

MICRO-MORPHOLOGICAL STUDIES OF  
SOFT ROT FUNGI IN WOOD

A thesis presented in part fulfilment of the  
requirements for the Degree of Doctor of Philosophy  
of the  
University of London

by

(Mrs) Ayten Subghatalla Zainal, B.Sc., (Baghdad)

Department of Botany and Plant Technology  
Imperial College of Science and Technology  
London, S.W.7.

NOVEMBER 1975

To my life, my beloved husband

Mohammed Jamil

unique in the world, whose inspiration, encouragement, understanding and ceaseless sacrifice of himself for our three children and myself, accomplished this work.

#### ABSTRACT

Previous workers had suggested that absence of cavities in softwood colonised by Phialophora fastigiata compared to the typical soft rot cavities formed in hardwood was due to the high lignin content of the former. The present work was initiated to test this theory. Investigations are described which lead to the conclusion that the presence of high lignin content is not solely responsible for lack of cavity formation, but could be considered as a delaying factor.

The effect of lignin in normal and delignified Scots pine was studied using scanning electron microscopy and transmission electron microscopy.

Detailed studies were also carried out using both types of microscopy to investigate the very early penetration of the fungal hypha into birch cell walls, which suggested a localized enzymatic action at the penetration point. The penetration hyphae were highly constricted at the point of penetration through the  $S_3$  layer and the middle lamella. The constant appearance of septa in the penetration hypha at the  $S_3$  layer and the middle lamella were seen in almost every case examined.

A new explanation for the erosion decay and the cavity formation have been suggested, and ideas are suggested for further studies in this field.

Techniques are described for the preparation of ultra-thin sections of sound and decayed cell walls for examination in the transmission electron microscope. Fixative techniques have been developed to differentiate the fine structure of the wood cell wall and the hyphal cell contents in these sections.

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Key to abbreviations

|                |                                                              |
|----------------|--------------------------------------------------------------|
| am             | amorphous matrix                                             |
| an             | annulus                                                      |
| c              | cavity                                                       |
| ce             | cavity edge                                                  |
| cm             | compound middle lamella (two primary walls + middle lamella) |
| dg             | dark granular material                                       |
| ed             | electron dense plugging                                      |
| h              | hypha                                                        |
| hw             | hyphal wall                                                  |
| l              | lumen                                                        |
| ls             | lomasomes                                                    |
| m              | middle lamella                                               |
| ma             | mitochondria                                                 |
| ms             | microfibrillar skeleton of the fungal wall                   |
| n              | nucleus                                                      |
| p              | pore                                                         |
| pa             | plasmalemma                                                  |
| pw             | primary wall                                                 |
| r              | wedge-shaped ring                                            |
| rc             | ray parenchyma colonized by hyphae                           |
| s              | septum                                                       |
| sp             | septal plate                                                 |
| S <sub>1</sub> | S <sub>1</sub> layer                                         |
| S <sub>2</sub> | S <sub>2</sub> layer                                         |
| S <sub>3</sub> | S <sub>3</sub> layer                                         |
| t              | transitional microfibrils                                    |
| tb             | T-branch                                                     |
| v              | vessel                                                       |
| w              | Woronin bodies                                               |
| SEM            | Scanning electron microscope                                 |
| TEM            | Transmission electron microscope                             |

PART I

INTRODUCTION

## 1. GENERAL

The U.K. annual imports of wood and wood products are of the order of £600,000,000. Some years ago the then Forest Products Research Laboratory estimated the annual cost of dealing with and restoring or replacing wood decay amounted to some £70,000,000. Both statistics clearly indicate the great benefit to the nation of a better, more effective and a wider application of wood preservatives to reduce this cost. For such preventative, remedial and curative treatments to be more effective, whilst, at the same time being no additional threat to man and his environment by way of pollution or toxic hazard, the toxic chemicals must be introduced into the wood in sufficient quantity and in the right place. Once in the wood they must be fixed and not removed by leaching, chemical reactions, sublimation, vaporization or other means.

These three points are the basic fundamentals of wood preservation and imply that (a) the chemicals will penetrate into the wood and throughout the cell wall layers; (b) the chemicals will be toxic to micro-organisms rather than deterrents; (c) the activity of all the organisms will be stopped; and (d) the toxic chemicals will remain stable and fixed in the wood. These implications, at best, are not proven and recent research is showing more and more clearly that a great deal of fundamental work must be done before a proper understanding can be possible which will enable man to know what is happening when a piece of wood is attacked by micro-organisms and how this may be prevented.

Timber in ground contact has been put into the exact place where evolution has produced a mass of micro-organisms for the express purpose of destroying organic remains by breaking up the complex molecules into simpler soluble substances. This serves two main purposes, firstly to rid the earth's surface of dead plants and animals so that space, light and soil are available to new living plants and animals; and secondly, in modern terms, to recycle the carbohydrates, fats and proteins into a form in which they can be re-used by the young and developing plants and animals.

This mass of micro-organisms contains a great variety of bacteria and fungi. An understanding of their complexity and interactions, particularly with respect to their colonisation and subsequent destruction of wood, must be a vital field of study before it is possible to even formulate accurately the problem that has to be overcome by wood preservation. A good deal of work has been done on this difficult subject, as will be discussed later. Until Findlay and Savory (1950) outlined the significance of the soft rot fungi in certain specific exposure conditions, it had been thought that Robert Hartig's categories of fungal decay (1878) were correct and that the majority of wood decay was due to white rot and brown rot organisms. The white rots were subsequently shown (Campbell, 1952) to destroy both cellulose and lignin, whilst the brown rots could only break down the cellulose (and hemicellulose) fraction of the wall leaving the lignin as a residue. Bravery (1972) showed the characteristic mode of destruction of the cell walls by each of these types of wood rotting fungi.

Savory's classic papers (1954a, b; 1955) changed this situation by establishing the soft rot fungi as being capable of causing considerable economic losses of timber in certain specific conditions of exposure. Very recently Sorkhoh (1975) has shown that the failure of hardwoods treated with a Copper Chrome Arsenate type wood preservative is due to a differential delay of the three main types of decay. The typical cavities formed by the soft rot fungi do not form for several weeks after the treated timber is exposed to the fungus, but severe decay does eventually occur. Decay by white and brown rot fungi is not initiated until some weeks after the first signs of cavity formation by soft rot fungi occurs. The action of the preservative is thus to defer the action of the fungal types in such a way that the soft rot fungi are able to attack first and thus become important as a source of decay of hardwoods. Sorkhoh (1975) has also shown that where the concentration of copper was high, i.e. in the walls of the ray parenchyma cells, there was no trace of decay by any of the fungal types, whereas a low loading in the lignified cell walls only served to delay fungal activity and give the soft rot fungi the first opportunity to destroy the wood.

It is, therefore, of fundamental importance to know precisely what is happening with each of the fungal types. Findlay (1970) carried out a detailed study of the cavities made by soft rot fungi in the cell walls of hardwoods. He examined sound apple wood and apple wood subject to the virus disease known as rubbery shoot and suggested that the lignin fraction of the cell wall was preventing the free diffusion of the enzyme system from the soft rot fungi and that this gave rise to the penetration of the hyphae into the

wall and subsequent cavity formation. Fuller (1970) working on the cellulase fractions of the enzyme systems of a range of fungi associated with wood decay came to a similar conclusion.

The work described in this thesis was designed to follow up this idea by reducing the lignin content of hardwoods and softwoods and then to investigate the effect of this on the activity of the soft rot fungi. In addition a more detailed study of the penetration of these fungi into the wood was undertaken by means of scanning electron and transmission electron microscopy.

## 2. SOFT ROT LITERATURE SURVEY

Timber has long been known to decay in different forms, but in spite of the importance to Britain in the early 19th century, when several hundred wooden ships were laid up at the end of the Napoleonic wars and the advent of the industrial revolution and the beginning of railways and the earliest patent for a wood preservative application (the Bethel process 1838), scientists were not attracted to study the subject, until Hartig published in 1878. *Earlier* Hartig thought that the hyphae arose from decaying wood fibres, so he attributed the primary cause of decay to be a natural process of metamorphosis during wood aging. Schacht (1850) observed for the first time that wood decaying fungi were responsible for rotted timber, he published this with a series of very beautiful and informative drawings of different types of cell wall degrade and even cavities which are now interpreted as having been caused by soft rot fungi. Robert Hartig (1878) (the founder of wood pathology) concluded that different micro-organisms are responsible for wood diseases and each one will show different symptoms in wood. He classified the various types of attack into white rot, brown rot, white pocket rot and the blue staining of wood.

Many others have tried to study decay of timber in natural environments, but it was not until Brefeld (1881-1908) succeeded in studying wood destroying fungi in the laboratory by developing the cultural methods, now commonly used, that a great step forward was made in timber decay research.

Very little attention was drawn to cavity formation. Dippel

(1898) referred to it with drawings as a result of examining several kinds of timbers.

In the beginning of this century, more people became interested in the subject. The great I.W. Bailey (1913) induced the presence of helical cavities which were formed by wood destroying fungi in the secondary wall of Pinus taeda L. Galloway (1931) gave the name "diamond spot" to fabric deterioration because of the square or rhombic shapes, his suggestion was that the hyphae preferred to grow in the same direction as the warp and weft of the cellulose. Bailey and Vestal (1937) gave an excellent interpretation of the cavity formation according to the orientation of fibrils of the secondary wall (this will be discussed in more detail under cavity formation section). Tamblyn (1937) noticed chains of cavities and named the decay "Black straw rot". His microscopical investigation led him to interpret the cavities in the secondary wall of the fibres as large bore holes containing unbranched fungal hyphae, "Vermiform hypha", which ran parallel to the long axis of the cell. Barghoorn and Linden (1944) isolated a number of pyrenomycetes and Fungi Imperfecti from submerged timber and rope in salt water. This kind of breakdown was characterized by softening of the wood surface. Many of the isolated fungi were characterized as cellulose destroyers. Barghoorn (1949) was studying timber buried in organic sediments and noticed the succession of the cell wall degradation, starting from the central layer of the secondary wall ( $S_2$ ) followed by the innermost layer ( $S_3$ ), then the outermost layer ( $S_1$ ), ending with the primary cell wall. According to him, all three cellulosic layers of the secondary wall are infiltrated to a certain degree with non-

cellulosic substances such as lignin, terpenes, resins or other "protective" chemical materials which delay the progression of biological and chemical degradation. His photomicrographs indicated the presence of cavities in the central layer of the secondary cell wall with some granular residue, his interpretation for this residue was that it was lignin because it had disappeared after delignification.

Varossieau's (1949) microscopical investigation on submerged timber in the foundations of demolished buildings in Rotterdam, indicates the presence of soft rot cavities in the cell wall, but due to the non-existence of fungal hyphae and bore holes, he considered the degradation as non-fungal.

Findlay and Savory (1950) introduced the wood scientist to the unusual deterioration of timber in cooling towers. From their microscopical examination of spruce, Scots pine and other timbers, they classified the zones - starting from the centre - in those timbers as follows :

1 - The central core

The timber was sound with presence of some hyphae of blue staining fungi.

2(a) - Zone of first attack

Little hyphae within the central layer of the secondary wall of tracheids, these hyphae had formed different types of chains of cavities as described by Bailey and Vestal (1937). The hyphae in these cavities were septate, empty and thin walled.

2(b) - Zone of general attack

This had severe attack with very large cavities and thick walled brown hyphae, finer branched hyphae were also present.

3 - The outer zone

Severely decayed and brittle, with almost completely degraded central layer of the secondary wall of the tracheids.

Findlay and Savory tried to investigate the decay in the laboratory but they were unsuccessful with Phialophora sp. They found Chaetomium globosum was a very active decay fungus with Sitka spruce blocks when they were impregnated with nutrient solution recommended by Abrams (1948). From their investigations, they concluded that Chaetomium globosum could be a very active cellulosic destroyer of the central layer of the secondary wall of tracheids, and they speculated as to whether the attack depended on partial heat hydrolysis or that the attack of Basidiomycetes might be necessary before soft rot degradation occurred.

Savory (1954a) found the previous suggestion was not true because a number of sound woods could suffer from soft rot. He called this type of decay "soft rot", and <sup>the term</sup> has become universally accepted. It is considered to be any attack on wood by certain cellulose attacking species of Ascomycetes and Fungi Imperfecti which produce distinct cavities with pointed-ends within the S<sub>2</sub> layer of the secondary xylem walls. Their effect on a wood block is to soften the wood surface and this can be easily scraped away. Savory isolated many soft rot fungal species (including Phialophora sp. and

Chaetomium) from decayed timbers, and some of these fungi gave positive results under laboratory conditions. Softwoods were more resistant to soft rot decay than hardwoods, as he suggested because of high lignin contents in the first group (this will be discussed in part II).

Savory (1954b) was using beech wood (Fagus sylvatica) attacked by Chaetomium globosum. His results indicated that the rate of decay varied according to the amount of nutrient salts available for the fungus and these could be rate limiting factors. This was confirmed by Findlay (1954). Savory succeeded in producing soft rot attack in softwood with some difficulty in pure culture and he suggested that this was due to the high lignin content which plays a protective role to the cellulose. This, also, may be applied to the S<sub>3</sub> layer both in hardwoods and softwoods.

Savory (1955) made more observations on the physiological factors (nutrient, temperature, moisture etc.) relating to soft rot decay by microfungi. He also investigated the decay in preserved wood which showed evidence of soft rot to a certain extent. The decay in preserved hardwood was greater than that in preserved softwood because of the susceptibility of hardwoods and the resistance of microfungi to preservatives. He recommended that the soft rot fungi should be included in laboratory tests for wood preservatives. Pratt (1956) and Behrens (1958) published reports about the maintenance of red wood in water cooling towers. They found that heavy chlorine (algecide) and alkaline circulating water increased the surface deterioration by cellulolytic organisms. They considered it was due to chemical "white deterioration (delignification)"

which had left the wood surface covered with cellulose fibres exposed to cellulolytic organisms. Baker (1959) disagreed with Pratt and Behrens because he found deterioration in timbers in non-flooded zones. Oliver (1959a) measured the depth of softened surface caused by soft rot fungi in both salt and fresh water, there was an indication of slightly higher deterioration in salt water. This was later the subject of a number of papers by Gareth Jones and his co-workers who have raised two points :

- a) Softwoods show active soft rot with mixed cultures in the laboratory, where mono-cultures are very slow to act.
- b) CCA treated wood and sea water undergo some sort of ion exchange process which detoxifies the wood and allows decay (mainly soft rot) to go ahead.

Oliver (1959b) summarized the knowledge to date (physiological factors, media, microscopical techniques etc.) concerning soft rot decay.

Timbers in mines and fence posts attracted several scientists to investigate the types of deterioration that occurred there. Levy and Lloyd (1960) isolated soft rot fungi from softwood species. Levy (1962) and Corbett and Levy (1963b) expanded their studies to wooden fence posts in soil (untreated and treated with preservative). Levy (1963a, b; 1964) became more involved with soft rot decay in mines. Merrill and French (1963) found soft rot decay in fibre building materials which had sufficient environmental conditions for decaying.

Wood preservation is one of the most important aspects in the field of timber and has attracted many people through the ages to investigate and find the best preservative and better ways of applying it. Many people studied the soft rot decay in preserved wood. (Duncan 1960a, b, Thomson 1968, Levi 1969, Savory and Bravery 1970 and Levy 1971). Sorkhoh (1975) showed that in the ray parenchyma walls where the concentration of the preservative was high, there was no indication of decay by any fungal type, but in the lignified cell wall where the concentration of the preservative was relatively low, there was a delay in the fungal activity which gave more chance to the soft rot fungi than the other types of decay fungi to cause degrade.

Studying the morphology of soft rot fungi might raise several questions. How and why does the fungus invade the wood ? How does the fungal hypha penetrate into the cell wall ? Why does it attack the middle layer of the secondary cell wall forming the peculiar biconical ended cavities ? How do the cavities run parallel to the microfibrils of the cell ?

Many workers have made intensive studies to attempt to answer some of the questions that could be raised in this field. There is still dispute in answering some of the questions. Therefore, it is more convenient to consider the literature covering the extensive research carried out in this field under the following sections :

## 2.1 Colonization

Corbett (1963) investigated the pathway of a fungus

into an orientated centimetre cube of wood by sealing two pairs of opposite faces with araldite. Her results indicated the fastest colonization occurred with the transverse faces exposed, then the tangential and the slowest was with the radial faces. The fungus started by colonizing the ray cells when the infection was originated from a tangential face. Greaves and Levy (1965) found that the fungus Chaetomium globosum colonized the ray cells of Scots pine after one week. The hyphae started to cross into tracheids via simple pits in the second or third week, some time later the fungus started to penetrate through bordered pits or using active penetration directly through the cell wall. More work was carried out in this field by Corbett and Levy (1963b), Greaves (1966), Kaarick (1967), Banerjee (1969), Banerjee and Levy (1971) and Dwyer (1975), who investigated the fungal colonization and succession in wood. They reached the conclusion that bacteria and moulds were the first invaders of wood, followed by stainers and soft rot fungi which were isolated from greater depth. This last group of fungi isolated would provide suitable conditions for the wood-rotting Basidiomycetes to penetrate deeply and degrade the cell wall as a nutrient source.

## 2.2. Penetration

All the knowledge about the very early penetration of the cell wall by a fungal hypha has not given the ultimate and undisputed answer to the means of penetration. There are

two schools of thought, each one has its own supporters. One of them says the penetration is enzymatic, the second group support the hypothesis of penetration being mechanical.

Cartwright (1930) noticed the smoothness of the sides of bore holes in Sitka spruce caused by Trametes serialis. He concluded that either the penetration was very rapid or that mechanical force had to follow the enzymatic activity which produced a general softening in the cell wall. The first step of penetration was a v-shaped notch cut in the cell wall at the point of contact of thread like advancing hypha. His other evidence for penetration being enzymatic was that in the very early stage there was a deep staining reaction at the part of the wall which was surrounding the bore hole. He suggested this could be due to the increase of lignin content after the cellulose breakdown.

Procter (1941) noticed the presence of two sizes of hyphae, a small one which was active, penetrating the cell wall without noticeable reduction in hyphal diameter, while a larger hypha reduced its diameter when it came into contact with the cell wall (this has been noticed in the present work). Both hyphae had produced the same sizes of bore holes. His explanation for the penetration phenomenon was that the first touch of the hypha against the highly lignified wood cell wall (even before the chemical action), caused a resistance to the hypha stimulating it to form a minute hypha. The touching of this minute hypha with the wood cell wall stimulated the hypha to produce enzyme to

hydrolize the cell wall and the mechanical force followed. The present work showed not only a constriction of the hypha, but a septal formation in the hypha at the point of crossing the  $S_3$  and middle lamella regions. Therefore, Procter's suggestion about the mechanical force which was caused by highly lignified wall might bring to light the cause of septum formation as well.

Liese and Schmid (1961) showed in their E M work that the hypha in blue staining fungi can penetrate into the pores of the bordered pits. Their observation showed there was no influence of the pits on the direction of hypha because they found that the hypha often grew near the bordered pits without penetration. They supported the hypothesis of mechanical penetration and no enzymatic reaction was involved whatsoever, although they found a slimy zone surrounding the tip of some hyphae which was attributed to be substances secreted by the hyphae to accelerate the mechanical penetration. The penetration was happening by a boring hyphae which does not grow from the apex but from the appressorium (a swelling at the tip of the hypha). Corbett (1963) described the passive penetration of the fungal hypha through the bordered pits in softwood which occurs more easily between ray cell and tracheid, and tracheid to ray cell, than from tracheid to another tracheid. In hardwood the simple pits were the pathway for the main passive penetration from one cell to the adjacent cell. The conclusions of Corbett were confirmed later by Greaves and Levy (1965), Levy (1965a) and

Levy (1967).

Liese and Schmid (1964) confirmed the appressorium formation by testing the ability of four blue-staining fungi to penetrate the cell wall as well as metal foils. They found some fungi were able to grow through the silver foil which confirmed their previous hypothesis of mechanical penetration. The boring hyphae grew from a swelling head part of the hypha which was called "Transpressorium".

Levy (1965a) pointed to simple pits in Beech wood which appeared to be used by the fungus as a passive penetration path. He also presumed the presence of plasmodesmata in the secondary wall with the remaining cytoplasm or protein residue which attracted the branched hypha to attack it enzymatically (this was noticed in the present work in one case when Chaetomium globosum was colonizing the ray cells). Liese (1970) assumed that the penetration of the blue stain fungi into the cell wall was due to both localized enzymatic force at the tip of the transpressorium and mechanical force impulsing it into the cell wall. The penetration of soft rot fungi into the softwood tracheids occurred by means of a small perforating hypha which was similar to the transpressorium in blue stain fungi. The perforation hypha penetrated by enzymatic action either the  $S_3$  layer or the pit chamber into the  $S_2$  layer. After approaching the  $S_2$  layer either it changed its direction by about  $90^\circ$  forming a cavity or continued growing by crossing many tracheids with constricted penetration hyphae, resuming its original diameter in each

lumen. Nilsson (1974a) does not agree with Levy (1965a) and Lundström (1972) that the presence of plasmodesmata is a stimulus to be used as a pathway by the penetrating hypha, because he found bore holes formed by the fungus in viscose fibres and cellophane.

### 2.3 T-branch and cavity formation

The unique behaviour of soft rot fungi in forming a T-branch was rediscovered by Corbett and Levy (1963a). They described the initiation of cavity formation as the hypha lying in the lumen of the tracheid parallel to the long axis contiguously to the cell wall. This hypha formed a very fine lateral branch which penetrated the cell wall, by forming a bore hole then it crossed all the cell wall layers of the first cell until it reached the  $S_2$  layer of the second cell wall, where it turned to the vertical position and branched at  $180^\circ$  giving the T-branch which is the initial base for cavity formation. Corbett (1963) described a "proboscis" hypha which is a very fine hypha growing from the tip of a cavity along the lie of the microfibrils in the  $S_2$  layer giving rise to a very narrow cavity.

Levi (1964 and 1965), Levi and Preston (1965) postulated three hypotheses for the change in direction of penetrating hypha to form a T-branch :

- 1) Blind end of plasmodesmata and other cell wall capillaries.
- 2) The fine hypha penetrated from the lumen of the

first cell proceeding through  $S_3$ ,  $S_2$ ,  $S_1$  and compound middle lamella by bore holes which were formed mechanically or enzymatically, then reaching the  $S_2$  layer of the second cell wall and facing the barrier  $S_3$  layer, forced the hypha to change its direction and to grow in the  $S_2$  layer.

3) Presence of "weak microfibrils" or low resistant areas in the  $S_2$  layer, which caused a diversion in the hyphal direction when it approached such a region in the wall. Levi and Preston (1965) modified Roelofsen's theory (1956) about the conical ends of cavities. They suggested it was due to the longitudinal diffusion of enzymes between the microfibrils which is easier and faster than transverse diffusion through the lignin in the amorphous region of microfibrils, therefore, the rate of decomposition was governed by lignin modification rather than cellulose decomposition. The constriction in between the cavities in their opinion was due to the lack of enzyme secretion in the septal area of hypha.

Levy and Stevens (1966) suggested that the T-branch is acting as haustorium because if soft rot fungi cannot obtain their nutrient by direct erosion of the enzyme complex through the cell wall because the presence of the boundary layers forms a barrier, the fungus will penetrate that barrier by mechanical force forming haustorium-like hypha (T-branch) in the  $S_2$  layer of the cell wall, which could be degraded enzymatically.

Liese (1970) attributed the variation in cavity size and shape to several factors such as : fungal species, duration of

attack, wood species, cell types and ecological factors. He also suggested that ectoenzymatic activity at the tip of the hypha fluctuated : a minimum of activity produced the pointed-end of the cavity, and a resumption of activity, and secretion of more enzyme, resulted in formation of another cavity.

"Lundstrom (1970 and 1972) estimated that the time for cavities to appear in birch fibres after being attacked by Phialophora sp. was between two to six days. His in vitro studies indicated that two days were enough for the hypha to form a new cavity.

Nilsson (1974a) tried to correlate his work with the hypotheses which had been suggested by Levy (1965a), Levi (1965) and Lundstrom (1972) about the change in direction of penetration hypha within the wood cell wall. He disagreed with the previous hypotheses because the T-branch occurred in all tested fibres, including the cellophane and viscose fibres, and also because cavities were formed in thin transverse sections of birch wood by penetration hypha without change in the direction, therefore, he agreed with Levy and Stevens (1966) that the hypha in the cavity could be considered functioning as a haustorium. Nilsson succeeded in producing cavities by Hemicola alopallonella in fibres which contained no or very little lignin, such as cotton and Avicel. He suggested that the rate of decomposition of cotton fibres was influenced by the cellulose decomposition and not by lignin decomposition (Levi and Preston, 1965), because of

the absence of lignin in cotton fibres. As a result the shape of the cavities were dependent on the cellulose structure and not on lignin or hemicellulose. The last opinion was also suggested by Wardrop and Jutte (1968), Jutte and Wardrop (1970). Nilsson explained the reason for formation of chains of cavities instead of a single long cavity. He postulated firstly that it is unlikely that the enzyme secretion was decreased in the septal region (Levi, 1965) giving rise to constrictions between the cavities, because septa were also observed inside the hypha within the cavity; secondly, the inhibition of enzyme secretion by toxic phenolic groups produced during the degradation of wood (Liese, 1970) does not apply in the lignin free fibres as in cotton. Therefore he concluded that : (1) the inhibition of enzyme synthesis could be due to breakdown restraint by the cellobiose or glucose accumulated around the hyphae during the cell wall hydrolysis; (2) the enzymes might be produced only when the hypha is growing in the cell wall parallel to the microfibrils, but when there is a little diversion in hypha this would cause a reduction in the enzyme activity which ends up with a constriction. Nilsson (1974c) suggested a new definition for a "cavity" as being a zone of lysis of the cell wall substance produced by hyphae which are growing within the fibre cell walls parallel to the presumed direction of the cellulose microfibrils. Very recently Crossley (1975) has produced remarkable SEM micrographs of proboscis hyphae extending along the line of the microfibrils but in a much finer form and with a very small diameter cavity. (See Fig. A)

PROBOSCIS HYPHAE IN SOFT ROT CAVITY  
FORMATION BY CHAETOMIUM GLOBOSUM ON BIRCH

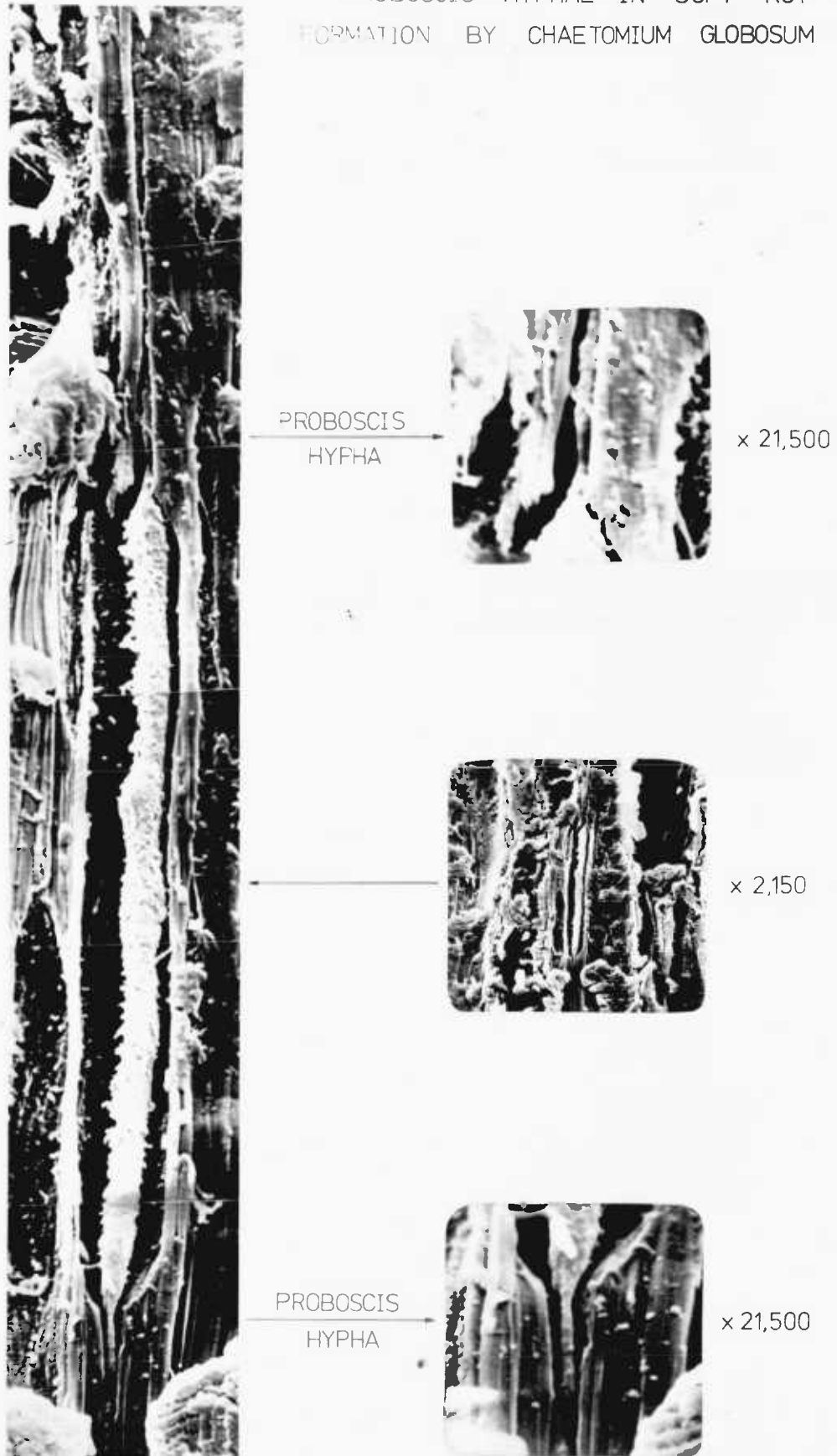


Figure A (Courtesy of Crossley)

#### 2.4 Interrelation of the secondary cell wall structure of wood with soft rot fungi

Many workers have made an investigation of the structure of the wood cell wall by using micro-organisms and their enzymes. Bailey and Vestal (1937) explained the regular cavities with conical ends by the suggestion that there were two planes of enzyme flow, one following on the long axis of cellulose molecules, and the other at an angle of 20 - 25 degrees to this axis. Both would result in cavities oriented either helically or parallel to the long axis of the cell, in other words parallel to the orientation of microfibrils in the central layer of the secondary wall. Meier (1955) found that Chaetomium globosum attacked the S<sub>2</sub> layer of hardwood forming cavities without any lignin residue being left behind. This, he suggested, was because of the lack of lignin in the S<sub>2</sub> layer. He concluded that Chaetomium could not extensively attack the more heavily lignified wall in softwood because the fungus could not decompose the lignin. In the course of the present work, Chaetomium globosum has been shown to attack the S<sub>2</sub> layer of softwood Pinus sylvestris and cause typical soft rot cavities as in hardwood. Cowling (1958) considered the lignin as a "protective barrier" against degradation by any except specialized wood-destroying organisms which, with their enzymes, are capable of degrading the wood cellulose. In pulp (delignified) wood, the cellulose became less resistant to degradation. Savory and Pinion (1958) investigated the

chemical aspect of soft rot in beech caused by Chaetomium globosum and found, that a small amount of lignin was consumed when the cellulose and pentosan were almost finished. They concluded that this suggested the presence of more than one enzyme system. Courtois (1963a, b) compared several forms and patterns of infection and their form groups in both softwood and hardwood attacked by soft rot fungi. He postulated three different enzymatic decompositions : (a) From the cell lumen or the erosion type. (b) From the  $S_2$  layer of the secondary wall forming cavities in this layer, these cavities varying according to their ends, length, size .... etc. He also found that the  $S_3$  layer was decomposed simultaneously with the  $S_2$  layer in the latter stages (this will be discussed in Part III and IV). (c) From the  $S_1$  and primary wall which forms some cavities in the  $S_1$  layer additional to cavities in the  $S_2$  layer. He also believed that hypha secreted enzyme when it was within the cavity but not in the constricted region of the cavity. He investigated the ability of more than one species of fungus on one species of wood giving him results of similar effect. He postulated that the nature of the cell wall is an important factor. Liese (1964) observed the  $S_3$  layer of softwoods was more difficult to be attacked by ectoenzymes than that in hardwoods because of the heterogeneous enzymatic complex of soft rot fungi. The cavities varied in their size and shape according to the chemical and morphological structure of the cell wall layers, and was also influenced by cell types, wood species, fungal species

and ecological factors. Corbett (1965) classified the cell wall destruction in soft rot decay into two types :

Type 1 : (The present work is involved with this type). This was the only type to be observed in the sapwood of Scots pine infected with Chaetomium globosum. It originated in the  $S_2$  layer by T-branching which began to degrade enzymatically the cellulose molecules of one microfibril from one end. These cellulose molecules were believed to be arranged parallel to the microfibrils (Roelofsen, 1959), so the degradation would end with cavity formation.

