum	5.1	7.0 ***	3.6 ***
Trichoderma viride 'B'	10.7	14.6 **	5.3 ***
'C'	11.9	10.0	0
Mucor hiemalis 'A'	18.2	21.9	0
'B'	20.9	14.4 **	0
Thysanophora penicilloides	7.0	11.2 ***	10.0 ***
Dothichiza pityophila 'A'	14.6	31.2 ***	21.2 ***
'C'	9.6	39.4 ***	13.2 **

a) All measured after 16-18 h incubation except for

M. hiemalis 'B' 20 h

C. herbarum 24 h

T. penicilloides 29 h

D. pityophila 'A' & 'C' 26 h

For significance levels see Table 1.2

The Theoretical Colonisation Index, defined by Mitchell (1973) has been calculated (% germination x germ hyphal length) for each isolate under each treatment and the values are given in Table 1.4. This index is a measure of the total growth that a fungus makes under given conditions, and there is a very large range of control values from 38970 (D. rostrata) to 45 (T. penicilloides). Excluding Dreschlera rostrata the effect of 200 µg ferulic acid is to reduce the range considerably (3811 to 45 in control, 1927 to 326 with 200 µg ferulic acid) and the leaf saprophytes no longer predominate as the species with the highest values of the Theoretical Colonisation Index.

In Table 1.5 the different isolates have been grouped according to the direction of the effects of 200 µg ferulic acid on percentage germination, germ hyphal length and theoretical colonisation index, and the percentage change relative to control has been given (control - treatment/control x 100). A. pullulans has been shown with a depressed theoretical colonisation index. From Table 1.4 it can be seen that fungi showing a significant stimulation of percentage germination and of germ hyphal length, and a greater colonisation index belong exclusively to the specialised needles saprophytes and soil fungi (group 'B' in the preliminary experiments); while only Mucor hiemalis 'B' and Trichoderma viride 'C' are included among the leaf saprophytes and Penicillium expansum (comprising group 'A' above) which respond with significant inhibition to 200 µg ferulic acid.

Under the influence of 600 µg some fungi are able to maintain a fairly high level of percentage germination

TABLE 1.4 Effect of ferulic acid on the Theoretical Colonisation Index of microfungi from litter and soil.

	Theoretical Colonisation Index[a]		
	Ferulic acid per block (µg)		
	0 (Control)	200	600
Dreschlera rostrata	38,970	29,839	899
Stemphylium dendriticum	3,811	1,462	0
Botrytis cinerea	2,320	1,541	0
Cladosporium herbarum	1,322	657	0
Penicillium expansum[b]	527	327	0
P. janthinellum	653	969	788
P. spinulosum	381	689	17
P. Funiculosum	180	527	11
Trichoderma viride 'B'	830	1,240	174
'C'	920	591	0
Mucor hiemalis 'A'	1,640	1,927	0
'B'	543	828	0
Thysanophora penicilloides	45	326	948

a) Computed from values of % germination and germ hyphal growth given in Tables 1.2 and 1.3.

b) After 17 h incubation

TABLE 1.5 Effect of Ferulic acid at 200 µg per block on the germination and germ hyphal growth of microfungi.

Fungi grouped according to the direction of the effect on percentage germination, germ hyphal length and Theoretical Colonisation Index relative to control with appropriate percentage changes.

Percentage Germination

REDUCTION*		INCREASE*		NO EFFECT*	
B.cinerea	10.0	P.spinulosum	45.8	D.rostrata	+ 0.4
C.herbarum	42.3	P.funiculosum	113.3	S.dendriticum	-35.8
P.expansum	20.4	M.hiemalis 'B'	121.2	P.janthinellum	- 0.4
T.viride'C'	23.4	T.penicilloides	354.7	T.viride 'B'	+ 9.4
				M.hiemalis 'A'	- 2.3

Germ Hyphal Length

REDUCTION*		INCREASE*		NO EFFECT*	
D.rostrata	23.8	P.janthinellum	54.1	C.herbarum	-13.9
S.dendriticum	40.1	P.spinulosum	23.9	T.viride 'C'	-16.0
B.cinerea	26.2	P.funiculosum	37.3	M.hiemalis'A'	+20.3
P.expansum	22.1	T.viride 'B'	36.5		
M.hiemalis'B'	31.1	T.penicilloides	60.0		
		D.pityophila'A'	113.7		
		D.pityophila'C'	310.4		

Theoretical Colonisation Index

REDUCTION		INCREASE	
D. rostrata	23.4	P. janthinellum	48.4
S. dendriticum	61.6	P. spinulosum	80.8
B. cinerea	33.6	P. funiculosum	192.8
C. herbarum	50.3	T. viride 'B'	49.4
P. expansum	38.0	M. hiemalis 'A'	17.5
T. viride 'C'	35.8	M. hiemalis 'B'	52.5
Aureobasidium pullulans	(see text)	T. penicilloides	624.4

* Significant $p < 0.05$

(Table 1.2) and germ hyphal length (Table 1.3) while others have not germinated. Using the Theoretical Colonisation Index (Table 1.4) three fungi have a value greater than 780, one isolate of value 174, two isolates have values of 11 and 17 and all the rest have zero values. P. expansum has not germinated at 17 hours but after 26 hours of incubation its Theoretical Colonisation Index is 318, suggesting that one of the main effects of this high concentration of ferulic acid is an exaggerated delay in the onset of germination. The percentage germination of Thysanophora penicilloides is actually greater at this higher level of ferulic acid than in either of the other treatments, while the hyphal lengths of T. penicilloides and Dothichiza pityophila (both isolates) are still stimulated with respect to the control.

Generally speaking, the conifer litter and soil inhabiting species are fungi with slower hyphal growth in control than the leaf saprophytes, a feature corresponding to observations made in the preliminary experiments but there is no particular association of control percentage germination and ecological group. Bearing in mind the possible effect of delayed germination on the observed rate of hyphal growth - considered earlier in the time course studies - it is possible that fungi germinating rapidly in the control relative to the treatment and showing hyphal lengths inhibited by ferulic acid (e.g. Dreschlera rostrata) might have a stimulated level of hyphal growth beyond the experimental period used here. But the rate of hyphal growth of the leaf saprophytes is generally so rapid that hyphal lengths would be difficult

to measure at the time when stimulation by ferulic acid may be expected. However, from an ecological point of view, this consideration of possible effects that have been missed may not be so important. A rapid germination rate and/or a high rate of hyphal growth in the control treatment would seem to indicate an inherent capacity for successful colonisation of substrates and Hudson (1968) has commented upon the possession of this feature by the primary leaf saprophytes. But with the fungi from this group studied here there is the possibility of a diminution of this capacity in the presence of certain compounds in the environment. If one can consider the total potential hyphal growth, represented by the theoretical colonisation index, as the most important feature then the effect of phenolic acids on this potential could be due to either a direct inhibition of germ hyphal growth or an indirect effect by the inhibition of germination (initially at least). The fungi from the other ecological groups may have a lower intrinsic colonisation capacity but there is a stimulation of growth in the presence of phenolic acids, which has the effect of levelling out the disparity between the Theoretical Colonisation Indices of the leaf saprophytes and the conifer litter and soil fungi. As a result the balance of competitive advantage could be shifted towards the latter group in certain situations. And the behaviour of Dothichiza pityophila and Thysanophora penicilloides at very high levels of ferulic acid is worth recalling.

The results on which the above discussion is based are from a rather incomplete series of experiments. It had

been decided to study the effects of only ferulic acid in detail and time course effects have largely been assumed among the majority of the fungi tested. However, apart from the fact that ferulic acid seemed to be typical of a number of phenolic acids, this compound shows a very wide range of effects, which are complicated enough in themselves. As the results of the experiments with ferulic acid alone provide a pattern of behaviour of the fungi concerned which is strongly associated with the ecological distribution of these fungi, it was decided to investigate whether there was any differential ability of the fungi to decompose ferulic acid; and whether this ability was also correlated with ecological patterns.

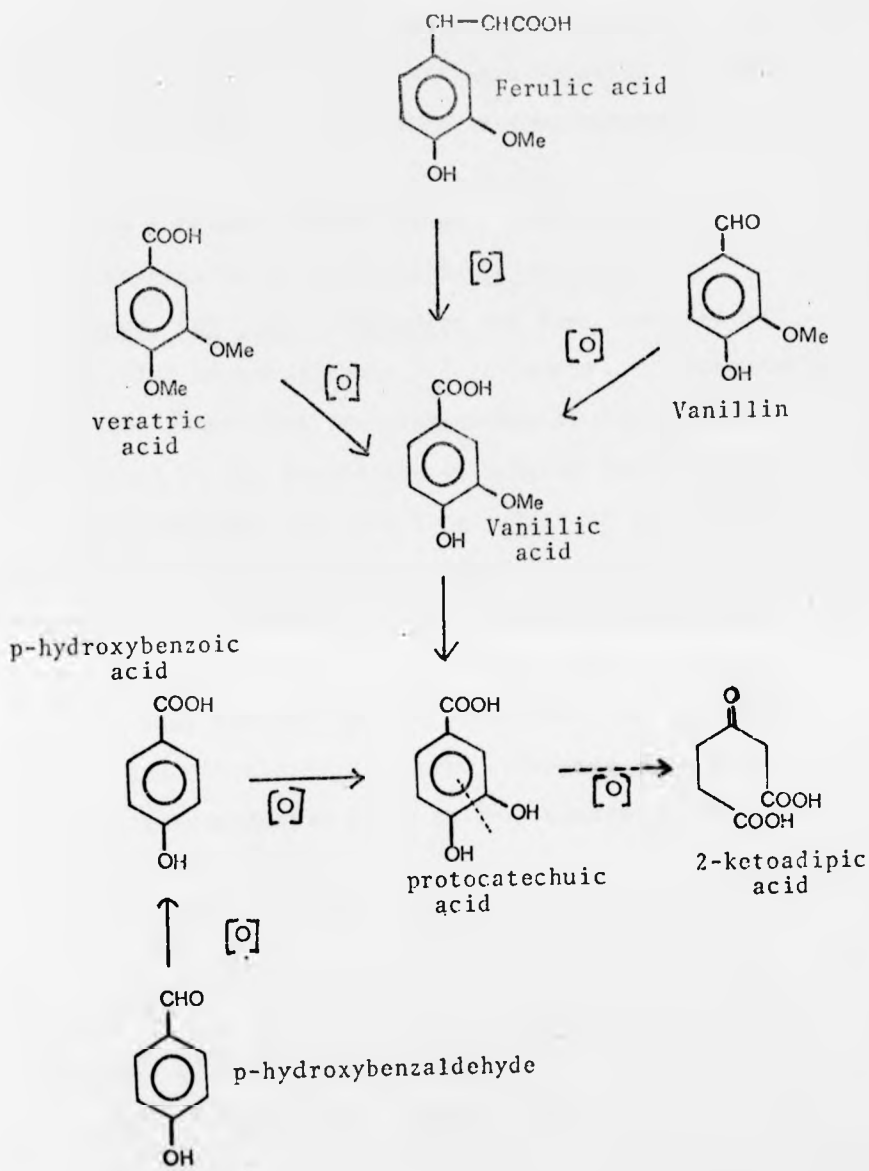
The work of Henderson's group, already referred to, established several important features in the metabolism of phenolic acids and aldehydes by microfungi and the capacity of these organisms to utilise them. Henderson & Farmer (1955) studied the utilisation of p-hydroxybenzaldehyde, vanillin and syringaldehyde and ferulic acid by 60 isolates of soil fungi, representing 28 genera, in still liquid culture. They showed that the disappearance of substrate over a period of 24 days was associated with the growth of mycelium from the agar plug inoculum. All isolates tested could decompose at least 90% of one or more of the p-hydroxyphenyl and guaiacyl compounds while syringaldehyde was less readily attacked. Only isolates of four species could make little or no impression on ferulic acid, and only one was unable to decompose vanillin, although several species were apparently unable to decompose the vanillic acid they formed from vanillin. Vanillic

acid was the only product formed from vanillin and ferulic acid, and syringic acid was the only product from syringaldehyde.

Subsequent work by Henderson (1956, 1961a, b) established pathways for the metabolism of these compounds via ring cleavage of protocatechic acid to 2-ketoadipic acid. Here detailed studies were carried out on a selection from the fungi she had used earlier and some other Fungi Imperfecti and some yeasts isolated by soil enrichment, with the aid of more sophisticated techniques for the isolation of intermediates. The most important features were a) the lag in the oxidation of the benzoic acids, to which the fungi were adaptable using the corresponding aldehydes (and also ferulic acid in the case of vanillic acid) and b) the characterisation of intracellular protocatechuic 3:4 oxygenase, which catalysed ring cleavage. The lag in this activity could be relieved by pre-incubation with p-hydroxyphenyl compounds but not by the syringyl equivalents. Thus, although protocatechic acid was not obtained as an intermediate (except from p-hydroxybenzoic acid in a few cases) a pathway could be proposed for the utilisation of the p-hydroxyphenyl and guaiacyl compounds with protocatechuic acid as the central intermediate (Fig. 1.11). P-hydroxybenzoate is hydroxylated to protocatechuate whereas vanillate is demethylated to the latter, and Henderson (1957) showed that para-methoxy groups are preferentially attacked to ortho- or meta groups in mono- or dimethoxylated compounds. Since vanillate oxidation (i.e. ortho-demethylation) requires adaptation vanillic acid

Fig. 1.11 Enzymatic oxidations involved in interconversion of some phenolic acids and aldehydes, and aromatic ring cleavage, by microfungi. For the types of enzymatic activity see Flaig & Haider (1961b).

Me = CH_3


CH—CHCOOH
Ferulic acid
OMe
OH
COOH
OMe
OMe
veratric acid
[O]
CHO
OMe
OH
Vanillin
COOH
OMe
OH
Vanillic acid
p-hydroxybenzoic acid
COOH
OH
COOH
OH
OH
protocatechuic acid
O
COOH
COOH
2-ketoadipic acid
CHO
OH
p-hydroxybenzaldehyde

accumulates in the oxidation of veratric acid by microfungi (Henderson, 1957) although Nocardia produces a mixture of vanillic and 3-hydroxy-4-methoxybenzoic acids (Crawford et al., 1973) (see Fig. 1.11).

Flaig & Haider (1961a) found a similar pathway for the metabolism of vanillin by Polystictus (=Polyporus, Coriolus) versicolor and they isolated an extracellular protocatechuic 3:4 oxygenase. Subsequently it has been shown that protocatechuate is the central intermediate in the metabolism of benzoic and cinnamic acids and aldehydes for most fungi (Cain et al., 1968) and for bacteria (e.g. Cain & Farr, 1968) and for a Nocardia (Crawford et al. 1973). But the mechanism varies as not all bacteria, and perhaps not all fungi, split the ring between the two hydroxyls (the 'ortho' route); and the bacterial 'ortho' cleavage involves different intermediates prior to 2-ketoadipate (Cain & Farr, 1968, Cain et al. 1968).

Syringyl and sinapyl compound metabolism probably goes via gallic acid (Flaig & Haider, 1961b) but there is little in the literature about the mechanism of the utilisation of this compound rather than polymerisation. Flaig (1964) reported that coniferyl units are split off from wheat straw lignin in preference to sinapyl units and that syringyl compounds were not found among decomposition products. This probably reflects preferential oxidation of the benzyl carbons attached to syringyl nuclei (see above) with the release of the nuclei as 3:5-dimethoxy-benzoquinone (Young & Steelink, 1973) which can be isolated from soil. Perhaps the comparatively poor

ability of fungi to metabolise syringyl compounds reflects their minor status as decomposition products of lignin as well as purely mechanistic factors.

It was the intention of the work described below to relate the utilisation of ferulic acid by microfungi to the effects of this compound on spore germination and germ hyphal growth, so that it is necessary to study this utilisation over the period of germination and initial growth. By contrast the method of Henderson & Farmer (1955) probably determined the ultimate ability of fungi to metabolise the phenolic compounds because the experiments were carried out over 24 days. It is therefore inappropriate to use agar plugs as inocula, as in that study or preformed mycelial mats, as used by Henderson (1961b); rather, spore suspension must be employed as inocula. Respirometry with spore suspension inocula was used by Henderson (1956, 1961a,b) for metabolic studies and the metabolism of vanillin by bacteria in soil samples was investigated by Kunc (1971a) using this technique. However, it was decided to employ shake cultures as they permit the identification of intermediates as well as determination of the activity of the inoculum.

The question of the initial concentration of substrate to be employed is a difficult one as several factors are involved. Measurement of fungal activity by means of mycelial dry weight is only suitable at relatively high concentrations if there are no other carbon sources but the concentrations necessary may bring problems of toxicity. Flaig & Haider (1961a) used concentrations up to 1 g l^{-1} of vanillin and other compounds in order to measure the dry weight of Coriolus versicolor. And a

concentration of 1 g l^{-1} was also used by Cain et al. (1968) in studies on the utilisation of a wide range of phenolic compounds including ferulic acid. Apart from the fact that this concentration seems impossible for ferulic acid, it should be noted that mycelial dry weights produced with several phenolic acids and aldehydes were often below 5 mg and sometimes below 1 mg, probably because of toxic effects. For these reasons the method of Henderson & Farmer (1955) was preferred, that is, the measurement of the disappearance of the substrate by ultra-violet spectrophotometry for the estimation of utilisation. Henderson and Farmer in fact used an initial concentration of 50 mg l^{-1} of ferulic acid to avoid toxicity, but here preliminary trials showed that S. dendriticum could readily decompose ferulic acid at an initial concentration of 100 mg l^{-1}.

In the standard conditions employed, the initial concentration of ferulic acid was 100 mg l^{-1} as the sole carbon source in a basal medium of Czapek salts, giving an initial pH of 4.5. 100 cm^3 of culture solution was inoculated with 1 cm^3 of spore suspension in 500 cm^3 and incubated at 25°C for five days on an orbital incubator. The concentration of ferulic acid remaining in the flasks was determined from the ultra-violet absorption of the culture filtrate at 314 nm. Analysis of the products of decomposition was carried out by chromatography of the residues left on evaporation of the culture filtrates under vacuum, either by two dimensional thin layer chromatography on silica gel, according to the method of Grabbe & Haider (1971a) or by gas liquid chromatography after silylation

of the residue with Bis-trimethylsilylacetamide. Identification of the substances eluted from the T.L.C. plates was carried out by ultra-violet spectrophotometry and gas liquid chromatography against authentic compounds. Other details of the experimental conditions and the methods of analysis are given in the Materials and Methods section.

Under the standard conditions 30 isolates (from 21 species) of microfungi were tested for their ability to decompose ferulic acid, including those fungi used previously and others isolated from Scots pine needles and from soil in the course of field experiments. There is also one isolate of the Proactinomycete Nocardia (see Materials and Methods for details of the isolates). The results of these experiments are given in Tables 1.6 and 1.7. The concentration of ferulic acid remaining in the flasks after five days incubation is shown in Table 1.6 and the products of decomposition found in the culture filtrates in table 1.7. These results are derived from replicate experiments. Two flasks were incubated for each fungus in each experiment.

The minimum concentration of ferulic acid that could be determined by its absorbance in the culture filtrates when other products were present was about 5 mg l^{-1} but from the absorbance of ferulic acid eluted from the T.L.C. plates and from direct gas chromatography of the filtrates concentrations of the order of 1 mg l^{-1} or less were indicated. In terms of the concentration of ferulic acid remaining after five days incubation, one might distinguish and define three levels of activity: very low activity (at least 90% of the ferulate remaining), moderate activity (90-60% remaining) and high activity (less than 10% remaining); and among those isolates with high activity some leave no ferulic

TABLE 1.6 Decomposition of Ferulic acid by microfungi. Concentration of Ferulic acid remaining in culture filtrates after 5 days incubation

	Ferulic acid concentration ($Mg\ L^{-1}$)
Uninoculated Control Culture	100
Dreschlera rostrata	< 5
Stemphylium dendriticum	< 5
Botrytis cinerea	99.0 ± 2.5
Cladosporium herbarum	71.0 ± 1.3
Aureobasidium pullulans	9.4 ± 3.5
Pencillium expansum	60.0 ± 3.9
P. janthinellum	0
P. spinulosum	0
P. funiculosum	0
Trichoderma viride 'A'	92.5 ± 3.8
'B'	87.0 ± 1.2
'C'	95.5 ± 1.7
'D'	90.5 ± 1.2
'E'	99.5 ± 0.4
'F'	99.5 ± 0.4
'G'	100.0 (± 0.0)
Mucor hiemalis 'A'	95.0 ± 2.8
'B'	61.5 ± 8.1
Mucor racemosus	90.5 ± 0.1
Mortierella ramanniana 'A'	99.0 ± 1.3
'B'	100.0 (± 0.0)
Thysanophora penicilloides	< 5
Dothichiza pityophila 'A'	< 5
'C'	< 5
Desmazierella acicola	0
Phoma eupyrena	0
Gliocladium roseum	< 5
Fusarium oxysporum	0
Coniella sp.	< 5
Gilmaniella humicola	0
Nocardia sp.	100 (± 0.0)

TABLE 1.7. Products identified by chromatography in the culture filtrates of microfungi after decomposition of Ferulic acid.

	VANILLATE	VANILLIN	OTHER PRODUCTS [a]	
Uninoculated Control	-	-	+	[b]
Dreschlera rostrata	-	-	-	[c]
Stemphylium dendriticum	-	-	-	[c]
Cladosporium herbarum	+	?	-	
Aureobasidium pullulans	+	?	+	
Penicillium expansum	+	-	-	
P. spinulosum	-	-	-	
Mucor hiemalis	+	+	-	
Dothichiza pityophila	-	+ [d]	+	
Gliocladium roseum	+	+ [d]	+	
Thysanophora penicilloides	+	-	-	

a) See text

b) Autoxidation of ferulic acid during extraction produced several coloured spots on the T.L.C. plates. Few of these fluoresced in UV-light and none gave spectra in the UV region.

c) These genera unusual in giving clear spectra of Ferulic acid at 5 mg L^{-1} but no other products.

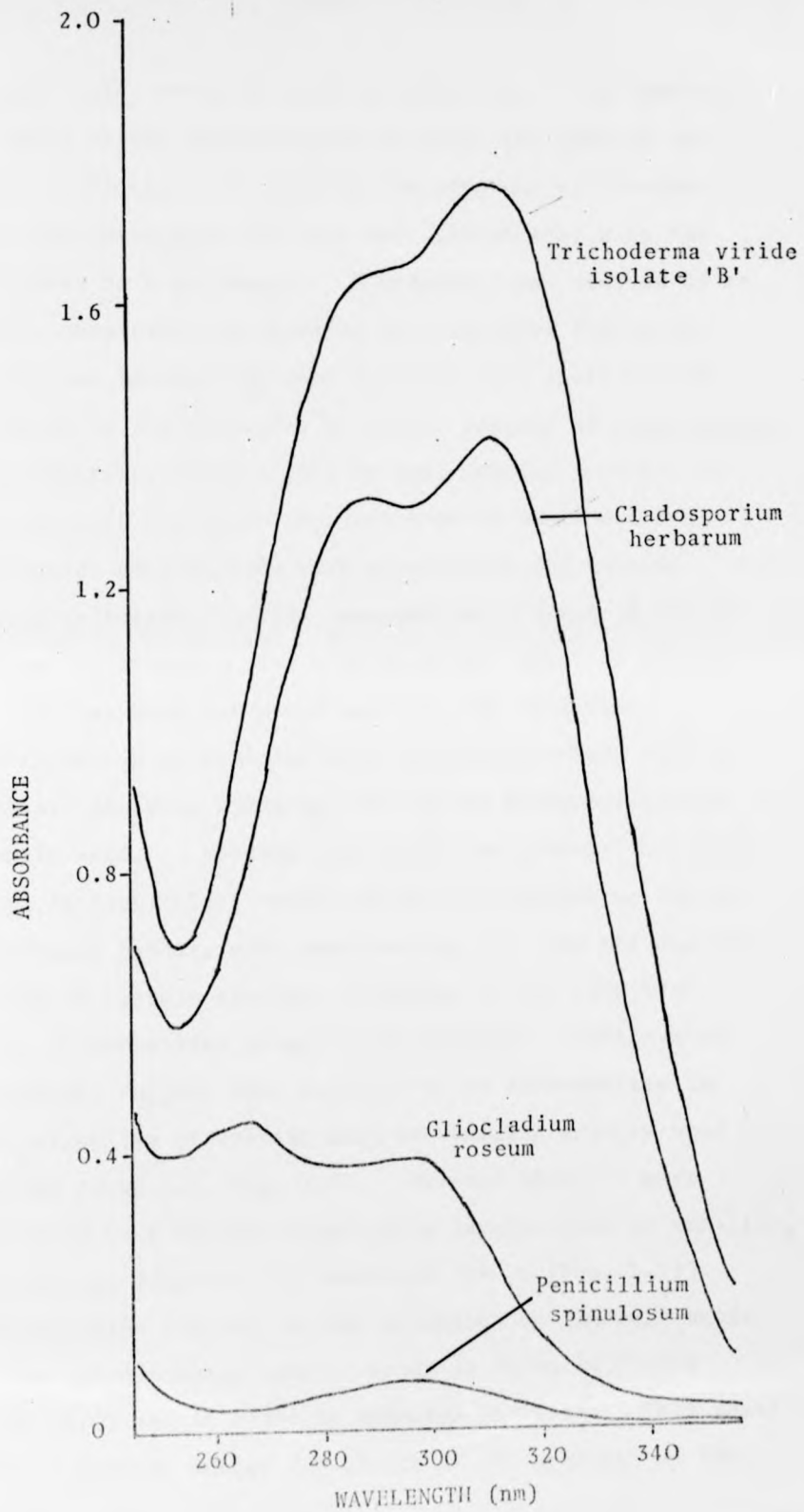
d) Vanillin eluted from spot on T.L.C. plate impure but spectrum with alkali confirmed presence of vanillin. A second peak was obtained in addition to vanillin on G.L.C. analysis of eluates.

acid and no other products, while others leave a minimal concentration of ferulate and additional products. The ultra-violet absorption spectra of culture filtrates from fungi typical of these levels of activity are shown in figure 1.12. Trichoderma viride 'B' and Cladosporium herbarum have moderate activity, Penicillium spinulosum has high activity, leaving no characteristic spectrum, while Gliocladium roseum has high activity, leaving products. Production of visible mycelium occurred only when there was some disappearance of the substrate. Thus some mycelium was produced by Trichoderma viride 'B' but not by any of the other isolates of this species.

There would seem to be moderate to high activity for decomposing ferulic acid among the majority of the isolates previously shown to be inhibited by ferulic acid - C. herbarum, P. expansum, A. pullulans, D. rostrata and S. dendriticum, only the latter two species being able to decompose ferulic acid readily, A. pullulans being rather variable. Among the soil and needle litter fungi, the isolates of Mucor spp., T. viride and Mortierella ramanniana stand out in being unable to make much impression on ferulic acid. All the other fungi of this group had high activity.

Vanillic acid was identified as an intermediate of ferulic acid decomposition in the culture filtrates of most fungi giving a residual spectrum, and some cultures produced vanillin (Table 1.7). Aureobasidium pullulans and Dothichiza pityophila were the only fungi to give spots on the T.L.C. plates of culture filtrates which gave reasonable spectra in the ultra-violet region, other than

Fig. 1.12 UV Spectra of the culture filtrates of several fungi after five days incubation with Ferulic acid. Dilution X4. An absorbance of 2.00 represents a concentration of 100 mg l^{-1} Ferulic acid in the indiluted filtrate.


2.0
1.6
1.2
0.8
0.4
0
ABSORBANCE
Trichoderma viride
isolate 'B'
Cladosporium
herbarum
Gliocladium
roseum
Penicillium
spinulosum
260
280
300
320
340
WAVELENGTH (nm)

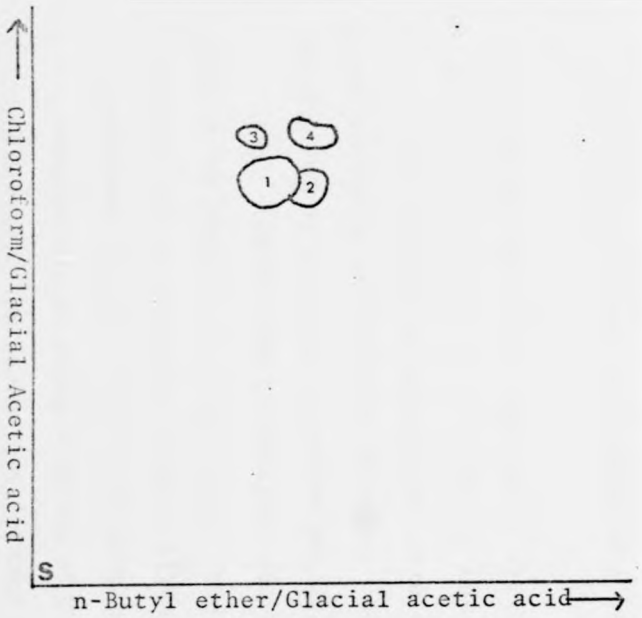
ferulic acid, vanillic acid and vanillin. The pattern of spots on the chromatograms of these two species are shown in Figure 1.13. showing the presence of Substance(s) A in the Dothichiza filtrate and Substance(s) B in the filtrates of both fungi. Substance A was present in an amount comparable to vanillic acid in other filtrates and it was interesting that vanillic acid could not be detected in the filtrates of either isolate of D.pityophila. Unfortunately, interference by autoxidation products of ferulic acid prevented the isolation of Substance A in sufficient amounts from bulk experiments for initial characterisation. This compound has a single λ max of 273 nm. in Ethanol, with a Bathochromic shift to 300 nm.

As has been discussed earlier, the oxidative demethylation of vanillic acid to protocatechuic acid is probably the rate limiting step in the decomposition of ferulic acid. Because this metabolic pathway is likely to be intracellular, vanillate probably builds up inside the fungal hyphae, with some leaking out into the culture medium of certain species, depending on the relative rates of successive steps in the pathway. Ishikawa et al. (1963b) suggest that vanillin is an intermediate in the metabolism of ferulic acid to vanillic acid by wood rotting fungi (cf. fig. 1.3). However there is more likely to be a direct oxidation of ferulic acid to vanillic acid as was suggested by Henderson (1960) (Fig. 1.11). A β-oxidation pathway for the oxidation of cinnamic acids to the corresponding benzoic acids is known in plants (Hess 1970) and it might be expected in fungi. This would readily provide energy for growth as the products of the

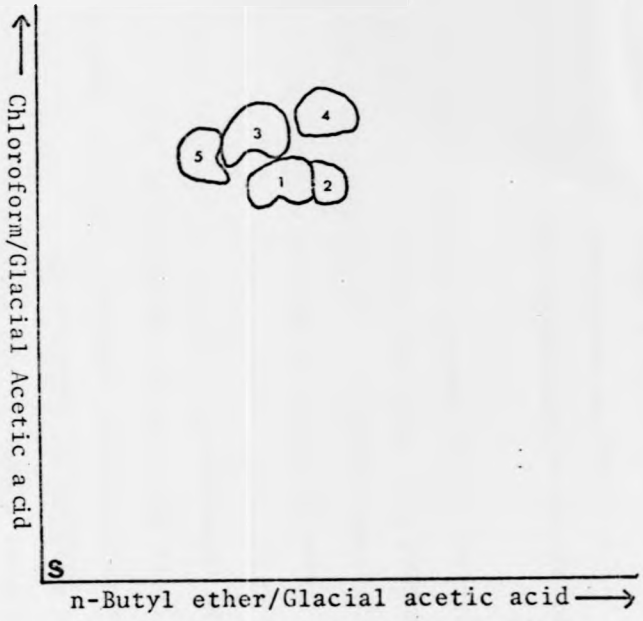
Fig. 1.13 Thin Layer Chromatograms of the culture filtrates of a) Aureobasidium pullulans and b) Dothichiza pityophila 'A'. The spots shown are those visualised under Ultra-violet light.

1 Ferulic acid
2 Vanillic acid
3 Vanillin
4 Substance 'B'
5 Substance 'A'

a) AUREOBASIDIUM



b) DOTHICHIZA

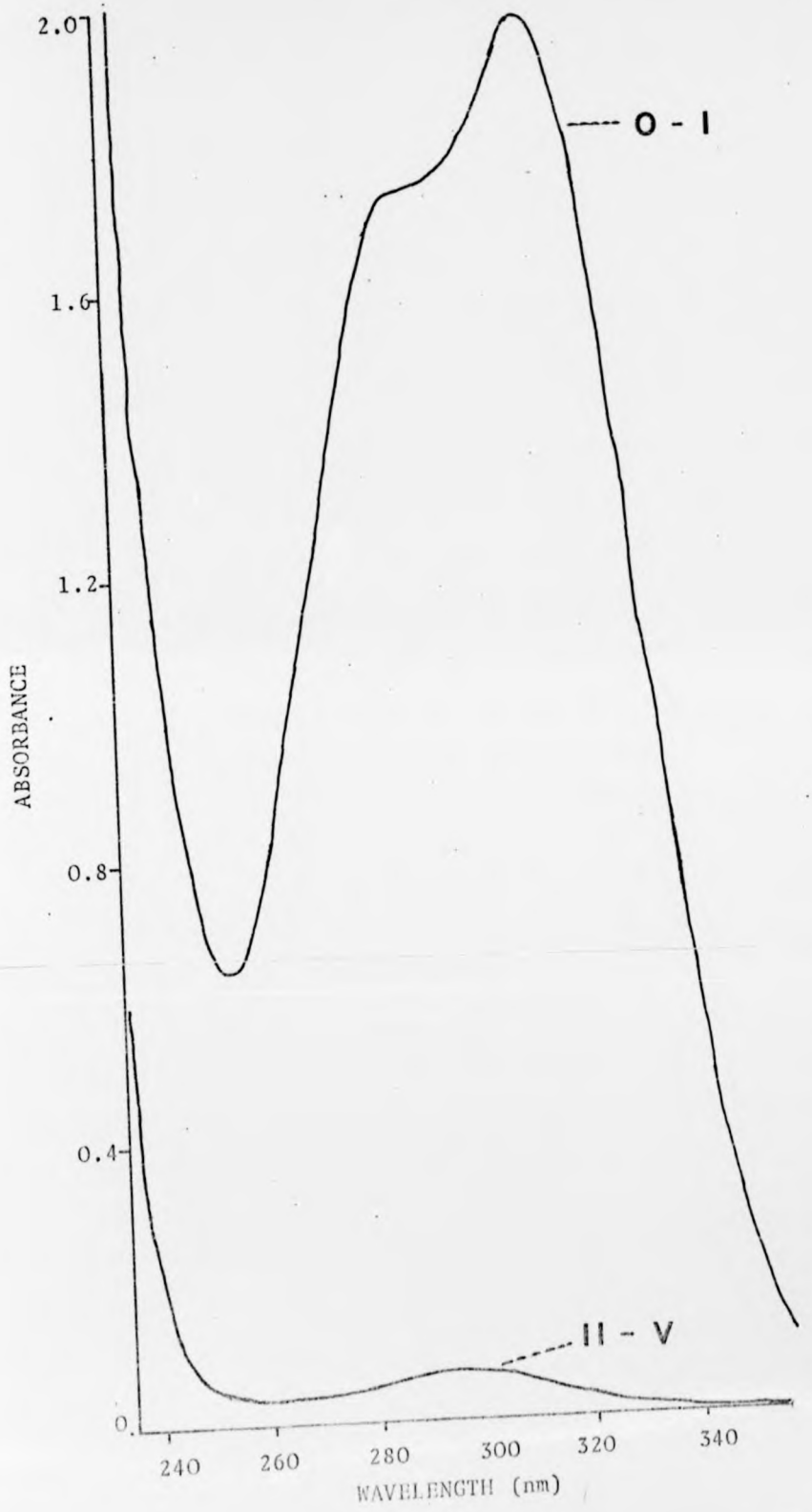


β-oxidation of ferulic acid would be vanillate plus acetyl CoA. The vanillin found in the culture filtrates would then be a product of the (enzymic?) reduction of vanillic acid, analogous to the vanillyl alcohol Henderson (1961b) found as a reduction product of vanillin in the cultures of some fungi. The concentration of the metabolic products in the culture filtrates do not represent significant accumulations in terms of the overall conversion of ferulic acid. However, they may be important as compounds released in the soil, and this aspect will be given consideration later.

