ECOLOGICAL STUDIES ON MICROBIAL DETERIORATION OF UNTREATED AND COPPER-CHROME-ARSENATE TREATED WOOD EXPOSED TO THE SOIL

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SUMMARY

Respirometry was employed to quantify the activity of microorganisms invading sapwood buried in the soil. Activity was related to the effect of extractable materials on the rate of colonisation of sapwoods of Pinus sylvestris (Linn) and Tilia vulgaris (Hayne). Extracted wood samples exposed to the soil respired more than those not extracted. Oxygen uptake in extracted blocks impregnated with some simple sugars and amino acids was greater than in control blocks. The ecological relevance of these studies were described in relation to early colonisation.

Differences in the subsequent decay of wood blocks as determined by weight loss was related to wood porosity and lignin content. The effect of partial delignification of wood on the rate of colonisation and decay was investigated.

Microscopy and respirometry were used to study the distribution of microbial activity in the invaded wood. In scots pine, respiratory activity as well as cavity formation was found to be greatest in the latewood. No preferential decay of the early or latewood was found in lime but the presence of both erosion form of attack and typical soft rot cavities were noted.

In CCA-treated wood oxygen uptake was positively correlated with weight loss both after four and eight weeks of soil burial. This correlation, however, was less perfect after eight weeks. The toxic limit of CCA in wood was found to be dependent upon the period of incubation. CCA inhibited cellulase activity in pure cultures and it was suggested that cellulase inhibition could be one of the

modes of action of the preservative.

Changes in pH, moisture content and respiratory activity in wood at different depths with period of incubation were investigated.

Treated wood extracted with culture filtrates of selected micro-fungi decayed more than controls and it was suggested that these fungi may also account for loss of the preservative from wood.

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CONTENTS

CHAPTER 1

Chapter 1.

General Introduction and Literature Review

The ecology of microorganisms invading wood in contact with the soil has received some attention in the recent years. Most of these investigations were primarily concerned with studies on the techniques of isolation (Greaves and Savory, 1965; Okigbo, 1966; Levy, 1967; Grant and Savory, 1969 and Butcher, 1970) and on the determination of successional trends of the microorganisms involved during the process of colonisation (Corbett and Levy, 1963; Merrill and French, 1966; Ueyama, 1965; Kaarik, 1967; Butcher, 1968 and 1970; Banerjee and Levy, 1971 and Sharp and Levy, 1974). Some aspects of the environmental and nutritional factors (Savory, 1954; Duncan, 1960; Levy, 1962; Corbett, 1963; Sharp and Eggins, 1970 and King, Oxley and Long, 1974), as well as antagonism and association of the organisms (Shields and Atwell, 1963; Hulme and Shields, 1972; Jacquiot, 1968, Greaves 1970 and 1972; and Sharp and Eggins 1970), have also been studied with the view to obtain a better understanding of the process of wood invasion. Wilcox (1970) has reviewed the literature on anatomical changes in wood cell walls attacked by fungi and bacteria and suggested various lines of direction for further ecological research.

While several attempts have been made and others are still being made to understand the ecology and physiology of microbial colonisation of wood in contact with the soil, there is still a dearth of information on the subject. The role which microfungi play in the deterioration of wood treated with a preservative and placed in contact with the soil, for

instance, is still not yet comprehensively clarified, nor has the activity of microorganisms present in a decaying piece of wood at a particular time been properly quantified.

i. Assessment of microorganisms in wood

As one of the most important pre-requisites to a clearer understanding of wood invasion, different techniques of isolation of microorganisms have been developed by several workers. In recent papers Levy (1971) and Butcher (1972) discussed the difficulties associated with such techniques particularly the problems concerning numerical assessment of microorganisms in wood. Up to date two approaches have been adopted; first, qualitatively, where the organisms were considered on a presence or on an absence basis and secondly, quantitatively, where the isolates from a known volume of a piece of wood were expressed numerically and subjected to a statistical analysis. (Butcher, 1972).

Many fungi, however, are not readily isolated from test blocks despite the fact that they may be frequently observed on wood. Others do not normally fruit under laboratory conditions in pure cultures and this may account for their low frequency recorded from timber when only isolation techniques have been adopted. Besides, different distribution patterns in wood may influence the chances of isolating particular groups (Butcher, 1972). The influence of isolation techniques and culture media on the number and type of microorganisms isolated have been discussed by Greaves and Savory (1965), Kerner-Gang (1966), Grant and Savory (1968) and Banerjee and Levy (1970). Chou, Preston

and Levi (1974) employed anatomical studies to quantify the degree of fungus colonisation which they expressed as the percentage of tracheid segments which contained hyphae over the total number of tracheids examined per unit area of the section. Harley (1971) considered that the methods used in studying microorganisms in ecological situations must combine techniques of isolations and every effort be made to distinguish those which are present as dormant spores from those present as active vegetative mycelia. Nevertheless, the use of different isolation techniques and several media have resulted in the tabulation of a large number of microorganisms colonising wood. The broad outline of fungal succession on timber in contact with the soil has thus been established. Corbett and Levy (1963) suggested the following pattern: Moniliales group I, which includes Trichoderma viride; Penicillium spp and Botrytis spp. These are followed by the Sphaeropsidales which include several soft-rot fungi, and then the Moniliales group II including Gliocladiopsis and Cylindrocarpus spp and finally the basidiomycetes. A similar pattern has been described by Merrill and French (1966). Other patterns have also been reported but they differ in such minor details as the fungal species. Butcher (1971) compared colonisation of preservative treated and untreated pine stakes and found that the pattern of succession was similar in both stakes in the below-ground zones except that colonisation was slower in the treated stakes and fewer fungi were detected.

These taxonomic successions have been thought to be reflections of a biochemical sequence because the order

in which the components of a complex material such as wood and other plant parts are degraded starts from soluble sugars through hemicelluloses and celluloses to lignin (Garret, 1951, quoted by Griffin, 1972). Whatever the names of the organisms involved in this sequence may be, the different functions they all perform lead to ultimate destruction of the wood. Butcher (1968) believed that in addition to characterizing the changing fungal flora, it is equally important to know whether the fungi isolated were in physiologically active or inactive state within the wood and continued lack of this essential information would mean that a particular organism might be assigned more importance than is warranted. More attention should therefore be placed on the build-up of activities of microorganisms in wood rather than on their apparent continued presence. Therefore, in addition to periodic isolations and anatomical studies on the distribution of microorganisms in wood, it is desirable to obtain measurements of either oxygen uptake or carbon dioxide release to provide further information on the build-up of activities of microorganisms in wood cells.

ii Factors affecting initial colonisation and build-up of microoganisms in wood

Information on factors which influence colonisation of wood in contact with the soil is not sufficiently documented (Garrett, 1955; Savory, 1955; Cartwright and Findlay, 1958; Duncan, 1960; Findlay, 1965; Butcher, 1970; and Banerjee and Levy, 1971). Besides factors

such as suitable soil temperature, moisture content of the wood and the soil, and adequate aeration of the soil, the one noteworthy factor which influences early invasion and survival of the organisms is the availability of nutrients.

a Nutrients in wood and initial colonisation

Banerjee and Levy (1971) reported that the presence or an absence of surface nutrients is an important factor which affects colonisation of the surface and eventually below the surface layers. They found that the number of fungal species present on the surface of posts reached a climax by the fourth and fifth months of exposure and thereafter declined and explained that with the depletion of nutrients, not only is the activity of existing "sugar" fungi in the surface layers greatly curtailed, but also, the surface layers are rendered uninhabitable for new fungi; which generally colonise the wood by deposition of spores. King, Oxley and Long (1974) have shown that during drying of wood soluble nutrients become deposited in the surface layer cells and this increased colonisation and decay of this layer by soil microorganisms. Very recently Long (1975) has shown by paper chromatography that there was accumulation of larger quantities of simple sugars in the surface layer cells of laboratory dried wood planks and smaller quantities from the interior cells of the same plank.

Nutrients also play a major role in the susceptibility of different timber species to microbial attack. The sapwood of hardwoods, for instance, is known to be more susceptible to softrotting than that of softwoods (Duncan, 1960; Levy, 1965). This phenomenon is reflected in the

general observation that preservatives are usually significantly less effective in the sapwood of hardwoods than in the sapwood of softwoods (Smith, 1971). Whether these observations are due to differences in the nutritional status, toxic contents, if any, or in the molecular structure of the wood cell walls is not yet clarified. Smith (1969) who reported that there was a greater attack by soft-rot fungi on hardwoods treated with copper-chrome-arsenate than on softwoods and found that this difference was less noticeable when creosote was used to treat the different species, suggested that the location and fixation of the preservatives in the wood could be contributory factors responsible for this difference.

Differences in the amount of the lignin content and its association with the cellulose have also been suggested (Wilcox, 1968).

Bailey, Liese and Rösch (1968) suggested that the varying ability of individual organisms to degrade the different hemicelluloses in hardwoods and softwood may be an important factor in determining their capacity to utilise such woods. Whilst this appears to be so George, (1973)(quoted by Levy, 1973), Takahashi and Nishimoto (1973) in Japan, have found that xylose, which occurs to a greater extent as part of the hemicellulose content of hardwoods, and mannose, occurring in the hemicellulose of softwoods, were equally utilised as the carbon sources in pure cultures by Chaetomium globosum, a fungus which causes considerable soft rot in hardwoods. Mannose utilisation, therefore, cannot be responsible for the greater resistance of softwoods to soft rot. Further studies on the biochemistry of wood decay will, no doubt, be necessary to explain these phenomena.

From the point of view of wood preservation, however, it is important to understand the factors that influence the initial resistance of an untreated wood species before applying the preservative since DaCosta and Osborne (1968) reported that the nature of timber substrates appear to have some effect on the subsequent performance of wood preservatives.

b Moisture content of wood and its colonisation

As a general rule decay of wood can occur only if its moisture content exceeds 20% of its oven dry weight and severe damage may result when the moisture content is above the fibre saturation point, that is about 30%. Water, apart from its need as a reactant in hydrolytic reactions, is also required for creating a continuum between the invading organisms and the wood substrate and within this, diffusion of enzymes to the site of reaction occurs. By causing swelling of the wood polymers, water enlarges the openings that are almost closed in the dry wood. Physical exclusion of water from wood has therefore been long recognised as a means of controlling decay, and the use of water repellent additives in wood preservation has also received much attention (Belford, 1968). Nevertheless, little is known about the minimum moisture requirements of fungi causing soft rot, but laboratory and field observations indicate that for rapid attack many soft rotters require more moisture than do most decay fungi (Savory, 1955 and Duncan, 1960). Butcher (1968) pointed out that differences in the moisture content of wood above ground, ground line and below ground zones appeared to be one of the main factors influencing the course and the speed of colonisation

in those regions of test stakes. He explained that timber above ground was too dry for the establishment of soft rot fungi and basidiomycetes whereas the high moisture content in the below ground zones favoured the development of soil-borne moulds, and soft rot fungi. Yazawa (1960) (quoted by Ueyama 1966) has pointed out that Ceratocystis moniliforme can tolerate a high moisture content (up to 80% of the oven dry weight) in the timber it invades, while the slow growing decay fungi are limited by a moisture content of 65-70%. In the course of time, however, with a decline in concentration of available nutrients and a corresponding drop in moisture content the substrate becomes more suitable for growth of cellulose and lignin decomposing fungi which become dominant. Thus, in terms of colonisation of wood, changes in moisture content and decline in readily available nutrients have been considered as two of the major physical factors which determine succession of fungi on timber in ground contact. These factors invariably influence the total microbial activity and the consequent decay of wood.

c pH relations and wood colonisation

The role that pH plays in the colonisation of wood has not been examined. It is, however, apparent that soil pH influences the growth of fungi. Duncan (1960) noted that generally soft rot organisms grow at pH4-5 and reach their maximum rate at pH6-7 but a pH of 9 has a retarding effect on their growth. Cartwright and Findlay (1958) wrote that fungi may change the pH of their substrate possibly in the direction most suited to their growth and the extent of this change varies according to the species of fungus. Since the

fungicidal activity of many wood preservatives is pH dependent (Wessel and Adema, 1968), such changes may affect the protective ability of the preservatives. Butcher (1968) did not find any trend in pH changes in wood in relation to frequency or type of organisms isolated but Good, Basham and Kadzielawa (1968) associated stain reaction in wood by certain members of the Fungi Imperfecti and bacteria with high pH, high moisture content and relatively high respiratory activity. They observed that as the stain is destroyed the pH and the water content fell and the primary decay organisms became dominant. It has not been determined whether colonisation by sapstain fungi lowers the pH of the wood substrate to inhibit the growth of other fungi but permit the development of basidiomycetes.

No conclusive correlation can therefore be made between the pattern of succession and pH of wood. In preserved wood, however, a record of the pH changes might help in some way to understand the mechanisms involved in its failure over a long period of exposure to the soil microflora.

d Soil temperature and microbial colonisation of wood

Literature on the effect of temperature on the rate of microbial colonisation of wood in soil contact is almost lacking. Temperature, as a general rule affects the growth rates of different organisms in different ways. It might therefore be reasonable to mention that the rate of wood colonisation and the variety of organisms invading the wood will be markedly affected. In the tropics and subtropical regions where soil temperatures are usually high throughout the year, activities of microorganisms invading wood in soil contact would be profoundly affected. This, coupled with high

humidities, would provide ideal conditions for the development of microorganisms on wood especially in the immediate vicinities of the soil surface where soft rot organisms are most active. Changes in temperature have not been related to successional trends in the colonisation of wood but Duncan (1960) noted that soft rot organisms tolerate a fairly wide range of temperature optima.

iii Biotic factors

Colonisation of wood in soil contact is also affected by the presence and activities of other organisms. Some fungi, notably, Trichoderma viride, Gliocladium spp and Penicillium spp have been shown, in vitro, to be quite effective in inhibiting the growth of some wood decay organisms (Kerner-Gang, 1970) and behave similarly when inoculated onto wood (Shields and Atwell, 1963, and Etheridge, 1971), possibly as a result of their rapid depletion of simple nutrients (Hulme and Shields, 1970) or their abilities to grow at faster rates than other wood invading organisms or because of their secretion of inhibitory substances. Bacteria and some actinomycetes have also been reported to produce antifungal compounds (Greaves, 1970).

Whilst these observations were based on experiments using pure cultures, the behaviour of the organisms may vary in their natural habitats; for it is now clear that the activities of microorganisms and their interactions can be profoundly affected by the physical and chemical framework together with the biological conditions within the soil (Harley, 1971).

The introduction of a piece of wood into the soil creates an ecological situation. The small crevices provided by the aggregates of soil inadequately adhering to the wood surfaces constitute different ecological niches. What, for instance, happens at the soil-wood interface? Do nutrients leach out from the wood surface cells into this area to promote germination of dormant spores, activate the hyphae and other microbial propagules and hence cause their subsequent invasion of the wood? Or, alternatively, do nutrients move from the soil into the wood and thereby help to alleviate the deficiences (especially nitrogen) which characterise wood? Toole (1970) found that the sapwood of pine promoted germination of spores and germ tube elongation to a greater extent than the sapwood of sweetgum and suggested that the wood substrate may have some effect on the initiation of germination of basidiospores.

Knowledge on the ecological factors which govern decay of wood in contact with the soil is still not adequate and further information is therefore needed.

iv Influence of preservatives on intial colonisation

Petrenko (1969), in discussing the role of bacteria and fungi in the decomposition of preserved wood in ground tests, pointed out that wood preservatives washed out of the wood accumulate in the biologically active upper layers of the soil thus creating a protective zone which prevents microorganisms from reaching the wood specimens. With the passage of time, due to the action of physical and biological factors, this protective capacity diminishes and microorganisms gain access into the wood. Concentration of preservatives at the surface layer of wood, no doubt, affects colonisation and decay. The migration of preservatives to the surface layer of treated wood during drying has been investigated by Smith and Cockcroft

(1961). In practical terms such migration appears to be desirable because it fortifies the groundline zones against microbial and insect attack. When cracks or splits do occur in the wood, however, the interior layers containing lesser amounts of preservative become accessible to attack and deterioration of the wood quickly occurs.

The role that initial colonisers play in a preserved wood has yet to be clarified. Duncan (1960) tabulated some data on the tolerance of mould and staining fungi to multi-salt preservatives and Butcher (1971) also noted that mould fungi can withstand high concentrations of copper-chrome arsenic preservatives. Soft rot fungi, both Ascomycetes and Fungi Imperfecti, have also been reported to be tolerant to preservatives (Savory, 1955; Duncan, 1960; Da Costa and Kerruish, 1963; and Gorshin and Krapivinia, 1969). Tolerance is also well known for some basidiomycets (Da Costa and Kerruish, 1964 and Eusebio, 1964) but in general these fungi are more sensitive to preservatives than the soft rot fungi.

It is now being suggested that soft rot fungi together with mould and some staining fungi act in some ways to modify the preservative, lower its effectiveness and allow the entry of less tolerant decay fungi (Duncan and Deverall, 1964). It has also been found that fungi, especially basidiomycetes, colonising wood secrete large amounts of organic acids which react with the bound preservative and render it more soluble or less toxic (Levi, 1969; Chou, Preston and Levi, 1974). A search through the literature available did not reveal any comprehensive report on the mechanism that exists within these early colonisers which enables them to evade the toxic action of the preservatives since most of these compounds

are either inhibitors of oxidative phosphorylation or are enzyme inactivating agents. It is probable that initial colonisers of treated wood adopt shunt metabolic pathways in order to evade the toxic action of the preservatives and consequently produce large amounts of organic acids which react with the preservatives to detoxify them. Detoxification of preservatives could also result from oxidation by enzymes, particularly the oxidases (Lyr, 1963).

Cserjesi (1967) investigated the tolerance of Cephaloascus fragrans to pentachlorophenol in relation to different strains of the fungus and found that one isolate which originally had low tolerance became adapted to higher concentrations of the fungicide and remained as tolerant as the other isolates of the same species. Similarly, continuous presence of an organism on a preservative treated wood may result in it becoming adapted to the preservative.

It is evident from the above discussion that moulds, staining fungi and soft rot organisms play a part in the failure of wood treated with preservatives and any further information on them will help to understand the problems associated with the protection of timber in use.

v Aim of the present investigation

In the studies on the ecology of microorganisms colonising wood in contact with the soil, much attention has been given to isolation of fungal species and determination of successional trends. Research on the quantitative distribution of the activities of such organisms in relation to environmental factors and nutritional status of the wood substrate has been somewhat neglected. The work set out in this thesis, therefore, attempts to employ respirometry as a direct method to

quantify the active microbial population in an invaded wood and to use this method to extend information on the effect of some nutritional and anatomical factors and on the influence of low concentrations of copper-chrome-arsenate preservative on initial colonisation and subsequent decay of the sapwoods of Pinus sylvestris and Tilia vulgaris.

Chapter II

Materials and General Methods

a Wood species

Sapwood of Pinus sylvestris (Linn) and Tilia vulgaris (Hayne) were used for this project. Pine was obtained from a fifty year old tree from the New Forest in the United Kingdom. After felling, three feet bolts were cut from the log at breast height level, quarter-sawn into planks and covered with polythene sheets. They were transported to the laboratory and some were immediately stacked in a ventilated oven maintained at a temperature of 50°C for drying. Those that were not immediately required were stored in a deep freezer and when needed they were removed, defrosted and dried as before. Lime, Tilia vulgaris, was obtained from the Building Research Establishment, Princes Risborough Laboratory, in the form of planks already dried. These were, however, dried again before conversion into test blocks. They have been described as air seasoned samples in this work. At a later stage in the course of this project fresh lime (green) planks were obtained from the same source as above. The difference between these and the already seasoned samples with respect to initial colonisation have been discussed in Chapter 3. In both cases wood which was exclusively sound and without knots and has straight grain was employed for test block preparation throughout this project. In the case of pine those with little resin were chosen.

b Conversion of dried planks into test blocks

The dimensions of the test blocks used varied in the different experiments set up and these will be described in the relevant sections. Generally, the planks were converted into test blocks with a small laboratory band saw and further planed to give the correct sizes required. Particular attention was paid to blocks which were treated with preservative solutions since non-uniformity of test block dimensions could affect preservative retentions.

c Extraction of test blocks

Extraction with warm distilled water

Blocks were first impregnated with warm distilled water under vacuum and placed in conical flasks containing 150 mls of distilled water. The flasks were attached to a flask shaker and set up in such a way that the former were half immersed in water baths thermostatically controlled at 30°C. This set-up was very convenient because shaking could be carried out at higher speeds. Shaking was carried on for two weeks, the water in the flasks being changed twice daily for the first week and once daily for the second week. When loss in weight as a result of extraction was to be known, the same blocks were dried to constant weights in the oven set at 80°C for two days before and after extraction and the percentage loss in weight was calculated upon this basis.

d Hot water extraction

Severe extraction in hot water was carried out with the soxhlet leaching apparatus as described in the BS 838.

Extraction was carried out for 48 hours and the blocks were dried as before.

c Solvent extraction

Successive extraction using the soxhlet apparatus was carried out with the following solvents in the following order:

Ethanol:Benzene (1:2)	24 hours
Ethanol	24 hours
Distilled water	24 hours

After extraction the blocks were further impregnated with hot distilled water ($70^{o}C$ - $80^{o}C$) under vacuum to enable the cells to swell and then washed with further portions of the hot distilled water. This was done to avoid any traces of the solvent being trapped in the cell walls. They were dried as before.

f Treatment with copper-chrome-arsenate preservative

The C.C.A., Tanalith CT 106, used for this work was supplied by the Building Research Establishment, Princes Risborough Laboratory. It was analysed by Dr. J.W.W. Morgan who calculated the nominal composition to be:

$CuSO4.5H_2O$	31.4%
$K_2Cr_2O_7$	43.4%
$AS_2O_5.2H_2O$	20.6%
Total salts	95.4%

This fell within the minimum requirements of BS 4072 specifications. Serial concentrations of the treating solution were prepared on mass/mass basis with distilled

water. The test blocks were impregnated with the solutions using the vacuum desiccator described in BS 838. The set-up was, however, slightly modified. A separating funnel containing the treating solution was fitted to the desiccator using a quickfit adaptor having a vent on its side. The vent was joined to a vacuum rubber tubing leading to a valve to which was fitted a pressure gauge. The test blocks were packed and weighted down with a piece of metal in a plastic container centrally placed in the desiccator. Vacuum pressure was created in the desiccator by attaching firmly the vacuum valve to a water ejector until the gauge registered a vacuum of 25 in (i.e. about 4 in pressure of mercury). The stop cock on the tubing was turned off to separate the vacuum source. The treating solution was then run through a plastic tubing attached to the lower end of the separating funnel.

The residual vacuum was released slowly and the blocks were allowed to soak for 2 hours. They were then lightly dried on filter papers and weighed. The amount of preservative solution absorbed into the blocks was calculated from the original dry weights before treating and the final weights after treating. Control blocks were impregnated with sterile distilled water. The retentions of the preservative in the blocks were calculated upon the following basis according to I.R.G. document No. 229:

$$\frac{W \times 100 \times C}{N \times V \times 100} = kg/m^3$$

where:

W = total absorption of the preservative in grams

C = concentration of preservative solution (% mass/mass)

n = number of replicates

V = volume of each block in cu cm.

g Fixation of the preservative and drying of treated test blocks

Treated blocks were open-piled on glass rods in a single layer in covered glass vessels. A small dish containing xylene was placed in the vessel to prevent mould growth on blocks treated with lower concentrations of the preservative. They were kept wet in the closed containers for 2 weeks in the laboratory to allow the preservative to fix into the wood cell walls. To allow for equal distribution of the preservative in the wood, the test blocks were turned on their sides once every day throughout the fixation period. At the end of two weeks the lids were slightly opened on the first day to allow drying of the blocks to begin. On the second day they were placed on glass plates and allowed to dry under normal laboratory temperature conditions (between 18-20^{o}C). Turning of the blocks on their sides was continued until they were dried.

h Source of soil

The soil used in this work was also obtained from the Princes Risborough laboratory. It was a sandy loam and was removed from the same site as samples used by Smith (1971). Its water holding capacity was determined by the method described in the IRG document No. 208. In all the soil burial studies described in this thesis, the soil was maintained at this capacity, namely 30%. It was checked periodically by weighing the culture containers and spraying the soil surface with sterile distilled water to make up for any loss.

i Culture containers for soil burial

Rectangular plastic containers (22 cm x 15 cm x 10 cm) length x width x depth) with lids were employed for soil burial. Each container was filled with 6 to 7 Kg of soil. The number of test blocks per container varied throughout the project since this depended on the dimensions of the test blocks. Generally, however, it varied from 6 to 9 blocks per container for the larger sized blocks (15 cubic centimeter) and 20 to 30 blocks for the smallest sized blocks (one cubic centimeter). In all cases the blocks were buried at least 3 cm apart and the upper surfaces were not less than 4 cm below the soil surface. They were orientated so that the transverse face was horizontal with the soil surface. The soil was not amended with nutrients and reliance was placed on the natural soil microflora for infection. A batch of soil was used only once.

j Incubation temperature

Culture containers were placed in incubators maintained at a temperature of $30^{o}C$ throughout the period of incubation. Shallow vessels containing water were placed at the bottom of the incubators to keep the humidity very high around the soil surface. The period of incubation varied and this will be mentioned in the specific experiments.

k Assessment of Results

Measurement of Respiration

Test blocks were 'exhumed' and wiped clean of soil particles and any fungal growth at the completion of incubation. Warburg respirometers in a circular water bath

were employed in all the determinations of oxygen uptake and hence of aerobic microbial activity in the deteriorating test blocks. The temperature of the bath remained constant at 30°C throughout the determinations. The main compartment of each flask held wood specimens cut approximately into 5 x 10 x 2 mm (tang x long x rad) billets. This dimension ensured rapid exchange of gas within the wood cells. The central well contained 0.5 mls of 20% potassium hydroxide and a small roll of filter paper to increase the absorbing surface. A thermobarometer was included in the determinations and this measured the changes caused by the atmosphere. A correction was made for the actual readings with the thermobarometer. After an initial 2-hour period during which the system was left opened to allow the test specimens and the flasks to attain to the temperature of the bath, the outlets were closed and thereafter readings were taken each hour. Usually four readings were taken. The gas exchange was calculated in the standard manner (Umbreit, Burris and Stauffer, 1964).

After measuring their respiration the volumes of wood pieces used per manometer flask were determined by displacement of water in a graduated cylinder and in a 50 ml pipette closed at one end, and especially adapted for this purpose. The wood pieces were first saturated with water by vacuum impregnation to enable them to sink. In some experiments accurately cut one cubic centimeter wood blocks were used per manometer flask and the above determination was not carried out. Rates of oxygen consumption were calculated from the mean of the third and fourth hour readings at which time the mycelia growing in the wood blocks respired actively. The results were expressed as µl of oxygen/hour/cubic centimeter of wood.

Respiration per volume of wood rather than per weight was used because in comparing activity at different stages of wood colonisation, the important criterion is what happens in a specific volume of wood and not in a specific weight, since the latter is dominated by moisture which is variable. Even the dry weight which could be calculated is undesirable as a base because it declines as wood substance is lost and hence gives a false impression of increasing respiration rate.

Since contrasting views on the use of respiration to study decay and preservative assessment in wood have been reported, it was thought necessary to perform some preliminary studies to assess the suitability of the Warburg manometer and reproducibility of results in the measurement of oxygen uptake in wood invaded by soil microorganisms.

1 Isolation and culturing of fungi

The isolation method employed was the drill technique described by Levy (1967). A 3 mm twist drill was inserted into a carpenter's brace. The wood block was clamped in a vice and the whole set-up together with the drill was swabbed with cotton wool soaked with alcohol and then flamed. A sterile petri dish was placed underneath the wood block and the drill was inserted and twisted into the wood. The resultant particles fell directly into the petri dish below. The particles were spread on nutrient agar in plates under sterile conditions. Eggins and Pugh cellulose medium and 2% malt agar were used for all the isolations. The plates were incubated at 25^{o}C and observed from time to time until all the growing fungi were identified. Those that did not

bear fruiting bodies immediately were subcultured and later identified.

Other methods specific to individual experiments are discussed in the appropriate sections of the thesis.

ii Preliminary studies on the use of the Warburg manometer to assess respiration in wood colonised by soil microorganisms

a Introduction

Several methods have been employed for determination of microbial activities in different environments but the use of respiration has been applied to very few wood decay studies. Smith (1967) and Good, Basham and Kadzielawa (1968) used a flowing gas system for collecting respired carbon dioxide in sodium hydroxide which was then measured by a conductometric method while Klingston (1965) titrated the absorbed carbon dioxide in sodium hydroxide with an acid and estimated the residual alkali to give a measure of the activity of fungi in decaying wood blocks. Good and Darrah (1967), Halabisky and Ifju(1968) and Behr (1972) used the Warburg respirometer to estimate oxygen uptake in decaying wood and to test the toxicity of wood preservatives. By adding certain phenolic reagents to respiring decayed wood Hintikka and Laine (1970) were able to demonstrate the presence of lignin-decomposing basidiomycetes by the use of the Warburg manometer. Recently Smith (1973) described the automation of a gas chromatographic method which he developed for monitoring carbon dioxide and then applying it to study rhythms in fungal respiration in decaying wood

blocks.

For the purposes of this project, the Warburg respirometer was used because measurement of oxygen exchange provides a more direct assessment of metabolic reactions coupled to the production of adenosine triphosphate than does carbon dioxide exchange.

Respirometric assessments reported in the literature employed methods which involved, first, exposure of the wood blocks to pure cultures of fungi growing on agar or on presterilised soil and then measuring the activity after definite periods. It is, however, likely that the pattern of colonisation of the test blocks may not be the same when pure cultures are used as they will be in an unsterilised soil where the wood blocks are exposed to the natural soil microflora. The minimum period of exposure of the test blocks to obtain measurable values on the Warburg respirometer may therefore vary with the culture medium as well as the wood species used and the type and concentration of the preservative under test. Behr (1972) found that three weeks exposure period was inadequate to obtain measurable values (5.6 µl of O_2/hr) on the respirometer when Poria monticola was exposed to wood blocks treated with 0.11 pounds per cubic foot of copper-chromium arsenate preservative, whilst it was most appropriate to obtain respirometric values (67µl of O_2/hr) when Lenzites trabea was exposed to wood blocks treated with 0.058 pounds per cubic foot of pentachlorophenol. When Lentinus lepideus was exposed to wood blocks treated with 1.4 pounds per cubic foot of creosote the amount of oxygen respired when measurements were taken after 3 weeks was 128 µl per hour. The measurable value on the respirometer therefore depends not only on the period of

exposure to the fungi but also on the species of fungi, the type and concentration of the preservative and the conditions under which the test blocks are exposed.

Halabisky and Ifju (1968) noted that the Warburg respirometer was a more sensitive technique for the detection of fungal activity in wood than the weight loss method. Behr (1972) used the Warburg to determine the threshold values of creosote and pentachloropherol but was not successful with copper-chrome-arsenate. When Smith (1967) used the conductometric method to measure carbon dioxide release in wood blocks treated with different concentrations of C.C.A., he obtained unsatisfactory results. He found that the test fungus grew in wood blocks with higher preservative retentions equally as well as in blocks with lower retentions. This was probably due to non-uniform distribution of the C.C.A. in the test blocks after drying. Smith and Cockcroft (1961) have shown that the present techniques of drying blocks impregnated with preservatives may result in a very uneven distribution of the latter throughout the test blocks with greatest concentrations occurring at the surface layers. This will, no doubt, affect toxicity levels determined by standard laboratory tests since high surface loadings could affect colonisation, hence the inconclusive results obtained by Smith.

It is therefore the intention of these preliminary experiments to:

(1) determine the period required for respiration to become measurable in wood blocks exposed to the soil microflora.

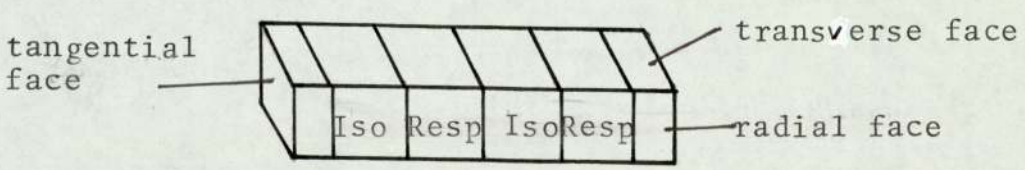
(2) assess the suitability of oxygen uptake as a measure of invasion of C.C.A.-treated blocks colonised by soil

microorganisms.

b Method

Period of minimum activity in relation to the microflora present in colonised wood

Wood blocks of both pine and lime measuring 10 x 50 x 10 mm in the tangential, radial and longitudinal directions, respectively, were prepared. All the surfaces except the two radial faces were coated with araldite, an epoxy resin, and allowed to dry. This ensured colonisation of the wood from the radial surfaces only. The wood blocks were buried in the soil and incubated as described earlier. Eight blocks per species were buried. After specific periods, two blocks per wood species were removed, the sealant peeled off and measurement of uptake of oxygen and isolations were carried out after dividing the blocks into four parts, shown in the diagram below.



The results are presented in Table 1.

c Suitability of oxygen uptake as a measure of invasion of C.C.A.-treated blocks

Wood blocks of lime with similar dimensions as above were prepared and treated with the following concentrations of C.C.A: 0%; 0.01%; 0.05%; 0.10%; 0.25%; 0.50%; 1.00%; 1.50% and 2.00% to give different retentions (Table 2). The preservative was

Table 1. Uptake of oxygen in wood blocks in relation to the microorganisms present

Days of Soil Burial	Name of Microorganism	Pine		Lime	
		Presence	Oxygen uptake in µl/hr/cc of wood	Presence	Oxygen uptake in µl
5	Mucor sp	X	3.5	X	9.6
	Bacteria	X		X	
	Fusarium sp			X	
10	Penicillium sp	X	10.2	X	14.6
	Cephalosporium sp			X	
	Humicola sp			X	
	Chaetomium sp			X	
	Fusarium sp	X		X	
	Bacteria	X			
17	Fusarium sp	X	12.9	X	20.8
	Cephalosporium sp	X		X	
	Penicillium sp	X		X	
	Chaetomium sp			X	
24	Bacteria	X	23.3		42.3
	Penicillium sp	X		X	
	Aspergillus sp	X			
	Arthrobotrys sp			X	
	Fusarium sp	X		X	
	Chaetomium sp			X	

allowed to fix into the wood and then dried. They were buried in the soil and incubated as before. Twelve replicate blocks per each concentration of the preservative were set up. At intervals of 3, 4 and 8 weeks, four blocks per treatment were removed, cleaned and their oxygen uptakes determined. The results are presented in Figures 1-4 and Table 2.