Type 2 : When the hypha came into contact with the wall of a fibre from the lumen, either it gave a very fine hyphal branch which degraded the cell wall in the shape of V, or, more often, the cell wall would be eroded along the hyphal length which lies contiguously to the cell wall. Levy (1965a) attributed the variation in the behaviour of Chaetomium globosum in each species of wood to the fine structure and chemistry of the cell wall; he also noticed that the tip of the hypha appeared to stain more intensely than the rest of the hypha. Levy (1965b) attributed the two distinct types of destruction to the chemical nature and variation of the cell wall in softwoods and hardwoods. He attributed the difficulty of Chaetomium sp. in penetrating the secondary cell wall of softwoods from lumen of the tracheid to the  $S_3$  layer, either to its different chemical nature or the thickness of this layer; or postulated that it might be due to some deposited material at the lumen surface which acts as a physical

barrier. He also showed that the type of destruction depended on the species of wood, so that a fungus might produce cavities or breakdown the cell wall from the lumen by removing the  $S_3$  and  $S_2$  layers. Liese (1970) explained the behaviour of soft rot fungi in wood as being purely enzymatic and this was more obvious in hardwood than in softwood because of the direct contact of the  $S_3$  layer in hardwood to the enzymatic attack by hypha growing on the wall. The  $S_3$  layer in softwood was more resistant because of its chemical composition which contained either a different type of polysaccharide or the presence of a different type of incrustation material. He also pointed to the high lignin content in softwood. Findlay (1970) studied the behaviour of the fungus Phialophora fastigiata in normal and delignified wood specimens comparing them with rubbery shoot which contains abnormal lignin. He reached a conclusion that the lignin in the cell wall was a barrier for the free diffusion of the enzyme from the fungal hyphae which resulted in the fungal hyphae penetrating into the  $S_2$  layer and forming cavities. Nilsson (1973) made an intensive investigation of 160 different species of fungi for their cellulolytic activity, the type of the decay produced in each wood and their decay capacity. He classified the type 2 (Corbett, 1965) decay in birch into two patterns : 1) dissolution of the cell wall in discontinuous areas which were mostly contiguous to the hypha giving rise to a perforated appearance of the fibre. (He did not observe this type of erosion in the  $S_2$  layer of the tracheids of Scots pine. The

present work showed this type of erosion in Scots pine only when it was attacked by Phialophora fastigiata S6A which was supplied by P.R.L.).

2) Striated erosion of the cell wall formed by enzyme diffusion over the whole wall surface. He called this type of erosion a true erosion and it was not observed in softwood. (The present work showed this type of erosion in Scots pine attacked by Phialophora fastigiata 731-1-3b which was supplied by Nilsson). He classified the fungi into four groups according to the patterns of degradation in birch wood:

1. Bore hole forming fungi.
2. Fungi producing only cavities.
3. Fungi producing only erosion.
4. Fungi producing cavities and erosion.

In 1973 a visit by Nilsson to the department resulted in the suggestion of exchanging strains of the fungus, which stimulated the present author to find differences in the behaviour of the different strains of Phialophora when cultured in two different media (as will be explained further in detail under Part II). This also stimulated Nilsson to carry out an investigation which led him to succeed in producing cavities in Scots pine with Phialophora sp., so he attributed the failure of Findlay (1970) in obtaining cavities in Scots pine either to the methods of decay testing or to the differences in the strain of the fungi. Nilsson (1974a) expanded his work to test the cavity formation by Hemicola alapallonella

in various lignified and nonlignified cellulose fibres and found cavity formation in all the tested fibres of natural origin. Nilsson (1974c) observed no or very little cavities in delignified wood or in cotton fibres, he attributed the reason for sparse cavity formation in the delignified wood to be due to the disorganisation of the microfibrils caused by the delignification (Findlay, 1970). (This was later shown to be unlikely because the orientation of the microfibrils in the delignified Scots pine was very similar to that in normal Scots pine, Zainal (1975)). Because of a greater variation in the shape of the cavities formed in certain cellulosic fibres than was observed in the decayed wood, he postulated that the cavity formation and its shape was influenced by the cellulose structure instead of being influenced by lignin and hemicellulose.

From this brief historical review it is clear that many investigations have been made in this field in an attempt to solve the problems and questions that have been mentioned. Still there are many problems unsolved and questions unanswered. Closer detailed observations at higher magnifications might well provide more information and this clearly suggests the electron microscope as an important tool for further investigations.

### 3. ELECTRON MICROSCOPICAL STUDIES OF FUNGI

#### ASSOCIATED WITH WOOD

Anatomy of wood has always been an attractive subject for investigation in detail. The discovery of wood mycology or wood pathology could be attributed to the first pioneer wood anatomists Schacht, R. Hartig, I.W. Bailey, Preston, Frey-Wyssling, Wardrop and many others.

Findlay (1970) made an excellent literature survey for the ultrastructure of the wood cell wall, its chemical constituents and the effect of the micro-organisms enzyme on each constituent. The present work is confined to the morphological studies of soft rot fungi behaviour in wood cell wall using the electron microscope, and does not cover the chemistry of the wood cell wall. There appears to be a correlation between the chemical ultrastructure of the wood cell wall and the degradation caused by fungal enzymes which needs more research to be done, using more sophisticated instruments, which might help to answer all the questions that could be raised in this field. Simmer et al. (1975) is an excellent example of such work.

The early studies of the wood cell wall was based on the light microscope concluding that a typical wood fibre or tracheid wall is not homogeneous and consisted of different layers, (Diagram 1).

The heterogeneity of the cell wall layer is mostly due to the differences in the orientation of microfibrils, Preston (1974). Microfibrils are chains of cellulose molecules grouped together in bundles of fairly constant supra-molecular but sub-microscopic thickness which form the fairly open framework of the wall, Jane (1970).

The middle lamella was first named by German workers, but

Kerr and Bailey (1934) used the term which became internationally accepted. The middle lamella is followed by the primary wall in which the orientation of microfibrils are irregular and mainly transverse. This layer is followed by the secondary wall which is divided into three layers arranged successively starting from the primary wall towards the lumen,  $S_1$ ,  $S_2$  and  $S_3$  (named by Bailey). The microfibrils in the  $S_1$  layer are organised in a flat helix "S" almost transversely to the cell axis and some in "Z" helices (Jane 1970). Many workers suspected the presence of two sets of planes of microfibrils in this layer and this was confirmed by Meier (1955), Frei, Preston and Ripley (1957) Wardrop (1964) and Harada (1965), who showed an electron micrograph for the  $S_1$  layer of conifer tracheid. In the  $S_2$  layer, the broadest layer - which is concerned in the studies of soft rot fungal attack - the microfibrils are arranged more regularly in steeply "Z" helices, while in the  $S_3$  layer two flattish helices of the microfibrils are running in opposite sign, Wardrop (1964) and Preston (1974), Diagram 1.

Many wood anatomists, Fengal (1962 and 1967), Côté and Day (1965), Jutte (1969) and several others used the electron microscope to study ultra-thin sections of wood. At the same time the wood microbiologists were trying to apply the electron microscope in their field. Most of the work carried out in the early days used the replica technique which is excellent for studying the intact wood layered surface but not so good for the degraded wall especially with soft rot cavities. Meier (1955) was the first who succeeded in producing some electron micrographs of soft rot cavities with smooth edges by applying replica technique. He also published electron

micrographs of decayed wood with brown rot fungus which indicated a porous structure within the secondary wall which was obviously attributed to the breakdown of cellulose. He suggested the gross distribution pattern of lignin but he doubted the size of the pore shown in the secondary wall which did not correlate with the knowledge of the cell wall constituent ultrastructure. In 1958 Cowling's electron micrographs showed clear differences between cell wall layers in non-decayed wood, these differences disappeared after brown rot fungal attack and did not indicate a porous structure of the secondary wall as seen in Meier's (1955) electron micrographs. He interpreted these results as a collapse of the lignin framework of the secondary wall during the preparation procedure. He suggested that, brown rotted wood could be taken as an experimental material to study the ultrastructural relation between lignin and microfibrils. Liese (1964, 1965b and 1970) applied the replica technique to study soft rot attack in Scots pine caused by Chaetomium globosum and also to study brown and white rot attack in wood.

The extreme difficulties in finding the right technique applicable and suitable for fixing and embedding the decayed wood cell wall because of the heterogeneity of the fungus and the wood cell wall layers led the investigators to use the replica technique. (These difficulties will be explained further in detail in Part IV). Levi (1964) using ultra-thin sections produced the most impressive micrographs for hyphae within cavities in the  $S_2$  layer of the cell wall. The investigators, Chou (1970 and 1971), Findlay (1970) and Bravery (1972) began to use the ultra-thin sections exclusively. Findlay (1970) contributed superb electron micrographs for ultra-thin sections of birch wood fibre wall attacked by Phialophora

fastigiata. He showed the cell wall penetration, T-branch and cavity formation. He was unfortunately unable to obtain (as in the present work) thin sections of the very early penetration of the cell wall which is an observation that might help to achieve great progress in the present knowledge in this field.

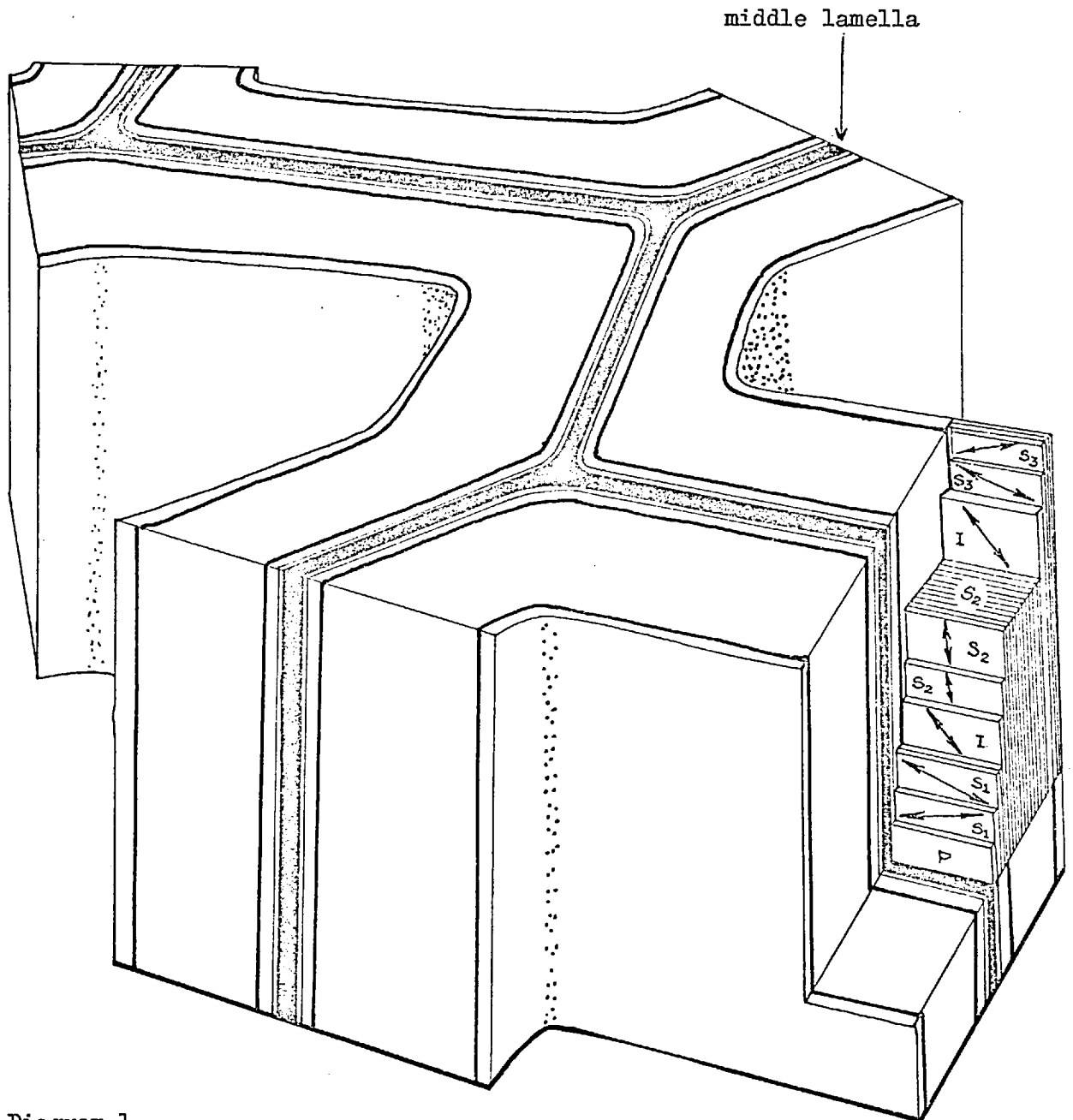


Diagram 1

Diagrammatic representation of a segment of conifer tracheids with the wall as revealed by electron microscopy. Double arrows show the general run of the microfibrils in the various layers. The intermediate layers (I) are not always present, and  $S_3$  is absent in some species. (Modified from Preston, 1974).

## PART II

### PHYSIOLOGICAL BACKGROUND

## 1. INTRODUCTION

In recent years intensive studies have been carried out on the physiology of wood decaying micro-organisms. Several investigators have discussed the effect of the amount of nutrients on the rate of decomposition of wood caused by soft rot fungi. Findlay (1934) found an increase in the rate of decay in Sitka spruce when the wood specimens were treated with low concentration of ammonium nitrate, while the decay capacity of the fungus was greater when there was an organic source of nitrogen. Savory (1954a, b) obtained results indicating a higher rate of decay in beech wood attacked by Chaetomium globosum when inorganic nutrients were present. He correlated his results in the laboratory with the decay of timber in water cooling towers. Duncan (1960a) noticed an increase in the decay caused by soft rot fungi by applying mineral nutrients and vitamins to the wood or by adding them to the agar substrate. She obtained similar results by adding organic matter to the soil. Cowling and Merrill (1966) obtained significant decomposition of hardwood and softwood sawdust by soil-inhabiting fungi with additional nitrogen. Findlay (1970) found no significant weight loss by Phialophora fastigiata grown on agar medium containing filter paper as a carbon source, when different concentrations of inorganic nitrogen were used for impregnating softwood blocks. He observed low weight losses with higher concentrations of inorganic and organic sources of nitrogen. He postulated that the high concentration of nitrogen might be inhibiting the growth and the attack of the fungus. Lundstrom<sup>"</sup> (1973) and Butcher (1975a) have also investigated the effect of nitrogen source in culture media on the behaviour of soft rot fungi. They found that

higher nitrogen concentration caused a decrease in the attack.

Banerjee and Levy (1970) subcultured Chaetomium globosum several times in malt agar medium and Abrams plus cellulose medium. The decay capacity of the subcultured fungus in both birch and Scots pine was higher when it was grown on Abrams plus cellulose substrate than malt agar medium. Butcher (1975b) attributed the differences in the growth to the malt extract used as a carbon source which he claimed was inhibiting the decay. Bravery (1968) and Nilsson (1973 and 1974a) concluded that the presence of an additional carbon source to cellulose such as glucose or asparagine can react as an inhibiting factor for cellulase production. Nilsson used both carbon sources widely in his experiments. Butcher and Drysdale (1974) found that fungi need adaptation to be able to utilise the wood cell wall cellulose: this could be achieved by culturing them on cellulosic media before the preparation of inocula. Butcher (1975a) postulated the unimportance of an additional carbon source such as glucose to induce cellulase and enhance the decay.

All previous workers investigated the effect of nutrients on the rate of decay of normal wood by fungi. Many investigators attributed the greater resistance of softwood to soft rot decay to its higher lignin content, meanwhile the study of the rate of decay and the effect of nutrients in the delignified wood was neglected. Savory and Farmer (1959) were the first investigators to examine the effect of the delignification of wood on the rate of decay in the laboratory by immersing beech and Scots pine wood blocks into chlorinated water. The decay capacity of the fungus in untreated Scots pine after six weeks' attack was 1.5%, but in the

blocks treated with chlorinated water it was 16.4%. Thompson and Kaustinen (1964) used small chips or shavings to get rapid delignification using sodium chlorite and buffer for the process. Their test showed that some carbohydrate was lost during delignification and in the washing stage after delignification. Bailey, Liese and Rösch (1968) obtained an increased weight loss when delignified softwood was exposed to Chaetomium globosum. They postulated that the delignification effect was similar to the "pre-cellulolytic" stage of the brown rot fungi. Findlay (1970) delignified the softwood specimens by approximately 5% loss in weight which caused the enhancement of the attack of Phialophora fastigiata without cavity formation. He described the type of decay in delignified wood as an erosion type. Nilsson (1974c) noticed sparse cavity formation in the fibres of sulphate pulp which contained 10.8% lignin. He explained this phenomenon as being due to the disorganisation of the microfibrils caused by the delignification. Such disorganisation was not observed during the studies described in this thesis.

## 2. MATERIALS AND METHODS

### 2.1 The effect of culture media on the fungal growth

#### 2.1.1 Media

Several media were prepared as follows :

##### 2.1.1.1 Malt agar medium

1.0 l distilled  $H_2O$

25.0 g malt extract agar

Malt extract agar was mixed thoroughly with one litre distilled water, poured into medical flats and autoclaved at 15 p.s.i. for 20 minutes. After autoclaving the medium was left to cool to approximately  $45^{\circ}C$  and then poured into sterilized plastic petri dishes 9 cm diameter.

##### 2.1.1.2 Abrams mineral salts medium (Abrams 1948)

1.0 l distilled  $H_2O$

3.0 g  $NH_4NO_3$

2.0 g  $K_2HPO_4$

2.5 g  $KH_2PO_4$

2.0 g  $MgSO_4 \cdot 7H_2O$

15.0 g Agar

All the constituents were mixed together, autoclaved at 15 p.s.i. for 20 minutes and poured into the petri dishes in a similar manner to the malt agar medium already described.

2.1.1.3 Mixture of birch sawdust + malt agar medium

1.0 l distilled  $H_2O$   
50.0 g birch sawdust (60 - 85 mesh)  
50.0 g malt extract agar

These constituents were mixed thoroughly whilst being heated, autoclaved at 15 p.s.i. for 20 minutes, and then left to cool to approximately  $45^{\circ}C$ . This medium was then poured into sterilized plastic petri dishes which had been previously cooled in a deep freeze for 15 minutes. When pouring the medium into the cooled petri dishes, the dishes were continuously, gently shaken until the medium started to solidify. This method of pouring prevented the separation of the malt agar from the wood sawdust.

2.1.1.4 Modified Eggins and Pugh medium (Bravery 1968, after Eggins and Pugh 1962)

1.0 l distilled  $H_2O$   
1.0 g  $KH_2PO_4$   
0.5 g KCl  
0.2 g  $MgSO_4 \cdot 7H_2O$   
0.1 g  $CaCl_2$   
15.0 g Agar  
10.0 g cellulose CF 12  
0.543 g  $(NH_4)_2SO_4$   
0.001 g Thiamine Hydrochloride

2.1.1.5 Abrams + Cellulose medium

1.0 l distilled  $H_2O$   
3.0 g  $NH_4NO_3$   
2.0 g  $K_2HPO_4$   
2.5 g  $KH_2PO_4$   
2.0 g  $MgSO_4 \cdot 7H_2O$   
15.0 g Agar  
10.0 g Cellulose CF 12

2.1.1.6 Cellulose agar medium (Nilsson 1973)

1.0 l distilled  $H_2O$   
1.0 g Asparagine  
1.0 g  $NH_4NO_3$   
1.0 g  $KH_2PO_4$   
0.5 g  $MgSO_4 \cdot 7H_2O$   
0.01 g  $FeSO_4 \cdot 7H_2O$   
0.01 g  $ZnSO_4 \cdot 7H_2O$   
0.5 g Yeast extract (Difco)  
2.5 g Glucose  
15.0 g Agar  
10.0 g Cellulose CF 12

The cellulose was prepared by macerating Whatman's cellulose CF 12 in a high speed macerator for 30 minutes. The constituents of each medium (2.1.1.4, 2.1.1.5, and 2.1.1.6) were mixed and steamed without the cellulose, which was then mixed in before autoclaving at 10 p.s.i. for 20 minutes. The method of pouring the medium was

similar to the one applied in medium 2.1.1.3. The media were poured into sterilized plastic petri dishes in a lamina-flow bench and allowed to cool. Each petri dish was then inoculated with a small piece ( $2\text{ mm}^2$ ) of inoculum of each fungus individually. The inoculum subcultures were prepared in advance from stock cultures which were kept in McCartney bottles at  $4^{\circ}\text{C}$ . The inoculated petri dishes were kept in polythene bags and incubated at  $25^{\circ}\text{C} \pm 2$ . They were examined every three days until the fungal growth was seen to be well established.

#### 2.1.2 Fungi

##### 2.1.2.1 Chaetomium globosum S70B (F.P.R.L.\*)

An active soft rot fungus in both hard and softwoods and well recommended for its activity in laboratory experiments.

##### 2.1.2.2 Phialophora fastigiata S6A (F.P.R.L.\*)

Krapivina (1960) was the first author to show that some of the staining fungi could produce soft rot in hardwoods. Karkanis (1966) observed that Phialophora fastigiata can behave as a soft rot and a stainer fungus in hardwood, but as a stainer only in softwood. Findlay (1970) arrived at a similar conclusion.

\* From Princes Risborough Laboratory, Buckinghamshire, England

2.1.2.3 Phialophora fastigiata 731-1-3b

Supplied by Nilsson (1973)

2.1.3 Results:

The results of the fungal growth are tabulated in table 1 and plates 1 and 2, indicating that the mycelium of Chaetomium globosum usually needed less than a week to cover the petri dish. The fungus Phialophora fastigiata needed a longer time to grow on some media, but did not show any growth on other media even after three weeks' of incubation.

Plate 1 shows the growth of Phialophora fastigiata S6A and plate 2 Chaetomium globosum S70 B on the following media.

1. 2.5% malt agar
2. Abrams mineral salts
3. Mixture of birch sawdust + malt agar
4. Modified Eggins and Pugh
5. Abrams + cellulose

TABLE 1

| MEDIA                                   | Phialophora fastigiata S6A |                       | Phialophora fastigiata<br>731-1-3b |                       | Chaetomium globosum S70B |                        |                       |
|-----------------------------------------|----------------------------|-----------------------|------------------------------------|-----------------------|--------------------------|------------------------|-----------------------|
|                                         | <u>mycelium</u>            | <u>rate of growth</u> | <u>mycelium</u>                    | <u>rate of growth</u> | <u>mycelium</u>          | <u>fruiting bodies</u> | <u>rate of growth</u> |
| Malt agar                               | ++                         | 2                     | +                                  | 2                     | +++                      | 00                     | -1                    |
| Abrams                                  | -                          | -                     | NT                                 |                       | ---+                     | -                      | 1                     |
| Mixture of birch<br>sawdust + malt agar | +++                        | 1-2                   | NT                                 |                       | +++                      | 0                      | -1                    |
| Modified Eggins and<br>Pugh             | -                          | -                     | NT                                 |                       | ---+                     | 000                    | -1                    |
| Abrams + cellulose                      | -                          | -                     | NT                                 |                       | +                        | 000                    | -1                    |
| Cellulose<br>(Nilsson 1973)             | +                          | 2                     | ---+                               | 2                     | ++                       | 0                      | -1                    |

- = No growth at all
- + = Very little growth
- + = Fairly good growth
- ++ = Good growth
- +++ = Very good growth
- 0 = Few fruiting bodies
- 00 = More fruiting bodies
- 000 = Covering the plate
- 1 = Less than a week
- 1 = One week
- 1-2 = One to two weeks
- 2 = Two weeks
- NT = Not tested

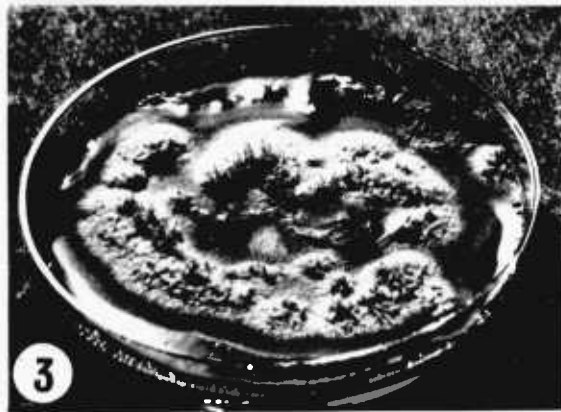
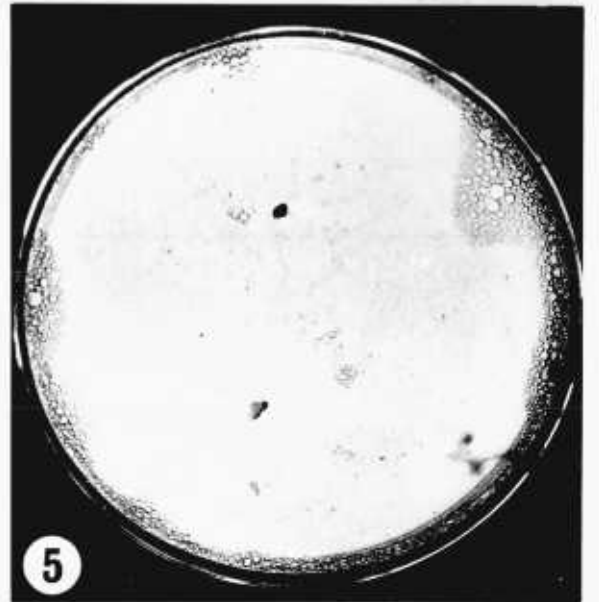
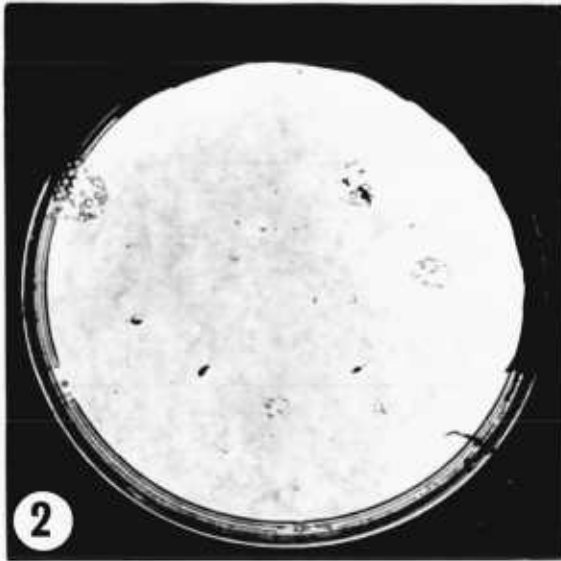
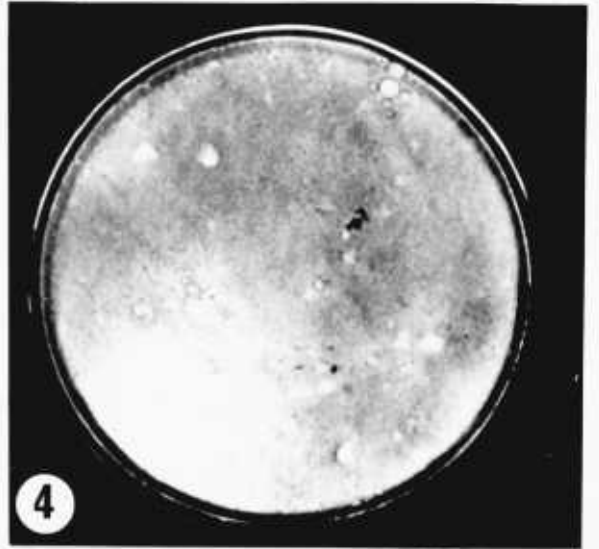
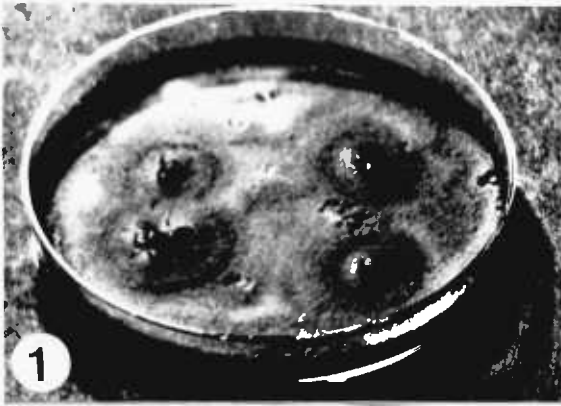


PLATE 1

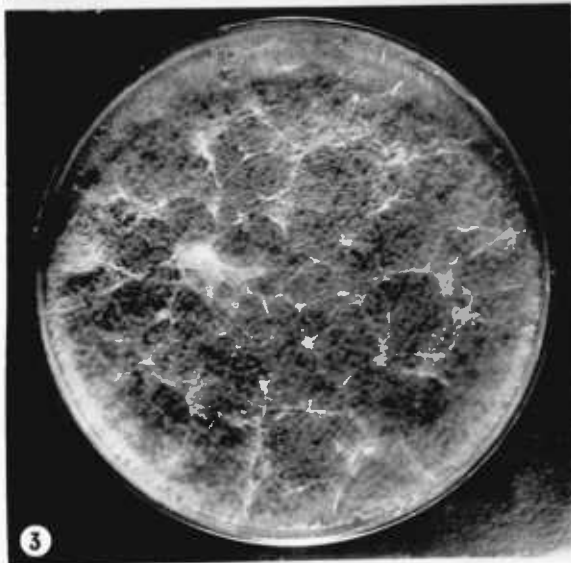
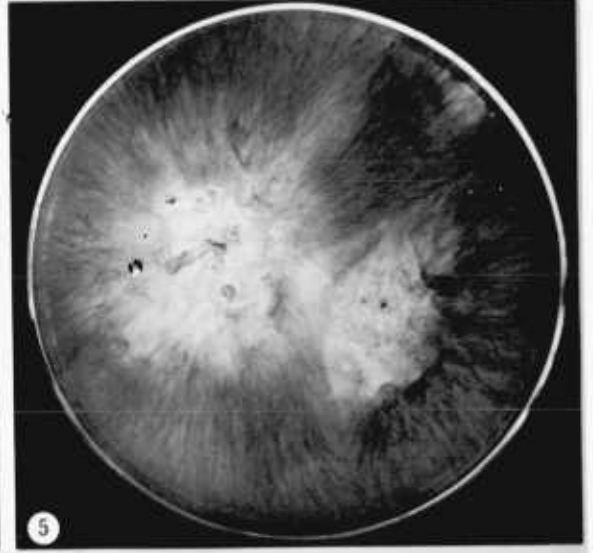
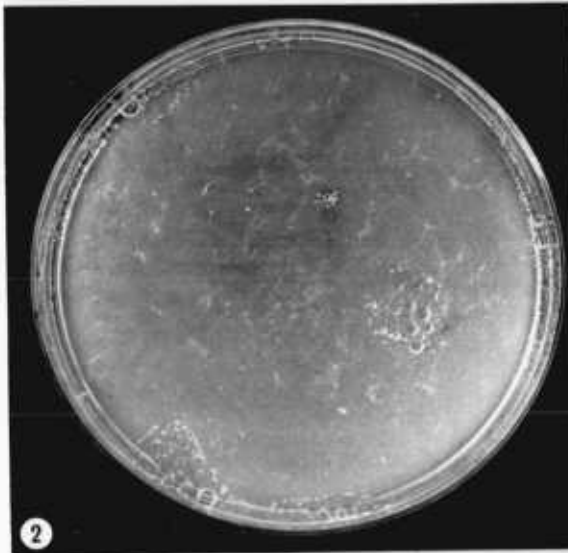
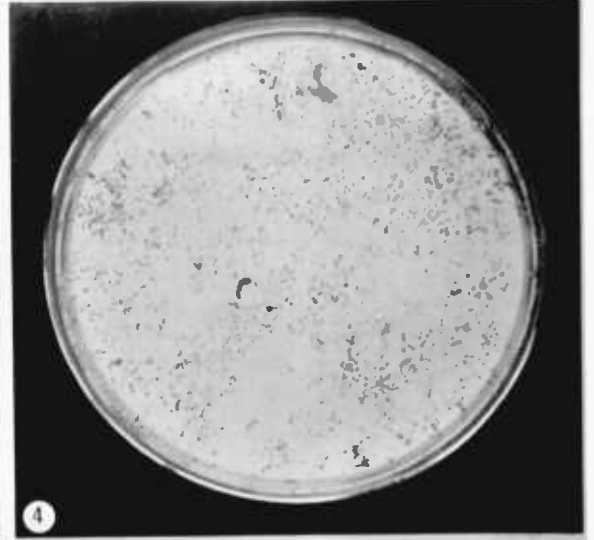
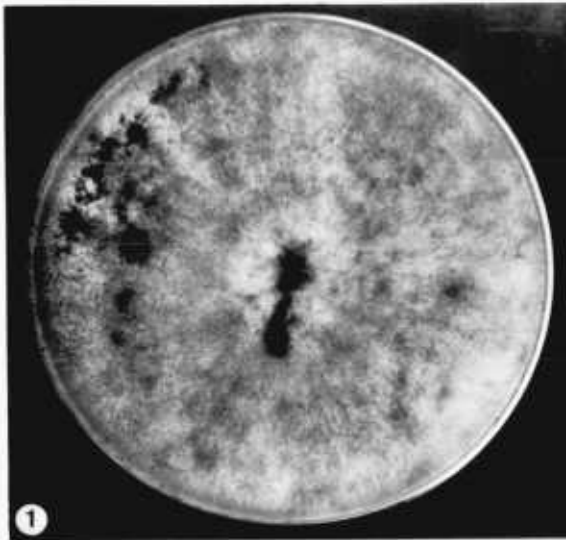


PLATE 2

## 2.2 Decay capacity (weight loss)

An intensive investigation was carried out in the present work on normal and delignified wood specimens to clarify the role of lignin as a barrier in softwood to soft rot attack as postulated by Findlay (1970) and Fuller (1970). Also the effect of two different media on the decay capacity and the behaviour of the fungi used was investigated with the aid of the electron microscope. (This investigation will be discussed in Parts III and IV.)