In further experiments the time course of the attack of ferulic acid was investigated. Ferulic acid was provided at an initial concentration of 100 mg l^{-1} for three species able to decompose it readily over a period of five days - Penicillium janthinellum, Dothichiza pityophila and Stemphylium dendriticum. The UV-spectra of the culture filtrates on successive days are shown in Figures 1.14, 1.15 and 1.16. P. janthinellum (Fig. 1.14) removes all the substrate in a matter of two days while D. pityophila (Fig. 1.15) seems to reduce the ferulic acid to a residual level in three to four days, with the accumulation of other products. S. dendriticum (Fig. 1.16) shows a pronounced lag, which may be the expression of initial inhibition, but ultimately, removes nearly all the ferulic acid.

A limited series of experiments were carried out with ferulic acid under different conditions and with protocatechuic acid and p-coumaric acid, using a selection of fungal

Fig. 1.14 Time course of the decomposition of Ferulic acid by Penicillium janthinellum. UV Spectra of culture filtrates, diluted fourfold. Roman numerals indicate time after inoculation (in days).


2.0
1.6
1.2
0.8
0.4
0.
ABSORBANCE
O - I
II - V
240
260
280
300
320
340
WAVELENGTH (nm)

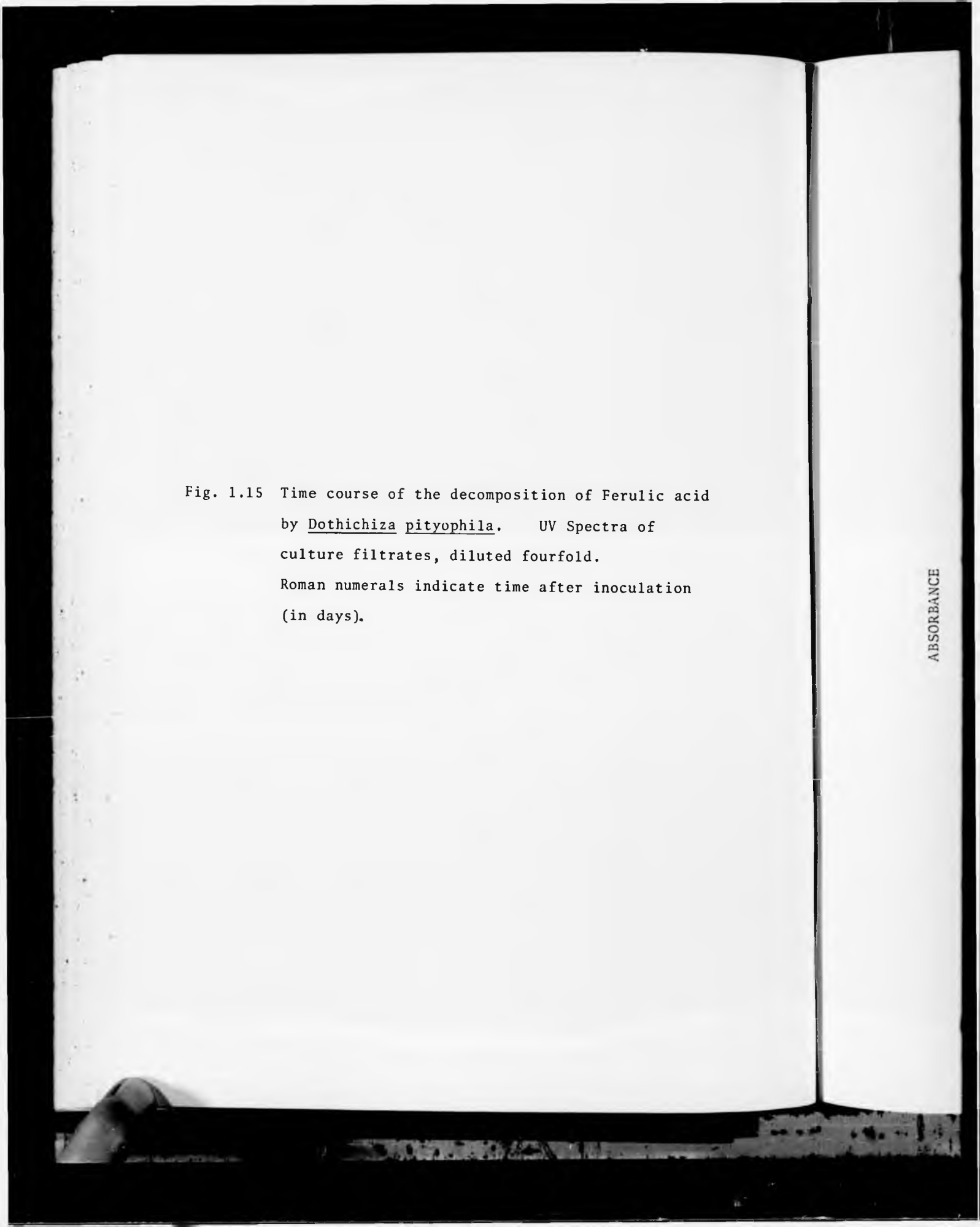
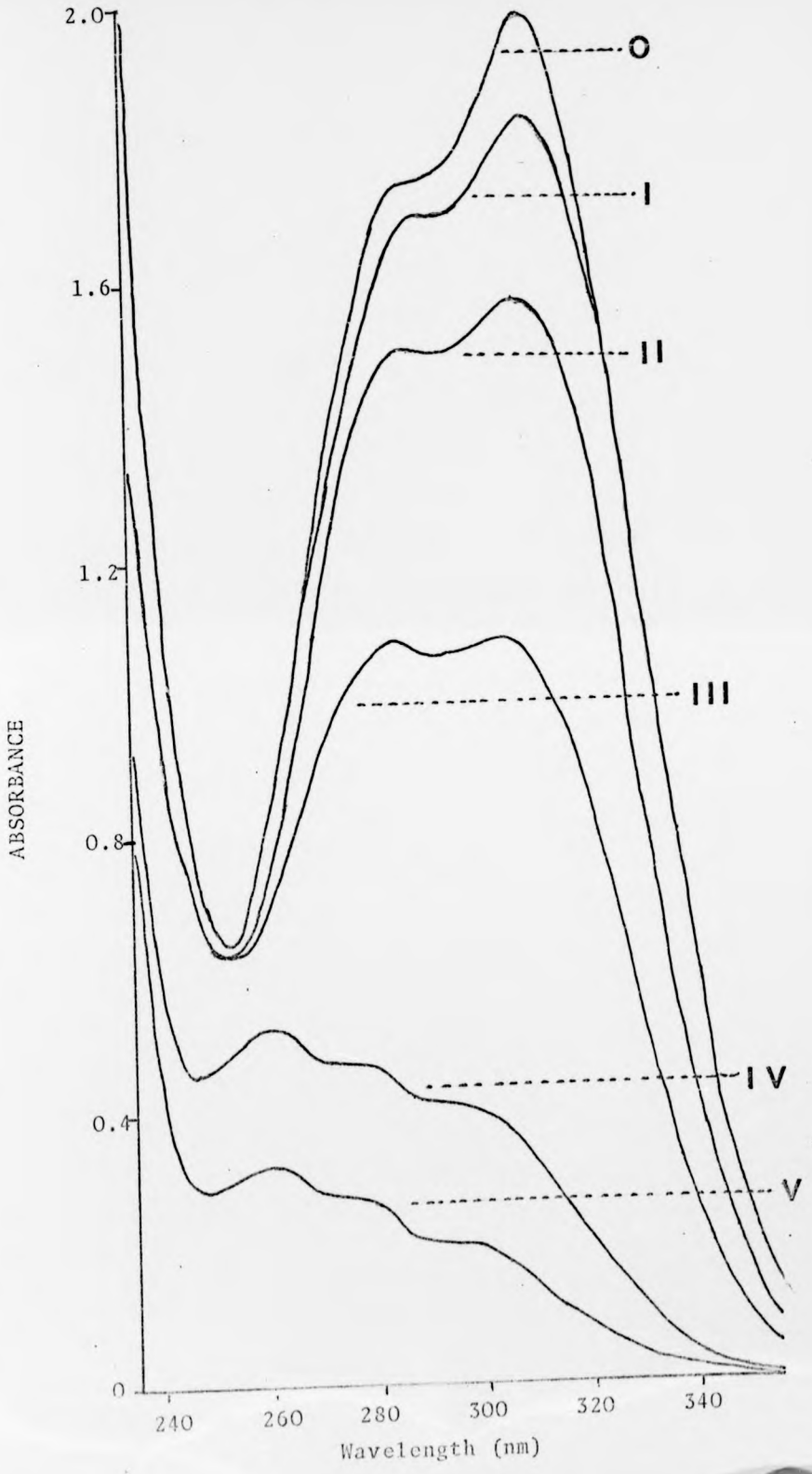


Fig. 1.15 Time course of the decomposition of Ferulic acid by Dothichiza pityophila. UV Spectra of culture filtrates, diluted fourfold. Roman numerals indicate time after inoculation (in days).


2.0
1.6
1.2
0.8
0.4
0
ABSORBANCE
240
260
280
300
320
340
Wavelength (nm)
O
I
II
III
IV
V

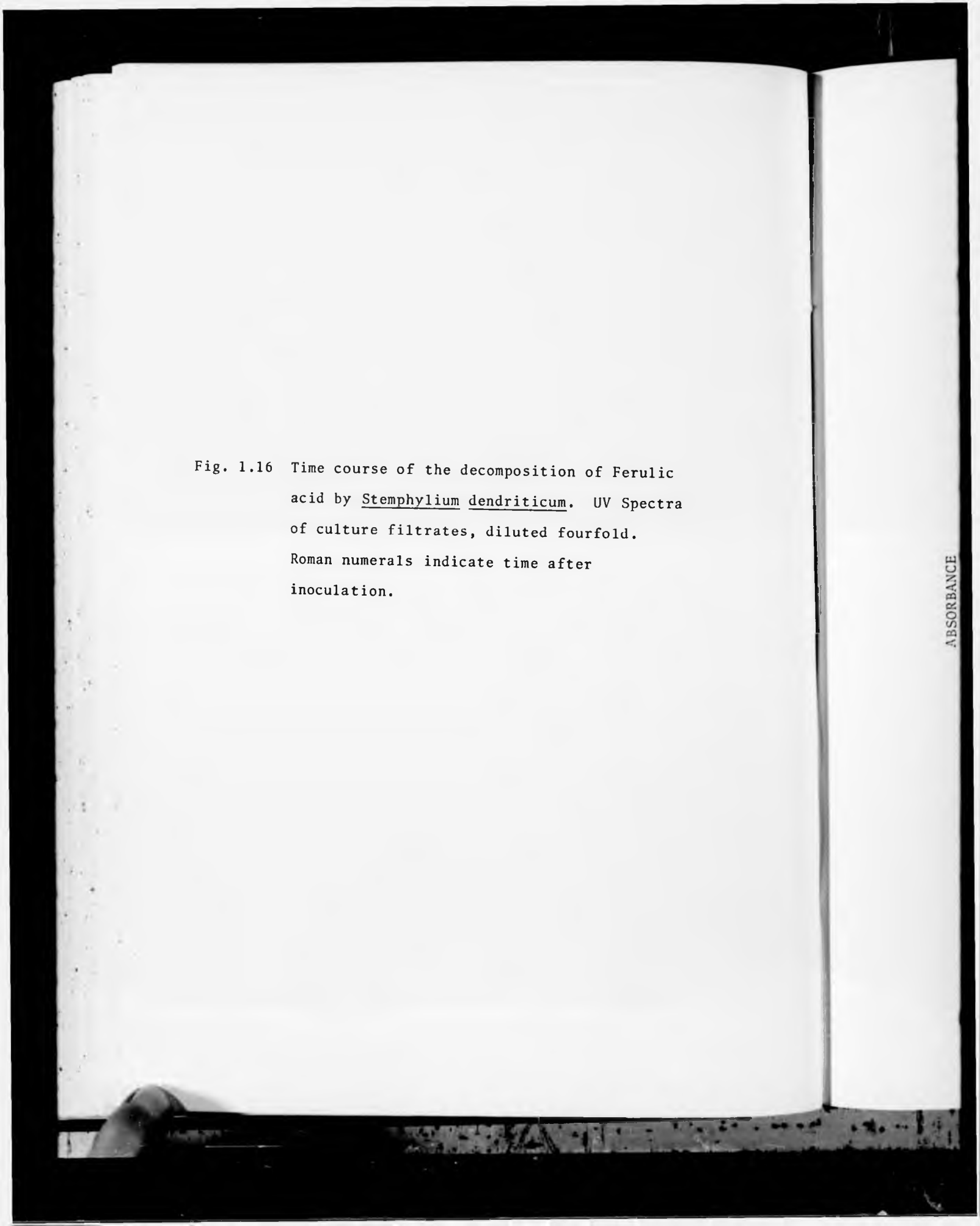
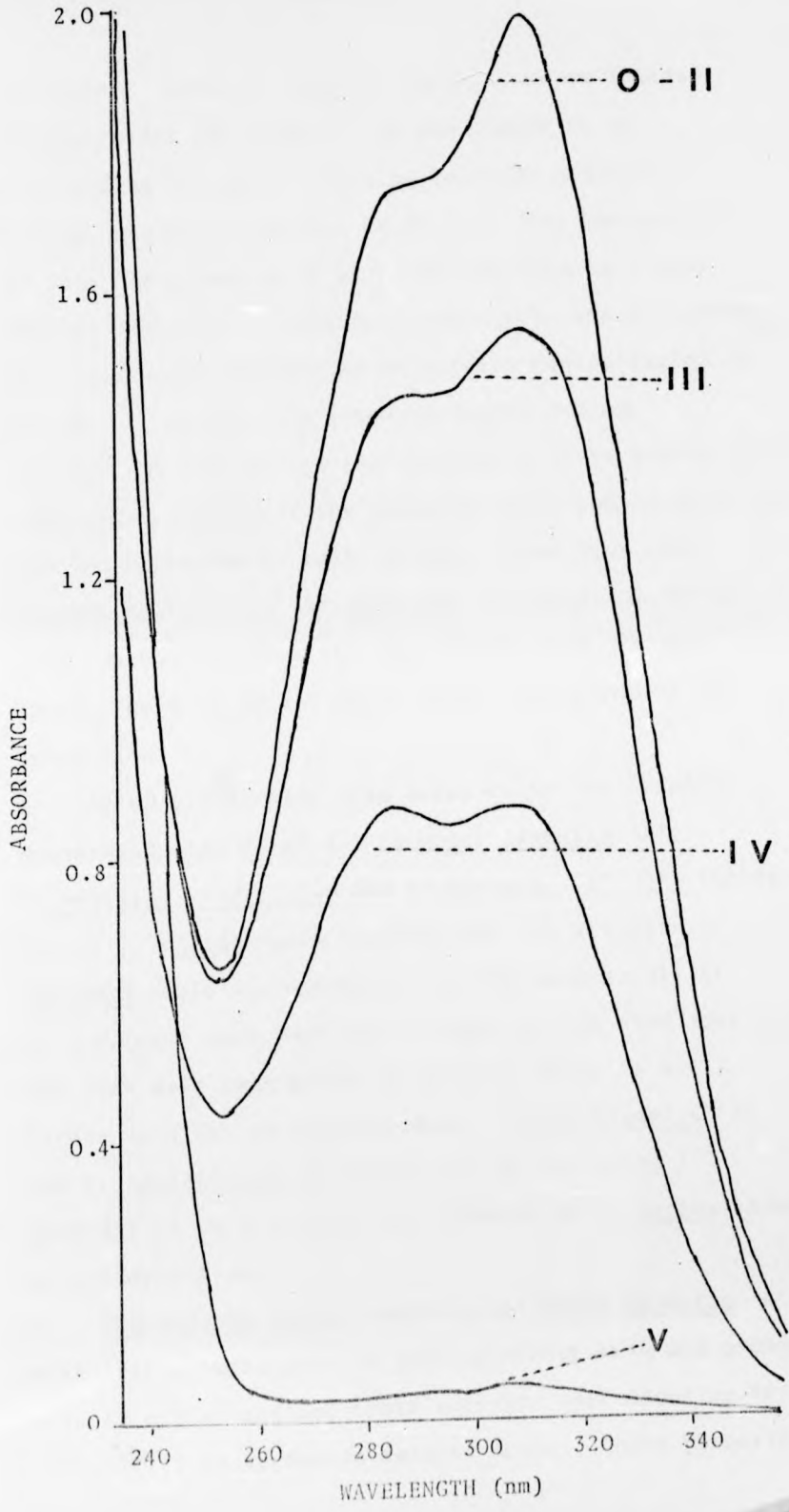


Fig. 1.16 Time course of the decomposition of Ferulic acid by Stemphylium dendriticum. UV Spectra of culture filtrates, diluted fourfold. Roman numerals indicate time after inoculation.


2.0
1.6
1.2
0.8
0.4
0
ABSORBANCE
O - II
III
IV
V
240
260
280
300
320
340
WAVELENGTH (nm)

isolates. Ferulic acid was employed at an initial concentration of 50 mg l^{-1} in the Czapek at pH 4.5, and at 100 mg l^{-1} with an ammonium nitrate medium, giving an initial pH of 5.5. (the composition of this basal medium is given in the Materials and Methods section). Protocatechuic acid and p-coumaric acid were each employed at an initial concentration of 100 mg l^{-1} in both the standard Czapek medium (initial pH 3.7) and in the ammonium nitrate medium (pH 5.5). The concentrations of the phenolic acids remaining in the culture filtrates of each isolate, after five days incubation (10 days for Nocardia) are given in Table 1.8 with the results from the experiments with 100 mg l^{-1} ferulic acid at pH 4.5 (from Table 1.6) provided for comparison.

At pH 5.5 ferulic acid seems to be less readily decomposed than at pH 4.5 by Mucor hiemalis 'B', Stemphylium dendriticum and Trichoderma 'B' with isolate 'C' of T. viride again showing very low activity. Nocardia could not decompose ferulic acid at all at pH 4.5 (even when left for a longer period than five days) but does make some growth at pH 5.5, which is still rather acid for an Actinomycete. Mucor hiemalis 'B' and S. dendriticum can remove all of the 50 mg l^{-1} provided at pH 4.5, with the isolates of T. viride showing no decomposition.

Trichoderma viride isolates and Mucor hiemalis 'A' make little impression on protocatechuic acid and p-coumaric acid in either medium, their activity corresponding to their low ability to decompose ferulic acid. These phenolic

TABLE 1.8 The decomposition of ferulic, protocatechuic and p-coumaric acids by microfungi

	Concentration of phenolic acid remaining after five days ($Mg\ L^{-1}$)						
	FERULIC			P-COUMARIC		PROTOCATECHUIC	
Initial Phen. acid Concentration	50	100	100	100	100	100	100
Medium	1	2	1	1	2	1	2
			From Table (1.6)				
Trichoderma viride 'B'	50.0	96.8	87.0				
'C'	50.0	95.8	95.5		99.2		100.0
'E'			99.5		98.4		97.1
'G'			100.0	78.8		95.2	
Mucor hiemalis 'A'			95.0	93.2		93.2	
'B'	0	97.9	61.5				
Stemphylium dendriticum	0	15.4	<5		0		0
Dothichiza pityophila 'C'			<5	1.8		0	
Nocardia (10 days)		64.0	100.0				

Medium 1 Czapek salts

Medium 2 NH_4NO_3 medium

acids are all decomposed readily by D. pityophila isolate 'C' and S. dendriticum but S. dendriticum leaves no trace of protocatechuic acid and p-coumaric acid and D. pityophila no trace of protocatechuic acid, in contrast to their behaviour with ferulic acid. No evidence of intermediate products were seen in the UV-spectra of the culture filtrates. It is concluded from these results that the pattern of behaviour of microfungi to protocatechuic and p-coumaric acids is essentially similar to the pattern observed with ferulic acid, with respect to species differences in decomposing ability.

In general there is no correspondence of the level of ability to decompose ferulic acid, defined above, and the occurrence of stimulation or inhibition of spore germination and germ hyphal growth by ferulic acid. However, in the case of the four species of Penicillium investigated there is correspondence. The three soil inhabiting species, P. spinulosum, P. janthinellum and P. funiculosum all decompose ferulic acid readily, and are stimulated on agar. By contrast, P. expansum shows only moderate ability to decompose ferulic acid and is inhibited.

The low activity of Mucor spp. and T. viride, among the soil fungi, is paralleled to some extent in the data of Henderson & Farmer (1955) in the decomposition of vanillin and ferulic acid. Although the experiments reported here have not directly tested for the absolute ability of fungi to decompose and utilise a substrate, the results for Trichoderma and Mucor (and Mortierella)

do seem to suggest a lack of this ability, found in the results of Henderson and Farmer and which is expressed for more than one phenolic acid.

It is quite possible that the inability of several isolates to decompose ferulic acid to a significant extent was due to a requirement for a carbohydrate as an additional carbon source or because germination is poor in liquid medium. Phenolic acids were used throughout these experiments as the sole carbon source because the effects of a carbohydrate carbon source are apparently very complicated, depending on the relative levels of the phenolics and carbohydrates and there may be two-way interactions in their utilisation (Flaig & Haider, 1961a; Kunc, 1971b; Mangenot, 1971). Henderson (1956) used yeast extract to stimulate spore germination in her respirometry work. This aspect of the problem was investigated here by following the progress of spore germination and germ hyphal growth in the standard liquid medium used for ferulic acid - 100 mg l^{-1} at pH 4.5. The fungi used were S. dendriticum, D. pityophila isolate 'C', T. viride isolates 'B' and 'C' and M. hiemalis isolate 'B'. Details of the method are given in Materials and Methods. The control treatment used the blank medium.

Germination was considerably delayed and the final levels reached were low in all cases. Tangled masses of hyphae tended to form which made the determination of percentage germination inaccurate after a certain time. Nevertheless, much relevant information was provided by these experiments.

Mucor 'B' did not germinate at all in the controls in this experiment but did on agar. In both the control

and ferulic acid treatments Trichoderma 'C' did not germinate until about 36 hours inoculation, finally reaching a level of about 20%. The germ tubes never exceeded about 10 µm in length (i.e. about twice the diameter of the swollen conidia). The results for Stemphylium, Trichoderma 'B' and Mucor 'B' are given in Table 1.9. With Dothichiza 'C' the concentration of cells was measured to assess the extent of budding, and hyphal lengths (Fig. 1.17).

The germination of Stemphylium was severely depressed but there was an initial inhibition by ferulic acid. The hyphal lengths are very close in both treatments until the end of the experimental period when the hyphae in the ferulic acid solution appear to maintain their growth. This suggests that the lag in the decomposition of ferulic acid by Stemphylium may be due to the initial growth of hyphae on reserves in the spores. Tricoderma viride 'B' shows inhibition of percentage germination but stimulation of hyphal growth by ferulic acid. However, the concentration of hyphae dropped very dramatically in both treatments by 39 hours and they had practically disappeared by 48 hours. The same thing happened when the experiment was repeated and there were signs of lysis in the hyphae. There was no evidence of bacterial contamination.

Dothichiza pityophila showed stimulation of germ hyphal growth and the concentration of blastospores by ferulic acid. It had been observed how the hypha produce blastospores as they cease growth, which is delayed by ferulic acid, and in liquid culture most of the growth is

TABLE 1.9 Effect of Ferulic acid on spore germination and hyphal growth of microfungi in liquid culture.

Percentage Germination

STEMPHYLIUM DENDRITICUM			TRICHODERMA VIRIDE 'B'			MUCOR HIEMALIS 'B'		
Time (hours)	Control	Ferulic Acid	Time (hours)	Control	Ferulic acid	Time (hours)	Control	Ferulic acid
6	0	0	9	0	0	33	0	2.6
12	12.5	3.8 *	12	12.3	7.1 *	39	0	32.5
18	15.7	12.8	15	44.3	16.5	45	0	42.0
						56	0	43.1

Hyphal Lengths (arbitrary units at x10 magnification)

STEMPHYLIUM DENDRITICUM			TRICHODERMA VIRIDE 'B'			MUCOR HIEMALIS 'B'		
Time (hours)	Control	Ferulic Acid	Time (hours)	Control	Ferulic acid	Time (hours)	Control	Ferulic acid
12	6.4	6.7	21	7.9	6.8	33	-	8.1
18	9.5	9.3	33	8.9	11.0 *	39	-	8.9
24	13.0	13.7	39	8.5	11.6 ***	45	-	12.5
30	14.6	18.4	45	10.9	11.9	56	-	12.7

* Significant $p < 0.05$

*** Significant $p < 0.001$ t-test

Fig. 1.17. Effect of Ferulic acid on the growth of Dothichiza pityophila 'C' in liquid culture.

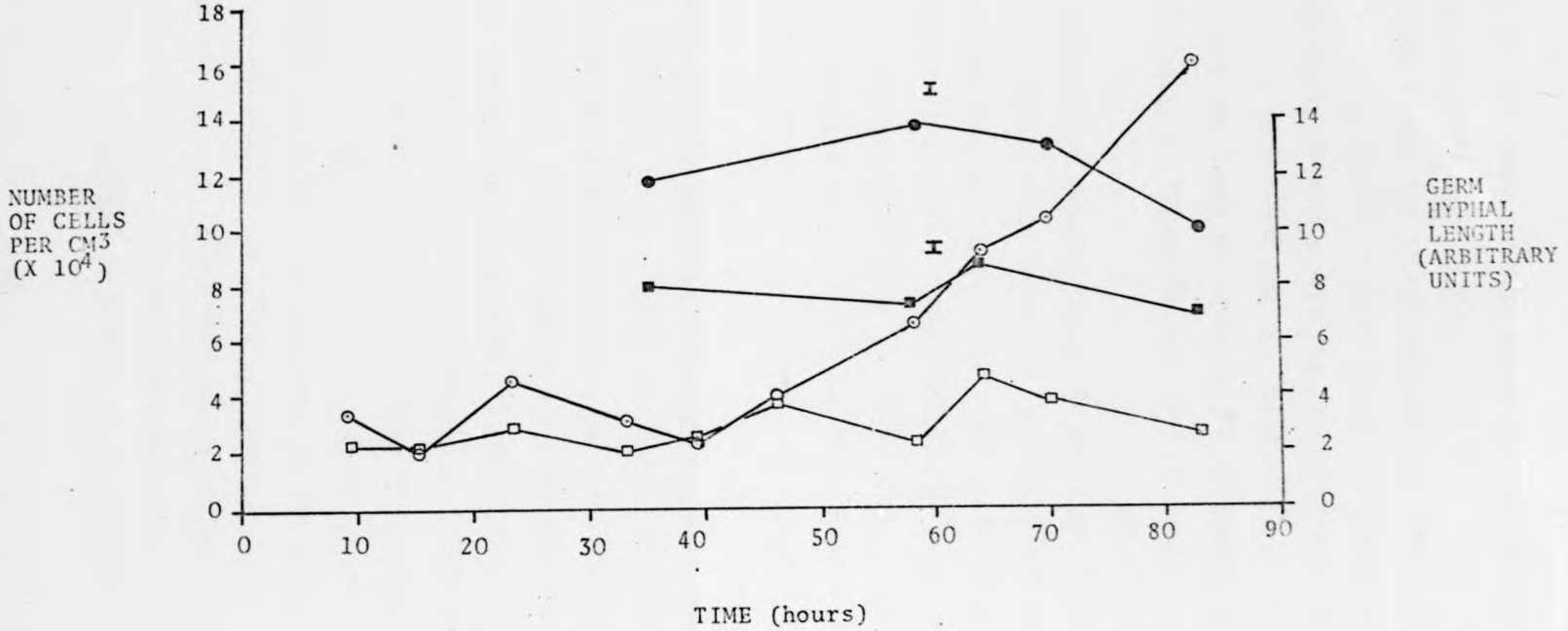
—■— Hyphal length in control culture

—●— Hyphal length in Ferulic acid solution (100 mg l^{-1})

⊥ Least significant difference at p = 0.05

—□— Concentration of blastospores in Control culture

—○— Concentration of blastospores in Ferulic acid solution


NUMBER OF CELLS PER CM3 (X 10^4)
GERM HYPHAL LENGTH (ARBITRARY UNITS)
H
H
TIME (hours)
0
2
4
6
8
10
12
14
16
18
0
10
20
30
40
50
60
70
80
90

by budding, as it is with A. pullulans. Hyphal lengths appear to reach a maximum level in the case of Mucor 'B', Trichoderma 'B' and Dothichiza 'C'. It should be noted that the isolates of Mucor and Trichoderma only make a limited use of ferulic acid compared to Dothichiza where the main growth is by blastospore production. Referring again to the data of Henderson & Farmer (1955), Trichoderma and Mucor were among the genera of fungi unable to metabolise vanillin beyond vanillic acid. It is possible that vanillate was accumulating at toxic concentrations but in some cases there was almost quantitative conversion of vanillin to vanillic acid. It is therefore suggested that some of the isolates of these genera have a special capacity for making limited growth by the initial oxidation of vanillin or ferulic acid to vanillic acid, which appears as a stimulation of growth in the presence of the phenolic acid. Thus Mucor 'B' only germinates in the ferulic acid solution and Trichoderma 'B' eventually lyses in the liquid medium after the cessation of hyphal growth. In the same way Dothichiza 'C' maximises hyphal growth before using the energy obtained by the metabolism of ferulate in blastospore production.

There is some correspondence of these results with the behaviour of the fungi on agar, particularly in the case of Trichoderma 'B' and Dothichiza 'C'. Comparing the performance of the five isolates in these experiments and their ability to utilise ferulic acid, it cannot be said that poor germination is the reason for low decomposing activity. In fact, ferulic acid seems to stimulate the germination and growth of some fungi independently of

complete metabolism of this compound.

These results suggest an hypothesis in which soil fungi and certain saprophytes of conifer litter which are exposed to phenolic acids, are adapted to use the presence of these compounds as a stimulus to one or more parameters of spore germination; this is not necessarily associated with the ability to utilise the compound readily but is a strategy in the colonisation of substrates (the release of phenolic acids may be used as a 'signal' for the availability of other substrates, for example). Other parameters may be inhibited, but fungi not showing this adaptation are inhibited more generally, as might be expected from previous work on phenolic compounds.

It is also suggested, on the basis of rather limited data that the behaviour of fungi towards phenolic acids, and cinnamic acids in particular, may be analogous to the behaviour towards ferulic acid. Clearly there is need for further work on this aspect, and on the effects of compounds like vanillin, particularly as ferulic acid is not encountered in a free state in soil as frequently as p-coumaric and vanillic acids and vanillin. Also only a limited number of isolates were examined for germination behaviour in liquid culture, due to the nature of the apparatus used.

Nevertheless, the rather complicated series of interactions between fungi and phenolic acids that has been described above, can be regarded as operating independently of the validity of the latter as model compounds for lignin decomposition. The significance

of actual ferulic acid decomposing ability is a subject of the next two chapters.

CHAPTER TWO

CHEMICAL AND MICROBIOLOGICAL FIELD STUDIES ON THE NEEDLE LITTER OF PINUS SYLVESTRIS

The mycoflora of the needle litter of Scots Pine (Pinus sylvestris) comprises several distinct populations of fungi which are interesting in their own right as material for comparative functional studies of fungal growth. In addition, the mycoflora of Scots Pine litter, and perhaps conifer litter in general, may have features distinguishing it from the 'typical' pattern of fungal colonisation of Angiosperm litter, which has considerable significance in view of the fact that conifer litter forms Mor humus, in contrast to the litter of most broad-leaved trees.

There have been several studies of the decomposition of Scots Pine litter as an example of a Mor forming system in comparison to Mull forming litters (Nykvist, 1959, 1963; Davies et al., 1964; Jaquin & Bruckert, 1965, Bruckert & Jaquin, 1966, Vedy & Bruckert, 1970). Particular attention has been paid to the water soluble substances released during decomposition, especially aliphatic and phenolic acids because of their implication in the mobilisation of iron in the soil and therefore podzolisation. Although the emphasis of these studies has been largely on pedological differences between Mull and Mor forming litter, these differences have been attributed directly to the lower microbiological activity in Mor compared to Mull. In view of the peculiarities of both the decomposition of Scots pine needle litter and its mycoflora there would appear

to be much that could be learned from a consideration of these two aspects together.

The needle litter of conifers is characteristic of Mor forming litters in consisting of three well defined layers, differing in microbiological and textural characteristics. These are the 'Litter' (L) layer, the 'Fermentation' (F) layer and the 'Humus' (H) layer. The L layer comprises intact needles (needle pairs in the case of Scots Pine), brownish grey but showing few signs of decay. Most workers distinguish two layers to the F horizon, the F1 layer of blackened and obviously decayed but largely intact needles, and the F2 layer of needle fragments in the process of physical disintegration by the microfauna. The H layer results from the completion of the comminution of the needles and the mixing up of the amorphous residues with material of animal origin. Below the H layer is the A1 horizon which is best regarded as being transitional between the litter layers (AO) and the A2 mineral horizon. (Kendrick & Burges, 1962; Duchaufour, 1965; Bridges, 1970). The whole litter horizon and the A1 can together be called Mor (Jaquin & Bruckert, 1965). Kendrick & Burges (1962) estimated that the time spent by needles in each of the L, F1 and F2 layers was approximately six months, approximately two years and seven to ten years, respectively. Because of the slow decomposition of the F2 layer the mass of the whole litter can be regarded as constant throughout the year (Bruckert & Jaquin, 1966).

By contrast, the litter of rapidly decomposing leaves of a broad-leaved tree, Oak (Quercus spp) for example, consists of an L layer overlying an A1 horizon of Mull, which is a layer intimate incorporation of humus into the

mineral soil, under the action of earthworms. How rapidly the decomposition takes place depends upon the type of leaf and the climatic conditions, but characteristically there is an annual cycle of litter mass. Moder sometimes accumulates under deciduous trees, particularly Beech, under adverse conditions, consisting of L and F layers, but no H layer.

Initial interest in litter centered on the decomposition of freshly fallen leaves and needles, parallel to work proceeding at the same time on living leaves which showed the presence of leachable chelating agents (e.g. Coulson et al. 1960; Muir et al. 1964). Nykvist (1959, 1963) showed that air dried needles of Scots Pine and Spruce (Picea abies) yielded far less water soluble substances, and yielded them more slowly, than deciduous leaves. He established that the concentration of water solubles in the leachate was determined by the balance between extraction and decomposition, since anaerobic extraction tended to augment the concentration extracted while aerobic leaching led to decomposition of the extracted substances. Nykvist concluded that, under experimental conditions regarded as being natural, the balance was more towards decomposition in needle litter than in leaf litter due to the slower rate of extraction of water solubles from the former litter.

Jaquin & Bruckert (1965) determined the concentration of free vanillic and p-hydroxybenzoic acids in freshly fallen litter (L layer) and in the A1 of a Mull under Oak (Quercus petraea) and of a Mor under Scots Pine, as well as aliphatic and mineral acids. Leaching the material with cold water they found lower concentrations of all these

substances in the Pine litter than in the Oak leaves, but here the effect of aerobic decomposition reducing the concentrations in the leachate was more pronounced in the latter litter. The technique of these authors allowed aerobic incubation of moist litter prior to extraction, which is probably more realistic than the method of Nykvist (1959, 1963) which involved aeration during extraction.

Following the seasonal pattern of phenolic acid concentration in litters on the same site, Bruckert & Jaquin (1966) found an abrupt fall in the levels of p-hydroxbenzoic, vanillic and p-coumaric acids in the Oak litter during the months following leaf fall. In Pine litter, however, the levels of the phenolic acids remained steadier through the year, although initially lower; and in April the vanillic acid concentration of Pine litter exceeded that of Oak litter. Incubation experiments confirmed that decomposition of the free phenolic acids (and other compounds) was less marked in the Pine litter than in that of Oak, and also there was a tendency for new production of water soluble forms from the litter in the former material but not the latter. In the mineral horizons the concentrations of phenolic and aliphatic acids were much reduced but to a lesser extent in the Mor A1 than in the Mull A1. The net result of these processes is that in a Mor vanillic acid and citric acid, particularly, persist and provide a potential for podzolisation. Only under very wet conditions do succinate and lactate accumulate in a Mull (Bruckert & Jaquin, 1966 and Jaquin & Bruckert, 1965).