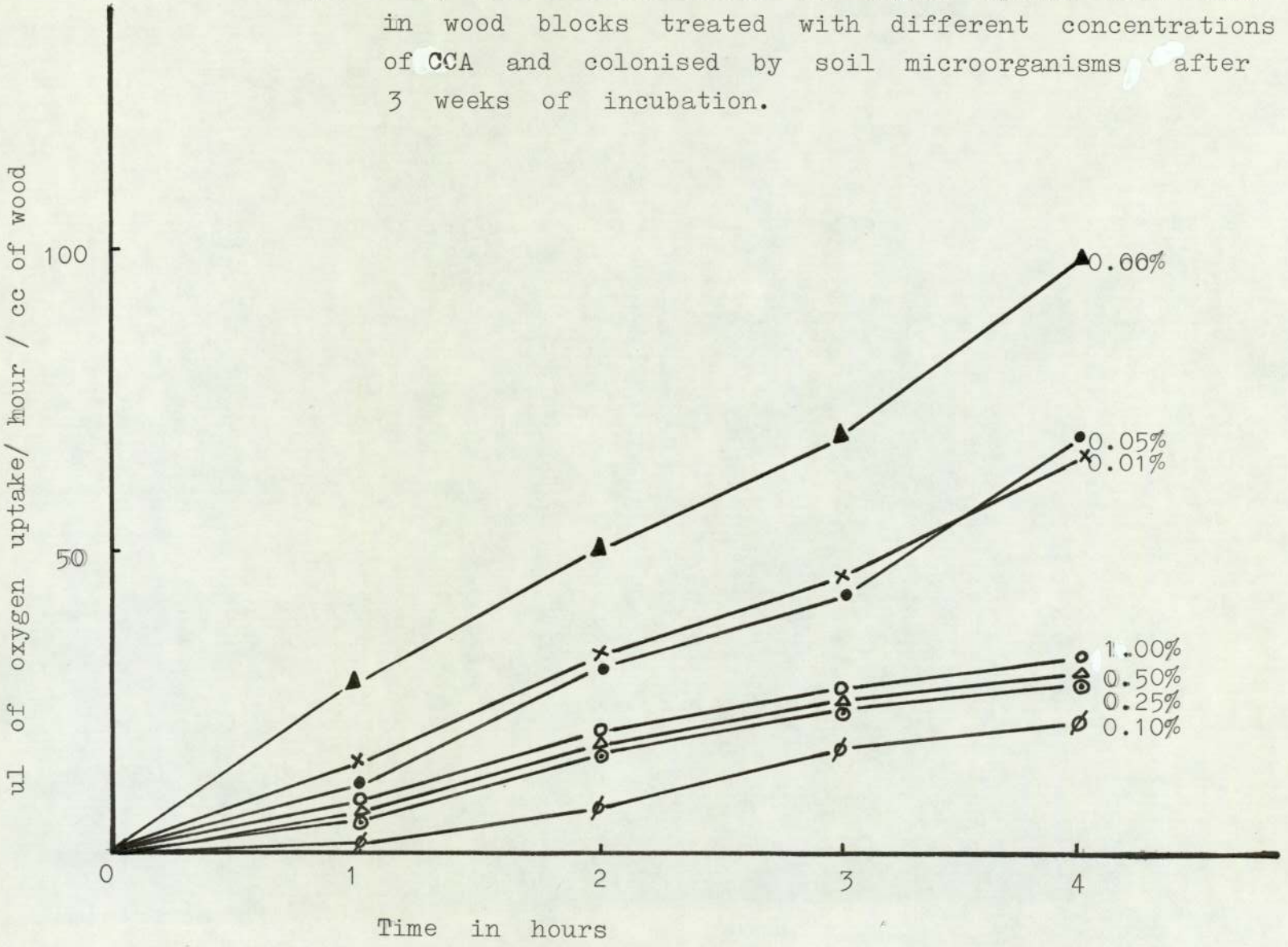
d Results

(i) The results in Table 1 show that even as early as five days of soil burial, the wood blocks were colonised and gave measurable values of respiration on the Warburg manometer. Lime was colonised more than pine and this increased with the duration of soil burial. The frequency of isolating organisms was greater on the lime blocks than it was on pine.

(ii) The results of preservative-treated blocks showed that the higher the concentration of C.C.A. in the test blocks, the lower the rates of their colonisation and the less the uptake of oxygen by the invading organisms. Figure 3 showed that there was a consistent increase in the rate of oxygen uptake at all the preservative concentrations with time except at zero and the lowest concentrations after eight weeks where there was a slight reduction. This may probably be due to the beginning of depletion of soluble nutrients within the cells and hence reduction in metabolic activities of the invading organisms. This aspect of colonisation is described later in the thesis.

Blocks treated with 1.5 and 2.0 per cent concentrations of the C.C.A. did not show any evidence of respiration when they were sampled after 3 weeks of soil burial but showed some respiration after the fourth week and this increased after

Fig.1 Progressive measurements of oxygen uptake with time in wood blocks treated with different concentrations of CCA and colonised by soil microorganisms after 3 weeks of incubation.



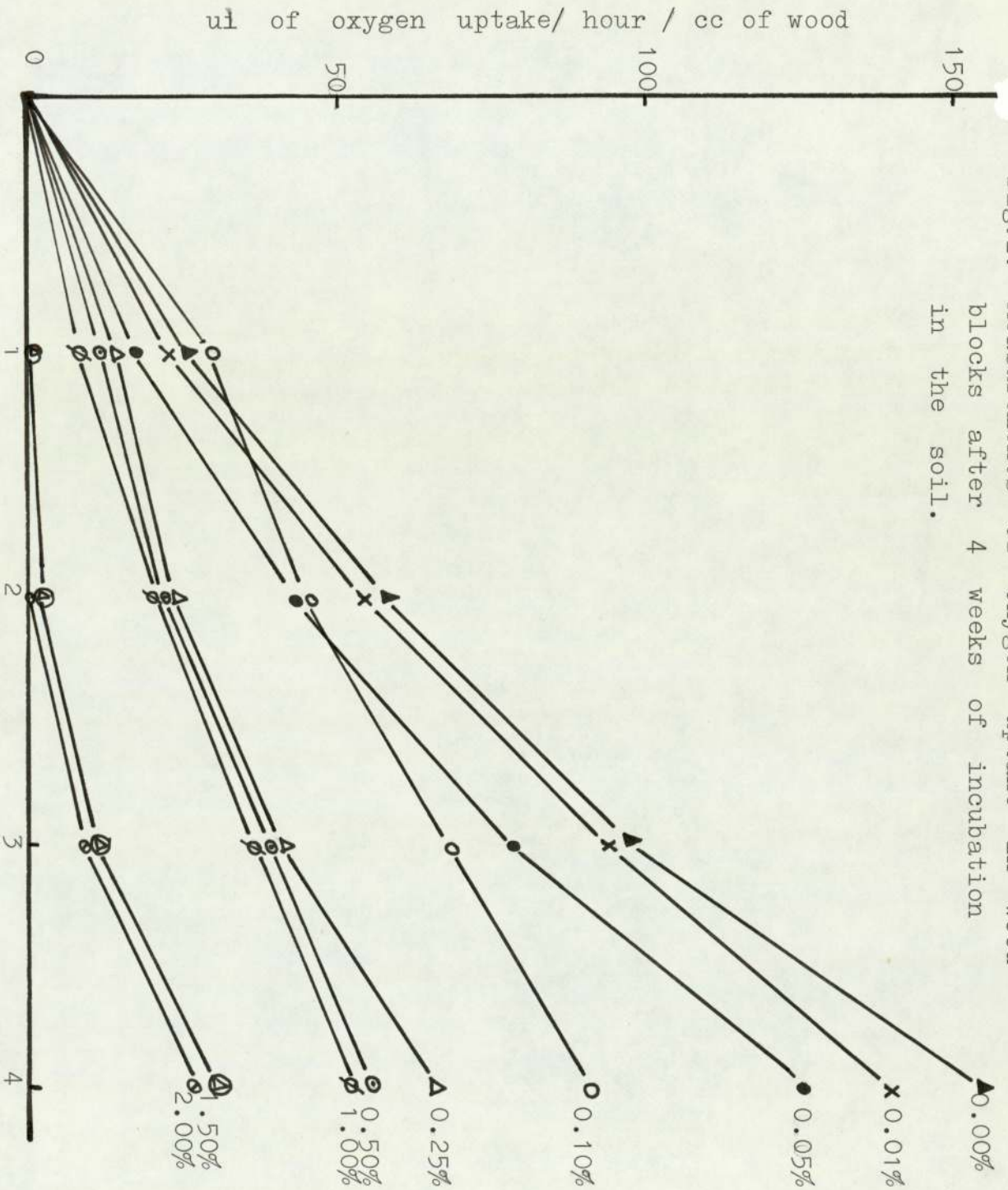


Fig.2. Measurements of oxygen uptake in wood blocks after 4 weeks of incubation in the soil.

Table 2. Rate of oxygen uptake in wood blocks treated with different concentrations of CCA and exposed to soil microorganisms for 4 weeks.

Concentration of CCA in %	Retention of kg/m^3	Rate of O_2 uptake in µl/hr/cc of wood block	mean	Standard deviation
0.00	0.00	44.0 31.4 37.2 36.5	37.2	5.20
0.01	0.08	39.4 37.6 36.5 43.0	39.1	2.18
0.5	0.39	37.1 34.4 28.9 34.1	33.6	3.38
0.10	0.82	27.3 23.5 25.4 21.8	24.5	2.94
0.25	1.50	12.6 11.8 17.6 19.9	15.5	1.26
0.50	3.96	16.9 13.2 15.6 15.6	15.2	1.54
1.00	7.80	14.1 14.2 16.1 15.1	14.9	0.93
1.5	9.60	9.6 9.4 10.3 9.8	9.7	1.23
2.00	11.50	5.3 8.0 8.0 5.6	6.7	1.46

Fig.3 Relationship between oxygen uptake and concentration of CCA in wood after different periods of incubation.

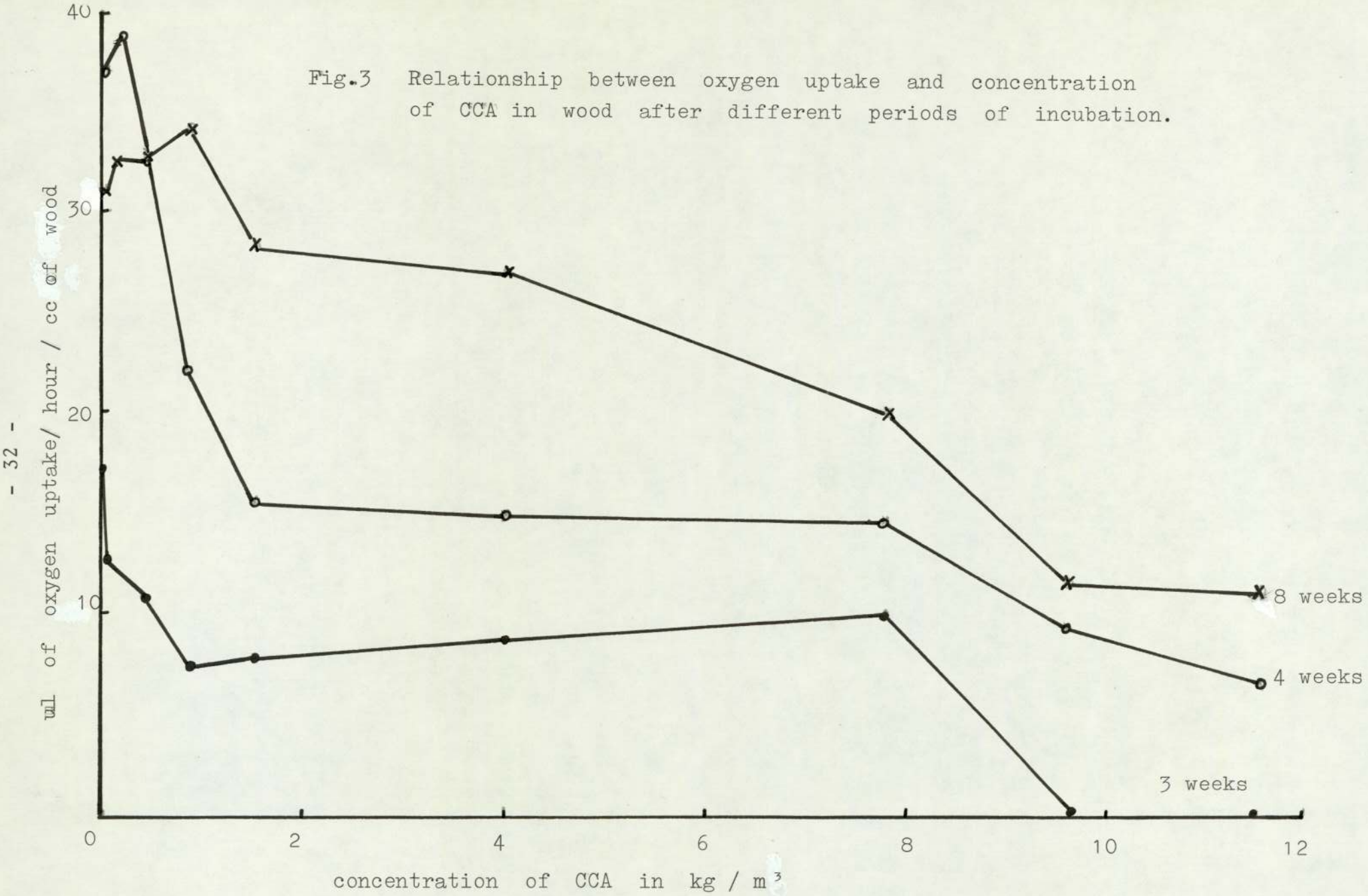
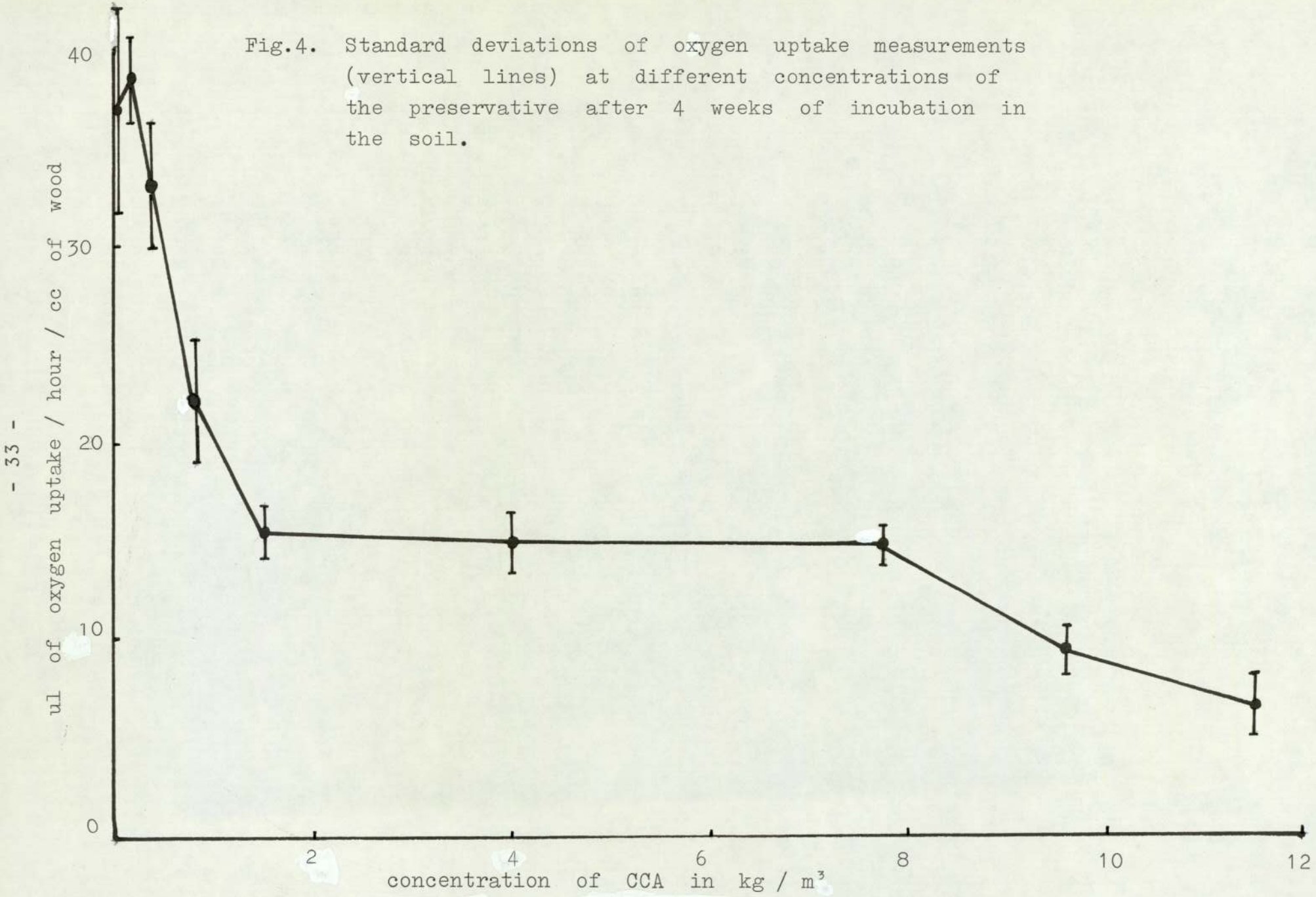


Fig.4. Standard deviations of oxygen uptake measurements (vertical lines) at different concentrations of the preservative after 4 weeks of incubation in the soil.



eight weeks. The duration of soil burial thus affected the rates of colonisation of the treated blocks. In Table 2 and Figure 4, the variations about the means are shown for each concentration after 4 weeks. It is noted that these variations are much reduced at the higher concentrations of the preservative where most probably only the more tolerant microorganisms grew and competition has been considerably reduced or became non-existent.

c Conclusions

The Warburg respirometer has been shown in these preliminary investigations to measure effectively the degree of colonisation of treated and untreated wood blocks. Although it was realised that the range of treating solution concentration used was inadequate for the length of exposure of the test blocks to soil microorganisms, yet it became evident from Figure 3 that measurement of the rate of respiration could be effectively used for preservative threshold value determinations and less time is required for this than for the more usual methods. Since all respiration, ultimately, must be at the expense of wood material, oxygen uptake determination is a measure of wood decay. The relationships between respiration and weight loss as measurements of wood decay have been described in a greater detail at the later parts of the thesis.

Chapter III Effect of wood substrate on intial colonisation and subsequent decay.

Introduction

Initial invasion of wood in ground contact by microorganisms depends, among other factors, on availability of nutrients present on the surface layers. Their subsequent establishment and survival within the wood, however, depends to a large extent on their ability to utilise the cell contents and to degrade and mobilise the structural components of the wood for growth and reproduction. That the heartwood of a timber species contains toxic and inhibitory substances which influence its susceptibility to decay by microorganisms, has long been recognised. The sapwood, however, contains very little or no such substances and the effect of its nutrient content on the growth and reproduction of organisms have also been reported in many timber species (Cartwright and Findlay, 1958). Olofinboba and Lawton (1968), for instance, identified maltose, raffinose, glucose and sucrose in the sapwood of Antiaris africana and noted that these substances were highly utilised by the blue-stain fungus, Botryodiplodia theobromae which rapidly invades the logs after felling.

Studies on the colonisation of sapwood in contact with the soil have shown that the microfungi are the first group of organisms in the succession and it is they rather than the higher fungi, basidiomycetes, that are influenced by the more easily available substances at the initial stages of invasion. Activities of the higher fungi result in substantial degradation and loss of wood substance. Methods used in estimating such losses are well established and accepted as standards. Methods for assessing initial

colonisation range from visual estimations through anatomical studies to periodic isolations and frequency determinations. The limitations of the last method have been discussed briefly in the general introduction.

Occurrence of colonisation as estimated visually may be scored by different observers either in terms of intensity or merely as growth or no growth. There is no way of standardising these estimations. Besides, in certain cases, particularly in stained wood where the surface may show no evidence of the attack as a result of rapid drying off of the outer layers after penetration, whereas the inner layers may be heavily discoloured, it is difficult to assess the degree of attack. In anatomical studies a bias may be placed on the selection of a heavily stained area for sectioning as opposed to an area with a clean surface but which otherwise has been heavily stained within.

In this investigation measurement of oxygen uptake of colonised wood and weight loss determinations have been employed to determine and distinguish between initial invasion and subsequent destruction of the wood substance by soil microorganisms and an attempt has been made to relate these to the nutritional status and anatomical differences in the species of wood used for this project.

Although oxygen uptake and weight loss give no information on the form or manner of invasion, or on the species or groups of microorganisms involved, yet what they lose in this way these measures gain by being quantitative, objective and rapid.

III:i Respiratory assessment of colonisation of extractive-free sapwood

a Method

Wood specimens measuring 5 x 50 x 10 mm in the tangential, radial and longitudinal directions, respectively were prepared from laboratory dried scots pine and air seasoned lime sapwoods. They were divided into three batches for each species. Each batch contained ten blocks. The blocks were treated as follows:

(1) Water-extracted at 30°C

(2) Solvent-extracted by the Soxhlet apparatus

(3) Non-extracted controls

Before treatment, blocks to be extracted with water and 'solvent' were dried to moisture-free weights. These original dry weights were noted and the blocks were marked. After extraction the blocks were similarly dried and the percentage loss in weight due to extraction was calculated, (table 3). Four blocks per treatment per wood species were selected at random and buried in the soil. Incubation was carried out at 30°C. After thirty days of burial, the blocks were removed, cleaned and the oxygen uptakes of the invading microorganisms were determined. The remaining blocks were kept for decay studies.

b) Results

The results presented in tables 4 and 5 show that when extractives were removed from sapwood blocks either with a warm distilled water or with a mixture of organic solvents and water, colonisation by soil microorganisms was affected

Table 3. Average and standard deviation (S.D.) of percentage weight loss due to extraction of sapwood blocks of Scotspine and Lime

Treatment	Pine		Lime	
	Mean	S.D.	Mean	S.D.
Water extraction	2.04	1.16	3.50	1.80
Solvent extraction	4.50	2.53	9.63	3.51

Table 4. Effect of extraction on the degree of microbial colonisation measured by respiration of pine sapwood buried in the soil for 30 days.

Treatment	Uptake of O_2 in µl/hr/cc of wood	mean	standard deviation
Not extracted	20.8, 19.9, 20.2, 20.0	20.1	0.17
Water extracted	14.4, 16.7, 17.8, 18.8	16.9	1.88
Solvent extracted	14.8, 11.7, 14.4, 14.2	13.8	1.40

Test of significance

	't'	Difference in O_2 uptake
Not extracted versus water extracted	8.5	significant at $p < 0.001$
Water extracted " solvent extracted	7.6	significant at $p < 0.001$
Not extracted " solvent extracted	12.6	highly significant when $p < 0.001$

Table 5. Effect of extraction on the degree of microbial colonisation measured by respiration of lime sapwood buried in the soil for 30 days.

Treatment	Uptake of O_2 in µl/hr/cc of wood	Mean	Standard deviation
Not extracted	33.9, 44.3, 46.5, 51.9	44.2	7.54
Water extracted	33.7, 42.8, 40.6, 52.7	42.4	7.85
Solvent extracted	33.6, 56.3, 39.6, 46.2	43.9	9.72

Test of significance

	't'	Difference in O_2 uptake
Not extracted versus water extracted	0.112	not significant
Water extracted " solvent extracted	0.77	not significant
Not extracted " solvent extracted	0.157	not significant

in the case of pinebut not in the case of lime. The mean oxygen uptakes in the solvent and water extracted pine were 13.8µl and 16.9µl respectively whereas the mean oxygen uptake for the unextracted control blocks was 20.1 µl. Using the student's 't' test to determine the difference between the means of two small samples, it was found that the differences in mean oxygen uptakes in non-extracted versus water-extracted; water extracted versus solvent extracted; and non-extracted versus solvent extracted pine were significantly high (t = 8.5, 7.6 and 12.6 respectively, p = 0.001). Solvent extraction removed more materials from the wood blocks than water extraction (table 3).

In the lime blocks extraction had no effect on the rate of colonisation. The mean oxygen uptakes in the non-extracted, water extracted and solvent extracted blocks were 44.2 µl, 42.4 µl and 43.9 µl respectively. The degree of invasion of the lime blocks was generally much greater than that of the pine blocks; for instance, whilst the mean oxygen uptake of non-extracted pine was 20.1 µl, that for lime was 44.2 µl and solvent extracted pine registered 13.8 µl of oxygen uptake while that for pine respired 43.9 µl of oxygen after 30 days of soil burial.

]]I:ii Progressive colonisation of extractive-free and unextracted lime sapwood

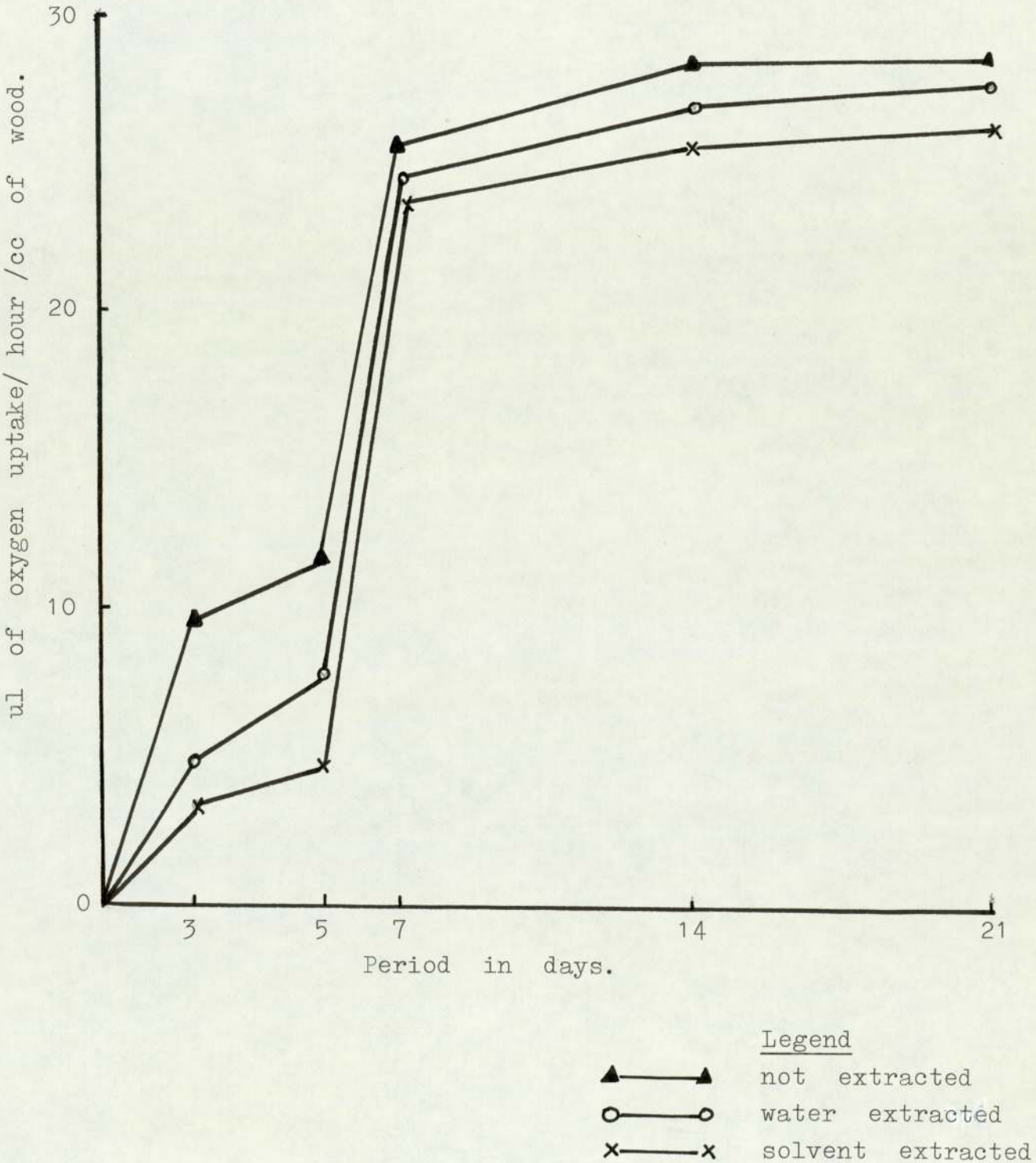
a) Method

Because of the rapid colonisation of lime blocks, it was thought that the incubation period in the soil was too long so that the effect of nutrients, if any, in the non-extracted blocks on the rate of colonisation was masked. Therefore, a fresh batch of lime blocks prepared from air-seasoned planks were treated as before and buried in the soil for 21 days. Each treatment contained 20 blocks. At shorter intervals four blocks per treatment were removed and assessed for activity in the respirometer.

b) Results

Figure 5 shows the progress of colonisation as measured by oxygen uptake of the extracted and non-extracted lime blocks. Though there appeared to be a slight difference between them, this difference was significant only up to the fifth day of measurement after which very little difference was observed. The reason for this observation could be explained on the basis of the nutritional status of the wood before conversion into the experimental blocks. The lime planks used for these experiments were obtained as already dried material and possibly have lost a great deal of their stored nutrients by wood-cell respiration during the seasoning period. The pine planks on the other hand were obtained in the 'green' state and were immediately dried at 50°C. Living parenchyma cells were therefore killed before they had

Fig.5. Effect of removal of water and solvent soluble materials from lime sapwood on the rate of initial colonisation.



time to respire away the stored nutrients. The susceptibility of pine and lime to decay in relation to structure have been described elsewhere in the thesis. Migration of soluble nutrients to the surface layer of wood during drying have been investigated and their loss during conversion of planks into test specimens was suggested by King, Oxley and Long (1974).

At a later date during the course of this project, 'green' lime wood was obtained from a freshly felled tree and it was, therefore, decided to assess its nutrient value on the rate of colonisation by soil microorganisms. This is described in the next experiment.

III:iii Further experiment on the effect of extractive-free laboratory-dried lime sapwood on colonisation by soil microorganisms

a) Method

Laboratory dried sapwood of lime was converted into 5 x 50 x 10 mm (tang. x rad. x long.) blocks. The blocks were subjected to different extraction treatments as in experiment one. The mean percentage weight loss due to extraction was 4.6 for blocks extracted with distilled water and 10.8 for solvent extracted blocks. In all fifteen blocks per treatment were prepared and divided into two batches, of five and ten. The second batch of ten blocks per treatment was kept for weight loss studies. The other blocks were buried in the soil as before. At the end of 14 days they were removed, cleaned and the oxygen uptake of the invading microorganisms were determined. The results are tabulated in table 6.

b) Results

It is evident from the results that extraction removed soluble nutrients necessary for initial colonisation. The degree of invasion of the treated blocks followed the same order as that of laboratory dried scotspine sapwood blocks subjected to similar treatment; that is to say: non-extracted blocks were the most colonised and solvent-extracted blocks were the least colonised. Using the student's 't' test it was found that the differences between the mean uptakes of oxygen of the differently extracted blocks and the non-extracted blocks were significantly high (table 6).

Table 6. Effect of extraction of laboratory dried lime wood on microbial colonisation after 14 days of soil burial.

Treatment	Uptake of O_2 in µl/hr/cc of wood	Mean	Standard deviation
Not extracted	43.8, 50.9, 47.6, 40.6, 53.7	47.32	5.27
Water extracted	22.1, 27.6, 30.1, 28.7, 31.2	27.94	3.53
Solvent extracted	17.8, 21.6, 22.4 21.6, 26.8	22.04	3.20

Test of significance

	't'	Difference in O_2 uptake
Not extracted versus water extracted	6.87	significant when $p < 0.001$
Water extracted " solvent extracted	9.22	significant at $p < 0.001$
Water extracted " solvent extracted	2.78	significant between $p = 0.05$ and $p = 0.02$

The conclusion to be drawn from these short experiments is that laboratory dried sapwood contains more soluble nutrients than air seasoned sapwood and these nutrients are responsible for the greater susceptibility of unextracted sapwood to passive colonisation by soil microorganisms.

]]I:iv Effect of addition of some animo acids and simple sugars to wood on the rate of initial colonisation

Introduction

It is generally known that when wood contains added sugar the rate of decay is sometimes greater than that of wood without added sugar (Findlay, 1941).

GÄumann(1938) (quoted by Southam and Erlirch, 1943) has shown, however, that the susceptibility of wood to decay is apparently not affected by its natural sugar content but if nitrogenous compounds are removed by leaching the rate of decay is lower in the leached than in the unleached sapwood blocks. It has also been shown that addition of nitrogenous compounds sometimes (Peterson and Cowling, 1964) but not always (Findlay, 1941) increases the rate of decay.

In the above reports, wood blocks were normally exposed to pure cultures of single decay organisms, usually, basidiomycetes. In a complex ecological situation such as is found in an unsterile soil where there will be a succession of organisms on the wood, added nutrients will influence the activity of fast growing early colonisers and will become quickly depleted by them. Therefore, the rate of destruction of the wood material may not necessarily depend on the utilisation of these nutrients by the early colonisers since many of these organisms do not cause substantial weight loss. It was therefore, decided to find out, firstly, whether addition of nutrients to extractive-free sapwood influences the rate of initial colonisation by soil microorganisms and secondly, to find out whether other factors such as the non-extractable component of wood has

more influence on decay than the natural nutrient content of the wood.

b) Method

Pine sapwood blocks measuring 5 x 50 x 10 mm (tang. x radial x long.) were chosen for this experiment. They were extracted with warm distilled water in the manner described in Chapter 2. The blocks were then dried at 80°C in an oven for 3 days after which they were impregnated under vacuum pressure with 2% solutions of either glucose, mannose, alanine or aspartic acid or a mixture of all the four solutions. Thirty blocks per nutrient solution were set up. The blocks were then slowly dried under laboratory conditions and later incubated in the soil at 30°C for five weeks. Non-extracted and extracted blocks with no nutrients added were used as controls. At weekly intervals six blocks were removed from each of the nutrient-block samples and the activity of the microorganisms in the invaded blocks were determined by the Warburg manometer.

c) Results

It is evident from the results presented in Figure 6 that all the added nutrients accelerated the rate of colonisation of the blocks. Alanine promoted the greatest growth and aspartic acid the least. Both glucose and mannose promoted lesser colonisation than alanine but more than aspartic acid. There was also a marked difference between the extracted and the non-extracted, the latter being least in supporting colonisation. Wood blocks impregnated with a mixture of all the nutrient solution

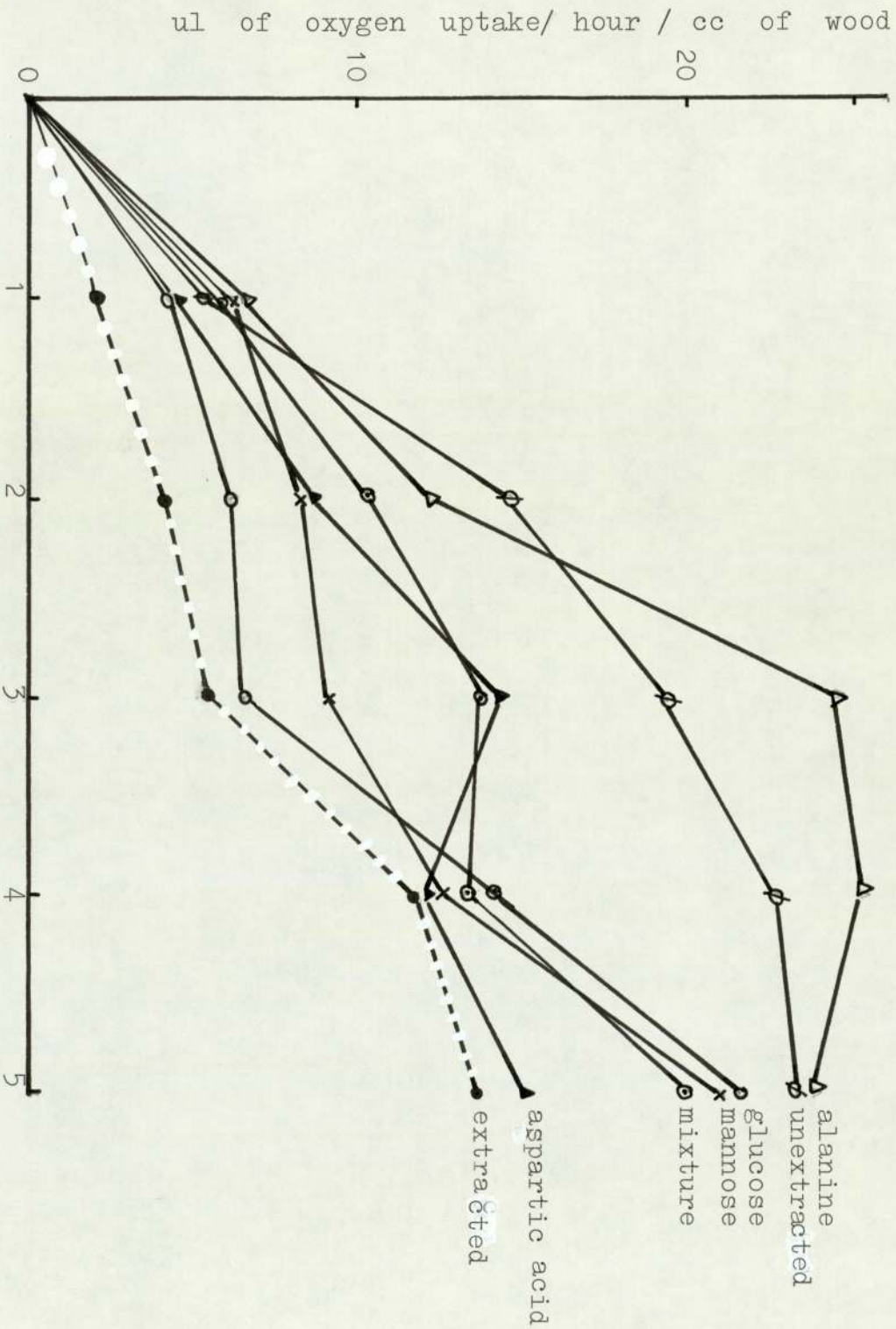


Fig.6. Effect of simple nutrients on the rate of colonisation of pine sapwood by soil microorganisms.

also increased the rate of invasion.