### 2.2.1 Media used :

- (a) 2.5% malt agar
- (b) Cellulose agar (Nilsson 1973)

### 2.2.2 Fungi used :

- (a) Chaetomium globosum S70B (F.P.R.L.)
- (b) Phialophora fastigiata S6A (F.P.R.L.)
- (c) Phialophora fastigiata 731-1-3b (Nilsson)

### 2.2.3 Wood specimens

All the experiments involved in this thesis were carried out by using sapwood of birch (Betula sp.) representing a hardwood, and Scots pine (Pinus sylvestris) representing a softwood. Birch is one of the timbers attacked aggressively by soft rot fungi. The soft rot cavities formed in this wood by Phialophora fastigiata are relatively larger than the ones formed by Chaetomium globosum, which is a great help in the <sup>Preparation for the</sup> investigation under electron microscope. Scots

pine was chosen to follow up the work carried out by Findlay (1970), and to investigate the reasons for his failure in obtaining soft rot cavities in this wood when it was attacked by Phialophora fastigiata.

The wood specimens were cut into sticks of 15 cm long and 3 x 3 mm cross section, these were orientated with the grain parallel to the long axis and two faces in the radial plain and two in the tangential plain. The sticks were cut into orientated cubes of 3 x 3 x 3 mm. Blocks of this size were used in order that the delignification fluids could penetrate readily throughout the block and produce a comparatively uniform delignification. All the wood samples were examined microscopically before use in order that any disorganisation of the microfibrillar structure could be observed.

#### 2.2.3.1 Normal wood

As mentioned before, orientated 3 mm cubes of birch sapwood and Scots pine sapwood were used in this work. For the convenience of not repeating the name of the wood the term "wood block" is generally used henceforth. As the wood blocks were small in size two blocks of each sample were used in each culture for each period of incubation tested; and three replicas were prepared for each test to obtain relatively accurate results of the average of these.

#### 2.2.3.2 Delignified wood

The delignification was carried out by digesting the 3 mm cubes in a solution of 33 g sodium chlorite, 41 g sodium acetate, 30 g acetic acid and 1000 ml of distilled water (Browning 1967). The mixture was prepared by dissolving sodium chlorite in 300 ml of water and then mixing it with the rest of the constituents.

The delignification of Scots pine 3 mm<sup>3</sup> wood blocks was carried out as follows :

1. Each two blocks were dried at 105°C for 24 hours.
2. The blocks were then weighed to four places of decimals.
3. The blocks were placed into a small conical flask containing the delignification solution, covered and heated to 75°C  $\pm$  5 on a stirrer/hot plate for one hour. The solution was then changed to a fresh delignification solution and this was repeated twice at hourly intervals.
4. At the end of three hours of treatment, the blocks were removed from the delignification solution, washed in running water for 30 minutes to get rid of the extracted lignin and dried at 105°C for 24 hours.
5. Blocks were weighed and the percentage difference in weight between the initial and the delignified blocks calculated. This indicates the lignin loss as a result of the treatment.

#### 2.2.4 The procedure for measuring the decay capacity

The procedure to measure the decay capacity of the different strains of fungi in normal and delignified wood was as follows :

1. The wood blocks were dried at  $105^{\circ}\text{C}$  for 24 hours.
2. Weighed to four places of decimals in gram (in the final calculation converted to mg).
3. Sterilized at 15 p.s.i. for 15 minutes in a nutrient solution of  $\text{NH}_4\text{NO}_3$  3.0 g,  $\text{KH}_2\text{PO}_4$  1.5 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g, and 1000 ml  $\text{H}_2\text{O}$ . This solution was recommended by Nilsson (1973) to activate the fungal attack in wood.
4. The sterilized wood blocks were placed on the growing mycelium of each of the three strains of the fungi grown on both malt agar and cellulose agar slopes individually in large test tubes. To prevent drying out, the cotton wool plugs of test tubes were covered with aluminium foil then the test tubes were kept in perforated polythene bags and incubated at  $25^{\circ}\text{C} \pm 2$  for 3 days, 1, 3, 6, 12 and 20 weeks.
5. The wood blocks removed from cultures after each incubation period was over, adhering mycelium carefully scraped off, dried at  $105^{\circ}\text{C}$  for 24 hours and weighed.
6. The percentage difference in weight between the unexposed and exposed blocks was a measure of the decay capacity of the fungus or the weight loss of the wood blocks after exposure to the fungal strain for a period of time.

Two extra tests for 1 week and 20 weeks were prepared by placing the weighed, sterilized wood blocks on media with-

out fungal mycelia to measure the uptake of mineral salts from the media by wood blocks and the subsequent increase in weight.

Another set of normal and delignified wood blocks were prepared for the microscopical examination. These blocks were freeze dried after each fungal incubation period was over. Freeze drying was used in an attempt to prevent the hyphae in the wood cell wall from collapsing, (George 1974).

#### 2.2.5 Results

The results are tabulated in four Tables showing a great difference in the decay capacity of the two strains of Phialophora fastigiata in normal Scots pine and in delignified Scots pine.

##### Key to abbreviations

|                               |                                                           |
|-------------------------------|-----------------------------------------------------------|
| I.D.W.                        | initial dry weight                                        |
| F.D.W.                        | final dry weight after fungal attack                      |
| % W.L.                        | % weight loss due to fungal attack                        |
| F.D.W.A.D.                    | final dry weight after delignification = I.D.W.D.W.       |
| I.D.W.D.W.                    | initial dry weight of delignified wood = F.D.W.A.D.       |
| % L.L.                        | % weight loss due to delignification = loss of lignin.    |
| F.D.W.D.W. & F.A.             | final dry weight of delignified wood after fungal attack. |
| C <sub>1</sub>                | control of 1 week                                         |
| C <sub>20</sub>               | control of 20 weeks                                       |
| }                             |                                                           |
| not exposed to fungal attack. |                                                           |
| +                             | weight gain.                                              |
| -                             | no change in the weight.                                  |
| C                             | contaminated.                                             |
| *                             | birch blocks.                                             |
| L                             | experiment lost.                                          |

TABLE 1

Weight losses in birch attacked by Phialophora fastigiata 731-1-3b and S6A grown in different media

(Average of three replicas)

| 731-1-3b     |                 |              |              |              | S6A          |              |              |
|--------------|-----------------|--------------|--------------|--------------|--------------|--------------|--------------|
| <u>MEDIA</u> | <u>TIME</u>     | <u>I.D.W</u> | <u>F.D.W</u> | <u>% W.L</u> | <u>I.D.W</u> | <u>F.D.W</u> | <u>% W.L</u> |
| M            | 3 days          | 27.50        | 27.20        | 1.09         | 25.00        | 24.60        | 1.60         |
| A            | 1 week          | 38.30        | 37.50        | 2.09         | 33.10        | 32.60        | 1.51         |
| L            | 3 weeks         | 34.20        | 32.30        | 5.55         | 33.20        | 28.60        | 13.86        |
| T            | 6 weeks         | 31.70        | 26.60        | 16.09        | 37.20        | 31.20        | 16.13        |
| A            | 12 weeks        | 36.20        | 30.00        | 17.13        | 32.50        | 23.20        | 28.62        |
| G            | 20 weeks        | 26.30        | 20.00        | 23.95        | 31.30        | 22.20        | 29.07        |
| A            | C <sub>1</sub>  | 37.30        | 38.30        | 2.61+        | 37.40        | 37.70        | 0.80+        |
| R            | C <sub>20</sub> | 33.80        | 34.50        | 2.03+        | 36.40        | C            |              |
| C            | 3 days          | 32.10        | 31.60        | 1.56         | 35.70        | 35.80        | 0.28+        |
| E            | 1 week          | 40.40        | 38.90        | 3.71         | 33.40        | L            |              |
| L            | 3 weeks         | 29.60        | 25.40        | 14.19        | 34.80        | 30.40        | 12.64        |
| U            | 6 weeks         | 33.60        | 28.40        | 15.48        | 29.10        | 24.20        | 16.84        |
| L            | 12 weeks        | 34.30        | 25.10        | 26.82        | 33.00        | 27.50        | 16.67        |
| O            | 20 weeks        | 23.50        | 14.90        | 36.60        | 35.80        | 25.00        | 30.17        |
| S            |                 |              |              |              |              |              |              |
| E            |                 |              |              |              |              |              |              |
| (Nilsson     | C <sub>1</sub>  | 30.30        | -            |              | 33.90        | 34.10        | 0.59+        |
| (1973)       | C <sub>20</sub> | 35.20        | 35.40        | 0.57+        | 36.70        | 37.20        | 1.34+        |

TABLE 2

Weight losses in Birch and Scots pine attacked by Chaetomium globosum S70B grown in different media

(Average of three replicas)

| BIRCH        |                 |              |              |              | SCOTS PINE   |              |              |
|--------------|-----------------|--------------|--------------|--------------|--------------|--------------|--------------|
| <u>MEDIA</u> | <u>TIME</u>     | <u>I.D.W</u> | <u>F.D.W</u> | <u>% W.L</u> | <u>I.D.W</u> | <u>F.D.W</u> | <u>% W.L</u> |
| M            | 3 days          | 31.30        | 32.00        | 2.19+        | 31.30        | -            |              |
| A            | 1 week          | 30.80        | 31.30        | 1.59+        | 29.90        | 29.40        | 1.67         |
| L            | 3 weeks         | 33.40        | 27.70        | 17.06        | 26.50        | 25.50        | 3.77         |
| T            | 6 weeks         | 37.90        | 23.90        | 36.93        | 31.60        | 30.00        | 5.06         |
| A            | 12 weeks        | 33.80        | 11.00        | 67.46        | 21.00        | L            |              |
| G            | 20 weeks        | 32.20        | 9.70         | 69.88        | 27.80        | 24.80        | 10.79        |
| A            | C <sub>1</sub>  | 27.80        | 28.00        | 0.71+        | 31.00        | C            |              |
| R            | C <sub>20</sub> | 36.80        | 37.90        | 2.98+        | 31.90        | 32.60        | 2.19+        |
| C            | 3 days          | 26.00        | 25.00        | 3.85         | 28.90        | 28.20        | 2.42         |
| E            | 1 week          | 33.20        | 28.00        | 15.66        | 28.00        | 27.00        | 3.57         |
| L            | 3 weeks         | 30.00        | 17.60        | 41.33        | 31.40        | 29.40        | 6.37         |
| U            | 6 weeks         | 25.00        | 10.00        | 60.00        | 30.20        | 25.40        | 15.89        |
| L            | 12 weeks        | 27.10        | 7.90         | 70.85        | 27.00        | 20.80        | 22.96        |
| O            | 20 weeks        | 23.40        | 6.20         | 73.50        | 29.80        | 21.00        | 29.53        |
| S            |                 |              |              |              |              |              |              |
| E            |                 |              |              |              |              |              |              |
| ( Nilsson    | C <sub>1</sub>  | 29.80        | -            |              | 32.20        | 32.30        | 0.30+        |
| 1973)        | C <sub>2</sub>  | 34.70        | 35.20        | 1.42+        | 33.40        | 33.50        | 0.29+        |

TABLE 3

Weight losses in normal and delignified Scots pine attacked by *Phialophora fastigiata* S6A

| MEDIA    | TIME            | NORMAL WOOD |       |       | DELIGNIFIED WOOD |              |       |               |                     |       |
|----------|-----------------|-------------|-------|-------|------------------|--------------|-------|---------------|---------------------|-------|
|          |                 | I.D.W       | F.D.W | % W.L | I.D.W            | F.D.W<br>A.D | % L.L | I.D.W.<br>D.W | F.D.W.<br>D.W & F.A | % W.L |
| M        | 3 days          | 23.50       | 23.50 | -     | 34.50            | 26.80        | 22.32 | 26.80         | 22.80               | 14.93 |
| A        | 1 week          | 32.70       | 33.50 | 2.39+ | 26.00            | 20.50        | 21.15 | 20.50         | 15.90               | 22.44 |
| L        | 3 weeks         | 33.70       | 33.80 | 0.29+ | 30.30            | 24.50        | 19.14 | 24.50         | 15.20               | 37.95 |
| T        | 6 weeks         | 33.50       | -     | -     | 32.00*           | 25.60        | 20.00 | 25.60         | 13.50               | 47.27 |
| A        | 12 weeks        | 26.00       | 25.60 | 1.53  | 29.00            | 23.80        | 17.93 | 23.80         | 13.00               | 45.38 |
| G        | 20 weeks        | 33.10       | 32.20 | 2.72  | 35.70            | 28.80        | 19.33 | 28.80         | 11.80               | 59.03 |
| A        | C <sub>1</sub>  | 29.60       | 30.80 | 3.89+ | 31.00            | 24.90        | 19.68 | 24.90         | 25.30               | 1.58+ |
| R        | C <sub>20</sub> | 32.80       | 33.60 | 2.38+ | 27.70            | 22.00        | 20.58 | 22.00         | 22.90               | 3.93+ |
| C        | 3 days          | 30.30       | -     | -     | 24.60            | 19.80        | 19.51 | 19.80         | 19.70               | 0.51  |
| E        | 1 week          | 31.00       | 31.10 | 0.32+ | 36.50            | 29.60        | 18.90 | 29.60         | 23.40               | 20.95 |
| L        | 3 weeks         | 28.60       | 28.70 | 0.38+ | 37.80            | 29.00        | 23.28 | 29.00         | 13.00               | 55.17 |
| L        | 6 weeks         | 29.10       | 29.30 | 0.68+ | 29.60            | 23.20        | 21.62 | 23.20         | 7.70                | 66.81 |
| U        | 12 weeks        | 31.90       | 31.40 | 1.57  | 28.70            | 22.90        | 20.21 | 22.90         | 7.00                | 69.43 |
| L        | 20 weeks        | 32.50       | 31.80 | 2.15  | 32.70            | 26.60        | 18.65 | 26.60         | 6.80                | 74.44 |
| O        | C <sub>1</sub>  | 26.70       | -     | -     | 30.80            | 24.60        | 20.12 | 24.60         | C                   | C     |
| S        | C <sub>20</sub> | 28.80       | 29.00 | 0.69+ | 34.00            | 27.80        | 18.24 | 27.80         | 28.20               | 1.42+ |
| E        |                 |             |       |       |                  |              |       |               |                     |       |
| (Nilsson |                 |             |       |       |                  |              |       |               |                     |       |
| 1973)    |                 |             |       |       |                  |              |       |               |                     |       |

TABLE 4

Weight losses in normal and delignified Scots pine attacked by Phialophora fastigiata 731-1-3b

| MEDIA                                                           | TIME            | NORMAL WOOD |       |       | DELIGNIFIED WOOD |              |       |              |                    |       |
|-----------------------------------------------------------------|-----------------|-------------|-------|-------|------------------|--------------|-------|--------------|--------------------|-------|
|                                                                 |                 | I.D.W       | F.D.W | % W.L | I.D.W            | F.D.W<br>A.D | % L.L | I.D.W<br>D.W | F.D.W<br>D.W & F.A | % W.L |
| M                                                               | 3 days          | 32.00       | 32.20 | 0.62+ | 27.80            | 22.10        | 20.50 | 22.10        | 19.00              | 14.03 |
| A                                                               | 1 week          | 30.00       | -     |       | 37.30            | 29.80        | 20.11 | 29.80        | 25.80              | 13.42 |
| L                                                               | 3 weeks         | 33.30       | 33.40 | 0.29+ | 38.20            | 29.80        | 21.98 | 29.80        | 21.20              | 28.86 |
| T                                                               | 6 weeks         | 25.10       | 24.90 | 0.79  | 28.20            | 22.20        | 21.28 | 22.20        | 12.00              | 45.95 |
| A                                                               | 12 weeks        | 31.18       | 33.30 | 4.71+ | 30.10            | 23.50        | 21.92 | 23.50        | 12.00              | 48.93 |
| G                                                               | 20 weeks        | 29.40       | -     |       | 27.00            | 21.00        | 22.22 | 21.00        | 9.40               | 55.24 |
| A                                                               | C <sub>1</sub>  | 30.70       | 31.00 | 0.97+ | 28.50            | 22.60        | 20.70 | C            | C                  | C     |
| R                                                               | C <sub>20</sub> | 35.20       | 37.20 | 5.68+ | 34.00            | 26.70        | 21.47 | 26.70        | 27.00              | 1.11+ |
| C<br>E<br>L<br>L<br>U<br>L<br>O<br>S<br>E<br>(Nilsson<br>(1973) | 3 days          | 33.90       | -     |       | 23.40            | 18.70        | 20.08 | 18.70        | 15.40              | 17.65 |
|                                                                 | 1 week          | 29.20       | -     |       | 28.90            | 22.50        | 22.14 | 22.50        | 15.40              | 31.56 |
|                                                                 | 3 weeks         | 25.70       | 25.40 | 1.17  | 34.50            | 28.00        | 18.84 | 28.00        | 13.10              | 53.21 |
|                                                                 | 6 weeks         | 27.50       | 26.90 | 2.18  | 24.20            | 19.40        | 19.83 | 19.40        | 6.10               | 68.56 |
|                                                                 | 12 weeks        | 31.70       | 30.90 | 2.52  | 32.70            | 25.30        | 22.63 | 25.30        | 6.40               | 74.70 |
|                                                                 | 20 weeks        | 30.50       | 29.50 | 3.27  | 33.90            | 26.50        | 21.82 | 26.50        | 5.50               | 79.25 |
|                                                                 | C <sub>1</sub>  | 31.80       | -     |       | 28.70            | 23.30        | 18.81 | 23.30        | -                  |       |
|                                                                 | C <sub>20</sub> | 25.90       | 26.20 | 1.14+ | 30.40            | 24.60        | 19.07 | 24.60        | -                  |       |

### 3. DISCUSSION

The results of the first experiment using six different media to test the rate of growth of Phialophora fastigiata S6A were unexpected. This strain needed almost two weeks to grow on malt agar medium and cellulose agar medium (Nilsson 1973); the growth was slow and never covered the whole petri dish. This strain showed very good growth in the mixture of birch sawdust plus malt agar. This might suggest that the fungus prefers the natural source of carbon which is available in the birch sawdust to that available in malt. This strain of Phialophora fastigiata did not show any indication of growth even after three weeks' of incubation in Abrams, Abrams plus cellulose and modified Eggins and Pugh media. A possible explanation for this phenomenon could be that, the fungus requires an additional carbon source in the media, which is present in the medium of Nilsson (1973) as glucose and yeast. George (1974) found a sparse growth of Chaetomium globosum on agar with mineral salts medium, where an additional carbon source was absent.

Phialophora fastigiata 731-1-3b had fairly good growth in malt agar medium but very little in Nilsson (1973) medium. To explain the variation in the behaviour of the growth of the two strains of Ph. fastigiata in different media, the effect of each strain on the wood was examined by electron microscopy. The microscopical results of the decayed wood by the two strains showed a great variation in the decay pattern which will be discussed in Part III.

The results of the weight loss experiments indicated that Ph. fastigiata S6A in malt agar medium is more aggressive in attacking normal birch than the strain 731-1-3b. The maximum weight loss with

S6A in birch after 20 weeks attack was 29%; while the strain 731-1-3b achieved approximately 24% weight loss in a similar time. The decay capacity of the strain S6A in normal birch and Scots pine did not show any difference when it was cultured on Nilsson (1973) medium from malt agar medium; while the strain 731-1-3b showed higher capacity in birch when cultured on Nilsson's medium. Apparently the strain 731-1-3b is more aggressive in birch on Nilsson's medium than on malt agar medium. Comparing these results with the results of other workers, raises a question : Does the strain 731-1-3b need additional carbon or nitrogen sources by comparison with the strain S6A ?

Savory (1954b) found a direct relationship between the nitrogen concentration and the weight loss caused by Chaetomium globosum at concentrations up to 3 times standard Abrams salts. Findlay (1970) obtained low weight losses in higher concentrations of nitrogen which he interpreted as causing an inhibition of the growth and decay. Findlay also found the addition of asparagine enhanced the decay of beech by Ph. fastigiata, this was interpreted as meaning that the fungus could assimilate the organic nitrogen more than <sup>it could from</sup> inorganic nitrogen. According to the results of the present work with the same strain of Ph. fastigiata as used by Findlay but in birch, the presence of asparagine did not show any significant differences. A possible explanation for these differences could be the effect of using different wood species..

As the addition of nitrogen and its concentration has always been in dispute among investigators the investigations on the importance of carbon source and its concentration show disagreement

amongst them. Several workers suggest the addition of inducers (the end products of enzyme degradation) in small percentage, while others believe that the inducers inhibit enzyme production, Bravery (1968) and Nilsson (1973 and 1974c). If Nilsson was satisfied of the inhibiting effect caused by the additional source of carbon for the enzyme production he would have omitted it from his substrate.

Butcher (1975a) postulated from the results of Banerjee and Levy (1970) that the carbon source in malt extract is responsible for obtaining lower weight loss by the fungus, so he did not find it necessary to add glucose to the media either for enzyme induction or to intensify the decay. Here we may ask by analysing the results of the present work, what caused the enhancement of decay by

Ph. fastigiata 731-1-3b in birch when it was cultured on Nilsson's medium ? Why did this not occur with the strain S6A ? This increase in the decay capacity of the strain 731-1-3b in normal Scots pine was relatively higher than in S6A. Was this enhancement due to decreased carbon source, although glucose is also present ? Is it because the medium contains a higher nitrogen source than the malt agar medium ? The answer could well be the lower carbon content or the higher nitrogen concentration or a combination of the two. There is another factor, since the carbon source varies in its effect according to the fungal species, Nilsson (1974c), which helps to explain the variation of the results in the two different strains of Ph. fastigiata which were used in the present work. The effect of carbon or nitrogen on the fungal decay capacity might also depend on the ratio of the other constituents of media and on the wood species because of their variation in the constituents of the cell wall layers. One

of these constituents is the lignin which until 1970 was believed to be solely responsible for the susceptibility of hardwoods to soft rot attack, the resistance of softwoods, and cavity formation.

The results of delignifying Scots pine to 20%  $\pm$  2% weight loss and exposing it to the two strains of Ph. fastigiata was very striking even after three days' incubation. The results of the weight loss for both strains were almost similar when they were cultured on malt agar medium, while both proved to be more destructive on Nilsson's medium, which caused a weight loss of almost 75%, this again proves that this medium increases the decay capacity of the fungus. These results raise several questions. Why is the fungus behaving so destructively? Is it because of the removal of lignin? Are the other wood cell wall constituents affected? Is the removal of lignin caused by free enzyme diffusion?

To be able to give an adequate answer it is necessary to analyse the investigations of the previous workers.

Savory and Farmer (1959) noticed the remarkable increase in the weight loss of Scots pine after chlorination. They attributed this to lignin loss by the treatment. Bailey et al. (1968) pointed out that the delignification is similar to the "pre-cellulolytic" stage of brown rot fungi. Findlay (1970) found an increase in the weight loss in delignified Scots pine attacked by Phialophora fastigiata, but there was no indication of cavity formation. He suggested, delignifying wood samples to different levels which might help the investigator to know the right level of lignin for cavity formation. His suggestion was postulated from his belief that the presence of lignin is responsible for the cavity formation. The

present work showed that this is unlikely because the cavities were observed in normal Scots pine after longer exposure to the fungus p. 101. The delignification in this work was higher than Findlay's experiment and still did not show cavity formation although its early stages (i.e. T-branch) was seen. Findlay (1970) agreed with Levi and Preston (1965) that the demethoxylation of lignin is the initial stage of cell wall degradation by soft rot fungi. This could well be explained by the hypothesis of Bailey et al. (1968) and the delignification in the present work could therefore be acting as an enzyme acts in brown rot. The hypothesis of Bailey et al. might be applicable to the delignification process. By delignification of the wood specimen, the lignin removal has been achieved gradually. In other words the breaking of the linkage between the lignin and the cellulose is achieved by the delignification treatment which possibly happened by modifying the lignin into another phase. At the end of delignification treatment the cell wall might contain little unchanged links with cellulose, some modified lignin and some extracted lignin. When the wood specimen is in this stage it could be ideal for the fungal attack which enhances the decay.

Nilsson (1974c) observed a sparse cavity formation in the fibres of sulphate pulp which obtained 10.8% lignin, he concluded that the delignification led to an increase of the erosion type attack but the type 1 (cavity formation) was decreased. Nilsson explained the results of Courtois (1963b), Bailey et al. (1968) and Findlay (1970) about the increase in weight loss of delignified wood as being due to the fungi which had been used in the tests which were able to produce Corbett's type 2 attack. This conclusion of

Nilsson is unlikely to be applied with the Ph. fastigiata strain which was used by Findlay (1970) and in the present work. This fungus causes decay type 1 and type 2 (Corbett 1965) in normal birch, type 1 and a porous decay in normal Scots pine (this was observed in the present work). The microscopical results of delignified Scots pine did not suggest any type 2 attack, and as a matter of fact the fungal hypha attacked the cell wall layers by occupying and penetrating through them but not by enzyme diffusion from the lumen (cf. Part III). Nilsson (1974c) drew the attention of the investigators to the role of hemicellulose as a structural component giving the fibre walls a certain structure which is convenient for cavity formation. He suggested that delignification increases the porosity of the cell wall which helps the cell wall degrading enzymes to diffuse more freely longitudinally along the cellulose microfibrils. Nilsson (1974b) suspected the enzymes xylanase and mannanase to be very likely involved in the cell wall degradation additional to their function as hemicellulose degraders. George (1974) agreed with Nilsson that hemicellulose composition had a significant role in the soft rot decay. Sinner et al. (1975) found that the pretreatment of the holocellulose with xylanase enhanced the cellulose degradation. The importance of hemicellulose in the cell wall degradation is obvious from their results. The delignification might be acting as the xylanase acts on the holocellulose. They also emphasised that the cellulose cannot be degraded unless the xylan is partly removed. Again here the delignification could be interpreted as being responsible for removing or destroying some of hemicellulose which then exposes the cellulose and increases the opportunity for cellulose

degradation.

The results of Sinner et al. (1975) on the holocellulose of spruce wood is surprising. As they recorded that mannan in delignified spruce was hardly degraded by mannanase. If this is likely to happen, one would assume that the delignification had removed a certain amount of mannan which was inhibiting the cellulase diffusion. When this inhibitor is removed, the cellulase has more freedom to flow through the cell wall causing the degradation of available cellulose.

From the results of the previous workers, it is more likely that hemicellulose has an important role in the soft rot decay. The soft rot fungus in normal wood with high lignin content needs longer time until the hemicellulose is attacked by hemicellulases which opens up the way for the cellulase to attack the cellulose. In the delignification process, some of the hemicellulose could have been also extracted Thompson et al. (1964), the lignin has been demethoxylated or modified by breaking the linkage between the lignin and cellulose. In this stage the cellulose microfibrils are available for the fungal enzyme which attacks it destructively causing the high weight loss.

Findlay (1970) tried to compare the results of delignified softwood attacked by soft rot fungus with wood specimens attacked by white rot fungus (lignin destroyer) before exposure to a soft rot fungus. He did not obtain significant weight loss, his explanation was that the white rot fungus had utilized the freeing cellulose after the lignin utilization (Levi 1964) which resulted in no cellulose being available to be utilized by soft rot fungus. This experiment would be more interesting and worthwhile to

investigate microscopically, to find out if the cavities are present. Are the hyphae inside the cavity surrounded by the black granules which is assumed to be lignin residues? If this material is absent then the hypothesis of it being lignin residue is more likely.

Only two blocks of birch wood were delignified<sup>and</sup> the microscopical work indicated the presence of cavities. This might lead us to doubt the role of lignin and its responsibility in cavity formation. It would be very interesting to carry out an investigation of "delignification" to find out the presence of hemicellulose or other cell wall constituents additional to lignin. This might give an answer to a question of the role of each cell wall constituent in the cavity formation.

The results in the present work indicate a very high weight loss in the delignified Scots pine specimen, by comparing these results with the results of the previous workers suggests that the differences might be due to several reasons: (a) The size of the blocks which are much smaller than those used by Findlay (1970) and the one suggested by Nilsson (1973) 10 x 20 x 20 mm. (b) The blocks being small gave better chance for the delignification solution to penetrate through the cell wall which caused higher lignin loss (approximately  $20\% \pm 2$ ). (c) The delignification was not destructive to the cell wall layers or did not cause any visible disorganisation in the orientation of the cellulose microfibrils (see Part III). (d) By extracting more lignin a higher percent of hemicellulose might have been removed also which gave the wood cell wall porosity. This helped to raise the decay and increase the decay capacity. (e) Cellulose agar medium Nilsson (1973) has proved to increase the weight loss remarkably.

PART III

SCANNING ELECTRON MICROSCOPY

## 1. INTRODUCTION

The advent of scanning reflection electron microscope stimulated the botanist to use this instrument as a new tool to investigate the sub-structure of plant material. In 1965 the Cambridge "Stereoscan" electron microscope became commercially available and is now widely used in most research fields.

The scanning reflection electron microscope SEM is characterized in comparison with the light microscope by a great depth of focus, higher resolution, higher magnification, and a three dimensional <sup>perspective</sup> ~~image~~. Furthermore, the maximum specimen size around  $1\text{ cm}^3$ , can be scanned at low magnification and could be orientated during observation. In addition, specimen preparation is simple and rapid by comparison with preparation for transmission electron microscopy. Thus, investigators in the wood research field have used this instrument widely after Findlay (1970) had pioneered its application to fungi in wood.

The application of the SEM in wood decay field started with Findlay and Levy (1969), who demonstrated the usefulness of this tool not only to study the wood anatomy but also the decayed wood. Similar work carried out by Findlay (1970), Bravery (1971), Jutte and Levy (1971a & b), Jutte (1973), George (1974), Jutte and Zabel (1974), Levy (1974) and Sach et al. (1974), all concluded that the SEM is an excellent instrument for studying the sub-structure of wood degraded by fungi or bacteria.

Collett (1970) reviewed the historical background of applying the SEM in wood science. He also studied the effect of different techniques of preparation of the wood specimens on the

appearance of the bordered pit. Scurfield and Silva (1970), Meylan and Butterfield (1972) and Greaves (1973) used the SEM to investigate the internal structure of wood. Echlin (1974) gave an excellent detailed bibliography for the application of this tool in the plant sciences.

## 2. MATERIALS AND METHODS

### 2.1 Wood specimens

As mentioned in Part II, two specimens of wood, birch and Scots pine were used in the present work. Both woods were exposed individually to Phialophora fastigiata S6A, Ph. fastigiata 731-1-3b and Chaetomium globosum S70B, for different periods of time. The effect of different culture media on the behaviour of each strain of Ph. fastigiata in Scots pine was investigated. Furthermore, an investigation was carried out on the behaviour of Ph. fastigiata in delignified Scots pine.

The wood specimens were either fixed and stored in F.A.A. (Formalin acetic alcohol, 90:5:5), or freeze dried. Jutte and Zabel (1974) fixed the wood specimens in 3.5% buffered phosphate glutaraldehyde, this was not applied in the present work because the results of the fixation methods used were satisfactory. The procedures of the specimen preparation were as follows :

1. The 3 mm<sup>3</sup> blocks of wood specimens which had been stored in F.A.A. were removed from the fixative, washed under running water for two hours and cut under a stereo binocular microscope with a new safety razor blade (cleaned with acetone to remove the wax) to a size approximately 3 x 3 x 1 mm to the desired plane (Radial, tangential and transverse). The razor blade was used mostly when it was desired to study the transverse face, splitting the wood was quite satisfactory for studying the other planes, (Jutte and Levy 1971a). The freeze dried wood specimens were split and sectioned in dry condition which was extremely difficult especially with sound softwood, but easier with decayed hardwood.

2. The wet sections were placed on filter paper in a closed petri dish and kept for 48 hours in room temperature to dry.
3. The wood sections were mounted onto a clean metal stub with "Durofix". The sections being small in size, more than one sample was mounted onto the stub to show the three planes of the wood. This had the additional advantage of not having to change the stubs so frequently during the microscopical investigation. A thin layer of glue containing silver was applied on the edges of each section to provide a good conducting layer between the stub and the wood specimens, Greaves (1973).
4. The mounted wood specimens were fixed on a rotating device and coated with a thin ( $300 - 500 \text{ \AA}$ ) layer of gold/palladium in a vacuum coating unit.

Absolute cleanliness is required during specimen preparation to avoid production of artefact which would effect the interpretation of observations. The razor blade should be cleaned with acetone, the samples should be covered while drying or after coating and the surface of the coated specimens should not be touched.

## 2.2 Microscopy

A Cambridge scanning reflection electron microscope (2A) was used to carry out the investigation in the present work. The accelerating voltage of 20 kilovolts (KV) was used with aperture  $100 \mu$  or  $200 \mu$ , while the working distance was approximately 14 mm. The stub was tilted to an angle of  $45^\circ$  so that specimens were scanned at an angle of  $45^\circ$ . 35 mm Pan. F. Ilford film was used for photography.