Vedy & Bruckert (1970) analysed for a greater number of free phenolic acids in litter but they found that only p-hydroxybenzoic, vanillic and p-coumaric acids remained throughout the year in a Mull forming litter; and in a Mor developed under the same climate, minor amounts of ferulic and protocatechuic acids in addition. Alkaline hydrolysis of the water extracts also showed the presence of phenolic acids bound in soluble polymers. The polymers are produced in the litter by microbial activity and Vedy & Bruckert observed a greater tendency for polymerisation in the litter over Mull than in the Mor, and although the polymers are essentially transient substances this difference between the litters was clearly associated with more active humification (i.e. synthesis of insoluble, more stable components of the soil organic matter) in the litter over Mull. Polymerisation also occurred in the mineral horizon (A1), which resulted in the phenolic acids being retained in the upper layers of the soil whereas the aliphatic acids are leached right through. Toutain (1972) also observed a corresponding difference in humification in two Beech (Fagus sylvaticus) litters over a Mull and a Moder.

Greater microbiological activity in the more favourable environment of a Mull litter thus leads to a greater degree of metabolism or insolubilisation of phenolic acids leached out of the plant remains than in coniferous needle litter forming a Mor. The free phenolic acids remaining in the litter or the soil are what are left from the opposing factors of production from the plant matter and loss by

leaching, metabolism and humification.

The first systematic and detailed account of the mycoflora of Scots Pine was by Kendrick & Burges (1962). They suggested a model for the fungal succession, different from that of other litters, which has subsequently been subjected to scrutiny of several workers. But perhaps an equally valuable result of this study was the relationship between the pine needle as a fungal substrate to the physical stages of decay in the litter. This was made possible by a combination of cultural studies and observations on fungal growth in the needles.

In the L layer, Kendrick & Burges (1962) found little to suggest decomposition of the needles which they saw primarily as a substrate for the sporulation of parasites of living needles and weak parasites or primary saprophytes of senescent material. In the F1 layer the needles are blackened by the superficial mycelial network and the staining of internal cell walls by secondary saprophytes, accompanied by extensive breakdown of internal tissues, particularly the mesophyll (cf. Gremmen, 1957). F2 layer needles showed further evidence of decay, with collapse of vascular tissues. The surface reticulum of hyphae had also disappeared, and the needles, having been fragmented by the microfauna, were mixed up with exo-skeletal remains and faeces. However, Kendrick & Burges also regarded the F2 layer as a repository for needle fragments not previously attacked by fungi and which remain in this layer until completely comminuted by the microfauna. It should also be noted that this layer and the underlying H layer is frequently bound and

compacted by hyphal threads of Basidiomycetes.

One of the most important fungi in L needles is Lophodermium pinastri. This ascomycete is a parasite of needles of Pinus spp. often causing severe needle blight (e.g. Nicholls & Skilling, 1970). It produces hysterothecia on L needles, by which its presence is determined. L. pinastri produces a dematiaceous diaphragm across the needle, at the boundary of its zone of colonisation, preventing the growth of fungi along the needle (Kendrick & Burges, 1962); and Gremmen (1957) suggested that needles infected by L. pinastri show a different pattern of subsequent colonisation by secondary invaders than non-infected needles. However, there is no evidence from Kendrick & Burges (1962) to suggest that secondary invaders cannot directly colonise L. pinastri infected zones of the needle and subject the material to decomposition.

The presence of L. pinastri is detected by direct examination of the needle, but many other important fungi can be isolated by plating out the needles on agar. However, it is necessary to pre-treat the needles to distinguish between colonies obtained from superficial inoculum and colonies produced by mycelium growing from the needle interior (Parkinson & Kendrick, 1960). Most recent studies have involved needle washing to favour the growth of mycelium in or on the needle at the expense of the spore load adhering to the surface; and surface sterilisation is frequently used to suppress superficially colonising fungi and favour the growth of mycelium from within the needle.

Kendrick & Burges (1962) found that Pullularia (= Aureobasidium) pullulans and Fusicoccum bacillare were the two common weak parasites and/or primary saprophytes which grow on a nutrient medium from washed or surface sterilised L needles, A. pullulans being largely suppressed by surface sterilisation. Some of the colonies corresponding to A. pullulans may have been associated with the sphaeropsidaceous Dothichiza (= Sclerophoma) pityophila. Gremmen (1957) characterised L needles not infected by L. pinastri as belonging to the Sclerophoma stage. The predominance of A. pullulans and F. bacillare as primary saprophytes of needles of other conifers has been demonstrated by Parkinson & Balasooriya (1967) for Pinus nigra, using washing and surface sterilisation, and Reddy & Knowles (1965) for Picea mariana, using needle washing only (who consequently found only A. pullulans).

Hayes (1965a) investigated the mycoflora of litter of Abies grandis, Picea sitchensis and Pinus sylvestris in a mixed plantation, by means of direct observations and limited cultural studies of 'marked' needles undergoing decomposition. L. pinastri was restricted to Pine needles but F. bacillare was found on the needles of all three conifers, with Haplographium (= Thysanophora) penicilloides common on Abies and Picea but very rare on Pinus. A. pullulans was not, recorded, probably because needle washing was not employed, but a species of Aposphaeria was a common primary saprophyte not found in other studies. On other site Hayes (1965b) isolated fungi from needles of native Scots Pine, using untreated and surface sterilised

needles. Neither F. bacillare nor A. pullulans were isolated frequently; Graphium sp. and Geotrichum candidum appearing as the common primary saprophytes, accompanied by Penicillium funiculosum.

The impression gained from the six papers discussed above is that differences in the results are as much due to the methods of study (with different emphasis on direct observation and internal and superficial colonisation) as to other factors, including the species of conifer providing the needles, age of stand, climatic and pedological conditions and pre-infection by a parasite, which distinguish these studies. Some of the parasites and saprophytes are apparently restricted to certain species or genera of conifers, but with the exception of the ubiquitous A. pullulans they are specific to conifer litter. Parkinson & Balasooriya (1967) isolated Cladosporium herbarum from washed L needles in very low frequency, but otherwise there is an absence of the primary saprophytes which are commonly present on many other sorts of litter, Alternaria tenuis, Epicoccum nigrum and Botrytis cinerea. This was first noted by Kendrick & Burges (1962) and remarked upon by Hudson (1968) in his review. Moreover, not all the isolates recorded as A. pullulans can be said to have been necessarily representative of this very common saprophyte of leaves. An apparent connection between A. pullulans mycelium and the pycnidia of Dothichiza pityophila (ascogenous state Sydowia polyspora) on Pine needles had been noted by Batko et al. (1958) and Kendrick & Burges (1962). Hudson (1965), however showed that there was no taxonomic relationship between these two fungi, as

A. pullulans was related to another Ascomycete, and there were also consistent differences in colony type, A. pullulans being creamy at first then turning dark whereas D. pityophila has colonies which are initially almost black. On Pine wood, Butin (1963) distinguished D. pityophila from A. pullulans on the basis of pycnidial formation and features of mycelial morphology.

Clearly, both fungi can be isolated readily from Pine needles by agar culture and in spite of the distinction between the two species all isolates may be recorded as A. pullulans when both are present. This probably happened in the work of Kendrick & Burges (1962) and may be true of more recent work, particularly in North America (Millar, personal communication). As a result D. pityophila may be more common as a primary saprophyte of Pine needles than was previously thought. The relative frequencies of A. pullulans and D. pityophila are obviously important in view of the observed absence of other common primary saprophytes on conifer litter.

Recently, however, a study of the litter of Pinus strobus, P. contorta, P. resinosa and P. banksiana and the underlying mineral soils has revealed a mycoflora conforming to the 'typical' Angiosperm pattern (Widden & Parkinson, 1973). Along with Fusicoccum bacillare and a sterile form, frequently occurring saprophytes included Alternaria tenuis and Cladosporium herbarum on the needles of more than one species of Pinus, and also Epicoccum nigrum and very rarely Botrytis cinerea. This study used washed needles only. Widden & Parkinson (1973) suggest that climate may be important, together with the nature of the substrate, in determining fundamental features of the mycoflora. This

could account for the different results from their work in Canada, and that of other workers in Great Britain (Kendrick & Burges, 1962, Hayes 1965 a,b, Parkinson & Balasooriya 1967) and in The Netherlands (Gremmen 1957).

Mention should also be made of the work of Ishii (1967a,b) on the fungus flora of litter and soil under Pinus densiflora in Japan, using soil dilution plates. Along with unidentified Sphaeropsidales, Spondylocladium sp. and Aureobasidium pullulans, the primary saprophytes isolated from L needles were Cladosporium herbarum, Alternaria tenuis and Helminthosporium sp.

Secondary saprophytes are also seen in the L layer but these are characteristic of the Fl layer where they occur at a maximum frequency. Desmazierella acicola appears to be the most important internally colonising secondary saprophytes of Pinus sylvestris needles causing extensive breakdown and blackening. The striking apothecia of this fungus are unfortunately only seen rarely, being recorded by Gremmen (1957), Kendrick & Burges (1962) and Hayes (1965a,b) from its Verticladium trifidum conidial state. Kendrick & Burges (1962) attributed the surface reticulum of dark hyphae to Sympodiella acicola, and Helicoma monospora. These species are very slow growing in culture and are suppressed by faster growing fungi notably Trichoderma viride when washed needles are plated. Parkinson & Balasooriya (1967) noted the importance of S. acicola and H. monospora as superficial colonisers of Fl needles of Pinus nigra. Polyscytalum sp., Bisporomyces sp. and various sterile forms dominated the isolations from surface sterilised needles. Widden & Parkinson (1973)

did not find secondary saprophytes in a well defined phase in their investigation of the litter of various Pinus spp, although Coniothyrium sp. was frequent on F1 needles of P. strobus and Helicoma monospora was found on needles of this species. Since these authors did not employ surface sterilisation the lack of secondary saprophytes is perhaps not surprising as Trichoderma viride is obtained in abundance from washed F needles. This species is probably highly antagonistic to the growth of many other fungi on agar plates. However, Penicillium spp. and the Mucorales seem to be relatively insensitive to the antagonism of T. viride. All these fungi dominate the isolations from washed F1 and F2 needles, which marks the appearance of soil inhabiting fungi in the conifer litter system.

The interpretation of the data from the fungal isolations of the F2 is rather difficult because the surface sterilant can readily penetrate the porous needle material. Thus Hayes (1965b) obtained very few isolates from surface sterilised F2 needles of Scots Pine. Kendrick & Burges (1962) obtained evidence that Desmazierella acicola is largely replaced by Trichoderma viride in the interior of F2 needles, but in Pinus nigra only one of the major secondary saprophytes (a dark sterile form) appeared to decline in frequency from F1 to F2 surface sterilised needles (Parkinson & Balasooriya, 1967). In fact, Kendrick & Burges (1962) concluded that the soil fungi T. viride and Penicillium spp. were present largely as a passive spore potential. Thus the increasing incidence of these fungi on washed needles on passing from L to F1 to F2 needles, observed in all the studies, could be explained by increasing

proximity to the mineral horizons.

However, it should be noted that in those studies which have taken account of the mineral horizons below the litter (Ishii, 1967b; Parkinson & Balasooriya, 1967; Widden & Parkinson 1973), T. viride and Penicillium spp. are seen to occupy a zone of maximum frequency in the F2 and H. layers, with other fungi predominating in the mineral horizons. A successional pattern is therefore indicated among the soil fungi, the implication of which is active colonisation by T. viride and other soil inhabiters.

Among the Mucorales, the very common Mucor hiemalis and also Mortierella ramanniana may be characteristic of the F and H layers. The occurrence of M. hiemalis as a superficial fungus of F but not L needles, observed in most studies, is consistent with the role of this species as a "soil fungus colonising a substrate in association with cellulolytic and perhaps lignin decomposing fungi.." (Hudson, 1968). However, Hayes (1965a) regarded M.hiemalis as a 'constant', present in all layers of the litter.

The importance of Basidiomycetes, the mycelium of which can often be seen in the F2 and H layers, is quite uncertain, mainly because of the difficulties of isolation and characterisation of these fungi.

To sum up, the fungus flora of Scots Pine litter and other conifers is notable for the occurrence of several populations of fungi, giving rise to a successional pattern and to differences in the species colonising the surface and the interior of the needles. There appear to be certain peculiarities in the composition of the mycoflora, and the decomposition of the litter has some interesting features, particularly with respect to the release of phenolic acids.

These considerations led to the conjecture that the chemical characteristics of the litter may exert a selective effect on the microfungi colonising the Pine needles. It was clear that further study of this litter was warranted, in order to investigate the functions of the different fungal populations in the decomposition process. This was attempted by means of an integrated study of the phenolic acids in the litter, of the mycoflora and of the change in the substrate provided by the needles during decomposition.

The site previously used by Hayes (1965b) in the Blackwood of Rannoch, Perthshire, was available, and material was collected from here in a series of samples carried out from June until December, 1973. This site consisted of a stand of Pinus sylvestris var. scotica approximately 100 years old. Even though the 1968 gales had caused extensive thinning of the canopy, the ground flora was still very sparse.

The analysis of phenolic acids in Pine litter was hampered by technical difficulties which prevented much early progress in the development of the analytical procedures. Ultimately attention was directed to the determination of phenolic acids in the free water associated with the needles.

It is highly desirable to extract from needles which are in as natural a state as possible and therefore to avoid such procedures as air drying. To simulate natural leaching water should be used in the extraction of fresh needles and the extraction should be done in the cold to prevent decomposition of the substances extracted by microbial activity as well as by chemical changes. This method of extraction permits trends to be determined for the

release of water soluble substances on a comparative basis. However, the concentrations obtained can only be expressed in relation to the mass of the litter, which does not give any indication of the effective concentrations in the litter of compounds which may be biologically active. Litter samples taken for the analysis of phenolic acids had been frozen in the field and then stored in the deep freeze. It was thus possible to collect the melt water from these samples and to determine the concentration of phenolic acids in this liquid. The final aim was to reproduce these concentrations in experiments on the germination of fungal spores in liquid culture.

In a trial experiment 270 g of a sample of L needles collected from Devilla Forest, Fife, yielded 25 cm^3 of water on melting. After evaporation to dryness under vacuum the residue was silylated and analysed by gas liquid chromatography according to the methods used in the analysis of culture filtrates. A peak coinciding with p-coumaric acid was obtained, but the identity of this peak cannot be confirmed in the absence of corroborating data. This peak was also obtained from a water extract of the same litter. No other phenolic acids were detected, but there were peaks corresponding to aliphatic acids present in the chromatograms.

Samples of needles from L and F layers had been collected from the Blackwood in June and December, 1973, before the method had been fully developed. Unfortunately the size of the samples was too small for sufficient liquid to be collected for analysis, and no further work was done on this subject.

It has not been possible to determine the concentrations of phenolic acids that are likely to be encountered by fungi in Pine litter and to determine the likely effects of these concentrations in relation to the results obtained in Chapter One. It remains an open question whether the peculiarities of the conifer litter system observed in relation to the production of phenolic acids are important in the selective effects of the litter on the composition of its mycoflora.

The influence of the nature of the needles themselves on the composition of the mycoflora was investigated directly, however, in a series of field experiments involving the burial of material collected from the Blackwood in soil at two sites on the University Estate at Stirling. The idea of this study was prompted by an experiment of Ishii (1968) who buried mixtures of freshly fallen leaves and broad leaves, in varying proportion, in the litter of a stand of Pinus densiflora. As the proportion of broad leaves increased from zero to 100% the total counts of fungal isolates obtained by the dilution plate method increased by over four times. This increase was almost entirely due to the increase of secondary saprophytes and soil fungi characteristic of the F layer, while there was a decline in primary saprophytes to a steady level as soon as any Broad leaves were introduced into the mixture:

Fresh Weight Ratio Pine needles : Broad leaves	Fungal counts by dilution plate x 10^4 per g.		
	Primary Saprophytes	Sec. Saprophytes	Total
100 : 0	50.3	50.9	150
70 : 30	26.0	180.0	220
50 : 50	24.9	307.1	410
30 : 70	23.3	397.7	530
0 : 100	22.4	504.1	650

Some specialisation of the primary saprophytes is indicated whereas the secondary invaders are able to increase their inoculum potential through growth on the more readily available substrate of the broad leaves.

In the experiments to be described this procedure was more or less reversed, needles being taken from the Highland Pine forest and placed in a field soil and in a woodland soil. Fungal isolations were carried out in parallel from needles direct from the Blackwood litter layers and after burial in soil for about three months. The buried material consisted of fresh (non-sterilised) and sterilised (autoclaved) needles from the L and F1 layers, and before plating out these needles were surface sterilised to isolate the colonisers of the needle interior.

The basic procedure was as follows. Litter was collected from the Blackwood on 19 June, 24 September and 6 December 1973. Needles for direct isolation were surface sterilised immediately on returning to the laboratory with 0.1 % aqueous mercuric chloride plated out on 2% malt agar and incubated at 24^{o}-26^{o}C. The samples from each layer consisted of 10 needles (10 needle fragments with the F2 layer) from each of three replicate sub-sites. At the June and September sample dates, needles were buried the following day and removed on 3 September and 6 December respectively. In the first experiment L and F1 needles were buried, in the second only F1 needles. Samples of each replicate from each layer were sterilised by autoclaving and together with fresh needles were left in the cold overnight before burial. Each treatment sample was buried in soil at the two sites, one in a shallow acid

brown earth in mixed deciduous woodland, mainly Acer pseudoplatanus (the 'Hermitage Wood' site) and the other in a slightly acid, well fertilised field soil, used for a variety of experimental crops (the 'Experimental Gardens' site). Burial was achieved by sprinkling the pine needles on to layers of the appropriate soil prepared in seed trays (perforated for drainage) overlaying with more soil and finally burying the trays in holes dug in the soil at each site.(Further details of the soils and the means of burial will be found in the Materials and Methods section). The needles could be readily sifted from the soil, and so more intimate contact between needles and soil could be achieved than is possible using litter bags. In the first experiment (June - September) there were twelve trays at each site - two layers, two treatments, three replicates - buried in randomised blocks. In the second experiment (September - December) there were six trays.

Surface sterilisation was carried out in the apparatus described in Materials and Methods. The surface sterilisation involved two minutes immersion in still mercuric chloride solution (Kendrick & Burges, 1962), followed by washing in six changes of sterile distilled water, each of one minutes duration.

Needle samples from the Blackwood litter were plated out directly in order to obtain information about the resident populations being buried in the soil. Initially only surface sterilised needles were plated out but later the experiments were expanded to include washed needles. This provided a valuable set of data on the fungi in the natural litter system. Needle washing was carried out

by the method of Kendrick & Burges (1962), in 20 cm^3 screw-top vials shaken mechanically; five washings in 1% sterile Teepol followed by five washings in sterile distilled water, two minutes each washing, was found to be satisfactory. The apparatus employed for surface sterilisation could not be used, because of excessive foaming of the detergent through the top of the apparatus.

The technique of Association Analysis, devised for the study of distribution of species in higher plant ecology, was used to assist in the analysis of the distribution of fungal species. A computer program for Phytosociation work written by Dr. Peter Bannister (University of Stirling) was used unmodified. (This Algol program can be run in several different ways but only the way it was used in these studies will be described). Data are compiled as the fungal species isolated from each needle, which is therefore equivalent to a quadrat sample or an individual stand. At each stage in the analysis the computer calculates the Chi-squared values for the frequency of each species compared to each other species, and the Chi-squared values for each species are summed. The fungal species with the highest summated Chi-squared is used to divide the needles on the basis of the presence and absence of this species. The analysis is continued until the maximum Chi-squared value is no longer significant at a predetermined level ($p = 0.05$ was used throughout). An association arises when a division produces a set of needles, all with two or more species in common, but analysis for each species is carried out independently, in accordance with Phytosociation theory. A print out is

by the method of Kendrick & Burges (1962), in 20 cm^3 screw-top vials shaken mechanically; five washings in 1% sterile Teepol followed by five washings in sterile distilled water, two minutes each washing, was found to be satisfactory. The apparatus employed for surface sterilisation could not be used, because of excessive foaming of the detergent through the top of the apparatus.

The technique of Association Analysis, devised for the study of distribution of species in higher plant ecology, was used to assist in the analysis of the distribution of fungal species. A computer program for Phytosociation work written by Dr. Peter Bannister (University of Stirling) was used unmodified. (This Algol program can be run in several different ways but only the way it was used in these studies will be described). Data are compiled as the fungal species isolated from each needle, which is therefore equivalent to a quadrat sample or an individual stand. At each stage in the analysis the computer calculates the Chi-squared values for the frequency of each species compared to each other species, and the Chi-squared values for each species are summed. The fungal species with the highest summated Chi-squared is used to divide the needles on the basis of the presence and absence of this species. The analysis is continued until the maximum Chi-squared value is no longer significant at a predetermined level ($p = 0.05$ was used throughout). An association arises when a division produces a set of needles, all with two or more species in common, but analysis for each species is carried out independently, in accordance with Phytosociation theory. A print out is

obtained of needles grouped according to species which can then be examined for biological significance, i.e. the group of needles should correspond to a recognisable grouping based on needle type or treatment. The data for individual needles was contained on separate punch cards and so any combination of data or all the data can be analysed in any one run, as desired. Associations were not very common in the needle groups produced, particularly in the case of the 'natural' Blackwood samples, as there were rarely more than two species recorded from one needle, but this method of analysis was found to be a very useful adjunct to analysis of the distribution of individual species.

Notes on the identification of some of the isolates are given in Appendix 3. For the work on the litter plated out directly from the Blackwood, the results are given in Tables 2.1 and 2.2. General information on the isolations is given in Table 2.1, using three indices defined by Parkinson & Williams (1961). These indices are: the percentage of the needles colonised in each sample (the 'percentage colonisation'), the number of colonies per needle plated out (the 'colonisation quotient'), and the 'overall colonisation index' (% colonisation times colonisation quotient). The number of colonies obtained from a given number of particles of substrate plated out depends upon the intrinsic nature of the material and the pre-treatment, both these factors affecting the percentage colonisation and the 'colonisation quotient' of the needles. However, the effect of the pre-treatment, here surface sterilisation or washing, predominates in the determination

TABLE 2.1. Fungal colonisation of Pine needles in natural litter (Blackwood samples)

COLONISATION INDICES

	Surface Sterilised Needles			Washed Needles		
	L	F1	F2	L	F1	F2
JUNE SAMPLE						
% Colonisation	96.2	80.0	16.7			
'Colonisation quotient'	1.30	1.20	0.17			
'Overall Colonisation Index'	125.1	96.0	2.84			
SEPTEMBER SAMPLE						
% Colonisation		76.7	26.7		96.7	86.7
'Colonisation quotient'		1.00	0.23		1.77	1.20
'Overall Colonisation Index'		76.7	6.14		171.2	104.0
DECEMBER SAMPLE						
% Colonisation	96.7	73.3		96.7	96.7	
'Colonisation quotient'	1.17	0.93		1.57	1.80	
'Overall Colonisation Index'	113.1	68.2		151.8	174.1	

of the percentage colonisation. The decrease in the value of this parameter with depth in the case of the surface sterilised needles indicates the increasing penetration of the surface sterilant from the L to the F1 to the F2 layer, and there is a general suppression of isolations by surface sterilisation compared to washing. On the other hand, the 'colonisation quotient' indicates some effects of the needle layer, the value of this index decreasing with depth in surface sterilised samples but is at a maximum in washed F1 needles. The combined effect of these factors is indicated by the 'overall colonisation index'. Data on the occurrence of individual species are given in Tables 2.2a and 2.2b. In Table 2.2a are shown the number of times each species is isolated from each set of 30 needles (multiple colonies of one species on a single needle not being recorded), which gives an absolute measure of the frequency of that species in any one sample. Table 2.2b gives the percentage occurrence of each species out of the total number of isolates in each sample. This second measure (the 'percentage abundance') is more appropriate for the comparison of samples differing in the total abundance of isolates, as is the case here (Gams & Domsch, 1967, 1969, Gams et al. 1969).

The fungi isolated most frequently from L needles were Dothichiza pityophila, Fusicoccum bacillare, Desmazierella acicola and Penicillium spp. The incidence of Penicillium spp. on washed L needles was associated specifically with the colonisation of the needle petioles. Other species were of minimal importance in this layer.

TABLE 2.2. Fungi isolated from Pine needles collected from Blackwood

a) Frequency of isolation of individual species from each sample of 30 needles

	SURFACE STERILISED			WASHED	
	JUNE	SEPT.	DEC.	SEPT.	DEC.
L NEEDLES					
Dothichiza pityophila	11		1		3
Fusicoccum bacillare	14		23		17
Desmazierella acicola	7		11		2
White Sterile Form	4		-		-
Paecilomyces sp.	1		-		-
Mucor spp	1		-		2
Trichoderma viride	1		-		8
Penicillium spp.	-		-		15
F1 NEEDLES					
Dothichiza pityophila	6	3	4	-	-
Fusicoccum bacillare	1	1	-	-	-
Desmazierella acicola	15	13	11	4	3
White Sterile Form	-	1	3	-	-
Brown Sterile Form	9	4	2	-	-
Oidiodendron sp.	1	-	-	-	-
Pyrenochaeta sp. 1	1	-	-	-	-
Coniothyrium sp.	-	1	-	-	-
Fusarium sp.	1	1	2	-	-
Mucor spp.	-	-	-	20	18
Trichoderma viride	1	-	3	19	13
T. polysporum	-	2	-	-	-
Penicillium spp.	-	3	1	6	10
Mortierella ramanniana	1	1	1	4	9
Trichocladium sp.	-	-	1	-	1
F2 NEEDLES					
Fusarium sp	-	1		-	
Mucor spp.	2	1		9	
Mucor racemosus	1	-		-	
Trichoderma viride	1	-		19	
Penicillium spp	-	4		6	
Mortierella ramanniana	-	-		2	
Mortierella sp.	1	1		-	

TABLE 2.2 Fungi isolated from Pine needles collected from Blackwood.

b) Percentage abundance of individual species (based on total number of isolates).

	SURFACE STERILISED			WASHED	
	JUNE	SEPT.	DEC.	SEPT.	DEC.
L NEEDLES					
Dothichiza pityophila	28.2		2.9		6.4
Fusicoccum bacillare	35.9		65.6		36.2
Desmazierella acicola	17.9		31.4		4.3
White Sterile Form	10.3		-		-
Paecilomyces sp.	2.6		-		-
Mucor spp	2.6		-		4.3
Trichoderma viride	2.6		-		17.2
Penicillium spp.	-		-		32.0
F1 NEEDLES					
Dothichiza pityophila	16.7	10.0	14.3	-	-
Fusicoccum bacillare	2.8	3.3	-	-	-
Desmazierella acicola	41.6	43.3	39.3	7.5	5.6
White Sterile Form	-	3.3	10.7	-	-
Brown Sterile Form	25.0	13.3	7.1	-	-
Oidiodendron sp.	2.8	-	-	-	-
Pyrenochaeta sp. 1	2.8	-	-	-	-
Coniothyrium sp.	-	3.3	-	-	-
Fusarium sp.	2.8	3.3	7.1	-	-
Mucor spp.	-	-	-	37.5	33.4
Trichoderma viride	2.8	-	10.7	35.8	24.1
T. polysporum	-	6.7	-	-	-
Penicillium spp.	-	10.0	3.6	11.3	18.5
Mortierella ramanniana	2.8	3.3	3.6	7.5	16.7
Trichocladium sp.	-	-	3.6	-	1.9
F2 NEEDLES					
Fusarium sp.	-	14.3		-	
Mucor spp.	40.0	14.3		13.9	
Mucor racemosus	20.0	-		-	
Trichoderma viride	20.0	57.1		52.8	
Penicillium spp.	-	-		16.7	
Mortierella ramanniana	-	-		5.6	
Mortierella sp.	20.0	14.3		-	

F. bacillare appears to decline in importance as the needles age,this corresponding with its decreased abundance among isolates from the F1 layer. Dothichiza pityophila shows the reverse trend in isolations from surface sterilised needles and it is more abundant in the F1 layer than F. bacillare. Desmazierella acicola increases in abundance from the L to the F1 layer and dominates isolations from surface sterilised F1 needles. The Brown Sterile Form appears in isolations from these needles in quite high frequency but apart from this there are no important isolations of internal colonisers of F1 needles other than Desmazierella acicola. The frequencies of occurrence and percentage abundances of Desmazierella acicola and Dothichiza pityophila from surface sterilised F1 needles in successive samples are very stable.

Isolations of Trichoderma viride and Mucor spp. (Mucor hiemalis group) are almost entirely confined to washed needles of the L and F layers with a marked increase in incidence occurring from the L layer to the F1 layer. Penicillium spp. are less abundant than Mucor spp. and T. viride and have a lower frequency of isolation from the two samples of washed F1 needles than from the single sample of L needles treated in this way. Mortierella ramanniana occurs in isolations from washed F needles with about the same frequency as Penicillium spp. Because of the porosity of the F2 needles very few isolates were obtained from surface sterilised F2 needle fragments but none of the species characteristic of F1 surface sterilised needles were isolated from these fragments,

except Fusarium sp. No attempt had been made to reduce the efficacy of the surface sterilisation in this study, in contrast to that of Kendrick & Burges (1962).

The F layers present a picture of seasonal stability in the fungal isolations in contrast to the L layer, which reflects the rapid change taking place in the L needles through the year compared with the slow decomposition of F needles. Previous work by Parkinson & Balasooriya (1969) and Widden & Parkinson (1973) had failed to find any significant seasonal variation in species composition but Widden & Parkinson (1973) and Parkinson et al (1968) have shown seasonal variation in fungal activity which is highly dependent on moisture content. The L layer is more subject to drying out and the restriction of fungal activity, if not complete cessation of growth, than the F layers (Kendrick & Burges, 1962).

From the results presented above it appears that Fusicoccum bacillare and Dothichiza pityophila are the principal primary saprophytes of this litter of Scots Pine. There is very little correspondence with the results of Hayes (1965b) from this site who isolated Graphium sp., Geotrichum candidum and Penicillium funiculosum as the primary saprophytes of surface sterilised needles, except perhaps in the occurrence of Penicillium spp. on a sample of washed L needles noted above. What the reason is for this discrepancy is not clear, although Hayes did surface sterilise the needles with 0.1% silver nitrate solution. The importance of F. bacillare as a primary internal saprophyte of the needle litter of various conifers has already been noted and the results presented here confirm this. In fact the

study of Hayes in the Blackwood is one of the few not to have shown F. bacillare as an important isolate. This suggests that Hayes' methods were responsible for the different results, the alternative being that the mycoflora of the litter changed very dramatically in 11 years. The other common primary saprophyte of Pine needles, Aureobasidium pullulans was entirely lacking from isolations in this study and was also not of any importance in Hayes' study. Although Dothichiza pityophila may have been recorded as A. pullulans in some work, Kendrick & Burges (1962) noted A. pullulans as a superficial coloniser, which is not the case for D. pityophila here, and, furthermore, D. pityophila does not decline as readily as A. pullulans in F1 needles.

The complete absence of the common primary saprophytes supports the conclusions from previous European work that the early stages in the fungal succession on the needle litters of conifers is unusual and the absence of A. pullulans in this study reinforces this view. It was noted in Chapter One that phenolic acids had a generally inhibitory effect on the germination and hyphal growth of some microfungi that were saprophytes of Angiosperm leaves, with varying substrate specificities, whereas there was a stimulatory effect on specialised saprophytes and soil fungi that colonise Pine litter. The behaviour of two specific saprophytes of the litter of a number of conifers, Dothichiza pityophila and Thysanophora penicilloides, to ferulic acid provides a particularly

strong contrast to the behaviour of the widespread Cladosporium herbarum, Botrytis cinerea and Aureobasidium pullulans. It has not been possible to relate the effects of phenolic acids on agar to the situation in Scots Pine litter because of the limited results from the analysis of phenolic acids in the litter. However, this pattern strongly suggests that phenolic acids may be important factors in the adaptation of fungi to their environment.

With regard to the secondary saprophytes, Desmazierella acicola is clearly the major representative of this group to be found as a coloniser of the needle interior, in agreement with previous work on Pinus sylvestris (Gremmen, 1959; Kendrick & Burges, 1962; Hayes, 1965a,b). The Brown Sterile Form is also important and it is possible that it is associated with Sympodiella acicola, having very dense mycelium like this species and not spreading far from the Pine needle over the agar.

The increase in abundance of the isolations of Trichoderma viride and the Mucorales with depth from washed needles reflects the occurrence of these species as soil inhabiting fungi. Penicillium spp. on the other hand do not show any preference for washed F needles over L needles, but the reverse if anything. This was confirmed in the Association Analysis, in which Penicillium spp. were absent as 'Divisor' species in any combination of data except when washed L needles were run separately, whereas D.pityophila, T. viride, Mucor spp. Desmazierella acicola and the Brown Sterile Form do act as Divisors. This indicates that Penicillium spp.

were not common and were without any heterogeneities in distribution, except with respect to washed L needles.

Kendrick & Burges (1962) considered T. viride and Penicillium spp. together as soil fungi present largely as a high spore potential. The presence of T. viride in high frequency in isolations from washed F needles was therefore taken as a sign that the washing technique had failed to remove adequately the high spore load and to favour the growth of superficial mycelium. However, the conclusions from the results presented here are that needle washing can distinguish between superficial mycelium and spores; and that T. viride and Mucor spp. should be taken together as representatives of an actively colonising but superficial population of fungi, in contrast to the Penicillia which are probably passive. This is not to say, however, that the role of T. viride and the Mucorales in the decomposition of the needles is any clearer.

In the burial experiments, the behaviour of the different populations of fungi comprising the mycoflora under different environmental conditions is subjected to further examination.

At the end of the first experiment in September 1973 the L layer needles that had been buried in June were mostly intact but blackened and softened, i.e. they had the appearance of Fl needles. The Fl needles buried in both experiments showed no change.

The 'overall colonisation indices' for each sample are given in table 2.3. The colonisation index of

TABLE 2.3 Fungal colonisation of Pine needles buried in soil

'Overall Colonisation Index'

L NEEDLES				F1 NEEDLES			
Hermitage Wood		Experimental Gardens		Hermitage Wood		Experimental Gardens	
NA	A	NA	A	NA	A	NA	A
			JUNE - SEPTEMBER				
78.3	109.3	167.0	145.0	63.3	99.0	76.7	132.3
				SEPTEMBER - DECEMBER			
				69.6	113.1	128.7	153.0

A = Needles autoclaved before burial

NA = Needles not autoclaved before burial

autoclaved needles is higher than that of non-sterilised needles for each treatment combination except L needles buried in the Experimental Gardens where the reverse is true. The colonisation index is consistently higher with needles buried in the Gardens than in Hermitage Wood. Table 2.4a shows the frequency of occurrence of each species isolated out of a total of 30 needles. In table 2.4b the 'percentage abundance' of the isolates have been calculated from the data in 2.4a, with the mean values for the two samples of F1 needles being used.

Among species isolated from only non-sterilised needles Dothichiza pityophila, Desmazierella acicola and Fusicoccum bacillare have clearly survived from inoculum present at the time of burial. This is probably also true of the Brown Sterile Form, which was indistinguishable in culture from the isolates obtained direct from the Blackwood. Comparison of the results in tables 2.2 and 2.4 suggest that it is possible that the White Sterile Form, Pyrenochaeta sp. 1 and Trichoderma polysporum may also be survivors. No conclusions can be made regarding Mortierella ramanniana.

Isolates from autoclaved needles buried in Hermitage Wood belong to only five types and T. viride is by far the most common species. Ten species were isolated from autoclaved needles from the Experimental Gardens and more are common relative to T. viride, particularly Gliocladium roseum. T. viride is the only soil fungus characteristic of Pine litter to be common throughout the samples of needles, Mucor hiemalis being frequently isolated from needles buried in the Gardens but isolates

TABLE 2.4. Fungi isolated from Pine needles buried in soil.

a) Frequency of isolation of individual species from each sample of 30 needles.