It is therefore concluded that removal of soluble nutrients from sapwood lowers the rate of its colonisation by soil microorganisms and addition of nutrients enhances it. The ecological implication of these studies is that added nutrients are likely to leach from the wood into the soil to influence microbial activity in the wood-soil interface and the natural soluble nutrients in the surface cells of the wood may behave similarly. The effect that these nutrients have on surface colonisation is described in the next experiment.

III:v Effect of soluble nutrients from the wood surface cells on the activity of microorganisms in the soil

a) Introduction

Nutrients present in the surface-cells of sapwood have been reported to promote germination of fungal spores *in vitro* (Morton and French, 1966; and Toole, 1970). It might be supposed that they will similarly influence microbial spores in the soil although it is not yet known whether initial penetration of wood in the soil is by germinating spores or by hyphal contact. Whichever unit of infection is influenced by the wood substrate, the respiratory activity of the growing organisms on the surface of the wood in contact with the soil, will be high. It was therefore decided to find out whether such surface nutrients from the wood cells leach out into the soil to influence microbial activity.

b) Method

It was assumed that the wood specimens used in this experiment contain high levels of soluble nutrients. They were cut from the first 10 mm layer from the surface of a laboratory dried plank of scotspine since it has been shown that soluble nitrogenous compounds and sugars are redistributed during drying of wood and that these nutrients accumulate in greater proportions on the evaporating surfaces. (King, Oxley and Long, 1974)

Both 'green' and laboratory dried pine were converted into 10 x 50 x 10 mm (tang. x rad. x long.) blocks and prepared as follows:

(1) Dried sapwood blocks in which soluble nutrients have migrated to the radial surface were sealed with

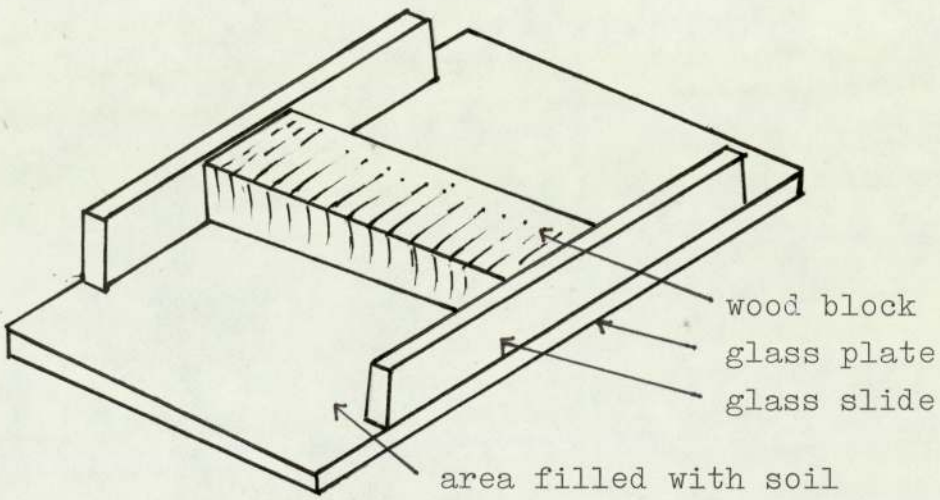


Fig.7. Diagram showing arrangement of wood block and soil on glass plate used for studying the effect of soluble nutrients in wood on the activity of soil microorganisms.

araldite on all the other surfaces except the two radial faces.

(2) Water extracted blocks were also sealed with araldite on all faces except the two radial faces.

(3) Unextracted blocks with all the surfaces sealed.

(4) Green sapwood blocks with all the surfaces except the two radial faces were covered with a polythene sheet and fastened with cellotape.

The blocks were each placed on a glass plate which had two glass slides securely cemented on two opposite sides with araldite to form embankments (see Figure 7). Soil was passed through a 40 mesh sieve to homogenize it and then adjusted to its water holding capacity. It was then gently packed on the exposed sides of the wood block on the glass plate. This set-up was placed on a V-shaped glass rod in a plastic container with water at the bottom to maintain a high humidity around the soil. Four replicate blocks per treatment were set up and this provided eight replicate exposed surfaces per treatment.

Assessment of microbial respiration in the soil and in the wood was undertaken at the end of the seventh and the twenty-first days of incubation. Two replicate blocks were removed at each sampling time. The presence of fungal mycelium in the soil-wood interface was observed with the microscope. To measure respiration in the soil, the blocks were gently pushed towards the edge of the glass plate with a knife and soil sections (3mm in thickness) were cut parallel to the wood surface at marked distances away from the exposed surfaces. The soil sections were placed in previously weighed manometer flasks and then weighed again.

The weight of the soil per flask was noted. Oxygen uptake was determined as before but the reaction flasks were first allowed to equilibrate for 4 hours in the water bath. Hourly readings were taken for 3 hours. After this, the potassium hydroxide solution was blotted off the central wells of the flasks and the latter together with the soil were dried overnight in an oven maintained at 110°C. The dry weights of the soil samples were calculated and the results are presented as mean oxygen uptake in µl per hour per gram dry weight of soil.

c) Results

The results are presented in table 7. Comparison of the means of the treatments is shown by analysis of variance in appendix 1 and 2. These indicate that there is no variation in microbial activity in the soil sections at the various distances away from the surface of the wood both after seven days of incubation between treatments ($F = 0.32$, $p > 0.05$) and within the soil section ($F = 1.18$, $p > 0.05$). After 21 days of incubation no significant difference was observed both between and within treatments ($F = 0.32$, $p > 0.05$ and $F = 2.39$, $p > 0.05$ respectively).

It can be concluded that nutrients in the wood surface cells had no influence on the activities of the soil microorganisms. This result seemed surprising since it was found by microscopic examination *in situ* that fungal mycelia and some sporulating fungi (identified as *Mucor sp* and *Aspergillus sp*) were present in the crevices in the soil-wood interface and it was thought that these fungal growths may result in high oxygen uptake, at least, in the soil in

Table 7 The effect of sapwood substrate on the activity of microorganisms in the soil in the immediate vicinity of the wood

Treatment	µl of O_2 uptake in the 1st mm layer of wood	Mean µl of oxygen uptake/hr/gm dryweight of soil		
		Distance away from the exposed surface of wood		
		3 mm	6 mm	9 mm
7 DAYS				
Green wood	10.8	150.6	148.6	156.2
Dried non-extracted wood	6.9	144.7	138.9	152.1
Water extracted wood	4.6	156.2	145.6	139.8
Non-extracted wood with all surfaces sealed(control)	0	152.4	153.6	148.2
21 DAYS				
Green wood	22.6	595.9	404.6	496.1
Dried non-extracted wood	19.8	526.5	462.8	501.6
Water extracted wood	15.7	358.4	399.6	482.9
Non-extracted wood with all surfaces sealed(control)	0	348.2	446.2	424.8

contact with the wood surface. It may be postulated that either these mycelia were confined to the soil wood interface and therefore did not enter the first layer to any appreciable extent or that the soluble nutrients in the wood surface cells were so low in quantity that their effect could not be detected by the respiratory method used. It was not possible to make photographic records of fungal growth in the crevices in the soil-wood interface because of the difficulty in obtaining concentrated illumination. No further attempts were made to proceed with this aspect of the influence of wood substrate on microbial activity in the soil. It is sufficient to say that more sensitive methods are required to assess the influence of minute quantities of nutrients from wood on the activity of soil microorganisms.

III:vi Decay of extractive-free and non-extracted sapwood.

a) Introduction

Susceptibility of sapwoods of scots pine and lime to colonisation has been quantified by measuring the respiration rates of the invading organisms in the previous experiments. It became evident that this was enhanced by the presence of soluble nutrients in the wood. Subsequent decay may, however, depend to a greater extent on the non-extractable portion of the wood. A knowledge of the initial resistance of the non-extractable portion may provide some information before application of preservatives to the wood. It was therefore decided to find out whether removal of soluble nutrients from the wood affects the rate of decay as measured by loss of weight.

b) Method

The remaining batch of water and solvent extracted blocks of scotspine and lime used for experiments I:i and I:ii respectively were employed for this experiment. They were marked and dried to moisture free weights. Non-extracted blocks similarly dried provided controls. Six replicate blocks per treatment were used for each wood species. The blocks were buried in the soil in a large plastic box. They were arranged in a 6 x 6 latin square design to reduce as much variation in the distribution of soil microorganisms that would colonise the wood blocks as possible. The position of each block in the container was noted and incubation was carried out at $30^{o}C$ for 6 weeks. After this period the blocks were exhumed, brushed clean of any adhering soil, weighed and then dried to moisture-

free weights as before and weighed again. The results are presented in tables 8 and 9.

c) Results

The results indicate that there is no significant difference between the mean percentage weight losses of unextracted and water extracted sapwood blocks of scots pine ('t' = 1.3, p = 0.1) and of lime ('t' = 1.16, p = 0.1). This suggests that removal of soluble nutrients by water extraction did not affect decay of sapwoods of pine and lime after 6 weeks of soil burial.

The difference between the mean weight losses of non-extracted and solvent extracted samples is, however, highly significant for lime ('t' = 7.4, p = 0.001) and barely significant for pine blocks ('t' = 2.27, p = 0.1). Similarly, solvent extracted samples decayed faster than water extracted blocks ('t' = 5.7, p = 0.001, for lime; and 't' = 2.75, p = 0.05, for pine). Solvent extraction, therefore, increased decay susceptibility of the sapwood blocks. This result is not consistent with what is generally thought, namely, that extraction of sapwood with organic solvents does not increase decay susceptibility; considering that sapwoods generally do not contain inhibitory substances. A close examination of table 3 indicates that more extraneous materials were removed from both wood species by solvent extraction than by water extraction. Therefore it is probable that the greater percentage decay of the solvent extracted blocks is due to the indirect effect of removal of some deposited materials such as gums, resins or tanniniferous compounds which resulted in an increase in

Table 8. Effect of water and solvent extraction of Scots pine sapwood on weight loss after 6 weeks of decay.

Treatment	% weight loss	Mean	Standard deviation
Not extracted	16.2, 14.4, 16.1, 15.5, 14.4, 15.1	15.3	0.79
Water extracted	14.6, 15.1, 14.1, 14.9, 15.4, 14.8	14.8	0.44
Solvent extracted	16.3, 24.4, 15.8, 16.1, 16.7, 17.9	17.9	3.27

Test of significance

	't' value	Difference in Weight loss
Not extracted versus water extracted	1.3	not significant
Not extracted versus solvent extracted	2.27	Significant at p = 0.1
Water extracted versus solvent extracted	2.75	Significant between p=0.05 and p=0.02

Table 9. Effect of water and solvent extraction of Lime wood on weight loss after 6 weeks of decay.

Treatment	% weight loss		Mean	Standard deviation
Not extracted	17.7, 18.5, 18.5, 17.5	17.7, 18.5,	18.1	0.15
Water extracted	23.5, 18.7, 18.0, 21.5	17.4, 16.9,	19.3	2.60
Solvent extracted	18.8, 23.3, 19.9, 20.2	36.3, 30.0,	24.8	6.96

Test of significance

	't' value	Difference in wt. loss
Not extracted versus water extracted	1.16	not significant
Not extracted versus solvent extracted	7.4	highly significant at $p < 0.001$
Water extracted versus solvent extracted	5.7	significant at $p < 0.001$

porosity of the blocks and subsequently enhanced the rate of ingress of moisture from the soil into the wood blocks thus providing them with sufficient moisture to enable decay to progress.

The moisture contents of the test blocks after decay are presented in tables 10 and 11. All the moisture contents of the differently treated wood blocks were very high indicating that moisture is not a limiting factor. Tests for any significant differences between the mean moisture contents of the blocks, however, showed some difference. Solvent extracted blocks absorbed more moisture than either water extracted or unextracted blocks in both wood species. Since the percentage weight loss due to decay is greater in solvent extracted samples than in water and non-extracted samples it appeared that moisture content may be related to weight loss. Scatter diagrams in figures 8 and 9 and calculations of coefficient of correlation suggest some slight relationships ($r = +0.685$, $p = 0.01$ for pine and $r = +0.523$, $p = 0.01$ for lime).

Table 10. Effect of water and solvent extraction on the final moisture uptake of pine sapwood blocks after 6 weeks of decay in the soil.

Treatment	% moisture content	Mean	Standard deviation
Not extracted	138, 138, 152, 155, 147, 146	146	7.15
Water extracted	147, 154, 161, 141, 161, 153	152	7.85
Solvent extracted	171, 180, 175, 172, 170, 172	173	3.67

Test of significance

	't'	Difference in m.c.
Not extracted versus water extracted	0.40	not significant
Not extracted versus solvent extracted	4.73	significant at p = 0.001
Water extracted versus solvent extracted	2.92	significant at p = 0.02

Table 11 Effect of water and solvent extraction on the final moisture uptake of lime sapwood blocks after 6 weeks of decay in the soil

Treatment	% Moisture content	Mean	Standard deviation
Not extracted	165, 175, 159, 172, 165, 174	168	6.32
Water extracted	166, 162, 168, 166, 177, 178	169	2.05
Solvent extracted	184, 182, 185, 191, 186, 182	185	3.34

Test of significance

	't' value	Difference in m.c.
Not extracted versus water extracted	0.12	not significant
Not extracted versus solvent extracted	3.09	Significant at p=0.02
Water extracted versus solvent extracted	2.46	Significant at p = 0.05

Fig.8. Relationship between weight loss and moisture content in sapwood blocks of scots pine after 6 weeks of soil burial.

r=+0.685, p=0.01

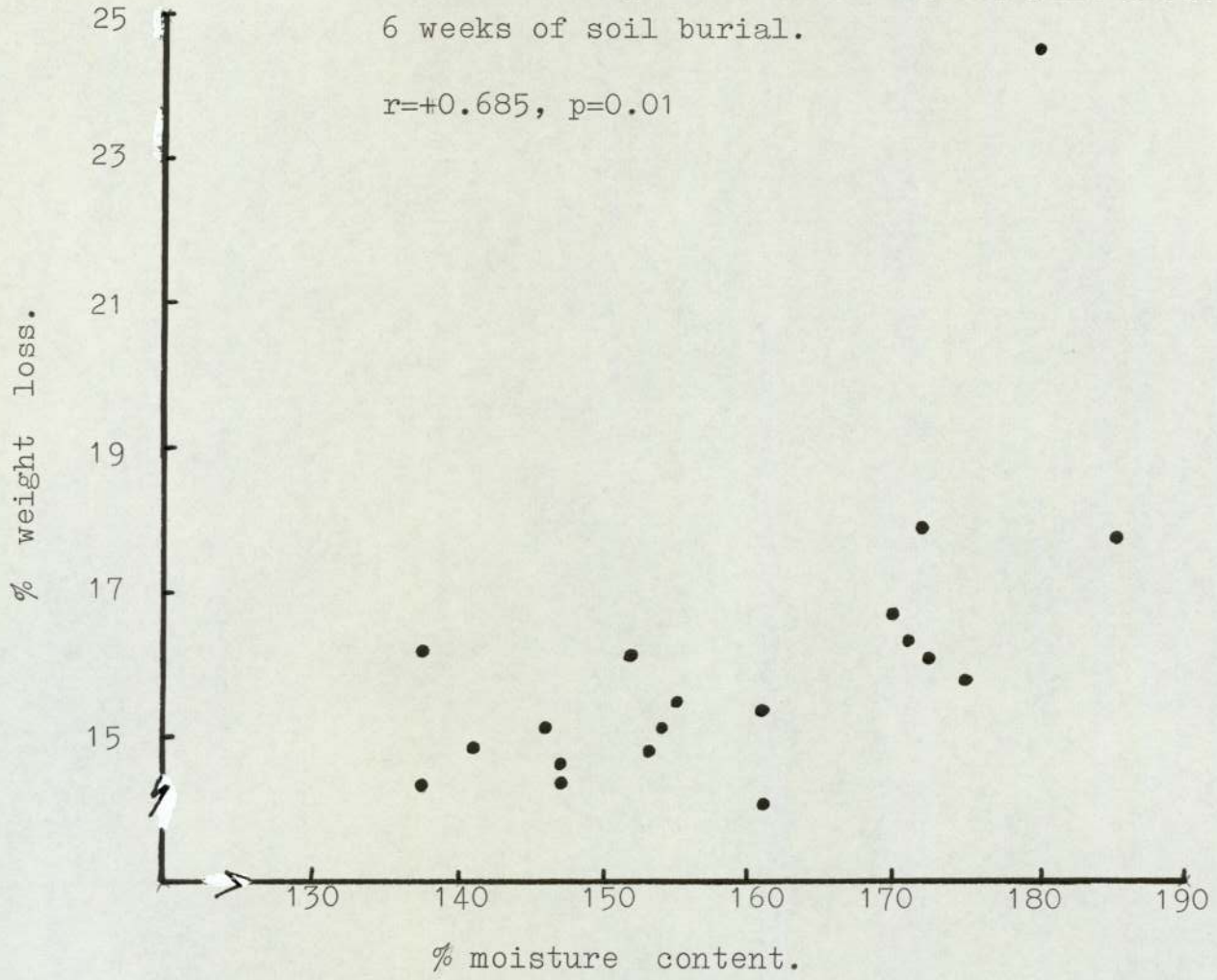
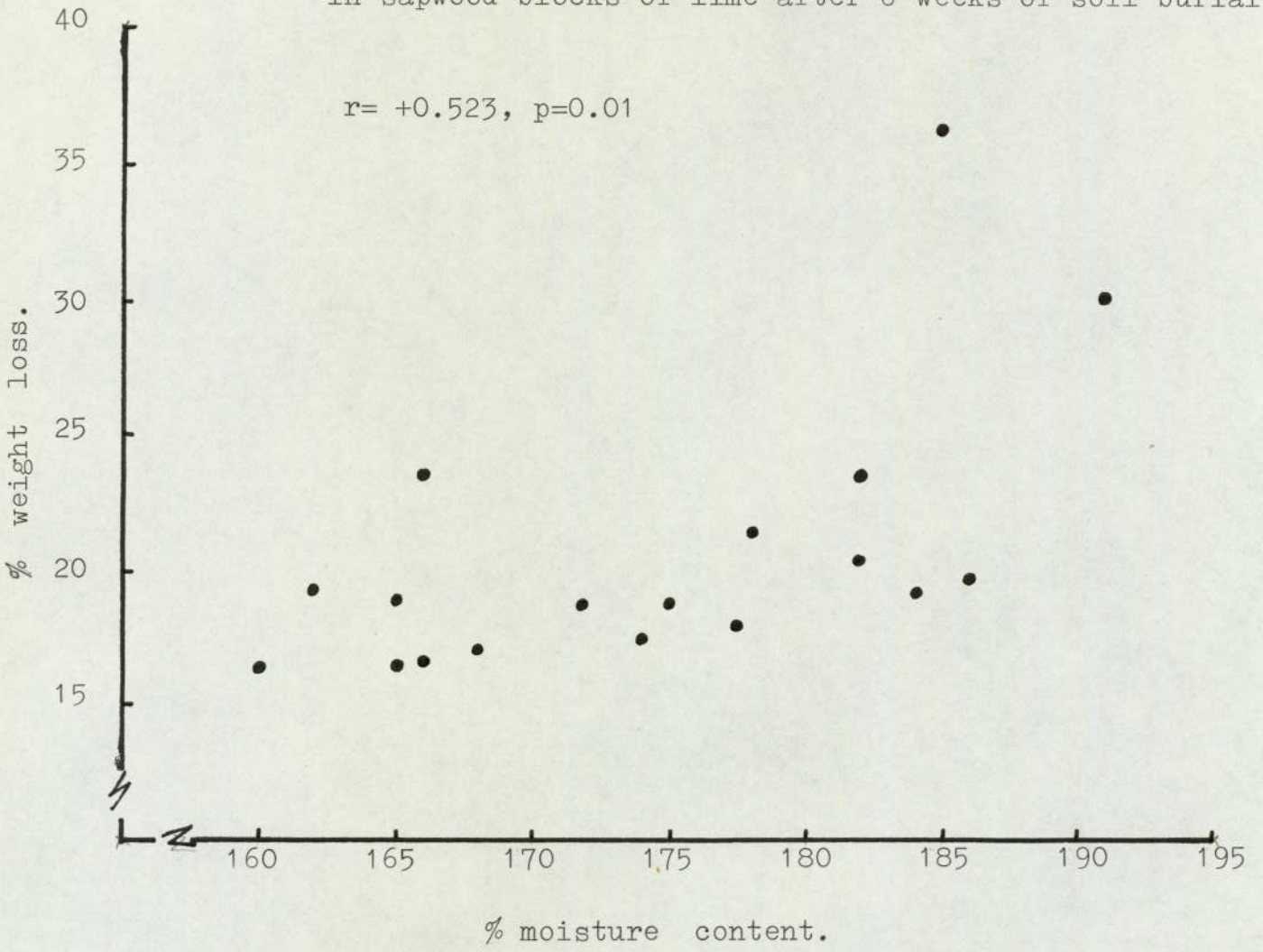


Fig.9. Relationship between weight loss and moisture content in sapwood blocks of lime after 6 weeks of soil burial.



I:vii Effect of removal of soluble nutrients from sapwood on the type of fungi that utilise it

a) Introduction

In a search for further explanation for the greater susceptibility of solvent extracted wood blocks to decay, it was thought that removal of soluble nutrients from the wood might have affected the nutritional groupings of microorganisms that utilised it. If this is so,then it would mean that the 'sugar' fungi, for instance, would colonise the non-extracted blocks in the first instance and cellulolytic organisms would utilise the solvent extracted wood blocks. It was, therefore, decided to isolate microorganisms, particularly fungi from unextracted and solvent extracted scotspine sapwood.

b) Method

The dimensions of wood blocks were the same as in experiment I:i. Five replicate blocks were extracted with the solvent solution as before and another five unextracted blocks were used as controls. They were sterilised by drying for 24 hours in the oven at a temperature of 100^{o}C. They were then buried in the soil for 30 days. Isolations were carried out in the manner described in the section under materials and general methods in chapter 2. The species of fungi and the frequency of their occurrences are tabulated in table 12.

Table 12 Percentage frequency of isolation of microorganisms from solvent-extracted and unextracted blocks

	Unextracted	Solvent extracted
Mucor sp	3	1
Cephalosporium sp	10	8
Fusarium sp	45	31
Penicillium sp	2	6
Cladosporium herbarum	8	3
Chaetomium globosum	10	12
Arthrobotrys sp	2	1
Aspergillus niger	12	20
Bacteria (colonies)	8	18

c) Results

Isolation of fungi showed that both unextracted and solvent extracted blocks were colonised by fungi of similar species. The frequency of isolation was, however, greater on unextracted specimens than on the extracted ones. Although isolation techniques alone cannot be used to quantify the activity of microorganisms in wood, it can be concluded that the individual fungal species did not select the differently treated wood blocks with respect to colonisation. The fact that there was greater frequency of isolation from the unextracted blocks, however, provides further evidence to prove the greater influence of soluble nutrients on the degree of colonisation.

Discussion

The experiments described in this chapter were set out in an attempt to evaluate the nutritional status of the sapwoods of Scots pine and lime used in this project and to find out whether the Warburg respirometer could be used to quantify the activity of invading microorganisms with respect to changes in the wood substrate.

It was found that removal of water and solvent soluble materials decreased the rate of colonisation of both sapwood species as indicated by the lower oxygen uptake measurements. This influence was more noticeable in solvent extracted blocks than in water extracted samples and in laboratory dried lime than in air seasoned samples. It was suggested that this difference in the lime blocks could have resulted from the initial nutritional state of the sapwood.

Impregnation of extractive-free sapwood blocks of pine with glucose, mannose, alanine and aspartic acid resulted in increased rates of colonisation suggesting that these substances or others of similar molecular weights are necessary to promote greater rate of growth of invading microorganisms.

After a longer period of soil burial, it was found that extracted wood samples have decayed to a greater extent than the non-extracted blocks. This effect was statistically significant in the solvent extracted samples but not in the water extracted ones in both species. The present observation is not consistent with the general belief that extraction with organic solvents does not increase decay susceptibility since very little or no

inhibitory substances are present in sapwood. Variations in wood species, however, do exist and to explain my observations three hypotheses can be put forward:

(1) Selective colonisation and utilisation of the un-extracted and the solvent extracted blocks by invading 'sugar' and cellulolytic microorganisms respectively.

(2) Increase in porosity of the wood blocks as a result of opening up of the pit apertures and removal of resins and other deposited materials to facilitate ingress of water into the blocks to promote decay.

(3) Removal of some inhibitory compounds from the blocks by solvent extraction.

The first hypothesis was suggested because it has been shown in vitro that cellulase production by microorganisms does not start until the glucose in a nutrient medium has been consumed (Bermiller, Tegtmeier and Pappelis, 1969: Nilson, 1974). Complete inhibition of initial cellulolytic activity by ancilliary carbon sources has also been reported (Bravery, 1968a). This phenomenon of inhibition of initial cellulase production by microorganisms colonising the wood was thought to have operated in the non-extracted blocks where there was a large amount of sugars. Thus the delay in cellulase production resulted in a lower rate of decay of the non-extracted blocks. In the extracted blocks there was no such inhibition of cellulolytic activity and the blocks registered greater percentage decay. Besides if there had been a selective utilisation of the wood substrates, it would have been expected that microorganisms with different nutritional requirements would have shown

preference in colonising the differently treated wood blocks; for instance, cellulolytic microorganisms would have predominated on the extracted blocks and the 'sugar' fungi would have been found mostly on the unextracted blocks. Isolation tests, however, failed to confirm this and it was then concluded that soil microorganisms do not show any selectivity in colonising the differently treated wood blocks. It must be mentioned that emphasis is not being placed on the results of the isolation since the frequency of occurrence of a particular fungus in the culture plates may merely be a measure of the ease of its isolation.

Increase in porosity of the wood blocks as a result of removal of deposited materials may also account for the greater percentage decay of the extracted blocks. The percentage loss in weight as a result of solvent extraction in both wood species was greater than that resulting from water extraction and this effect was greater in lime than in scotspine. The solubility of the extracts in alcohol and benzene suggest that they are organic in nature.

Although it became evident that all the wood blocks were at considerable moisture contents to permit decay, it was found that solvent extracted blocks absorbed more moisture than either water extracted or unextracted samples. A plot of scatter diagram and calculation of coefficient of correlation showed a sign of some correlation between moisture content and weight loss. Increase in moisture content as a result of increase in permeability is therefore associated with increase in

decay rate of the wood blocks.

Another probable explanation for the difference in decay of the extracted and unextracted wood blocks is that extraction removed inhibitory compounds as well as soluble nutrients from the wood blocks. In the unextracted blocks both inhibitory compounds and nutrients would act together to determine the extent of colonisation and decay. When microorganisms enter the wood their growth will be enhanced initially by the soluble nutrients within the cells. Removal of these nutrients by extraction would slow down their activity but they would not be inhibited because of the absence of toxic materials. In the unextracted samples when nutrients have been exhausted by respiration of the invading organisms, there is still present in the wood toxic substances which will exercise some inhibitory effect on the invaders, thus resulting in reduced decay ability. Inability of water to extract these inhibitory substances has been shown by the lack of significant difference in weight loss between the unextracted and water extracted samples. The greater loss in weight found in the solvent extracted samples indicates that the inhibitory substances have been removed by the alcohol and benzene mixture. It appears, therefore, that large amounts of soluble nutrients in the unextracted wood initially override the effect of the inhibitory component.

The effect of inhibitory factors in Scots pine on the germination of basidiospores of Lenzites trabea has been reported by Baker, Miller, Morgan and Savory, (1973). In these investigations it was found that spores which came into contact with pine sapwood which was initially dried and then rewetted failed to germinate and this was followed by

their disintegration. Spores that come into contact with green sapwood taken from freshly felled trees, on the other hand, germinated readily. The suggestion put forward was that changes which occurred in the wood during drying gave rise to some factor which inhibited germination of the spores but exceptionally rapid drying (as found in other experiments) minimised development of the inhibitory factor. In the light of these observations and the present studies the following conclusions can be drawn:

(1) Soluble nutrients as well as inhibitory substances are present in the sapwood of Scots pine.

(2) Slow drying of wood results in the depletion of nutrients possibly as a result of respiration of moribund parenchyma cells.

(3) Decrease in nutrient content by microbial utilisation resulted in increase in the ratio of inhibitory substances to nutrient content in the unextracted control blocks.

(4) Extraction increases porosity and hence permeability of wood blocks.

In a very limited experiment it was found that nutrients in the wood surface cells had no stimulatory effect on the activity of microorganisms in the soil at distances remote from the surface of the wood. It may be suggested that reactivation of microbial activity in the soil prior to colonisation of the wood blocks was due rather to the effect of the incubation temperature and the moisture content of the soil than to the effect of soluble nutrients diffusing out of the wood. The reason for this seemingly

non-stimulatory effect of nutrients is not clear but it is suggested that other more sensitive methods are required to measure microbial activity in the soil.

Chapter IV

IV:1 Distribution of microbial activity in wood

a Introduction

It was found in the previous chapter that the nutritional status of sapwood had some influence on the rate of its colonisation by soil microorganisms. This was indicated by the higher oxygen uptake of organisms invading the non-extracted wood samples. Upon entering the wood, however, the course of growth of the invading organisms will be determined by their ability to overcome the structural barriers of the wood cells. This will depend partly on their capacity to produce degradative enzymes and partly on the accessibility of energy giving substrates. From the viewpoint of micro-ecological studies, the individual cells of a wood species, as well as the various tissue systems, constitute different ecological niches upon which their initial invasion and final decomposition will, among other factors, depend. Therefore detailed studies of the microscopic changes in wood undergoing deterioration should include, first of all, the recognition of microbial material and activity in the various cells and tissue systems of the wood since this will be important in understanding the progress of decay. Corbett (1965) comprehensively studied micromorphological changes in deteriorating lignified cell walls by microscopy and suggested that other techniques would be required for quantitative estimations of the activity of the invading microorganisms.

In a wood species with a marked difference in cell wall thickness such as occurs in the earlywood and latewood

of conifers, it would appear that microorganisms would invade the wood through areas of least resistance and would proliferate in cells containing considerable quantities of nutrients. Indeed sporulating fungi have been observed in the tracheids, vessels and parenchymatous cells of deteriorating wood (Savory, 1954; Levy, 1965; and Sharp and Levy 1974).

In more detailed studies, Corbett (1963) found that the rate at which Chaetomium globosum grew through wood blocks was related to which of the surfaces of the wood (transverse, tangential or radial) is exposed to the fungus. She found that in both Pinus sylvestris and Fagus sylvatica, hyphae emerged on the upper unexposed surface sooner when a transverse face was exposed than when a tangential face was exposed and the latter sooner than when a radial face was exposed to the fungus mycelia. It suggested by her that the fungus grew through areas of least resistance taking advantage of any space or aperture that existed to enable the hyphae to grow through the wood block; hence penetration from the transverse face occurred primarily through the lumen of tracheids, vessels and fibres and penetration from the tangential face occurred along the rays. With the radial face exposed penetration appeared to have occurred through the pits or possibly by enzymatic degradation of the longitudinal wlls of the cells. It seems therefore that the radial face is the most difficult point of entry and the rate of growth of a fungus through wood from this surface might indicate its potential as a wood deteriorating organism.

Krapivina (1962) reported differences in distribution of hyphae of mould fungi within the annual ring, with species of Fusarium fairly uniformly distributed within both early-

wood and latewood while species of Penicillium and Verticillium were limited primarily to the earlywood. Savory (1954), Duncan (1960) and Courtois (1963) reported that soft rot cavities were more conspicuous in the latewood than in the earlywood of softwoods while Levy and Stevens (1966) reported that they were more prevalent in the earlywood than in the latewood of hardwoods. In a review, Wilcox (1970) outlined conflicting reports on decay resistance of the various regions within the annual ring of wood to basidiomycete attack. In one instance it was reported that the earlywood of pine and spruce was more resistant to brown rot than the latewood whilst in another the latewood of spruce was found to be more resistant to the attack of brown rot fungi than was the earlywood. Comparing with other types of decay, Liese (1970) noted that only a few hypae are visible in wood attacked by brown rot fungi and since cell wall lysis can be observed at some distance from the hyphae, the enzymes are not apparently restricted close to the hyphae but must diffuse to a certain extent. The distribution of hyphae probably bear little relationship to the progress of decay in a wood attacked by a brown rot fungus. In wood attacked by a white rot fungus the latewood is deteriorated first and more rapidly than the earlywood.

In soft rot fungi degradative enzymes remain close to the hyphae as they are secreted, hence the resultant formation of cavities have a direct bearing on the distribution of hyphae in the wood. Location of soft rot cavities is one way of studying the distribution of microfungal activity in wood but this must be complemented by other methods which

provide direct measurements of physiological activities of the invading microorganisms in the different tissue systems of the wood with the progress of decay.

It was therefore the purpose of this experiment to use

(1) respirometry and microscopy to examine the distribution of microbial activities in the early and latewood of Scotspine sapwood blocks invaded by pure cultures of selected microfungi and also by the general micro-organisms occurring in the soil and

(2) to study the location and formation of soft rot cavities in lime sapwood.

b Respiratory methods

The sapwood of pine with the early and latewood rings 2-3 mm wide was chosen for the respirometric studies. It was converted into 5 x 50 x 5 mm (tang. x radial x long.) test blocks; the annual rings being perpendicular to the radial face. They were divided at random into six batches, each containing at least four replicate blocks. The blocks in the first batch were coated with araldite on all the faces except the two radial faces. This enabled penetration to occur only through the radial face and also reduced variability due to penetration from the transverse face. They were buried in the soil. The blocks in the rest of the batches were not coated and they were either buried in the soil or exposed separately to pure cultures of Chaetomium globosum, Cephalosporium sp. and a Penicillium sp. in petri dishes containing Abram's medium. The wood blocks were first sterilised by autoclaving for 10 minutes at 15 pounds per square inch and then placed on sterile filter papers con-

taining actively growing cultures in the plates.

Incubation was carried out at 30°C for 12 weeks. Sterile distilled water was added to the culture plates at two weeks intervals to prevent drying of the agar and to keep the humidity around the blocks high. At the end of the incubation period all the blocks were harvested, cleaned free of soil or mycelia and the sealant peeled off the relevant blocks. Each block was dissected from the transverse plane to separate the earlywood and the latewood bands. Though there was no sharp demarcation between them, it was hoped that a fair amount of accuracy was obtained in their separation. Oxygen uptake was determined by the Warburg respirometer and the results are expressed in µl of O_2 per hour per cubic centimeter of either earlywood or latewood. (Table 13.)