### 3. RESULTS

#### 3.1 Orientation of microfibrils<sup>aggregates</sup> in sound wood (Figs. 1 to 7)

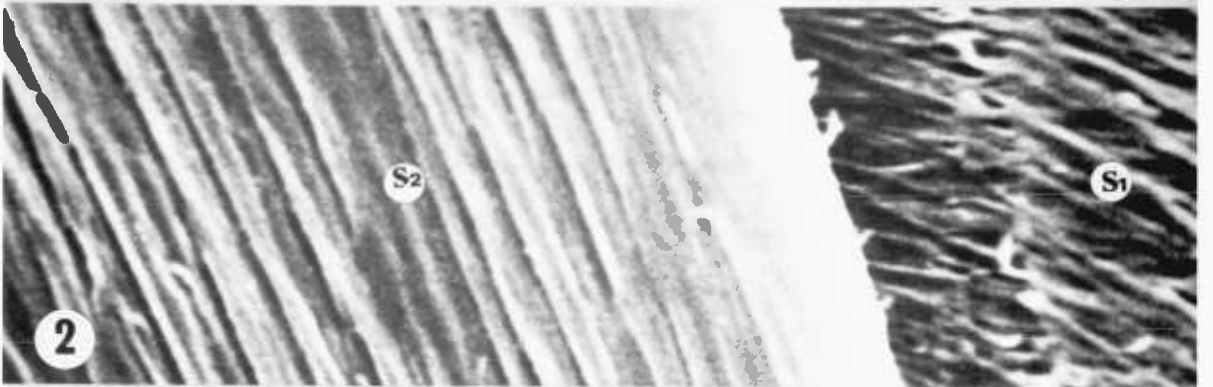
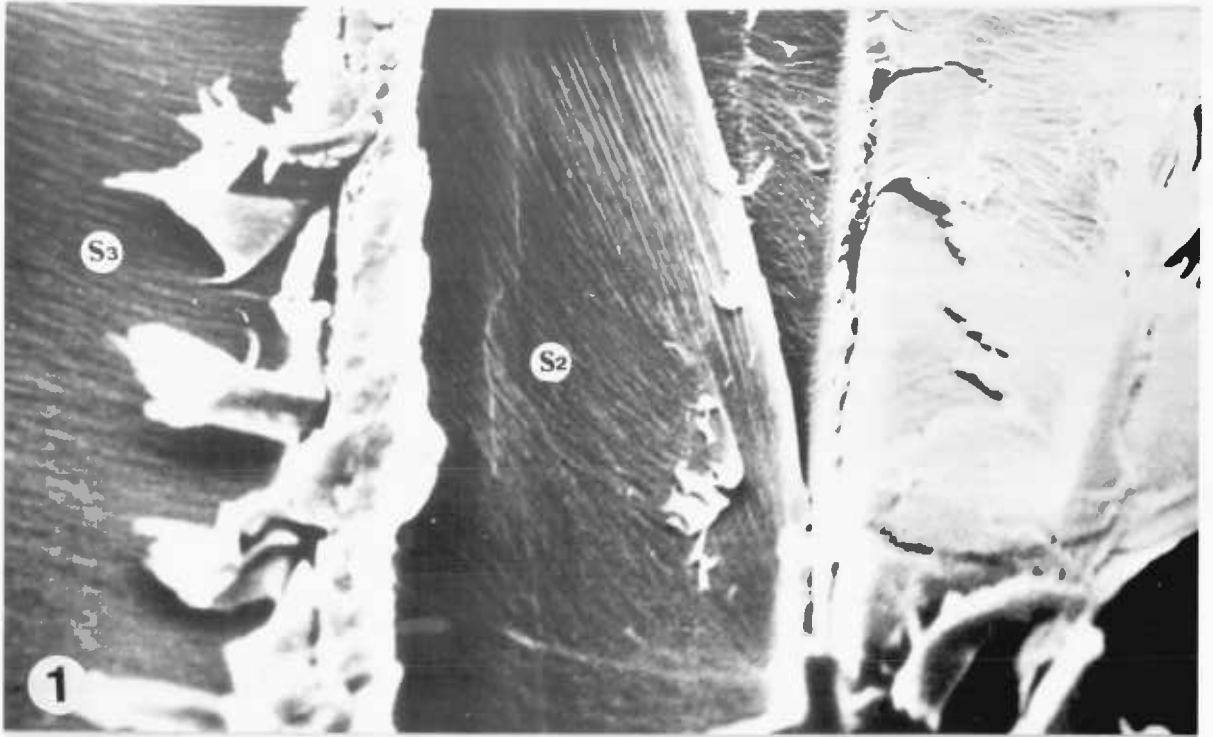
##### 3.1.1 In normal Scots pine (Pinus sylvestris) (Figs. 1 to 3)

The figures 1 to 3 are tangential longitudinal sections of tracheids showing the orientation of microfibrils in each layer of the secondary cell wall.

Fig. 1 (x2,080) shows the  $S_2$  layer split along the microfibrillar orientation revealing the  $S_1$  layer; while the orientation of microfibrils of  $S_3$  layer is apparent in the tracheid wall on the left of the micrograph.

Fig. 2 (x10,675) is a high magnification of a part of Fig. 1, showing the steep microfibrillar orientation of the  $S_2$  layer which has split revealing the  $S_1$  layer with its flat microfibrillar orientation, almost perpendicular to the axis.

Fig. 3 (x4,100) shows the flattish helices of the microfibrils of the  $S_3$  layer covered with the warty layer. The top of the micrograph shows a gradual change in the orientation of microfibrils from the  $S_3$  layer into the  $S_2$  layer, which probably represent the transitional microfibrils, Harada (1965).



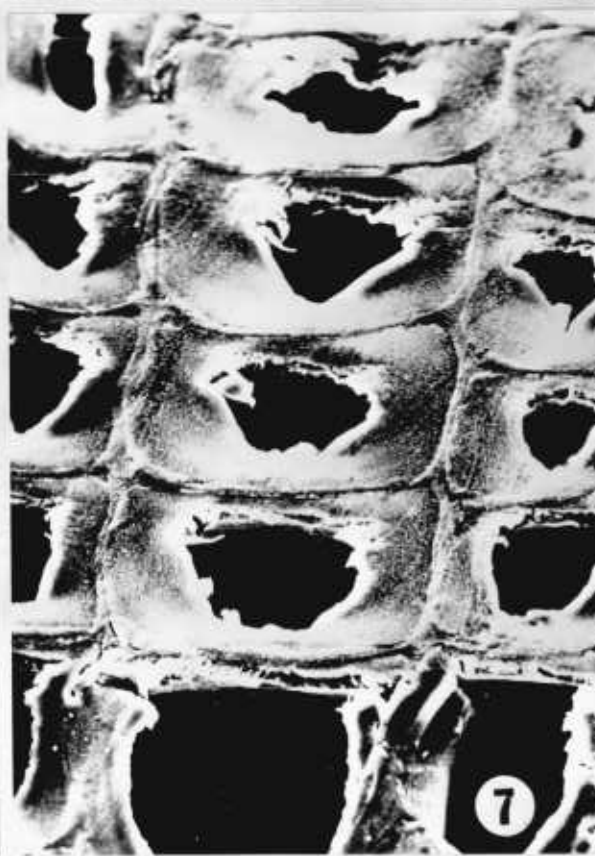
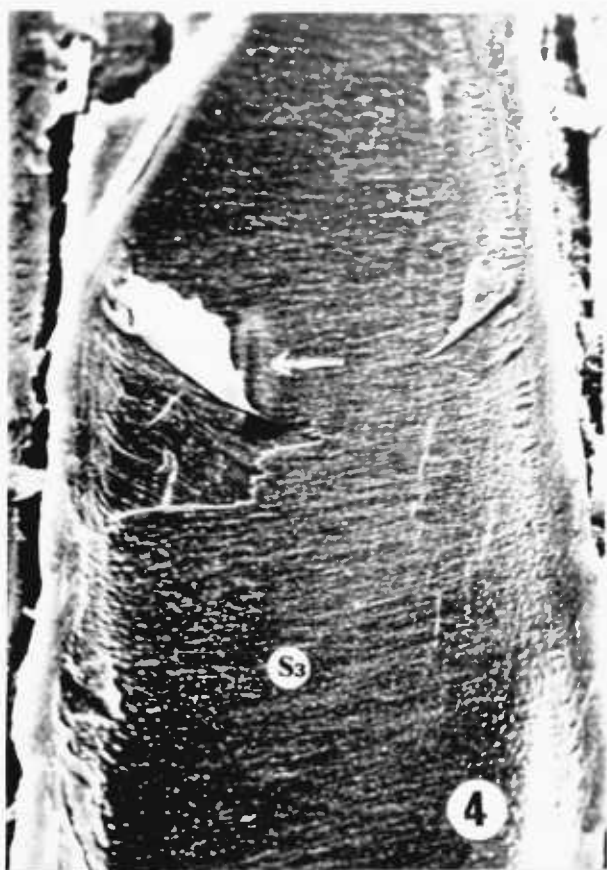
### 3.1.2 In delignified Scots pine (Figs. 4 to 7)

Fig. 4 (x3,045) shows the general appearance of delignified cell walls. The effect of delignification is more apparent in the middle lamella than in any other cell wall layer. This effect is represented as a sort of disorganisation which has produced a slight separation of the cells in this layer. While the orientation of microfibrils in each layer of the secondary cell wall (Figs. 5 (x 2,000) and 6 (x 2,707)) has not been affected by the delignification process and is similar to that in normal wood (Figs. 1 to 3).

Fig. 7 (x1,141) is a transverse section of tracheids showing a little distortion in the cell walls which occurred during the specimen preparation.

Comparing Figs. 4 to 7 with Figs. 1 to 3 (normal wood), very fine white dots on the surface of all cell wall layers are apparent. Undoubtedly this is not an artefact produced by specimen preparation for SEM, but is due to the delignification, as both specimens were prepared in the same procedure.

The cell wall of delignified wood was very sensitive to the electron bombardment during focussing which caused obvious damage (arrowed), in Fig. 4. This was not observed in normal wood in the magnification x 2500 approximately.



### 3.2. Normal wood attacked by fungi

3.2.1 Birch attacked by Phialophora fastigiata S6A cultured on 2.5% malt agar medium (Figs. 8 to 20).

Fig. 8 (x1,004) is a general view of the fungal hyphae growing on the surface of the wood block, showing hyphae of different diameters. The wood surface in some areas has a rough appearance which is due to heavy degradation of the cell wall. This appearance was not observed in the sound wood.

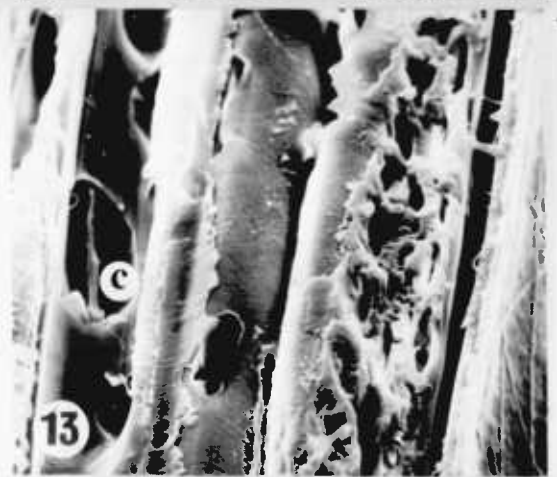
Fig. 9 (x 272) shows the heavy colonization of two vessels by fungal hyphae.

Fig. 10 (x6,021) shows the penetration of hyphae through the scalariform perforation plate of the vessel.

Fig. 11 (x1,210) is a general view of fibre cell wall degradation with several soft rot cavities. The arrows are pointing to active penetrations with constrictions of fungal hyphae through the cell wall, giving rise to T-branches and then to subsequent cavities.

Fig. 12 (x614) shows a series of pointed-end soft rot cavities in the S<sub>2</sub> layer of the fibre wall in which one of the cavities contains a hypha. On the right of the micrograph is a vessel colonized by fungal hyphae.

Fig. 13 (x825) is a high magnification of Fig. 12 revealing more clearly the shape of the cavities and the hypha present in the middle one.



In Fig. 14 (x3,080) a part of the  $S_3$  layer which was covering the cavities has split and ruptured the edges of the cavities, while the original smooth edge of diamond shaped cavities is apparent as a shadow under the covering  $S_3$  layer. The hypha within the cavities is constricted between the two cavities and is surrounded by granular material, while two hyphae one on the right and the other on the left of the cavities lying in the lumen have a smooth appearance. The micrograph also shows several cracks in the wall above the cavities which may well be due to the drying of the specimen.

Fig. 15 (x7,030) is a higher magnification of Fig. 14 showing the constriction of the hypha between two cavities to which a part of the wall is still attached. The granular surrounding of the hypha in the cavity is more distinct while the other two hyphae in the lumen have smooth walls.

Fig. 16 (x2,933) reveals a large diamond shaped cavity enclosing a fungal hypha, while on the left of the cavity is a hypha with three constrictions. The cavities which the constricted hypha belongs to have been destroyed during the preparation of the specimen except a very small part of the cell wall which is present in the constricted area between the first and the second swelling of the hypha from the top. The shape of this constricted hypha indicates the presence of a chain of three pointed-end, diamond-shaped cavities.

Fig. 17 (x6,695) is a high magnification of the constricted hypha of Fig. 16 showing the granular surrounding of the hypha. A small area on the left of the micrograph is swollen (arrowed) which is due to the electron beam damage during focusing.

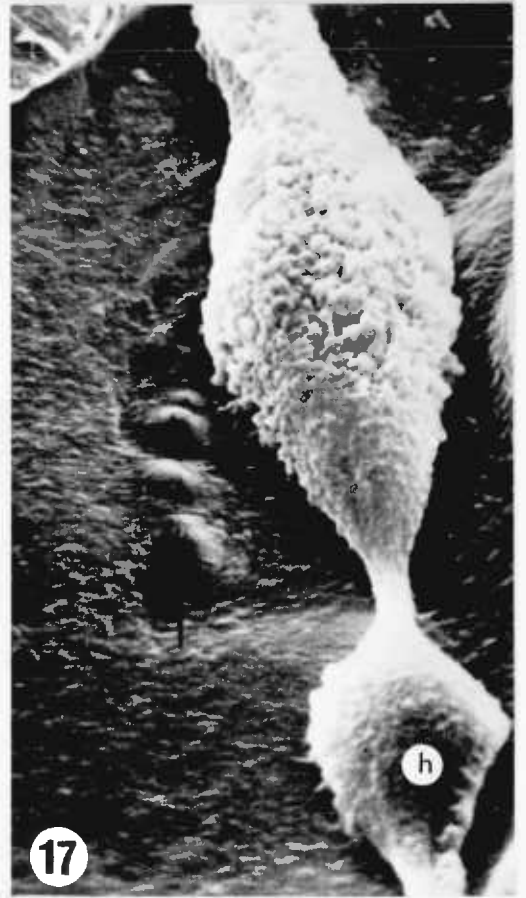
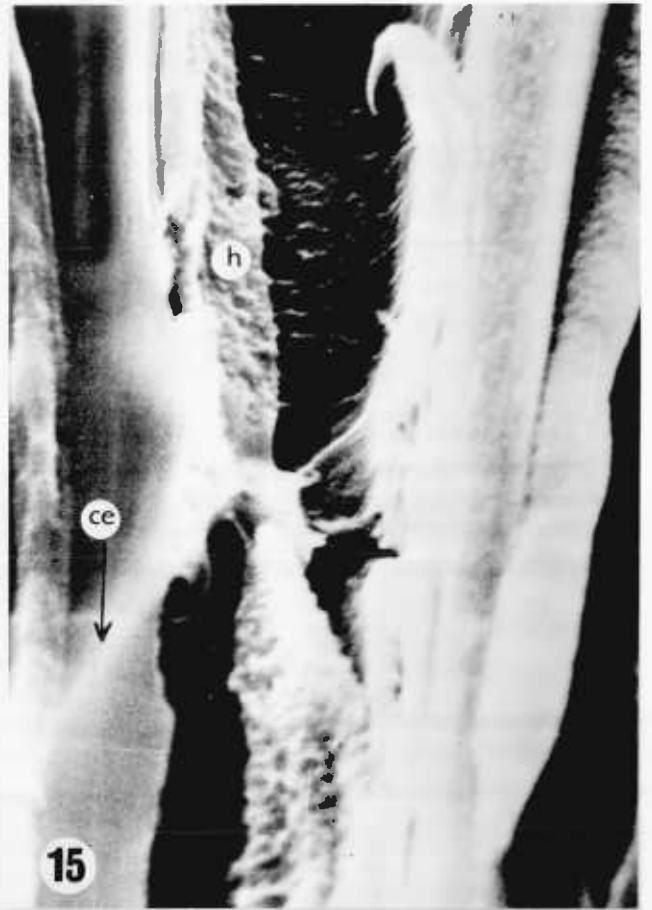
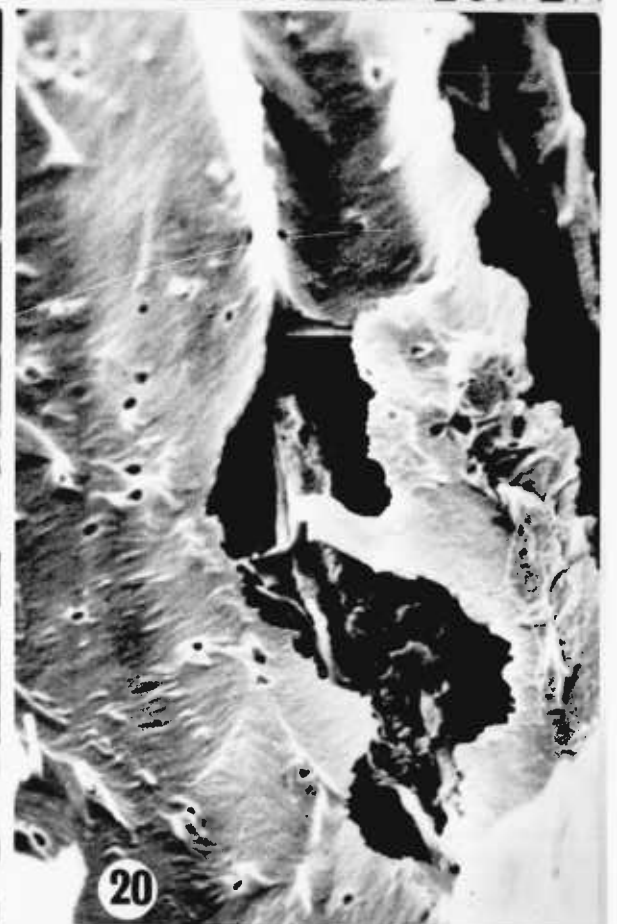
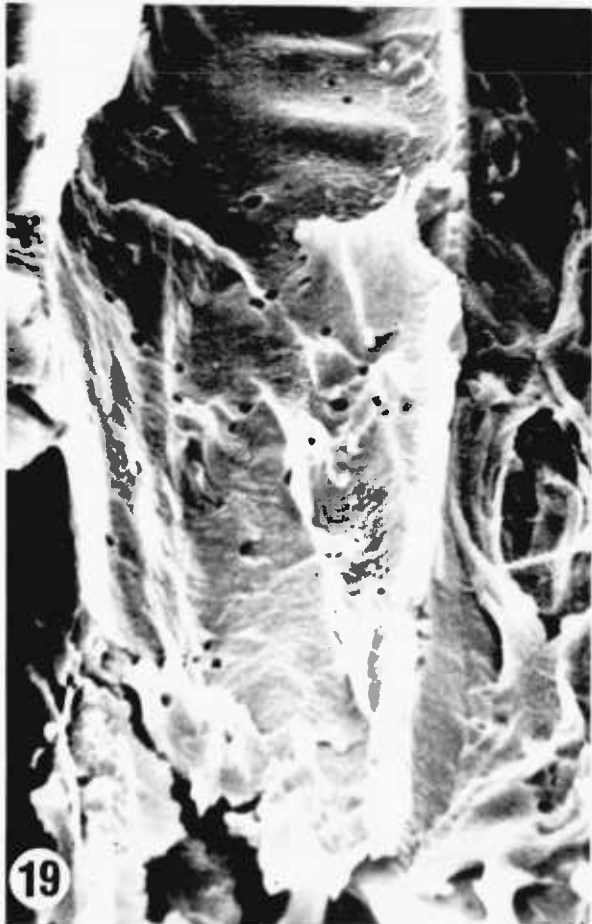
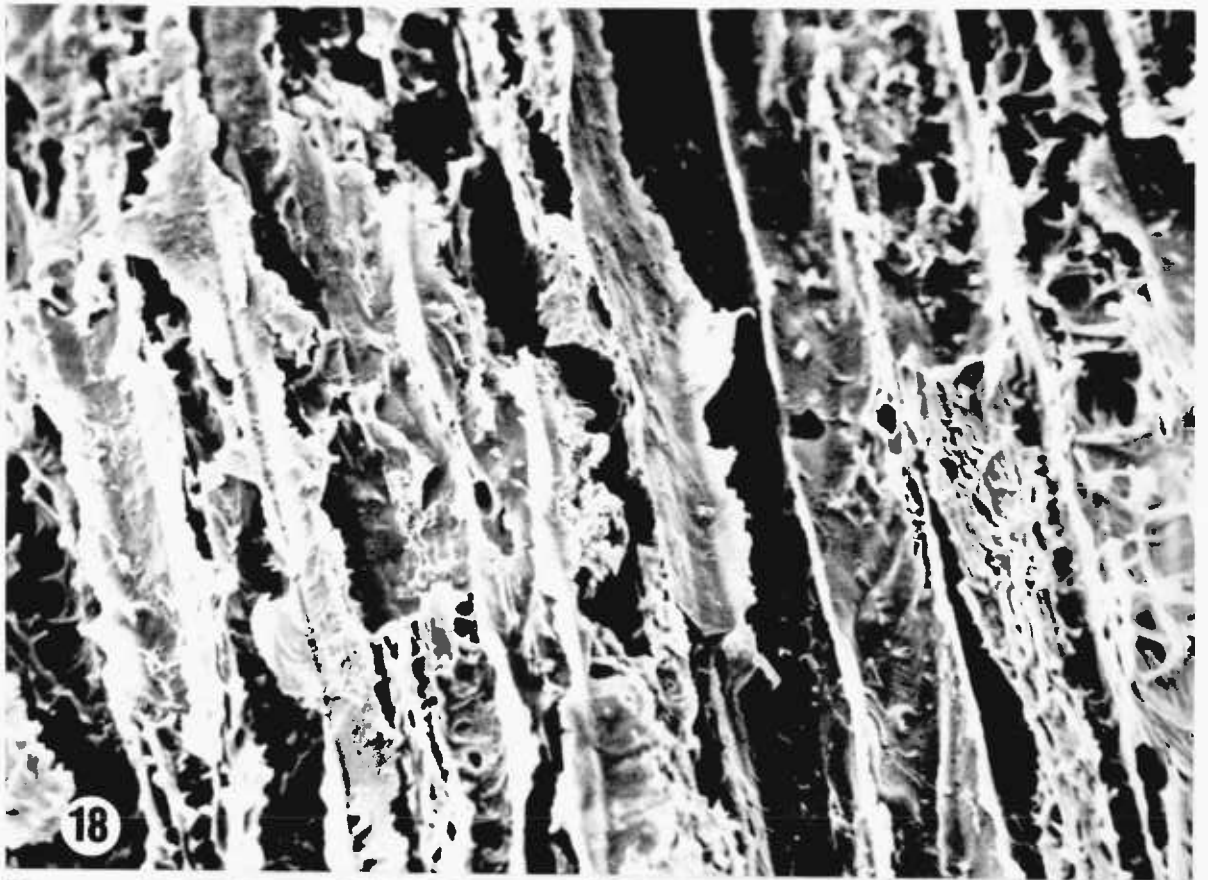


Fig. 18 (x865) represents a severely degraded specimen after twenty weeks' exposure to the fungus, with very large cavities and ruptured cell walls at different planes. This rupturing is due to the extreme brittleness of the specimen in the advanced stage of the decay.

Fig. 19 (x3,383) is a higher magnification of a part of Fig. 18 which demonstrates a great change in the appearance of the cell wall. In this stage it was noticed that a great number of small holes spread through the cell wall, these holes could well be bore holes.

Fig. 20 (x3,571) represents a part of the cell wall covered with bore holes, while a part of the cell wall is ruptured revealing an empty space (which might be a cavity) with a hypha inside.



3.2.2 Birch attacked by Chaetomium globosum S70B cultured  
on 2.5% malt agar medium (Figs. 21 to 28)

Fig. 21 (x 727) shows the invasion and colonization of a vessel by fungal hyphae which are very well branched and different in their diameter.

Fig. 22 (x 761) presents the colonization of another vessel by fungal hyphae. The interesting feature of this micrograph is the network between the bars of the scalariform perforation plate. This network is extended between the branched fungal hyphae.

Fig. 23 (x 2,767) shows the network more closely and it appears to represent the "cotton wool-like" hyphae which were seen and described by George (1974).

Fig. 24 (x 768) is a transverse section of degraded birch showing the vessels colonized by the fungal hyphae which cross through the bars of the perforation plate to the next cell. A part of the  $S_3$  layer of one of the vessels is ruptured and pulled away with the fungal hypha from the rest of the cell wall. The surrounding fibres are not very clear because during photography the focusing was aimed at the inside of the vessel, but all are very thin walled, suggesting active fungal decay.

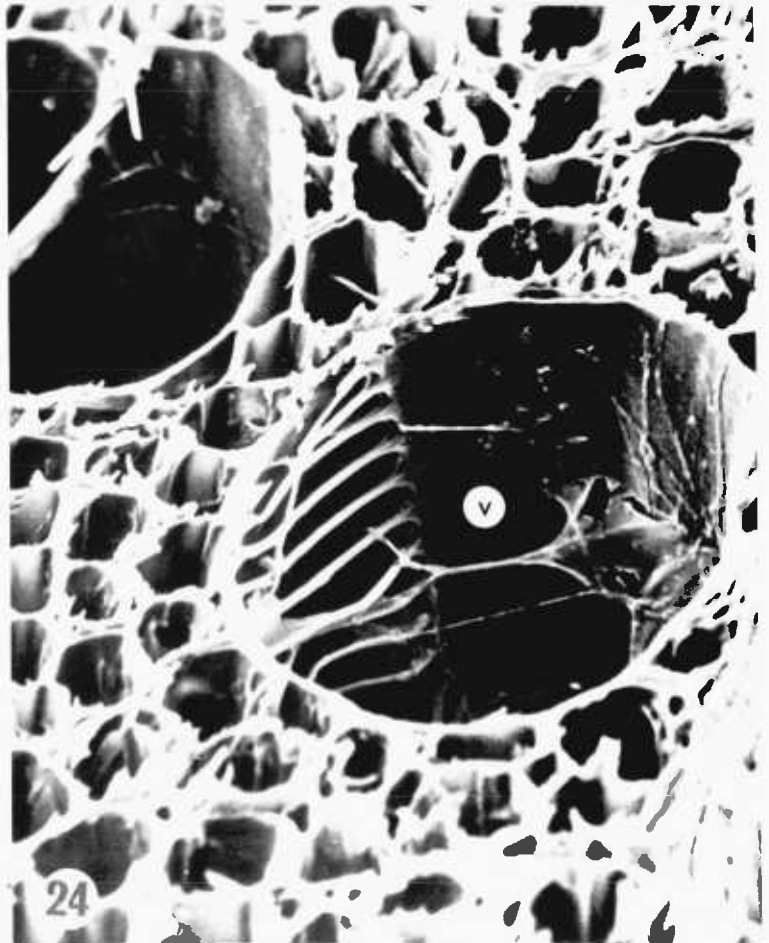
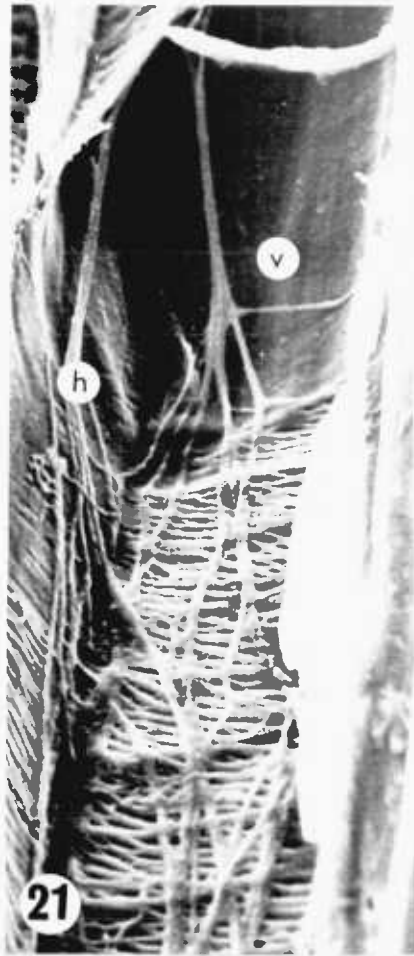


Fig. 25 (x 687) is a general view of fibres with young soft rot cavities.

Fig. 26 (x 687) demonstrates the decay of fibres in the late stages (12 weeks), revealing very large soft rot cavities.

Fig. 27 (x 1,666) shows the decayed fibre wall with a porous (perforated) appearance; also an end of a cavity is clear on the bottom right of the micrograph.

Fig. 28 (x 2,800) reveals a chain of pointed-end cavities enclosing a hypha in the middle cavity and the fibre wall is degraded and has a porous (perforated) appearance. The appearance of the fibre wall on the right of the micrograph is changed, which could be due to enzyme diffusion from the fungal hyphae lying on the surface of this layer of the cell wall.



3.2.3 Scots pine attacked by Chaetomium globosum S70B  
cultured on 2.5% malt agar medium (Figs. 29 to 33)

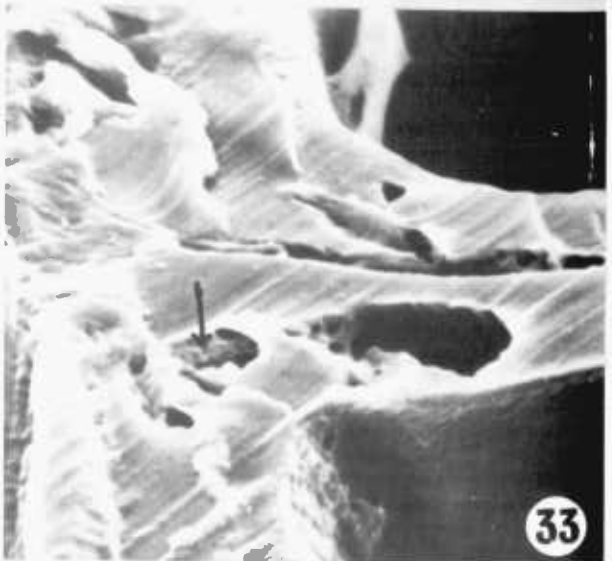
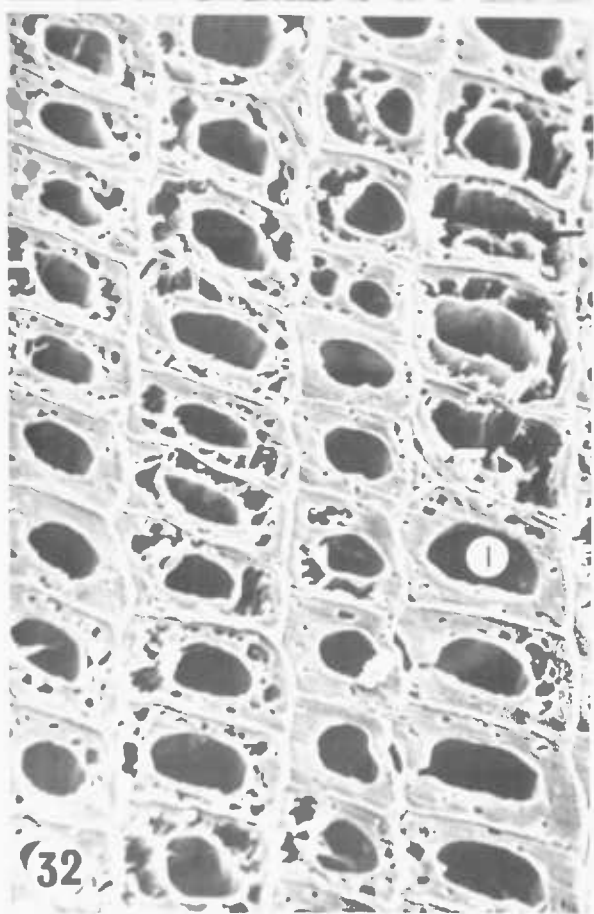
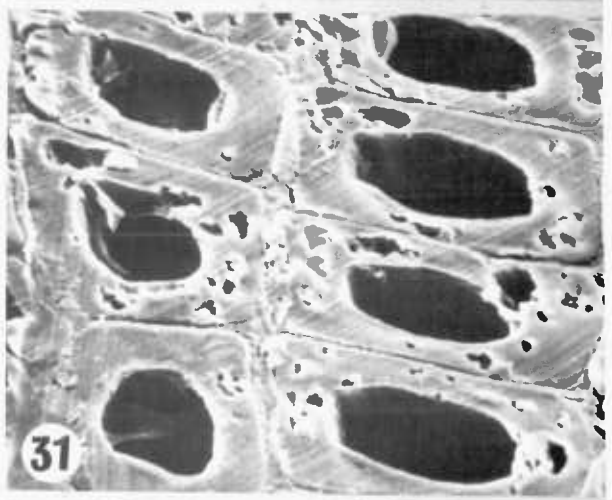
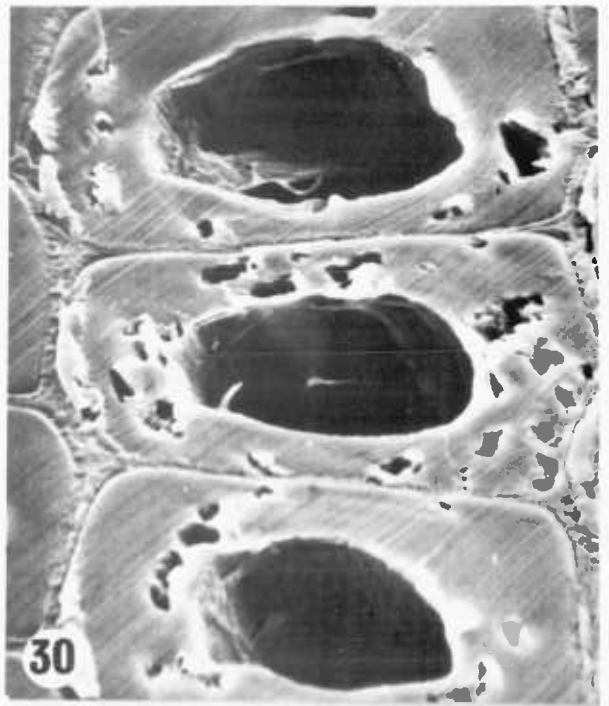
Fig. 29 (x1,478) is a longitudinal face of a degraded tracheid showing a large chain of ruptured cavities in the middle of the micrograph. The remaining cell wall to the right and the left of the ruptured cavities has a type of porous appearance.