	HERMITAGE WOOD		EXPERIMENTAL GARDENS	
	N.A.*	A*	N.A.	A.
L NEEDLES				
Dothichiza pityophila	1	-	15	-
Desmazierella acicola	7	-	1	-
White Sterile Form	2	-	-	-
Brown Sterile Form	2	-	1	-
Pyrenochaeta sp. 2	1	-	1	5
Fusarium sp.	1	-	4	4
Mucor spp.	-	3	1	4
Trichoderma viride	9	26	5	17
Penicillium spp.	1	-	1	1
Gliocladium roseum	1	-	12	14
Nocardia	-	2	9	1
Trichocladium sp.	1	3	-	-
Rhizopus sp.	-	-	-	1

	N.A.		A		N.A.		A	
	Sept.	Dec.	Sept.	Dec.	Sept.	Dec.	Sept.	Dec.
F1 NEEDLES								
Dothichiza pityophila	1	-	-	-	3	-	-	-
Fusicoccum bacillare	1	-	-	-	-	-	-	-
Desmazierella acicola	9	7	-	-	5	8	-	-
White Sterile Form	-	1	-	-				
Brown Sterile Form	1	2	-	-				
Pyrenochaeta sp. 1	1	-	-	-				
Pyrenochaeta sp. 2	1	-	-	-	-	4	3	-
Fusarium sp.	-	1	5	-	1	1	7	1
Mucor spp.	1	-	-	-	-	7	3	6
Trichoderma viride	8	10	27	29	7	16	18	28
T. polysporum	-	4	-	-	-	1	-	-
Penicillium spp.	-	-	1	2	3	3	-	-
Gliocladium roseum					1	-	8	8
Nocardia					10	-	5	-
Trichocladium sp.	-	1	-	4	-	-	-	1
Rhizopus sp.					-	-	1	-
Mortierella ramanniana					-	-	-	1
Absidia orchidis					-	-		
Phoma eupyrena					-	-	1	-

* A - Needles autoclaved before burial
NA - Needles not autoclaved before burial

TABLE 2.4. Fungi isolated from Pine needles buried in soil.

b) Percentage Abundance of individual species (based on total number of isolates).

	HERMITAGE WOOD		EXPERIMENTAL GARDENS	
	N.A.*	A*	N.A.	A
L NEEDLES				
Dothichiza pityophila	3.8	-	30.0	-
Desmazierella acicola	26.9	-	2.0	-
White Sterile Form	7.7	-	-	-
Brown Sterile Form	7.7	-	2.0	-
Pyrenochaeta sp. 2	3.8	-	2.0	10.6
Fusarium sp.	3.8	-	8.0	8.5
Mucor spp.	-	8.8	2.0	8.5
Trichoderma viride	34.6	76.5	10.0	36.2
Penicillium spp.	3.8	-	2.0	2.1
Gliocladium roseum	3.8	-	25.0	29.8
Nocardia	-	5.9	18.0	2.1
Trichocladium sp.	3.8	8.8	-	-
Rhizopus sp.	-	-	-	2.1
F1 NEEDLES				
Dothichiza pityophila	2.1	-	4.1	-
Fusicoccum bacillare	2.1	-	-	-
Desmazierella acicola	33.3	-	17.8	-
White Sterile Form	2.1	-	-	-
Brown Sterile Form	6.3	-	-	-
Pyrenochaeta sp. 1	2.1	-	-	-
Pyrenochaeta sp. 2	2.1	-	5.5	3.4
Fusarium sp.	2.1	7.4	2.7	9.0
Mucor spp.	2.1	-	9.6	10.1
Trichoderma viride	18.7	41.2	31.5	51.8
T. polysporum	8.4	-	1.4	-
Penicillium spp.	-	4.4	8.2	-
Gliocladium roseum	-	-	1.4	18.0
Nocardia	-	-	13.7	5.6
Trichocladium sp.	2.1	5.9	-	1.1
Rhizopus sp.	-	-	-	1.1
Mortierella ramanniana	-	-	1.4	-
Absidia orchidis	-	-	2.7	1.1
Phoma eupyrena	-	-	-	-

* A - Needles autoclaved before burial
NA - Needles not autoclaved before burial

of Mortierella ramanniana and Penicillium not being abundant at all. By contrast, Gliocladium roseum is a species not previously isolated from Pine needles which is frequently isolated from needles buried in the Gardens and in the first experiment only a Proactinomycete was isolated from needles buried at this site, identified as a Nocardia. This is an interesting organism to be found colonising Pine needles. In addition, a second species of Pyrenochaeta was isolated at low frequency but fairly uniformly from autoclaved and non-autoclaved needles, and Trichocladium sp. was the only soil fungus not characteristic of Pine needles to be isolated more frequently from needles buried in Hermitage Woods than in the Experimental Gardens.

The statistical analysis of the distribution of individual species with respect to needle type (L or F1), pretreatment (autoclaved or non-sterilised) and soil type (Hermitage Wood or Experimental Gardens) was carried out using 2 x 2 contingency tables. This permitted the significance of any two treatments to be examined by the comparison of the frequency of isolation of species in two groups of needles. Either the total number of needles or total isolates could be used as the basis for the isolation frequency.

Proportion of needles (or isolates) on which species:	Needle group 1	Needle group 2
Present	a	b
Absent	c	d

$a + b + c + d = n$ Chi-squared = $\frac{n(ad-bc-n/2)^2}{(a+b)(c+d)(a+c)(b+d)}$

Almost identical results were obtained whether frequencies were based on needle numbers or total isolate numbers. Treatments can be compared at the level of individual samples (30 needles) or any combination of samples, e.g. all L needles against all F1 needles.

As might be expected Dothichiza pityophila was isolated more frequently from L than from F1 needles and the reverse is true for Desmazierella acicola, but these effects are only significant in isolations from needles buried in the Gardens. Actually, comparison of the results of the experiments with the frequency of Dothichiza pityophila originally present in the needles (table 2.2a) shows that the survival of this species was lower in F1 than in L needles. Dothichiza pityophila was isolated in significantly lower frequency from needles buried in Hermitage Wood than from those buried in the Experimental Gardens, and again the reverse is true of Desmazierella acicola.

T. viride has a significantly lower frequency in all samples of non-sterilised needles compared to the corresponding autoclaved needles. There is no significant difference between isolations from L and F1 needles but T. viride was significantly more common in isolations from needles buried in Hermitage Wood than in the Gardens.

Only isolations of G. roseum and Nocardia from needles buried in the Gardens were considered in the analysis. Autoclaving had no significant effect on the isolation of G. roseum from L needles but this fungus occurred with significantly lower frequency on non-sterilised F1 needles than on autoclaved F1 needles.

Nocardia was isolated with significantly greater frequency from non-sterilised L needles than from the autoclaved needles of this layer but there was no significant difference as regards isolations from F1 needles; as a result Nocardia is significantly more common among isolations from non-sterilised L needles than from non-sterilised F1 needles.

The remaining species were not isolated with sufficient frequency from any sample for the Chi-squared analysis to be reliable.

An example of the use of Association analysis is shown in figure 2.1 where there is a dendrogram produced from a run with the complete data from the burial experiments. The vertical scale represents the mean Chi-squared value of the dividing species. The first division is on the presence and absence of T. viride from these needles. Of those from which T. viride was isolated (+) there is a succession of divisions on Nocardia, Fusarium sp., Pyrenochaeta sp. 2 and Mucor spp., each division occurring on the needles from which the previous divisor was absent (-); this proceeds until a terminating group is formed where the Chi-squared value for any division would be below the minimum value. Of those needles without T. viride a division occurs on Desmazierella acicola; those needles with this species are further divided on the presence and absence of Dothichiza pityophila, while those without are successively divided on Nocardia, Pyrenochaeta sp. 2, Fusarium oxysporum, and the Brown Sterile Form, leaving a final group of the remaining

Nocardia was isolated with significantly greater frequency from non-sterilised L needles than from the autoclaved needles of this layer but there was no significant difference as regards isolations from F1 needles; as a result Nocardia is significantly more common among isolations from non-sterilised L needles than from non-sterilised F1 needles.

The remaining species were not isolated with sufficient frequency from any sample for the Chi-squared analysis to be reliable.

An example of the use of Association analysis is shown in figure 2.1 where there is a dendrogram produced from a run with the complete data from the burial experiments. The vertical scale represents the mean Chi-squared value of the dividing species. The first division is on the presence and absence of T. viride from these needles. Of those from which T. viride was isolated (+) there is a succession of divisions on Nocardia, Fusarium sp., Pyrenochaeta sp. 2 and Mucor spp., each division occurring on the needles from which the previous divisor was absent (-); this proceeds until a terminating group is formed where the Chi-squared value for any division would be below the minimum value. Of those needles without T. viride a division occurs on Desmazierella acicola; those needles with this species are further divided on the presence and absence of Dothichiza pityophila, while those without are successively divided on Nocardia, Pyrenochaeta sp. 2, Fusarium oxysporum, and the Brown Sterile Form, leaving a final group of the remaining

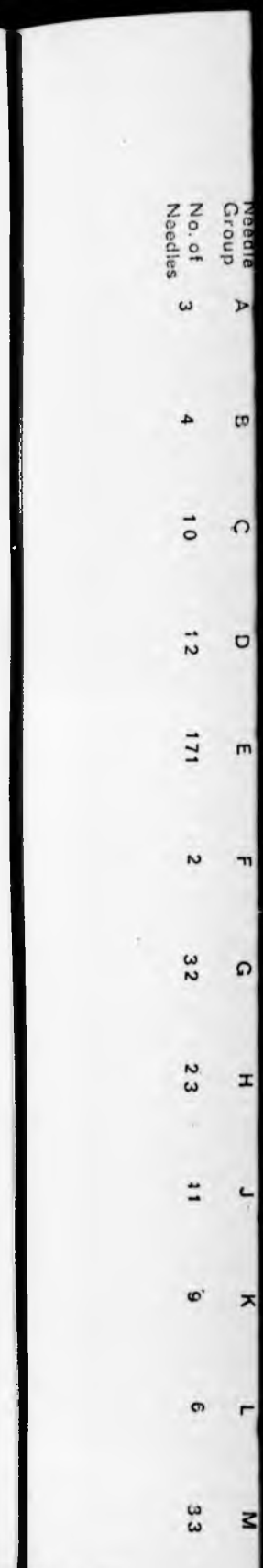


Fig. 2.1 Association Dendrogram from analysis of Fungal isolation data from buried Pine needles.

Dividing Species

1. Trichoderma viride
2. Nocardia
3. Pyrenochaeta sp. 2
4. Fusarium sp.
5. Mucor spp.
6. Desmazierella acicola
7. Dothichiza pityophila
8. Brown Sterile Form

Species common to all needles in each final group

A. T. viride + Nocardia

B. T. viride + Pyrenochaeta sp. 2

C. T. viride + Fusarium sp.

D. T. viride + Mucor spp.

E. T. viride

F. D. pityophila + Des.acicola + Nocardia

G. Des. acicola

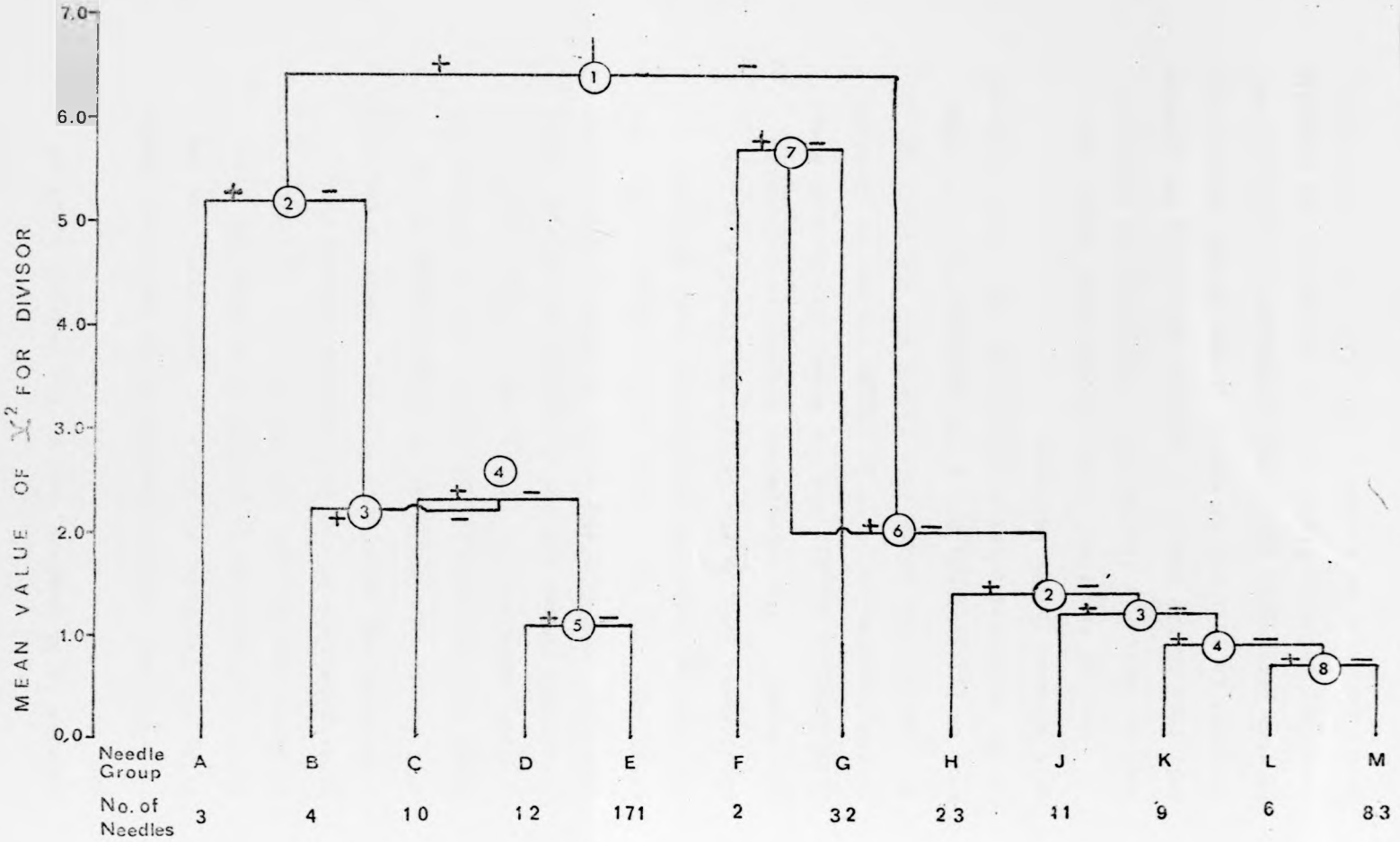
H. Nocardia

J. Pyrenochaeta sp. 2

K Fusarium sp.

L Brown Sterile Form

M (Remainder)


MEAN VALUE OF χ^2 FOR DIVISOR
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Needle Group
A B C D E F G H J K L M
No. of Needles
3 4 10 12 171 2 32 23 11 9 6 83

species in 83 needles, out of the initial total of 360.

The most obvious feature of the analysis, whether using all the data, as above, or only part, was the primary division on T. viride, this being the most frequent species, but also having very significant heterogeneities in its distribution between non-sterilised and autoclaved needles. Most of the other soil fungi, ie. Fusarium sp. Pyrenochaeta sp. 2, Mucor spp. and Gliocladium roseum, act as Divisors of needles containing T. viride, and so final needle groups are formed between T. viride and each of these species. Although the number of needles involved is often less than 10, these needle groups account for the greater number of Associations found in these experiments than in the isolations direct from the Blackwood. The saprophytes Dothichiza pityophila, Desmazierella acicola and the Brown Sterile Form never formed Associations with T. viride but act as Divisors for needles from which this fungus was not isolated, as well as Pyrenochaeta sp. 2 and Fusarium sp. As found in the Blackwood samples Penicillium spp. never occurred as a Divisor in the analysis.

Most of the final needle groups were formed from needles buried in the Gardens, indicating the effect of soil type on the number of species colonising the needles. Included in this set of needle groups is the Association between Dothichiza pityophila, Desmazierella aciocola and Nocardia, noted in Figure 2.1. D. pityophila and Nocardia were associated in a much larger number of needles when

L needles and F1 needles were run separately.

It is suggested that the lower frequency of T. viride on non-sterilised than on autoclaved needles was due to its active exclusion from the needles by the 'resident' saprophytes, particularly D. pityophila. The evidence for this is that firstly, the highest frequency of isolates of D. pityophila from L needles buried in the Gardens is inversely related to the distribution of T. viride. Secondly, the results of the Association Analysis show that T. viride forms needle groups with other soil fungi, but not with the needle saprophytes. Thirdly, when these saprophytes are not present T. viride is isolated far more frequently from non-sterilised needles than from the interior of the needles direct from the Blackwood. Finally, there is no indication from Infra-red spectroscopic data to be presented later that autoclaving effects the needles chemically. Any effect of autoclaving on the chemical nature of the needle as a substrate is therefore likely to be secondary to the effects of the new environment in improving the potential of T. viride to colonise the needles. It can be concluded that prior-colonisation by the needle saprophytes is an important factor limiting the colonisation by T. viride, as it is in the natural litter. It is interesting that Bruehl and Lai (1966) found that prior-colonisation of wheat straw by T. viride (among other fungi) prevented establishment of potential colonisers of the straw after burial in soil.

The behaviour of T. viride can be contrasted with that of Gliocladium roseum, and Nocardia, the distribution

of these species following that of D. pityophila in the non-sterilised needles according to needle layer and soil type. Autoclaving increases the frequency of isolation of G. roseum from F1 needles only, while Nocardia is actually more abundant in non-autoclaved L needles than in autoclaved material. The Association observed between Nocardia and D. pityophila on L and F1 needles shows directly the significant occurrence of both species on the same needles, which only became apparent in the Association Analysis. These results indicate that G. roseum and Nocardia are less limited by the prior-colonisation effect than T. viride and, in fact, Nocardia especially is affected positively by this factor.

It should be borne in mind that T. viride belongs to a genus of highly antagonistic fungi with the ability to produce antibiotics (Dennis & Webster, 1971a,b). Also,Mitchell (1973) found that an isolate of G. roseum was the fungus most inhibited by non-volatile antibiotics of T. viride among a wide range of test species. However, Mitchell also noted that the nutrient source affected not only growth of T. viride and its production of antibiotics but also the ability of G. roseum to outgrow T. viride and therefore to overcome or avoid antibiosis. In other words, the Competitive Saprophytic Ability (Garrett 1956) of both species is dependent upon the substrate, and so, as a result, is the outcome of the competitive interaction.

The composition of the mycoflora of the actual soils used in the burial experiments is not known but it is suggested that G. roseum would have a higher frequency

in the soil in the Gardens than in Hermitage Wood due to cropping (Domsch & Gams, 1972) and Nocardia would be more characteristic of the Gardens soil because this soil is almost neutral whereas the Hermitage Wood soil is quite acid. In the Gardens, therefore, the nature of the substrate gives G. roseum and Nocardia the advantage in the colonisation of Pine needles in the presence of the needle saprophytes.

Just what kind of substrate is provided by the Pine needles and how decomposition of the needle by fungi itself changes the nature of the substrate, was the subject of the final part of these studies on Pine litter.

Estimation of the substrates present in Pine needles by means of extraction of fractions of differential solubility, e.g. cellulose, lignin and xylans, and estimation of these by reagents was not attempted. It is possible to compare the activities of white rot fungi, for example, or the effect of different environmental conditions on decay by means of chemical analysis of lignin, even though the exact nature of the fractions reacting with a certain reagent (e.g. Thioglycollic acid) or left as a residue from extraction (by sulphuric acid for example) is uncertain. But in a naturally decomposing material such as Pine needle litter there is no comparative yardstick by which the reliability of the assay method can be judged. Moreover, in this case there seems to be no previous information regarding the composition of the litter as it decomposes, except for general observations on the rate of decomposition of lignified and non-lignified tissues

(Gremmen, 1957, Hayes 1965a).

However, there is a body of information available concerning the analysis of isolated lignins by infra-red spectroscopy in which certain characteristic absorptions have been assigned to structures known to be present in lignin. Furthermore changes that take place in lignin during decomposition and humification have been followed by infra-red analysis.

The examination of papers by several authors (Smith, 1955a,b; Durie et al. 1959; Flaig et al. 1959; Ziechmann, 1964; and Wojtas-Wasilewska et al. 1973) on the infra-red spectra of isolated lignins from hardwoods, softwoods, cereal straw and other materials revealed several consistent absorption maxima which can be assigned reliably to chemical groups in the lignin. Variations in wavelength due to the differences in material and the method of extraction are only very slight.

The most important absorptions by lignin are due to the aromatic groups; stretching of carbon - carbon bonds in the nucleus give rise to absorptions in two regions, 1600-1590 and 1500-1510 cm^{-1}, and absorption at 1660-1640cm^{-1} has been attributed to stretching of the double bonded side chain conjugated with the aromatic nucleus by Flaig et al. (1959), Ziechmann (1964) and Wojtas-Wasilewska et al. (1973). Bending of methoxyl and methylene groups, important as substituents to the benzene ring, gives rise to absorption at 1465 and 1425 cm^{-1}. Saturated oxygen containing groups absorb in the 1400-950 cm^{-1} region; correlation with exact structures is difficult but absorption by the phenoxy group, in phenolic ethers and phenolic hydroxyls, probably accounts for the peaks at

1265 and 1215 cm^{-1}; and the absorption maxima at 1130 and 1040 cm^{-1} can be attributed respectively to secondary and primary alcoholic groups. Carbonyl groups in lignin absorb in the 1730-1640 cm^{-1} region, the work of Smith (1955a,b) and of Flaig et al.(1959) suggesting that 1730-1700 cm^{-1} absorption being due to stretching of the COO-group present in esters and the absorption in the lower region to the carbonyl group stretching of aldehydes and ketones. These absorption frequencies are listed in table 2.5 for reference.

In wood (of Douglas Fir) Durie et al. (1959) found the absorption of the aromatic nuclei of lignin at 1510 cm^{-1}, the absorption of carbonyl groups at 1700 cm^{-1} and some of the saturated oxygen group maxima to be quite clear; but the broad cellulose band in the region of 1640 cm^{-1} merged with the lignin peak at 1600 cm^{-1} and there was also a broad region of absorption due to cellulose between 1200 and 900 cm^{-1} with a characteristic cellulose peak at 1160 cm^{-1}. Chemical removal of cellulose led to a sharpening of the lignin absorptions obscured by the cellulose bands.

Rotting of wheat straw from which lignin and humic acids were extracted for spectral analysis was followed by Flaig et al.(1959) and the humification of a Björkman lignin isolated from rye straw by soil microorganisms was studied by Wojtas-Wasilewska et al. (1973). The carbonyl absorption decreased in the study of the rye straw lignin but increased in lignin isolated from wheat straw after rotting. However, in both studies there was a broadening in the 1639-1600 cm^{-1} region probably due to the incorporation of nitrogen

TABLE 2.5. Characteristic Infra-red absorption frequencies of lignin (1750-850 cm^{-1} region).

Frequency (wave numbers) cm^{-1}		
	CARBONYL GROUPS	
1730 - 1700)	C = O Stretching	(COO - Esters
1700 - 1640)		(C = O Aldehydes, ketones
	AROMATIC GROUPS	
1600 - 1590)	C = C Stretching	
1510 - 1500)		
1660 - 1640	C = C Stretching, conjugated to benzene nucleus (possible)	
	ALIPHATIC GROUPS	
1465	$-CH_3$ Symmetrical bending)	$-OCH_3$
1425	$-CH_2$, $-OCH_3$ Bending)	
	SATURATED OXYGEN-CONTAINING GROUPS	
1265)	Phenoxy group	(Phenolic ether?
1215)	asymmetrical stretch	(Phenolic hydroxyl
1130	-OH Bending	Secondary alcoholic group
1040	C-O Stretching	Primary alcoholic group

compounds during humification. Bands in the 1400-950 cm^{-1} region became less intense, and a decrease in absorption attributed to the C=C group at 1660 cm^{-1} in the rye straw lignin was considered to reflect side chain cleavage.

The humic acids extracted from the wheat straw showed little change during the experimental period but they had the absorption due to the aromatic nuclei at 1510 cm^{-1} and several of the bands due to substituents at lower frequencies; and furthermore there was the broad 1640-1600 cm^{-1} characteristic of rotted lignin. Flaig et al. (1959) concluded from this that lignin is gradually transformed into humic acid like substances.

It was clear from these investigations that it was quite feasible to attempt the infra-red absorption analysis of suitably prepared but unextracted Pine needles, for measurement of the changes that take place during the decomposition of the litter.

Infra-red spectra could be obtained from Pine needles and Humus by preparing potassium bromide discs from material ground as finely as possible in a hammer mill. Before and after grinding the samples were freeze-dried and were kept in a desiccator thereafter to prevent reabsorption of moisture. The KBr discs were prepared by further grinding up the material in an agate pestle and mortar, mixing with KBr in a ratio of about six parts KBr to one part Pine needles (by volume) and pressing the mixture in a ten ton press under vacuum. The discs were basically transparent but it was not

possible to eliminate some clumping of the Pine needle material, which limited resolution. However, the resolution was sufficient to show very good uniformity between samples of the same needle type and to show that the spectra of each layer was characteristic. Needles collected from the main part of the Blackwood showed no differences with needles from the corresponding layer of the experimental site.

Typical spectra between 1900 and 850 cm^{-1} of L needles are shown in Figure 2.2, of F1 and F2 needles in Figure 2.3 and of H layer material in figure 2.4.

The spectrum of a sample of freshly fallen L needles collected in December (fig. 2.2a) shows a peak at 1730 cm^{-1}, a shoulder in the region of 1650-1600 cm^{-1}, a sharp peak at 1510 cm^{-1} and a more diffuse but quite intense absorption around 1160 cm^{-1}. It is considered that the peaks at 1730 and 1510 cm^{-1} are associated with lignin in the pine needle (carbonyl and aromatic absorptions respectively) and that the 1160 cm^{-1} region absorption is due to cellulose. There are also very minor peaks (but found consistently in L needles) at 1465 and 1425 which may be due to methoxyl groups in the lignin, and another consistent but small peak at 1660 cm^{-1} possibly due to conjugated side chains. The 1650-1600 cm^{-1} shoulder is thus attributable to the overlapping absorptions of lignin and cellulose in this region, as found in wood.

Older L needles, collected in June, show a very similar spectrum (Fig. 2.2b) but there is a rounding-off of the carbonyl group absorption at 1730 cm^{-1} and

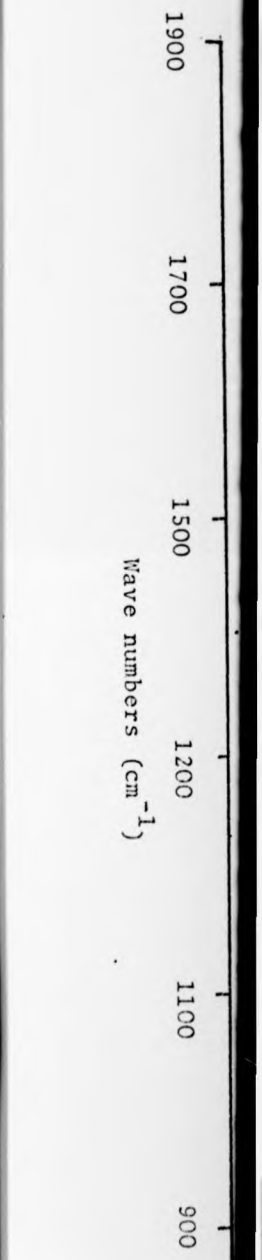


Fig. 2.2 IR Spectra of L layer needles from Scots Pine litter. a) Needles collected in December. b) Needles collected in June.

(a)
TRANSMITTANCE %
70
60
50
40
30
20
10
0
1900
1700
1500
1200
1100
900
Wave numbers (cm^{-1})
(a)
(b)
0
10
20
30
40
50
60
70
TRANSMITTANCE %
(b)

Pine

b)

this trend continues progressively in F1 and F2 material (Figs. 2.3a and 2.3b). In the F layer samples the lignin peak at 1510 cm^{-1} and the cellulose absorption at 1160 cm^{-1} are still visible, in addition to the carbonyl absorption. However the absorption in the region of 1660-1600 cm^{-1} has become rounded off. This probably represents absorption by Nitrogen compounds in the fungal mycelium (chitin) and perhaps also chemical incorporation of nitrogen into lignin, i.e. humification. And there is some indication of demethylation as the absorptions at 1465 and 1425 cm^{-1} have disappeared.

In the spectrum of material from the H layer the peak at 1730 cm^{-1} has now completely disappeared; instead there is a shoulder between 1730 and 1670 cm^{-1} which is characteristic of humic acids (Flaig et al, 1959). The absorption by lignin at 1510 cm^{-1} and by cellulose at 1160 cm^{-1} has disappeared. The lignin peak at 1510 cm^{-1} is generally more intense than that at 1600 cm^{-1} which suggests that there is little or no lignin and cellulose in the H layer and that the absorption between 1660 and 1610 cm^{-1} (which has a different appearance to that in any of the other spectra) is now due entirely to other substances, probably containing nitrogen. It will be recalled that the H layer contains as much animal material (faeces and exoskeletal fragments) as plant residues.

Autoclaved needles were compared with ordinary material to see if any changes were indicated, but samples of L and F1 autoclaved needles had spectra indistinguishable

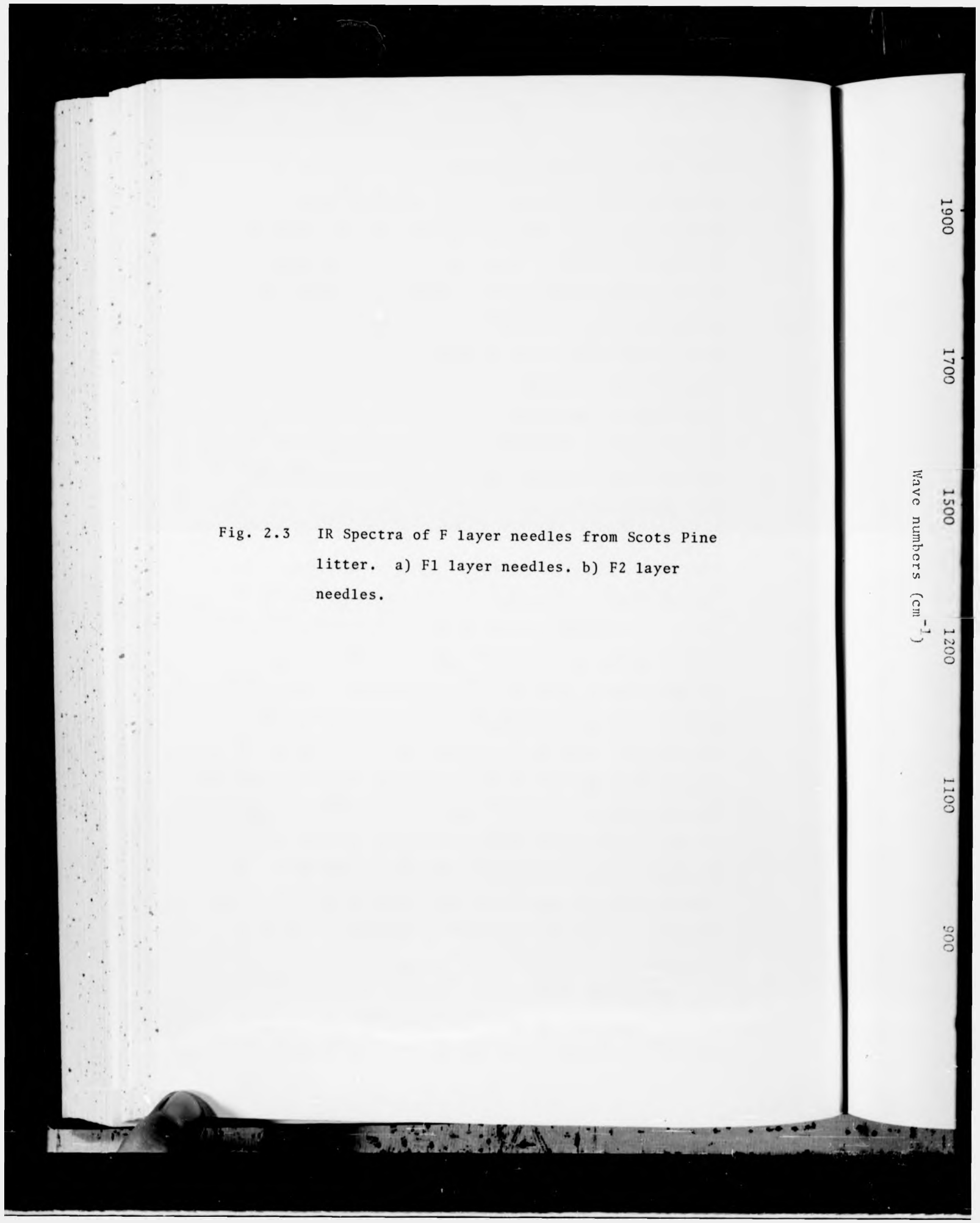
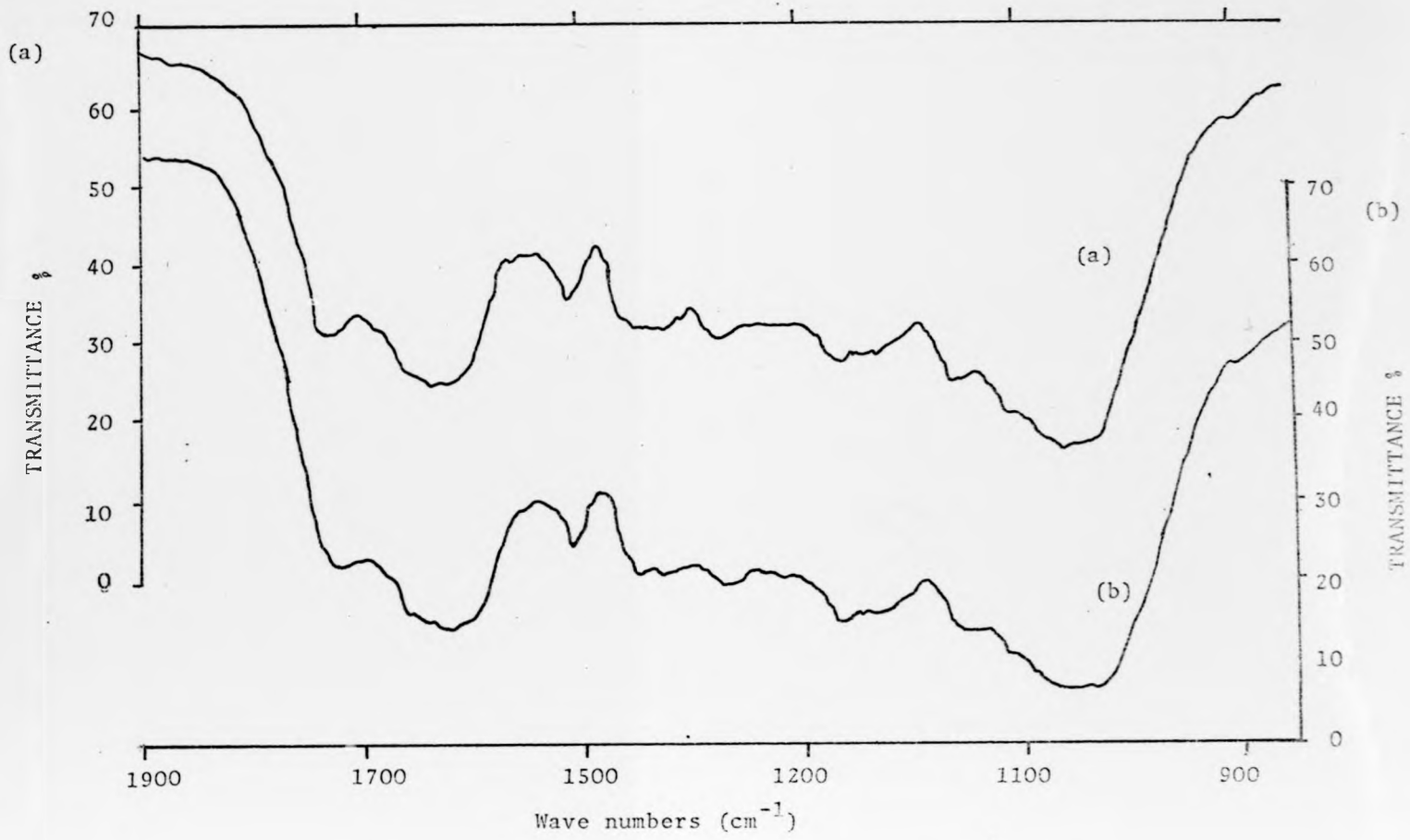
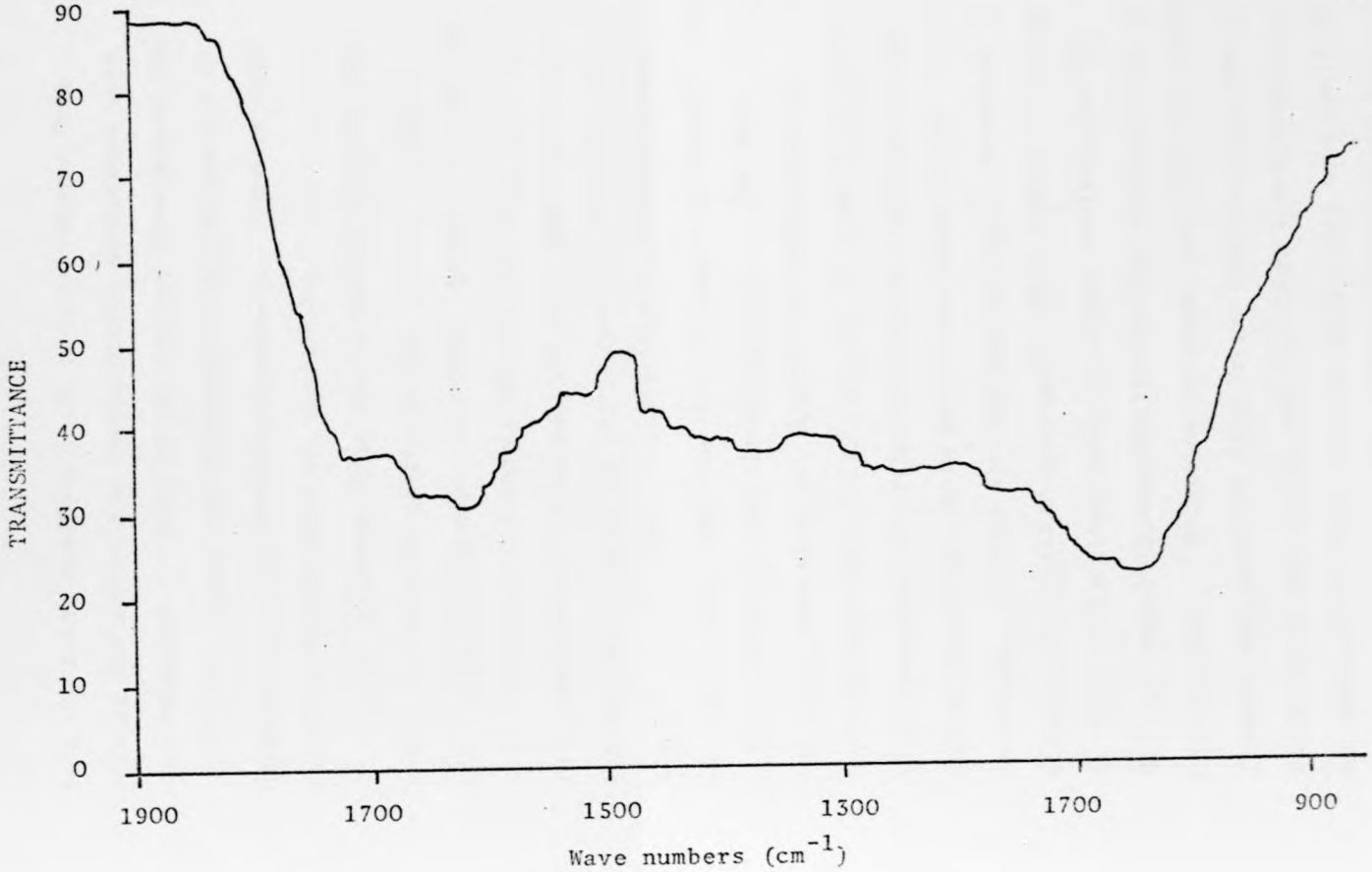


Fig. 2.3 IR Spectra of F layer needles from Scots Pine litter. a) F1 layer needles. b) F2 layer needles.