C Microscopic studies

Examination of soft rot cavities was carried out on Scotspine and lime sapwood after 12 weeks of burial in the soil and on lime sapwood inoculated with pure culture of Chaetomium globosum after the same period of incubation. The size of the wood blocks and the conditions of exposure were the same as before. After the incubation period the wood blocks were washed free of soil and fungal mycelium and boiled in tap water for 10 hours to soften them. Several transverse sections, 15µ and 30µ thick were cut with the sledge microtome and stained with safranin and picro-aniline blue. Permanent slides were prepared after dehydration of the sections in two changes of seventy percent ethanol-water solutions and finally in 100% ethanol and then mounted in

Table 13 Oxygen uptake in the early and latewood growth rings of *Pinus sylvestris* sapwood colonised by microfungi

Type of exposure	Region of wood	µl of O_2/hr/cc of wood	mean µl of O_2	Standard Deviation	Students 't'
Wood blocks with all surfaces exposed to the soil	Early wood	18.9, 18.6, 19.9 19.3, 20.2, 21.5	19.7	1.04	2.31 > 2.228
	Late wood	19.6, 22.0, 19.0, 22.1, 21.6, 23.6	21.3	1.71	sig-nificant at p=0.05
Wood blocks with only the radial face exposed to the soil	Early wood	19.4, 20.3, 17.6, 17.9	18.8	1.27	4.6 > 3.707
	Late wood	26.5, 29.2, 30.3, 26.1	28.0	2.03	sig-nificant at p=0.01
Wood blocks with all surfaces exposed to pure culture of *Chaetomium Globosum*	Early wood	11.6, 11.1, 11.5, 10.8	11.3	0.12	11.6 > 5.959
	Late wood	20.0, 21.9, 20.6, 21.7	21.1	0.29	sig-nificant when p<0.001
Wood blocks with all surfaces exposed to pure cultures of *Cephalos-porium sp.*	Early wood	9.3, 10.6, 8.3, 8.6	9.2	1.02	6.45> 5.959
	Late wood	11.7, 9.3, 11.7, 12.6	11.3	1.41	sig-nificant at p=0.001
Wood blocks with all surfaces exposed to pure cultures of *Penicil-lium sp*	Early wood	20.9, 20.7, 20.8, 23.4	21.5	1.3	7.4 > 5.959
	Late wood	19.5, 18.6, 19.7 18.0	19.0	0.79	Sig-nificant at p=0.001

Canada balsam after clearing in clove oil and differentiation in xylene. Observations were made under the high powers of the light microscope and the formation and distribution of soft rot cavities in the early and latewood regions were noted and recorded by photomicrographs (Plates 1-11).

d Results

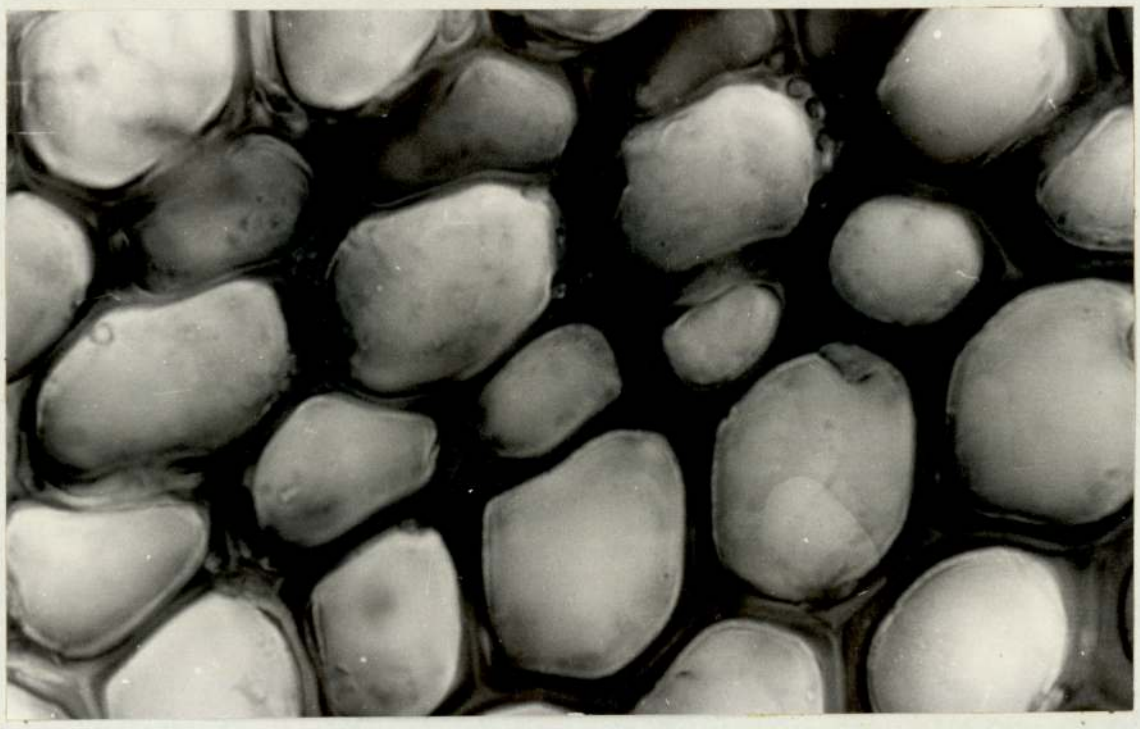
The results presented in Table 13 indicate that wood blocks incubated in the soil showed higher rates of colonisation than blocks exposed to pure cultures of the fungi tested. In all the blocks there were significant differences in the level of activity between the earlywood and the latewood growth rings. Blocks buried in the soil and those exposed to pure cultures of Chaetomium globosum and Cephalosporium sp showed higher respiratory activity in the latewood whilst Penicillium sp respired more actively in the earlywood.

Microscopic observations of sections taken from wood blocks buried in the soil showed that more soft rot cavities were formed in the latewood cells than in the earlywood of pine (Plates 1 and 2).

Tracheids bordering ray parenchyma cells were attacked more severely than those further away, Plate 3. The cavities were exclusively formed in the secondary wall, S_2 layer. At the more severely decayed regions, particularly in the cells at the surface layer of the wood, the S_2 layer was completely dissolved in the latewood, leaving the primary wall S_1 and the tertiary wall S_3 as two concentric rings (Plate 4). At this state the cell walls of the earlywood have become structurally indistinct due to deterioration.

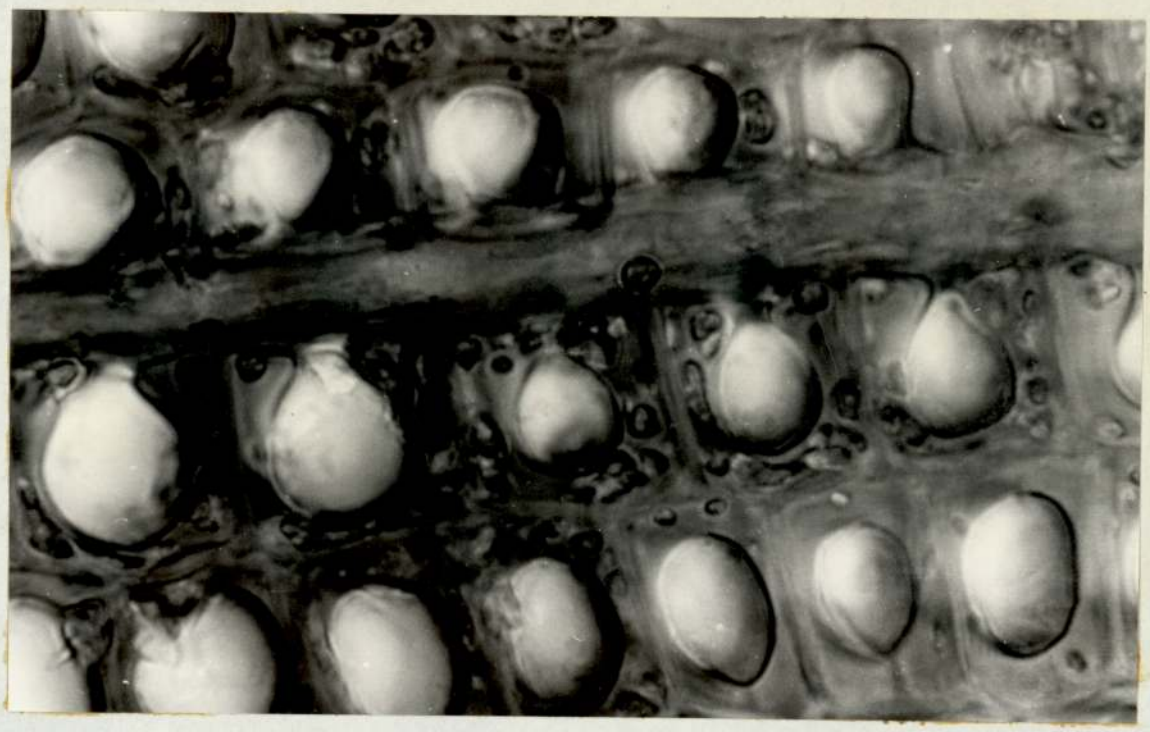
x 1000

Plate,1. Photomicrograph showing typical soft rot cavities (Corbett's type 1) in the latewood tracheids of Scots pine sapwood. (T.S)



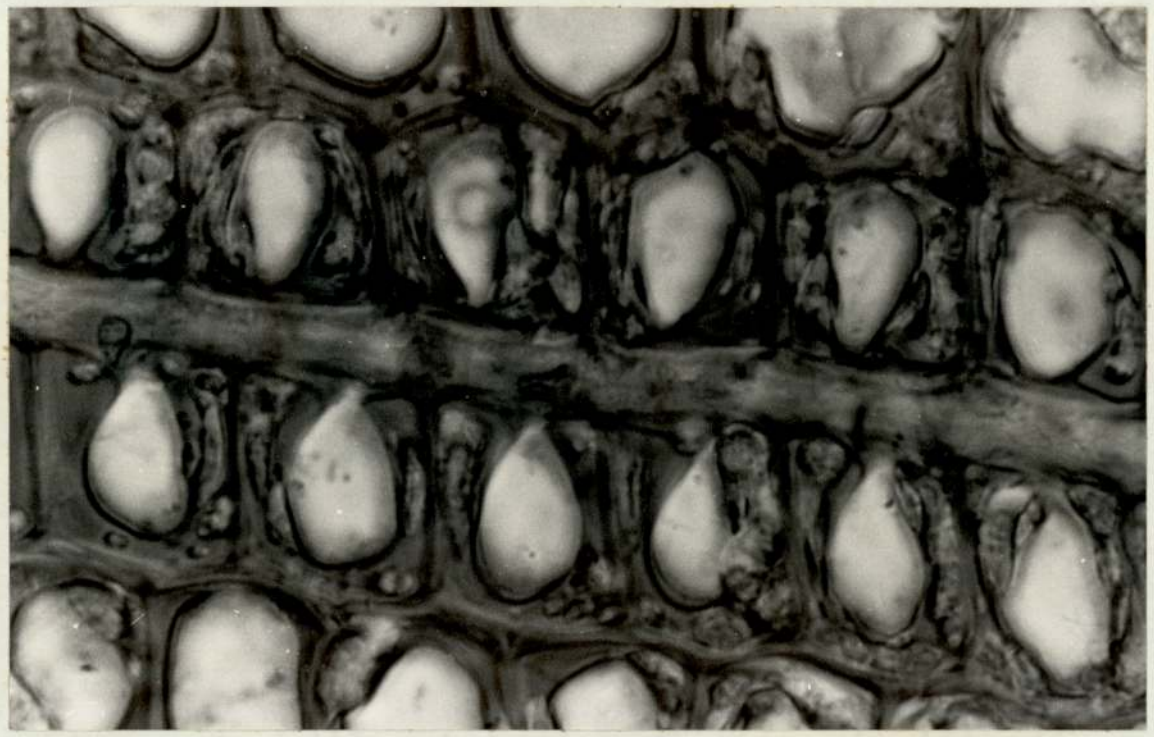
x 1000.

Plate, 2. Photomicrograph showing sparse formation of soft rot cavities in the earlywood tracheids of Scots pine sapwood. (transverse section)



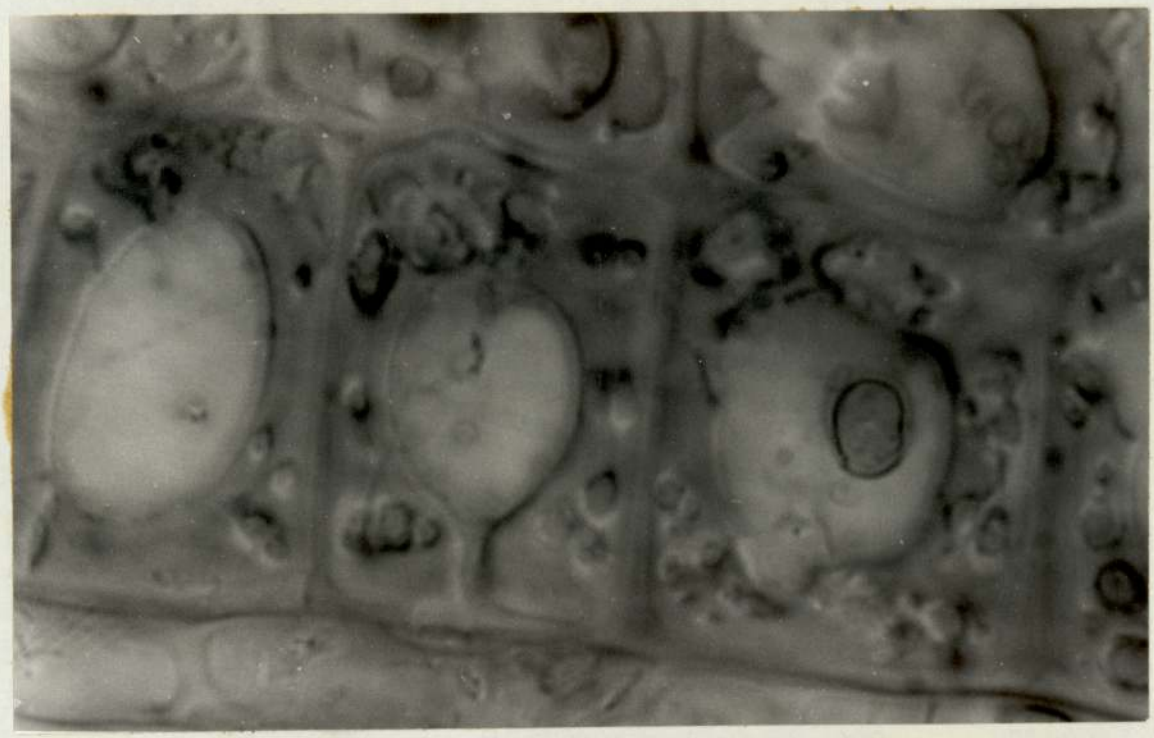
x 1000

Plate, 3. Photomicrograph showing intense formation of soft rot cavities in the tangential and radial walls of tracheids bordering a ray parenchyma in the latewood of Scots pine sapwood. (T.S)



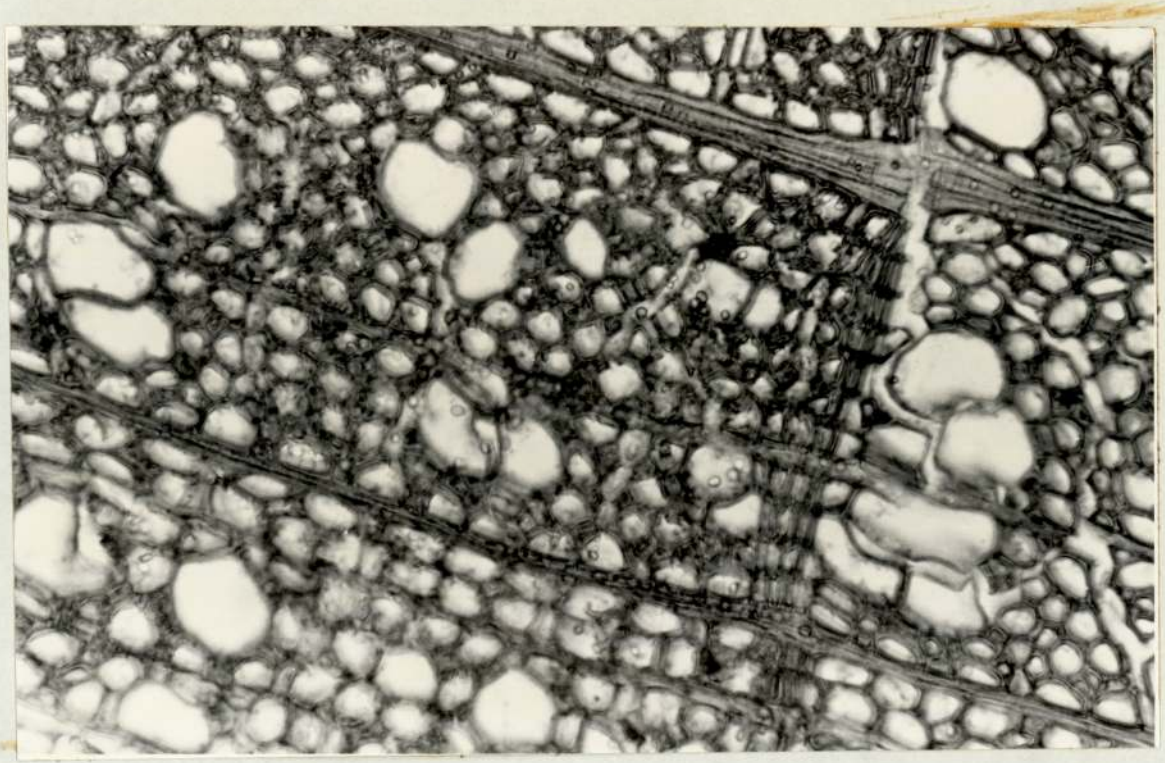
x 1000

Plate,4. Photomicrograph showing complete dissolution of the S_2 layer as a result of widening of the soft rot cavities in cells in contact with the soil. (T.S.)



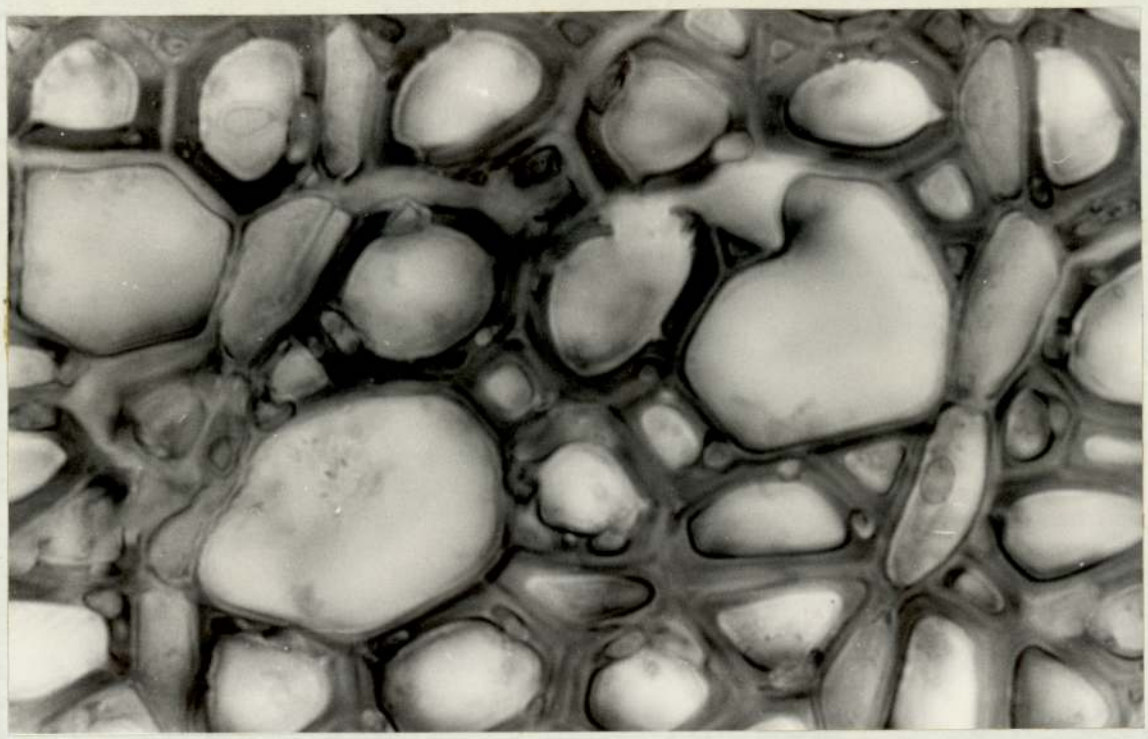
x 3500

Plate,5. Photomicrograph of soft rot cavities as seen under oil immersion. (T.S)



x 150 approx.

Plate,6. Photomicrograph showing ungerminating spores in decaying wood cells of lime sapwood after 12 weeks of soil burial. (T.S)

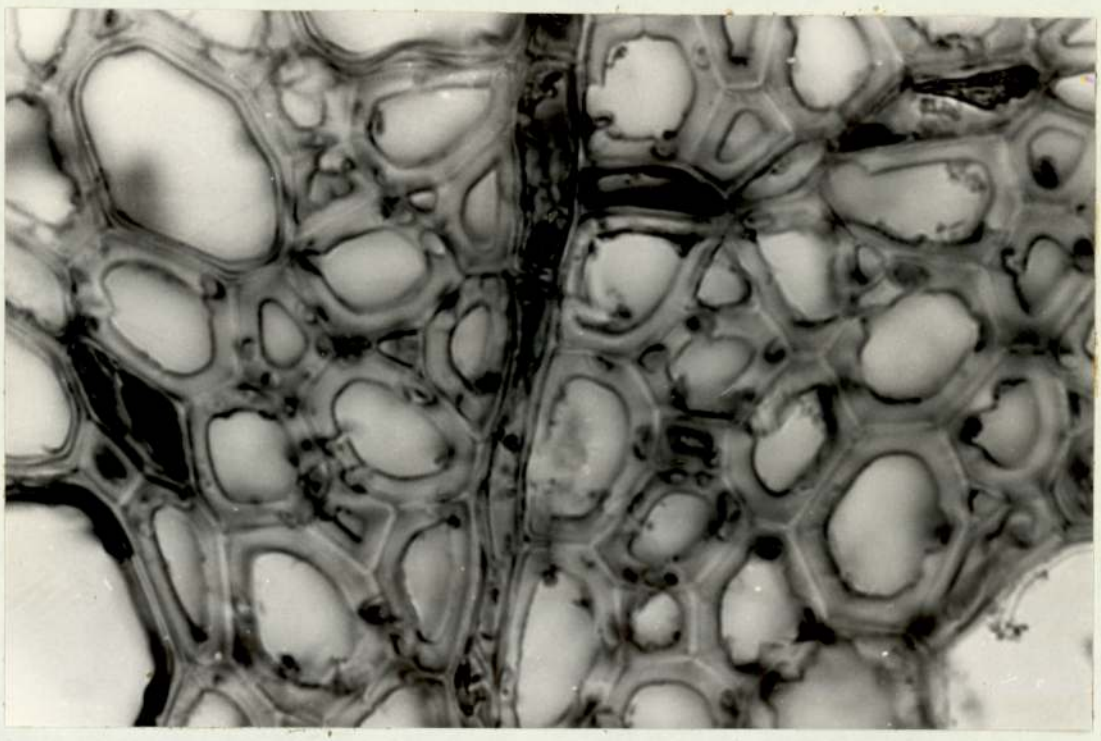


x 900

Plate,7. Typical soft rot cavities in lime sapwood cells after 12 weeks of soil burial. Note the thin S_3 layer. (T.S)

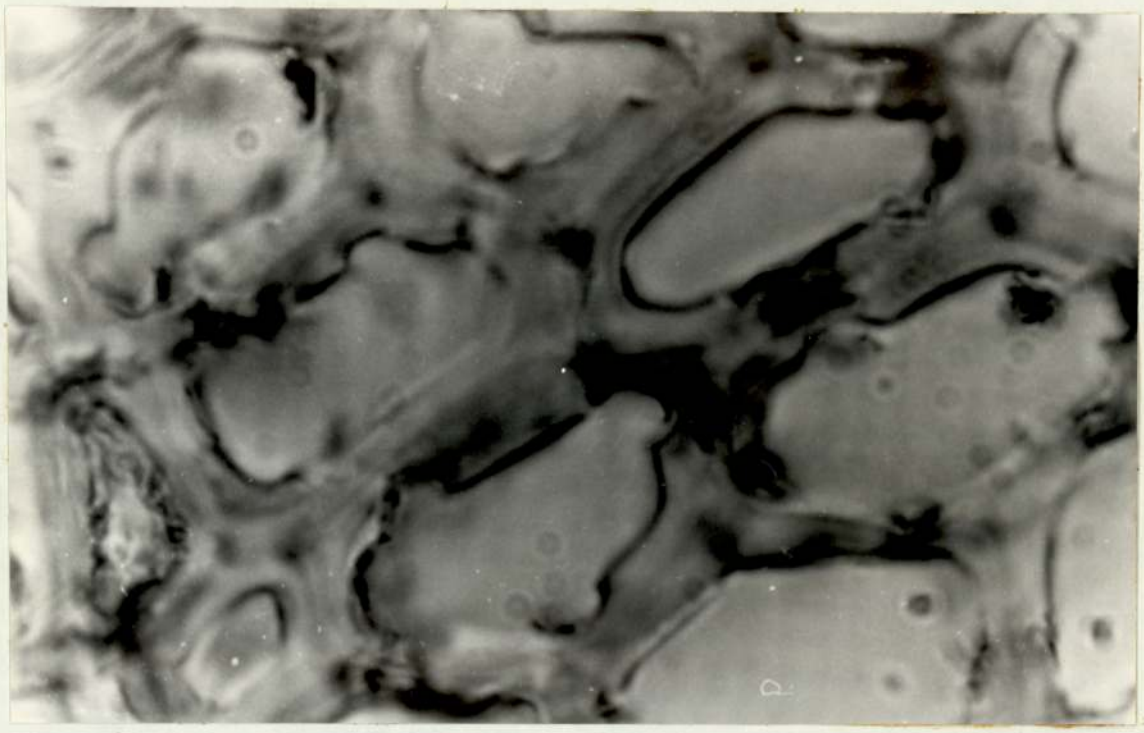
x900
approx.

Plate,8. Photomicrograph showing erosion of cell walls of lime from the lumen by Chaetomium globosum after 12 weeks of incubation. (T.S)



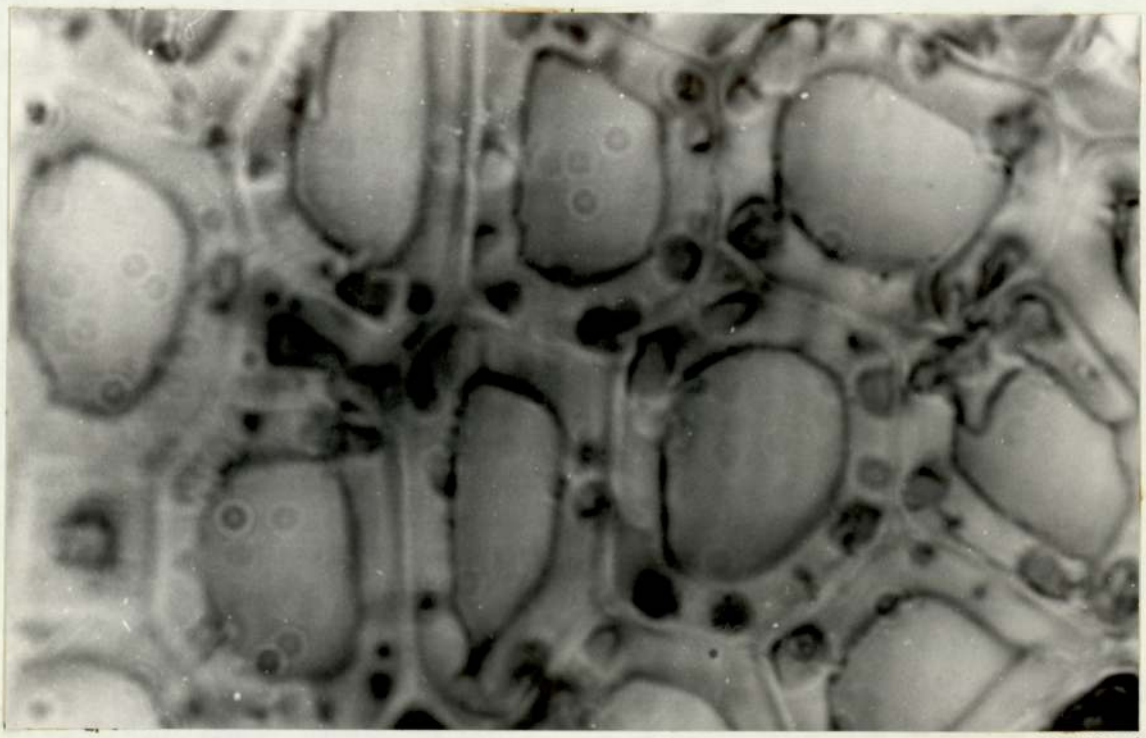
x 900

Plate,9. Photomicrograph of a section from decaying lime showing
(1) erosion of cell walls from the lumen and from the middle lamella region.
(2) typical soft rot cavities. (T.S)



x 3500.

Plate,10. Erosion of walls from the lumina of cells colonised by C. globosum. Picture taken under oil immersion. (T.S)



x 3500
approx.

Plate,11. Transverse section of lime sapwood attacked by C.globosum showing typical soft rot cavities. Picture taken under oil immesion.

In the lime blocks exposed to the soil a large number of spores were found in the decaying cells at the surface layer of the wood (Plate 5). Typical soft rot cavities were found in the secondary cell wall, s_2 layer. These, however, cannot be attributed to any specific microorganism. Erosion type of attack, i.e. dissolution of the cell wall from the lumen, was not found in the buried blocks (Plate 6).

Lime blocks exposed to pure cultures of C. globosum showed typical soft rot cavities and erosion type of attack (Plates 7 and 8). Erosion of the wall occurred both from the cell lumen and from the middle lamella regions (Plate 8).

Fibres bordering the vessels were more degraded than those further away from it, apparently because the former were the first to be attacked after the fungus had entered the wood through the vessels. There was no preferential formation of cavities in the earlywood or in the latewood.

c Discussion

The results presented in this section demonstrate the abilities of microorganisms to select areas within the wood microstructure for enhancement of growth. It also shows their preference for certain types of substrates. C. globosum and Cephalosporium sp are known to be cellulolytic microfungi; and their higher oxygen uptakes in the latewood of pine which contain a higher proportion of cellulose correspond with their ability to utilise this substrate. Penicillium sp was found to be more active in the earlywood than in the latewood. The reason for this is not quite clear but it may be due to its inability to penetrate the cell walls and thus its activity is confined only to the lumen, hence the

larger lumina of the earlywood accommodated more mycelium mass than the latewood which has smaller lumina.

The results for the soil burial studies are interesting. The greater oxygen uptake in the latewood correlated with the preponderance of soft rot cavities, supporting the evidence that location of cavities in wood can be used to study the distribution of soft rot fungi. The presence of more cavities in the tracheids bordering a ray parenchyma confirmed that the latter provided an easier path for growth of microorganisms through the wood and easier entry into the tracheids was achieved through the half bordered pits. In pine wood blocks with only the radial face exposed the ray parenchyma cells run parallel to the radial face and hence an organism upon entering the wood will spread with more ease radially along the rays than tangentially from tracheid to tracheid into the wood. Therefore the change from strongly decomposed peripheral region to a moderately infected region would be short and abrupt. This was noted in the pine wood blocks used in this experiment and also confirms the observation by Courtois (1963) who also reported that this formation is typical in softwoods. In lime, apart from the axial parenchyma, there are isolated parenchyma cells in short tangential lines among the fibre tissue (plates 8 and 9), thus colonisation of lime wood by microorganisms is faster and their distribution within the wood is diffuse.

Both types of cavity formation were observed in lime blocks inoculated with pure cultures of C. globosum. Erosion of the wall from the lumen was not observed in the blocks incubated in soil probably suggesting that other organisms with less cellulolytic capabilities are involved in the

deterioration of the wood in the soil. The fact that the cell wall of lime is destroyed from all directions, that is, from the lumen, from the middle lamella and from within the S_2 layer means that it needed adequate protection by preservatives to prevent soft rot attack.

Variability in the distribution of preservatives within the microstructure of the wood can also affect the extent of decay by soft rot fungi. There is, however, some evidence to show that the constituents of copper-chromium-arsenate preservative are deposited fairly uniformly within wood cell walls of conifers (Petty and Preston 1968). Rudman (1965) using electron microscopy has shown that the tertiary layer of the cell walls of eucalypt was penetrated by inorganic solutions during impregnation and the middle lamella also offered no resistance to flow consequently uniform distribution occurred in the cell walls. What has not been determined is just how much of the preservative within the cell walls would be sufficient to prevent cellulolytic enzyme production and hence cavity formation. The problems to be encountered in such studies would be enormous but an attempt has been made in the later parts of this thesis to investigate the influence of subtoxic concentrations of C.C.A. on the production of microfungal cellulases in vitro.

In wood the close association of lignin and cellulose within the cell walls affects the degree of attack of the latter by microorganisms. In the next section the effect of partial delignification of wood on the rate of initial colonisation and subsequent decay is described.

IV:ii Effect of partial delignification of wood on the rate of initial colonisation and subsequent decay

a Introduction

It is generally known that cellulolytic microfungi have a limited ability to utilise the cellulose in highly lignified cell walls (Cowling, 1963; Courtois, 1963; Liese, 1970; and Nilson, 1973). This phenomenon has been attributed to physical blocking of cellulose by the lignin and also to the different ways in which the two compounds may be chemically associated in wood cell walls (Bailey, Liese and Rösch, 1968). In softwoods the lignin content is commonly between 25 and 30 per cent of the dry weight while in temperate hardwoods it ranges from about 19 to 25 per cent of the dry weight (Jane, 1956). The higher lignin content together with the presence of a different type of polysaccharide in the cell wall of softwoods was therefore thought to be responsible for their resistance to soft rot attack (Liese, 1970). The preponderance of soft rot cavities in the S_2 layer in the latewood cells than in the earlywood indicate that the former is more susceptible to attack by cellulolytic microfungi than the latter. This is because the greater thickness of the latewood cells is due mainly to an increase in the thickness of the S_2 layer which contain a greater proportion of cellulose (Courtois, 1963). The walls of the earlywood cells on the other hand have, in relation to their thickness, a greater proportion of primary wall and middle lamella where the lignin content is higher (Jane, 1956), and thus could only be degraded to a larger extent by the highly specialised basidiomycetes, the white

rot fungi.

In order to understand more fully the mechanism of decay of wood species with different proportions of cellulose and lignin it was decided to modify chemically the wood cell wall by a process which partially delignifies the wood and to study the rate of deterioration and moisture relations of the invaded wood.

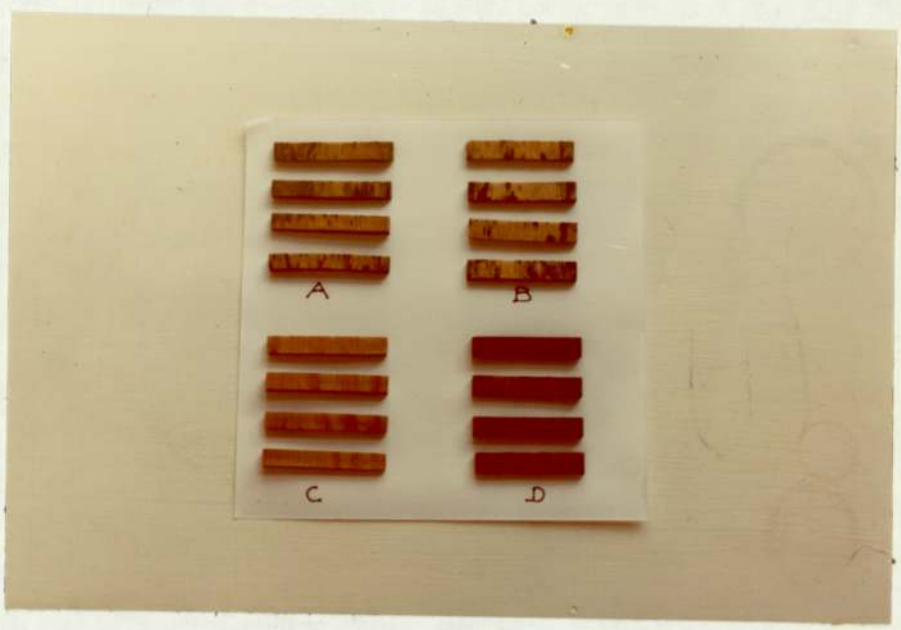
b Method

Sapwoods of both pine and lime were employed in this investigation. The blocks measured 5 x 50 x 5 mm in the tangential, radial and longitudinal directions respectively. They were dried to moisture-free weights, marked and the weights noted. The proceedure for partial delignification was similar to that described by Bailey, Liese and Rösch (1968) with slight modification. It consisted of impregnating wood blocks under vacuum with sodium chlorite and glacial acetic acid solution in the proportion of 5 grams of sodium chlorite to a hundred grams of wood in one litre of distilled water plus 10 mls of acetic acid. The blocks to be treated, chosen at random, were placed in conical flasks. Each flask contained five blocks and 100 mls of the treating solution. The flasks were attached to a shaker and arranged in such a way that the former were partially immersed in water baths maintained at 40°C. After 24 and 36 hours of shaking further portions of 0.5 mls of acetic acid were added to each flask. A total shaking time of 48 hours was allowed after which the blocks were removed, rinsed, vacuum impregnated with distilled water and further washed for 24 hours in distilled water. This second impregnation was necessary to remove any treating solution that might be trapped between

Table 14 Percentage weight loss after mild delignification of sapwood of pine and lime

Wood Species	Partially delignified		Control	
	Mean	Standard deviation	Mean	Standard deviation
Pine	3.64	1.06	2.04	1.08
Lime	4.01	2.06	1.50	1.4

Plate,12. Effect of partial delignification of sapwood of lime and Scots pine on surface colonisation after 2 weeks of soil burial.



a control lime blocks.
b partially delignified lime blocks.
c control pine blocks.
d partially delignified pine blocks.