Fig. 30 (x2,160) is a transverse section of degraded tracheids showing the intact compound middle lamella, the  $S_1$  layer, the broad  $S_2$  layer with several soft rot cavities of different sizes, the resistant  $S_3$  layer and the lumen containing hyphae contiguous to the  $S_3$  layer. Cavities are present in the  $S_2$  layer but there are a few close to the  $S_1$  layer.

Fig. 31 (x1,080) represents a more advanced stage of soft rot decay than in Fig. 30. Some of the small cavities in the  $S_2$  layer have begun to join together producing one large cavity; and a few cavities have appeared in the  $S_1$  layer; while the  $S_3$  layer is still very resistant.

Fig. 32 (x757 ) is an area representing severe soft rot decay with many small cavities in or close to the  $S_1$  layer. The interesting feature of this micrograph is the enormous size of some cavities almost the same size as some lumina, these cavities are likely to have been produced by the coalescence of several small cavities producing large holes in the  $S_2$  layer. Apparently the  $S_3$  layer is very resistant even at this stage of the decay, since there are only two cells (arrowed) that have ruptured  $S_3$  layers, which is very likely due to the preparation of the specimen.

Fig. 33 (x5,400) represents a pointed-end cavity in the  $S_2$  layer of tracheid in a transverse section. The arrowed structure in the micrograph could well represent a hypha within the cavity.



3.2.4 Scots pine attacked by Phialophora fastigiata S6A  
cultured on 2.5% malt agar medium

A. Early stages of the decay (Figs. 34 to 42)

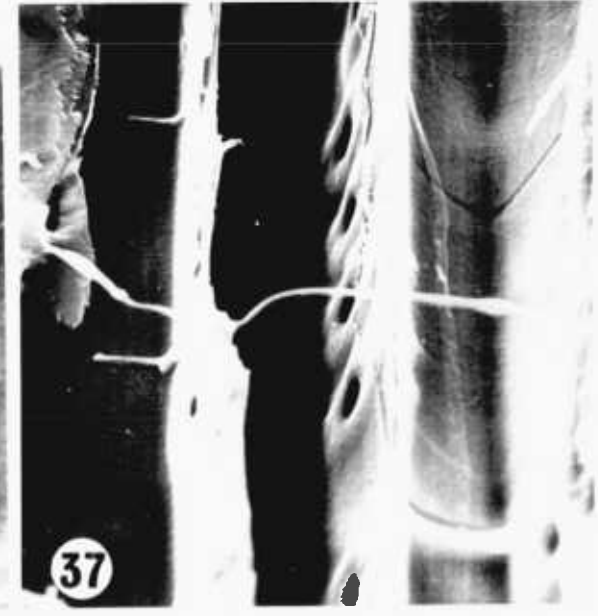
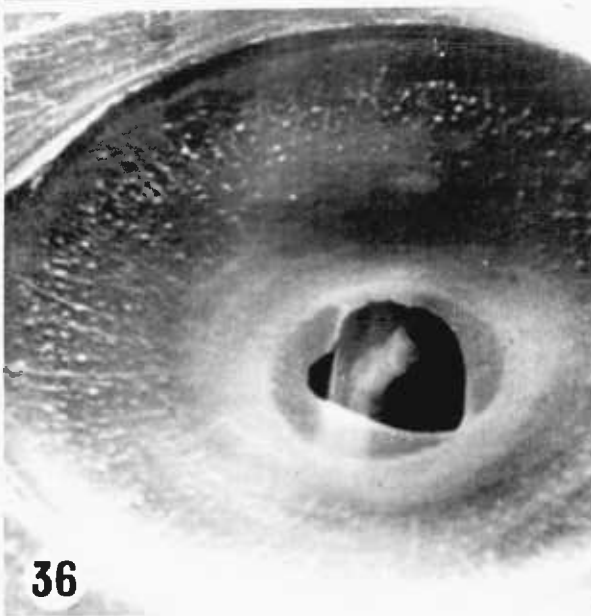
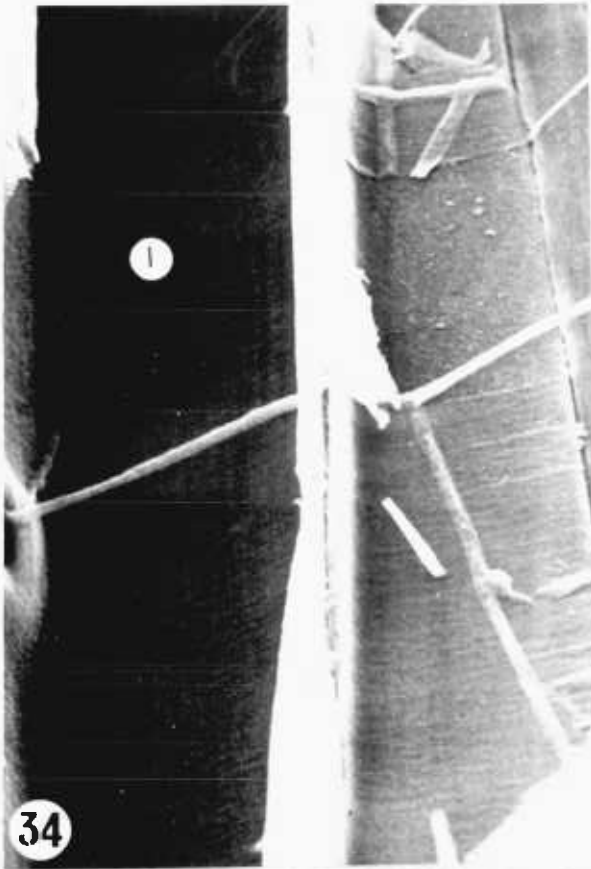
Figs. 34 (x1,320), 35 (x2,350) and 36 (x5,500) are representing the passive penetration of the fungal hyphae through bordered pits.

Figs. 34 and 35 possess two sizes of hyphal diameter, one is broad and flat lying contiguously to the  $S_3$  layer, while the other is narrow and crossing the empty space of the lumen. The narrowness of the hypha may be due to shrinkage of the tracheid walls during the drying of the specimen, causing the hypha which has both its ends attached to the tracheid walls to stretch.

Fig. 36 represents the lower chamber of a bordered pit after removing the upper chamber revealing a part of a fungal hypha passing from the under side of the pit. The pit membrane has been ruptured, either during the specimen preparation, or by a branch of the hypha which might have crossed the membrane to penetrate into the next cell and the branch lost during splitting leaving a small base on the original hypha. The margo and the torus are very clear in the micrograph.

Fig. 37 (x825 ) represents the stainer behaviour of the fungus by crossing three tracheid walls horizontally without the cell wall degradation. This hypha has two bulbous regions across the lumen of two tracheids which is difficult to interpret.

Figs. 37, 38 and 39 represent active penetration of the fungal hyphae through the cell wall in longitudinal faces.



In Fig. 38 (x2,300) the active penetration has happened at the arrowed areas, in the lower arrowed area the hypha has a rounded tip before touching the tracheid wall.

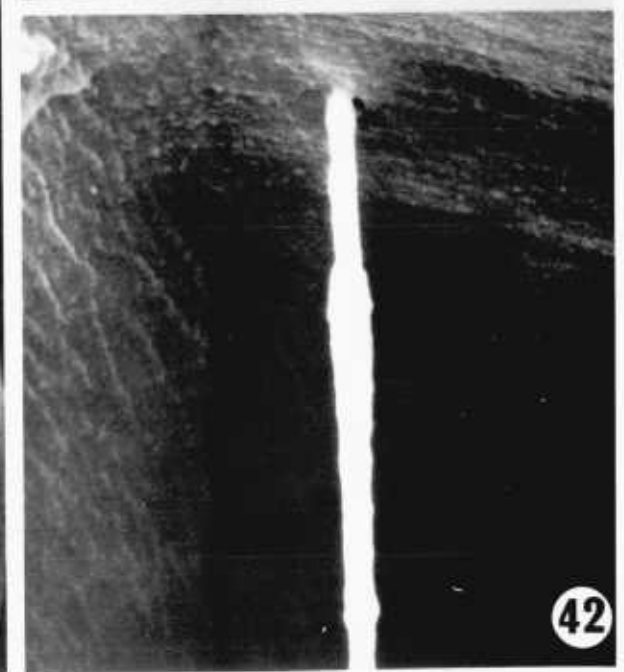
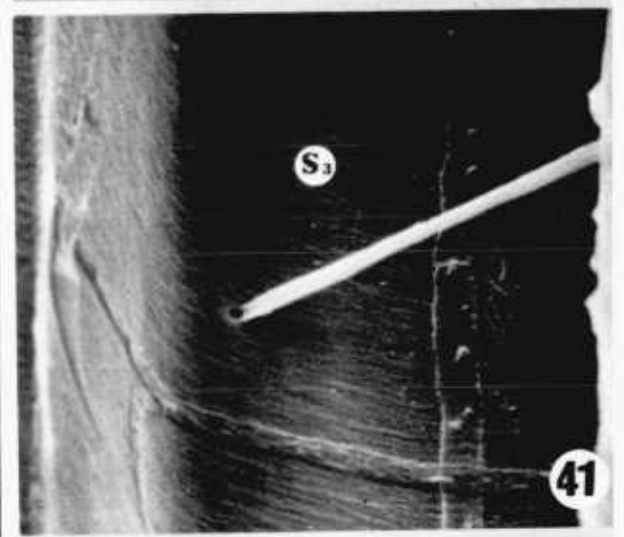
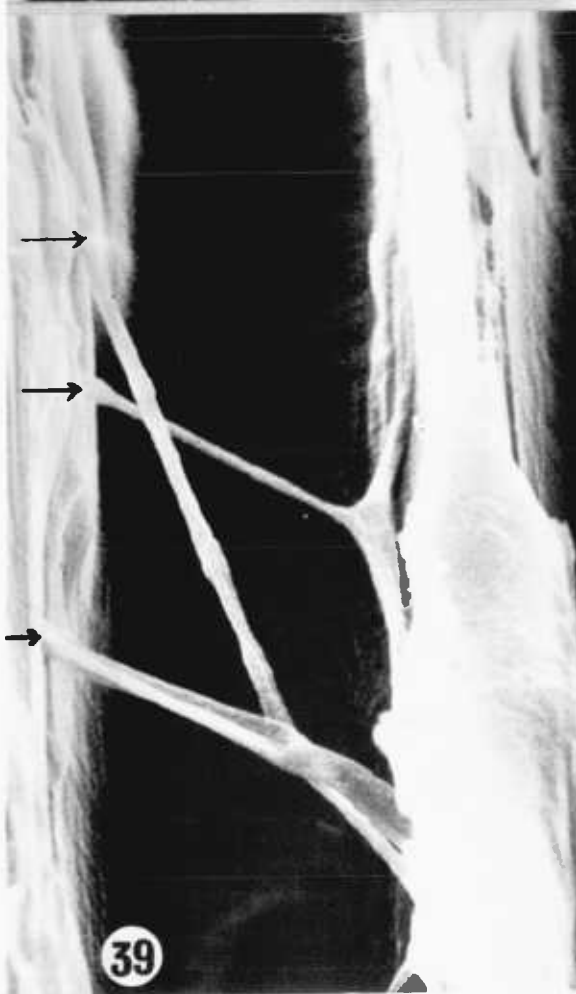
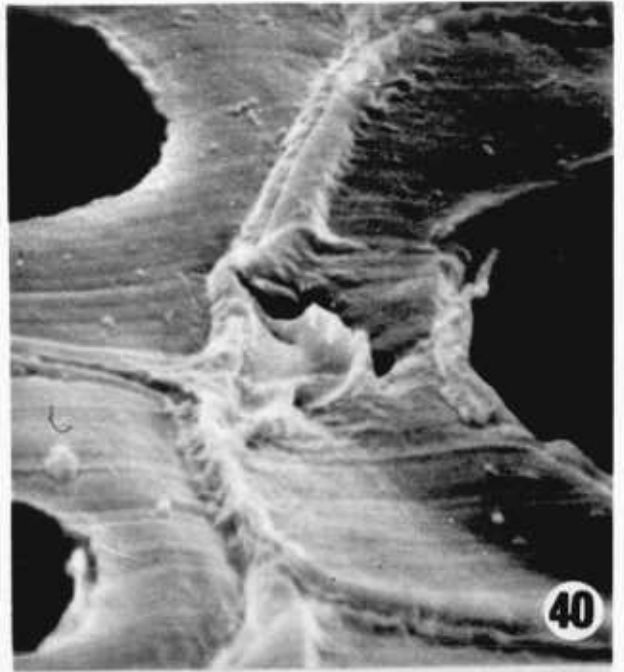
Fig. 39 (x3,226) shows again an active penetration at three points. The top two have bulbous structures before entering the cell wall. These structures could well represent "transpressoria".

Fig. 40 (x4,800) shows a transverse section of parts of several tracheids with a small hole in one of them very close to the  $S_1$  layer, and a tiny swollen part of a hypha is pushing the  $S_1$  layer and the middle lamella towards the tracheid on the left hand side. This swollen tip could well be a "transpressorium" trying to penetrate the cell wall.

The penetration point in all previous micrographs was not apparent because of the plane of which the micrographs were taken, while Figs. 41 and 42 show the actual penetration points.

In Fig. 41 (x5,400) the hypha originates from one cell wall, crosses the lumen and then enters the opposite cell wall. The penetration of the hypha occurs by bore hole formation, which is conspicuous in the  $S_3$  layer of the tracheid wall. The bore hole has the same diameter as the penetration hypha, but it appears to be larger because the hypha has probably contracted during the specimen drying, which had a similar effect on the  $S_3$  layer causing the two small cracks on the right of the vertically lying hypha.

Fig. 42 (x5,400) shows another bore hole with penetration hypha in a different tracheid.

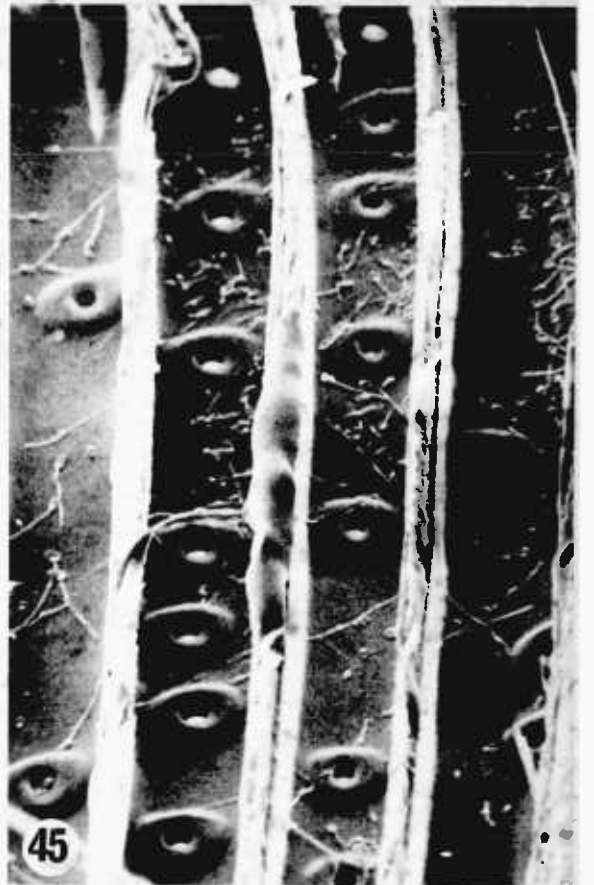
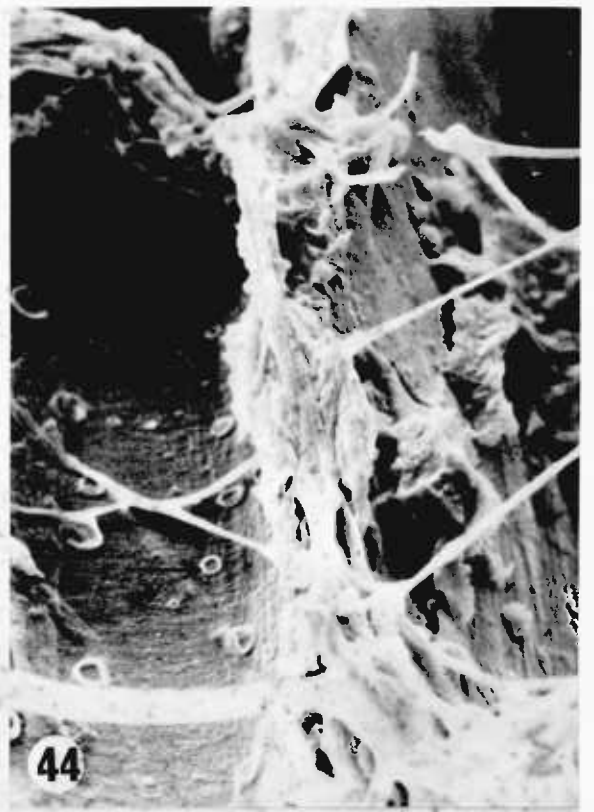


B. Late stages of the decay (9 to 20 weeks) (Figs.  
43 to 58)

Fig. 43 (x373) is a general view of the surface of the wood block colonized heavily and decayed severely by the fungal hyphae.

Fig. 44 (x2,708) is a high magnification of an area from Fig. 43 showing the stainer behaviour of the fungus and the cell wall break down. The spherical bodies (arrowed) could be spores but their appearance has changed during the coating of the specimen.

Figs. 45 (x666) and 46 (x2,666) show an unusual phenomenon of the fungal hypha. Apparently the fungus has formed spores on the  $S_3$  layer of the tracheids followed by their germination. These germinating spores have collapsed during the specimen preparation.



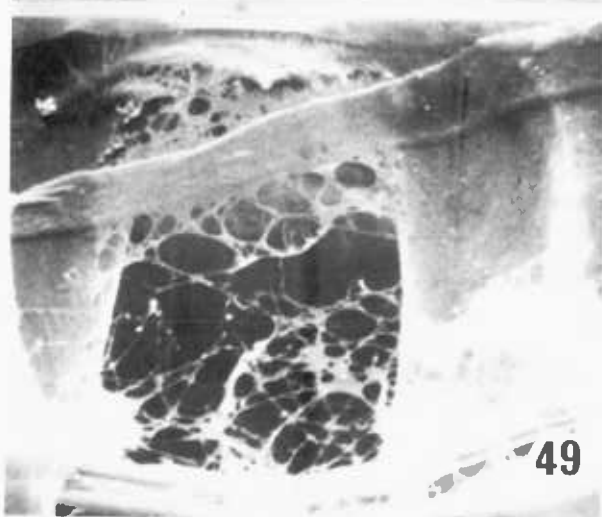
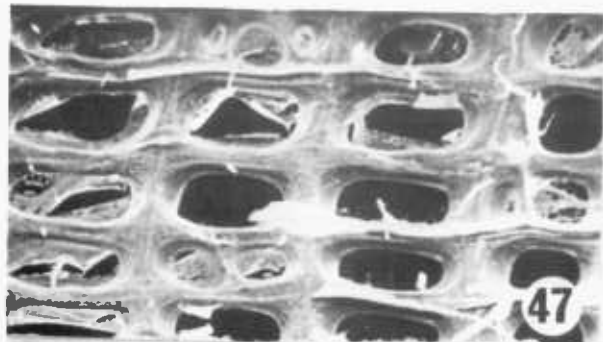
Figs. 47, 48 and 49 demonstrate the breakdown of the pit membranes of cross field pits. In Fig. 47 (x562) some pit membranes are completely missing and others have changed to a network (perforated) structure.

Figs. 48 (x2,314) and 49 (x3,680) show the network structure of pit membranes more clearly with fungal hyphae adjacent to the pit.

Another unusual phenomenon was noticed in the later stages of the decay which is clearly apparent in Figs. 50 and 51. These show heavy invasion and colonization of resin ducts by the fungal hyphae.

Fig. 50 (x1,055) shows a longitudinal section of resin duct colonized by the fungus, meanwhile several hyphae are crossing from neighbouring cells into the resin duct wall or vice versa.

Fig. 51 (x642) is a transverse section of the resin duct invaded by fungal hyphae.

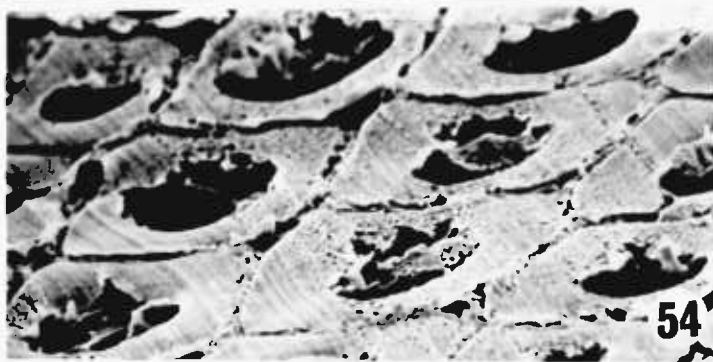
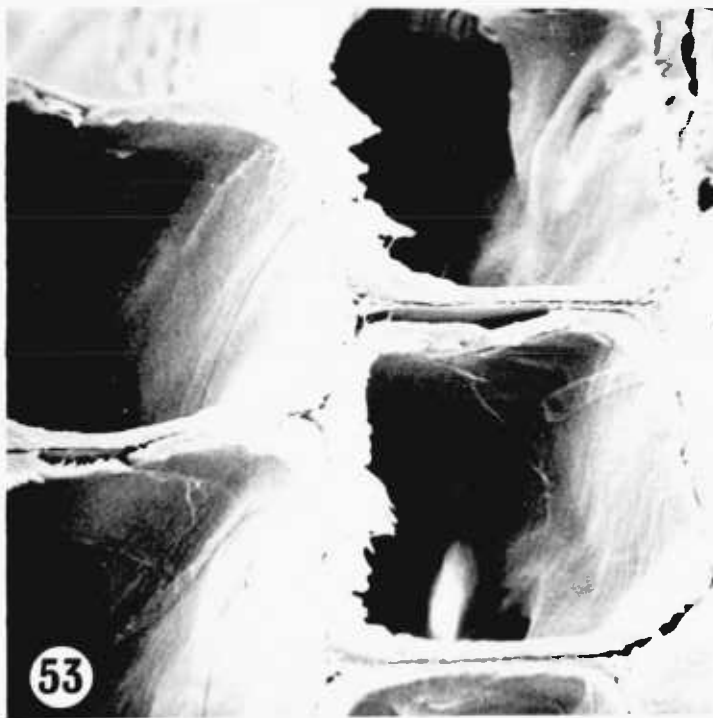
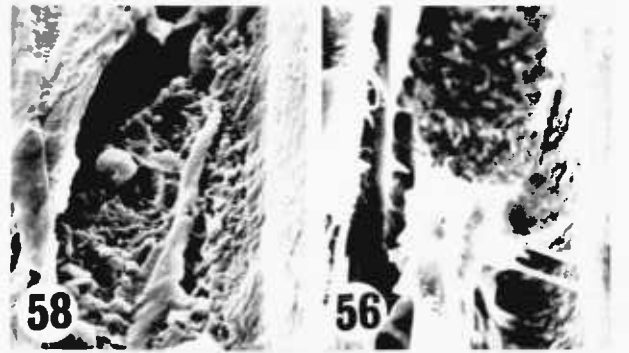
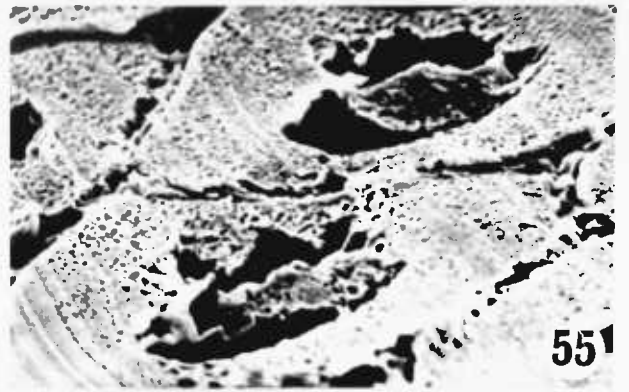


Exposing the wood specimen to the fungal attack for 20 weeks has produced a pattern of degradation in the tracheid walls which is different from soft rot decay (cavity formation).

Figs. 52 (x 654 ) and 53 (x1,292) are transverse sections of early wood tracheids showing the stainer behaviour of the fungus, while there is a change in the secondary cell wall of the tracheids, Fig. 53. This change in the secondary cell walls is clearer in the late wood, as shown in transverse section in Figs. 54 and 55.

Fig. 54 (x1,431) is a general view of an attacked area of the late wood showing a porous pattern of decay of the secondary cell wall. The porosity of the cell wall is well defined in Fig. 55 (x2,520). The interesting feature of this micrograph is that not only the  $S_2$  layer of the secondary cell wall, but all cell wall layers, are degraded including the middle lamella. The rupturing in the degraded  $S_2$  layer has occurred during sectioning which might be due to the brittleness of the degraded area.

Figs. 56 and 57 represent the previous type of degradation in longitudinal section. In Fig. 56 (x1,050) the tracheid in the middle of the micrograph is attacked by the fungus where the hyphae are apparent on the  $S_3$  layer of the lower half of the tracheid. The  $S_3$  layer of the top half has been peeled off during the specimen preparation, exposing the porous structure of the  $S_2$  layer which is defined in Fig. 57 (x2,400). This porous appearance was also noticed (seldomly) in the  $S_3$  layer which appears in Fig. 58 (x2,000).



3.2.5 Scots pine attacked by Phialophora fastigiata S6A  
cultured on cellulose agar medium (Figs. 59 - 65)

The early stages of the attack in Scots pine by the fungus cultured on cellulose agar medium (Nilsson 1973), were very similar to those early stages produced by the same strain of the fungus cultured on 2.5% malt agar medium. A great difference was observed in the mode of attack in the later stages (15 to 20 weeks' incubation), therefore, only these stages are represented by the figures that follow.

Fig. 59 (x 594 ) is a transverse section of early wood attacked by the fungal hyphae crossing from one cell to the next.

Figs. 60, 61, 62 and 63 represent the cell wall degradation in the late wood tracheids in a pattern of typical soft rot cavities.

Fig. 60 (x1,436) shows a number of small soft rot cavities situated mostly in the part of the  $S_2$  layer which is nearer to the  $S_3$  layer than the  $S_1$  layer.

Fig. 61 (x2,800) is an advanced stage of the decay in which more cavities are formed and have coalesced producing relatively large holes in the  $S_2$  layer.

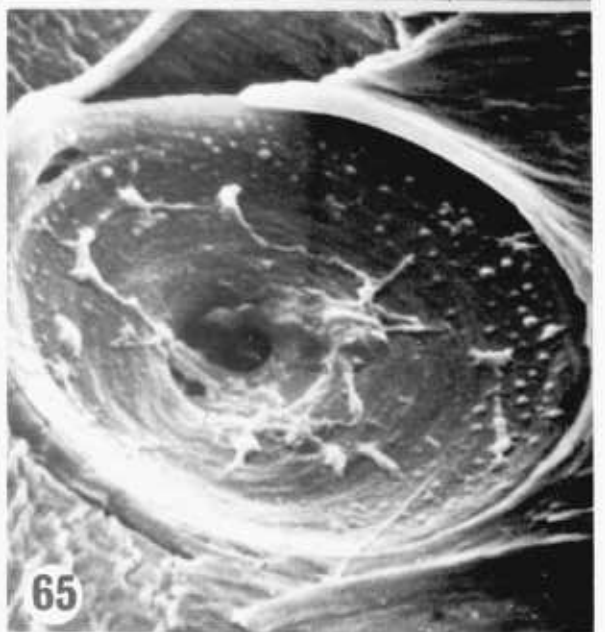
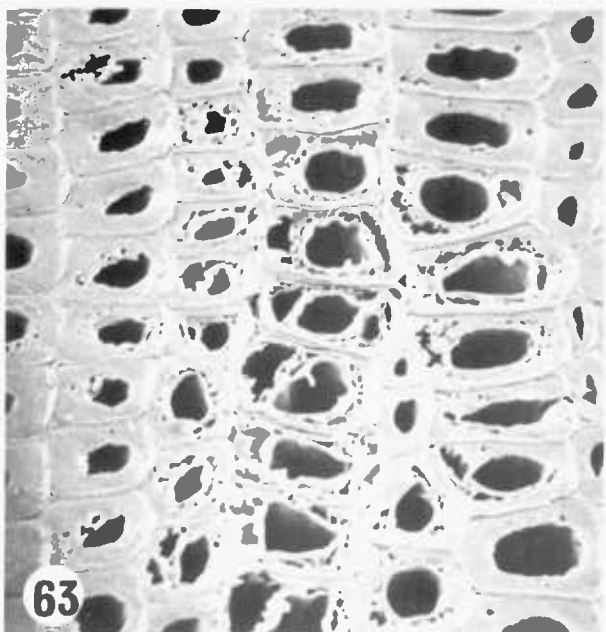
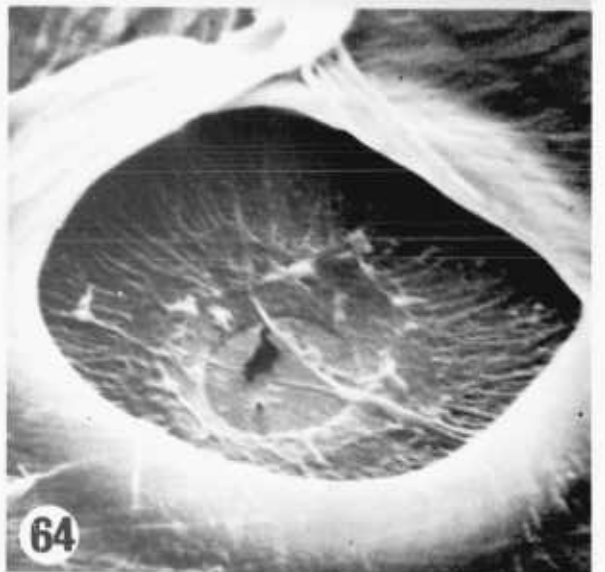
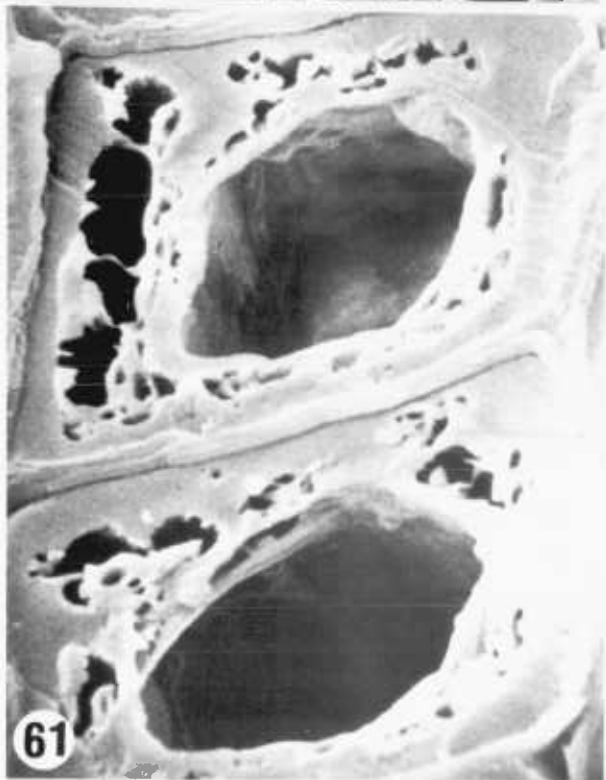
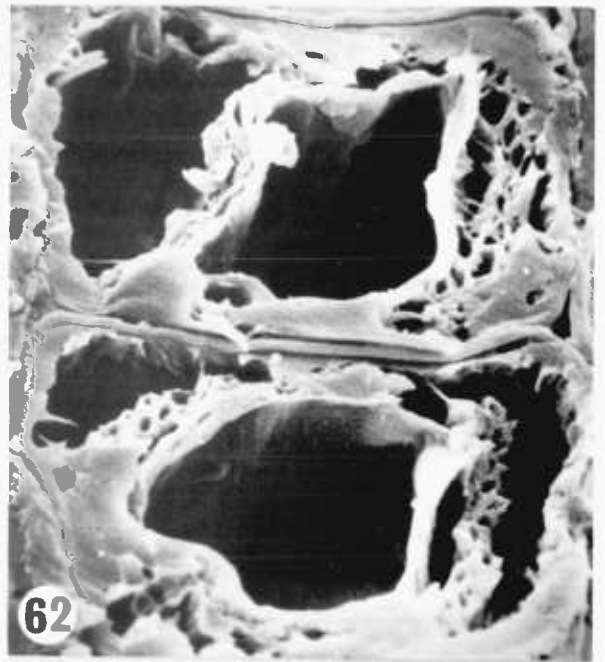
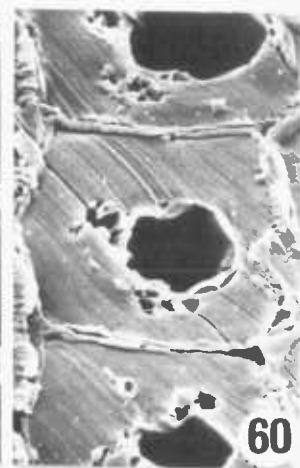
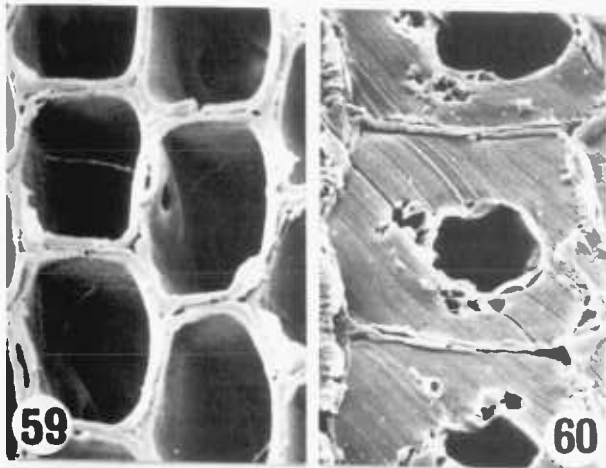
Fig. 62 (x2,223) is a more advanced stage of the decay characterized by very large holes in the  $S_2$  layer produced by coalescence of several soft rot cavities.