(a)
70
60
50
40
30
20
10
0
TRANSMITTANCE %
(a)
(b)
70
60
50
40
30
20
10
0
(b)
TRANSMITTANCE %
1900
1700
1500
1200
1100
900
Wave numbers (cm^{-1})

Pine

Fig. 2.4 IR Spectrum of H layer material from Scots Pine litter.


90
80
70
60
50
40
30
20
10
0
TRANSMITTANCE
1900
1700
1500
1300
1700
900
Wave numbers (cm-1)

from the fresh needles. F1 needles removed from soil at the end of the second burial experiment were also examined. None of the samples from any of the treatments showed any difference from the spectra of needles collected from the Blackwood. Needles from the first burial were not available.

It is concluded from the Infra-red analysis that there is a gradual change in the needles throughout their progress through the litter layers. It has not been possible to determine whether cellulose or lignin is preferentially attacked, but both seem to disappear between the F layer and the H layer. However, the changes taking place resemble humification, and this in itself would indicate that the material becomes less readily available for fungal growth. The data presented here does not support the contention of Kendrick & Burges (1962) that the F2 layer is in part an accumulation of fragments that have escaped fungal attack and which remain until comminuted by the microfauna, because the spectrum of the F2 material is clearly intermediate between F1 and H layers. Rather, it is suggested that there is fungal activity in the F2 layer which is responsible for the decomposition of the needles. A decline in fungal activity with depth through the layers of Pine litter has been observed by Parkinson et al. (1968) and Jorgensen & Wells (1973), as measured by respiratory activity, and this would be expected if the material becomes progressively more resistant to decomposition, a fact noted by the latter workers.

On a more speculative basis, if the absorption at 1730 cm^{-1} in the IR spectra of the Pine needles is associated with ester groups in lignin the gradual change in this absorption could be related to the release of esterified phenolic acids. Microorganisms decomposing the needles would therefore be involved in the continual production of phenolic acids, which is characteristic of Mor forming litter, without affecting extensively the structural integrity of the lignin. Probably the phenolic acids that can be extracted from the litter are only a fraction of the total release, the rest being metabolised by fungi. This phenomenon can be related to the differential ferulic acid decomposing activities of the various fungi isolated from Pine needles, which was noted in Chapter One.

Isolates of T. viride, Mortierella ramanniana and Mucor spp. isolated from Pine needles, or direct from the soils were unable to decompose ferulic acid readily or at all, in contrast to other soil fungi. The results of isolation experiments led to the conclusion that T. viride and the Mucorales are active but only superficial colonisers of the needles in the natural litter system. On the other hand, the specific saprophytes of the interior of the needles, Dothichiza pityophila, Desmazierella aciola and Thysanophora penicilloides have high ferulate decomposing abilities, as does Glicocladium roseum which has a greater capacity than T. viride to colonise the needle interior in the burial experiments, in the presence of D. pityophila.

Also an isolate of Fusarium oxysporum from the Gardens soil decomposed ferulic acid readily and although this genus is not a frequent coloniser of Pine needles, Fusarium sp. was isolated from surface sterilised needles only in the samples direct from the Blackwood. Therefore, there is an association of the ability to decompose ferulic acid readily (as defined in Chapter One) and the ability to colonise the needle interior in the natural litter or in soil. Furthermore, although Nocardia did not make much growth on ferulic acid in acid media, this organism is notable for its capacity to utilise a wide range of aromatic compounds, including phenolic acids (Waksman, 1967; Crawford et al. 1973).

Species of Penicillium from soil can decompose ferulic acid very readily but it has been shown how Penicillium spp. do not actually colonise the litter. Burges (1960) remarked how the Penicillia had a capacity for rapid growth on a wide variety of substances without extensive mycelial growth and the very rapid utilisation of ferulic acid by P. janthinellum in comparison with other fungi supports this view of the behaviour of Penicillium spp.

The data obtained from the Infra-Red analysis may shed some light on the actual role of T. viride and the Mucorales in Pine litter, a question of particular interest in view of the contrast of the physiology and ecology of these fungi with the Penicillia. Infra-red spectroscopy indicated a gradual change in the availability of substrates in the needles through the litter layers. If this is so the advantage of the specific needle

saprophytes given by prior-colonisation, could disappear and, together with the antagonistic behaviour of T. viride, this could lead to the eventual replacement of the specialised saprophytes by T. viride and other soil fungi in the needle interior. It was noted in the introduction to this chapter how T. viride and Mucor hiemalis form part of a population of soil fungi with a peak isolation from the F2 and H layers. It can be suggested, therefore, that these species along with Mortierella ramanniana and perhaps the Basidiomycetes are involved in the decomposition of the F2 needle fragments, which results in the accumulation of the plant-derived fraction of the H layer. Jorgensen & Wells (1973) found no significant difference between the respiratory activities of the lower layers of Oak litter and Pine litter in the same stands. This suggests that Pine litter is not particularly resistant to attack but that the accumulation of the F2 layer is due to other factors such as acidity.

In conclusion it can be noted that the ability of microfungi to decompose ferulic acid may have significance in a certain kind of saprophytic behaviour and in the functions of the different populations comprising the entire fungal flora of the Pine needle litter. It is not suggested that this is the only significance of ferulic acid decomposing ability, however, and further aspects will be considered in the following chapter. On the other hand, the importance of the differential effects on phenolic acids on the spore germination and hyphal growth of various species may lie in the overall

composition of the mycoflora. The involvement of phenolic acids in this ecological system comes about because of their continual liberation in the Pine litter from the decomposition of the substrate.

CHAPTER THREE

THE DECOMPOSITION OF FERULIC ACID BY NATURAL POPULATIONS OF MICROORGANISMS

An inevitable feature of all work on the isolation of microorganisms from their substrate or from soil is the involvement of selectivity in the techniques. Selectivity may unintentionally, but unavoidably, restrict the range of organisms that are isolated; for example, some saprophytes will not readily grow from the substrate on to normal agar media and slow growing fungi may be suppressed by more vigorous and antagonistic species. On the other hand, selective isolation media are frequently used, for example, to avoid bacterial 'contamination' of fungal isolation plates or to isolate only cellulose decomposing species. Furthermore, the pre-treatment of the substrate, e.g. washing or surface sterilisation, is a means of selective removal of certain fractions of the microbial populations on the substrates in order to obtain a meaningful picture from isolation of certain other populations.

In the isolation work from Pine needles reported in the previous chapter, washing of needles collected directly from the Blackwood was employed to remove the high spore potential from the needles in order to allow growth of mycelial forms, while surface sterilisation was directed at the isolation of internal colonisers of the needles.

A consequence of this is that certain superficial colonisers, originally characterised by direct observations, i.e. Sympodiella acicola and Helicoma monospora, were not isolated from washed needles, although probably present, because they are very slow growing. In isolations from surface sterilised needles that had been buried in soil, Trichocladium sp. and Gliocladium roseum appeared on the plates after a considerable length of time, possibly only after the antagonism of Trichoderma viride had lessened.

Furthermore, the burial of the Pine needles in soil can be considered as an example of the use of soil 'baiting' techniques, whereby a variety of natural materials have been added to soils for selective isolation (Warcup, 1960) as well as purified substances such as lignin or phenolic compounds (Jones & Farmer, 1967). The results of the experiments reported in Chapter Two present an apparent qualitative difference between the fungal populations of the two soils in Hermitage Wood and the Experimental Gardens. The principal fungi from non-sterilised and autoclaved needles buried at each site have been summarised in Table 3.1. Any species present in a particular square in this table was isolated from each replacate sub-sample of needles for that treatment. (L and F1 needles have been considered together). Many more species, including the actinomycete Nocardia, are characteristic of needles buried in the Gardens than in Hermitage Wood. However, it was noted in the

TABLE 3.1. SUMMARY OF FUNGAL ISOLATIONS FROM PINE NEEDLES BURIED IN SOIL.

	HERMITAGE WOOD	EXPERIMENTAL GARDENS		
NON-AUTOCLAVED	Desmazierella acicola	Desmazierella acicola		
		Dothichiza pityophila		
	Trichocladium sp.			
	Trichoderma viride	Trichoderma viride		
			Nocardia	Mucor hiemalis
			Gliocladium roseum	Penicillium spp.
			Pyrenochaeta sp. 2	
AUTOCLAVED	Trichocladium sp.			
	Trichoderma viride	Trichoderma viride	Nocardia	Mucor hiemalis
			Gliocladium roseum	Fusarium sp.
			Pyrenochaeta sp. 2	

previous paragraph that Trichocladium sp. was apparently suppressed by T. viride and the low isolation frequency of the former species may not give a true picture of its relative importance as a coloniser of the buried needles. In fact, the question may be asked of these isolations as a whole - how accurate a picture has been obtained in terms of the number of species isolated and their relative importance in the soil?

In order to answer this question use can be made of the data that have been accumulated on the ferulic acid decomposing activity of many of the fungi isolated. It is apparent that there are differences between needles buried in the Gardens and in Hermitage Wood, and between autoclaved and non-sterilised needles in these experiments, with respect to species that decompose ferulic acid readily - Desmazierella acicola, Dothichiza pityophila, Gliocladium roseum and Fusarium oxysporum (isolated directly from Gardens soil) - and those that do not - T. viride, Mucor hiemalis and Mortierella ramanniana. The same applies to the washed and surface sterilised needles in the natural Blackwood litter samples, which was considered in relation to the ferulic acid decomposing activity of superficial and internal colonisers of the needles in the previous chapter. The needles themselves, with their fungal populations, could be expected to have a certain decomposing activity, and therefore, by following the disappearance of ferulic acid from culture solutions incubated with Pine needles, an opportunity presented

itself to determine whether the activity of the fungi on the needles did in fact reflect the pattern expected on the basis of the isolation of individual species.

In addition, there seemed no reason why samples of the soils could not be examined for ferulate decomposing activity; it would then be possible to see if differences in the isolation data and any differences in the activity of the Pine needles corresponded with the activities of the two soils.

Finally, some preliminary attempts were carried out to relate the ferulic acid decomposing activity of natural populations of microorganisms in soil to the lignin decomposing activities of these poulations. Mangenot & Kiffer (1972) devised a method for the comparison of lignin decomposition in different soils by following the decomposition of lignin and total loss of weight in Beech sawdust achieved in soil samples (20 samples per soil) enriched with this substrate; and two soils were available from these studies which had been shown to differ greatly in the characteristics of lignin decomposition. Mangenot & Kiffer (1972) had found wide differences in the absolute capacity of different French and African soils to decompose lignin, but they were also able to distinguish soils in which lignin decomposition predominated in the total decomposition of the sawdust and those soils in which lignin decomposition did not stand out. Two types of lignin decomposition were therefore defined, and compared with white-rotting and brown-rotting of timber, although it should be noted that

significant decomposition of lignin took place in all the soils studied: and subsequently, they found (Mangenot, personal communication) that two soils being studied by Toutain (1972) were quite distinct in this respect. Both soils had developed under Beech in the same forest near Nancy (Meurthe et Moselle) but one ('Bezange') was a podzol with Moder and the other ('Sainte-Marie') was an acid brown earth with Mull. The podzol had most samples showing very low lignin decomposition in relation to total activity whereas the brown earth was one of the most active lignin decomposing soils examined, with all samples showing 'white rot' activity. Samples of the A1 horizons of these two soils were brought from France to be used with the A1 horizons of the soils from Hermitage Wood and the Experimental Gardens in ferulic acid decomposition experiments.

The metabolic activity of litter and the underlying mineral horizons had been studied on a comparative basis in previous work, using the endogenous respiration of samples (Parkinson et al., 1968; Jorgensen & Wells, 1973). Kunc (1971 a,b, 1974) has measured the respiratory activity of soil in the oxidation of vanillin and coumarin and has compared this activity to the respiration of the substrates by pure cultures of bacteria isolated from the soils. In the experiments reported below it was possible to compare the decomposition of ferulic acid by populations of microorganisms on Pine needles and in soil samples with the activity of pure cultures of fungi; Pine needles or soil samples were substituted

significant decomposition of lignin took place in all the soils studied: and subsequently, they found (Mangenot, personal communication) that two soils being studied by Toutain (1972) were quite distinct in this respect. Both soils had developed under Beech in the same forest near Nancy (Meurthe et Moselle) but one ('Bezange') was a podzol with Moder and the other ('Sainte-Marie') was an acid brown earth with Mull. The podzol had most samples showing very low lignin decomposition in relation to total activity whereas the brown earth was one of the most active lignin decomposing soils examined, with all samples showing 'white rot' activity. Samples of the A1 horizons of these two soils were brought from France to be used with the A1 horizons of the soils from Hermitage Wood and the Experimental Gardens in ferulic acid decomposition experiments.

The metabolic activity of litter and the underlying mineral horizons had been studied on a comparative basis in previous work, using the endogenous respiration of samples (Parkinson et al., 1968; Jorgensen & Wells, 1973). Kunc (1971 a,b, 1974) has measured the respiratory activity of soil in the oxidation of vanillin and coumarin and has compared this activity to the respiration of the substrates by pure cultures of bacteria isolated from the soils. In the experiments reported below it was possible to compare the decomposition of ferulic acid by populations of microorganisms on Pine needles and in soil samples with the activity of pure cultures of fungi; Pine needles or soil samples were substituted

for spore suspension inoculum in the standard culture solution containing initially 100 mg l^{-1} ferulic acid in Czapek salts.

Pine needles used in these studies belonged to samples collected in the Blackwood in September and December 1973 and to samples removed from soil at the end of the second burial experiment in December. Needles were taken from among the three replicate sub-samples after the washing or surface sterilisation appropriate to each sample. Before the September samples were examined trial experiments were carried out. F1 needles were collected from a site at Pendreich, near the University, and surface sterilised to determine the most suitable number of needles for inoculation of each flask. The decomposition of ferulic acid over a five day period was found to increase fairly regularly with the number of needles used as inoculum, but there was increasing interference in the ultra-violet spectra of the culture filtrates from 'tannins' extracted from the needles, which might have also interfered with the decomposition of the ferulic acid. Five needles were used as the standard inoculum because they did not produce significant amounts of 'tannins' while providing a representative sample of the mycoflora. To be able to compare the ferulic acid decomposing activities of the different samples the concentration of ferulic acid remaining would have to be in a suitable range for spectrophotometry and it was found that pre-incubation of these F1 surface sterilised needles for three days in damp chambers prior

to inoculation resulted in the decomposition of 50-70 mg l^{-1} ferulic acid in five days. F2 needle fragments were available for incubation with ferulic acid in the September sample. The needle fragments tend to occur in small clusters, and five clusters of fragments was arbitrarily chosen as the standard inoculum of F1 needles.

Only air-dried samples of the two French soils were available and these samples were employed with air-dried material from the Stirling sites in the main series of experiments involving washed soils and direct incubation of the air-dried soils. Fresh soil samples from the Stirling sites were also used. To minimise possible aerial contamination of air-dried samples soil crumbs or granules were used and the main intention behind the use of a light soil washing was to remove the spore potential from this contamination. The intact crumbs (granules in the case of the French soils) were obtained by sieving soil and taking the 10-20 mesh fraction after the soils had been sieved to 2 mm in the standard fashion to remove stones. It was found that the initial pH of the soil suspensions was markedly affected by the weight of the soil inocula, even in the range of 0.1 to 1.5 g of soil per 100 cm^3 of culture solution, which gave rise to appreciable differences in the pH of suspensions containing the various soils (Table 3.2). To minimise the differences in pH 0.1 g of each soil was used as inoculum, this being the minimum amount that could be weighed accurately, comprising about 10 crumbs or granules.

TABLE 3.2. Initial pH of Ferulic acid solutions inoculated with soil

Weight of soil inoculum in 100 cm^3 Ferulic acid solution	pH of soil suspension after equilibration			
	Hermitage Wood	Experimental Gardens	Bezange	Sainte-Marie
0.1	4.2	4.7	4.0	4.1
0.2	4.1	4.9	-	-
0.5	4.0	5.2	-	-
1.0	3.9	5.4	-	-
1.5	3.9	5.5	-	-

The air-dried crumbs were weighed aseptically into Petri dishes and transferred directly to the flasks of culture solution. Light washing of the samples was carried out according to the method of Gams & Domsch (1967) in a stream of water. A description of the Apparatus (modified) and the details of its use will be found by reference to the Materials and Methods section. The samples of soil are introduced into the washing chamber and the crumbs are retained there by the two steel meshes (0.15 mm holes) during the washing by 2 l of sterile water. The soil is then dried in a stream of sterile air and weighed and inoculated as before. Fresh soil was collected from Hermitage Wood and the Experimental Gardens and kept overnight at +4^{o}C while samples were air-dried for the determination of moisture content. The fresh material could then be weighed into samples to give the equivalent of 0.1 g air-dry inoculum. In each experiment six flasks were inoculated with each soil as appropriate.

In addition, washed soil particles (200 per soil) were plated out on Potato Dextrose Agar (Oxoid) for the isolation of fungi and bacteria.

After incubation at 25^{o}C on an orbital incubator the suspensions containing Pine needles or soil were filtered through membrane filters and were examined by ultra-violet spectrophotometry. The pH of the soil suspensions and of the filtrates from these were measured after incubation.

The results of the incubations of Pine needles

collected direct from the Blackwood in ferulic acid solution are shown in Table 3.3 which gives the concentration of ferulic acid remaining after five days incubation in each flask. The absorbances of the culture filtrates are given in Appendix 4. Needles incubated after washing have decomposed more ferulic acid than those incubated after surface sterilisation and this was associated with the more profuse growth of mycelium from the washed needles and smaller particles than from surface sterilised material. There were very few colonies in the suspensions not associated with particles of litter, suggesting that spores released into the culture solution were not an important source of inoculum for the decomposition of the ferulic acid. Penicillium spp. were more abundant among isolations from washed needles than from surface sterilised needles (Table 2.2) and it was shown in Chapter One that soil species of this genus can decompose ferulic acid very readily in contrast to the other fungi dominating isolations from washed needles. However the isolation of Penicillium spp. from washed needles was rather sporadic and the greater activity of washed needles is probably not due to these species principally. Instead, it is suggested that the increased decomposing activity and growth of mycelium in the cultures of washed needles is mainly from other ferulic acid decomposing species, e.g. Dothichiza pityophila and Desmazierella acicola, the growth of which is probably reduced in ferulic acid cultures in which surface sterilised needles are

TABLE 3.3. Decomposition of Ferulic acid by natural populations of fungi on Pine needles collected from Blackwood.

Inoculum 5 needles Initial concentration of Ferulic acid 100 mgL^{-1}

SAMPLE DATE	NEEDLE LAYER AND TREATMENT	OVERALL COLONISATION INDEX	CONCENTRATION OF FERULATE REMAINING MgL^{-1}			
			I	II	III	MEAN
SEPTEMBER	F1 S.STERILISED	76.7	71.5	74.5	28.0	58 ± 15
	F1 WASHED	171.2	9.0	12.0	8.0	10 ± 1
	F2 S.STERILISED	6.1	80.0	71.0	89.0	80 ± 5
	F2 WASHED	104.0	5.0	4.5	5.0	5 ± 0.2
DECEMBER	L S. STERILISED	113.1	30.0	63.5	63.0	52 ± 11
	L WASHED	151.8	14.0	7.0	5.0	9 ± 3
	F1 S. STERILISED	68.2	54.0	48.0	50.0	51 ± 2
	F1 WASHED	174.1	17.0	12.0	12.0	14 ± 3

5 DAYS INCUBATION

incubated. However, these fungi dominate the agar plate isolations from surface sterilised needles, in contrast to the situation with washed needles where their growth on agar is suppressed by other, rapidly growing fungi, rather than by the washing. In other words, the ferulic acid decomposing activity of washed Pine needles, as measured over five days, may reflect the activity of a population of fungi not fully represented in the agar isolations from these needles; whereas it is suggested that there is some correspondence between the decomposing activity and the isolation data in the case of surface sterilised needles.

It is also apparent that there is considerable uniformity within the replicate samples considering that no attempt was made to standardise the size of individual L and F1 needles comprising the samples and with F2 needles the number of needle fragments was determined by a rough approximation. In addition the mean absorbances with the two series of surface sterilised F1 needles are very similar. From this it is concluded that each needle (or each group of five) may be considered as a unit of inoculum independently of the size of the needle(s) but that the activity of this inoculum is dependent upon the pre-treatment of the needles. Furthermore, that this activity is determined by the litter layer from which the Pine needles are taken, i.e. by the nature of the fungi characteristic of the layers, can be seen by comparing the decomposition of ferulic acid by surface sterilised L, F1 and F2 needles in relation to the 'Overall Colonisation Index'

(Table 3.3). The 'Overall Colonisation Index' does not represent the total inoculum available for decomposition in the case of washed needles, from what has been said above; but it probably is a good guide to inoculum potential for surface sterilised needles, because there is no evidence for antagonisms in isolations from these needles or that some internal colonisers are unable to grow over the agar. F2 needles (surface sterilised) decomposed less ferulic acid than F1 or L needles, which behaved very similarly, as might be expected from the paucity of isolates from the F2 needles but the activity of the different needles is not proportional to differences in the inoculum potential given by the 'Overall Colonisation Index'.

The concentrations of ferulic acid remaining in the cultures of Pine needles removed from the soils after seven days incubation are shown in Table 3.4 with the Absorbances given in Appendix 5. (Inspection of cultures set aside for monitoring the course of the decomposition showed that there had been little decomposition of the substrate after five days). It will be recalled that all needles buried in soil were surface sterilised after removal. The quite good uniformity between replicates is to be noted again and the difference between the concentration of ferulic acid remaining after incubation of autoclaved needles from each soil is statistically significant (at $p=0.01$) as is the difference between incubations of non-sterilised and autoclaved needles buried in the Gardens (at $p = 0.05$).

TABLE 3.4 Decomposition of Ferulic acid by natural populations of Fungi on Pine needles buried in soil

Inoculum 5 needles

SITE OF BURIAL AND TREATMENT BEFORE BURIAL a)	'OVERALL COLONISATION INDEX'	CONCENTRATION OF FERULATE REMAINING Mg L^{-1} I	II	III	MEAN
Hermitage Wood					
Non-autoclaved	69.6	46.0	56.5	69.5	57 ± 7
Autoclaved	113.1	69.0	82.0	83.0	78 ± 5
Experimental Gardens					
Non-autoclaved	128.1	67.0	78.0	63.0	69 ± 4
Autoclaved	150.0	43.0	17.0	8.5	23 ± 13

7 DAYS INCUBATION

a) All needles surface sterilised before incubation

* Significant $p < 0.05$
** Significant $p < 0.01$

Whereas there is no significant difference between the activities of the non-sterilised and autoclaved needles buried in Hermitage Wood, the autoclaved needles buried in the Gardens decomposed more ferulic acid than the corresponding non-sterilised needles. If the 'Overall Colonisation Indices' given in Table 3.4 are taken into account, so that the extent of decomposition of ferulic acid is considered relative to the amount of inoculum, then for needles buried in the Gardens the activity of the inoculum on autoclaved needles is greater than on non-sterilised needles, whereas the opposite is true for needles buried in Hermitage Wood. The activity of the needles therefore reflects a difference in the pattern of colonisation of needles buried in the two soils which was apparent from the fungal isolation data, whether or not these isolations revealed the whole picture of colonisation. Furthermore, it can be tentatively concluded that the differences in the activity of the needles is reflected in the isolations of fungi known to decompose ferulic acid readily.

The results of the incubation of air-dried soil samples of the soils from the Hermitage Wood and Experimental Gardens sites are given in Figures 3.1 and 3.2 respectively, which show the UV-spectra of the culture filtrates after five days incubation. Also shown are the spectra of cultures of 'blank' medium without ferulic acid incubated with 0.1 g of soil. The pH of the suspensions and filtrates after incubation are given in Appendix 6, as are the

Fig. 3.1 Incubation of air-dried soil samples from Hermitage Wood with Ferulic acid. UV Spectra of undiluted filtrates after 5 days incubation with 0.1 g soil.

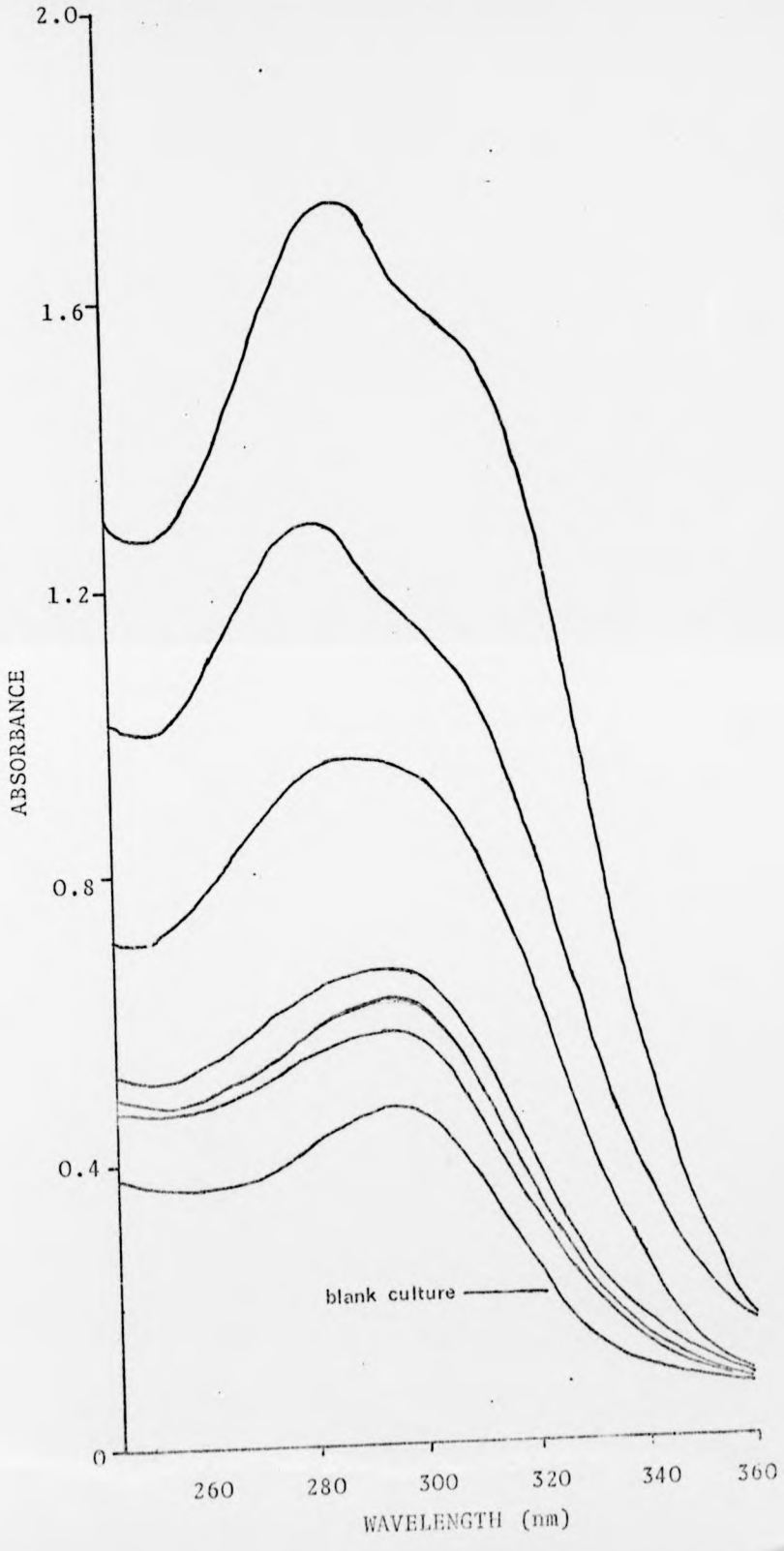
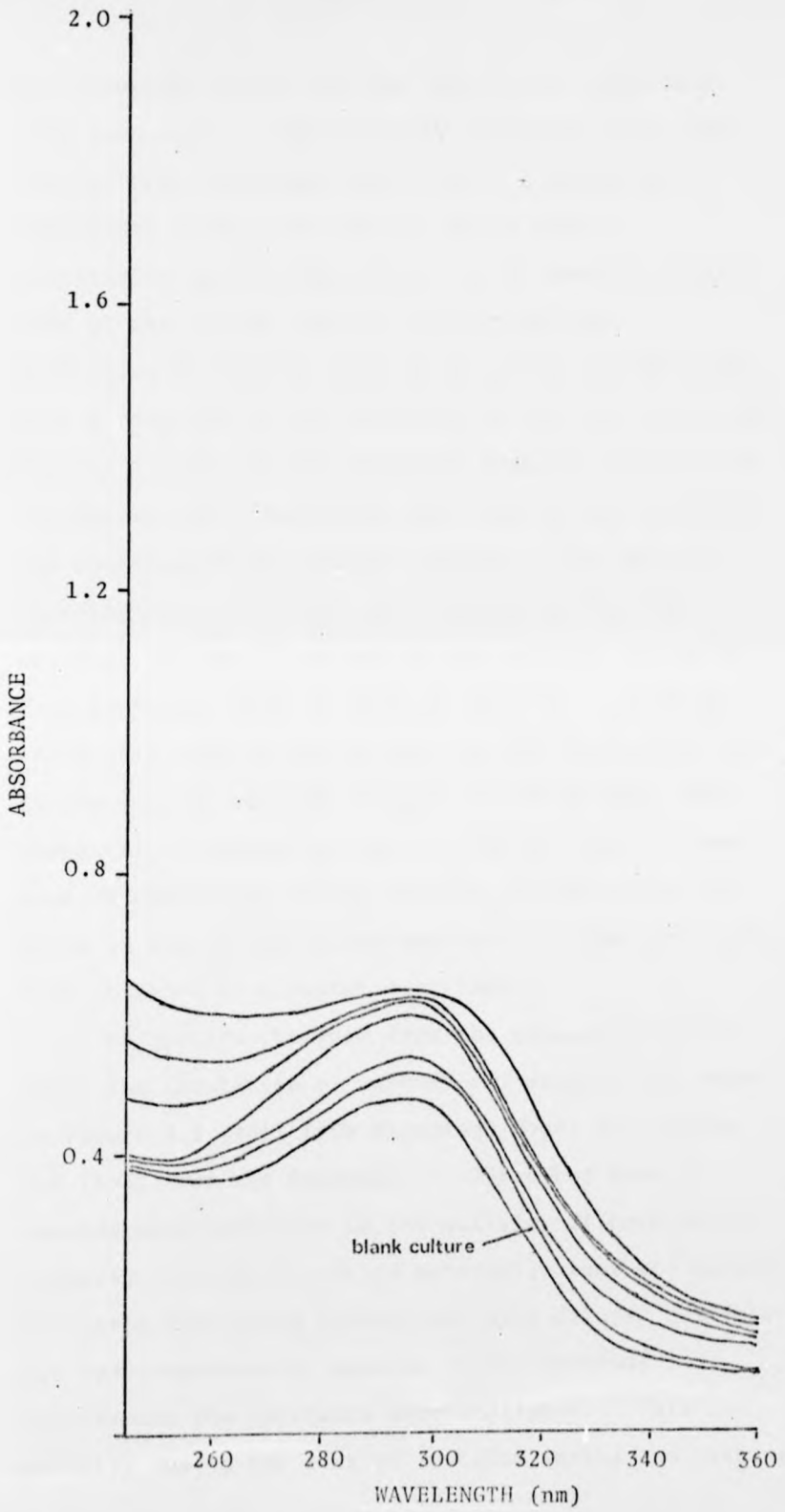

2.0
1.6
1.2
0.8
0.4
0
ABSORBANCE
blank culture
260
280
300
320
340
360
WAVELENGTH (nm)

Fig. 3.2 Incubation of air-dried soil samples from Experimental Gardens with Ferulic acid. UV Spectra of undiluted filtrates after 5 days incubation with 0.1g soil.

ABSORBANCE


2.0
1.6
1.2
0.8
0.4
ABSORBANCE
blank culture
260
280
300
320
340
360
WAVELENGTH (nm)

corresponding values for the subsequent experiments with each soil. The cultures incubated with soil samples from Hermitage Wood present a gradation in Absorbance between 250 nm and 340 nm with a progressive qualitative change in the spectra towards that of the 'blank' culture indicating that metabolism of ferulic acid has occurred, rather than just absorption of the substrate by the soil particles. All the spectra of the filtrates from the cultures of the Gardens soil incubated with ferulic acid resemble the spectrum of the 'blank' culture. The maximum concentration of ferulic acid remaining from the original 100 mg l^{-1} in any of the cultures of the soil from Hermitage Wood is about 21 mg l^{-1}. Although these soil samples can be said to have decomposed most of the ferulic acid the samples of the Gardens soil consistently removed all of it, and so there is some sort of difference in the activity of the soils, at least as regards air-dried material. Similar results were obtained in a repeat experiment.