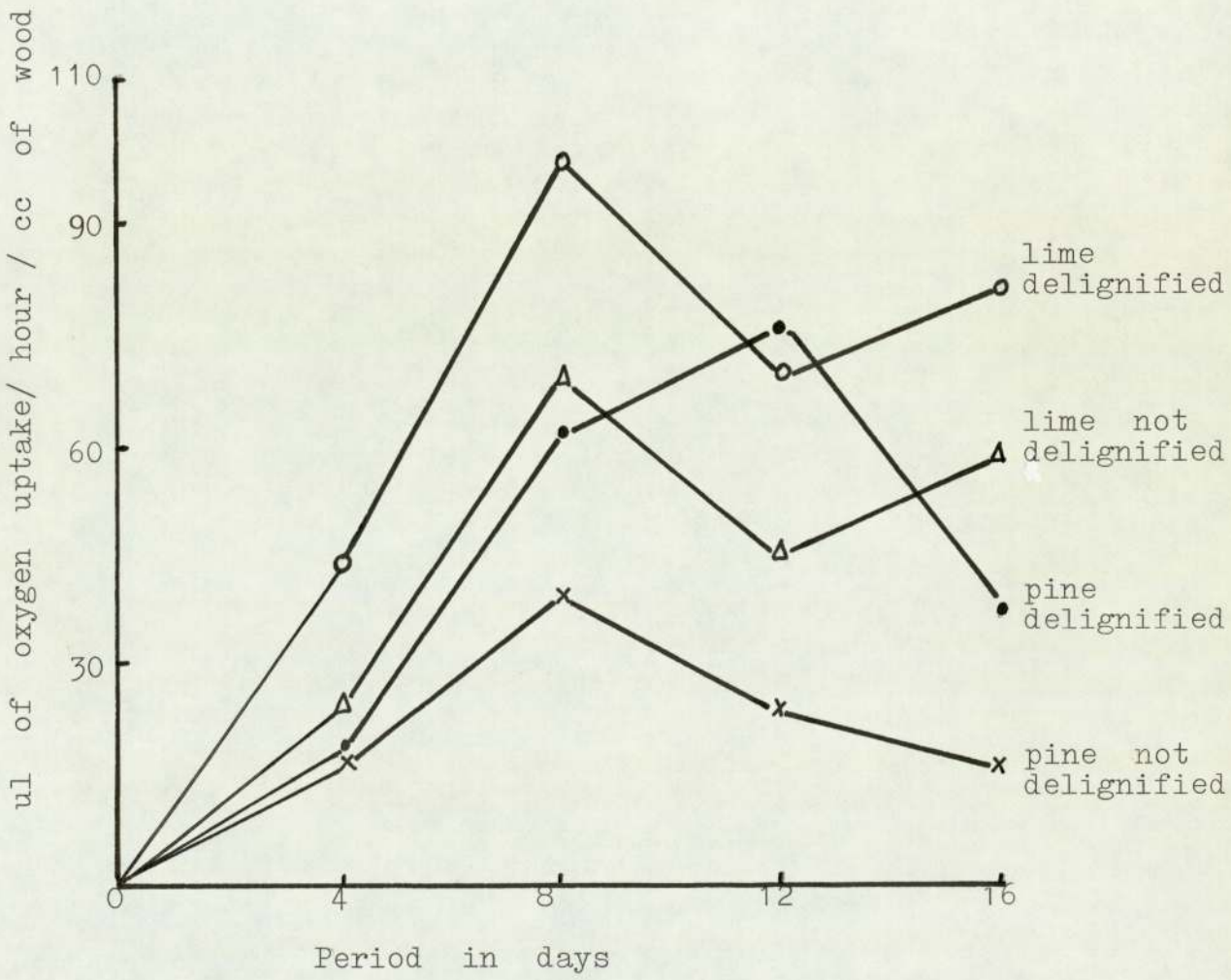
and within the cell walls. The blocks were then placed on filter papers to drain away the water and then dried to constant dry weights in an oven set at 100°C for 24 hours. The weights were noted and these were subtracted from the original dry weights to give a measure of the amount of materials lost from the wood blocks (Table 14), as a result of partial delignification.

Control blocks were similarly set up side by side with the experimental blocks except that they were impregnated and washed with distilled water for the same length of time as the experimental blocks. Sixty four blocks per wood species were treated in this way, half of which were partially delignified and the other half provided controls.

A preliminary observation indicated that surface colonisation of the partially delignified blocks was very fast (Plate 12). It was therefore decided to assess this at regular intervals by recording the rate of initial invasion with respirometry. The blocks were then divided into two batches so that for each wood species there were 16 partially delignified and 16 control blocks in a batch. They were incubated in the soil at 30°C and at intervals of four days, four blocks per treatment per species were removed and their respiration determined. The results are presented in Figure 10.

To assess the rate of decay by weight loss the second batch of treated blocks together with the controls were similarly buried in the soil. At intervals of three weeks, four blocks per treatment per species were removed and their percentage moisture contents and weight losses were determined. These were based on the oven dry weights after partial

Fig.10. Effect of partial delignification on the rate of colonisation of pine and lime sapwoods by soil microorganisms.



delignification and decay. The results are presented in Figures 11 and 12).

c Results

The results show that the rate of initial colonisation and subsequent decay were considerably increased in wood blocks with the lignin partially removed or modified and that such treatment caused pine sapwood to be as susceptible to decay as lime which did not receive the treatment. Partially delignified lime, however, decayed fastest registering a mean percentage weight loss of 17 after 3 weeks of soil burial whilst pine which had not been treated was the slowest to decay. The mean percentage weight loss after 3 weeks was only 2.5. Initial colonisation followed a similar pattern. It was gradual up to 8 days of soil burial in all the blocks after which it became irregular as a result, probably, of competition and interaction of freshly invading microorganisms. Moisture content was generally high in all the blocks but was highest in the blocks which were partially delignified. The rate of moisture uptake followed the same trend as the rate of decay.

The results for weight losses due to mild delignification showed that some lignin was removed from both pine and lime blocks but more of it was removed from the lime blocks although as a temperate hardwood they possibly had less to start with.

Fig.11. Effect of partial delignification on the rate of decay of pine and lime sapwoods by soil microorganisms.

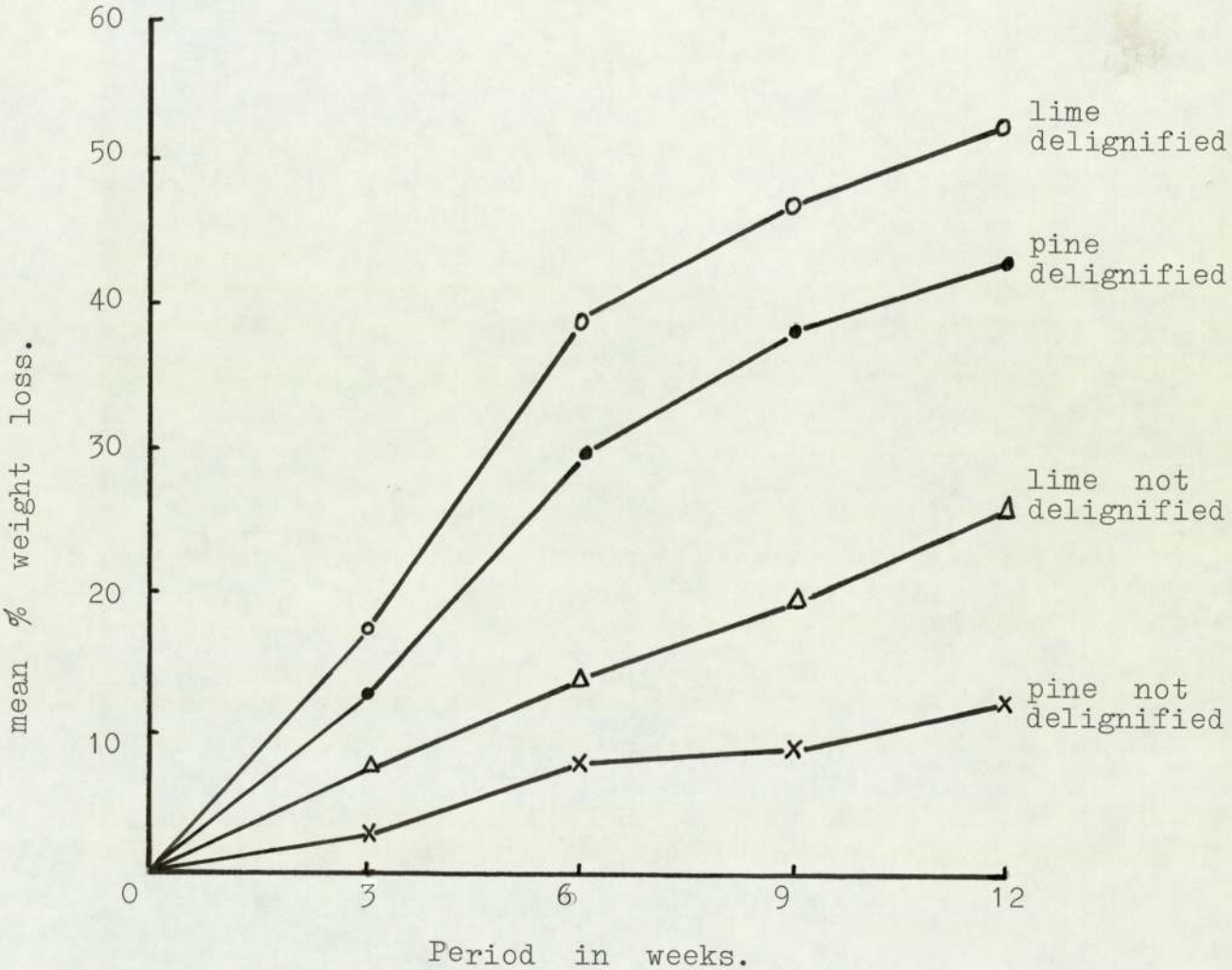
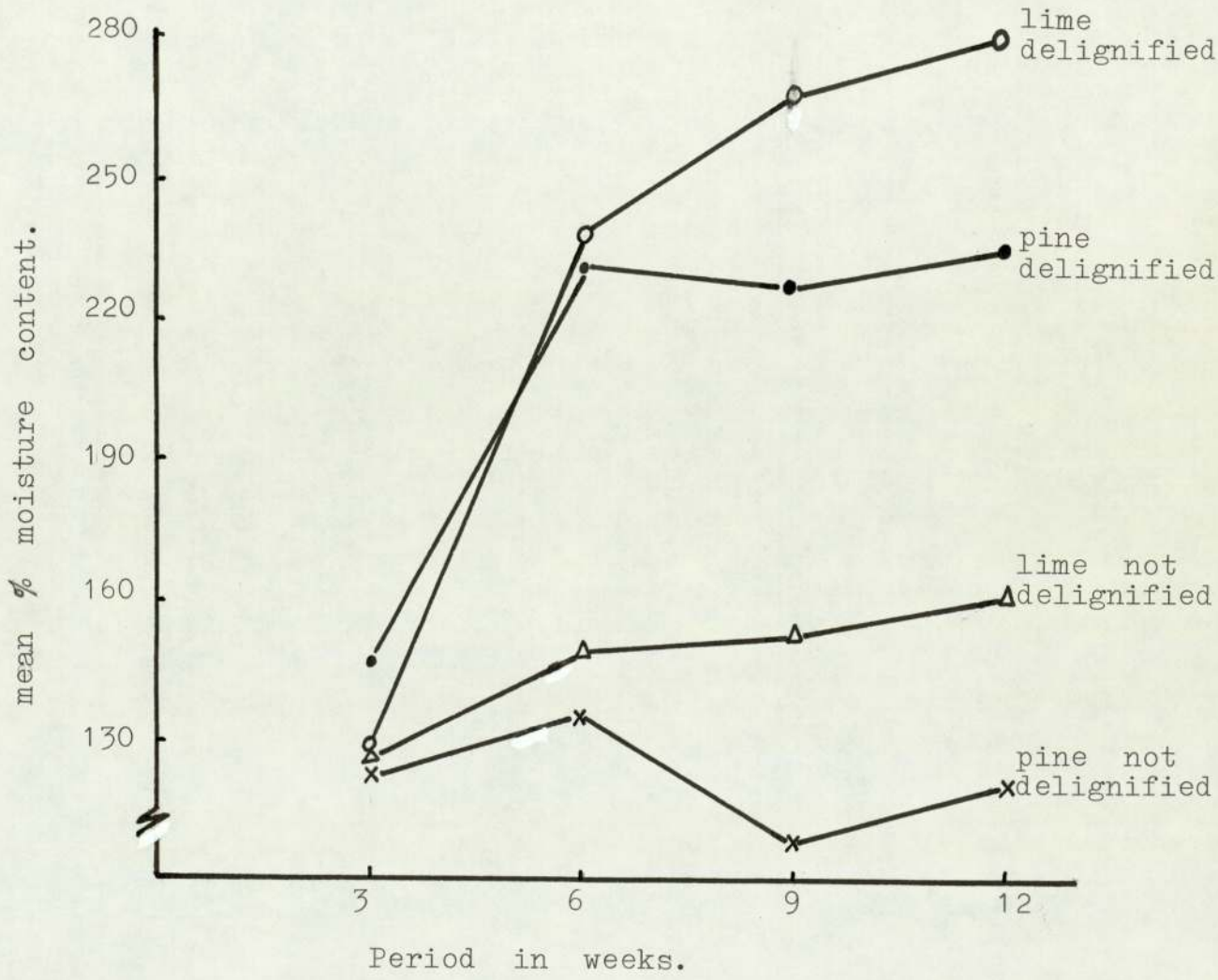


Fig.12. Changes in moisture content of partially delignified and untreated sapwood blocks with the progress of decay.



IV:iii Effect of partial delignification treatment on the general anatomy of the wood

a Method

It was thought that the sodium-chlorite-acetic acid treatment would initially bring about disorganisation of the wood cell walls. The effect of this treatment on the anatomy of the wood was therefore studied. Transverse and radial longitudinal sections, 15μ and 30μ thick, were cut with the sledge microtome from partially delignified wood blocks of pine and lime and also from untreated control blocks. Sections from control blocks were subjected to mild delignification treatment in test tubes immersed in a water bath at 40°C for the same period as before. Staining was carried out with either safranin and picro-alanine blue or with phloroglucinol hydrochloride which is a specific strain for lignin. Observations were carried out under the highest powers of the light microscope.

b Results

No disorganisation of the cell walls was observed in either wood species although the earlywood cells in pine were distorted in sections prepared from partially delignified blocks apparently due to weakening of the cell walls and pressure from the microtome knife.

c Discussion

Lignin is usually deposited in the compound middle lamella where it may constitute about 60-90% of the dry

weight of the cells and it is least abundant in the tertiary wall, S_3 layer, where it does not exceed 10% of the dry weight (Jane, Opt.cit). It has also been shown that the earlywood of a growth ring has a higher proportion of lignin in its cell walls than has the latewood cell walls.

Since the compound middle lamella acts as a cementing substance between the cells, any chemical reaction that disturbs its content would result in the disorganisation of the fine structure of the cell walls. The loss in weight of the wood blocks as a result of the delignification treatment indicate, at least, that a certain amount of lignin was lost through the sodium chlorite-acetic acid treatment. This amount, though small, could affect the molecular arrangement of the cellulose-lignin complex in the cell wall. It is also possible that some of the hemicelluloses would be hydrolysed to lower molecular weight compounds thus enabling the wood blocks to be more susceptible to colonisation and decay.

Microscopic observations, however, showed that there was no effect on the gross anatomy of the wood cells within the limits of resolution of the highest powers of the light microscope. Findlay (1970), on the other hand, produced electron photomicrographs to show that there was delamination of the cell wall microfibrils after partial delignification. This probably would increase the accessibility of the cellulose in the cell walls to enzymes of invading micro-organisms.

Nilsson (1974), studying the influence of physical structure of cellulosic substrates on the activities of soft rot fungi, found that these fungi appear to require an ordered fibrillar structure of the substrate in order to produce cavities. He

observed that there was rather sparse formation of cavities in delignified wood and cotton fibres whilst there was an increase in the erosion type of attack in these substrates and concluded that this could be due to disorganisation of the fibrillar structure after mild delignification.

Since erosion type of attack is described as advancing dissolution of the cell wall layers from the lumen of the cell (Corbett, 1963), it can be inferred that the large occurrence of this type of attack observed by Nilsson was the result of the S_3 layer having been modified by the delignification treatment thus enabling cellulolytic enzymes of the invading microorganisms to gain easy access to the S_2 layer. Consequently the rate of decay was found to be significantly greater in the partially delignified blocks than in the untreated control blocks in this experiment.

The fact that lime blocks lost more weight through delignification than the pine blocks probably suggest that lignin in lime is more easily removed by the sodium chlorite-acetic acid treatment, and also as a hardwood, lime was more accessible to treating liquids because of the open vessels.

Bailey, Liese and Rösch (1968) also studied the effect of partial delignification on the rate of decay of wood by selected microfungi and brown rot fungi in pure cultures and found a greater increase in decay rate of the treated blocks. They proposed a hypothesis which describes a pre-cellulolytic stage prior to cellulose decomposition in lignified wood cells. It basically includes all extracellular activity auxiliary to the breakdown of cellulose and it is concerned with increasing the accessibility of cellulose to cellulolytic enzymes. They suggested that this pre-

cellulolytic system could be similar to the effect of mild delignification and proposed that the ability of a fungus to effect an efficient pre-cellulolytic phase relates to its potential to utilise cellulose in the presence of lignin hence the brown rot fungi they used decayed control blocks as much as the partially delignified samples!

In the present investigation, the fact that the rate of decay in partially delignified lime was still greater than that of pine similarly treated suggests that differences in the cell walls still existed. If it is accepted that there is a chemical bonding between cellulose and lignin in wood then it may be suggested that this bonding is different in the two wood species. Therefore, when pine and lime are treated with a preservative that penetrates the cell wall, it is possible that the resultant chemical complexes formed may be different and this may account for different toxicity values.

Chapter V Colonisation and decay of CCA-treated wood

Introduction

Copper-chromium-arsenic is one of the commonly used water soluble preservatives with a very good performance in wood placed in contact with the soil. This property has been attributed partly to its ability to permeate the wood cell walls with the resultant formation of stable toxic complexes (Belford et al, 1959; Rudman, 1966). The chemistry of the formation of these complexes is not fully understood. Eadie and Wallace (1962) considered that in the fixation of CCA in pine sapwood, mixtures of copper-chromates and chromium-arsenate compounds are formed probably accompanied by the formation of a copper-cellulose complex (Preston, 1962; and Wilson, 1971). It appears therefore that accessibility of the cellulose in the wood cell walls to enzymes of invading soft rot fungi would depend on their ability to overcome the toxic complexes. *In vitro* studies have demonstrated the toxicity of soluble copper, chromium and arsenical salts and this has led to the assumption that the fungitoxic actions of copper and arsenic salts are responsible for the effectiveness of the preservative. Chromium has little or no toxic effect and it is added to aid fixation.

Dahlgren (1972) studied the course of fixation of CCA in wood and noted that there is an instant increase of pH when the preservative comes into contact with the wood because of ion-exchange and absorption reactions. As the elements are precipitated the pH of the wood continuously increases and reaches a maximum when all the chromium is

consumed.

The early compounds formed are unstable and are simultaneously converted into more stable ones. This is accompanied, further, by alternate increase and decrease of pH until the reaction ceases after several months. Wilson (1971) reported that the rate of fixation of CCA in both softwoods and hardwoods increased as the temperature was raised and maximum fixation was obtained at 30°C within forty two hours. He also noted that fixation generally occurred more rapidly in hardwoods than in softwoods and increasing the strength of the treatment solution led to faster fixation of arsenic and slower fixation of copper.

The rate of fixation of CCA in wood, therefore, does not only depend on the species of wood but also on the concentration of the preservative and on the temperature at which the wood is stored. These will also affect the length of time required to achieve complete fixation and hence the success or failure of the treated wood under exposed conditions.

Da Costa and Osborne (1968), for instance, found a wide variation in threshold values among different timber species and suggested differences in the mechanisms of fixation leading to the formation of different toxic compounds. Jones (1972) suggested that in an aquatic environment the ionic composition, for example, of brackish water, may be implicated in the leaching of CCA from treated timber. He found that arsenic was leached at a faster rate than chromium and copper was the most leach-resistant.

The loss of CCA from wood may not be due solely to changes in the physical conditions in the environment or inadequate fixation of the preservative in the wood but

also to biological processes in the ecosystem of which the treated wood forms part.

Tolerance of microfungi to CCA have been mentioned in the general introduction and literature review. Butcher (1971) found that among the early colonisers of CCA treated wood Penicillium frequentens and Cladosporium elatum were highly tolerant of both copper and arsenic whereas Cephalosporium sp. was tolerant only of copper. He further showed in soft rot capability tests of untreated wood that whereas Cephalosporium sp caused 29.2% weight loss, Cladosporium elatum produced only 2.5% and Penicillium frequentens produced none at all under the same conditions of exposure. To what extent does CCA affect cellulolytic activity of these and other initial colonisers of treated wood? Research along these lines has been ignored.

In a series of leaching experiments using a number of softwood and hardwood species Da Costa and Osborne (1968) have shown that decay occurred in wood treated with toxic amounts of CCA when exposed to basidiomycetes, both before and after leaching with water. They found that a large amount of decay occurred in treated blocks which were first leached, exposed to a fungus then leached again, and finally exposed to the same or other fungus. It suggested to them that the difference between the leached and the 'doubled leached' blocks was due to the action of the fungus on the preservative during the first decay period. Levi (1969) has shown that CCA can be extracted from treated wood by culture filtrates of some basidiomycetes grown on a glucose-peptone media and Chou, Preston and Levi (1974) confirmed these results by using

treated sapwood veneers of pine and X-ray fluorescence spectroscopy for analysis of the extracted copper, chromium and arsenic after decay. Levi (opt.cit) also found that Poria monticola precipitated copper from a copper sulphate solution as copper oxalate.

Whilst these results showed that wood destroying fungi, basidiomycetes, secrete certain metabolites which react with preservatives and render them more soluble and hence more readily removed by leaching under laboratory conditions, it is likely that similar conditions may not exist in service situations. For it is now known that generally basidiomycetes are relatively less tolerant of preservatives although some, like arsenic-tolerant Lenzites trabea and copper and arsenic-tolerant Poria vaillantii, are exceptions. Besides, since basidiomycetes have been shown to occupy the later stages in the succession during colonisation of both preservative-treated and untreated wood in contact with the ground (Butcher, 1971), it seems reasonable to suggest that by the time they gain access to the treated wood, the preservative might have already been modified to a less toxic form by the early colonisers.

It is therefore considered that any realistic approach to studies on detoxification of wood preservatives employed for wood to be used in contact with the ground should include studies on the action of the early colonisers.

The experiments which follow were designed to provide some fundamental information on some factors which may influence deterioration of CCA-treated wood in contact with the ground. It was hoped that the results obtained may throw some light on the mechanism of failure of wood treated with

varying concentrations of CCA.

V:i Effect of incubation period on the toxicity of CCA in wood as determined by oxygen uptake and weight loss measurements

Introduction

Methods for evaluating the toxicity of a preservative (usually expressed as the toxic value or the interval between the lower retention of the preservative which just allows decay to proceed and a higher retention which just prevents it) involves exposure of small blocks of perishable wood treated with graded concentrations of the preservative to pure cultures of test organisms under controlled conditions. The lowest concentration at which attack of the wood is prevented is taken as the toxic limit. Standard methods which have been adopted use weight loss as the criterion for determining the toxic limit and this has been shown to depend on the species of the test organism (Cowling, 1957), strain of the organism (Gersonde, 1958), species of wood (Duncan, 1958) and size and shape of the test block (Anon, C.S.I.R.O., 1963). Bravery (1968), in addition, reported on how values of toxic limits under such experimental conditions could be influenced by the incubation time. A number of workers (Halabisky and Ifju, 1968; Smith, 1969; Behr, 1972 and Damaske and Starfinger, 1975) investigated respirometry as a means of evaluating wood preservatives and the general conclusion was that it is less laborious and less time is required to achieve results. None, however, has compared

objectively this method with standard methods at different incubation times although after a specific period of exposure comparable results have been obtained. It was, therefore, decided to investigate the effect of incubation time on the toxicity of CCA in wood and to find out whether there is any correlation between oxygen uptake and weight loss measurements employed for such studies. For the rest of this project only the sapwood of lime was used since a faster rate of decay could be achieved.

b) Method

One cubic centimeter blocks, prepared from lime planks, were dried to moisture-free weights and treated with the following concentrations of CCA on mass/mass basis:0.01%, 0.05%, 0.10%, 0.25%, 0.50%, 1.00%, 1.50%, 2.00%, 3.00% and 4.00%. The following retentions expressed as kg/m^3 of the preservative in the wood blocks were obtained respectively; 0.08, 0.39, 0.82, 1.50, 3.96, 7.80, 9.60, 11.50, 15.90 and 24.40. Control blocks were treated with sterile distilled water. The preservative was allowed to fix into the wood and drying was carried out as described in the section under materials and general methods in chapter 2. The blocks were marked and dried again to moisture free weights. In all 352 blocks were treated. Each concentration contained 32 blocks. It was decided to study the effect of incubation time on toxicity both in pure culture and in the soil.

C. globosum and Leptographium lumbergii were selected for the pure culture studies. The former was used as a standard soft rot test fungus and the latter was chosen

because it was isolated several times in CCA-treated lime blocks in experiments described elsewhere in this thesis.

Soil burial test

Sixteen blocks per each concentration of CCA were buried in the soil in plastic containers and incubated at 30°C for eight weeks. Assessment of weight loss and oxygen uptake in the blocks were carried out at the end of the fourth and eighth weeks. At the end of each of these periods eight blocks per concentration were removed. Weight loss was determined in four blocks and the remaining four were used for measurement of oxygen uptake. In the determination of weight loss after four weeks, the moisture contents of the blocks were also noted. Since the soil surface had to be sprayed with water after the fifth week to prevent drying, moisture content of the remaining blocks were not determined at the end of the eighth week.

The mean weight loss, oxygen uptake and moisture content are plotted against concentration of CCA and presented in Figures 13 and 14.

The relationships between respiration and weight loss are shown in the form of scatter diagrams in Figures 15 and 16. These were shown for CCA concentrations at which significant weight losses (i.e. 3% and above) have been obtained. Similarly weight loss and oxygen uptake have been related to moisture content of the blocks in Figures 17 and 18.

Pure culture studies

The culture medium and the method described in B.S 838

Fig.13. Effect of period of incubation on the rate of oxygen uptake in wood blocks treated with different concentrations of CCA.

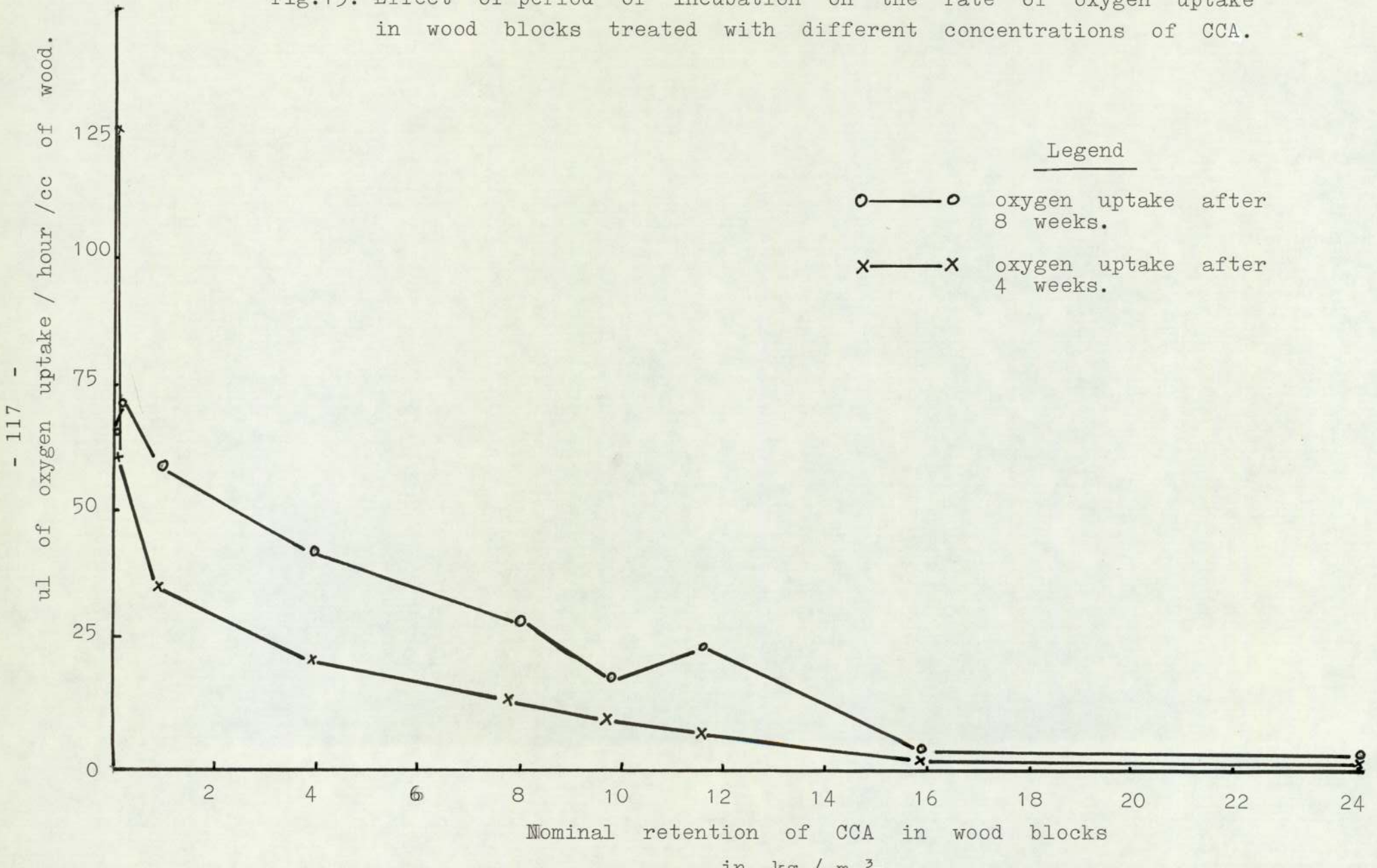


Fig.14. Relationship between % weight loss and moisture content of wood blocks treated with different concentrations of CCA after different periods of soil burial.

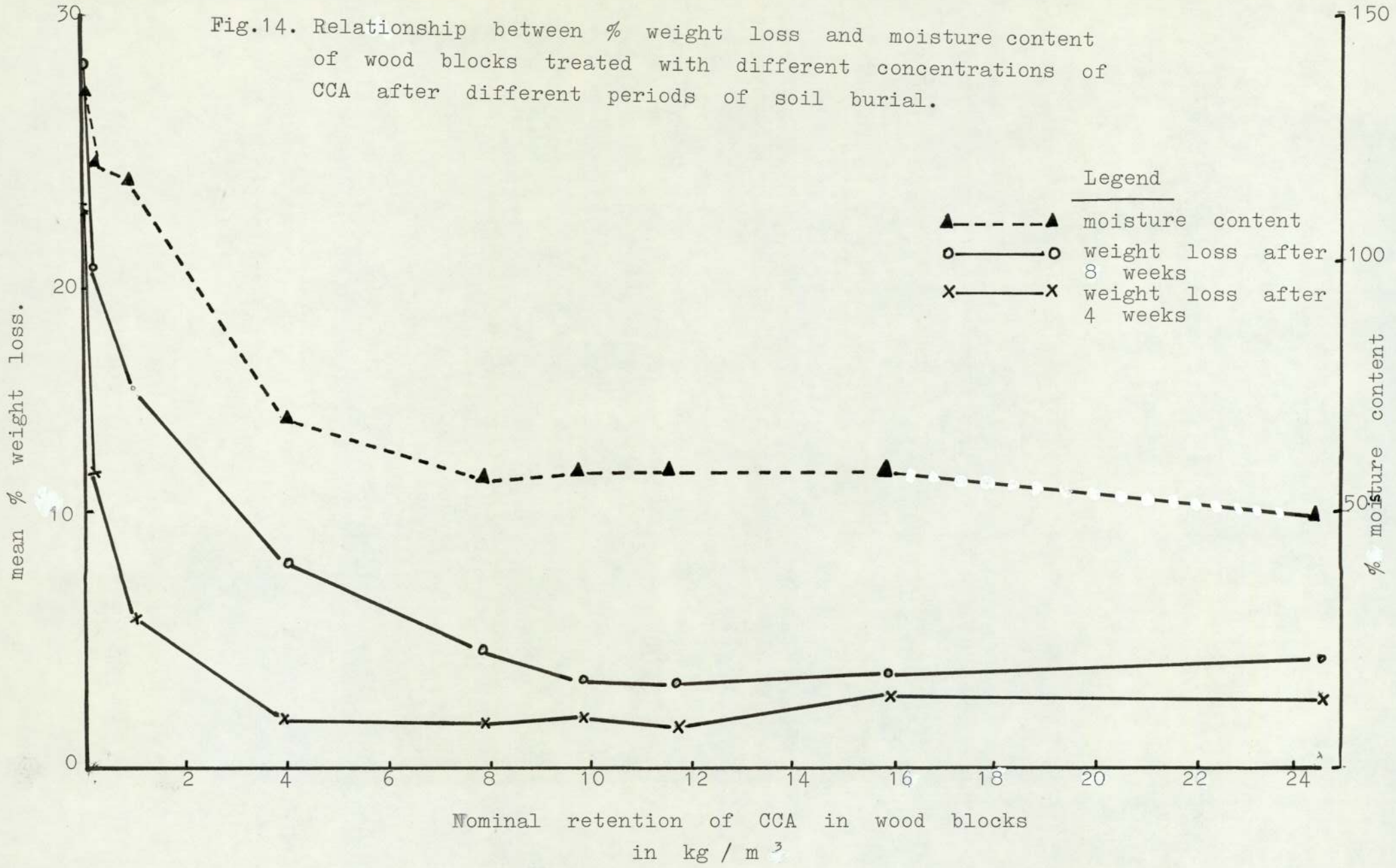


Fig.15. Relationship between % weight loss and rate of respiration in decayed wood blocks after 4 weeks of soil burial.