Fig. 63 (x 500 ) is a general view of the decay representing successive stages of development.

The interesting feature of Figs. 60, 61, 62 and 63 is the high resistance of the  $S_3$  layer to the decay. This layer remains intact with a slight change in shape which is due to the looseness of the  $S_2$  layer.

Fig. 64 (x5,115) shows the lower chamber of bordered pit with a fungal hypha which is probably starting the breakdown of the intact margo. The pit membrane is ruptured which is probably due to the specimen preparation.

Fig. 65 (x4,909) presents a view of the degraded margo and pit membrane with residue produced after the breakdown.



3.2.6 Scots pine attacked by Phialophora fastigiata 731-1-3b  
cultured on cellulose agar medium (Figs. 66 to 74)

The figures under this section represent the degradation of the cell wall of specimens of wood exposed to fungal attack for 20 weeks. The early stages of the decay was very similar to the ones produced in section 3.2.4.

Fig. 66 (xl,416) is a general view of the cell wall degraded by the fungus producing a change in its structure. The tracheid in the middle of the micrograph has a ruptured wall revealing probably the  $S_3$  layer (which is out of focus because the focusing was on the  $S_1$  layer). The outside wall of this tracheid shows probably the  $S_1$  layer with its microfibrillar structure changed, producing a thin layer with several holes.

Figs. 67 (x 750 ) and 68 (xl,371) are transverse sections of early wood degraded severely producing a kind of disorganisation of microfibrillar structure and causing separation of the layers.

Fig. 69 (x 800 ) presents a view of a transverse section of intensively degraded late wood. The appearance of the tracheid walls does not indicate a soft rot fungus decay pattern. On the contrary it is similar to a brown rot fungus decay. The tracheids adjacent to the rays are decayed more severely than the rest of the tracheids. Another interesting feature of this figure is, the presence of some holes mostly in the  $S_2$  layers of the secondary cell wall. These holes could well be soft rot cavities.

Fig. 70 (xl,400) is a high magnification of an area from Fig. 69 showing the degraded cell wall layers.

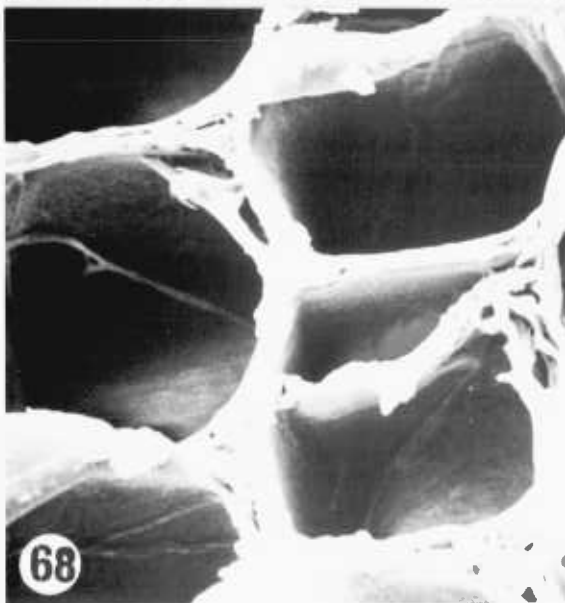
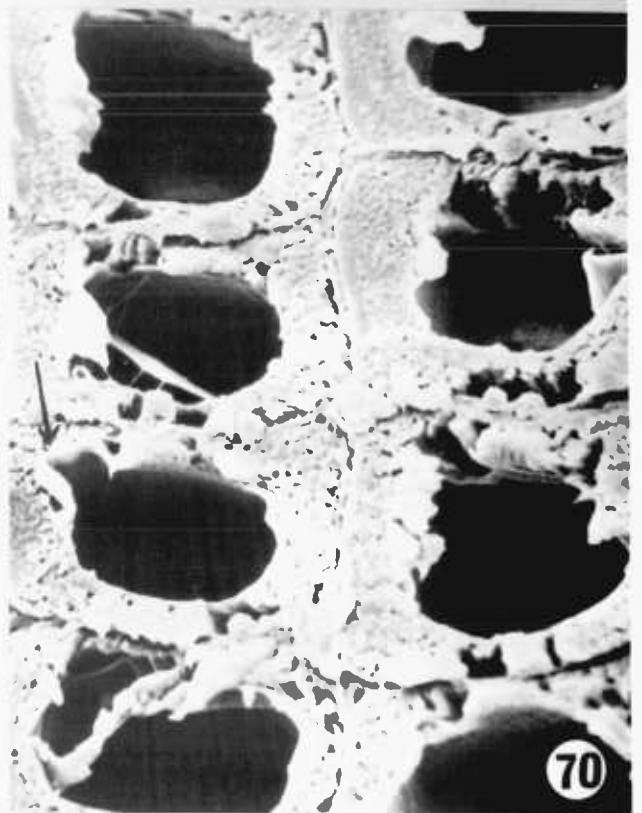
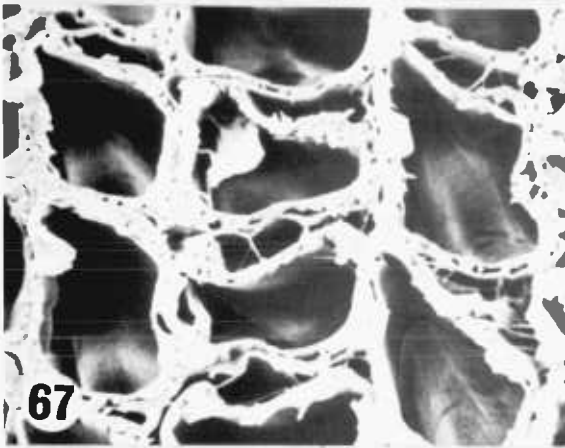
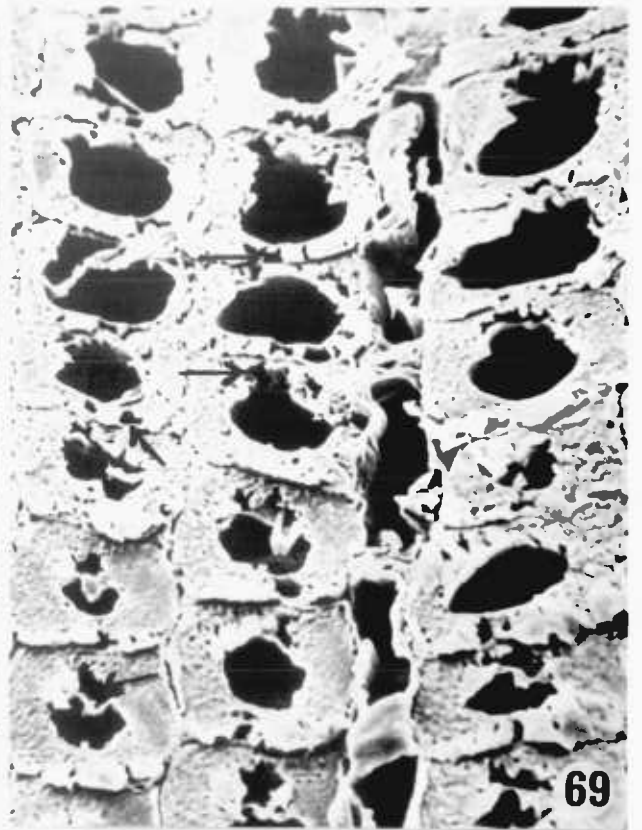
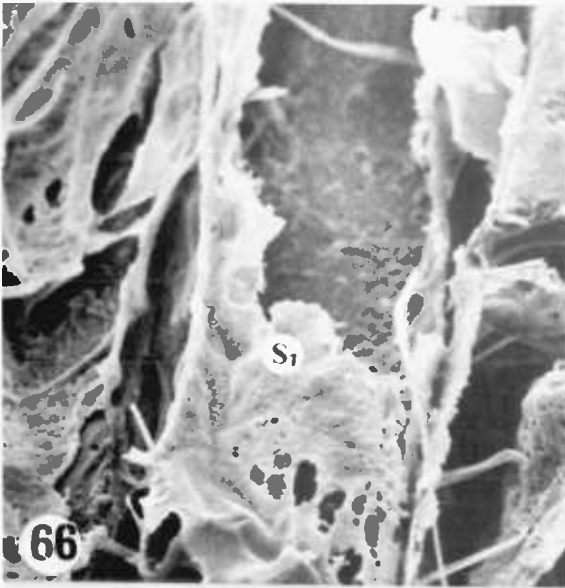


Fig. 71 (x 700 ) is another general view of the decay with more holes visible in the S<sub>2</sub> layer.

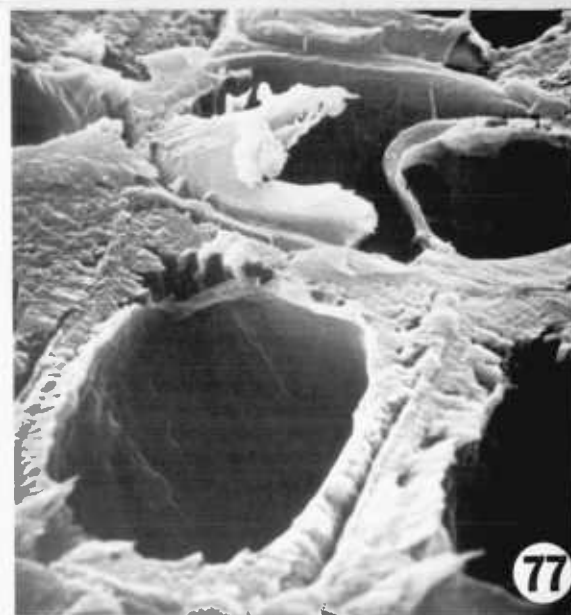
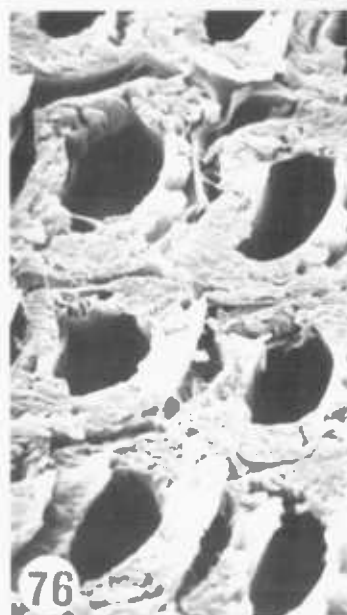
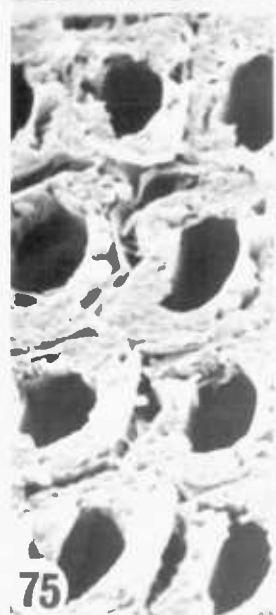
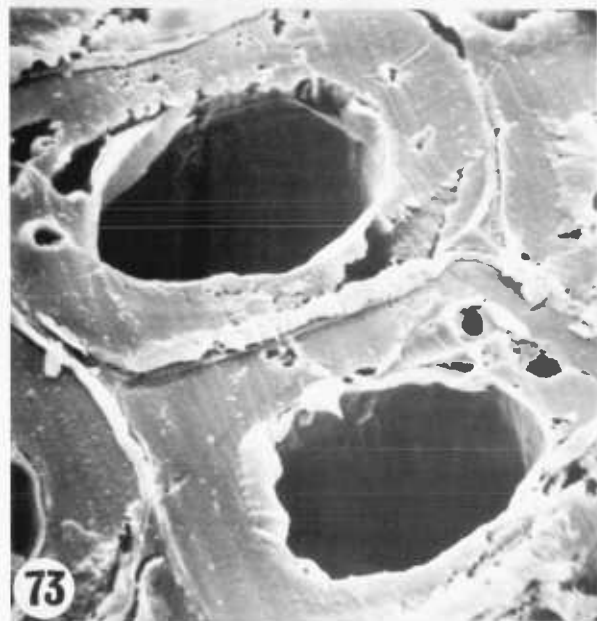
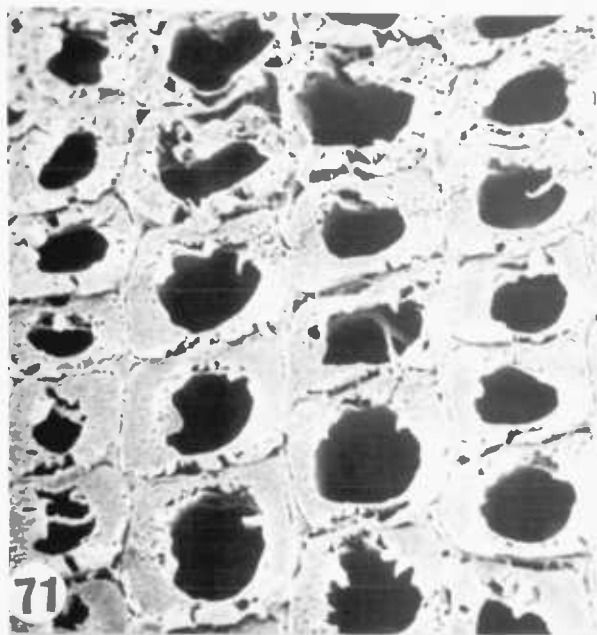
Fig. 72 (x2,208) shows two tracheids degraded and a fungal hypha is present in one of them.

A few microns away from the previous degraded area in the same specimen was observed a typical soft rot cavity formation in the S<sub>2</sub> layer of several tracheids. This is shown in Figs. 73 and 74.

In Fig. 73 (x1,766) both tracheids show a mixture of the type of degradation of soft rot cavities and that described in Figs. 69 and 72.

Fig. 74 (x2,245) shows a general view of the soft rot cavities more clearly.

An investigation was carried out on Scots pine attacked by Coniophora cerebella (Figs. 75 (x 917 ), 76 (x1,200) and 77 (x2,030)) to show the similarity in the decay pattern produced by this brown rot fungus and the pattern produced by Phialophora fastigiata 731-1-3b cultured on cellulose agar medium. The investigation showed a very close similarity in the decay pattern produced by both fungi.



3.3 Delignified Scots pine attacked by *Phialophora fastigiata*  
731-1-3b cultured on 2.5% malt agar medium (Figs. 78 to 83)

The figures under this section represent the degradation caused by the fungus after 6 weeks' incubation.

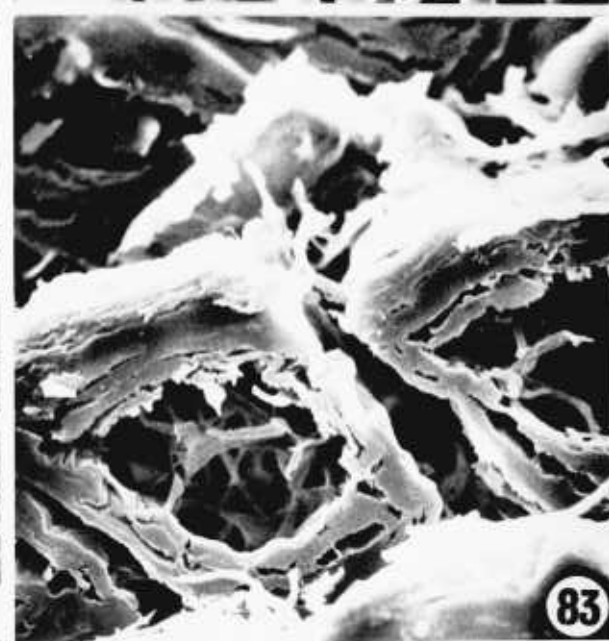
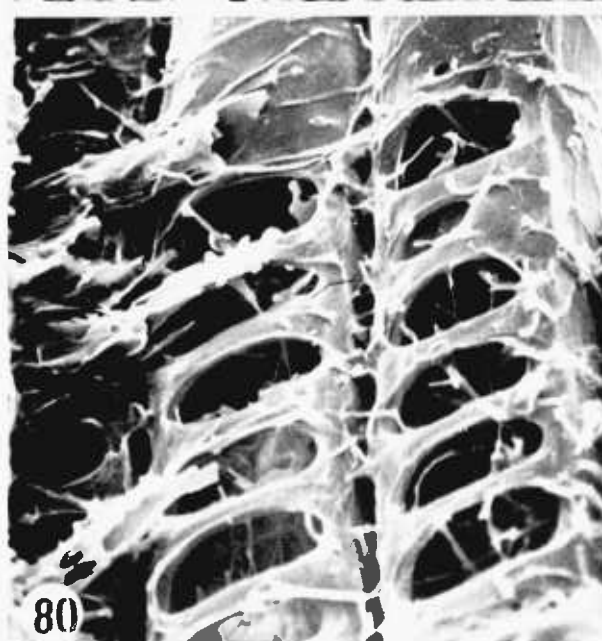
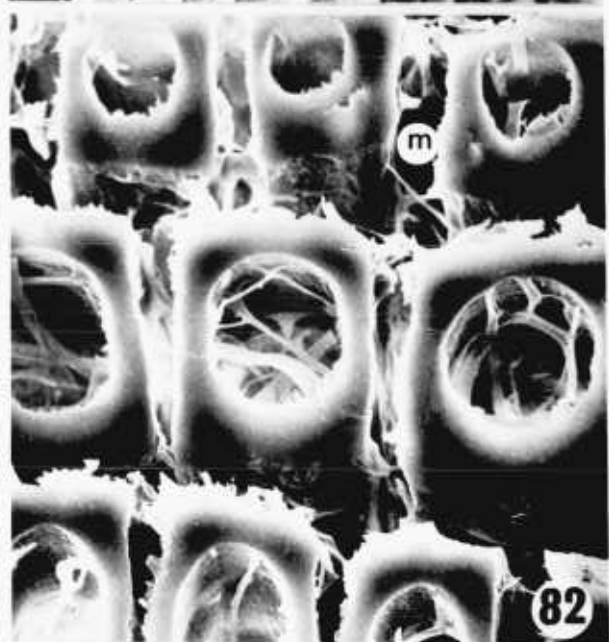
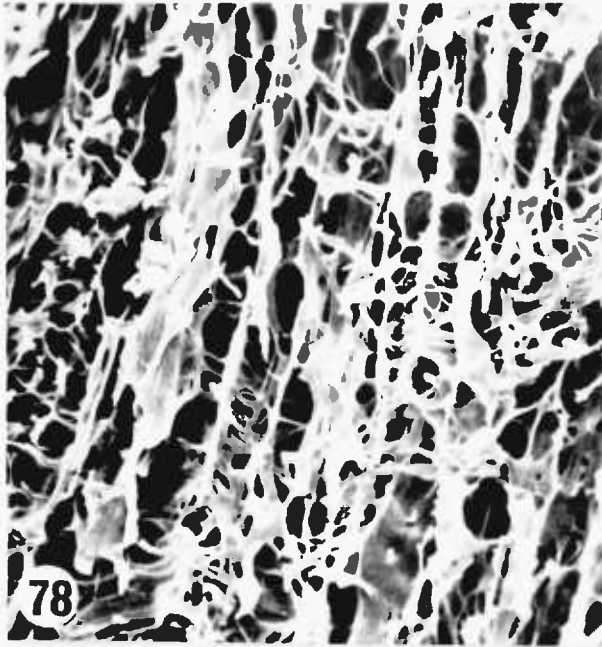
Fig. 78 (x 672 ) shows the general appearance of the surface of the block invaded by the fungus (compared with Fig. 43) causing severe decay with almost unrecognisable cell walls.

Fig. 79 (x 204 ) is a radial longitudinal split of Scots pine showing that the tracheids are almost separated from the middle lamella region resulting in a heavy invasion by the fungal hyphae.

Fig. 80 (x 676 ) shows the invasion of cross field pits by the fungus.

Fig. 81 (x 240 ) shows a transverse section and the radial splits of tracheids in which their lumina are completely colonized by hyphae. The separation of tracheids at the middle lamella causing invasion by the fungal hyphae is very conspicuous in this figure.

Figs. 82 (x1,096) and 83 (x1,388) are transverse sections of late wood tracheids showing heavy colonization by the fungal hyphae in the lumina of tracheids and in the separated middle lamella region. In Fig. 83 the fungal hyphae has a severe effect on all cell wall layers. There are also cracks in the cell wall which are due to the brittleness of the wall after delignification and decaying.



#### 4. DISCUSSION

Findlay and Levy (1969) pioneered the usefulness of scanning electron microscopy in the field of wood decay. Findlay (1970) concluded that this technique could be used to examine soft rotted timber when the surface under observation revealed the cavities, in other words when the S<sub>2</sub> layer is exposed. Figs. 14 and 15 where the S<sub>2</sub> was not exposed confirm this conclusion, since the cavity edges are revealed as shadow beneath the covering layer (the cavities with the hypha are apparent because the covering layer was ruptured during the preparation).

Findlay stressed the importance of using both SEM and TEM to confirm or to interpret specific results obtained from each. Both types of electron microscopy were therefore used extensively in the present work. The results which showed similarity using both microscopes will be discussed in conjunction with the TEM, while those results that were achieved by using the SEM only will be discussed in this part.

Vessels in normal birch proved to be the preferable xylem element for the fungal hyphae to start its invasion, (Corbett (1965; and Levy (1965a, b). The small diameter hyphae branch from the larger ones and could represent microhyphae (Liese and Schmid, 1962), or could be the "cotton wool-like" hyphae (George, 1974). The presence of these fine hyphae in the vessel (Figs. 22 and 23) could suggest their nutritive function, since the vessel is a conductive element in the xylem.

The presence of type 1 and type 2 decay patterns (Corbett, 1965) in the same fibre wall attacked by Chaetomium globosum was

unexpected (Figs. 27 - 29). Nilsson (1973) classified the type 2 into two kinds of erosion patterns : one was perforated which is obvious in the wall of the fibre with cavities (Fig. 28), while the other pattern was striated erosion. Nilsson suggested that the first type of erosion was produced mostly in the cell wall nearer to the fungal hypha; while the second was probably produced by enzyme diffusion on the whole wall surface. Nilsson did not specify the cell wall layer in which the erosion pattern occurred but he confirmed the resistance of the  $S_3$  layer. From Fig. 28 it appears that the porous (perforated) wall (the  $S_2$  layer) could have occurred in one of two ways. The first is that the perforation was produced by the fungal hypha which formed the cavity. This suggests the presence of either two kinds of enzyme system one producing cavities and the other producing porous erosion; or one kind of enzyme producing both types of decay pattern. The second is that the perforation of the cell wall occurred by diffusion of enzymes from the cell lumen over the whole cell wall, and most probably the cavity formed before the erosion decay occurred.

The pattern of decay in birch attacked by Ph. fastigiata was mostly type 1, which was described by Corbett (1965) and Findlay (1970) and will be discussed in conjunction with the TEM; and type 2 decay was not found very frequently. In the later stages numerous holes were observed in the cell wall (Figs. 18 - 20) which suggests that they are bore-holes. The presence of bore-holes confirms the penetration into cavities as a recolonization of existing cavities both from the lumen and the cavities in the neighbouring cells (Figs. 23, 45, 54, 63 in Part IV).

It was believed that the fungus Ph. fastigiata behaved as a soft rot and a stainer fungus in hardwood, but only as a stainer in Scots pine (softwood), (Karkanis (1966), Levy (1967) and Findlay (1970). This stimulated the present author to carry out a more detailed investigation into the behaviour of the fungus in Scots pine on two different media.

The early stages of the attack produced by the two strains 731-1-3b and S6A of the fungus were similar when they were cultured on both malt agar and cellulose agar media. The fungus behaved only as a stainer by crossing several cells in active penetration pattern but without cell wall degradation, Fig. 37. This figure also shows the effect of the method of specimen drying (air dried) on the appearance of decayed wood specimen. The wood cell walls showed signs of shrinkage during the specimen drying, resulting in longitudinal contraction of the fungal hypha which caused it to pull away the thin  $S_3$  layer.

The variation in the diameter of the fungal hyphae is apparent when they are occupying the lumen (Figs. 34, 35, 37, 38 and 39). The smaller diameter hyphae are frequently penetrating the cell wall in passive or active ways; while the larger diameter hyphae are frequently lying contiguous to the  $S_3$  layer. This variation in the diameter of hyphae could be interpreted in two ways: (a) The smaller hyphae could be younger and responsible for penetration because they can produce more effective enzymes (George (1974)). These smaller diameter hyphae appeared to be branches of larger hyphae (Fig. 23, 24 and 35) which suggests a possible similarity to the microhyphae (Schmid and Liese, 1965, and Chou, 1971). These hyphae

were observed first by Liese and Schmid (1962) using a replica technique who suggested their importance in bore-hole formation. This suggestion could well explain the function of the smaller diameter hyphae in bore-hole formation in the  $S_3$  layer in Figs. 41 and 42. (b) The smaller diameter hyphae are not contiguous to the  $S_3$  layer but are crossing the lumen of the cell from one wall to the opposite side. The cell wall might have contracted during the specimen drying, causing the hyphae to shrink in diameter.

Phialophora fastigiata in Scots pine showed several unusual phenomenon which are difficult to interpret.

(a) Spore formation and their germination (Figs. 44 - 46). This was observed only near the edges of the decayed wood block, and could well be due to the fact that in the early stages of the attack the hyphae which occupied the edge of the wood block were dependent on the diffusion of nutrient into the wood block from the medium. In the later stages when the diffusable nutrient decreased because of the consumption and drying of the medium, the fungal hyphae produced spores to adapt itself to the new substrate. After this adaptation occurred the spores germinated, resulting in formation of new hyphae. If this is so, it means a new aggressive invasion of the wood block began. This phenomena was also observed by Sorkhoh with the same fungal species in both Scots pine and birch (Sorkhoh, 1975).

(b) Invasion of resin duct (Figs. 50 and 51). This phenomenon was unexpected because one would assume that the resinous material in the canal would be toxic for the fungal hyphae. On the contrary there was a heavy invasion of the canal, but it is difficult

to know whether the change in the inner wall of the canal was caused by enzymic erosion or by the presence of the resinous material in the canal.

(c) Breakdown of the cross field pit membranes (Figs. 47 - 49). Bordered pits have been studied intensively by several investigators, Kerr and Bailey (1934), Harada (1954 and 1963) and Liese (1965a). Their investigations indicated that the pit membranes consist of the middle lamella and primary walls of two adjacent cells; and the microfibrils are orientated randomly in two planes. Therefore one would assume similar composition for the pit membranes in the cross field pits. So that when these membranes are attacked by fungal enzymes, perforations will be produced. Undoubtedly this postulation is hypothetical but it correlates well with recent observations by Harada (1975) on the orientation of the microfibrils in the layers of the membranes of the pit fields.

The fungus Ph. fastigiata showed a great variation in the decay pattern in Scots pine in the latter stages of the attack according to the strain and the media used. Nilsson (1973) observed in birch, that most fungi which grew well on malt medium produced type 1 decay (cavity formation); while type 2 decay predominated with those fungi which grew well on cellulose agar medium. He also observed only type 1 decay in Scots pine attacked by Ph. fastigiata 731-1-3b cultured on cellulose agar medium, while there was no indication of type 2 decay whatsoever.

The results of the present work did not support the conclusion drawn by Findlay (1970) that Ph. fastigiata behaves only as a staining fungus in Scots pine, nor Nilsson's results (1973).

Apparently Ph. fastigiata S6A which had a good growth on 2.5% malt agar medium, produced a kind of porous erosion pattern (Figs. 54 - 57), while the growth of this strain was sparse on cellulose agar medium and produced typical soft rot cavities (Figs. 59 - 63).

The strain 731-1-3b had fairly good growth on malt agar medium and very little growth on cellulose agar medium, resulting in a patched decay pattern when cultured on the latter medium. A few patches represented typical type 1 decay, while the rest were a mixture of general erosion and cavities (Figs. 66 - 74).

The failure of Findlay to obtain both type 1 and 2 decay, and Nilsson to obtain type 2 in Scots pine (although exactly the same strains of the fungus and the same media were used as in the present work), could be attributed either to the possibility that the incubation period was insufficient, or to mere luck that the sectioning was not in the decayed area. The latter is more likely as the decay pattern caused by this fungus in Scots pine is not as dominant as in birch, and therefore more frequent sectioning is likely to be necessary to achieve the observation of the decay pattern.

The variation of the decay pattern observed in Scots pine in the present work could be interpreted in two ways. Firstly, the porous pattern, or perhaps more correctly, "trans-S<sub>3</sub> erosion", which is produced by the strain S6A could be similar to the perforated erosion pattern in birch described by Nilsson (1973). Secondly, the type of decay produced by the strain 731-1-3b when cultured on cellulose medium could represent the second type of erosion described by Nilsson, and this also occurred in birch.

According to Nilsson (1974b) both kinds of erosion (type 2) decay did not occur in Scots pine attacked by Ph. fastigiata.

because the fungus was unable to degrade the  $S_3$  layer. This means that the degradation of the  $S_3$  layer was vital to obtain erosion decay. At the same time he emphasized the resistance of this layer which is supported by the present work. This suggests that erosion decay could occur without any visible breakdown of the  $S_3$  layer.

These observations suggest a possible new interpretation of Corbett's type 2 decay. She was using only the light microscope and what appeared at these magnifications to be a direct erosion from the lumen, similar to Bravery's (1972) pictures of white rot fungi, may in fact have been erosion of the  $S_2$  layer behind the nearly intact  $S_3$ . This might well be called trans- $S_3$  erosion. Variation of it may account for Nilsson's (1973) perforated erosion.

A similarity was observed between the pattern of the attack apparent in Figs. 54 - 57 and the pattern produced by Chaetomium globosum in birch Figs. 26 - 28. This indicates that the porous or perforated decay occurs in Scots pine when it is attacked by Ph. fastigiata. The interesting feature revealed in this work was that in the latter stages of this type of decay, the  $S_3$  layer was sometimes slightly altered but resistant (Figs. 56, 58 and 66), while in the early stages, the  $S_3$  layer was unchanged. Liese (1970) attributed the resistance of this layer in softwood to the differences in its chemical composition to hardwood. This could be a reason for limiting the effect of the ectoenzymes for a while, but not in the later stages when most of the polysaccharides of all cell wall layers except the  $S_3$  is consumed.