The spectra obtained from the culture filtrates after the incubation of washed soil samples are shown in Figure 3.3 (soil from Hermitage Wood) and Figure 3.4 (soil from the Gardens). There has been a considerable reduction in the activity of both soils, compared with the air-dried material, since the culture filtrates from these incubations were diluted fourfold for spectrophotometry whereas in the previous experiments the filtrates were undiluted. This is probably due to the loss of inoculum during the washing

Fig. 3.3 Incubation of washed soil samples from Hermitage Wood with Ferulic acid. UV Spectra of filtrates (diluted fourfold) after 5 days incubation with 0.1 g soil.

ABSORBANCE

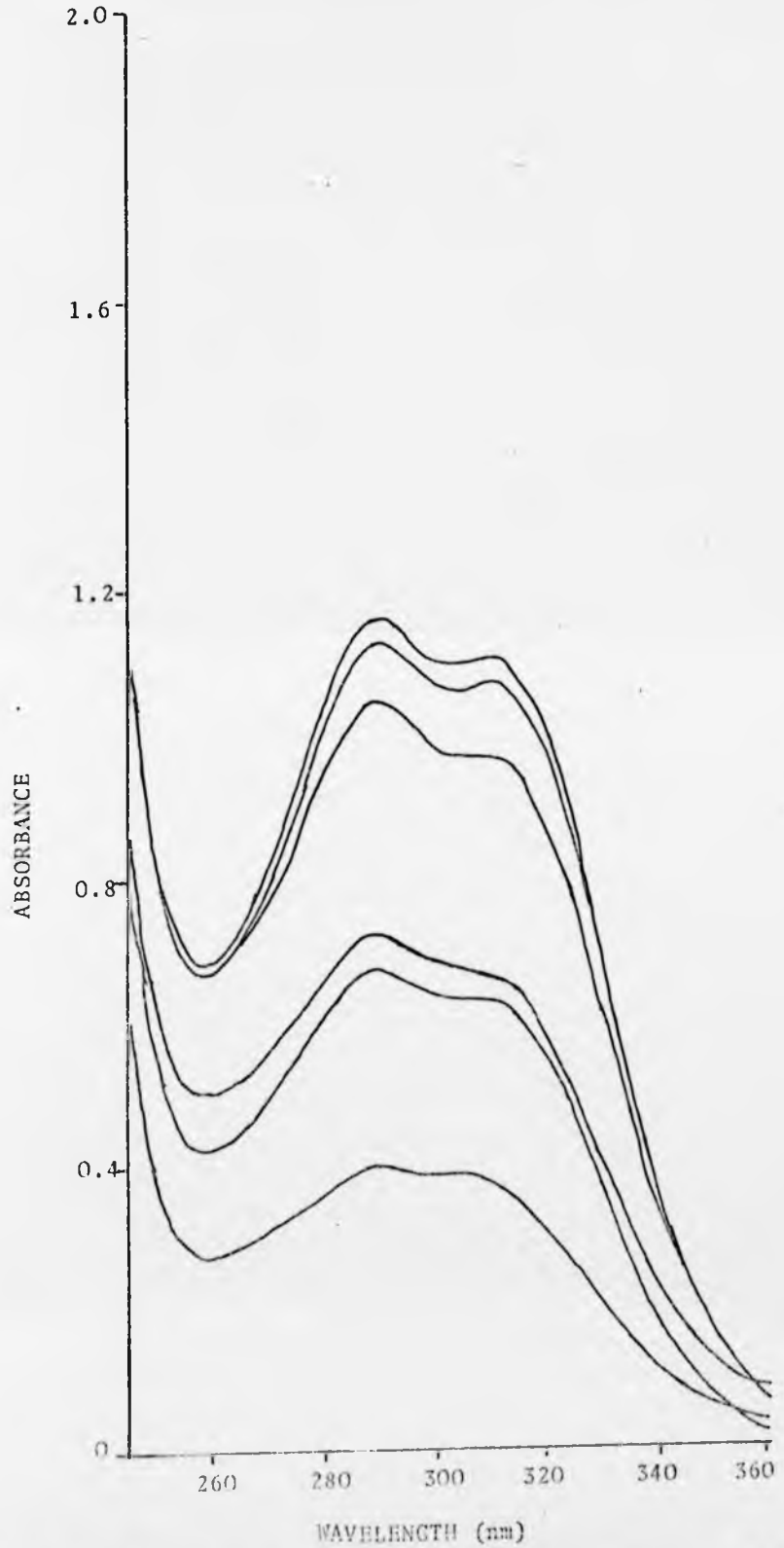
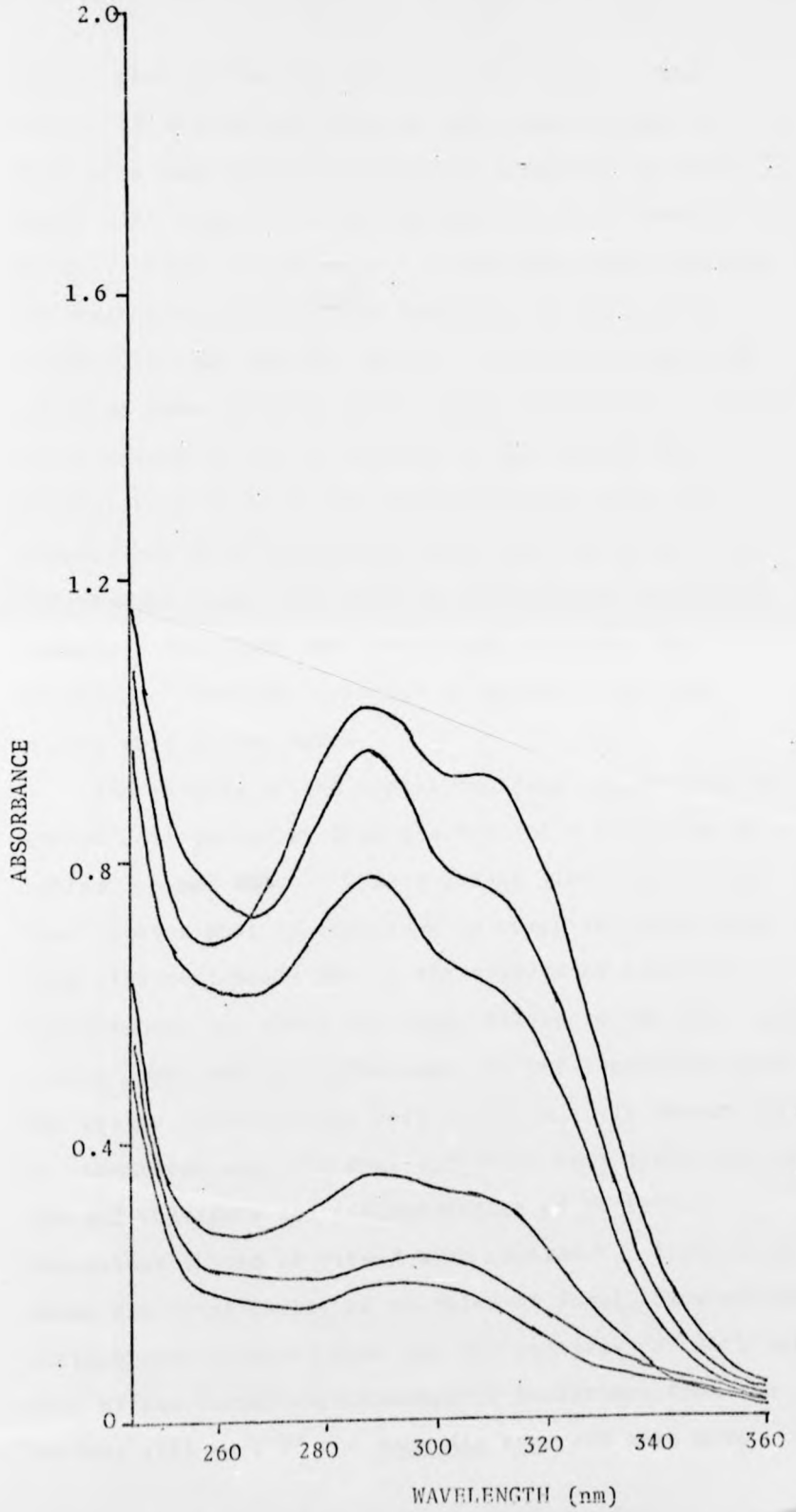

2.0
1.6
1.2
0.8
0.4
0
ABSORBANCE
260
280
300
320
340
360
WAVELENGTH (nm)

Fig. 3.4 Incubation of washed soil samples from Experimental Gardens with Ferulic acid. UV Spectra of filtrates (diluted fourfold) after 5 days incubation with 0.1 g soil.

erimental
filtrates
with



out of part of the clay and silt fractions. The effect of washing was greater than expected and it indicates that there is a limited potential on these small soil samples for the decomposition of ferulic acid. There is now only a slight difference between the mean concentrations of ferulic acid left after incubation with the two soils. Dilution plates were prepared from the suspensions after incubation. There was mycelium in all the flasks by the end of the incubation period and the dilution plates from the suspensions from the Gardens soil were overgrown with filamentous fungi, but from the suspensions containing Hermitage Wood soil very few fungal colonies were obtained. Instead, colonies of bacteria and pink yeasts were in abundance.

The results of the isolations from the plating out of washed soil particles from the two soils are given in Tables 3.5 and . Gams & Domsch (1967) point out that caution must be exercised in comparing isolations from different soils due to the effects of textural differences, but these two soils belong to the same class (silty loam) and the differences in the isolations from the washed particles are very striking. It should also be remembered that the soil particles were plated out on PDA and therefore the interpretation of bacterial isolations should be viewed with caution. Table 3.5a shows the total number of colonies of fungi, bacteria and actinomyctes isolated from the 200 particles of each soil. Most of the bacterial/actinomycete isolations from the Gardens soil were of the Nocardia type and were more

TABLE 3.5. Isolation of microorganisms from washed particles of soil from Hermitage Wood and Experimental Gardens

a) Overall colonisation by bacteria, actinomycetes and fungi.

	Total number of particles colonised	Number of particles colonised by: BACTERIA ALONE	BACTERIA (TOTAL)+ ACTINOMYCETES *	FUNGI	percentage colonisation by FUNGI	'Overall colonisation Index' FUNGI
Hermitage Wood	200	17	50	187	93.5	96.3
Experimental Gardens	200	-	166	78	39.0	151.0

* Actinomycete colonies were difficult to total separately as mixed colonies tended to occur

b) Species of fungi isolated

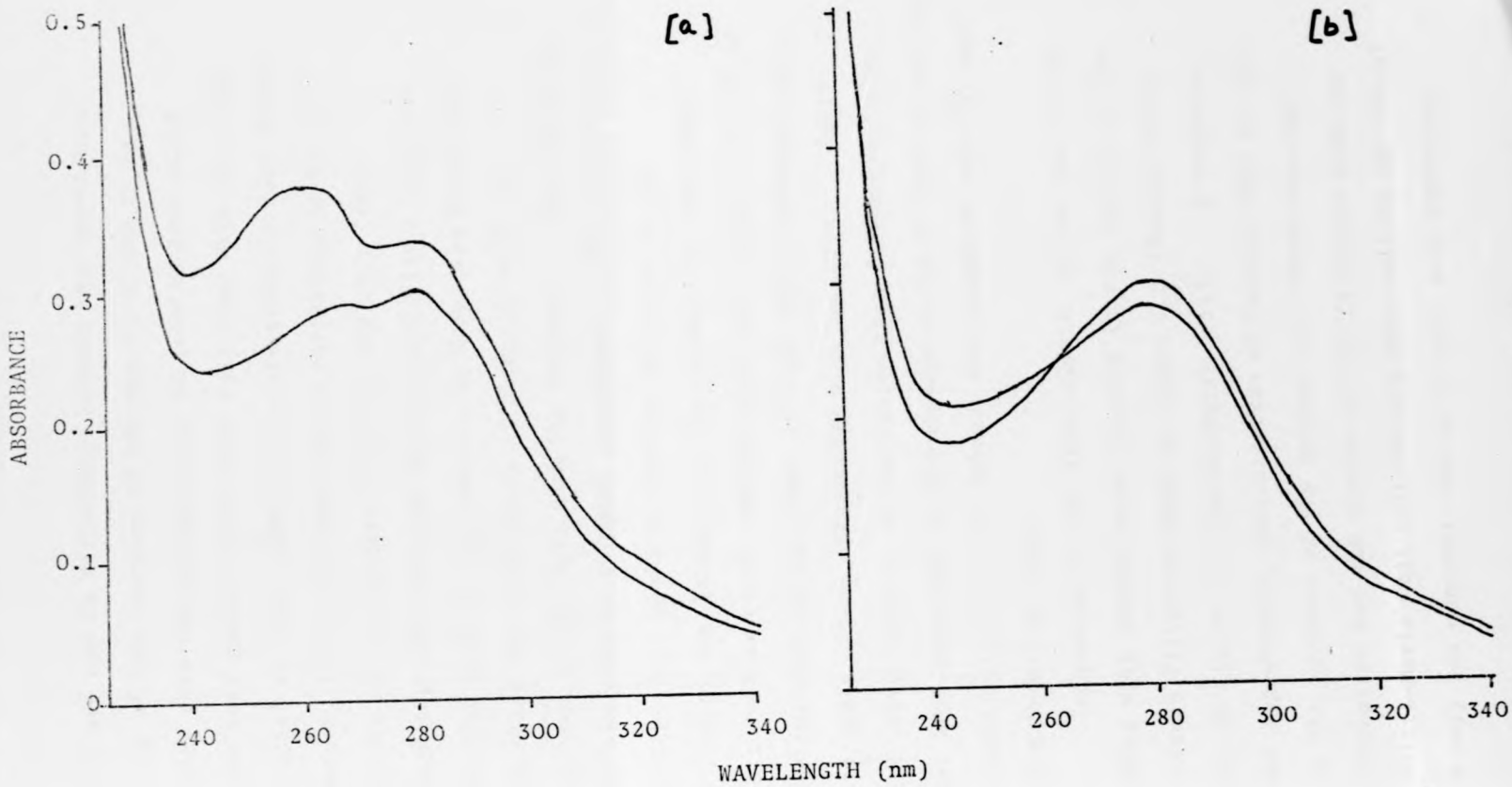
	Penicillium sp	Trichoderma viride	Mucoraccae **	Mortierella ramanniana	Fusarium oxysporum	Gilmaniella humicola	Coniella sp	Phoma eupyrena
Hermitage Wood	13	65	70	68	-	-	-	-
Experimental Gardens	-	4	30	-	3	8	2	31

** Mucor spp + Rhizopus

numerous than corresponding isolations from the other soil (mainly Eubacteria) but fungal isolations were much more numerous from this soil than from the Gardens soil. As can be seen from Table 3.5 the composition of the fungal populations is very different. The isolations from the soil from Hermitage Wood are dominated by Trichoderma viride and the Mucorales which appeared very rapidly on the plates whereas slower growing Dematiaceous forms are characteristic of the limited number of fungal colonies growing from the washed particles of the Gardens soil. The isolates of Gilmaniella humicola, Coniella sp., Fusarium oxysporum and the numerically important Phoma eupyrena were found to decompose ferulic acid readily as reported in Table 2.6 in contrast to the general behaviour of isolates of Mortierella ramanniana Mucor spp. and T. viride and although the results of the isolations from the suspensions by the dilution plate method are not directly comparable it is very interesting that the relative predominance of fungi and bacteria in the two soils should appear to be reversed. This may indicate that in the cultures of the washed Gardens soil it is the growth of filamentous fungi which is mainly responsible for the decomposition of ferulic acid but that in the case of the soil from Hermitage Wood bacteria and perhaps yeasts are more important.

The results of the incubation of fresh soil with ferulic acid are shown in the spectra of the culture filtrates given in Figure 3.5 a (Hermitage Wood) and Figure 3.5b (Experimental Gardens). Quantitatively

Fig. 3.5. Incubation of fresh soil samples from a) Hermitage Wood and b) Experimental Gardens with Ferulic acid. UV Spectra of undiluted filtrates after 5 days incubation.


[a]
[b]
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
240
260
280
300
320
340
WAVELENGTH (nm)

there are now no differences between the Absorbances of any of the cultures of the two soils and it is likely that the decomposition of ferulic acid would start more rapidly with fresh soil than with air dried material so that these results are probably 'off scale'. However, there is a qualitative difference in the UV spectra of the culture filtrates and Thin Layer Chromatography detected ferulic acid plus a trace of vanillic acid in the case of the Hermitage Woods soil but there was no evidence of phenolic acids in cultures of the soil from the Gardens. This difference was observed in a repeat experiment. The particles of Gardens soil produced abundant mycelium in the course of the incubation, the suspensions remaining more or less clear, whereas there was little mycelium in the cultures of the other soil and the suspensions were all quite cloudy, presumably with bacteria. Again therefore there is an indication that filamentous fungi are more important in the decomposition of ferulic acid in liquid culture by the Gardens soil compared with the soil from Hermitage Wood.

Incubation of air dried samples of the two French soils with ferulic acid resulted in the spectra of the culture filtrates shown in Figure 3.6 (Bezange soil) and in Figure 3.7 (Sainte-Marie soil). A variable, but appreciable, concentration of ferulic acid is left in the cultures of the Bezange soil after five days incubation but the spectra of the filtrates from the Sainte-Marie soil cultures are very uniform and reveal a very low concentration of ferulic acid remaining. It

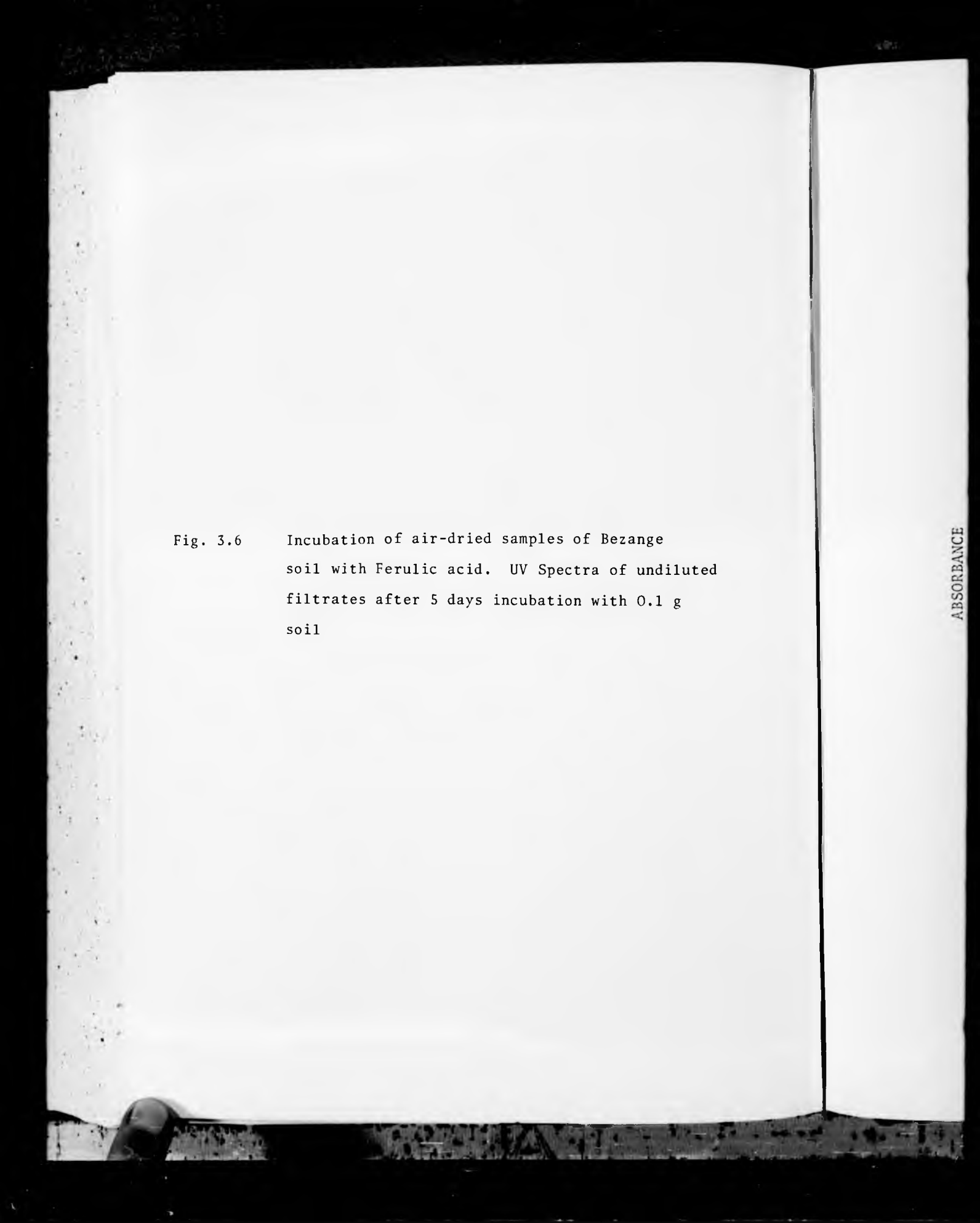
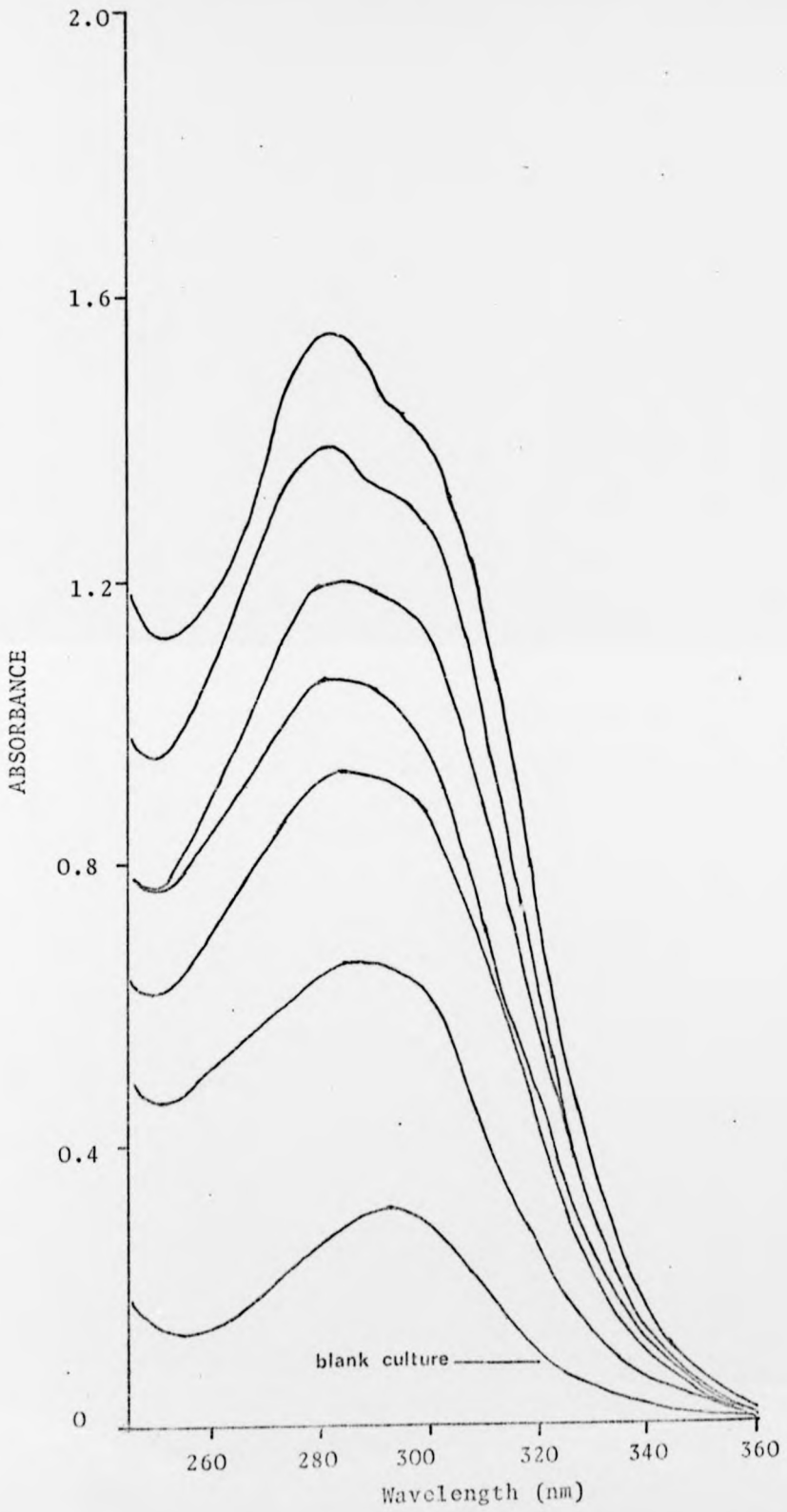


Fig. 3.6 Incubation of air-dried samples of Bezange soil with Ferulic acid. UV Spectra of undiluted filtrates after 5 days incubation with 0.1 g soil

luted

g


2.0
1.6
1.2
0.8
0.4
0
ABSORBANCE
blank culture
260
280
300
320
340
360
Wavelength (nm)

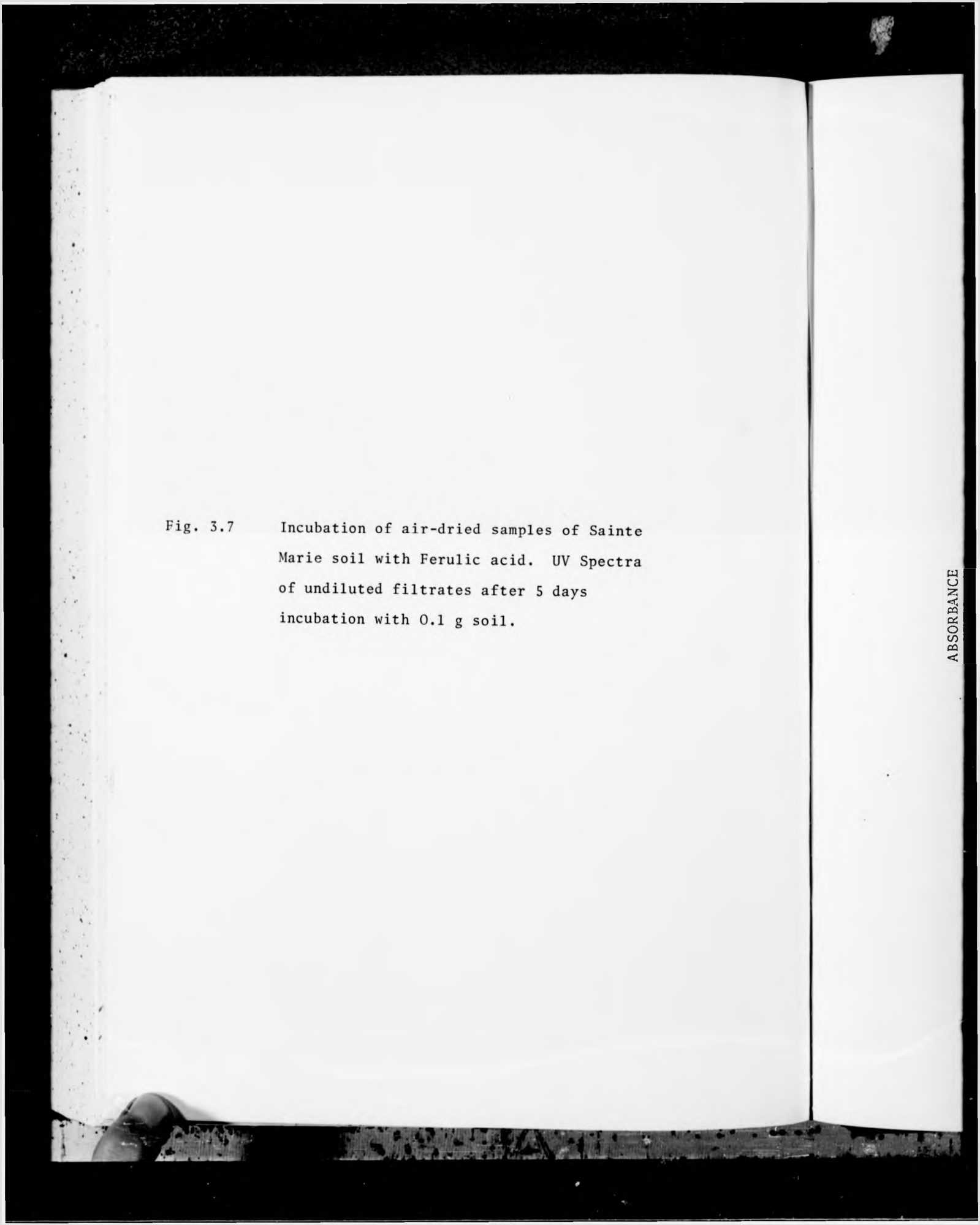
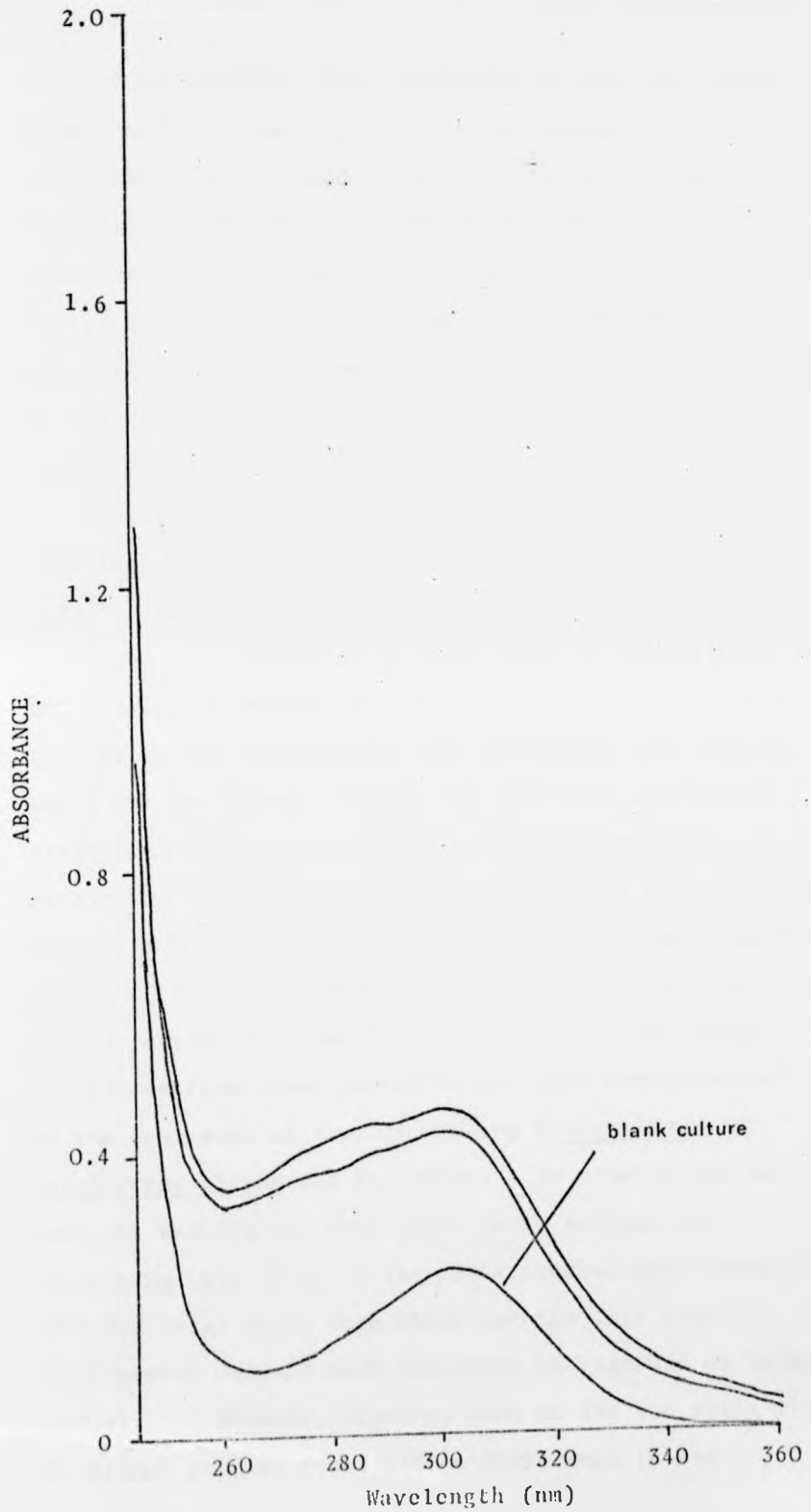


Fig. 3.7 Incubation of air-dried samples of Sainte Marie soil with Ferulic acid. UV Spectra of undiluted filtrates after 5 days incubation with 0.1 g soil.


2.0
1.6
1.2
0.8
0.4
0
ABSORBANCE
blank culture
260
280
300
320
340
360
Wavelength (nm)

is very interesting that a differential pattern should appear which is very similar to the results with the two soils from Stirling. The results of a repeat experiment indicated that this difference was consistent in that there was no ferulic acid left in any of cultures of the Ste.-Marie soil whereas there was a significant concentration of ferulic acid remaining in the cultures of the Bezange soil, though lower and more uniform than previously.

After washing of the soils there was no ferulic acid left in any of the cultures of either soil (Fig. 3.8). Both soils were sandy in texture having developed over sandstone so that there is little silt and clay to be washed out and the final result is that both soils are more active than previously for reasons which are not clear. Table 3.6 shows the results of isolations of fungi and bacteria from washed soil particles. Very few colonies of bacteria were obtained from either soil but this probably reflects the greater number of fungal colonies growing on the P.D.A. than in the isolations from the Stirling soils. The fungal isolations from these two soils are both characterised by the dominance of rapidly growing Penicillium spp. Trichoderma viride and Mucorales. In view of the fact that the washing was very light it is perhaps not surprising that three of the soils studied here produced very few fungi apart from these and the soil from the Experimental Gardens must therefore be regarded as being unusual. However, previous work on the two soils from the French studies found little difference in the

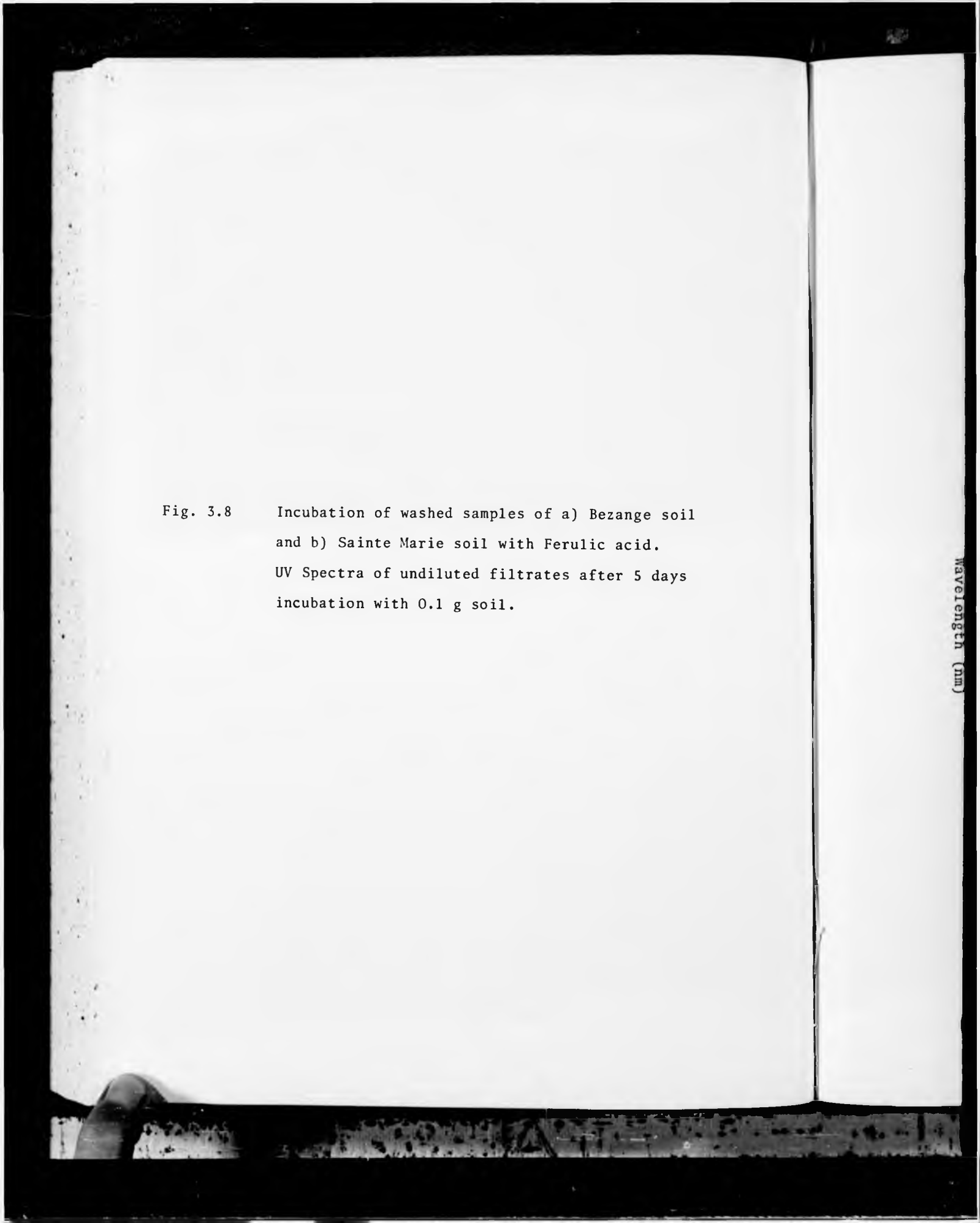


Fig. 3.8 Incubation of washed samples of a) Bezange soil and b) Sainte Marie soil with Ferulic acid. UV Spectra of undiluted filtrates after 5 days incubation with 0.1 g soil.