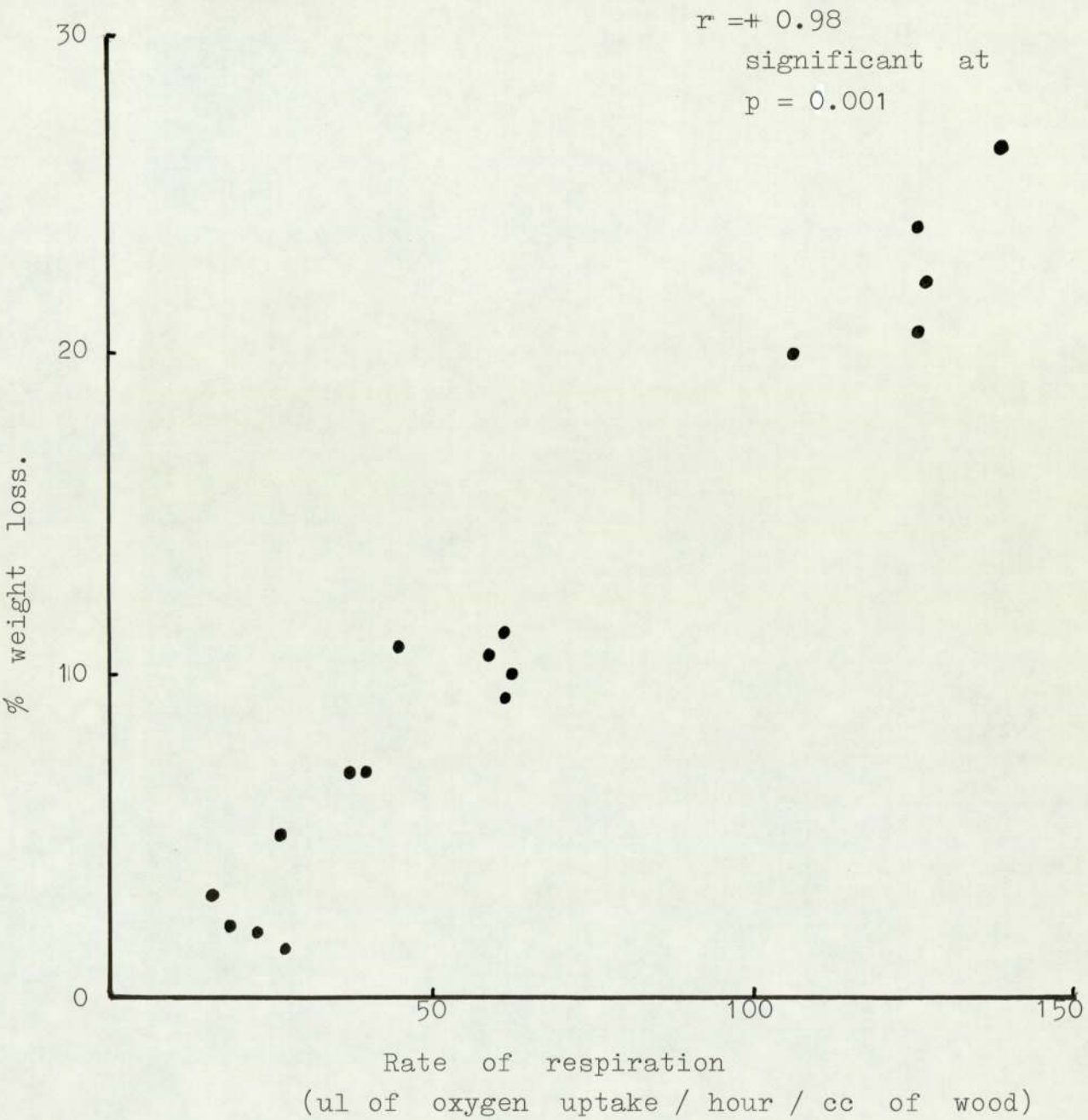


Fig.16. Relationship between weight loss and respiration after 8 weeks of incubation.

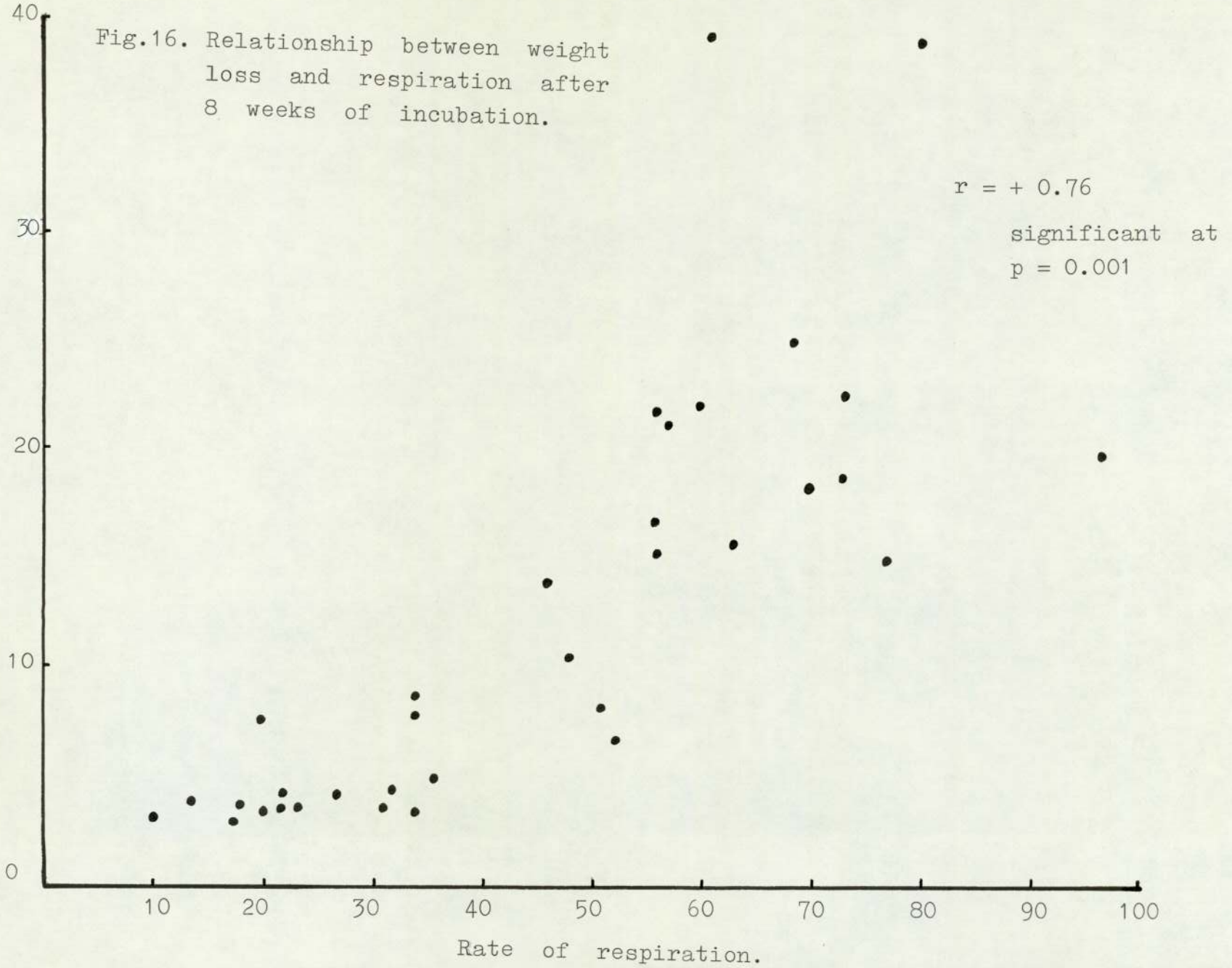


Fig.17. Relationship between rate of respiration and % moisture content of test blocks after 4 weeks of soil burial.

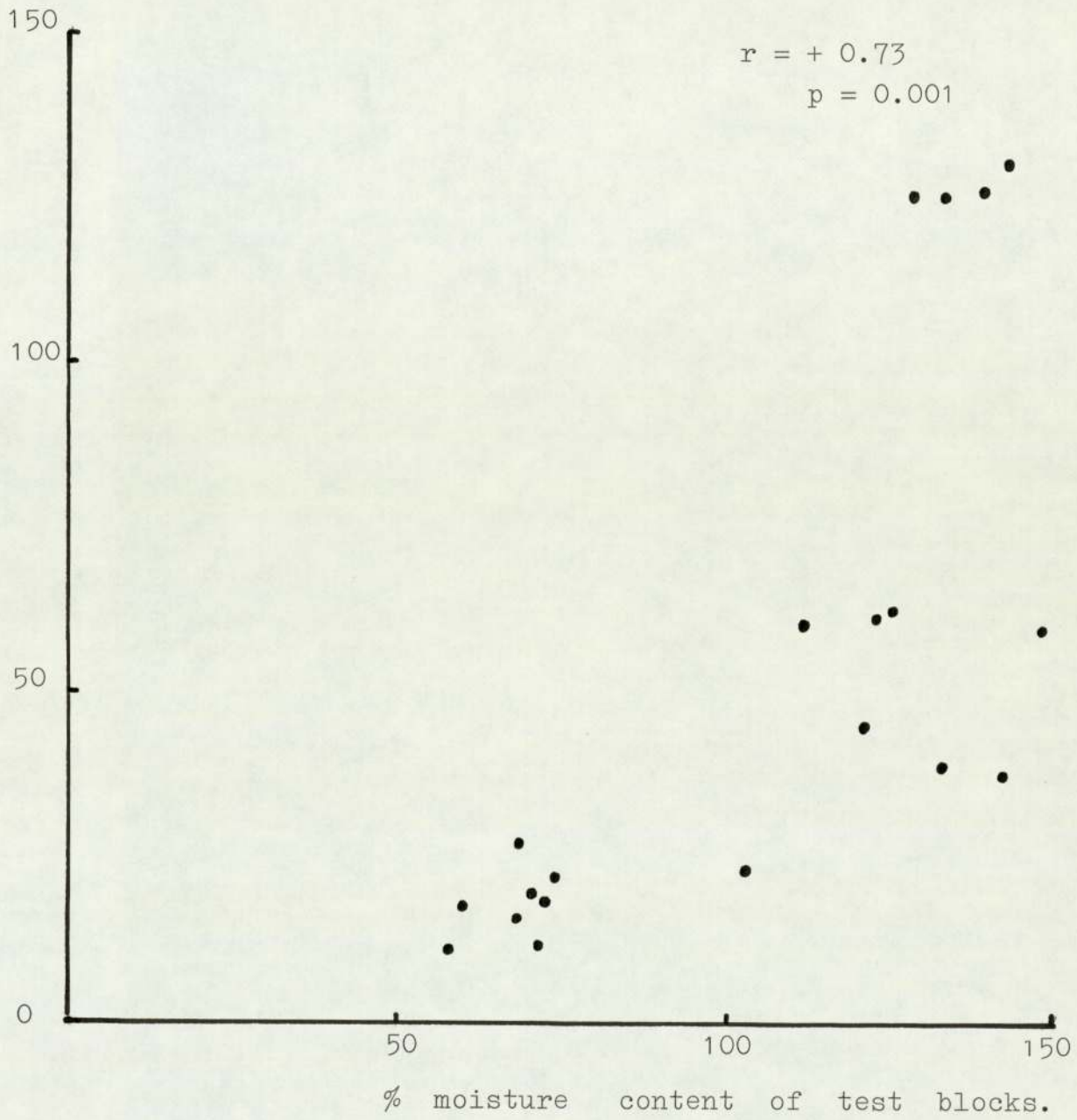
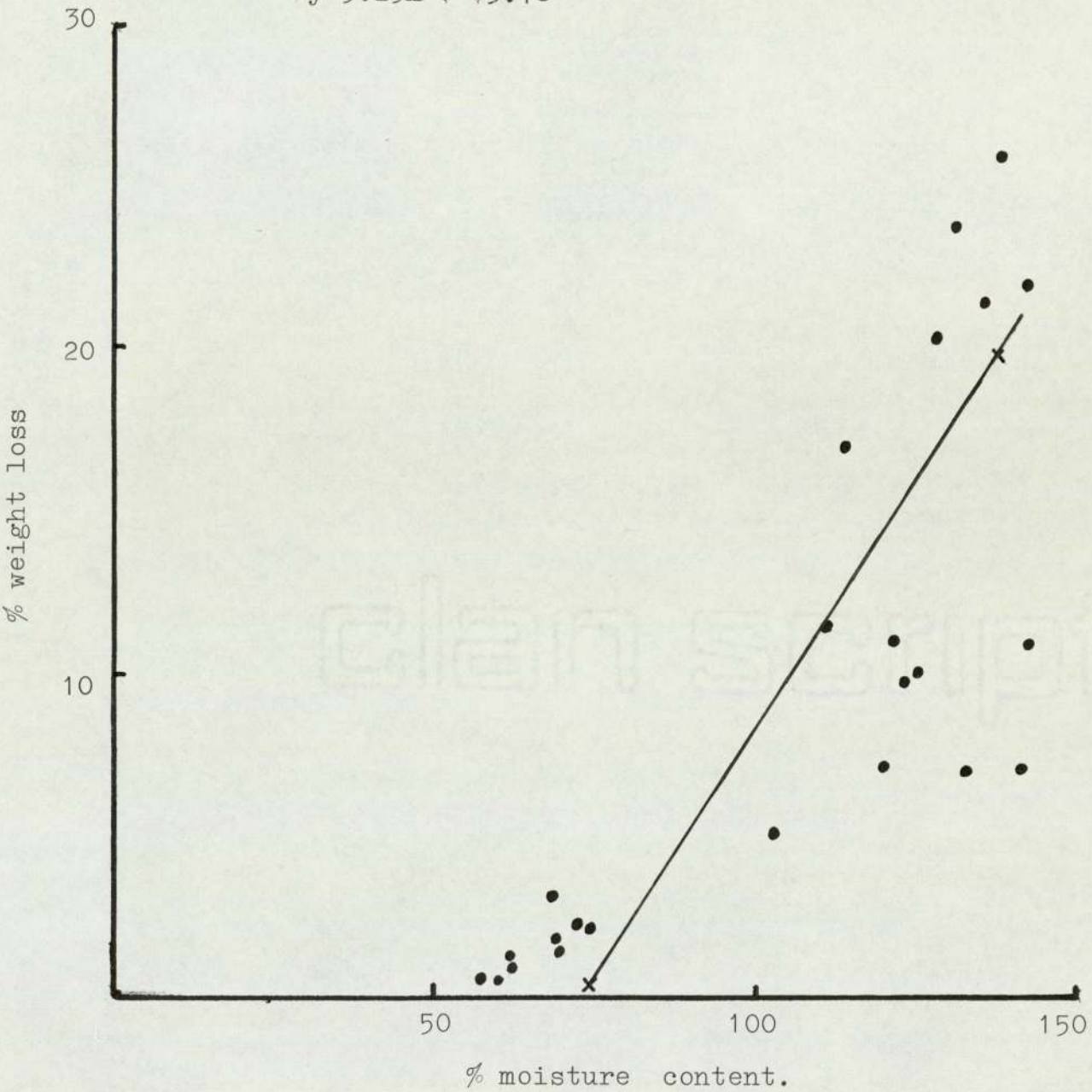


Fig. 18. Relationship between % weight loss and moisture content of treated wood blocks after 4 weeks of soil burial.

r=+0.84, p=0.001.

y=3.23x + 73.78



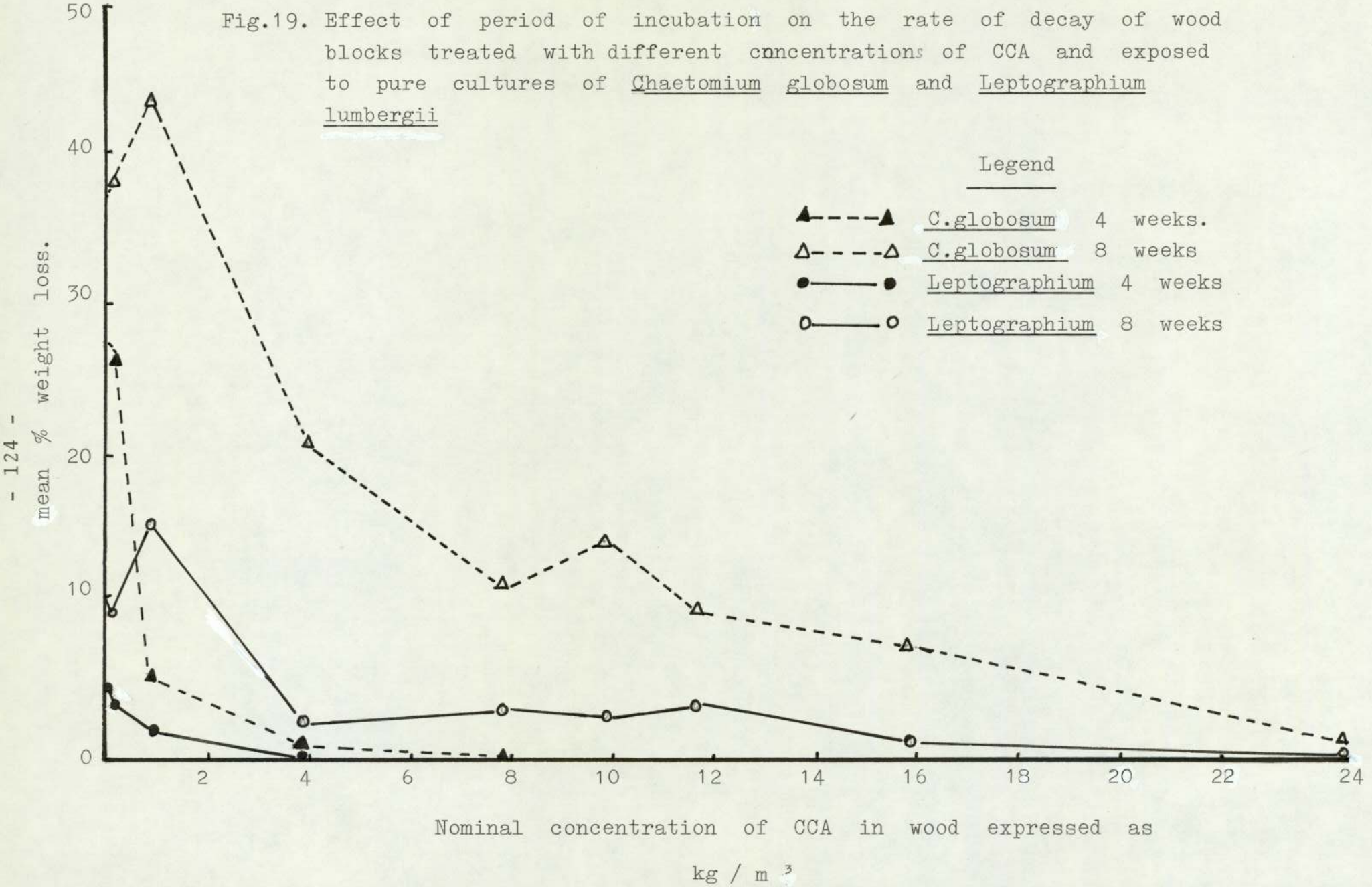
for test of toxicity using soft rot fungi was employed. Sterile petri-dishes were used instead of bottles as culture containers. Eight treated blocks per concentration of CCA were sterilised in the oven at 80°C for 24 hours. After cooling, they were weighed under sterile conditions and planted on actively growing mycelia of C. globosum and L.lumbergii on filter papers placed on the agar medium. Two petri-dishes were set up for each concentration of the preservative. Each petri dish contained four replicate blocks. Uninoculated control blocks were set up for each concentration and these were used to correct for any loss or gain in weight of the test blocks which may have resulted from loss of CCA from or uptake of minerals into the wood blocks. The blocks were incubated at 30°C for eight weeks. At intervals of two weeks 0.5 mls of sterile distilled water were added to the filter paper in the culture plates to maintain the humidity high around the blocks.

At the end of the incubation periods, (4 and 8 weeks) four blocks for each preservative concentration were removed at a time and dried to constant dry weights as before. The mean percentage weight losses were calculated and the results are presented in the form of a graph in Figure 19.

c) Results

After four weeks of incubation in the soil all the wood blocks were at sufficient moisture content (above 45% of oven dry weights) to enable decay to occur (Figure 14). Wood blocks treated with the lowest concentration of CCA

Fig.19. Effect of period of incubation on the rate of decay of wood blocks treated with different concentrations of CCA and exposed to pure cultures of Chaetomium globosum and Leptographium lumbergii



and control blocks, however, attained the highest moisture contents. Similarly, respiration rate and percentage weight loss were highest for wood blocks treated with low concentrations and in control blocks (Figures 13 and 14). It seems, therefore, that moisture content, rate of respiration and percentage weight loss may be related in some way.

Figures 15 and 16 show that there is a positive correlation between percentage weight loss and rate of respiration both after four and eight weeks of incubation at preservative concentrations where significant decay of the test blocks occurred ($r = +0.98$, $p = 0.001$; and $r = +0.76$, $p = 0.001$ respectively).

Figures 17 and 18 also show that there is a positive correlation between respiration and moisture content on one hand and between weight loss and moisture content on the other hand ($r = +0.73$, $p = 0.001$, and $r = +0.84$, $p = 0.001$ respectively).

One noteworthy point in Figure 13 is the drop in respiration rate in control blocks after 8 weeks of soil burial. The rate of respiration after four weeks was 125µl of O_2 whilst after 8 weeks it dropped to about half as much, 65 µl of O_2.

The effect of incubation periods on toxic limit is clearly shown in Figures 13, 14 and 19. After four weeks of incubation in the soil, weight loss was insignificant at preservative concentrations above 3.96 kg/m^3. After 8 weeks they were just significant at concentrations above 9.60 kg/m^3 (Figure 14). The level of significance used here is based on the 3% weight loss figure suggested by

B.S. 838 (1961). The toxic limit for the soil burial experiment can therefore be computed to be between 3 - 3.96 kg/m^3 after four weeks and 9.60 - 24.40 kg/m^3 after eight weeks of incubation. There is also an increase in oxygen uptake in wood blocks after eight weeks of incubation in the soil at all the preservative concentrations (Figure 13); for instance, at a concentration of 11.5 kg/m^3, the wood blocks respired only 8µl of oxygen.

The shift in toxic limit is also noticeable in the pure culture experiments. After 4 weeks of incubation the toxic value is 3.96-15.9 kg/m^3 of CCA in wood blocks inoculated with C.globosum. After eight weeks it has shifted to 15.90 - 24.4 kg/m^3. Treated wood blocks inoculated with L.lumbergii showed negligible weight losses but here again there was an increase after 8 weeks of incubation (Figure 19).

d Discussion

It became evident from the results in these experiments that extension of the incubation period from 4 to 8 weeks resulted in increase in weight loss and respiration rate at any given concentration of the preservative. Thus the toxicity of CCA in wood was affected by the length of exposure of the treated blocks to microorganisms both in pure cultures and under soil burial conditions.

Another striking observation was the reduction in oxygen uptake in control blocks after eight weeks of soil burial. This could be explained on the basis of nutrient depletion in the wood blocks. It may also be due to the onset of another phase of colonisation of the wood blocks during which the activity of the initial colonisers have been reduced by freshly invading antagonistic organisms. Bravery (1968) described three phases of fungal decay of wood blocks treated with different concentrations of a preservative and explained how these could be affected by time. The first stage is juvenile during which passive colonisation takes place. It is followed by a dynamic phase of active wood destruction and finally a mature phase where the bulk of the wood material has been utilised and decay rate is much slower. This concept of decay of preservative treated wood as affected by the period of incubation can be visualised very clearly in the results obtained in the above experiments. At each preservative concentration different phases of decay can be observed; for instance, the mature phase is reached soonest in wood blocks without any preservative and the juvenile stage is very much delayed in blocks with high concentrations of the preservative. Wood blocks with very low concentrations of the preservative can be said to be in

a dynamic phase of active wood destruction at the eighth week of incubation.

The conclusion to be drawn from these studies is that continuous presence of an organism or organisms in a preservative treated wood may result in changes in the latter to promote greater rate of growth of the invading organisms. Changes in pH of wood invaded by basidiomycetes over a period of time have been noted by Cartwright and Findlay (1958). In a treated wood such changes may affect the toxicity of a preservative which is pH dependent.

Another important result obtained in these experiments is the positive correlation between weight loss and respiration. It is therefore suggested that either method can be used to assess the rate of deterioration of preservative treated wood. Problems may however arise when weight loss method is used to determine decay rate after a short period of incubation during which very little or insignificant loss of wood substance may be obtained. At this stage assessment of respiration may indicate significant activity of the invading organisms.

The intensity of oxygen uptake in a decaying wood at a particular time reflect the activity of microorganisms in the wood during the time in question whereas a weight loss test gives the total loss of wood substance from the beginning of invasion to the time of assessment. It takes into account the effect of both primary and secondary invaders.

The very high positive correlation noted between percentage weight loss and rate of respiration after four weeks of incubation may probably indicate a dynamic stage of wood deterioration during which the amount of wood substrate

utilised is equivalent to the amount of oxygen consumed by the invading microorganisms. The less perfect positive correlation between weight loss and respiration after eight weeks of soil burial might suggest another stage in the process of decay during which certain changes have taken place in the wood. One such change may be nutrient limitation to the microbial population in the wood. Organisms which rely mostly on the presence of simple nutrients are the ones most likely to show a decline in activity. Another possibility is the introduction of other organisms which are unable to breakdown the wood but live on the byproducts of other organisms. At this stage although a greater weight loss may be obtained in the blocks the organisms present in it may not be wholly responsible for it.

The less perfect positive correlation between weight loss and respiration after 8 weeks reflects the fact that after this time a high weight loss has occurred in those samples with low CCA concentration (which had a high respiration rate after 4 weeks) as a result of the continuing high rate of consumption of wood material even though that rate may have increased a little, remained steady or even declined by this time. In fact, weight loss is produced as a result of integrating the respiration rate over the whole period and is not necessarily closely related to the actual respiration rate at the end of the period.

The higher moisture content obtained in wood blocks with low concentrations of the preservative was striking. This may be due to greater activity of the invading micro-organisms as a result of either

(a) metabolic processes according to the equation

$C_6H_{12}O_6 + 6O_2 \rightarrow 6H_2O + 6CO_2 + Energy$, or

(b) conduction of water from the soil by fungal hyphae into the wood (although the distance for such conduction may be short, or

(c) increase in wood permeability as a result of breakdown of cell walls and formation of cavities, or

(d) the interaction of all the above processes, or

(e) reduction in wood absorbancy as a result of CCA impregnation.

By analysing the data a positive correlation has been established between moisture content and weight loss (Figure 18). From a calculated regression line it seems that a moisture increase from 74% to 138% is accompanied by a weight loss of 19%. Assuming that the loss in weight of the wood has resulted solely from utilisation of the carbohydrate component of the wood, then starting with 100 gm of carbohydrate, 19 grams of carbohydrate will be lost. If the weight of wood decreases by 19 gm, the amount of water which will be produced is $\frac{138 \times (100-19)}{100}$ = 109.8 or 110 approx. But only (110-74)gm = 36 gm has been released. From the equation above the ratio of carbohydrate to water is 180:108 or 5:3 and starting with 19 gm of carbohydrate $\frac{19 \times 3}{5}$ = 11.4 gm of water will be produced; but 36 gm of water is needed. In addition, the wood cell wall is made up of other constituents and it can therefore be concluded that the higher moisture content in the control blocks and blocks containing low concentration of the preservative must be due to factors other than moisture resulting from the metabolism of wood constituents.

The presence of fungal mycelia usually observed on decaying wood removed from the soil might also suggest that conduction of water from the soil into the wood is likely, and the presence of soft rot cavities will no doubt increase the permeability of the wood to moisture.

It has also been observed in this experiment as well as in the preliminary experiment described in Chapter 2, that wood blocks treated with higher concentrations of the preservative contained less moisture and were more difficult to convert for respirometric measurements than control blocks and samples with lower concentrations. It was then thought that the preservative had perhaps, altered the hydrophilic properties of the wood such that less moisture was absorbed into the treated wood. The hypothesis put forward was that the extent to which the cell walls was altered depended on the amount of CCA in the wood, hence the higher the concentration of the preservative in the wood the greater the reduction in hydrophilic properties of the cell walls and the less moisture can be absorbed. The CCA used in this project did not contain any emulsion additives and a search through the literature also did not reveal any chemical explanations to support this hypothesis. It was therefore decided to study the uptake of moisture by CCA treated wood blocks under sterile conditions. This is described in the next experiment.

V.ii Uptake of moisture by treated wood blocks under sterile conditions.

a. Method

One cubic centimeter sapwood blocks of lime were treated with 0.1, 1.0 and 3.0 per centage concentration of CCA. They were conditioned to allow fixation of the preservative to take place and dried as described in Chapter 2. Control blocks were treated with sterile distilled water and were dried in the same way as the treated blocks. Five blocks each per concentration of the preservative were set up. The blocks were marked and the dry weights were checked and noted after further drying in the oven at 80°C for 24 hours. They were then buried in a sterile plastic container filled with vermiculite which had been moistened with distilled water to its water holding capacity(208% of oven dry weight) and sterilised by autoclaving twice at 15lb/sq inch for 30 minutes. The container was closed tight with its lid and incubation was carried out at 30°C for four weeks. At the end of the incubation period the blocks were removed, cleaned and immediately weighed. They were dried to constant moisture-free weights and the dry weights were noted. The percentage moisture content based on the final dry weights of the blocks were calculated. The results are presented in Table 15.

b. Results

The results clearly indicate that wood blocks treated with the preservative absorbed less moisture than control blocks. The higher the concentration of CCA used in treating the blocks the lower the moisture content of the treated

Table 15 Uptake of moisture by treated wood blocks under sterile conditions after 4 weeks of burial in wet vermiculite

Concentration of CCA	% moisture content in wood blocks.	Mean	Standard error of mean
0 (0)	138, 125, 124, 122, 121	126.0	3.02
0.1(0.82)	119, 113, 115, 113, 113	114.5	1.18
1.0(7.80)	102, 101, 95, 97, 105	100.0	1.79
3.0(15.90)	101, 99, 98, 98, 97	98.6	0.70

Figures in brackets are CCA retentions in kg/m^3

blocks. Greatest variability of moisture content was obtained in the control blocks (standard deviation = 6.74).

c. Discussion

These results therefore prove the hypothesis that CCA reduced moisture uptake in the treated blocks perhaps by altering water absorbancy of the wood cell walls where the preservative is deposited. Burial in wet vermiculite is a more severe test than burial in wet soil since the former had greater water holding capacity. It is, however, likely that a similar result may be obtained when soil is used although it is difficult to obtain a sterile soil. By comparing the moisture content of the inner layers of treated and untreated wood blocks where no microorganisms can be found, it may be possible to study this phenomenon when the test blocks are buried in an unsterile soil.

In the next experiment changes in moisture content as well as changes in respiration rate and pH of wood blocks at different depths after different periods of incubation are described.

V:iii Changes in pH, moisture content and respiration in wood blocks at different depths after different periods of incubation.

a. Introduction

Käärik (1967) discussed some hypotheses to explain the mechanism of fungal succession in the deterioration of wood in ground contact and put forward that one of these factors is the dependance upon certain changes in the wood substrate such as moisture content and pH. Good, Basham and Kadzielawa (opt.cit) associated stain reaction by some members of the Fungi Imperfecti and bacteria with high moisture content, a high pH and a relatively high respiratory activity of the invaded wood. Butcher (1968) recorded a drop in pH in wooden stakes below the ground level from 5.4 to 4.9 after six months of burial. His assessments were based on discs removed from a cross section of his stakes without particular attention to the depth to which the fungi had penetrated. On the other hand, King (1975) who also made comparable observations in an untreated wood, related the drop in pH to depth of fungal penetration after a specific period of incubation.

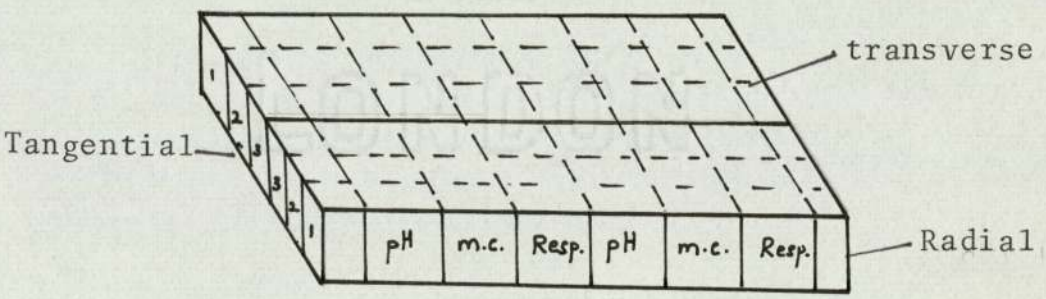
These experiments, however, were of short duration so that only micro-fungi were involved and these organisms have less effect on pH than basidiomycetes.

To extend these studies further it was decided to find out whether changes in pH and moisture content both in the treated and the untreated wood are associated with microbial activity at different depths in the wood with the progress of decay.

b. Method

Accurately cut sapwood blocks of lime measuring 30 x 50 x 10 mm in the tangential, radial and longitudinal planes were treated with either 0.25% or 1.5% of CCA solution. For each concentration there were eight replicate blocks. Untreated blcoks provided controls.

After treatment the preservative was allowed to fix into the wood and the blocks were dried under laboratory temperature conditions for three weeks. All the surfaces except the two radial faces in each block were coated with araldite. After the sealant had set and dried the test blocks were buried in the soil. Incubation was carried out at 30°C for four months. At monthly intervals two blocks per preservative concentration were removed, cleaned and converted for pH, moisture content and respiration determination. In order to sample the wood blocks for the above parameters without bias the scheme adopted for the conversion of the blocks is illustrated in the diagram below:



Conversion of the blocks was carried out with a stanley knife and after cutting out the various portions, respiration and moisture content determinations were immediately carried out. After oxygen uptake measurements,

Fig.20. Respiration measurements.

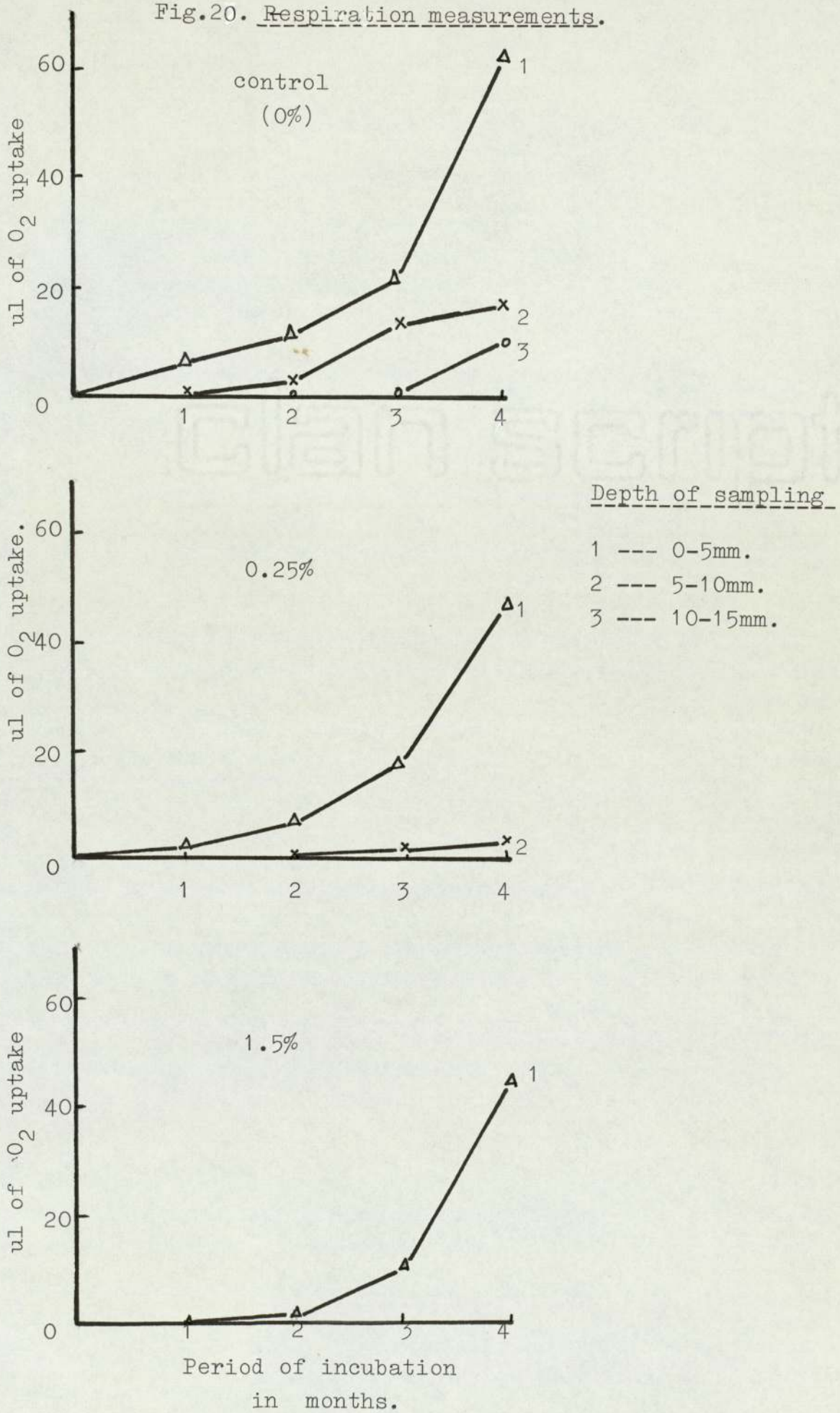


Fig. 21 . pH measurements.