The porous structure of the secondary cell wall in late wood (Figs. 54, 55 and 69 - 72), and in early wood (Figs. 67 and 68), suggests a great similarity in the pattern of decay present in these

figures and figures 75 - 77. The latter three figures represent the decay pattern produced by brown rot fungus Coniophora cerebella.

Therefore one can assume that Ph. fastigiata in certain conditions can behave in the same way as brown rot fungi, by diffusing enzymes from the hypha in the cell lumen which penetrate but do not break the  $S_3$  and destroy the  $S_2$ . Subsequent rupture of the  $S_3$  layer as a drying artefact gives the appearance of type 2 decay. Brown rot fungi are characterized by secretion of enzymes while the hypha are lying in the lumen. The enzymes diffuse from the lumen through the  $S_3$  layer into the  $S_2$  layer to decompose the polysaccharides. The hyphae of brown rot fungus seldom penetrate into the cell wall, except in the later stages. This means that the enzymes in brown rot fungus are not restricted by the vicinity of the fungal hyphae. Comparison of the decay pattern produced by Coniophora cerebella in Scots pine and type 2 decay produced by any soft rot fungus, raises the question of whether enzyme secretion and diffusion in the latter is reliant on the invading hyphae being in the close vicinity to the cell wall. A possible explanation could be that the enzyme secretion and diffusion from the soft rot fungus could be limited when the fungal hypha is orientated along the microfibrils in the  $S_2$  layer, but not when it is in the lumen. Another possibility could be that the enzyme produced by the fungal hypha when it is in the lumen might be different from that secreted by the hypha when it is in the  $S_2$  layer. This emphasizes the importance of the alignment of the fungal hypha along the microfibrillar orientation in the  $S_2$  layer which may be necessary before the fungal hypha is able to produce cavities.

The holes in all cell wall layers in Figs. 54, 55 and 67 - 72, are probably soft rot cavities which have been produced.

(a) Before the erosion occurred the fungal hypha's decomposing of the cell wall by cavity formation was probably interrupted (for some reason or other) and the cell wall was subsequently decomposed by enzyme diffusion from the fungal hypha in the cavity (Levi and Preston, 1965). This could be the case when fully-formed cavities and the beginning of perforation is observed (Figs. 73 and 74).

(b) The cavities were produced after erosion occurred, but the hypha which formed the cavity was unable to form more cavities because the cellulose microfibrils were already decomposed by erosion and their orientation altered.

(c) They were produced simultaneously with the erosion.

From the preceeding explanation, one would conclude that the type 2 decay produced by Ph. fastigiata in Scots pine could be considered very similar to the decay caused by brown rot fungus. In other words that this fungus can behave as a stainer, as a soft rot fungus and as a brown rot fungus.

Cavity formation was only observed in Scots pine attacked by Ph. fastigiata S6A when it was cultured on cellulose medium (Figs. 59 - 63). It is difficult to give an explanation for this behaviour of the fungus, because erosion was well as cavities occurred with the strain 731-1-3b when it was cultured in similar medium. Here one might suggest that this variation could be attributed to the differences in the strain of the fungus, or could well be due to the sectioning not occurring in the eroded areas.

The variation in decay patterns which occurred in Scots pine, with both strains S6A and 731-1-3b of Ph. fastigiata, is unlikely to be due to the mineral salt which was used as a sterilizing solution

for the wood blocks during autoclaving. Although Savory (1954b) succeeded in producing cavities in Scots pine attacked by Chaetomium globosum only when he impregnated wood blocks with Abrams salt solution, Findlay (1970) was unsuccessful in inducing cavities in Scots pine attacked by Ph. fastigiata by using the same solution in different concentrations. The present work also showed abundant cavity formation without using any mineral salts (Figs. 29 - 33). The effect of the mineral salts could be to enhance the activity of the fungus in attacking wood species, because of its additional nitrogen source (this was discussed in Part II).

Undoubtedly it is worthwhile to expand and to carry out more detailed investigations into the behaviour of different species of Ph. fastigiata by culturing them on different media. The investigation could include chemical studies on fungal enzymes to discover whether the enzymes which are responsible for producing type 1 decay could produce type 2 decay. It is also necessary to investigate the similarity of type 2 decay to the decay produced by a brown rot fungus by using TEM. Furthermore it would be interesting to determine whether the part of the cell wall remaining after erosion is unchanged lignin residue, or if the lignin has been modified or demethoxylated. If the latter has occurred it would indicate a closer similarity between the behaviour of this fungus and brown rots.

PART IV

TRANSMISSION ELECTRON MICROSCOPY

## 1. INTRODUCTION

Wood is one of the most difficult materials to prepare for transmission electron microscopy, therefore the early investigations in wood science was built up on replica techniques (Preston [1952,1974] Meier [1955] and Liese and Côté [1960]). This technique can be excellent to study the surface structure of the wood cell wall, but is not as good as ultra-thin sections for investigating decayed wood. Replication was used in the early days because the modern sophisticated ultra-microtomes were not available and the embedding resins were not very well known and developed.

The technique of specimen preparation for transmission electron microscope TEM is still a developing art, relying on experiment and based on experience. The ability of each biological tissue to<sup>act</sup> with the fixative, embedding resin and the stain, varies from one tissue to another. No one procedure can be applied for all the plant tissues or all the animal tissues. This variation in the technique is due to the differences in the chemical constituent of each component of the living cell; the reaction of each component to the fixative or to the resin or to the stain; the purpose of the study (whether it is morphological or histochemical) and the condition of the tissue when fixed (i.e. whether it was normal or not). Sometimes, while using one kind of tissue, for instance wood, one would expect if the procedure was ideal that the results would have been the same with all specimens used in that experiment, but it is possible to find some of the specimens giving very good results and others not. This could well be due to the size of the specimens, to the conditions of the cells in each specimen, to the poor fixation .... etc.

## 2. MATERIALS AND METHODS

The techniques applied in this work were extracted from different references : Pease (1964), Kay (1965), Hayat (1970), Dawes (1971) and Meek (1973). Modification to the techniques were required because of the heterogeneity of the wood cell wall. The major steps of preparation of ultra-thin sections for TEM observations were as follows :

### 2.1 Specimen preparation

The 3 mm<sup>3</sup> blocks of birch and Scots pine, whether decayed and freeze dried or taken directly from the fungal cultures were fixed in the sledge microtome and a few sections 20  $\mu$  thick were cut. The sections were examined under phase contrast microscope to locate the decayed areas. Those sections were cut into smaller pieces approximately 0.5 mm<sup>3</sup> with a very sharp razor blade that had been cleaned with acetone. The minute wood specimens were placed in "embryo cups" full of the chosen fixative solution, the cups covered with lids.

A drop of water was used as a medium for sectioning and cutting the wood specimens when KMnO<sub>4</sub> was planned to be used as a fixative, while a buffer solution was used with the specimens for other fixatives (see section 2.2).

### 2.2 Fixation

"Fixation is to preserve every detail of cellular ultra-structure right down to the molecular level exactly as it was in life the instant before the cell was killed" (Meek (1973)).

All the reference books on the techniques of fixation deal mostly with animal tissue and sometimes with soft plant tissue. The problem in the present work was the specimen to be examined. Not only is wood a difficult substance in the microscopy field, it was decayed to various degrees by different fungi. The difficulty in the procedures of this part of the work was to find the right fixative applicable and suitable for both the fungus and the wood cell wall. Therefore several fixatives were used, some giving negative results and some positive.

#### 2.2.1 Phosphate buffered glutaraldehyde fixative

(Sabatini, Bench and Barnet, 1963)

The buffer was prepared as follows :-

##### Stock solution

- A. 0.4M dibasic sodium phosphate  
( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ) 35.598 g/500 ml dist.  $\text{H}_2\text{O}$
- B. 0.4M Monobasic potassium phosphate  
( $\text{KH}_2\text{PO}_4$ ) 27.217 g/500 ml dist.  $\text{H}_2\text{O}$
- C. 14% commercial glutaraldehyde

##### Fixative constituents

The mixture of the phosphate buffer was prepared by mixing 70 mls of solution A with 30 mls of solution B, producing a buffer of 0.4M with pH 7.2 which was checked by pH meter. The final desired 0.2M fixative at pH 7.2 with 3.5% glutaraldehyde was prepared by mixing : 1 volume of 14% glutaraldehyde, 1 volume distilled water and 2 volumes of the mixed 0.4M phosphate buffer.

##### Procedure

The wood specimens were placed in "embryo cups" full of the fixative and placed in a vacuum desiccator then the vacuum was

pulled with a water pump for 30 minutes. The whole desiccator with its contents under vacuum was kept at  $4^{\circ}\text{C}$  for 6 hours. After the fixation period was over, the specimens were washed with 0.2M buffer solution 3 times each for 15 minutes at  $4^{\circ}\text{C}$  to get rid of the excess glutaraldehyde.

This fixative is one of the best fixatives to preserve the details of the structure of the cytoplasm but not the cell membrane, although it has a disadvantage in that the tissue fixed with glutaraldehyde lacks contrast, Millonig (1961 and 1962) and Hayat (1970). To achieve better contrast and fixation, the specimens were post fixed with osmium tetroxide ( $\text{OsO}_4$ ) 1% buffered with 0.2M phosphate buffer at  $4^{\circ}\text{C}$  for 2 hours. Before the dehydration stage it is essential to wash the specimens thoroughly with 0.2M buffer to avoid the excess  $\text{OsO}_4$ . This was carried out in three stages, each for 15 minutes at  $4^{\circ}\text{C}$ .

Great care was taken in handling osmium tetroxide because it is volatile and toxic and can cause damage to the epithelial tissues of the eyes or the nose. Therefore the work must be carried out in a fume cupboard. Cleanliness is one of the important factors in the technique, all the glassware which was contaminated with osmium was washed first with 30% alcohol and then in water in a fume cupboard. All the fixative solution was prepared freshly and used immediately, the changes of the solutions was done with pasteur pipette.

2.2.2 McClean and Cook (1952), Sorensen's phosphate buffer and %  
osmium (Dawes, 1971)

Stock solution

- A. 0.4M dibasic sodium phosphate  
( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ) 35.598 g/500 ml dist.  $\text{H}_2\text{O}$
- B. 0.4M monobasic potassium phosphate  
( $\text{KH}_2\text{PO}_4$ ) 27.218 g/500 ml dist.  $\text{H}_2\text{O}$

or

- 0.4M monobasic sodium phosphate  
( $\text{NaH}_2\text{PO}_4$ ) 23.996 g/500 ml dist.  $\text{H}_2\text{O}$
- C.  $\text{OsO}_4$  osmium tetroxide 4%

Fixative constituents

70 mls of solution A was mixed with 30 mls of either of the B solutions giving rise to a phosphate buffer of 0.4M at pH 7.2. Furthermore, 1 volume of 4%  $\text{OsO}_4$  was mixed with 1 volume of distilled water and then with 2 volumes of 0.4M buffer. The product was a fixative of 0.2M sorenson's phosphate buffer at pH 7.2 + 1% osmium.

Procedure

The small wood specimens were placed in an "embryo cup" full of the fixative and kept for 24 hours at  $4^\circ\text{C}$  under vacuum in a desiccator. The following day the specimens were washed with 0.2M buffer solution three times. Each wash took 30 minutes at  $4^\circ\text{C}$ . This was followed by dehydration.

2.2.3 Potassium permanganate ( $\text{KMnO}_4$ ), Luft (1956)

Investigating the comparison of the orientation of micro-fibrils in normal and delignified wood cell wall layers, stimulated the author to investigate the possibility that some alteration in the microfibrils might have happened during the decay of the wood

by fungal penetration through the cell wall. This investigation was not possible to achieve with the previous two fixatives especially with silver or gray sections (see section 2.7). Therefore 1% and 2% potassium permanganate was used as a fixative. This fixative is characterized as being excellent in depicting the clarity of membranes and it was hoped that it might have a similar effect on the cellulose microfibrils.

The fixative was prepared by dissolving thoroughly 1 g or 2 g of potassium permanganate in 100 ml of distilled water the solution was filtered five times through Whatman No. 1 filter paper and it was then ready to be used as a fixative.

#### Procedure

The wood specimens were placed in "embryo cups" full of the fixative and kept at 4°C without vacuum. A series of fixation periods (5, 10, 15, 30, 45 minutes, 1 and 1½ hours) were used to ascertain the most suitable fixation period for wood specimens. After each period of fixation, the specimens were washed with cold distilled water about five times (each for 15 minutes) until the washing water was completely colourless. The specimens after washing were ready for the subsequent dehydration.

Specimens which were fixed in 2%  $\text{KMnO}_4$  for 45 minutes gave the most successful results.

### 2.3 Dehydration

Dehydration is removing all the free water from the specimen and replacing it by an organic solvent miscible with the embedding media. In the present work this was carried out by replacing the

fixed and washed wood specimens in a series of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% and 90% alcohol for about 15 - 20 minutes, in each step at 4°C (in an ice bath). The final dehydration was done with absolute alcohol, changing it three times for one hour each, except the second change which was kept overnight, Findlay (1970).

Great care was taken during the changing of the alcohol series, which was carried out with a pasteur pipette, to ensure that the specimens were not exposed to the air or contaminated with water.

## 2.4 Embedding

### 2.4.1 Plastic media

Two types of plastic media have been used in the present work.

#### 2.4.1.1 Methacrylate + styrene

Methacrylate was introduced as an embedding medium by Newman et al. (1949). A mixture of n-butyl methacrylate and styrene was used to embed highly vacuolated or damaged plant specimens by Kusida (1961), Mohr and Cocking (1968), Chou (1970) and Findlay (1970).

The preparation and application of the plastic monomer for embedding the wood specimens in the present work was exactly as described by Findlay (1970).

The results with methacrylate embedded material were unsatisfactory because :

1. Uneven polymerization occurred.
2. Air bubbles formed after polymerization which produced un-

uniform sectioning.

3. Shrinkage after polymerization could be as high as 20% which changes the dimensions of the cell wall.
4. Unstable under electro bombardment, especially in thin sections. This instability caused contamination of the lenses of the microscope.
5. Methacrylate vapour is inflammable and causes irritation in the respiratory system (even when a fume cupboard is used).

Methacrylate has some advantages, such as a low viscosity, which helps in easy infiltration and it is relatively easy to section. In spite of this, epon (epoxy resin) was used extensively in the present work.

#### 2.4.1.2 Epon (Epoxy resin)

Epoxy resins were first introduced as an embedding media by Maaløe and Birch-Anderson (1956). Improvements in epon were made by Glauert and Glauert (1958), Luft (1961) and Glauert (1965). The hardness of the polymerized block can be adjusted by changing the proportions of the constituents.

Medium hardness of epon was used in the present work, the constituents being as follows.

|                                             |   |         |
|---------------------------------------------|---|---------|
| Epikote 812                                 | - | 230 g   |
| Benzyl dimethyl amine (B.D.M.A) accelerator | - | 5.5 g   |
| Methyl Nadiz Anhydride (M.N.A.) Hardener    | - | 114 g   |
| Dodecenyl succinyl anhydride (D.D.S.A.)     |   |         |
| Hardener                                    | - | 123.6 g |

#### 2.4.2 Infiltration and embedding procedure

This stage was found to be one of the most critical stages in electron microscopy. The least carelessness may damage the

whole results. Great care, cleanliness and patience was applied to insure good infiltration and, therefore, good results.

The infiltration and embedding procedures were as follows.

1. After the final stage of dehydration, the wood specimens were placed in a mixture of 2:1 absolute alcohol and propylene oxide (epoxy propane) for 30 minutes at 4°C.
2. The solution was changed to a mixture of 1:2 absolute alcohol and propylene oxide for 30 minutes at 4°C.
3. The specimens were changed to fresh propylene oxide for 30 minutes at 4°C.
4. The solution was changed to a mixture of 2:1 propylene oxide and epon medium hardness for 30 minutes at 4°C.
5. The specimens were transferred to a mixture of 1:2 propylene oxide and epon for one hour at 4°C. (For all the stages where propylene oxide was used, the "embryo cups" were covered with lids because the reagent is highly volatile. Mixing the proportions of epon with propylene oxide was carried out in screw-topped small bottles immediately before using it).
6. The specimens were changed to a fresh mixture of 1:2 propylene oxide and epon. The "embryo cups" were kept at 4°C and half-uncovered overnight to allow the propylene oxide to evaporate gradually.
7. The next morning, the specimens were picked up from the resin one by one with a toothpick, dried on filter paper gently (without pressure) to get rid of the surplus resin then transferred to other "embryo cups" containing enough resin to cover the specimens. The old resin was poured into

- a disposable bottle to be polymerized before disposing.
8. The "embryo cups" were placed in a vacuum desiccator and the vacuum pulled for 30 minutes. The evacuated desiccator was kept at 4°C.
  9. The last procedure was repeated in the evening and kept overnight at 4°C.
  10. Steps Nos. 7, 8 and 9 were repeated for five consecutive days.
  11. The last step was followed by three days, during each of which the resin was changed twice daily to a fresh resin at 4°C but without using a vacuum.
  12. After the final stage of changing resin, the specimens (approximately 8 in each) were placed in plastic embedding dishes (40 mm diam x 5 mm depth) containing fresh epon resin. A stereo-binocular light microscope was used to place the specimens in the desired orientation.
  13. A small label carrying the serial number of the specimen was placed into the resin, in the middle of the embedding dish. At this stage the dishes were left at room temperature for 1-2 hours.
  14. The orientation of specimens were checked before putting the embedding dishes in an oven at 55-60°C for 44 hours to allow polymerization to take place. After the first three hours in the oven, the arrangement of the specimens in the resin were checked to ensure the final desired orientation of each specimen. After the polymerization was achieved, the disc of resin containing the polymerized wood specimens

was removed from the embedding dish and left for a few hours at room temperature before the mounting stage. It was not necessary to use propylene oxide as an intermediate stage for these wood specimens which were to be embedded in methacrylate/styrene, because of the miscibility of absolute alcohol with methacrylate. Therefore the procedure was carried out exactly as that followed in the infiltration of epon resin from the stage No. 4 until the polymerization, using absolute alcohol instead of propylene oxide and methacrylate instead of epon resin. The embedding was carried out in gelatine capsules instead of embedding dishes.

## 2.5 Mounting

The shape of gelatine capsule which was used as containers to embed the wood specimens in methacrylate, produced an ideal shape of mounted specimen. The gelatine capsule was peeled off when the specimen was ready for subsequent trimming.

Epon discs were examined in stereo-binocular light microscope to select the desired orientation of specimens (L.S. or T.S.). The embedded specimens which had been fixed with sorensen's buffer and osmium or glutaraldehyde, were transparent so that it was possible to see the direction of the fungal hypha inside the wood.

Care was taken in cutting out the resin area surrounding and containing the specimen. This was achieved gently by using a fine coping saw, leaving approximately 2 mm of polymerized resin around each specimen.

The side of the block which was going to be fixed on the "perspex" rod, was sometimes found to be oblique, this was re-orientated by using a metal file. This side was mounted with "Durofix" on a short piece of "perspex" rod, left either in room temperature for 24 hours or at 60°C for one hour followed by several hours in room temperature before the subsequent trimming.

It should be kept in mind that the embedded specimen should be nearer to the top face of the mounted block, since this helps very much in the trimming.

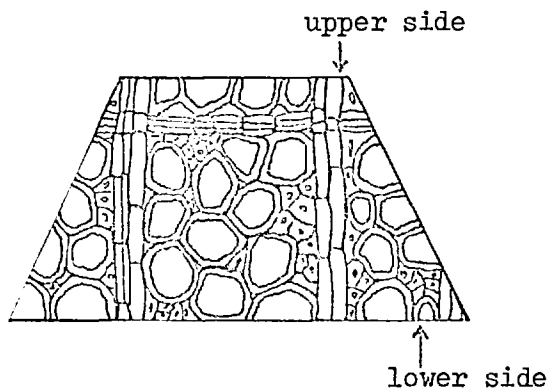
## 2.6 Trimming

Trimming is as important a stage as any other in the preparation of material for electron microscopy. The first indication of having well fixed and good embedded specimens could be recognized at the trimming stage.

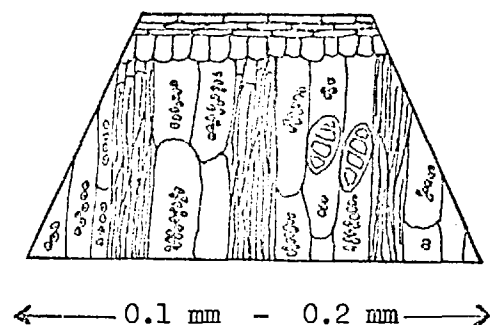
The "perspex" rod with its mounted specimen was replaced in the chuck (block holder) and the first rough trimming carried out by removing all the excess embedding medium around the wood specimen, or the specimen itself when it was too large. This step was done by cutting very thin sections gently with a sharp razor blade cleaned with acetone, producing a pyramid shape with trapezoid face. Very much care was taken in the final trimming, where a steady hand and a sharp razor blade are necessary to get very smooth shiny steeply sloping sides. The lower side which was going to touch the knife first, was parallel and double the length(at most 0.2 mm) of the upper side. To ensure the specimen sectioning in the ultra-microtome the face of the trapezoid was trimmed down to the specimen.

It was found that it is essential to select the right sides of the final pyramid according to the orientation of the cells in the face of the block. For instance when the specimen was embedded longitudinally (in other words the cutting face was in the longitudinal plane), the block was trimmed in a way which produced a right angle between the longitudinal plane and the lower side of the block. This meant that the sectioning was running parallel to the axis. This orientation in the trimming was very helpful in producing full and serial sections.

Transverse sectioning in wood specimens is one of the most difficult planes to section because the knife will be running against the axis.



Trapezoidal face of the trimmed block showing the T.S.



Trapezoidal face of the trimmed block running parallel to the longitudinal axis of the cell wall

## 2.7 Sectioning

Successful and undamaged ultra thin sectioning is the most

difficult stage in the whole of electron microscopy, whatever kind of material used. Wood is one of the most difficult specimens to handle in this field. The difficulties in obtaining good ultra-thin sections in wood were summarized by Fengel (1967). Production of successful sections depends on several factors : the ultra-microtome; the sharpness of the knife; the knife angle; fixation and embedding; the hardness of the embedding resin; the dehydration; the infiltration and polymerization; the trimmed block and its face; the level of fluid in the trough; the temperature in the room; the air movement in the microtome room; and last but not least the patience and experience of the sectioner.

Those points were carefully followed in the present work, resulting in the production of beautiful silver or grey almost undamaged ribbons of sections.

A glass knife was used at the start of this work but did not prove very satisfactory. A diamond knife with a water trough was then used extensively and gave excellent results.

A Reichert "Om U2" ultra-microtome was used and proved very satisfactory, although it suffers from poor illumination.

The grey and silver ribbon of sections (60 - 90 nm, Peachey 1958) were picked up very carefully from the surface of the water in the trough by 100 or 200 mesh formvar (polyvinyl formal) coated copper grids, Drummond 1950).

The method of picking up the ribbon of sections with the grid was by holding a clean and dry grid horizontally with the coated face downwards, touching it gently with a steady hand onto the ribbon which was floating on the water. The electrostatic

charge on the grid surface attracted the ribbon to the grid. The grid was dried by touching the edge of it with filter paper, then kept in a special clean grid holder ready for staining.

Expansion of sections was required with methacrylate/styrene embedded specimens, this was achieved with a toothpick dipped into xylene and held one centimeter above the water trough. Epon embedded material did not need any expansion, which can be considered another of the advantages of using epon as an embedding medium.

It was found in the present work that the most useful size of grid was 100 mesh, because the distance between two successive bars of the grid is larger than the 200 mesh, and therefore the chance of the bar covering the critical point of the section was less.

## 2.8 Staining of sections

The wood specimens which were fixed with potassium permanganate and cut into relatively thick sections could be examined in the TEM without staining, but the thin sections and the epon embedded material required further staining. The aim of the present work was to investigate the alteration in the wood cell wall layers caused by the penetration of the fungal hyphae, which required very thin sections (silver or grey) to be studied under higher magnification. Therefore double staining was essential, and was done with uranyl acetate and lead citrate, Watson (1958) and Reynolds (1963). The staining was carried out as follows :

1. Drops of 2% freshly prepared uranyl acetate were placed in grooves on a polythene sheet in a petri dish. Grids with

mounted sections were immersed in a drop of stain and the petri dish with the lid on was kept at 60°C for 15, 20, 30 and 45 minutes.

2. Grids were picked up from the stain after each period of exposure, washed under a stream of double (glass) distilled water and then soaked for five minutes in double distilled water.
3. After soaking, the grids were washed again under a stream of double distilled water, dried by touching the edge of the grid with filter paper, then immersed in drops of freshly prepared lead citrate placed in grooves on polythene sheets in a petri dish containing granules of NaOH (to reduce CO<sub>2</sub>). The petri dish was covered and kept at room temperature for 10, 15, 20 and 30 minutes.
4. The grids were picked up after each time period, washed under a stream of double distilled water, soaked for five minutes and washed again with double distilled water.
5. The grids were dried and kept in the grid holder box ready for the subsequent microscopical examination.

At least two grids from each specimen were kept unstained for comparison.

After trying different periods of exposure of the specimens to both stains, it was determined that potassium permanganate fixed materials needed approximately 30 minutes in uranyl acetate at 60°C and 20 minutes in lead citrate. While glutaraldehyde and osmium fixed specimens required 45 minutes in uranyl acetate at 60°C, another 15 minutes at room temperature, and 30 minutes in

lead citrate was sufficient.

## 2.9 Microscopy

At the first stage of the present work an A.E.I. EM6 transmission electron microscope was used; later, most of the work was carried out using the model A.E.I. EM6B. The latter is characterized by better resolution ~~at high magnifications~~; 24 plates can fit into the camera; the grid holder carries three grids; and it incorporates a better cooling device with liquid nitrogen.

### General advice to a timber technologist attempting to use transmission electron microscopy

Wood is characterized not only by the heterogeneity of the cell wall layers, but also by the variation of the chemical composition of each layer. This raises several extreme difficulties in the TEM field.

From the little experience of the present author in this field, these difficulties could be overcome as follows :

1. Absolute cleanliness, precaution, punctuality and carefulness are highly recommended in each step of the preparation for microscopy.
2. Examination of a section of the wood specimen a few microns thick is essential to find out the area of the specimen which is required for the investigation.
3. Small specimens approximately  $0.5 \text{ mm}^3$  give better results.
4. Cutting up the specimens should not be done in a dry condition.

5. Always use new razor blades wiped clean with acetone in the cutting and trimming stages so as to avoid damage of the cell wall which would occur with a blunt razor blade.
6. Rapid and gentle cutting of the specimens is required followed by immediate fixation, since it is important to preserve the cell structure in its original condition.
7. Freshly prepared fixatives, alcohol series, resins and stains are necessary.
8. A fume cupboard is essential when using methacrylate, osmium tetroxid and glutaraldehyde.
9. Some of the chemicals are expensive, therefore economical use of those chemicals is recommended.
10. The temperature in the whole experiment should be nearly constant. For instance an ice bath is recommended to be used in changing the solutions or the resins of the specimen.
11. Vacuum impregnation during fixation, infiltration and embedding is highly recommended. If some of the specimens float on the surface of the fixative or alcohol, they are unlikely to give good results, therefore these should be excluded from the experiment.
12. Great patience is required to pick up the specimens one by one with a toothpick from the used resin, drying them gently on filter paper and replacing in fresh resin. If you do confuse the specimens, it is advisable to throw them away, otherwise the results will be misleading.
13. The final desired orientation of wood specimens must be achieved before the solidification of the resin.

14. The glasswear contaminated with resin should be wiped with tissue then turned upside down for several hours to drain all the resin and then washed thoroughly with washing powder in cold water. Epon resin is carcinogenic, therefore disposable gloves should be used during handling of the resin. All the contaminated tissue, toothpicks, filter papers, gloves and the used resin should be polymerized in an oven before disposing of them.
15. A single sided safety razor blade can be used for the rough trimming, while an ordinary "Gillette" type razor blade (divided into six pieces and used in a holder) is recommended for the final trimming. Trimming should always be done with great care by cutting very thin slices. The edges (sides) of the pyramid should be smooth shiny without even a single particle adhering to them. If the trimming of the specimen is producing a powder-like offcut, or not going smoothly, it is very likely that the specimen is not fixed and infiltrated properly with the resin, therefore this specimen should be avoided. It was found that trimming of the block should be orientated according to the plane of the wood cell wall in the longitudinal direction. This helped very much in the sectioning.
16. When one reaches the final trimming, the operator will be very tense, and a slight wrong movement of the razor blade will damage the whole block. In this case it is better just to leave the trimming for a while, relax and start fresh again, otherwise one will damage most of the blocks

and this will cause more frustration.

17. Sectioning is the most time and nerve consuming stage. The person should be very patient and determined and never give up if the sections are not obtainable. If you are sure of your fixation, embedding and trimming, find out the reason of this poor sectioning, which might be due to the knife or to the microtome. Never be frustrated or surprised even if you cannot obtain sections for several days. Try to change the specimen block, do not insist on one block because even the block gets tired. The most difficult sectioning on wood is the transverse plane.
18. Wood ultra thin sections require a longer period of time in uranyl acetate and lead citrate.
19. If one is investigating decayed wood and demanding a certain particular part of the decayed cell wall, it is likely not to appear in TEM observation, because it might have been chopped away in the trimming stage. Therefore be hopeful and optimistic and investigate other grids belonging to specimen blocks.
20. Last, but not the least, be patient, try several ways in each stage, practice trimming and sectioning in all your spare time. The more you practice the more you discover your faults and the more you learn. Be determined and never give up because one day you will achieve your desire and this could well be in only one grid of one specimen.

### 3. RESULTS

#### 3.1 The effect of three different fixatives used for the specimen preparation for TEM observations

The three fixatives used, Sorensen's buffer with osmium, potassium permanganate and glutaraldehyde, gave different results; the first two named were very effective in quite different ways, whilst the third named was not satisfactory.

##### 3.1.1 Sorensen's phosphate buffer and osmium.

This fixative gave excellent details of the fungal cytoplasm as shown in Figs. 1, 2, 11, 12, 13, 14, 15, 16, 17, 18, 21, 22, 23, 28, 29, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 63, 69, 70, 71, 72 and 73. In most of these figures, the nucleus with its double membrane and nucleolus, mitochondria, lomasomes, plasmalemma and the fungal wall are very conspicuous.

##### 3.1.2 Potassium permanganate

Figs. 5, 6, 7, 8, 9, 10, 19, 20, 24, 25, 26, 27, 30, 31, 32, 33, 34, 61, 62, 64, 65, 66, 67, 68 and 74 to 93 show that one effect of this fixative is to destroy the cytoplasmic content of the fungal cell, but it also exhibits a high contrast of the cellulose microfibrils and their orientation in different layers of the wood cell wall.

##### 3.1.3 Phosphate buffered glutaraldehyde

Sections obtained from specimens fixed with this fixative

were not very satisfactory, because of the poor fixation of the cytoplasmic content of the fungal cell, Figs. 3 and 4 (although this fixative is considered one of the best). The poor fixation might be due to the presence of the fungal hyphae within the wood cell wall.

Of the three fixatives used, one (glutaraldehyde) was not satisfactory for decayed wood. The other two, however, proved very satisfactory, but for different purposes. Where detail of cell content was required Sorensen's fixative was used; but for detail of the wood cell wall potassium permanganate was in a class of its own. These two fixatives were used throughout the work for these specific purposes.

3.2 Fungal hyphae (*Phialophora fastigiata* S6A) in culture media  
(Figs. 1 to 4)

3.2.1 Cellulose medium (Nilsson, 1973)

The cell wall of filamentous fungi have been investigated by several workers with the conclusion that chitin forms a microfibrillar framework embedded in an amorphous layer (Aronson and Preston, 1960a,b; and Bartnicki-Garcia and Nickerson, 1962).

Fig. 1 (x13,616) shows a part of almost senescent fungal hypha surrounded by a wall in which the amorphous matrix is clear while the microfibrillar skeleton is not very obvious (this microfibrillar skeleton is shown clearly in Figs. 33, 34, 41, 46 and 47). The nucleus, mitochondria and lomasomes are apparent in the micrograph.

In Fig. 2 (x25,463) the amorphous surrounding of the cell wall is well defined.