ABSORBANCE
0.5
0.4
0.3
0.2
0.1
0
240
260
280
300
320
340
Wavelength (nm)
[a]
[b]

oil

ys

TABLE 3.6 Isolation of microorganisms from washed soil particles

Bezange and Ste-Marie soils

	Total number of particles colonised	Number of particles colonised by BACTERIA	% colonisation by FUNGI	'Overall colonisation index' FUNGI	Number of colonies of fungal species				
					PENIC.	TRICHO.	MUC.	MORT	DEM.*
Bezange	200	2	99.0	130.7	42	71	24	127	1
Ste-Marie	200	6	100.0	151.0	77	66	46	101	1

* PENIC. - Penicillium spp
TRICHO. - Trichoderma viride
MUC. - Mucoraceae
MORT. - Mortierella ramanniana
DEM. - Dematiaceous forms

species composition of the fungal flora (Kiffer, personal communication).

These experiments using soil inocula for the decomposition of ferulic acid can at best be regarded as providing a glimpse into some aspects of the metabolic activity of the populations of soil microorganisms. For one thing the use of air-dried material is not very satisfactory and the experimental conditions were not adjusted to take account of the higher activity of the Fresh soils. However, in the case of the Stirling soils, where both sorts of material were available, there was an indication that the behaviour of air-dried and fresh soil did correspond, in that there was complete decomposition of ferulic acid by the soil from the Gardens but incomplete decomposition by the Hermitage Wood soil, with the air-dried samples exaggerating the difference. And the air-dried samples of the soils of Bezange and Ste-Marie showed a similar difference.

The results of the incubation of the washed soil particles in Ferulic acid and the plating out of these particles are more difficult to interpret than in the case of the air-dried samples. The activity of the Stirling soils was affected more than expected by washing and so slight variation in the washing conditions from one soil to another could result in artificially produced differences (or similarities), if not perhaps affecting the results of the isolations very much. And three of the soils produced few fungi apart from isolates of rapidly growing species of Penicillium, Mucorales and

Trichoderma viride as is characteristic of washed Pine needles. And so the same conclusion could be drawn about these isolations as from those from the needles, that the species isolated do not represent the available potential for the decomposition of ferulic acid. However, in the case of the Stirling soils it did appear that it was not the filamentous fungi that were mainly responsible for the decomposition of the phenolic acid by the Hermitage Wood soil samples in contrast to the cultures of the Gardens soil. And so, the relative activities of the fungi from the two soils did correspond to the difference in the isolations from washed particles according to the ferulic acid decomposing ability of the fungi concerned.

Although soil washing leads to difficulties in the interpretation of differences in isolation data for soils of varying texture, it would seem that washing could be of use in the study of the localisation of microorganisms and the overall activity of soil samples on the different size fractions of the mineral matter. This would complement previous studies on the differential distribution of fungi on particles of organic matter and mineral matter within the mineral horizons (for example, Balasooriya & Parkinson, 1967 and Gams & Domsch, 1969).

The Bezange and Ste-Marie soils attracted attention because they were undergoing a considerable divergence in development in spite of forming on the same parent material and under almost identical conditions of vegetation cover, climate and topography (Toutain, 1972).

Differences were revealed in the characteristics of lignin decomposition by the (laboratory) method of Mangenot & Kiffer (1972) which were associated with the slower decomposition of litter in the moder (Bezange) than in the mull (Ste-Marie). However, Toutain (1972) was able to identify differences more precisely. He found that there was very rapid decomposition of lignin and complete and rapid humification of the Beech leaf litter in the brown earth (Ste-Marie) which contrasted with the persistence of intermediate products of humification of lignin, such as the Fulvic acid fraction of the soil organic matter, in the Bezange podzol. Furthermore, the process of polymerisation of water soluble substances taking place in winter, characterised by Vedy & Bruckert (1970), was different in the two soils; and alkaline hydrolysis yielded much more p-hydroxybenzoic acid from the polymers of the Bezange soil than from the Ste-Marie soil.

It is therefore very significant that the degree of completeness of lignin decomposition and of phenolic acid metabolism in these two soils should be paralleled in the pattern of ferulic acid decomposition by the air-dried samples of these soils.

These latter experiments cannot be regarded as conclusive but similar differences were noticed in the other set of soils using both air-dried and fresh material.

With regard to these two soils from Stirling the activity of the fungi in samples incubated with ferulic acid can be said to correspond with the activity of

fungal inoculum on the Pine needles buried in each soil and ultimately with the differences in the fungal isolation data from these needles. Such differences in the microbial populations obviously reflect the fact that one soil is an acid woodland soil and the other an almost neutral field soil, but it is interesting to speculate on whether there are differences in lignin decomposition and humification analogous to the situation in the two French soils.

GENERAL DISCUSSION

The decomposition of lignin must be regarded as one of the major factors influencing the colonisation of plant remains by microorganisms. It is the material most resistant to chemical and enzymic attack found in higher plants, and it is widely held that during lignin decomposition phenolic compounds are released and these are potentially biologically active. Originally certain phenolics were used in microbiological studies on lignin decomposition because they are structurally related to lignin but eventually it became clear that some of these substances do occur free in soil although their place in the overall decomposition of lignin is uncertain. In previous work on the metabolism of lignin-related phenolic compounds by soil fungi (Henderson & Farmer, 1955) and on the fungistatic implications of the release of phenolics in soil (Lingappa & Lockwood, 1962) important general features were established concerning the biological significance of these compounds. However, there has been little regard for comparative aspects of physiology in relation to the detailed ecology of fungi involved in the decomposition of plant remains, although abundant information exists on the distribution of species on different types of litter and in different soil types. This is particularly true of the primary saprophytes of leaf litter; while many of these are specific to the litter of certain taxonomic groups e.g. Dreschlera spp. on Gramineae, several are very widespread and almost ubiquitous, e.g. Cladosporium herbarum and Aureobasidium pullulans and others, e.g. Alternaria tenuis, are more restricted in distribution but do not show any preference for specific litter types.

A priority for further studies on ecological aspects of lignin decomposition was, therefore, the investigation of the behaviour of a range of microfungi from different leaf litters and from soil towards some phenolic acids of special interest as lignin model compounds.

Particular attention was directed to the mycoflora of the needle litter of Scots Pine because it consists of a range of primary and secondary saprophytes and soil fungi suitable for comparative studies in pure culture. Also, previous work has shown that many of the widespread saprophytes, common on Angiosperm leaves, were lacking, and being a Mor forming system this litter has interesting features in lignin decomposition and phenolic acid production. An integrated study of this litter in relation to lignin decomposition was therefore merited, using a combination of microbiological and chemical techniques.

The primary need seemed to be to complement the valuable survey of the utilisation of lignin-related phenolic acids and aldehydes by soil fungi carried out by Henderson & Farmer (1955) with studies of the effects of phenolic acids on the spore germination and germ hyphal growth of microfungi.

Screening of six p-hydroxy-substituted benzoic and cinnamic acids carried out using the overall production of hyphae from spore inoculum indicated that there was a range of effects correlated with the ecological origins of the fungal species tested. The hyphal production of a group of primary saprophytes of Angiosperm leaf litter was inhibited relative to control by one or more phenolic acids while there was stimulation with a group of soil fungi and two species of saprophytes restricted to conifer needle litter.

The differential behaviour towards ferulic acid and sinapic acid of twelve species representing these groups of fungi was resolved into the effects on spore germination and hyphal growth. At concentrations inhibitory to the germination of some of the leaf saprophytes ferulic acid stimulated the final percentage germination of some of the soil fungi and a conifer needle saprophyte. These latter fungi were among those with intrinsically slower germination rates compared to the rest of the test fungi. With all but two isolates of the soil fungi and conifer needle saprophytes there was ultimately significant stimulation of the mean germ hyphal length during the experimental period. Experiments with an isolate of Trichoderma viride showed that ferulic acid could also stimulate the rate of germ hyphal growth but this was obscured by the delay in the onset of germination caused by this compound relative to the control (Final percentage germination was not affected). The germ hyphal growth of all but one of the Angiosperm litter saprophytes was significantly depressed by ferulic acid. Sinapic acid produced stimulations of the same nature as ferulic acid but was less inhibitory than the latter compound to the Angiosperm saprophytes, features which were indicated in the preliminary tests.

The value of the Theoretical Colonisation Index, defined by Mitchell (1973) as measure of the total potential growth, can be seen in determining the overall effects the phenolic acids have on microfungi. This becomes especially important when the two parameters determining this index are affected in opposite directions, as is the case with Mucor hiemalis 'B' for example, or when one parameter is more sensitive to the

The differential behaviour towards ferulic acid and sinapic acid of twelve species representing these groups of fungi was resolved into the effects on spore germination and hyphal growth. At concentrations inhibitory to the germination of some of the leaf saprophytes ferulic acid stimulated the final percentage germination of some of the soil fungi and a conifer needle saprophyte. These latter fungi were among those with intrinsically slower germination rates compared to the rest of the test fungi. With all but two isolates of the soil fungi and conifer needle saprophytes there was ultimately significant stimulation of the mean germ hyphal length during the experimental period. Experiments with an isolate of Trichoderma viride showed that ferulic acid could also stimulate the rate of germ hyphal growth but this was obscured by the delay in the onset of germination caused by this compound relative to the control (Final percentage germination was not affected). The germ hyphal growth of all but one of the Angiosperm litter saprophytes was significantly depressed by ferulic acid. Sinapic acid produced stimulations of the same nature as ferulic acid but was less inhibitory than the latter compound to the Angiosperm saprophytes, features which were indicated in the preliminary tests.

The value of the Theoretical Colonisation Index, defined by Mitchell (1973) as measure of the total potential growth, can be seen in determining the overall effects the phenolic acids have on microfungi. This becomes especially important when the two parameters determining this index are affected in opposite directions, as is the case with Mucor hiemalis 'B' for example, or when one parameter is more sensitive to the

active compound than the other, which applies to germ hyphal growth here. Ferulic acid depressed the Theoretical Colonisation Index of all the Angiosperm leaf saprophytes and only one isolate of a soil species while stimulating the values of this index for all the rest of the soil fungi and the conifer saprophyte in which percentage germination could be measured. This pattern parallels quite closely the results from the preliminary tests. With the number of replicates employed the method used for the latter was rather crude due to the variability within treatments but with further refinements it could be used to give a meaningful picture of the behaviour of fungi towards phenolic acids.

When the control values for the Theoretical Colonisation Index are considered the Angiosperm leaf saprophytes have the highest values. This corresponds with the capacity of these fungi for rapid colonisation of many substrates, which was referred to by Hudson (1968). Many of the soil and conifer litter species are very slow growing in control, resulting in a considerable disparity between the Theoretical Colonisation Indices of many of these fungi and the rapidly growing leaf saprophytes. In the presence of ferulic acid there is a levelling out of the differences in growth potential and there would be a concomitant reduction in the relative competitive advantage of the common leaf saprophytes over the soil fungi and conifer saprophytes. Only two specific saprophytes of conifer needle litter were studied, Dothichiza pityophila and Thysanophora penicilloides, because of the poor sporulation of other species from this litter after repeated transfers in pure culture. However, it is highly significant that the percentage germination of T.penicilloides

and the germ hyphal lengths of both species were still stimulated with 600 mg l^{-1} of ferulic acid in contrast to the marked inhibition of all the other saprophytes at this high concentration. T. penicilloides barely germinated in control but reached approximately 45% germination at this concentration of ferulic acid, compared with 29% germination with 200 mg l^{-1} ferulic acid. This suggests an important adaptation to the presence of ferulic acid.

There was sufficient data available from the experiments on the effects of phenolic acids other than ferulic acid to conclude that the behaviour of microfungi towards ferulic acid was probably representative of a general pattern, with perhaps ferulic acid showing more marked differential effects with respect to ecological groups of fungi. Further work was therefore concentrated on other aspects of the physiology of these fungi using ferulic acid rather than continuing spore germination studies with more detailed work on other compounds.

The ability of soil and litter fungi to utilise ferulic acid as a sole carbon source was studied. By measuring the utilisation of ferulic acid over a five day period in culture solution inoculated with spore suspensions the ferulic acid decomposing ability in the initial growth period could be defined. The variation in this ability found among the fungi studied did not correspond with the effects of ferulic acid on spore germination or hyphal growth. Species of Angiosperm leaf saprophyte inhibited by ferulic acid on agar showed a full range of ferulate decomposing activity, as defined, from very low (Botrytis cinerea) to high (Stemphylium dendriticum) and Dreschlera rostrata). A wide

range of soil fungi and three specific saprophytes of conifer needles had high ability to decompose the substrate, some removing all traces, others leaving a minimal concentration of ferulic acid and various intermediate metabolites, the exceptions being isolates of Mucor spp., Mortierella ramanniana and Trichoderma viride which generally had a very low capacity to utilise ferulic acid. There were indications from experiments with protocatechuic and p-coumaric acids using selected isolates that this pattern of decomposing ability would be expected generally with phenolic acids. Henderson & Farmer (1955) found that isolates of Trichoderma and Mucor were among a limited number of fungi out of a total of 28 genera to be unusual in showing only a limited utilisation of vanillin and ferulic acid over a period of 24 days. The lower ferulate decomposing activity of Mucor and Trichoderma found here was not due to poorer ability to germinate in the liquid medium compared to other fungi decomposing ferulate readily; and in fact with the isolate of Mucor hiemalis and one of the isolates of T. viride used in the spore germination studies in this medium there was stimulation of spore germination and germ hyphal growth by ferulic acid, corresponding with the situation on agar. Evidence was provided by Henderson & Farmer (1955) that the limited utilisation of vanillin in their experiments was due to oxidation of vanillin to vanillic acid but vanillic acid could not be metabolised. The stimulation of Mucor and Trichoderma by ferulic acid compared with the control suggests a specific adaptation to the presence of ferulic acid as a strategy in the colonisation of substrates, achieved by the limited utilisation of ferulic acid over a period of five days, the preliminary oxidation of ferulic acid to vanillic acid

providing energy for this. By an analogous process, hyphal growth of Dothichiza pityophila was greater in the ferulic acid solution than in the blank medium, but the main growth occurs by production of secondary blastospores, hyphal growth being maximised before this occurs. Decomposition of the ferulic acid is therefore not associated principally with the stimulation of hyphal growth, a fact confirmed by the delay in the disappearance of ferulic acid in the time course experiments with D. pityophila compared to Penicillium janthinellum.

In general, it can be suggested that the occurrence of phenolic acids in litter and soil has led to the adaptation of certain fungi to their presence by a stimulation of growth potential. This, taken with the inhibition of the growth potential of other fungi by these compounds, could be of advantage in the colonisation of substrates in environments where phenolic acids are particularly important. The validity of this hypothesis depends upon how far the differential behaviour of fungi towards ferulic acid is a general phenomenon with other phenolic compounds more frequently occurring (in detectable quantities) in litter and soil and on the concentrations of these compounds in the fungal environment. Since the response of spore germination and initial hyphal growth is independent of ferulic acid decomposing ability, the hypothesis does not depend upon any postulated relationship between phenolic acid decomposing ability and lignin decomposing ability, or on phenolic acids being important intermediates in the overall turnover of lignin.

Further indications of the importance of phenolic acids

in the ecology of fungi in litter and soil was provided by studies on the needle litter of Scots Pine in conjunction with information available from previous work.

First of all, however, consideration must be given to the general factors determining the overall composition of the mycoflora of a litter and the growth of the fungi therein. The primary factors are probably the chemical nature of the substrate, the climate and the soil conditions, interacting in a complex fashion. To the extent that all leaf litters consist of certain materials such as cellulose and lignin it is not surprising that several primary saprophytes are very widespread and some others are more limited in range but not obviously specifically adapted to any particular litter types. However, the rate of breakdown of the litter depends upon the climate, affecting the fungal activity, as well as the nature of the material itself. Phenolic compounds leached out of living leaves are probably the major cause of Mor formation but the phenolic content of leaves depends upon the physiological state of the plant which is influenced by soil conditions, poor nutrient status increasing the phenolic content (Coulson et al., 1960, Davies et al. 1964). Pedological and climatic conditions should thus be considered as being responsible for the realisation of a potential for Mor formation inherent in the leaf material itself. The formation of Mor often associated typically with conifers is thus at least partly due to the predominance of conifers on nutrient poor soils and in poorer climates.

There is no evidence to suggest that the species composition of the mycoflora of a typically Mull forming

litter is altered when the litter is subjected to conditions which result in Moder or Mor formation (although this does not seem to have been investigated as such). It has been assumed therefore that the absence of most of the otherwise widespread primary saprophytes from coniferous needle litter, reported in European studies, is due to the unique character of the needles on top of a general tendency for Mor formation, leading for example to an exceptional acidity. However, the studies of Widden & Parkinson (1973) in Canada and of Ishii (1967a,b) in Japan showed that the widespread Angiosperm saprophytes could be important in the decomposition of conifer litter under certain conditions. As a result it is not clear whether conifer litter is generally atypical in its mycoflora, but perhaps depending upon certain climatic conditions for full expression of the peculiarities, or whether the European pattern is a local aberration in a world wide occurrence of a small group of primary saprophytes on many litters due to the ubiquitous provision of certain basic substrates, this second situation being alluded to by Widden & Parkinson (1973).

The results of fungal isolations from the needle litter of native Scots Pine confirmed the general impression from previous European work that the common primary saprophytes of Angiosperm leaf litter were not imporant in the initial decomposition of the litter. The principal primary saprophytes were Fusicoccum bacillare and Dothichiza pityophila. The former species had been recorded in nearly every previous study of conifer litter, but D. pityophila has not been recorded previously as such in isolation data, and the ubiquitous Aureobasidium pullulans which may have included some isolations of D. pityophila was entirely absent.

The results presented here, then, reinforce the view that the needle litter of Scots Pine has an 'atypical' primary mycoflora, and it is possible to make an important contrast between the differential effects of ferulic acid on a number of primary saprophytes in relation to their ecological distribution - Cladosporium herbarum, Botrytis cinerea and Aureobasidium pullulans against Dothichiza pityophila and Thysanophora penicilloides. In view of these differences apparent from the agar block tests it is legitimate to consider the possible involvement of phenolic acids released during the decomposition of the litter in the selective effects of the Pine needles on fungal colonisation because physical environmental factors and the initial nature of the substrate alone are insufficient.

If it had been possible to test the effects of phenolic acids at natural concentrations some direct evidence may have been obtained to support the role postulated for these compounds. The question of concentration is one of the most crucial aspects of any work on biologically active substances in soil, this environment being very heterogeneous and so bulk measures of concentration are of limited usefulness. By determining the concentration of phenolic acids in solution in the free water in the litter it had been hoped to achieve a meaningful measure of concentration but this object was not realised. Para-coumaric acid was tentatively identified, however, in the melt-water of litter from a different site suggesting that further attempts along this line would be worthwhile. Beck et al. (1969) have found inhibitory effects on bacterial growth in water extracts of the litter of a number of deciduous trees and a number of conifers including Pinus sylvestris. There was no direct analysis of these

extracts and there have been other studies on the inhibitory effects of litter, antifungal (Tousson et al, 1969), as well as antibacterial (Melin & Wiken, 1946). However, what is interesting about the results of Beck et al. (1969) is that they found that the inhibitory effect was markedly increased at low pH and so for the same weight of leaf litter extracted, the extract from Spruce litter was more inhibitory than those from Oak or Beech litter suggesting that the biological activities of water soluble substances is of greater consequence in conifer litter than in deciduous litter. With deciduous leaf litter there was an abrupt detoxification of the extracts as the litter aged after leaf fall, parallelling the decline in phenolic acids particularly, noted by Bruckert & Jaquin (1966) and Vedy & Bruckert (1970). It was unfortunate that Beck et al. (1969) did not test the behaviour of inhibitory effects of conifer litter in this way.

It must be remembered that the results of the agar block tests and the liquid culture work indicates that both stimulation and inhibition are to be expected from phenolic acids, depending upon the concentration and the fungus. Therefore, the possible involvement of phenolic acids in the ecology of fungi in the litter is not to be visualised on an analogy with soil fungistasis, which was the motivation for Lingappa & Lockwood's (1962) investigation into the effects of lignin-related phenolic compounds. Instead, phenolic acids must be viewed as factors affecting the relative competitive advantage of different fungi. With natural concentrations of phenolic acids in solution it could be possible to investigate the effects of phenolic acids on the outcome of competition for substrates. For example, leaf litter could be soaked in appropriate concentrations of phenolic

acids and subjected to mixed inoculum, on the lines of the experiments by Wilkinson & Lucas (1969a,b) which studied the effects of 'Gramoxone W' on competition for saprophytic colonisation of plant residues; these workers showed that only subtle differences in the sensitivity to the herbicide of the pairs of fungi inoculated was needed to alter radically the outcome of the competition. The primary colonisation of Pine litter is thus one area where evidence from *in vitro* studies suggests a possible involvement of phenolic acids but where much more work is needed.

Colonisation of needles by secondary saprophytes and soil fungi and the subsequent course of the decomposition is another aspect of fungal ecology where the differential behaviour among a range of fungi towards phenolic acids may have ecological significance. One of the most important questions concerning the nature of the succession, and if indeed there is a succession at all, relates to the isolation of soil fungi from washed F layer needles. *Mucor hiemalis* and *Trichoderma viride* are two common soil inhabiting species isolated from such Pine needles and from other types of litter where there is a gradual transition from intact leaf material to soil, e.g. *Carex* (Pugh, 1958), *Eucalyptus* (Macauley & Thrower, 1966), *Pteridium aquilinum* (Frankland, 1966). It is interesting that these very widespread soil species should be common on Pine litter when the widespread primary saprophytes are absent. *M. hiemalis* is generally regarded as a 'sugar fungus' but one which appears late in the succession. Thus, Frankland (1966, 1969) showed that *M. hiemalis* appears after the main cellulose and lignin decomposers in the succession on *Pteridium* while Harrison

(1971) suggests that M. hiemalis makes a delayed appearance in Oak litter, after the inhibitory effects of leaf tannins have lessened.

From the results of the experiments reported here T. viride and Mucorales, including M. hiemalis, are considered as active colonisers of the surface of the F needles and there was evidence that T. viride colonises aging L needles as well. Therefore, a successional pattern in the distribution of these fungi is indicated, in line with the peak distribution in the F2 and H layers indicated by previous work (Parkinson & Balasooriya, 1967; Widden & Parkinson, 1973), even if the porosity of the F2 needles makes assessment of the colonisation of the needle interior in this layer difficult. The Infra-red spectroscopic data on the change in the needles a fungal substrate can be viewed in this context. The IR-spectra of the Humus (H) layer material had certain features characteristic of humic acids and there was a gradual transformation of the needles to a substrate in this state through the layers of the litter. As a result of these substrate changes the advantage of the needle saprophytes in prior-colonisation of the needle interior over M. hiemalis and T. viride could be lost with the consequence that the latter fungi could play a role in the decomposition of the F2 layer. Both primary and secondary saprophytes could be replaced by the soil fungi, since, for example, the primary saprophyte D. pityophila persisted in quite a high frequency in F1 needles.

It must be remembered that there was probably interference in the spectra of the unextracted Pine litter from Nitrogen

compounds not involved in humification, e.g. Chitin, which is presumably not a feature of spectra of humic acids and lignin extracted after the decomposition of plant material. However, the resolution in the Infra-red analysis of unextracted material was quite adequate for trends to be determined in the decomposition. Both lignin and cellulose disappeared rather abruptly between the F2 and H layers and probably neither cellulose nor lignin decomposition predominated in the L and F layers. Nevertheless, the change in carbonyl absorption indicated that major changes were occurring in the substrate during decomposition, as well as the physical reduction in the mass of the needles. The resolution could certainly have been improved if a better mill had been available and the potential of this technique in decomposition studies is very great. In other litters which decompose rapidly it is the replacement of the widespread primary saprophytes by specialised secondary saprophytes that is of most interest. Hudson (1968) pointed out that the common primary saprophytes appear to be successful colonisers of very many substrates so that their disappearance would seem to involve substrate changes specific to each type of litter. Infra-red analysis could be of considerable use in this field.

Turning now to the Pine needle burial experiments, the burial of the needles in soil would seem to increase the potential for fungal colonisation; the overall colonisation index was increased relative to that of the natural litter; there was colonisation by species of soil fungi not characteristic of Pine litter and also by an Actinomycete; and T. viride was isolated more frequently from the interior of the buried needles than from the natural litter, indicating

a limitation imposed by the natural environment on colonisation by this species. Also T. viride formed needle groups with most of the soil fungi in the Association Analysis, which could be taken to indicate a greater number of niches available within the total substrate. However, there was survival of the specific saprophytes of the needles, and prior-colonisation by these species, particularly D. pityophila, still restricted the colonisation by T. viride of the interior of the non-autoclaved needles compared to the colonisation of the autoclaved needles, although the adverse effects of the natural environment had been removed. By contrast Gliocladium roseum and Nocardia were able to colonise the needle interior more readily in the presence of the needle saprophytes than T. viride; Nocardia in fact showed a preference for needles already colonised by D. pityophila.

It is in the colonisation of the needles that the ferulic acid decomposing activity of the fungi involved may be important. It is significant that Mucor spp. and T. viride are not able to decompose this compound readily in contrast to Gliocladium roseum and to the needle saprophytes. Previous work had also indicated that Nocardia has a high capacity for the utilisation of phenolic acids in the appropriate medium. Phenolic acids are liberated in Pine litter throughout its decomposition (Bruckert & Jaquin, 1966) and a mechanism can be postulated on the basis of Infra-red data for the release of phenolic acids which were esterified to lignin, from the change in carbonyl absorption; phenolic acids so released would largely be metabolised by the fungi involved on the attack on the Pine needles. If phenolic acids are released from ester linkages

it is possible that changes in the lignin-carbohydrate complex are taking place, in which may lie the significance of the substrate changes observed in the Infra-red analysis.

Pine needles buried in soil, then, are selective for a certain kind of saprophytic behaviour and with non-sterilised needles this behaviour is associated with certain physiological characteristics of the fungi. Furthermore, the Pine needles could be serving as 'model substrates' for wider aspects of saprophytism in connection with other systems. This is suggested because Dix (1964) showed that Fusarium spp. and Gliocladium roseum have a high capacity for invasion of moribund Bean roots although they are not the primary colonisers of the Rhizosphere. Both Fusarium oxysporum and G. roseum decomposed ferulic acid readily. Fusarium sp. was a minor isolate from the natural Pine litter in the Blackwood but was restricted to the surface sterilised needles. The behaviour of G. roseum on Pine needles has already been contrasted with that of T. viride. It is also worth noting that Trichocladium sp. was the only soil species to be characteristic of needles buried in Hermitage Wood apart from T. viride. Trichocladium was the most important fungus associated with the decomposition of lignin in soil in the experiments of Wojtas-Wasilewska et al.(1973).

Some fungi not involved in Pine needle colonisation, however, have high ferulate decomposing activity. Penicillium spp. were distinguished from Mucorales and T. viride in not being active colonisers of the needle surface but the ability of the Penicillia to decompose ferulate rapidly fits in with their characteristic pattern of behaviour as fungi existing largely as spores in soil (Burges, 1960). Stemphylium

dendriticum and Dreschlera rostrata, among the Angiosperm leaf saprophytes tested, decomposed ferulic acid readily in contrast to Cladosporium herbarum and Botrytis cinerea. It is interesting that the first two species are dematiaceous because Rich & Horsfall (1954) associated the dark mycelium of Stemphylium sarcinaeforme with the ability of mycelial extracts to oxidise and detoxify a wide range of phenolics and quinones which were toxic to a hyaline fungus (Monilinia fructicola) but not to S. sarcinaeforme.

The selective nature of Pine needles as a substrate led to differences in the species isolated from the two soils in Hermitage Wood and the Experimental Gardens. The isolation technique was also selective, involving surface sterilisation and plating out on malt agar. However, when the Pine needles were incubated in ferulic acid solution there was a uniformity of ferulic acid decomposing activity within samples sufficient to show a correspondence between the activity of surface sterilised needles selected from the natural litter and from soil with the ferulate decomposing activity of the individual species isolated. As a result the Pine needles can be said to be useful model substrates for indicating differences in the mycoflora of different soils as well as selecting for a certain kind of saprophytic behaviour as defined above. Differences between soils detected by the use of this soil baiting technique could possibly be extended to differences in the decomposition of the substrate by Infra-red analysis if left in the soil for longer than three months.

The situation with the incubation of the soil samples in ferulic acid solution was complicated by the activity of other microorganisms not dealt with in pure culture studies - Bacteria and Yeasts. However, the filamentous fungi seemed to be

mainly responsible for the decomposition of ferulate in the incubation of the Gardens soil samples, but it was Bacteria that were active in cultures of the soil from Hermitage Wood. This corresponded with the fact that ferulic acid decomposing isolates of fungi were more important among the fungal isolations from needles buried in the Gardens than in Hermitage Wood. More importantly, there were differences in the total activity of the soil samples which paralleled differences found with the two French soils. In the latter case, the degree of completeness of ferulic acid metabolism in the soil cultures corresponded with the degree of completeness of lignin decomposition and also of phenolic acid metabolism associated with humification in the soils themselves. This correspondence is probably not fortuitous because the decomposition of ferulic acid by microfungi is probably representative of other phenolic acids more frequently found in soil - p-hydroxybenzoic, vanillic and p-coumaric acids.

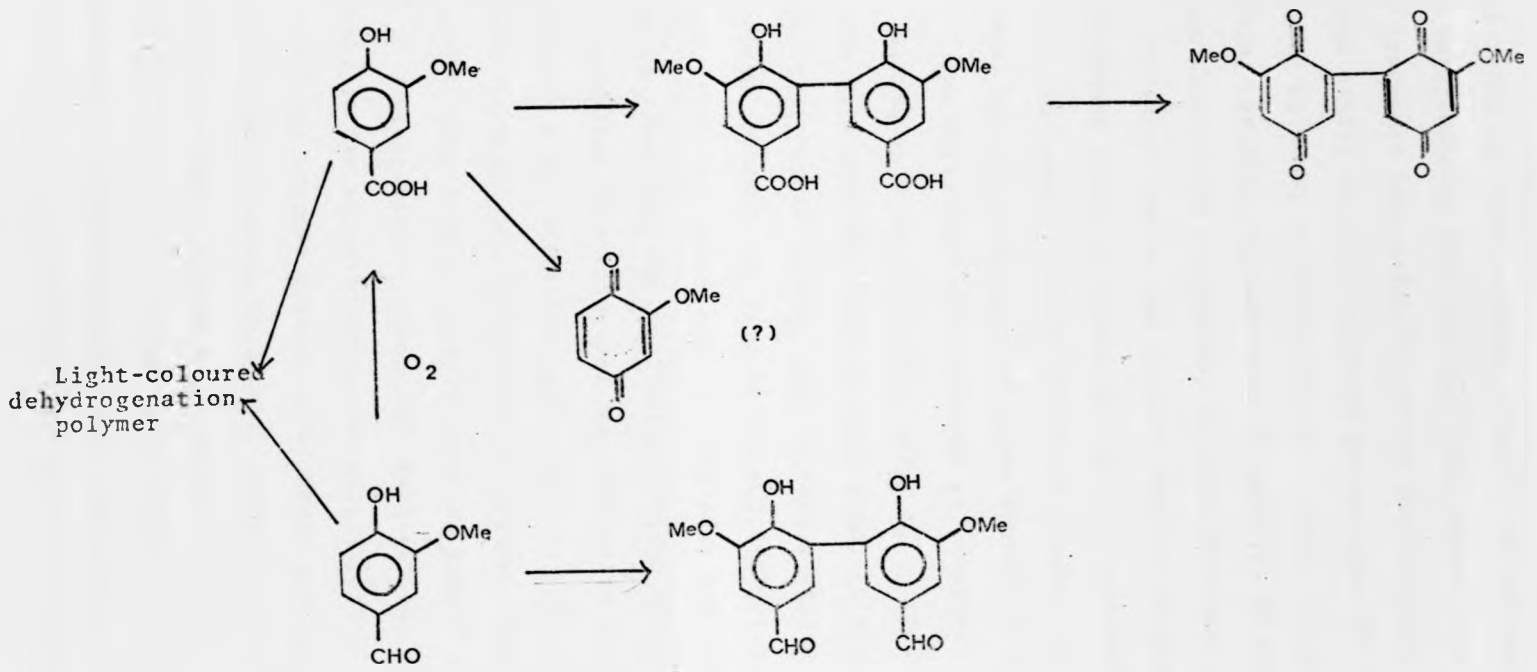
In the pure culture experiments some soil fungi decomposing ferulic acid readily, e.g. Gliocladium roseum, left residual amounts of ferulic acid and intermediates while others, e.g. Penicillium spp. left no traces of substrate or intermediate metabolites. It is productive to contemplate the analogy of this situation with the presence or absence of ferulic acid left after incubation of the soils. In Chapter One it was suggested that since the metabolism of ferulic acid and other phenolic compounds is likely to be intracellular, the appearance of vanillic acid and vanillin in some culture filtrates may be due to an intracellular build-up of these compounds in some fungi to such levels that

some leak out into the culture medium. As an alternative it could be that the presence or absence of intermediates and ferulic acid in the culture filtrates depends upon the extracellular phenol oxidising activity in cultures of the different fungi. Henderson & Farmer (1955) found no correlation between the ability of microfungi to metabolise phenolic compounds and the results of the Bavendamm test of the ability of these fungi to polymerise the compounds (and there is a general lack of coloured oxidation products in culture filtrates of microfungi compared to those of Basidiomycetes). A correlation was found, however, in the case of those fungi where no traces of substrate or any metabolic intermediates could be detected in culture filtrates. This suggests that extracellular enzymes may remove by polymerisation the small concentrations of substrates and intermediates leaking out into the culture medium and direct evidence of this comes from studies with Basidiomycetes decomposing lignin and lignin model compounds (Ishikawa et al.1963a,b).

In soil and litter the production of phenolic acids from lignin is depleted by microbial metabolism,by leaching and by humification (especially by the formation of transient polymers). The substances found and their concentration is therefore an expression of the balance between these opposing factors. The chemistry and biochemistry of humic substances is notoriously complicated but some approaches have proved useful. Vanillic acid and vanillin are known to form dimeric compounds by oxidative coupling in the presence of oxidising enzymes with further oxidation to quinones (Flaig & Haider, 1961b), as shown in Figure 1. These are the sort

Fig. 1 Oxidative coupling of Vanillic acid and Vanillin.