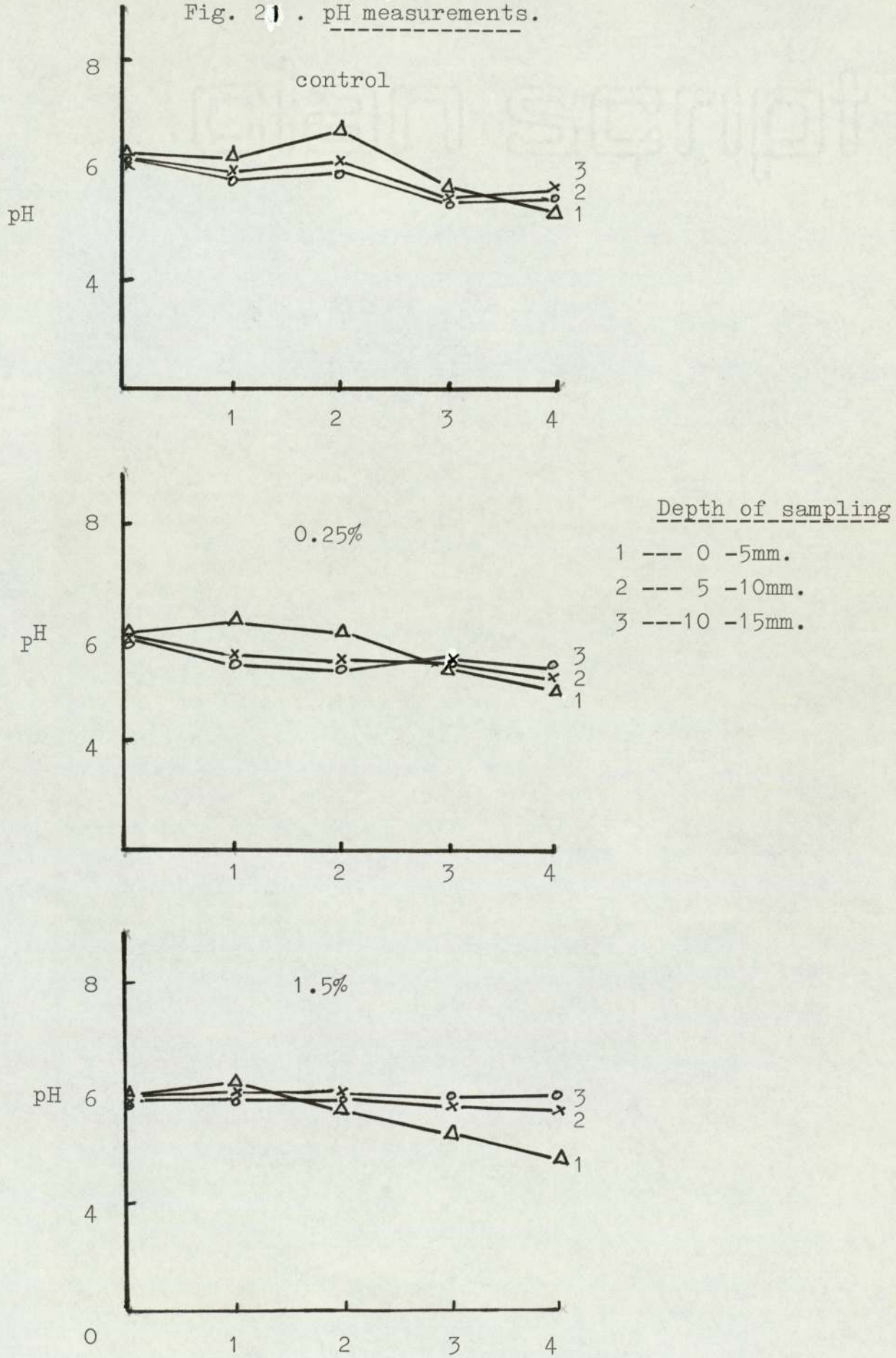
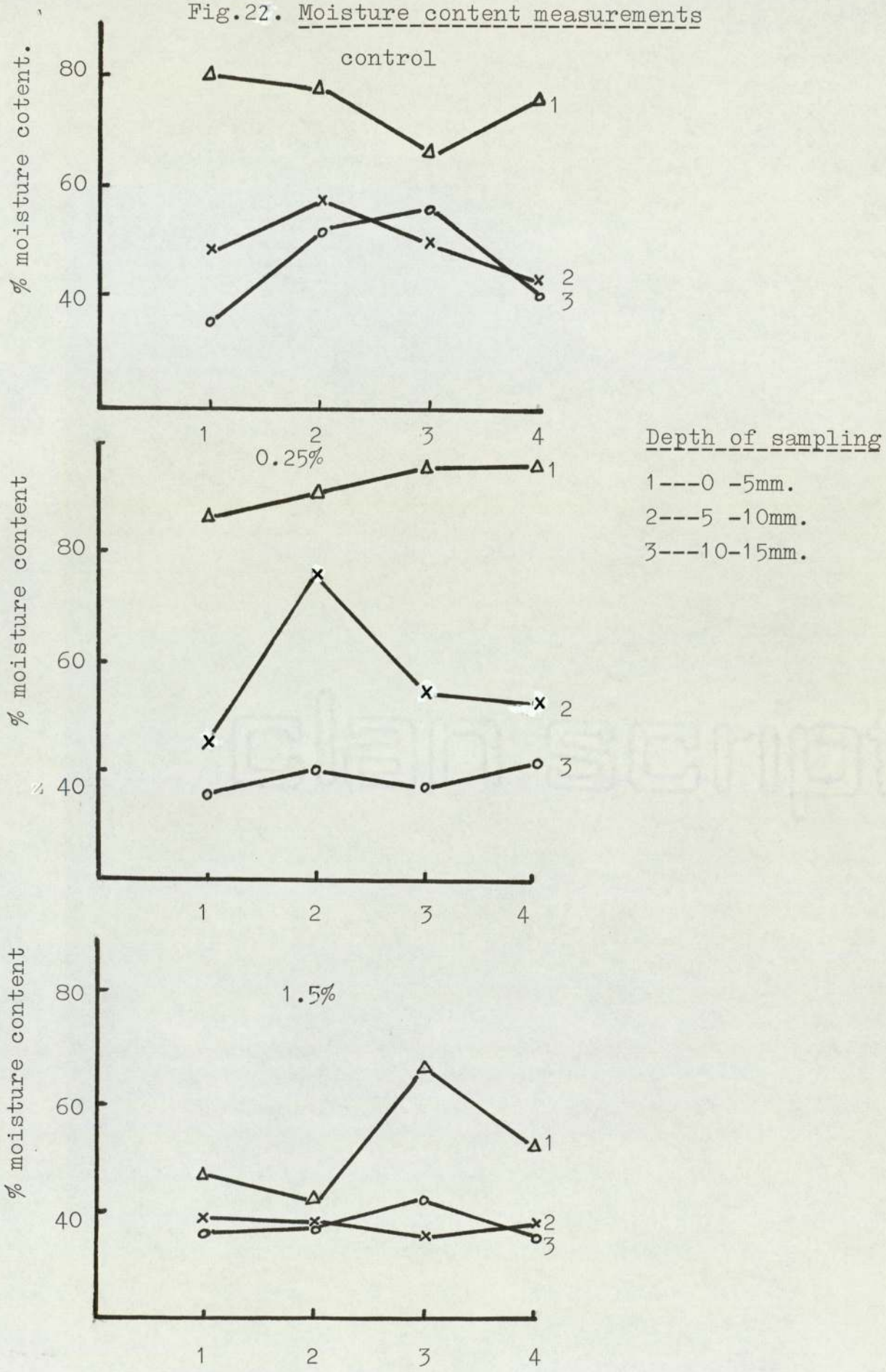


Fig.22. Moisture content measurements



samples obtained from blocks treated with 1.5% CCA were cut out into small billets and plated out onto either 2% malt agar or E and P cellulose medium and E and P cellulose medium adjusted to contain 1.5% of the preservative. In this way, fungi growing on the treated wood were isolated and used for pure culture toxicity studies. This is described in the next chapter.

To determine the pH, wood samples were dried and ground in a laboratory mill. One gram of the resultant powder was steeped in 10 mls of sterile distilled water for four hours with periodic agitation. pH of the suspension was measured with a Pye pH meter. The results are shown in Figure 20, 21 and 22.

c. Results

Respiration

As expected, the rate of colonisation and hence respiration increased with the period of incubation both in the control blocks and in the treated samples although the former had registered the highest rate of respiration at all the three depths sampled. Wood blocks treated with 1.5% CCA did not show any evidence of respiration beyond a depth of 5mm even at the end of four months of incubation. In these blocks, respiration was considerably high in the first layer suggesting that colonisation was confined to the surface layer and penetration of microorganisms into the wood was abruptly limited. Wood blocks treated with 0.25% CCA were penetrated up to a depth of 10 mm although respiration at this point was very low after four months. In the control blocks penetration had occurred to a depth

of 15 mm when the blocks were sampled after four months of incubation (Figure 20).

pH

pH of the wood blocks fell to a slightly more acid state. This was more evident in the outer layer than in the inner layer where the change did not appear to be significant. Since different blocks were sampled at different times variability in the test blocks could not be over-ruled and also overlapping of the layers was inevitable.

Moisture content

Like respiration, moisture content was highest in the surface layers where the wood was in contact with the soil although fluctuations were noticed in this layer. Generally, there was no progressive increase in moisture content as the period of incubation was increased, probably because by the end of the first month of burial the wood blocks had already reached equilibrium moisture content with the soil. This, however, does not appear to be so since the inner layers contain less moisture than the surface layers. Generally, also, the moisture content in wood blocks treated with CCA was low. This was particularly noticeable in the inner layers of 1.5% CCA-treated samples where the moisture contents did not exceed 45% of oven dry weight.

Exception to the above generalisations was the high moisture content recorded in the first 5 mm layer of wood blocks treated with 0.25% of the preservative.

d. Discussion

In the attempt to understand more fully the mechanism of deterioration of wood by microorganisms various methods such as isolation and identification of the invading organisms as well as microscopic studies and solubility studies of the decaying wood have been employed at one time or another. Certain other factors have also been recognised to be associated with the decay process and may even play some part in determining the course of decay of the wood.

Since the whole process of wood decay is complex it is usually not possible to assess the different parameters all at the same time but by putting together the relevant results it has been possible to obtain a fairly good picture of the possible mechanism of wood decay although a great deal is still unknown.

With reference to the present investigation it has been possible to assess respiration, pH and moisture content of decaying wood at different depths and after different periods of soil burial in one experiment. When these results are explained in terms of previous studies on the succession of fungi as determined by isolations, particularly with reference to the work of Banerjee and Levy (1971), the build-up of microbial activity in relation to the depth of penetration can be appreciated. The results obtained by these workers suggested that although many organisms could be found on the surface layer, relatively few had penetrated to a depth of 5 mm below the surface of the stakes and at 25 mm and 45 mm only very few fungi were isolated. This observation is in agreement with the respiration results obtained here although the depths of sampling were different

and the length of incubation was shorter. It is, however, evident that microbial activity decreased with depth and this decline with depth was more pronounced in the treated wood blocks where penetration was more or less abruptly limited.

The role which oxygen shortage may play in limiting the depth of penetration of the invading organisms cannot be overlooked but because the test blocks were considerably small in size oxygen limitation is thought unlikely to occur. Besides, there was no such limit in the control blocks. Therefore, it seemed likely that the sharp demarcation between the colonised surface and the sound inner parts of the treated blocks was a genuine preservative effect.

Increase in respiratory activity, particularly, at the surface layer is tied up with a corresponding decrease in pH as the period of incubation was increased. This change in pH in relation to growth of wood rotting fungi has long been recognised. (Cartwright and Findlay,1958). Basidiomycetes, in particular, have been shown to produce a large amount of acids as a result of their metabolism. Birkinshaw, Findlay and Webb (1940), for instance, found that Merulius lacrymans and Coniophora cerebella can both bring the pH of a malt solution from 6.8 down to a figure of 2. They pointed out that the brown rot fungi render the medium more acid than do the white rot fungi and that this acidity tends to persist longer than in the case of the latter. In this experiment since the drop in pH was not great it may be concluded that probably basidiomycetes were not present in the wood blocks or perhaps their activity was low at the time of sampling. Basidiomycetes were not encountered in the

isolations.

Distribution of moisture in the wood blocks showed that all the layers were at sufficient moisture content to promote decay. It is, however, striking to note that the inner layers of wood blocks treated with 1.5% had lower moisture contents than those of control blocks (refer to vermiculite experiment). In the latter some respiration had occurred at these layers but the magnitude of this respiration is much too low (Figure 20) to account for the additional moisture content in the control blocks. There was no respiration at these layers in blocks with 1.5% CCA.

The high moisture content in the surface layers of all the test blocks may be due to softening of these layers as a result of decay which consequently increased the permeability and perhaps the hydrophilic nature, of the wood. In similar studies but with stakes erected at an exposed site throughout the year, Greaves (1972) did not find any relationship between increase in moisture content of the stakes and the onset of the wet season and explained that such increase was possibly the direct result of increase in permeability as the surfaces became more softened by fungal attack.

The conclusion to be drawn from these experiments is that CCA not only minimises microbial colonisation of treated blcoks but also reduces the moisture absorbing capacity of the blocks and the degree to which this occurs depends on the concentration of the preservative solution used in impregnating the blocks.

Chapter VI Further experiments on the toxicity of CCA. Growth, survival and cellulase production of some selected microfungi in pure culture medium containing varying concentrations of CCA

Introduction

In the course of isolation of microorganisms from treated wood blocks used for the previous experiment it was noticed that Trichoderma viride, Chaetomium globosum, Aspergillus sp. Cephalosporium sp. Penicillium sp. and Leptographium lumbergii were isolated onto an agar medium at various times from wood blocks treated with 1.5 percentage solution of CCA but failed to grow on the same agar medium adjusted to contain the same amount of the preservative. It was therefore postulated that either (1) the toxicity of the CCA in the wood has been altered initially to a lower concentration by leaching or by the activities of other microorganisms such as bacteria and actinomycetes to allow subsequent invasion of the fungi or (2) the toxic properties of CCA in wood are fundamentally different from that in agar so that greater inhibition is obtained in agar than in wood when both are treated to contain the same amount of the preservative.

Sharp and Levy (1974) reported high percentage frequency of isolation of fungi from lime strips treated with 2.0% CCA as early as three days after soil burial. Greaves (1972) observed cell wall damage by bacteria in CCA-treated stakes exposed to the ground and concluded that bacteria are able to tolerate the preservative at retentions usually employed to prevent fungal attack. Irvine and Jones (1975) found

that in the presence of CCA in agar culture the inhibition point for most soft rot fungi occur between 0.0125 and 0.025 percent, whilst that for the marine bacteria they tested occurred between 0.1 and 0.5 per cent of the preservative. They suggested that the causative agents for the initial breakdown of treated timber under marine conditions are bacteria with fungal colonisation occurring later. Butcher (1971) on the other hand demonstrated that the inhibition points for mycelial growth of T.viride, Cephalosporium sp and C. globosum in agar culture containing CCA were 0.075%, 0.5% and 0.15% respectively. The above observations were based on the radial spread of mycelium or on germination of spores. None has reported on the effect of the preservative on fungal biomass production or on the degree of enzyme production.

In wood where the cell walls are penetrated by the preservative as in the case of CCA-treated wood, the ability of the invading microorganisms to decay the wood would depend on their capacity to produce sufficient cellulolytic enzymes in the presence of the toxic components of the preservative.

Fungal cellulases are generally regarded as being inducible and like all proteins their synthesis is affected by the presence of inorganic compounds. Little, however, is known about their activation or inhibition and the very few reports contain contradictory findings (Gascoigne and Gascoigne, 1960). A number of factors such as the type of substrate, the concentration of the salts used and the experimental conditions under which the test is carried out may play significant roles in the assessment of

their activity.

In this experiment the effects of varying concentrations of CCA in culture medium on the growth and cellulase activity of some selected microfungi are described.

VI.i Radial growth in agar plates and production of mycelial biomass in liquid culture

a) Method

Five litres of Eggins and Pugh cellulose agar medium were prepared and sterilised by autoclaving at 15 lb/sq.inch for 15 minutes. It was divided into seven portions, each containing 450 mls of the medium placed in sterile conical flasks and covered with cotton wool plugs. One per cent solution of CCA was also prepared and sterilised by passing it through a millipore filter (pore size 0.45 μ) under vacuum. The agar medium was allowed to cool to about 40°C and aliquots of the sterile CCA solution were withdrawn with a sterile hypodermic syringe and added, according to table 16, to the agar medium in the conical flasks. Corresponding amounts of sterile distilled were added to obtain equal volumes and the solutions were thoroughly mixed by shaking. Serial concentrations of the CCA in the agar medium were thus obtained (table 16). While still warm the mixtures were poured into sterile petri-dishes and into Cowling tubes which were then covered with cotton wool plugs. To ensure rapid jelling and even distribution of the cellulose, the tubes were immediately immersed in cold water. Three plates and 3 tubes per concentration per fungus were set up. The tubes were kept for the next experiment.

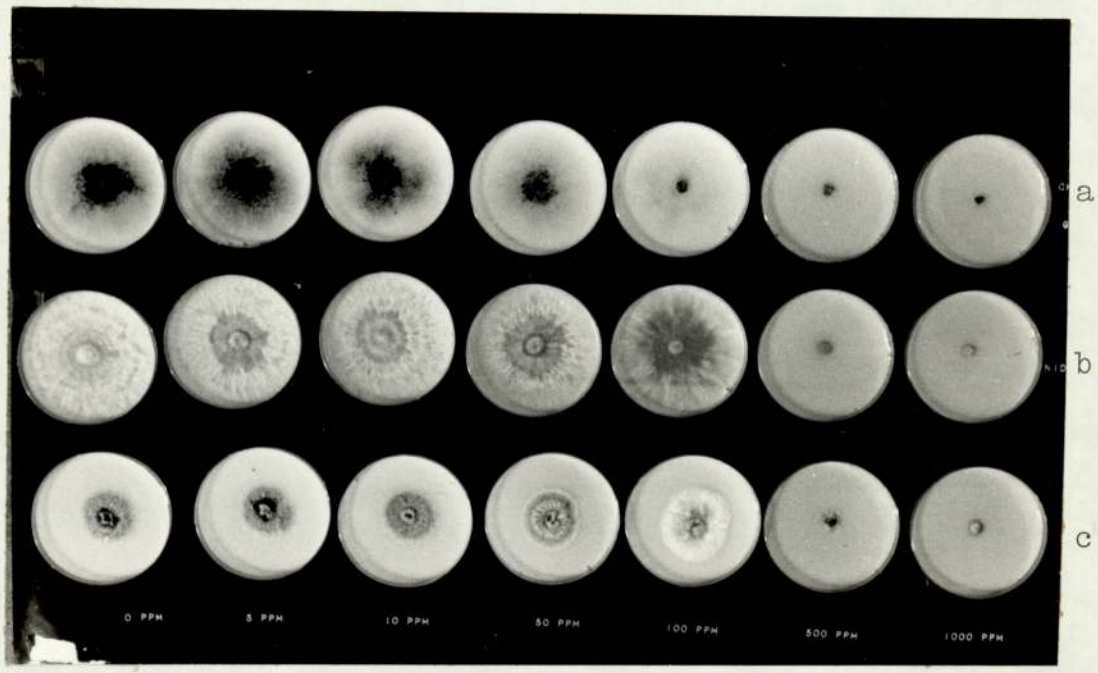
Table 16 Concentration of CCA in agar obtained from 1% stock solution of CCA

Concentration %	ppm	Vol. of agar medium in cc	Vol of CCA in cc	Vol. of distilled water in cc
0	0	500	0	0
0.0005	5	450	0.25	49.75
0.001	10	450	0.50	49.50
0.005	50	450	2.50	47.50
0.01	100	450	5.00	45.00
0.05	500	450	25.00	25.00
0.10	1000	450	50.00	0.00

Pure cultures of C. globosum, T. viride, L. lumbergii, Penicillium sp. Aspergillus sp. and Cephalosporium sp were maintained on agar plates containing E. and P. cellulose medium one week prior to the setting-up of these experiments. Mycelia inocula were removed with a 3 mm cork borer from the circumference of actively growing cultures of the fungi and were used to inoculate the plates. A plug was placed centrally in each plate with the mycelium in contact with the agar. Incubation was carried out at 30°C for one week.

Since T.viride, Penicillium and Aspergillus species produce spores very fast and discharge these at the slightest shaking of the plates which was inevitable due to daily observations made on the culture plates, spread of mycelium was not used as criterion for growth. Assessment was based on spore germination. In the case of C. globosum, Cephalosporium and L. lumbergii assessment was based on radial growth and mycelial characters. These were described and a photograph was taken to record the observations (plate 13). Culture plates which did not show any evidence of fungal growth in the CCA-cellulose agar medium plates at the end of one week were incubated further for another week. At the end of this period those in which growth was still inhibited were tested for viability. The inocula plugs were removed and subcultured onto fresh plates containing E. & P. cellulose medium and incubated at 30°C for a few days. The fungi were considered viable if they grew out of the inocula plugs.

To study the effect of CCA concentration on the production of mycelial biomass sterile E and P cellulose medium was prepared as before but without addition of agar.



Plate,13. Radial growth of C. globosum,(a), Cephalosporium spp ,(b), Leptographium lumbergii,(c), after 7 days of incubation on Eggins and Pugh's cellulose agar containing varing amounts of CCA.

Three concentrations 10, 100 and 1000 ppm, of the preservative in the growth medium was prepared as before. To ensure even dispersion of the cellulose particles the mixtures were thoroughly shaken and twenty-five millilitres of each solution were placed in conical flasks and covered with sterile cotton wool plugs. The flasks were each inoculated with a 3 mm mycelium plug from the circumference of actively growing colonies of C. globosum, L. lumbergii and Cephalosporium sp. These fungi were chosen because they showed variations in mycelium density as well as in radial spread on the agar cultures used in the previous experiment. Six replicate cultures were set up for each concentration per fungus. Incubation was carried out at 30°C for one week in an orbital (shaking) incubator maintained at 100 revolutions per minute. At the end of the incubation period the mycelia were filtered off and placed in weighed aluminium foil cups and dried overnight in an oven set at 100°C. The final weights were noted and the original weights of the aluminium foil cups were subtracted from these to give the weight of the mycelium produced. It was realised that since the weights of the cellulose particles and the agar plug might interfere with the actual weights of the mycelium, the weights of these were determined for a 25 ml medium after drying as before. This was subtracted from each of the weights determined earlier to give the actual mycelium wieghts produced at each concentration. The mean weights were calculated and the results are presented in table 17.

Cellulase production

Cowling tubes containing serial concentrations of the

Table 17 Weight of mycelium produced at different concentrations of CCA in culture medium

Species of Fungus	Mean weight in grams of mycelium at different concentrations of CCA			
	0%	0.001%	0.01%	0.10%
Cephalosporium sp	0.695 (0.211)	0.683 (0.162)	0.485 (0.101)	0.0
Leptographium lumbergii	0.610 (0.321)	0.575 (0.200)	0.490 (0.058)	0.0
Chaetomium globosum	0.596 (0.162)	0.586 (0.089)	0.216 (0.023)	0.0

(Figures in brackets are standard deviations)

preservative in cellulose agar medium prepared in the previous experiment were inoculated with 3 mm agar plugs of actively growing mycelia of the fungi. The level of the agar column in each tube was marked and the tubes were incubated at 30°C for 42 days. In order to prevent drying of the agar, the humidity in the incubator was kept high by placing a shallow vessel containing water at the bottom. The depth of clearance of the agar column at each concentration was measured to the nearest millimeter at the end of 21 and 42 days of incubation. The mean depth of clearance was calculated for each concentration and the results are presented in the form of histograms. (Figures 23, 24 and 25).

b) Results

Growth studies

After one week of incubation germination of spores and growth of mycelia of Aspergillus sp Penicillium sp. and Trichoderma viride had occurred at all the concentrations of CCA in the agar except at 500 and 1000 ppm, where there were complete inhibition. In C. globosum, L. lumbergii and Cephalosporium sp. there were variations in mycelial densities as well as radial spread. Growth of C. globosum was also inhibited at 500 and 1000 ppm hut mycelial spread was not affected below these concentrations. The density of perithecia was reduced at the higher concentrations of the preservative (Plate 13).

In Cephalosporium sp. mycelial growth was also inhibited at 500 and 1000 ppm, and the preservative had no effect on the form of growth of the mycelia below these concentrations although there were indications of cellulose

Fig.23. Depth of cellulose clearance in tubes containing different concentrations of CCA.

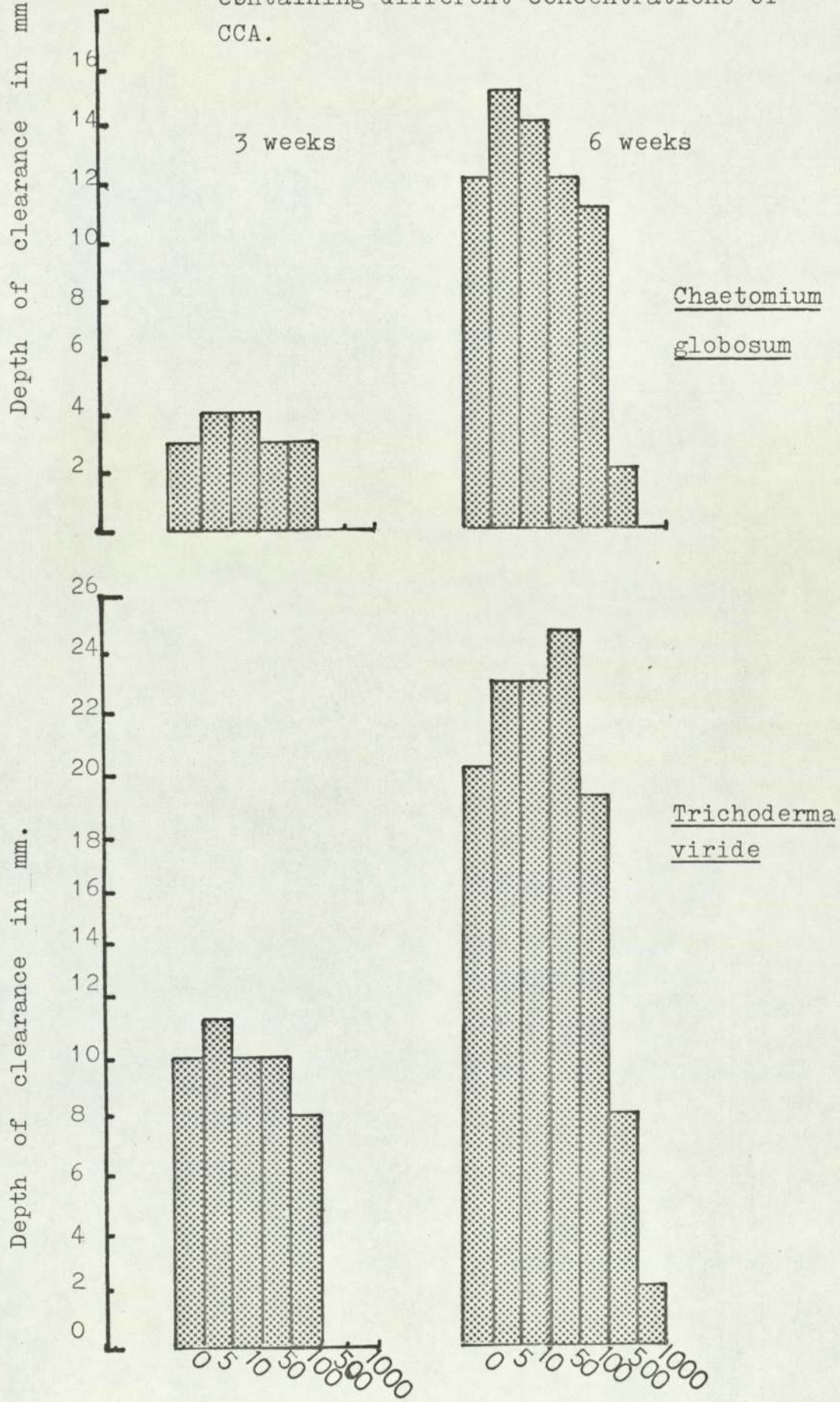


Fig. 24.

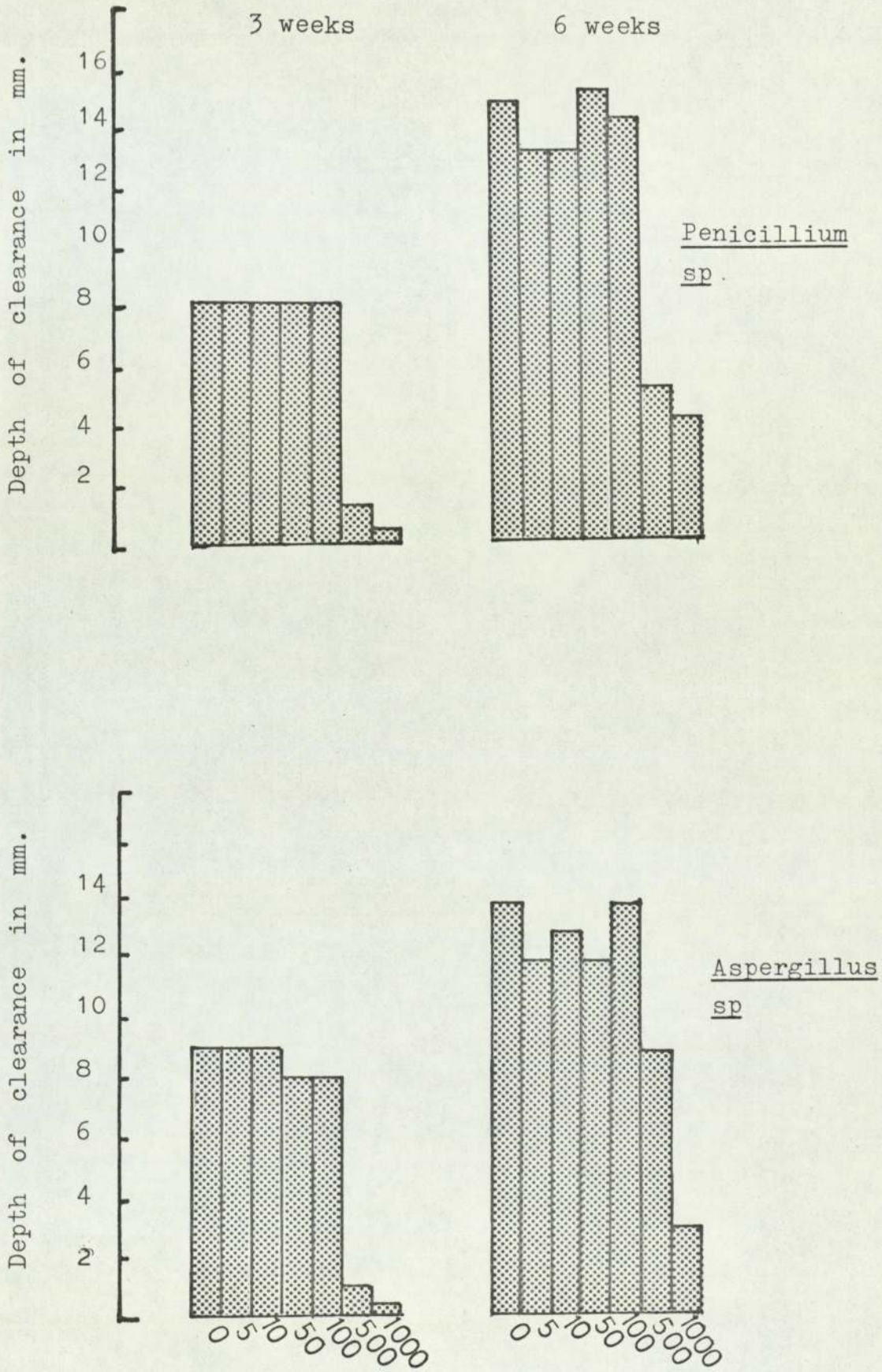
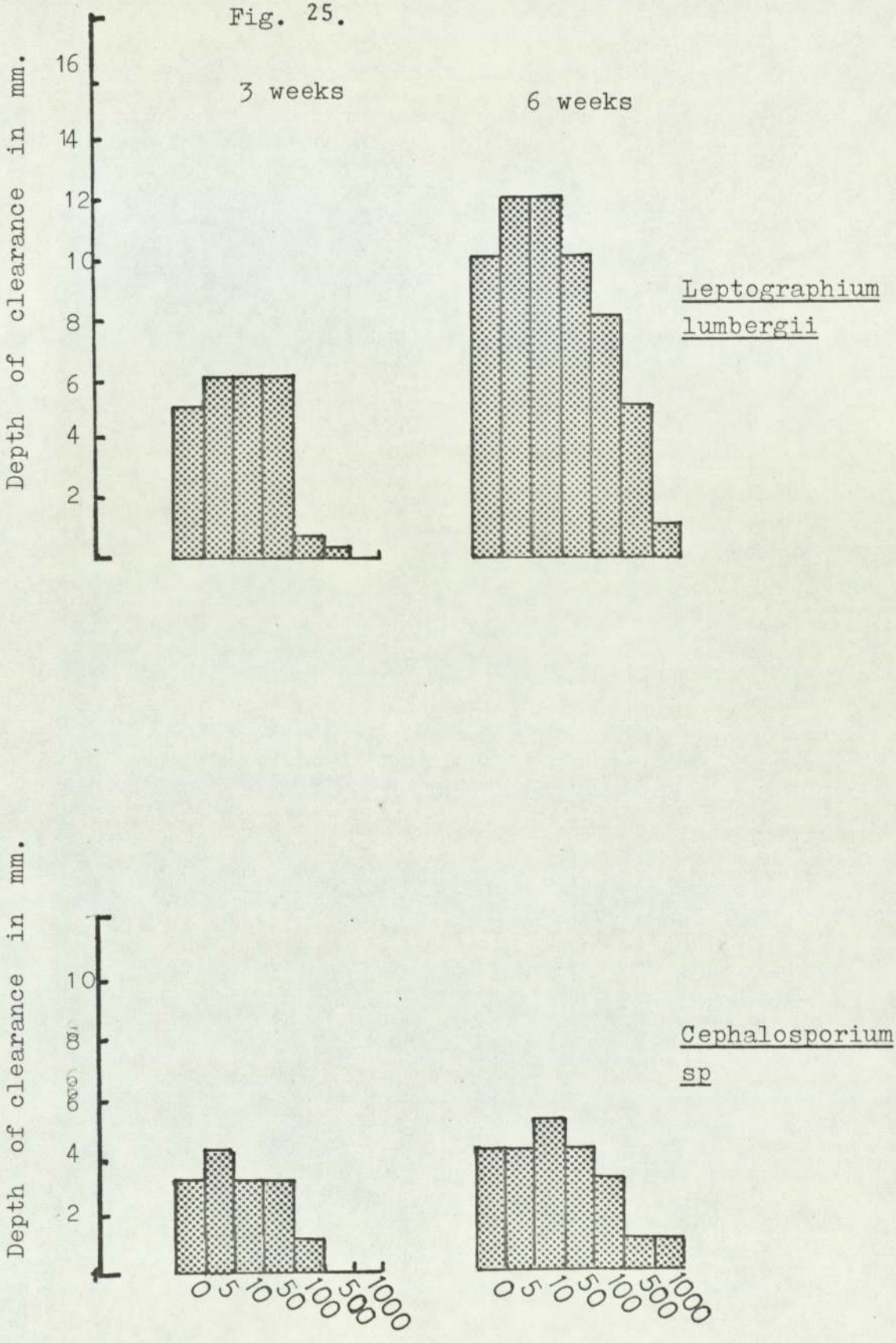

3 weeks
6 weeks
Depth of clearance in mm.
16
14
12
10
8
6
4
2
Penicillium sp.
Aspergillus sp
0
5
10
50
100
500
1000
Concentration of CCA in ppm.

Fig. 25.


3 weeks
6 weeks
Depth of clearance in mm.
16
14
12
10
8
6
4
2
Leptographium lumbergii
Depth of clearance in mm.
10
8
4
2
Cephalosporium sp
0 5 10 50 100 500 1000
0 5 10 50 100 500 1000
Concentration of CCA in ppm.

clearance in the plates with 5 to 100 ppm of CCA but not so much as in the plates without any preservative. In L. lumbergii the density of the mycelium formed increased with increasing concentration of the preservative up to 100 ppm and then sharply decreased at 500 ppm. Complete inhibition occurred at 1000 ppm. It was also noted that stain development in this fungus was less at the higher concentrations of the preservative.

After two weeks of further incubation C. globosum still showed complete inhibition at 500 and 1000 ppm. of the preservative. All the other fungi showed reduced growth at 500 ppm but still there was complete inhibition at 1000 ppm.

The test for viability showed that in no case was any of the fungi killed and even where complete inhibition of growth had been recorded the fungus still remained viable in the inocula plug.

The results for mycelium weight determination (table 17) indicate a reduction in the production of mycelium biomass with increasing concentration of the preservative. None of the fungi showed any growth at 1000 ppm. Cephalosporium sp. produced more mycelia at 0, 10 and 100 ppm of CCA than the other two fungi.

Cellulose clearance

After 3 weeks of incubation cellulase production in C. globosum and Cephalosporium sp. were inhibited at CCA concentrations of 500 and 1000 ppm. whilst in L. lumbergii it was inhibited only at 1000 ppm. In Aspergillus sp and Penicillium sp. there was no inhibition of enzyme activity

at any of the concentrations although very little activity was noticed at the highest concentrations.

After 6 weeks of incubation, only C. globosum was still inhibited at 1000 ppm. The rest of the fungi showed some cellulose clearance at this concentration.

C. globosum and L. lumbergii produced more clearance at CCA concentrations of 5 and 10 ppm than they did in the control tubes. T. viride produced the greatest clearance at 50 ppn whilst in Aspergillus sp. and Penicillium species the depth of clearance was random at concentrations lower than 1000 ppm.

c. Discussion

The results obtained in these experiments clearly indicate that not only was the growth of fungi affected by CCA but also cellulase activity was inhibited at the higher concentrations of the preservative. This information is relevant when considering the prevention of soft rot cavity formation in wood treated with CCA. The inhibition points for fungal growth and enzyme activity were much lower in pure agar cultures than the inhibition points in treated wood. Whereas in treated wood buried in the soil many variables such as the number and type and activity of organisms invading the wood and leachability of the preservative may be operating together to account for loss of its toxicity, in pure agar cultures the situation may be different. In the agar, the test fungi which are initially unable to grow at the higher concentrations of the CCA may become physiologically adapted to the preservative with time. Indeed, it was found that where there was no evidence

of mycelial growth the fungus still remained viable in the inocula plugs. In this case the effect of the preservative was fungistatic rather than fungicidal.

In view of the high fixation in wood reported to be characteristic of CCA it is improbable that reduction in its toxicity could be due to leaching at the initial stages of soil burial although it is possible that a certain amount may leach out after a longer period of exposure when extensive utilisation of the wood has taken place. Greaves (1971) postulated that bacterial slimes which contain polyssacharides may complex with some of the preservative, particularly the copper component after it has been released from the wood by bacterial enzymes. While it remained in this form the wood may be less toxic to decay fungi. Although this evidence is only circumstantial it has been reported that solubisation of CCA from wood by some basidiomycetes takes place in pure culture (Levi, 1969; Chou, Preston and Levi, 1974). It would be of interest to study the effect of bacterial enzyme on detoxification of CCA in treated wood since bacteria are reported to colonise CCA-treated wood in large numbers (Greaves, 1972).

In wood the components of CCA enter into chemical reactions with the celluloses to form complexes (Preston, 1962) whose properties may be different from the preservative solution. In the agar the preservative may be present as such and may be acting directly on the fungi in the inocula, a situation which may be similar to the germination of spores in a preservative solution where a larger surface area of the fungus is exposed to the presesvative. This might have accounted for the lower inhibition points

observed in the agar tests. The demerits of using agar medium in the determination of inhibition value of wood preservatives have been investigated fully by Rabanus (1931) who found that the methods usually employed for such determinations do not give consistent results and in many cases the inhibition values are dependent on the quantity and activity of the fungus mycelium. Such methods are therefore not directly applicable to practical situations.

Reduction in perithecia formation in C. globosum stain production in L. lumbergii and cellulase production in all the fungi at higher concentrations of CCA suggest that certain biosynthetic processes have been affected by the preservative. If this is so then the preservative has to be transported into the fungal cells to effect these changes. Greaves (1973) reported that CCA affects the permeability of bacterial cell membranes. It is possible that a similar situation may occur in fungi. Indeed, Chou, Preston and Levi (1974) found large amounts of copper, chromium and and arsenic within the hyphae of Poria monticola growing on CCA-treated wood. Although this fungus did not survive after two and a half weeks of incubation, other decay fungi they used in the test remained viable and showed evidence of some decay of the test blocks. It suggested to them that the protective action of CCA may be due not only to its fungistatic and fungicidal properties but also to its ability to inhibit cellulases. In this experiment inhibition of cellulase production by CCA has been shown to occur. Although assessment of cellulase production and activity was based on measurement of depth of clearance of

the agar column which depends largely on the diffusibility of the enzyme, it became evident that least activity was obtained at the highest concentrations of the preservative.

Evidence that the genetic apparatus may be affected by the preservative has been put forward (Ross, 1975). Copper taken up by yeast cells has been located in the nucleoli and in the chromosones (Lindegren, Miller and Kuo-chun Lui (1972).

The fact that greater depth of clearance was obtained in tubes containing 5 and 10 ppm of CCA in tubes inoculated with C. globosum and L. lumbergii and at 50 ppm in the case of T. viride than in control tubes suggested that stimulation of enzyme production had taken place at these concentrations. Activation of cellulose activity in the presence of low concentrations of certain ions has been discussed by Gascoigne and Gascoigne (1960).

Chapter VII The effect of microfungal metabolites on the solubilisation of CCA from wood. A bioassay test.

Introduction

In vitro studies have demonstrated the effect of basidiomycetous fungi on wood treated with CCA and have shown that Lenzites trabea affected the toxicity of the preservative without causing significant decay of the treated wood (Da Costa and Osborne, 1968). Culture solutions of Poria vaillantii and Poria monticola solubilised large proportions of CCA from treated veneers (Levi, 1969) and this was explained on the basis of the pH of the culture solutions of the fungi after growth which were 2.7 and 2.5 respectively. Chou, Preston and Levi (1974) found that components of the preservative were accumulated in large quantities in the hyphae of Poria monticola although the fungus was killed. It suggested to them that not only did the preservative affect the fungus but also the fungus affected the preservative.

Upon exposure of CCA-treated wood to the soil, microfungi, in their capacity as the initial colonisers, are also likely to affect and be affected by the preservative. Research in this direction has been ignored. In the concluding chapter of this thesis the effect of culture filtrates of some selected microfungi on the subsequent decay of CCA-treated wood was examined.

Plan of the experiment

The purpose of this experiment was to find out the leachability of CCA from wood by fungal culture solutions,

excluding mycelia, and without identifying the compounds present in the filtrate or estimating the amount of preservative in the leachate. What was required was the extent to which the culture filtrate extracted blocks would decay compared with distilled water-extracted blocks after subsequent exposure to soil microorganisms. Since the pH of the growth medium would vary as a result of different buffering activities of the fungi, solubilisation of the preservative in the wood, if any, might be explained in terms of pH effect of the culture filtrate although less pH change is expected with the microfungi than with basidiomycetes. Chemical reactions between the compounds present in the filtrate and the CCA-wood complex could not also be excluded.

b. Method

The following fungi, namely: Trichoderma viride, Scytalidium lignicorum, Penicillium funiculosum and Aspergillus fumigatus were selected from the culture collections of the Biodeterioration Information Centre and were subcultured onto 2% malt agar slopes. The composition of the liquid culture medium was based on the solution used by Levi (1969). It consisted of 20 gm, glucose; 0.5 gm peptone; 1 gm KH_2PO_4; 0.5 gm, $MgSO_4.7H_2O$; 1.0mg thamine; 1.0mg $FeCl_3.6H_2O$; 0.3mg, $MgSO_4.H_2O$ and 0.6mg $ZnSO_4.7H_2O$ in one litre of distilled water. The pH after autoclaving was 4.5. Portions 250 mls, were placed in sterile conical flasks and and then covered with cotton wool plugs. Inoculation was performed by withdrawing into a sterile hypodermic syringe and injecting 5 mls of either freshly prepared mycelial or

spore suspensions of the fungi into the conical flasks. The flasks were attached to shakers such that the former were partially immersed in water baths thermostatically controlled at 25°C. To provide for sufficient filtrate four flasks were set up for each fungus. Cultivation was carried on for a period of 21 days. The cultures were then centrifuged at 3500 r.p.m. for 10 minutes and the supernatants were immediately decanted and used for leaching the treated blocks. Some portions of the filtrate were retained and used for pH determinations.

Prior to culturing the fungi, miniature sapwood blocks of lime, measuring 10 x 30 x 5mm in the tangential, radial and longitudinal planes, respectively, were treated with either 1.5 or 3.0 per centage solution of CCA. The blocks were conditioned to allow for fixation of the preservative and dried as described in Chapter 2. For each concentration there were seven replicate blocks. Leaching of the blocks involved impregnation with the culture filtrates and shaking in further portions of the filtrate for 48 hours with final washing in four changes of warm distilled water. In this way it was hoped that small quantities of the culture solutions which might be trapped in the cells would be washed out. Control blocks were leached and washed in warm distilled water in the same way and for the same period as the test specimens. The pH of the culture filtrates were recorded before and after leaching. The leached blocks were then subjected to decay test in the soil to determine the residual effectiveness of the preservative. The blocks were dried, weighed and buried in the soil. They were incubated at 30°C for 12 weeks after which they were exhumed,

cleaned, dried and weighed again. The percentage loss in weight was calculated for each block. The results are presented in tables 18 and 19 and in Figures 26 and 27.

c. Results

Examination of the data in Table 18 indicate that the pH of the culture solution (initial pH is 4.5) has been altered to lower levels by all the fungi.

Penicillium funiculosum effected the greatest change by lowering the pH of the culture filtrate to a value of 3.20. Scytalidium lignicorum was least effective. The final pH of the culture solution after growth was 4.0. After leaching the blocks the pH of the filtrates had changed to values close to the original pH of the culture medium before growth. This change was more noticable after leaching wood blocks treated with the higher concentration of the preservative (Table 18).

The results shown in Table 19 and in Figures 26 and 27 indicate that there was a greater percentage decay in all the wood blocks leached with culture filtrates of the fungi than in the control blocks.

The order of effectiveness of the culture filtrates varied at the different concentrations of the preservative (Table 18), for instance, test blocks treated with 1.5% CCA and leached with filtrate from Trichoderma viride registered the highest weight loss whilst test blocks treated with 3.0% CCA decayed fastest after initial leaching with filtrate from A. fumigatus. In wood blocks treated with 1.5% CCA, the degree of decay was not affected by initial leaching with filtrate from P. funiculosum. Test blocks treated with 3.0% CCA and leached with culture filtrate from P. funiculosum,

Table 18 Changes in pH of culture filtrates after leaching treated wood blocks

Conc of CCA	Fungi in order of effectiveness	pH of Culture Filtrate	pH after leaching
1.5%	T.viride	3.56	3.82
	S.lignicorum	4.00	4.50
	A fumigatus	3.30	3.90
	P.funiculosum	3.20	4.35
	Control	7.2	5.00
3.0%	A.fumigatus	3.30	4.15
	P.funiculosum	3.20	4.50
	T.viride	3.53	4.32
	S lignicorum	4.00	4.65
	Control	7.2	5.95

Table 19 Decay of treated wood blocks leached with culture filtrate of selected microfungi

Test fungus	% Concentration of CCA used for treatment	% wt loss of culture filtrate extracted wood blocks after 12 weeks of soil burial	Mean	S.D.	Standard error of mean
Trichoderma viride	1.5	38.13, 37.85, 37.09, 35.84, 39.40, 29.55, 34.14	36.00	3.31	1.25
	3.0	3.63, 6.05, 5.78, 3.38, 3.38, 4.11, 5.37	4.52	1.20	0.45
Scytalidium lignicorum	1.5	27.19, 28.62, 31.80, 31.04, 30.77, 33.11, 27.73	30.03	2.32	0.88
	3.0	4.55, 4.55, 2.76, 3.87, 2.88, 3.29, 0.41	3.18	1.20	0.45
Aspergillus fumigatus	1.5	22.36, 18.74, 18.46, 22.24, 23.85, 26.93, 24.95	22.50	3.14	1.19
	3.0	20.47, 20.78, 16.81, 19.65, 19.58, 20.45, 16.55	19.18	1.82	0.68
Penicillium funiculosum	1.5	12.00, 10.91, 8.91, 9.90, 11.06, 10.97, 9.95	10.53	0.99	0.37
	3.0	6.52, 6.72, 5.44, 6.14, 6.24, 7.36, 6.24	6.38	0.58	0.21
Control	1.5	18.49, 13.73, 18.39, 17.83, 16.43, 17.19, 26.36	18.35	3.86	1.45
	3.0	3.14, 1.31, 0.01, 1.44, 0.40, 2.52, 0.70	1.36	2.79	1.05

Fig.26. % decay of culture-filtrate extracted wood blocks intially treated with 1.5% CCA. (vertical lines represent standard error of the means)

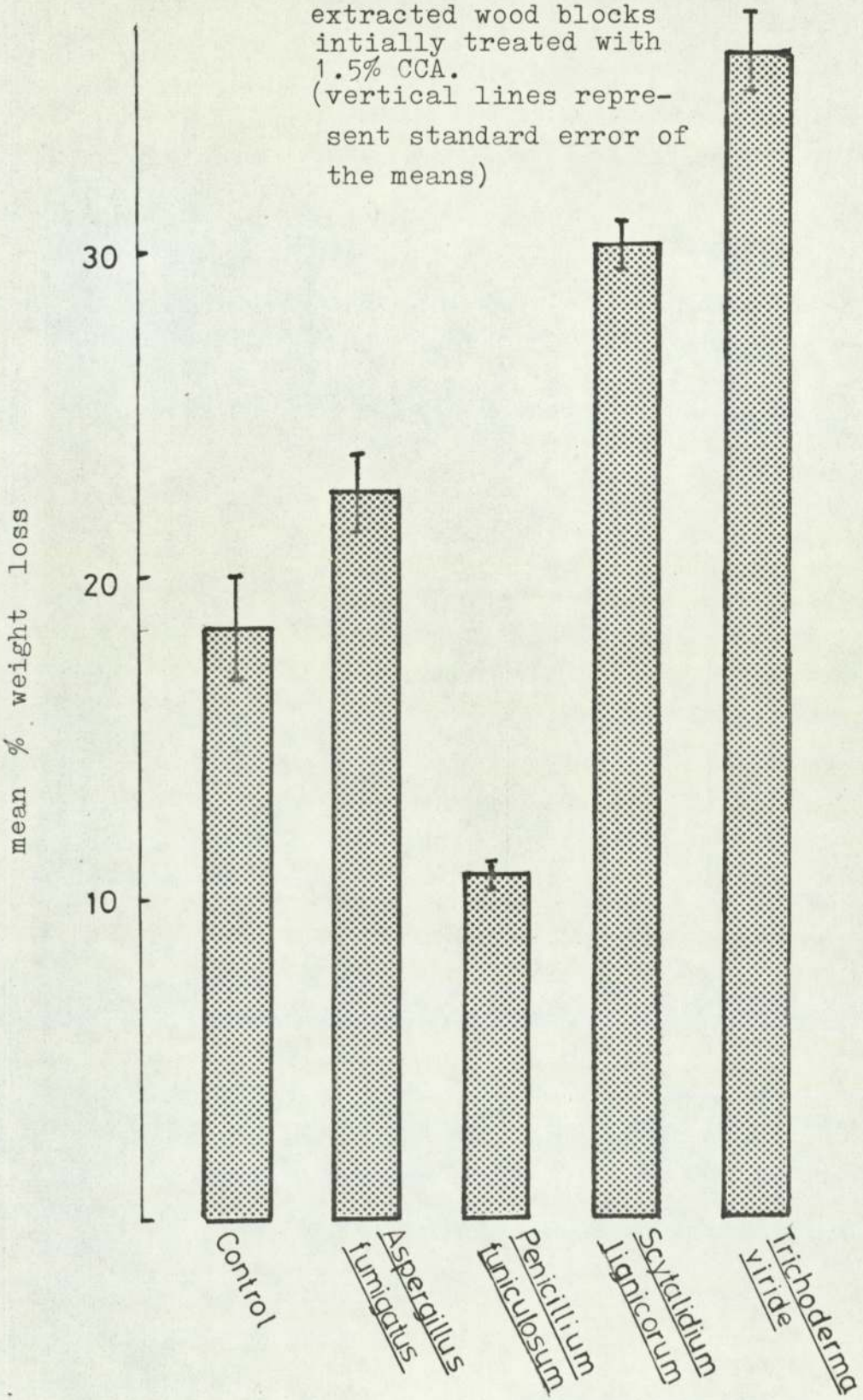
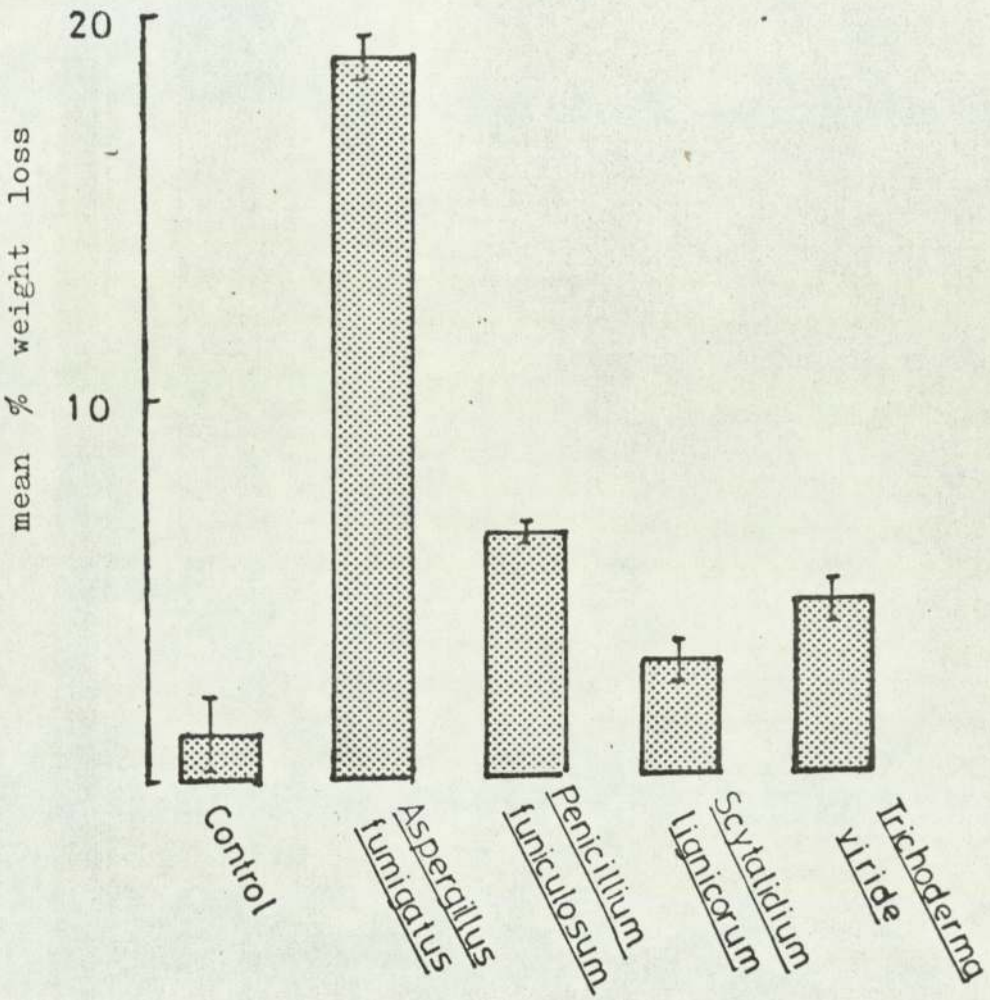


Fig. 27. % decay of culture filtrate extrated blocks initially treated with 3.0% CCA.
(vertical lines represent standard error of the means)



however decayed more than control blocks. The reason for this contrasting result is not clear but it could be due to variability in the exposure method.

d. Discussion

In the attempt to separate single factors to investigate with the view to understand the complex ecological processes during the decay of treated wood exposed to the soil, it is necessary to exercise some caution in the interpretation of data. CCA is a well proved preservative and it is being used in situations where decay hazards are high with considerable success. In the above experiment the test conditions are isolated and they may be optimum. These do not exist under normal conditions hence the results cannot be extended to service situations. Nevertheless, it is to be appreciated that the loss of CCA from wood does not only result from passive leaching but also from other reactions involving the activities of microfungi. Changes in the pH of the culture medium after growth of the fungi and in the filtrate after extraction suggest that under acid conditions some CCA could be lost from the treated wood. The effectiveness of the filtrates in solubilizing the preservative in the wood was more or less related to the final pH of the growth medium. Levi (1969) has also shown that Poria spp are able to precipitate soluble copper in a relatively non-toxic form from synthetic liquid medium. No check was conducted by leaching the blocks in pH buffer solution or in the growth medium and it is suggested these should be included in future studies.

The reason for the different order of effectiveness of

the fungal filtrates at different CCA concentrations is not clear.

Duncan and Deverall (1964) have also shown that some Ascomycetes and Fungi Imperfecti are capable of degrading toxic compounds into less potent forms thus rendering them less effective in protecting the wood from decay by less tolerant basidiomycetes. Although their method of testing subsequent decay after a prior steaming of the blocks to kill the original infection can be questioned, since such steaming could also affect the loss of the preservative and also pre-dispose the wood cellulose, their attempt provided a basis for further research. In this experiment fungal mycelium has been excluded and the effect of sterilisation by steaming has been eliminated. It would be of interest, however, to use pure culture tests for the second exposure in future experiments.

It is concluded from this experiment that the effectiveness of fungal culture filtrates in detoxifying CCA is related to the final pH of the culture solution.

Chapter VIII General Discussion and Conclusions

The experiments described in this project were designed to provide fundamental information on some ecological factors associated with the decay of CCA-treated and untreated wood buried in the soil.

To assess the results objectively a respiratory method was employed to quantify the activity of microorganisms in the invaded wood. No studies in the literature available have reported on the use of respiration to investigate the effect of wood substrate factors on microbial colonisation although a few had reported on its use for toxicity determinations of wood preservatives (Smith, 1966; Good and Darrah, 1966; Halabisky and Ifju, 1968 and Berh, 1972). Thus the work described in this thesis provides the first attempt in this area of wood ecology.

After repeatedly using the Warburg respirometer to measure oxygen uptake it was found in preliminary studies that comparable results could be obtained depending on the experimental conditions.

In Chapter 3, it was noted that sapwood blocks of both Pinus sylvestris and Tilia vulgaris buried in the soil after being extracted with either distilled water or a mixture of organic solvents and exposed to microorganisms in the soil consumed lesser amounts of oxygen than those not extracted suggesting that colonisation of and hence the activity of microorganisms in the test blocks was minimised by the absence of extractable materials. By impregnating extracted blocks with either glucose, mannose, aspartic acid or alanine, it was noted that the rate of oxygen consumption in these blocks increased to a greater extent as the period of burial

was increased. Control extracted blocks without any nutrients added consumed the least amount of oxygen while unextracted blocks used as a second set of control consumed as much oxygen as, and even slightly more than, some of the treated blocks. These studies suggest that simple sugars as well as amino acids present in sapwoods enhances colonisation by soil microorganisms. The method used confirmed established subjective observations but it also provided a more exact approach especially in objective comparative information on microbial utilisation of different sapwood species.

Using material from the same source as was employed in this project, Long (1975) identified tentatively by paper chromatography quantities of xylose, arabinose, fructose and glucose in the sapwood of Scots pine while King, Oxley and Long (1974) reported on the distribution of soluble nitrogen in laboratory dried wood and found that more nitrogen was present in the unextracted pine blocks than in the extracted samples. These results, in addition to the respiratory determinations described in this thesis confirmed that soluble nutrients in sapwoods enhance colonisation by soil microorganisms.

Another noteworthy aspect of nutrient effect on colonisation brought to light by respirometric determination was the influence of the source of wood samples and their precondition in relation to drying before conversion into test blocks. It was thought that the first batch of lime planks obtained from the Princes Risborough laboratory were slowly dried, presumably air seasoned, and in the process had lost some of their nutrient content as a result of respiration by moribund parenchyma cells. Other possibilities of such

nutrient depletion in wood had been mentioned elsewhere by Greaves (1971) although in this investigation no isolation was carried out to determine the presence of bacteria. Soluble nutrients have also been reported to migrate to the exposed surfaces of wood during drying (King, Oxley and Long, 1974) and it is possible that the samples obtained in the already dried state have had their surfaces planed off during conversion and thus did not contain redistributed nutrients.

Nevertheless, from the viewpoint of utilisation of wood it may be concluded that surface colonisation of slowly dried wood is likely to be less than that of wood rapidly dried when both are exposed to conditions suitable to microbial growth.

An attempt to use respirometry to determine any increase in activity of microorganisms in the soil which was in contact with the surface of wood yielded results contrary to expectation. Although soluble nutrients in the wood surface cells are likely to move into the soil to influence microbial activity in the immediate vicinity, and in the same way minerals and nutrients in the soil can move into the wood, the results obtained when the former was investigated proved to be negative. Measurement of microbial activity in a complex ecological unit such as the soil poses a number of problems particularly on the type of methods adopted and their reproducibility. Parkinson, Gray and Williams (1971) suggested that in studies on the effect of environmental conditions on microbial activity in mineral horizons of soil where very low rates of activity are likely to be found, it is necessary to amend the soil samples to allow high rates so that larger differences can be observed to make statistical analysis easier. This idea, however, is debatable.

While quantities of soluble nutrients in wood may be comparatively low, it is likely that outward diffusion of even minute quantities can trigger off the germination of microbial spores and propagules, hence the presence of sporing fungi noted in the soil-wood interface when direct observation was made with the microscope. The Warburg respirometer, on the other hand, failed to determine any such activity probably because in removing the soil sections for oxygen uptake measurements that bit of sporing fungi growing in the crevices between the soil and the wood blocks were not included in the samples removed.

Research on the ecology of wood decay in ground contact is mostly centred on the wood itself and on the invading microorganisms. It is suggested that if studies on the soil-wood inter-relationship is included valuable information may be obtained to help understand the complex process of wood decay in the soil.

Susceptibility of sapwood to decay is not always affected by its soluble nutrient content. In experiment III:iv it was noted that when decay was measured by the weight loss method the effect of extractable materials assumed less importance and the degree of decay was dependent on the gross anatomy and on the microstructure of the wood species. Thus extracted wood blocks decayed more than the unextracted samples and extracted lime blocks registered higher percentage decay than extracted Scots pine blocks. An hypothesis based on variations in the nature of the cell walls between wood species has been put forward to explain differences in decay resistance of softwoods and hardwoods. Physical blocking of enzymes by the higher lignin content in softwoods

was thought to be responsible for the lesser attach by soft rot fungi (Wilcox, 1970; Liese, 1970). In this work it was found that partially delignified Scots pine became as susceptible to decay as untreated lime. This result agreed with the work of Bailey, Liese and Rosch (1968). No general explanation can be put forward regarding the difference in the decay of solvent extracted and unextracted wood blocks for the two species since intra-specific variations do occur.

Three possible hypotheses have been put forward in Chapter 3 and these may have jointly accounted for the greater decay susceptibility of the solvent extracted wood samples. Whilst suggestions based on increase in porosity of the solvent-extracted samples and direct utilisation of the cellulose and possibly the lignin by the invading decay organisms may explain the greater decay rate of solvent extracted lime, in Scots pine removal of toxic compounds may, in addition, facilitate the activity of the colonising organisms.

Another important aspect of decay noted in Chapter 4 is the need to clarify the effect of wood microstructure on the distribution of activity of invading microorganisms. Such information, though fundamental, is very relevant to the study of soft rot control in preservative-treated wood since it has been reported that distribution of preservatives in wood also varies with the microstructure (Bravery 1972; Greaves, 1974). Previous studies on colonisation have revealed various pathways of entry of microorganisms into wood and their effect on micromorphological changes in the cell wall structure (Corbett, 1965). In Chapter 4 an attempt was made to use respiration methods to complement these

studies particularly to study the distribution of some microfungi in wood. Although the method applied in separating out the growth rings may be crude, since the early and latewood bands overlap, higher respiratory activity was recorded in the latewood in test blocks inoculated with Chaetomium globosum and Cephalosporium sp whilst Penicillium sp was most active in the earlywood. The only possible explanation is that microfungi which are able to utilise the wood tend to penetrate the cell walls and build up their activity in areas where the specific substrate they degrade abounds. Microscopic studies reported here confirmed the observation of previous workers and complemented the respiration results. Bravery (1972) reported on the preferential colonisation of latewood by C.globosum in a tri-n-butyltin oxide-treated birch wood and explained that this might possibly be due to the smaller lumina of the latewood cells which contained lower amounts of the preservative. If this explanation is extended to the present observation, on the basis of the size of lumina of the cells where untreated pine sapwood was used, questions may be asked as to why the larger lumina of the early wood cells could not support as much activity of Gglobosum as the latewood cells. Courtois (1963) and Butcher (1975) considered that the preferential attack of soft rot fungi on latewood tracheids suggests that cell wall thickness governs the development of soft rot in soft woods. Butcher (opt.cit.) further reported that even with exposed earlywood, soft rot developed much slower and cavities tended to be produced preferentially in those

tracheids adjacent to the wood rays possibly because the cell walls in these regions are almost as thick as those in the latewood. In the centres of his stakes, Butcher (opt. cit.) observed considerable attack in the latewood bands. The importance of uniform distribution of preservatives has usually been emphasized in treatment and drying schedules of timber but the initial effect of wood microstructure on the distribution of microbial activity cannot be overlooked. With respect to this, distribution of microbial activity in preservative-treated wood merits detailed studies.

A major conclusion arrived at in this thesis in Chapter 5 was the dependance of the level of toxicity of CCA on the period of incubation both in the soil and in pure culture. This result pointed out the inadequacy of standard laboratory tests for predicting the performance of wood preservatives. Similar observations were reported by Bavendamm (1956) (quoted by Cockroft, 1973) and Bravery (1968) who used weight loss methods but the work described here proved that a similar relationship existed when respiration method was used to assess colonisation of treated wood by soil microorganisms.

A further observation noted was the corresponding reduction in the coefficient of correlation between weight loss and respiratory activity as the period of incubation was increased, ($r = + 0.98$, $p = 0.001$, after 4 weeks and $r = + 0.76$, $p = 0.001$ after 8 weeks). The ecological and physiological implications of this observation are remarkable. From the viewpoint of succession of microorganisms on treated wood it may be suggested that at a certain stage in the decay of wood in contact with

the soil there is an active utilisation of the wood by the invading organisms such that the amount of wood matter degraded is equivalent to the amount of oxygen respired. At this stage respiration of the invading organisms is mostly aerobic and diffusion of gases in and out of the decaying wood is probably facilitated by active formation of cavities. With the passage of time due to depletion of substrate, reduction in microbial population or introduction of freshly invading organisms and their interaction, this correlation is reduced. The application of respiration as a measure of microbial activity in wood may be subjected to criticsms. The Warburg respirometer determines only aerobic respiration and since the system is closed it lacks the advantage of a continuous and constant supply of oxygen. The microorganisms grow in the complete absence of CO_2 and this could have some retarding effect on their metabolism.

Respiration measures the current rate of consumption of the wood and not the aggregate and it is totally unspecific in regard to the colonising organisms. Its advantages are that it is quick and easy to use; it measures small changes in oxygen consumption including respiration of bacteria in the wood, and also it provides a direct measure of total metabolism in the wood.

Further experiments in Chapter 5 pointed out that not only was the growth of fungi affected by the preservative but also enzyme activity was inhibited at the highest concentrations.

It would be of interest to assess cellulase activity by other methods but in the context of the present studies it is concluded that cellulase inhibition plays a part in the mode of action of CCA.

Another aspect of decay noted in Chapter 5 is the relationship between moisture content and weight loss or respiration of the invaded wood blocks. The importance of moisture content of wood has been mentioned in the colonisation of aerial and sub-soil sections of wooden posts (Banerjee and Levy 1971; Butcher, 1968 and Greaves, 1972), but its correlation with percentage decay of treated wood has never been described. In this work it was established that some form of relationship between these exists although water entering the wood from the soil may mask the effect of water resulting from the matabolism of the invading microorganisms (as suggested in the discussion in that Chapter). Duncan (1963) commented that this additional source of moisture resulting from respiration in wood decay is generally overlooked and suggested that from the viewpoint of preventing disfigurement of decorative coatings and deterioration of painted wood out of ground contact, water resulting from metabolism of fungi must be considered. Another major conclusion arrived at in this chapter is the reduction of water absorbancy in the CCA-treated wood blocks. This observation has never been reported anywhere in the literature available although Belford and Nicholson (1968) have found that when emulsion additives were added to CCA to treat sapwood blocks of Pinus sylvestris, Fagus sylvatica, Pinus radiata and Araucaria cunninghamii the rate of water absorption and the rate of drying were significantly reduced compared with untreated controls. In the present work it was found that even in the absence of any additives, CCA restricted absorption of water into the treated blocks and the degree of this reduction was dependent on the con-

centration of the preservative solution. Emulsion additives contain water repellent and pore filling materials which will prevent the ingress of water once the timber has dried. CCA on its own, has been shown to be deposited within the cell walls and not in the lumina (Rudman, 1966; Petty and Preston, 1968). The physical properties of the treated wood cell walls have not been fully investigated and it is suggested that more detailed information in this direction will be desirable.

It was, however, concluded that CCA did not only affect microbial colonisation but also water absorbancy of the treated wood.

Few studies have reported on the detoxification of CCA in treated wood. The observed tolerances of some wood decay fungi to CCA-treated wood have been linked to their ability to solubilize the copper, thereby allowing it to leach out of the wood (Levi, 1969). Other workers also reported on the accumulation of the components of the preservative in the hyphal cells thereby killing the fungi (Chou, Preston and Levi, 1974). Greaves (1973) also reported that CCA affects the permeability of bacterial cell membranes. Although these results were obtained from experiments conducted in vitro, they demonstrate the effects of the invading microorganisms on the treated wood, as well as the effect of the preservative on the microorganisms. The attempt made in the final chapter of this thesis to study the former aspect partly under soil burial conditions proved that microfungi are also able to remove some of the preservative from the treated wood and this results in the greater rate of decay of the latter. It would be of interest to include the bacteria in such studies since they

are known to tolerate preservatives at retentions usually employed to prevent fungal attack (Greaves,1972).

Appendix I

Analysis of variance of the soil data in Table 10

Source of Variation	Sum of squares	Degrees of Freedom	Mean square	F
Between blocks with different treatments (columns)	37.14	3	12.38	0.32
Between soil distances (soil sections) away from the wood surfaces (rows)	92.98	2	46.49	1.18
Residual	235.85	6	39.30	
Total	265.97	11		

Columns when $n_1 = 3$, $n_2 = 11$ at $p = 0.05$, $F = 0.32 < 3.6$

Rows when $n_1 = 2$, $n_2 = 11$ at $p = 0.05$, $F = 1.18 < 4.0$

Appendix 2

Analysis of variance of the data in Table 10

Source of variation	sum of squares	Degrees of freedom	Mean square	F Value
Between blocks with different treatments (Columns)	466.18	3	155.39	0.32
Between soil distances (soil sections) away from the wood surfaces (rows	2322.51	2	1161.25	2.39
Residual	2909.59	6	484.93	
Total	5698.28	11		

Columns when $n_1 = 3$, $n_2 = 11$ at $p = 0.05$, $F = 0.32 < 3.6$

Rows when $n_1 = 2$, $n_2 = 11$ at $p = 0.05$, $F = 2.39 < 4.0$

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