Me = CH_3


OH
OMe
COOH
MeO
OMe
COOH
COOH
MeO
OMe
O
O
O
O
OMe
(?)
O
O
O_2
Light-coloured
dehydrogenation
polymer
OH
OMe
CHO
MeO
OMe
CHO
CHO

of compounds to be expected as intermediates in humification along with quinones formed from simple phenols; and although the situation is complicated by autoxidation occurring in the formation of humic acids (Martin & Haider, 1969), the fact that the diphenol oxidases (requiring molecular oxygen) and the peroxidases are essentially interchangeable (Gierer & Opara, 1973) suggests that the final products are strictly determined by the starting compounds. Investigation of the extracellular phenol oxidase activity of microfungi could thus be of great benefit in relation to the polymerisation of phenolic compounds in soil. In particular, by a survey of litter and soil microfungi for the ability to polymerise products in their culture filtrates some idea could be gained of which fungi are important in this aspect of humification; the more detailed biochemical work on the lines of Haider's group (e.g. Martin & Haider, 1969, Martin et al. 1972, Grabbe & Haider, 1971a,b) could then be taken together with what is going on in soil. It might also be possible to relate differential patterns of phenolic acid production and humification in various soils to differences in the fungi of the soils with respect to phenol oxidase production

The experiments on the incubation of mixed populations of microorganisms on Pine needles and in soil particles with ferulic acid can only be regarded as preliminary but there is much of interest in this work to stimulate further work along these lines. In particular it could be said that herein lies the validity of phenolic acids as lignin model compounds, representing overall decomposition processes of lignin in soil, rather than phenolic acid decomposition by microorganisms in pure culture being a model for their ability to decompose lignin itself.

In the work presented here phenolic acids have been implicated in various aspects of the ecology of microfungi in litter and soil. The key-stone for these ideas is the connection observed between the patterns of behaviour of these microfungi towards phenolic acids and the ecological distribution of the fungi. In this respect it is not important how great a proportion of lignin decomposition takes place through the production of phenolic acids, these compounds being significant as compounds released by lignin decomposition into the fungal environment. The acceptance of the diverse conclusions concerning the importance of phenolic acids is limited by the number of fungi and the number of phenolic compounds that have been studied. However, it is hoped that the value of this study in part comes from demonstrating the use of a wide range of techniques in an attempt to investigate some ecological principals in the decomposition of lignin.

BIBLIOGRAPHY

BALASOORIYA, I. & PARKINSON, D. (1967). Studies on fungi in a Pine-wood soil. II. Substrate relationships of fungi in the mineral horizons of the soil. Revue d'Écologie et de Biologie du Sol. 4 639-643.

BATKO, S., MURRAY, J.S. & PEACE, T.R. (1958). Sclerophoma pityophila associated with needle cast of pines and its connexion with Pullularia pullulans. Trans. Br. mycol. Soc. 41 126-128.

BECK, G., DOMMERGUES, R. & VAN DEN DRIESSCHE, R. (1969). L'effet litiere. II. Étude experimentale du pouvoir inhibiteur des composes hydrosolubles des feuilles et des litières vis-à-vis la microflora tellurique. Oecologia Plantarum 4 237-266.

BICHO, J.G., ZAVARIN, E. & BRINK, D.L. (1966). Oxidative degradation of wood. II. Products of alkaline nitrobenzene oxidation by a methylation - gas chromatographic technique. Tappi 49 218-226.

BJÖRKMAN, A. (1956). Studies on finely divided wood. I. Extraction of wood with neutral solvents. Svensk Papperstidn. 59 477-485.

BLOOMFIELD, C. (1963). Report Rothamsted Experimental Station

BÖRNER, H. (1960). Quoted in Patrick & Tousson (1965).

BRIDGES, E.M. (1970). World Soils. Cambridge University Press.

BROWN, S.A. (1964). Lignin and Tannin Biosynthesis. In Biochemistry of Phenolic Compounds. Harborne (ed.). Academic Press.

BRUCKERT, S. & JAQUIN, F. (1966). Relation entre l'évolution des acides hydrosolubles de deux litières forestières et les processus pédongénetiques. Bull. École Nationale Superieure Agronomique 8 95-112.

BRUEHL, G.W. & LAI, P. (1966) Prior colonisation as a factor in the saprophytic survival of several fungi in wheat straw. Phytopathology 56 766-768.

BURGES, N.A. (1960). Dynamic equilibria in the soil. In The Ecology of Soil Fungi. Parkinson & Waid (eds.). Liverpool University Press.

BURGES, N.A., HURST, H.M. & WALKDEN, BERYL, (1964). The phenolic constituents of humic acid and their relation to the lignin of the plant cover. Geochim. Cosmochim. Acta 28 1547-1554.

BUTIN, H. (1963). Über Sclerophoma pityophila (Corda) v. Höhn als Blauepilz an verarbeitetem Holz. Phytopathologie Zeitschrift 48 298-305.

CAIN, R.B., BILTON, R.F. & DARRAH, J.A. (1968). The metabolism of aromatic acids by micro-organisms. Metabolic pathways in the Fungi. Biochemical Journal 108 797-828.

CAIN, R.B. & FARR, D.R. (1968). Metabolism of Aryl-sulphonates by micro-organisms. Biochemical Journal 106 859-877.

CASIDA, L.E., Jr. (1972) Interval scanning photomicrography of microbial cell populations. Applied Microbiology 23 190-192.

CHRISTIE, T. (1965). The effects of some phenolic compounds upon growth of two species of Phytophthora. N. Zealand J. Agric. Res. 8 630-635.

COULSON, C.B. DAVIES, R.I. & LEWIS, D.A. (1960) Polyphenols in plant, humus and soil. I. Polyphenols of leaves, litter and superficial humus from mull and mor sites. Journal of Soil Science 11 20-29.

CRAWFORD, R.L., McCOY, ELIZABETH, HARKIN, J.M., KIRK, T.K. & OBST, J.R. (1973). Degradation of methoxylated benzoic acids by a Nocardia from a lignin-rich environment: Significance to lignin degradation and effects of chloro-substituents. Applied Microbiology 26 176-184.

DENNIS, C. & WEBSTER, J. (1971a). Antagonistic properties of species-groups of Trichoderma. I. Production of non-volatile antibiotics. Trans. Br. mycol. Soc. 57 25-39.

DENNIS, C. & WEBSTER, J. (1971b). Antagonistic properties of species-groups of Trichoderma II. Production of volatile antibiotics. Trans. Br. mycol. Soc. 57 41-48.

DAVIES, R.I., COULSON, C.B. & LEWIS, D.A. (1964). Polyphenols in plant, humus and soil. IV. Factors leading to increase in biosynthesis of polyphenols in leaves and their relationship to mull and Mor formation. Journal of Soil Science 15 310-318.

DEVERALL, B.J. & WOOD, R.K.S. (1961). Chocolate spot of beans (Vicia faba L.) - interactions between phenolase of host and pectic enzymes of the pathogen. Ann. appl. Biol. 49 473-487.

DIX, N.J. (1964). Colonisation and decay of Bean roots. Trans. Br. mycol. Soc. 47 285-292.

DIX. N.J. (1972). Effect of soil fungistasis on spore germination and germ-tube growth in Penicillium species. Trans. Br. mycol. Soc. 58 59-66.

DOMSCH, K.H. & GAMS, W. (1972). Fungi in Agricultural Soils. Longmans. Duchaufour, P. (1965). Precis de Pedologie. Masson & Cie.

DURIE, R.A. LYNCH, B.M. & STERNHILL, S. (1959). Comparative studies of brown coal and lignin. I. Infra-red spectra. Australian J. Chem. 13 156-168.

FALCK, R. & HAAG, W. (1927). Der Lignin und der Cellulose-Abbau des Holzes, zwei verschiedene Zersetzungsprozesse durch holzbewohnende Fadenpilze. Chemische Berichte 60 225-232.

FISCHER, G. (1953). Quoted in Jones & Farmer (1967).

FLAIG, W. (1964). Effects of microorganisms in the transformation of lignin to humic substances. Geochim. Cosmochim. Acta 28 1523-1535.

FLAIG, W. & HAIDER, K. (1961a). Die Verwetung phenolischer Verbindungen durch Weissfäulepilze. Archiv für Mikrobiologie 40 212-223.

FLAIG, W. & HAIDER, K. (1961b). Reaktionen mit oxydierenden Enzymen aus Mikroorganismen. Planta Medica 9 123-139.

FLAIG, W., SCHOBINGER, U. & DEUEL, H. (1959). Umwandlung von Lignin in Huminsäuren bei der Verrottung von Weizenstroh. Chemische Berichte 92 1973-1982.

FRANKLAND, JULIET C. (1966). Succession of fungi on decaying petioles of Pteridium aquilinum. Journal of Ecology 54 41-63.

FRANKLAND, JULIET C. (1969). Fungal decomposition of Bracken petioles. Journal of Ecology 57 25-36.

FRIEND, J., REYNOLDS, S.B. & AVEYARD, MURIEL A. (1973). Phenylalanine ammonia lyase, chlorogenic acid and lignin in potato tuber tissue inoculated with Phytophthora infestans. Physiological Plant Pathology 3 495-507.

GAMS, W. & DOMSCH, K.H. (1967) Beiträge zur Anwendung der Bodenwaschtechnik für die Isolierung von Bodenpilzen. Archiv für Mikrobiologie 53 134-144

GAMS, W. & DOMSCH, K.H. (1969). The spatial and seasonal distribution of microscopic fungi in arable soils. Trans. Br. mycol. Soc. 52 301-308.

GAMS, W., DOMSCH, K.H. & WEBER, E. (1969). Nachweis signifikant verschiedener Pilzpopulationen bei gleicher Bodenutzung. Plant and Soil 31 439-450.

GARRETT, S.D. (1956) Biology of Root-infecting Fungi. Cambridge U.P.

GIERER, J. & OPARA, A.E. (1973). Studies on the enzymatic degradation of lignin. The action of peroxidase and laccase on monomeric and dimeric model compounds. Acta Chemica Scandinavica 27 2909-2922.

GRABBE, K. & HAIDER, K. (1971a). Die Huminstoffbildung und die Stickstoffverteilung bei der Strohrotte in Beziehung zur mikrobiellen Phenolbildung. Z. Pflaznenernährung und Bodenkunde 129 202-215.

GRABBE, K. & HAIDER, K. (1971b) Die Huminstoffbildung und der Stickstoffumsatz bei der Bereitung des Kultursubstrats und während des Wachstums von Agaricus bisporus. Z. Pflanzenernährung und Bodenkunde. 139 216-226.

GREMMEN, J. (1957). Microfungi decomposing organic remains of pines. Fungus 27 34-42.

GUENZI, W.D. & McCALLA, T.M. (1966). Phenolic acids in Oats, Wheat, Sorghum and Corn residues and their phytotoxicity. Agronomy Journal 58 303-304.

GULYAS, F. (1967). On the role played by several soil fungi in the microbiological decomposition of lignin. Agrokémia es Talajtan 16 137-150.

HAIDER, K. & DOMSCH, K.H. (1969). Abbau und Umsetzung von lignifiertem Pflanzenmaterial durch mikroskopische Bodenpilze. Archiv fur Mikrobiologie 64 338-348.

HARKIN, J.M. (1966). Recent developments in lignin chemistry. Fortschritte der Chemischen Forschumg 6 150-158.

HARKIN, J.M. (1967). Lignin - a natural polymeric product of phenol oxidation. In Oxidative Coupling of Phenols. Taylor & Battersby (eds.). Edward Arnold.

HARRISON, A.F. (1971). The inhibitory effect of Oak leaf litter tannins on the growth of fungi in relation to litter decomposition. Soil Biology and Biochemistry 3 167-172.

HATA, K. (1966). Investigations on lignins and lignification XXXIII Studies on lignin isolated from Spruce wood decayed by Poria subacida B11. Holzforschung 20 142-147.

HAYES, A.J. (1965a). Studies on the decomposition of coniferous leaf litter. II. Changes in external features and succession of microfungi. Journal of Soil Science 16 242-257.

HAYES, A.J. (1965b). Some microfungi from Scots Pine litter. Trans. Br. mycol. Soc. 48 179-185.

HENDERSON, MOIRA E.K. (1956). A study on the metabolism of phenolic compounds by soil fungi using spore suspensions. J. Gen. Microbiol. 14 684-691.

HENDERSON, MOIRA E.K. (1957). Metabolism of methoxylated aromatic compounds by soil fungi. J. Gen. Microbiol. 16 686-695.

HENDERSON, MOIRA E.K. (1960). Studies on the physiology of lignin decomposition by soil fungi. In The Ecology of Soil Fungi. Parkinson & Waid (eds.). Liverpool University Press.

HENDERSON, MOIRA E.K. (1961a). Isolation, identification and growth of some soil Hyphomycetes and Yeast-like fungi which utilise aromatic compounds related to lignin. J. Gen. Microbiol. 26 149-154.

HENDERSON, MOIRA E.K. (1961b). The metabolism of aromatic compounds related to lignin by Hyphomycetes and Yeast-like fungi of soil. J. Gen. Microbiol. 26 155-165.

HENDERSON, MOIRA E.K. (1966). Enrichment in soil fungi which utilise aromatic compounds. Plant and Soil 23 339-350.

HENDERSON, MOIRA E.K. & FARMER, V.C. (1955). Utilisation by soil fungi of p-hydroxy-benzaldehyde, ferulic acid, syringaldehyde and vanillin. J. Gen. Microbiol. 12 37-46.

HESS, D. (1970). Pflanzenphysiologie. Ulmer.

HIGUCHI, T., ITO, Y. & KAWAMURA, I. (1967a). Para-hydroxyphenylpropane component of grass lignin and the role of tyrosine in its formation. Phytochemistry 6 875-881.

HIGUCHI, T., ITO, Y., SHIMADO, M. & KAWAMURA, I. (1967b). Chemical properties of milled wood lignin of grasses. Phytochemistry 6 1551-1556.

HIGUCHI, T. & KAWAMURA, K. (1953). Biochemical study of wood rotting fungi II. Relation between Bavendamm's reaction and tyrosinase. J. Japanese Forestry Soc. 35 350-354.

HUDSON, H.J. (1965). An ascomycete with Aureobasidium pullulans- type conidia. Nova Hedwigia 10 319-328.

HUDSON, H.J. (1968). The ecology of fungi on plant remains above the soil. New Phytologist 67 837-874.

ISHII, H. (1967a). Studies on the distribution of microfungi in Pine Forest soil. I. Vertical distribution in a soil profile. Bull. Simane Agric. College 15 15-21.

ISHII, H. (1967b). Studies on the distribution of microfungi in Pine Forest soil. II. Distribution in organic horizon. Bull. Faculty of Agriculture Shimane University 1 60-70.

ISHII, H. (1968). Studies on the distribution of microfungi in Pine Forest soil. IV. Influence of needles and broad leaves on the micro-fungus flora. Bull. Faculty of Agriculture Shimane University 2 51-56.

ISHIKAWA, H., SCHUBERT, W. & NORD, F.F. (1963a). Investigations on lignins and lignification. XXVII. The enzymatic degration of softwood lignin by white rot fungi. Archives Biochem. Biophys. 100 131-139.

ISHIKAWA, H., SCHUBERT, W. & NORD, F.F. (1963b). Investigations on lignins and lignification. XXVIII. Degradation by Polyporus versicolor and Fomes fomentarius of aromatic compounds structurally related to softwood lignin. Archives Biochem. Biophys. 100 140-149.

ISHIKAWA, H., SCHUBERT, W. & NORD, F.F. (1963c). Investigations on lignins and lignification. XXX. Enzymatic degradation of Guaiacylglycerol and related compounds by white-rot fungi. Biochemische Zeitschrift. 338 153-163.

ISHIKAWA, T. & MIYAZAKI, M.(1972). Quoted in Craford et al.(1973).

JAQUIN, F. & BRUCKET, S. (1965). Identification et évolution des acides hydrosolubles de deux litières forestières. Compte Rendues Acad. Sci. Paris 260 4556-4559.

JONES, D. & FARMER, V.C. (1967). The ecology and physiology of soil fungi involved in the degradation of lignin and related aromatic compounds. Journal of Soil Science 18 74-84.

JORGENSEN, J.R. & WELLS, C.G. (1973). The relation of respiration in organic and mineral soil layers to soil chemical properties. Plant and Soil 39 373-387.

KENDRICK, W.B. & BURGES, N.A. (1962). Biological aspects of the decay of Pinus sylvestris leaf litter. Nova Hedwigia 4 313-342.

KIRK, T.K. (1971). Effects of microorganisms on lignin. Annual Review of Phytopathology 9 185-210.

KIRK, T.K. & KELMAN, A. (1965). Lignin degradation as related to the phenoloxidases of selected wood-decaying Basidiomycetes. Phytopathology 55 739-745.

KUNC, F. (1971a). Decomposition of vanillin by soil micro-organisms. Folia Microbiologica 16 41-50.

KUNC, F. (1971b). Effect of glucose on the decomposition of vanillin by soil microorganisms. Folia Microbiologica 16 51-57.

KUNC, F. (1974). Microbial decomposition of coumarin in soil. Folia Microbiologica 19 209-217.

KUSAKARI, S-I., & TAKAGI, Y. (1973). Fungistatic activity of soil sterilised by gamma radiation. Can. J. Microbiology 19 1333-1334.

LARSSON, S. & MIKISCHE, G.E. (1967). Gas chromatographic analysis of lignin oxidation products. The diphenyl ether linkage in lignin. Acta Chemica Scandinavica 21 1970-1971.

LARSSON, S. & MIKISCHE, G.E. (1969). Gaschromatische Analyse von Ligninoxidationsprodukten. III. Oxidative Abbau von methyliertem Björkman-Lignin (Fichte). Acta Chemica Scandinavica 23 3337-3351.

LARSSON, S. & MIKISCHE, G.E. (1971). Gaschromatographische Analyse von Ligninoxidationsprodukten. V. Zweitrimere Abbauprodukte aus Fichtenlignin. Acta Chemica Scandinavica 25 673-679.

LEWIS, J.A. & PAPAVIZAS, G.S. (1968). Survival of root-infecting fungi in soil. VII. Decomposition of tannins and lignins in soils and their effects on Fusarium root rot of bean. Phytopathologische Zeitschrift 63 124-134.

LI, C.Y., LU, K.C.,NELSON, E.E., BOLLEN, W.B. & TRAPPE, J.M. (1969). Effect of phenolic compounds on growth of Poria weirii in vitro. Microbios 3 305-311.

LI, C.Y., LU, K.C., TRAPPE, J.M. & BOLLEN, W.B. (1972). Poria weirii - inhibiting and other phenolic compounds in roots of Red Alder and Douglas Fir. Microbios 5 65-68.

LINDEBERG, G. (1946). On the decomposition of lignin and cellulose in litter caused by soil-inhabiting Hymenomycetes. Arkiv für Botanik 33 A 1-16.

LINGAPPA, B.T. & LOCKWOOD, J.L. (1962). Fungitoxicity of lignin monomers, model substances and decomposition products. Phytopathology 52 295-299.

LUNQUIST, K. (1970). Acid degradation of lignin. II. Separation and identification of low molecular weight phenols. Acta Chemica Scandinavica 24 889-907.

MACAULEY, B.J. & THROWER, L.B. (1966). Succession of fungi in leaf litter remains of Eucalyptus regnans. Trans. Br. mycol. Soc. 49 509-520.

MACKO, V., STAPLES, R.C., RENWICK, A.A. & PIRONE, J. (1972). Germination inhibitors of rust uredospores. Physiological Plant Pathology 2 347-355.

MANGENOT, F. (1971) Trans. Int. Symp. 'Humus et Planta VI 37-42

MANGENOT, F. & KIFFER, E. (1972). Pouvoir ligninolytique des sols de la R.C.P. 40. Revue d'Écologie et de Biologie du Sol 9 21-39.

MARTIN, J.P. & HAIDER, K. (1969). Phenolic polymers of ~~Stachybotrys~~ Stachybotrys atra, ~~Stachybotrys~~ Stachybotrys chartarum and Epicoccum Nigrum in relation to humic acid formation. Soil Science 107 260-270.

MARTIN, J.P., HAIDER, K. & WOLF, D. (1972). Synthesis of phenolic polymers by Hendersonula toruloidea in relation to humic acid formation. Soil. Sci. Soc. Amer. Proc. 36 311-315.

MAURER, C.L. & BAKER, R. (1964). Ecology of plant pathogens in soil. I. Influence of chitin and lignin amendments on development of Bean root rot. Phytopathology 54 1425-1426.

MELIN, E. & WIKÉN, T. (1946). Antibacterial substances in water extracts of Pine Forest litter. Nature 158 200-201.

METLISKI et al. (1973). Abstracts of Mycology February 1973 53-22004.

MILLER, H.J. (1949). Modifications of the slide-germination method of evaluating fungicides including the use of Venturia inaequalis and Phytophthora infestans. Phytopathology 39 245-259.

MITCHELL, C.P. (1973). Antagonistic interactions involving Trichoderma species. Ph.D. Thesis, University of Stirling.

MOLOT, P.M. (1970). Influence de l'acide p-coumarique sur l'activité des pectinases et des celullases et sur leur secrétion par Fusarium roseum var. graminearum (Hypocreacées, Sphaeriales). Comptes Rendues Acad. Sci. Paris, Ser. D. 270 2097-2100.

MORRISON, I.M. (1974). Structural investigations of the lignin-carbohydrate complexes of Lolium perenne. Biochemical Journal 139 197-204.

MUIR, J.W., MORRISON, R.I., BROWN, C.J. & LOGAN, J. (1964). The mobilisation of Iron by aqueous extracts of plants. I. Composition of the amino acid and organic acid fractions of an aqueous extract of Pine needles. Journal of Soil Science 15 220-225.

NARAYANARAO, A. MANIBHUSHANRAO, K. & SURYANARAYANAN, S. (1972). Effect of some phenolic compounds on sporulation and growth of Pyricularia spp. Proc. Indian Acad. Sci. Sect. B 75 140-144.

NICHOLLS, T.H. & SKILLING, D.D. (1970). Lophodermium pinastri outbreak in Lake States Forest Nurseries. Plant Disease Reporter 54 731-733.

NIEMANN, G.J. (1969a). Phenolic constituents from Larix needles. III. Benzoic and Cinnamic acids of L. leptolepis. Acta Botanica Neerlandica 20 397-400.

NIEMANN, G.J. (1969b). Phenolic glucosides from needles of Larix laricina. Phytochemistry 8 2102-2103.

NIEMANN, G.J. (1972). Phenolics from Larix needles. IV. Constituents of L. laricina. Acta Bot. Neerl. 21 549-552

NYKVIST, N. (1959). Leaching and decomposition of litter. II. Experiments on needle litter of Pinus sylvestris. Oikos 10 212-224.

NYKVIST, N. (1963). Leaching and decomposition of water soluble organic substances from different types of leaf and needle litter. Studia Forestalia Succica 3 3-31.

PARKINSON, D. & BALASOORIYA, I. (1967). Studies on fungi in a Pine-wood soil. I. Nature and distribution of fungi in the different soil horizons. Revue d'Écologie et de Biologie du Sol. 4 463-478.

PARKINSON, D. & BALASSOORIYA, I. (1969). Studies on fungi in a Pine-wood soil. IV. Seasonal and spatial variations in the fungal populations. Revue d'Écologie et de Biologie du Sol 6 147-153.

PARKINSON, D., BALASOORIYA, I. & WINTERHALDER, K. (1968). Studies on fungi in a Pine-wood soil. III. Fungal growth and total microbial activity. Revue d'Écologie et de Biologie du Sol 5 637-645.

PARKINSON, D. & KENDRICK, W.B. (1960). Investigations of soil microhabitats. In The Ecology of Soil Fungi. Parkinson & Waid (eds.). Liverpool University Press.

PARKINSON D. & WILLIAMS, S.T. (1961). A method for isolating fungi from soil microhabitats. Plant and Soil 13 347-355.

PATRICK, Z.A. & TOUSSON, T.A. (1965). Plant residues and organic amendments in relation to biological control. In Ecology of Soil-Borne Plant Pathogens.Baker & Snyder(eds.)Murray.

PERO, P.W. & OWENS, R.G. (1971). A microtechnique for evaluation of antifungal activity. Phytopathology 61 132.

PUGH, G.J.F. (1958). Leaf litter fungi found in Carex paniculata L. Trans. Br. mycol. Soc. 41 185-195.

PUGH, G.J.F. & BUCKLEY, N.F. (1971). The leaf surface as a substrate for colonisation by fungi. In The Ecology of Leaf Surface Microorganisms. Preece & Dickinson (eds.) Academic Press.

RICH, S. & HORSFALL, J.G. (1954). Relation of polyphenol oxidases to fungitoxicity. Proc. Nat. Acad. Sci. (U.S.A.) 40 139-145.

REDDY, T.K.R., & KNOWLES, R. (1965). The fungal flora of a boreal raw humus. Can. J. Microbiol. 11 837-843.

SCHUBERT, W.J. (1968). Metabolism of Lignins. In Comprehensive Biochemistry, Vol. 20. Florkin & Stotz (eds.). Elsevier.

SIEGEL, S. (1956) Non-parametric statistics for the behavioural sciences. McGraw-Hill

SINHA, M.K. (1972). Organic matter transformation in soils. IV. Aromatic compounds in fulvic acid of soil incubated with C^{14}- tagged Oat roots under aerobic and anaerobic conditions. Plant and Soil 37 273-281.

SKIDMORE, A.M. & DICKINSON, S.H. (1973). Effect of Phylloplane fungi on the senescence of excised Barley leaves. Trans. Br. Mycol. Soc. 60 107-116.

SMITH, D.C.C. (1955a). Para-hydroxybenzoate groups in the lignin of Aspen (Populus tremulus). Journal of the Chemical Society 2347-2351.

SMITH, D.C.C. (1955). Ester groups in lignin. Nature 17b 267-268.

SMITH, F.B. & BROWN, P.E. (1935). Quoted in Jones & Farmer (1967).

SRIDHAR, R. & OU, S.H. (1974). Phenolic compounds detected in Rice blast lesions. Biologia Plantarum 16 67-70.

SRINIVASAN, K.V. & NARASIMHAN, R. (1971). The effect of certain phenolic and related compounds on spore germination and appressorial formation in Colletotrichum falcatum Went. Proc. Indian Acad. Sci. Sect. B 72 81-91.

SUNDMAN, VERONICA, KUUSI, T., KUHAHEN, S. & CARLBERG, G. (1964). Microbial decomposition of lignin by various micropopulations. Acta Agriculturae Scandinavica 14 229-248.

TOUSSON, T.A., MENZIGER, W. & SMITH, R.S., Jr. (1969). Role of conifer litter in the ecology of Fusarium: stimulation of germination in soil. Phytopathology 59 1396-1399.

TOUTAIN, F. (1972). Étude comparée de deux Hêtraies sur grès rhétien: divergances pédoclimatiques et biochimiques. Bull. d'E.N.S.I.A. de Nancy. 14 103-116.

VAN SUMERE, C.F., VAN SUMERE - De PRETER, C., VINING, L.C. & LEDINGHAM, G.A. (1957). Coumarins and phenolic acids in the uredospores of Wheat Stem Rust. Can. J. Microbiol. 3 847-862.

VEDY, J.C. & BRUCKERT, S. (1970). Évolution saisonnière de composés organiques soluble en relation avec divers processus biochimiques d'humification. Pedologie. Gand 20 135-152.

WAKSMAN, S.A. (1967) The Actinomycetes. Ronald.

WARCUP, J.H. (1960). Methods for isolation and estimation of activity of fungi in soils. In The Ecology of Soil Fungi. Parkinson & Waid (eds.). Liverpool University Press.

WHITEHEAD, D.C. (1964). Identification of p-hydroxybenzoic, vanillic, p-coumaric and ferulic acids in soils. Nature 202 417-418.

WHITEHEAD, D.C. & TINSLEY, J. (1963). The biochemistry of humus formation. J. Sci. Food Agric. 12 849-857.

WIDDEN, P. & PARKINSON, D. (1973). Fungi from Canadian coniferous forest soils. Can. J. Botany 51 2275-2290.

WILKINSON, VALERIE & LUCAS, R.L. (1969a). Influence of herbicides on competitive ability of fungi to colonise plant remains. New Phytologist 68 701-708.

WILKINSON, VALERIE & LUCAS, R.L. (1969b). Effects of herbicides on growth of soil fungi. New Phytologist 68 709-719.

WILSON, D.M., NUOVO, G.J. & DARBY, W.B. (1973). Activity of o-diphenol oxidase in postharvest Apple decay by Penicillium expansum and Physalospora obtusa. Phytopathology 63 1115.

WINTER, A.G. & SCHÖNBECK, F. (1957). Untersuchungen über den Einfluss von Stickstoff auf den Abbau von Hemmstoffen aus Getreidestroh und Stoppeln. Z. Pflanzenernährung und Bodenkunde. 79 132-142.

WOJTAŚ-WASILEWSKA, MARIA, TROJANOWSKI, J. & STEPNIEWSKA, Zofia (1973). The model humification of a lignin preparation. Acta Microbiologica Polonica Ser. A 5 37-48.

YOUNG, MARIA & STEELIMK, C. (1973). Peroxidase catalysed oxidation of naturally occurring phenols and hardwood lignins. Phytochemistry 12 2851-2861.

ZIECHMANN, W. Spectroscopic investigations of lignin, humic substances and peat. Geochim. Cosmochim. Acta 28 1555-1566.

Personal Communications

KIFFER, E. (1974). Laboratoire de Botanique et de Microbiologie, University de Nancy-1, Nancy, France.

MANGENOT, F. (1974). Laboratoire de Botanique et de Microbiologie, University de Nancy-1, Nancy, France.

MILLAR, C.S. (1974). Department of Forestry, University of Aberdeen, Aberdeen, Scotland.

APPENDIX

APPENDIX

APPENDIX 1

Origins of fungi used in experiments with phenolic acids. Multiple isolates are designated by letters following the specific name.

		Substrate*	Source**
Mortierella ramanniana	'A'	Pinus sylvestris needle	
	'B'	Soil, H.W.	
Mucor hiemalis	'A'	Soil	CMI 113134
	'B'	Pinus sylvestris needle	
M. racemosus		" " "	
Desmazierella acicola		" " "	
Aureobasidium pullulans		Acer leaf	NJD
Botrytis cinerea		Vicia leaf	JWM
Cladosporium herbarum		Pisum leaf	JWM
Dreschlera rostrata		Hordeum leaf	NJD
Fusarium oxysporum		Soil, E.G.	
Gilmaniella humicola			
Gliocladium roseum		Buried Pine needle (E.G.)	
Penicillium expansum		Apple rot	JWM
P. funiculosum		Soil	NJD
P. janthinellum		Soil	NJD
P. spinulosum		Soil	NJD
Stemphylium dendriticum		Triticum leaf	CMI
Thysanophara penicilloides		Conifer needle	CBS 344.33
Trichoderma viride	'A'	Rhizosphere, Zea.	CPM
	'B'	Rhizosphere, Pisum	CPM
	'C'	Pinus sylvestris needle	
	'D'	" " "	

	'E' Buried Pine needle	(E.G.)
	'F' " " "	(H.W.)
	'G' " " "	(E.G.)
Coniella sp.	Soil, E.G.	
Dothichiza pityophila	Pinus sylvestris	CBS 128.64
	Pinus sylvestris needle	
Phoma eupyrena	Soil E.G.	

* H.W. = Hermitage Wood, University of Stirling.
E.G. = Experimental Garden, University of Stirling

** Sources other than from studies described in this thesis

CM Commonwealth Mycological Institute, Kew, England.

CBS Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

NJD Dr. N.J. Dix, University of Stirling, Dept. of Biology.

JWM Dr. J.W. Mansfield, University of Stirling, Dept. of Biology.

CPM Dr. C.P. Mitchell, University of Aberdeen, Dept. of Forestry

APPENDIX 2

Effect of phenolic acids on the production of hyphae by microfungi on agar blocks.

Test Fungus	Incubation Period	Hyphal numbers in each replicate						
		Control	FER	VAN	P-COU	P-HYD	SIN	SYR
Dreschlera rostrata		22	2	36	24	21	38	11
		17	1***	37**	14	19	39	29
	29 h	20	0	37	12	33	5	38
Stemphylium dendriticum		51	3	11	5	24	30	31
		46	0 *	23	5	30	33	25
	24 h	24	17	0	27	16	-	44
Botrytis cinerea		6	0	3	14	27	7	6
		17	1 **	12	9	2	17	0
	24 h	17	0	2	7	10	8	14
Cladosporium herbarum		36	3	7	6	27	32	32
		44	0 *	19 *	15*	50	11	26
	45 h	36	12	6	23	29	10	36
Penicillium expansum		12	6	8	0	5	6	27
		9	0	29	3 *	27	0	16
	24 h	11	6	11	3	48	5	11
Penicillium spinulosum		3	2	11	4	0	26	11
		4	34	32	11	21	63**	18**
	36 h	0	0	6	17	2	39	13
Trichoderma viride 'B'		5	71	218	51	129	173	139
		3	132 **	128***	57 **	290 **	80	*107**
	28 h	0	181	173	76	125	21	98
Mucor hiemalis 'A'		0	3	0	11	0	25	41
		0	2***	4	19 *	1	75***	3 *
	41 h	0	2	2	3	16	49	51
Mucor hiemalis 'B'		0	1	0	0	41	32	4
		1	8	0	0	0	14**	0
	40 h	0	12	0	0	0	34	30
Dothichiza pityophila 'A'		0	84	73	44	45	65	30
		11	50	53	44	68 *	10	9
	40 h	14	21	9	21	36	46	4
Thysanophora penicilloides		0	23	18	14	40	13	0
		0	37 *	5 *	5 **	19	1	23
	45 h	0	3	7	7	6	3	6

* Significant effect $p < 0.05$)
** Significant effect $p < 0.01$) t-test log transformation
*** Significant effect $p < 0.01$)

contd/....

FER	-	Ferulic Acid
VAN	-	Vanillic Acid
P-COU	-	P-coumaric Acid
P-HYD	-	p-Hydroxybenzoic Acid
SIN	-	Sinapic Acid
SYR	-	Syringic Acid

APPENDIX 3

Notes on some of the fungal isolations from the needle litter of Scots Pine.

Fusicoccum bacillare Sacc. et Penzig

Many of the colonies of this species produced pycnidia; those that did not could be identified by the characteristic sweet smell and morphological features of the mycelium.

White Sterile Form

Flocculose, slow growing mycelium, pure white at first, then turning grey.

Brown Sterile Form

Initially growing off the needles with fluffy aerial mycelium then slowly spreading into the agar with very dense surface hyphae.

Pyrenochaeta sp. 1

Isolated originally from material direct from the Blackwood and an identical form was isolated from the buried needles. Possibly *Pyrenochaeta acicola* (Lev.) Sacc.

Pyrenochaeta sp. 2

Light brown fluffy mycelium; the spiny pycnidia with an outer surface of globular cells.

Mucor spp.

Members of the *Mucor hiemalis* group including *M. hiemalis* Wehmeyer and *M. silvaticus* Hagem.

Paecilomyces sp.

This fungus was assigned to the *Paecilomyces*/*Gliomastix* complex. Conidia 14 um long.

Coniothyrium sp.

Pycnidia spiny with external hyphal net; spores ovoid, dark.

APPENDIX 4

Incubation of Pine needles collected in the Blackwood in Ferulic acid solution.

	Absorbance at 314 nm of culture filtrates after 5 days incubation.		
	Replicate samples		
	I	II	III
Control		2.00	
September sample			
F1 Surface sterilised	1.43	1.49	0.56
F1 Washed	0.18	0.24	0.16
F2 Surface sterilised	1.60	1.42	1.78
F2 Washed	0.10	0.09	0.10
December sample			
L Surface sterilised	0.60	1.27	1.26
L Washed	0.28	0.14	0.10
F1 Surface sterilised	1.08	0.96	1.00
F1 Washed	0.14	0.34	0.38

APPENDIX 5

Incubation of Pine needles removed from soil in Ferulic acid solution.

Needles	Absorbance at 314 nm of culture filtrates after 7 days incubation. Replicate samples I	II	III
HERMITAGE WOOD			
Non-autoclaved	0.92	0.13	1.39
Autoclaved	1.38	1.64	1.66
EXPERIMENTAL GARDENS			
Non-autoclaved	1.34	1.56	1.26
Autoclaved	0.86	0.34	0.17

APPENDIX 6

Incubation of soil samples in Ferulic acid solution

Origin of Soil	pH of suspensions and filtrates after five days incubation Nature of samples		
	Air-dried	Washed	Fresh
			Soil suspensions
Hermitage Wood	4.6	4.3	4.7
Experimental Gardens	5.1	4.8	5.1
Bezange	4.6	4.5	-
Sainte-Marie	4.8	4.8	-
			Filtrates
Hermitage Wood	4.9	4.4	4.8
Experimental Gardens	5.1	4.9	5.2
Bezange	4.6	4.6	-
Sainte-Marie	4.6	4.9	-