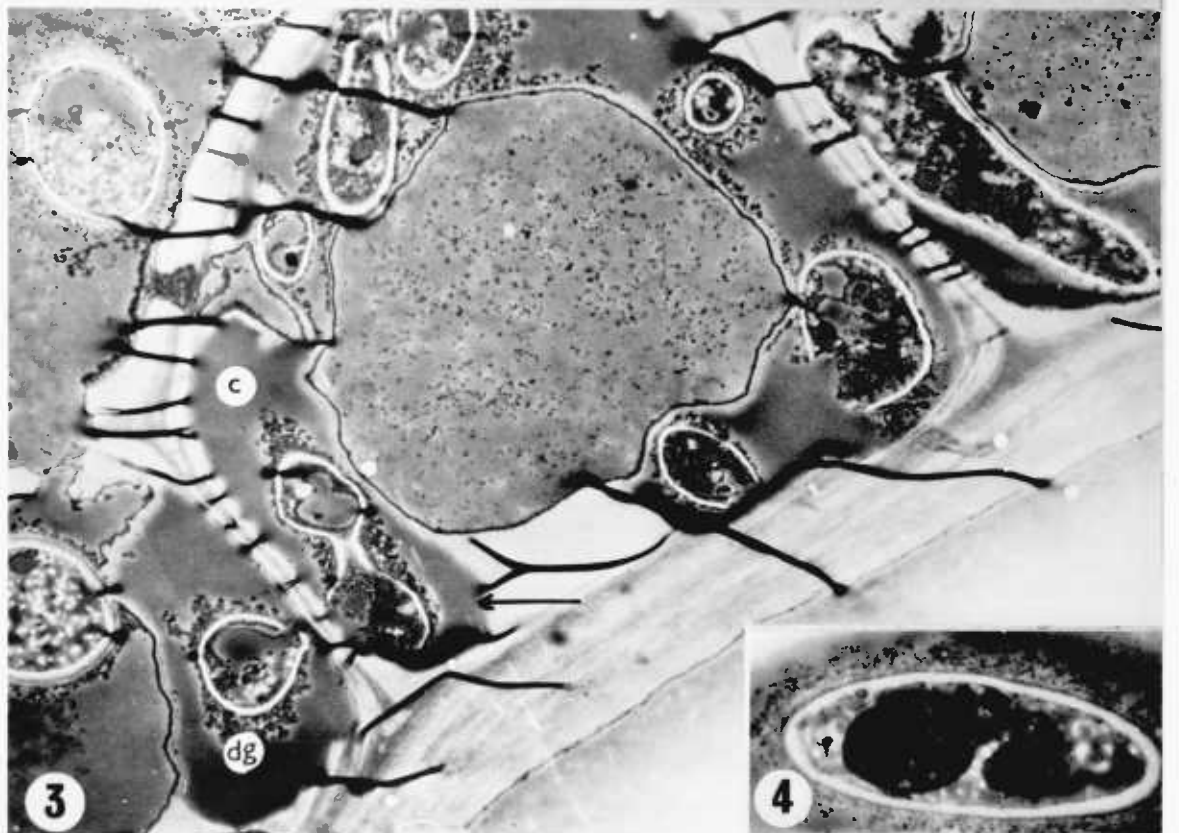
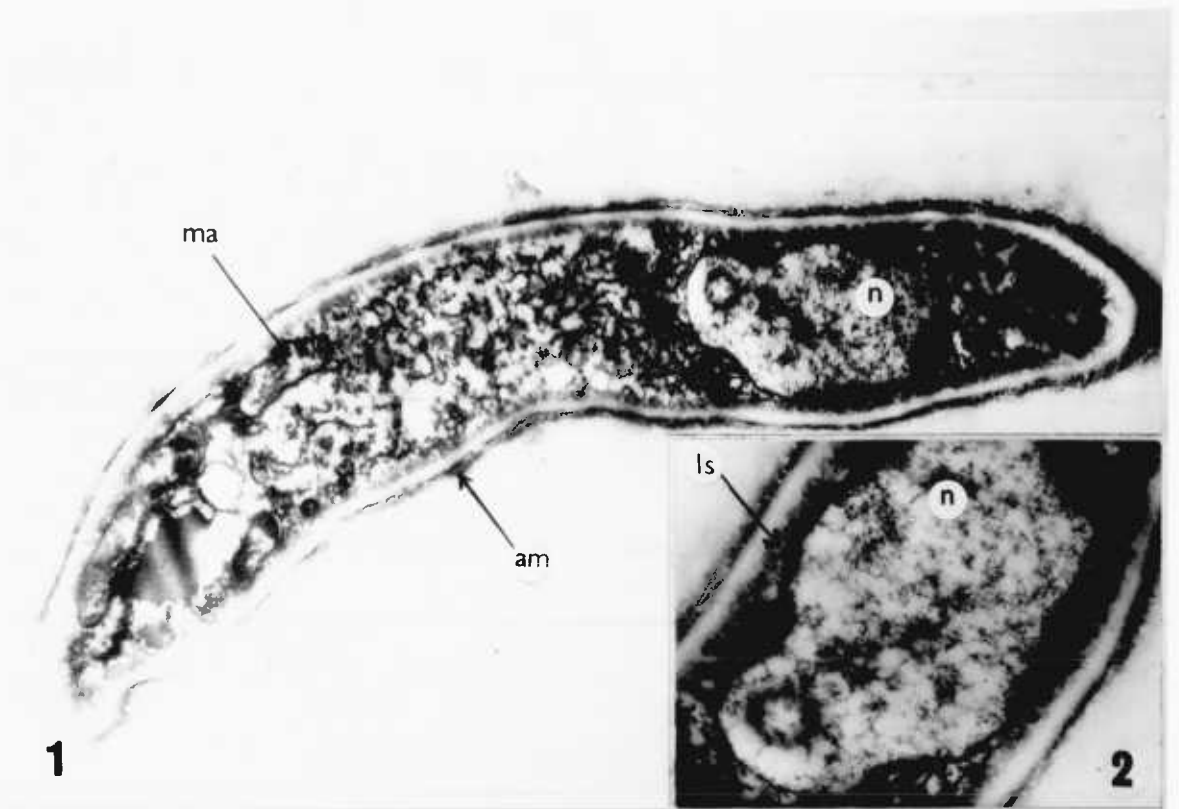
### 3.2.2 Mixture of birch sawdust and malt agar medium

A small piece of the medium was fixed and embedded to investigate the behaviour of fungal hyphae in birch sawdust.

Fig. 3 (x6,936) is a view of a transverse section of several cells of birch sawdust attacked by the fungus, causing enormous soft rot cavities in the  $S_2$  layer. Most likely these big cavities have been formed by the coalescence of several small cavities (see Figs. 32, 61 and 62 in Part III). The arrowed cavity has a very sharp pointed-end (see Fig. 33 in Part III). The interesting feature of this micrograph is the presence of dark granules surrounding the hyphae when they are in the  $S_2$  layer and in the lumen.

Fig. 4 (x11,877) shows an oblique section of fungal hypha in the medium surrounded by dark granules, which are not present in Figs. 1 and 2.

The specimens shown in Figs. 3 and 4 were embedded in methacrylate styrene which was found to produce folding (the black line in the micrograph) more frequently than epon embedded specimens. Thus epon resin was used extensively in the present work.



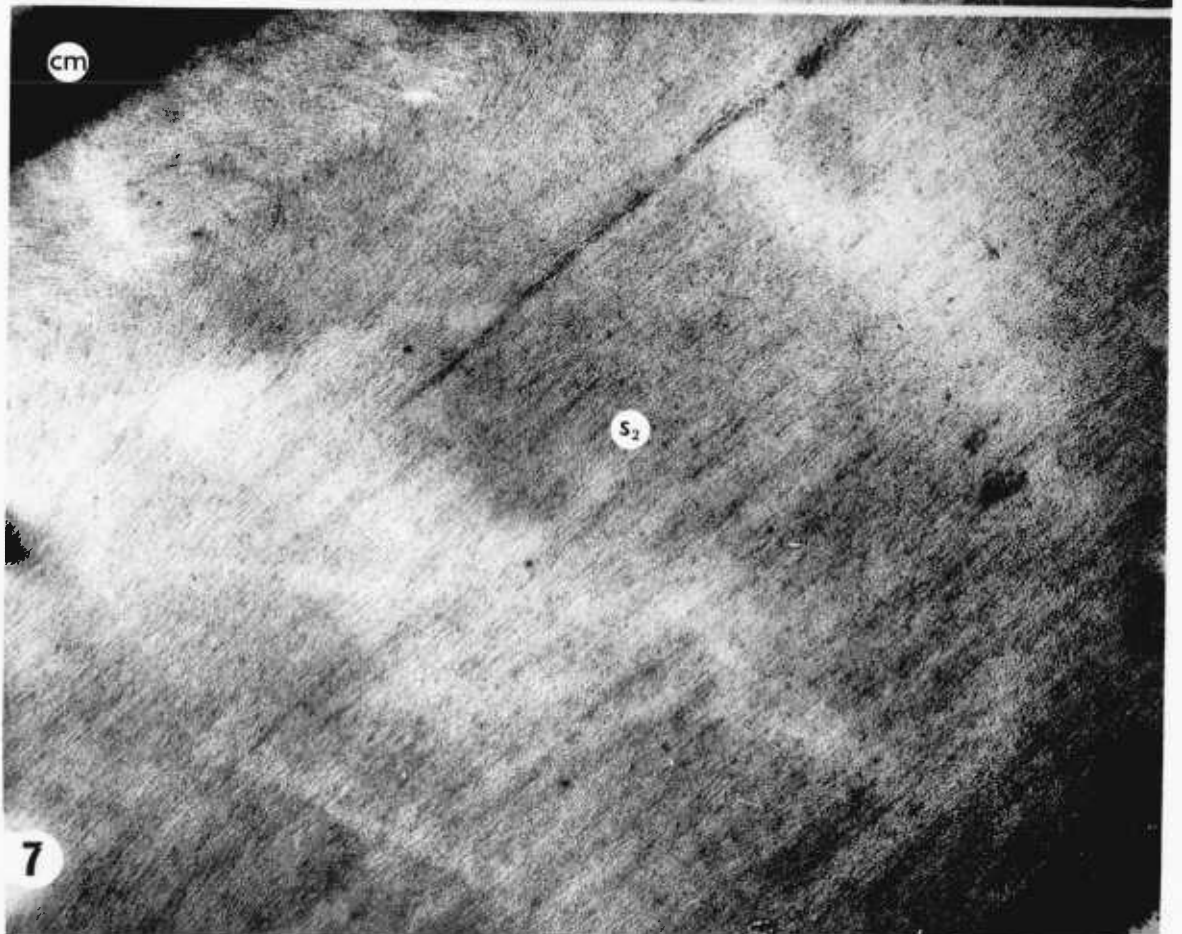
### 3.3 Orientation of microfibrils in sections of the wall

#### 3.3.1 In normal birch (*Betula* sp.) (Figs. 5 to 7)

The figures are representing longitudinal sections and show the orientation of cellulose microfibrils in each layer of the cell wall.

Fig. 5 (x35,620) shows the compound middle lamella (middle lamella and primary walls) and the  $S_3$  layer which have a darker colour than any other cell wall layer. These two layers are characterized by high lignin content per unit of volume (Berlyn and Mark, 1965; and Mark, 1967), therefore one would expect according to Hepler, Fosket and Newcomb (1970), that potassium permanganate which was used as fixative caused the dark staining of the lignin component of the cell wall. The white striations in the cell wall are artefact produced by uneven sectioning and staining.

In Fig. 6 (x41,230) the orientation of microfibrils are well defined, but it is difficult to draw a sharp line between the borders of two layers because of the presence of transitional microfibrils between the layers. These microfibrils are conspicuous in the micrograph between the  $S_1$  and  $S_2$  layers, and they are changing their orientation gradually, Harada (1965).

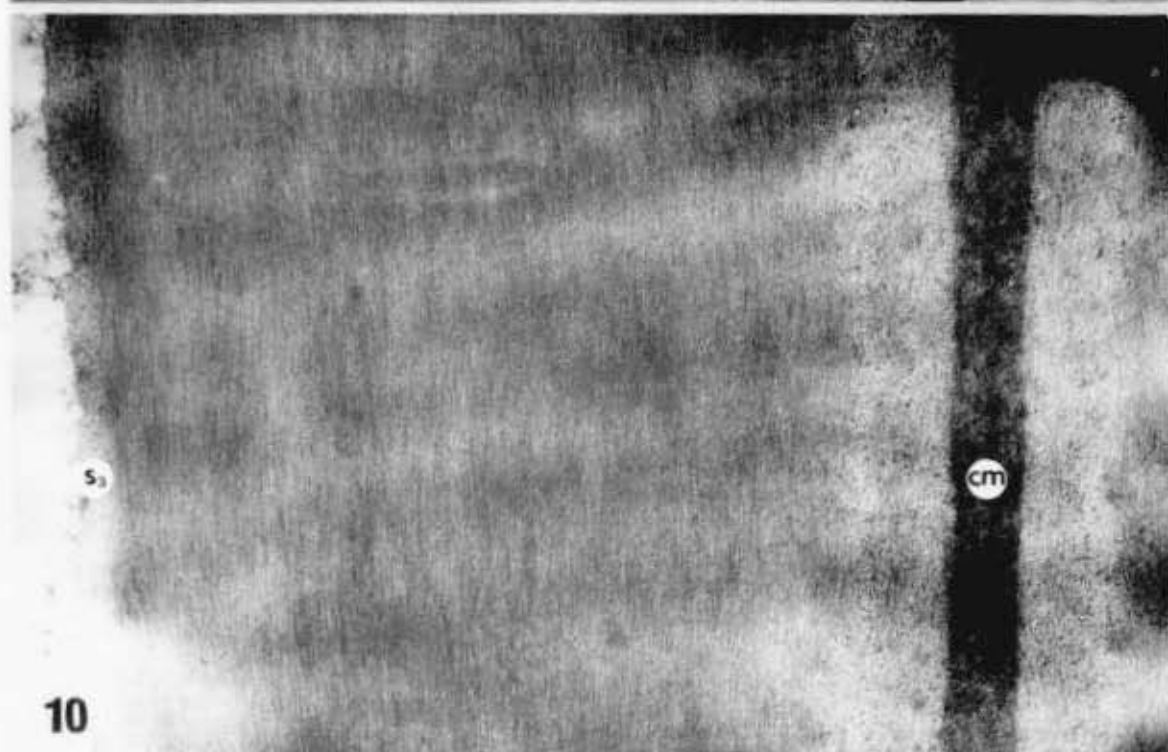
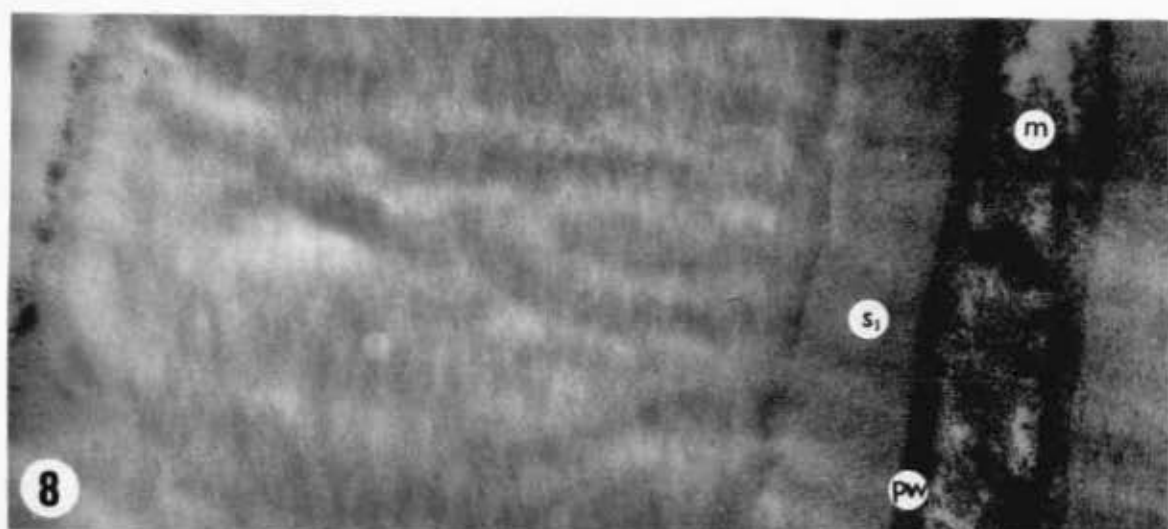


In Fig. 7 (x57,200) the microfibrils are very conspicuous as white dots against the dark background of the electron stain, (Heyn (1969)). The orientation of microfibrils in the  $S_1$  layer is not flat or perpendicular on the axis of the fibre (compare with Figs. 8 and 10), because of the oblique sectioning which has a similar effect on the orientation of microfibrils in the  $S_3$  layer. The microfibrils in the  $S_2$  layer are running in a steep helix which could be  $20^\circ$  to  $30^\circ$  to the axis. The microfibrils of the  $S_3$  layer in all the three figures are not as well defined as in delignified Scots pine (Figs. 8, 9 and 10) probably because this layer is not as well developed in birch as in Scots pine.

3.3.2 In delignified Scots pine (Pinus sylvestris)  
(Figs. 8 to 10).

Figs. 8 (x27,971), 9 (x47,207) and 10 (x45,000) show the effect of the delignification on the cell wall layers.

Apparently there are no visible differences in the orientation of the microfibrils of the cell wall layers in these figures by comparison with Figs. 5, 6 and 7, except that the middle lamella shows signs of disorganisation and is less dense than in normal wood.



### 3.4 Normal wood attacked by fungi

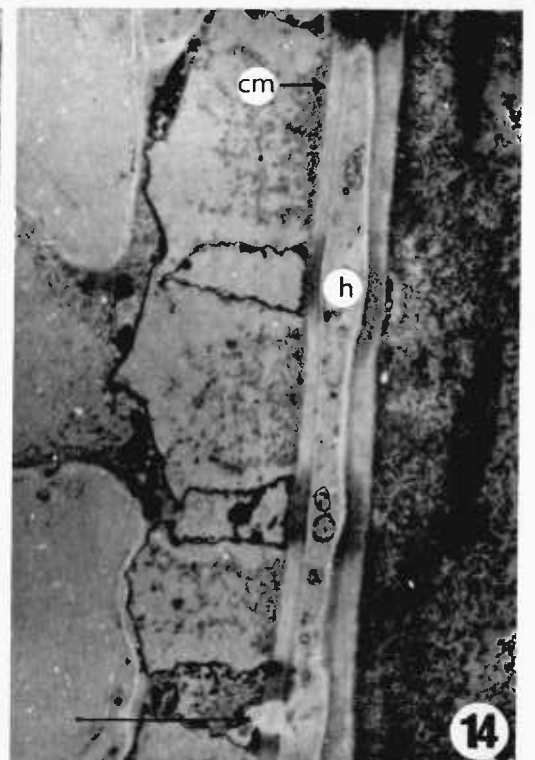
#### 3.4.1 Birch attacked by Chaetomium globosum S70B cultured on 2.5% malt agar medium (Figs. 11 to 14)

Fig. 11 (x4,781) shows a radial section of ray parenchyma cells with two pit pairs, through which plasmodesmata pass. From this figure one can assume that the cytoplasm of the ray parenchyma cells were still alive when the fixation of the specimen was carried out, therefore apparent plasmolysis occurred. The fungal hypha is penetrating through one of these areas of plasmodesmata to the adjacent cell.

Fig. 12 (x9,401) is a high magnification of the penetration of the hypha through a plasmodesma. The presence of the dark granules (arrowed) is difficult to interpret; they may or may not be an artefact.

Fig. 13 (x31,887) is a high magnification of a plasmodesma in which the continuation of the endoplasmic reticulum is apparent. Small portions of fungal hyphae are showing in the two cells which are connected by the plasmodesma.

Fig. 14 (x8,305) shows an unusual phenomenon which has been observed before by Findlay (1970) in birch wood attacked by Phialophora fastigiata S6A. The fungal hypha is lying in the compound middle lamella region without soft rot cavity formation. The cell on the left of the micrograph shows numerous pit canals, in the third one of which (arrowed), the fungal hypha has branched.



3.4.2 Birch attacked by Phialophora fastigiata 731-1-3b

cultured on cellulose agar medium (Figs. 15 to 68)

Sectioning of decayed wood to obtain the desired aspect is mere luck, because one can never predict whether the section will be in the desired area. Thus hundreds of specimens were sectioned to obtain the very early stages of penetration, T-branch and cavity formation, without much success. Nevertheless the advanced stages of soft rot decay brought to light new observations of the behaviour of soft rot fungus in the wood cell walls.

The mode of the decay caused by this fungus is represented by micrographs and are classified as follows :

- (a) Colonization of the lumen
- (b) Penetration of the cell wall
- (c) T-branching in the cell wall
- (d) Cavity formation in the cell wall
- (e) Examples of unexpected phenomenon which occurred during penetration, T-branching and cavity formation

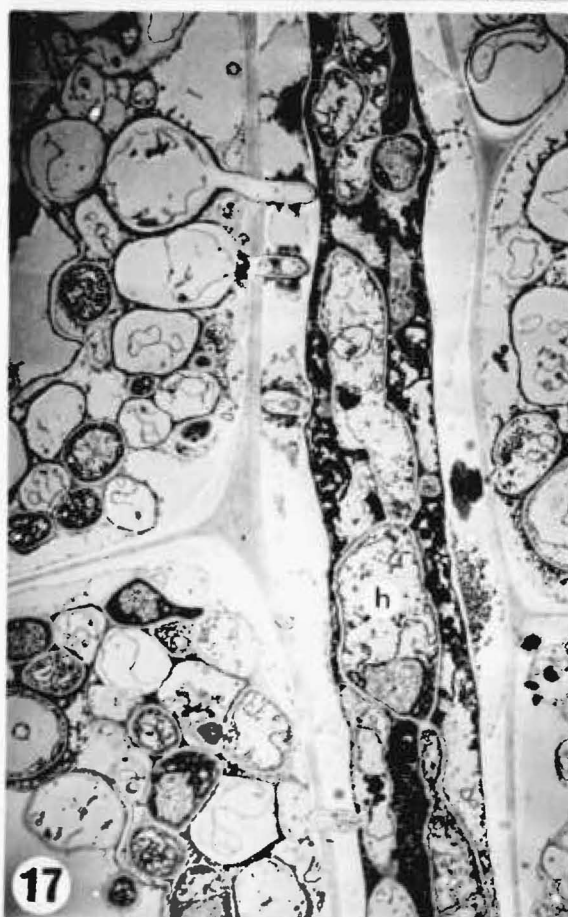
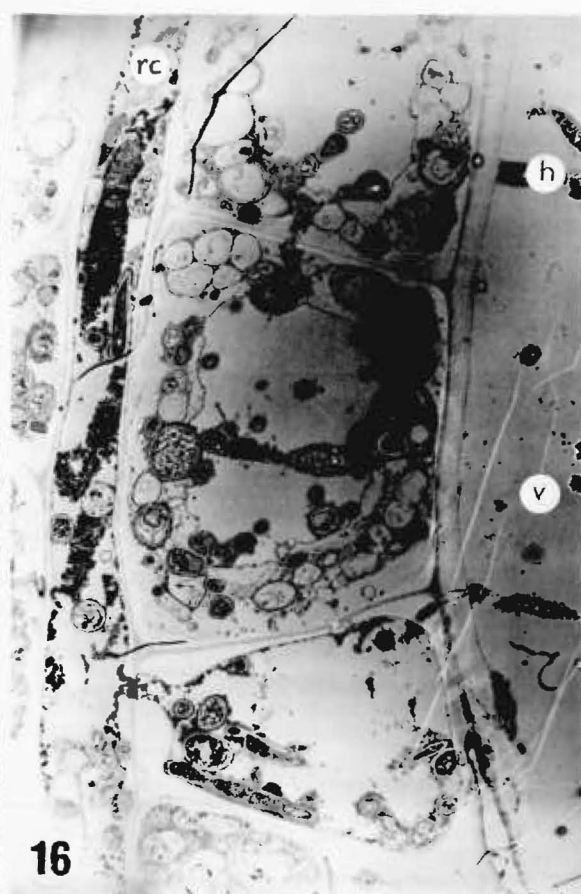
(a) Colonization of the lumen (Figs. 15 to 18)

Fig. 15 (x3,640) is a radial section of a group of ray parenchyma cells with severe plasmolysis. The cytoplasm of these cells are colonized by fungal hyphae. The hypha (arrowed) gave a branch which penetrated into the adjacent fibre wall and then continued to pass through the wall and into the lumen of the fibre. This hypha is not apparent in the  $S_2$  layer because of the non median-sectioning.

Fig. 16 (x2,139) is a transverse section of a part of a vessel on the right of the micrograph, the elongated ray parenchyma cells on the left and in between these are a few fibre cells. The ray parenchyma cells show heavy colonization by fungal hyphae and there are also some in the vessel lumen. It is difficult to interpret from this micrograph whether the initial infection and the degradation of the fibres by the fungus has originated from the vessel or from the ray cells.

Fig. 17 (x5,061) shows a ray parenchyma cell colonized heavily by the fungal hyphae in which several branches have occurred.

Fig. 18 (x11,356) is an enlargement of part of Fig. 17 showing two senescent hyphae which had formed penetration hyphae by reducing their diameter and then penetrated through the compound middle lamella and formed cavities in the  $S_2$  layer of the ray parenchyma cell wall.



(b) Penetration of the cell wall (Figs. 19 to 23)

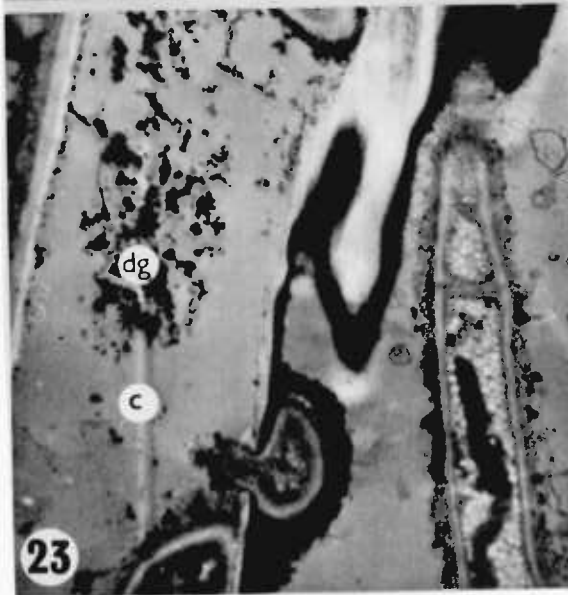
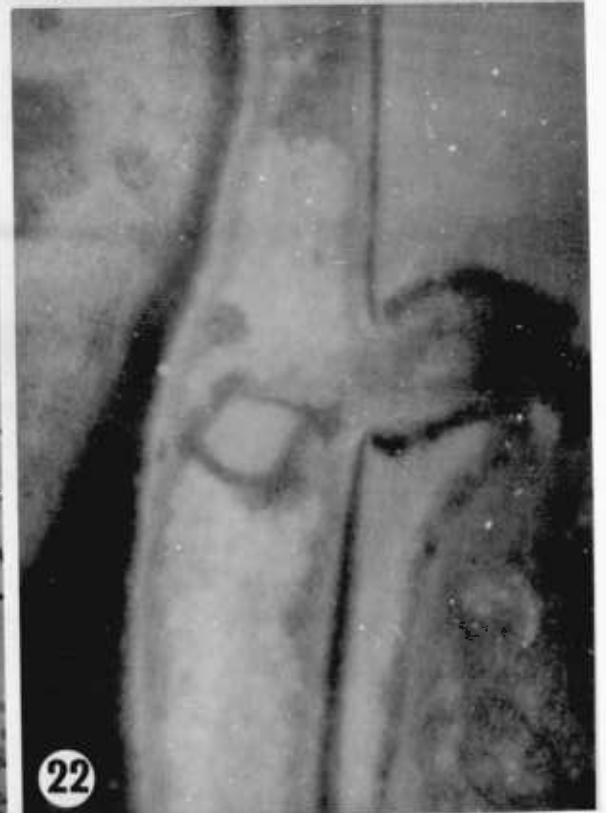
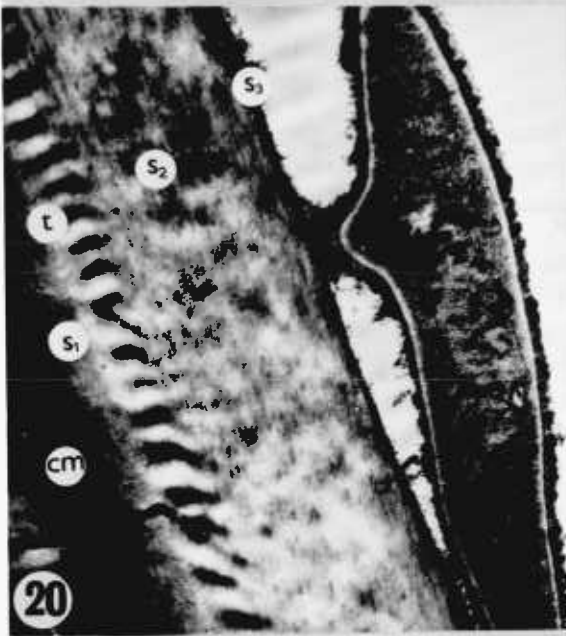
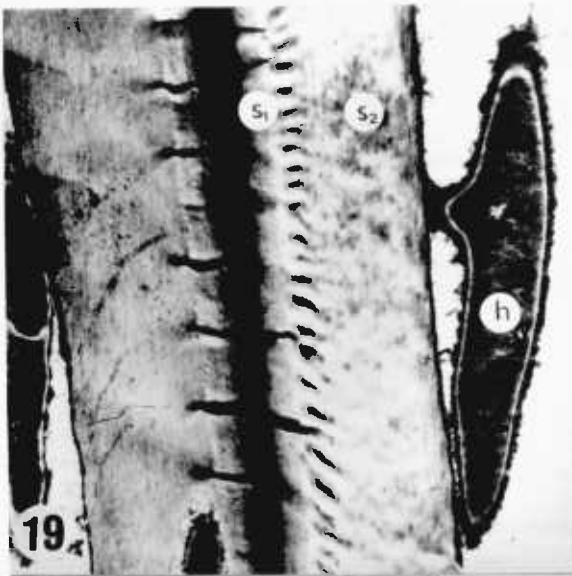
Fig. 19 ( $\times 8,386$ ) shows adjacent walls of two fibres with two hyphae each lying in the lumen of a fibre. There is a clarification in the  $S_2$  layer of the fibre on the right of the micrograph. This clarification is most probably due to uneven sectioning because of uneven infiltration of epon resin, especially into the microfibrils of the transitional area between the  $S_1$  and  $S_2$  layers. The hypha on the right of the micrograph has a small protrudence towards the fibre wall, almost touching the  $S_3$  layer.

Fig. 20 ( $\times 12,257$ ) is a high magnification of the previous figure showing the compound middle lamella,  $S_1$  layer, the transitional microfibrils, the  $S_2$  layer, the  $S_3$  layer and the hypha.

In Fig. 21 ( $\times 6,600$ ) the fungal hypha (h) is lying in the lumen of the fibre parallel to and contiguous with the long axis of the  $S_3$  layer. This hypha gave a very fine branch which has penetrated the  $S_3$  layer into the  $S_2$  layer nearly at a right angle through a small bore hole.

Fig. 22 ( $\times 27,655$ ) is a high magnification of the previous figure showing the penetration point of very fine hypha which is a branch from the hypha in the lumen.

Fig. 23 ( $\times 9,138$ ) presents another bore hole formation through the  $S_3$  layer into a cavity containing dark granular material in the  $S_2$  layer.



(c) T-branching in the cell wall (Figs. 24 to 27)

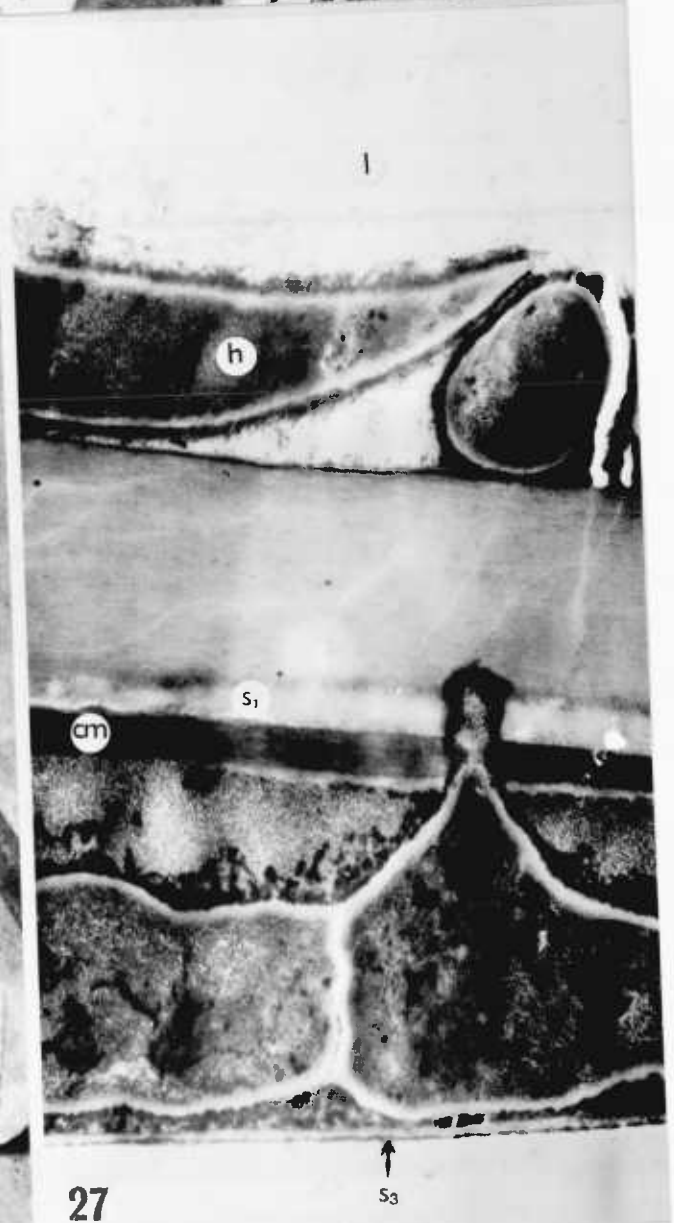
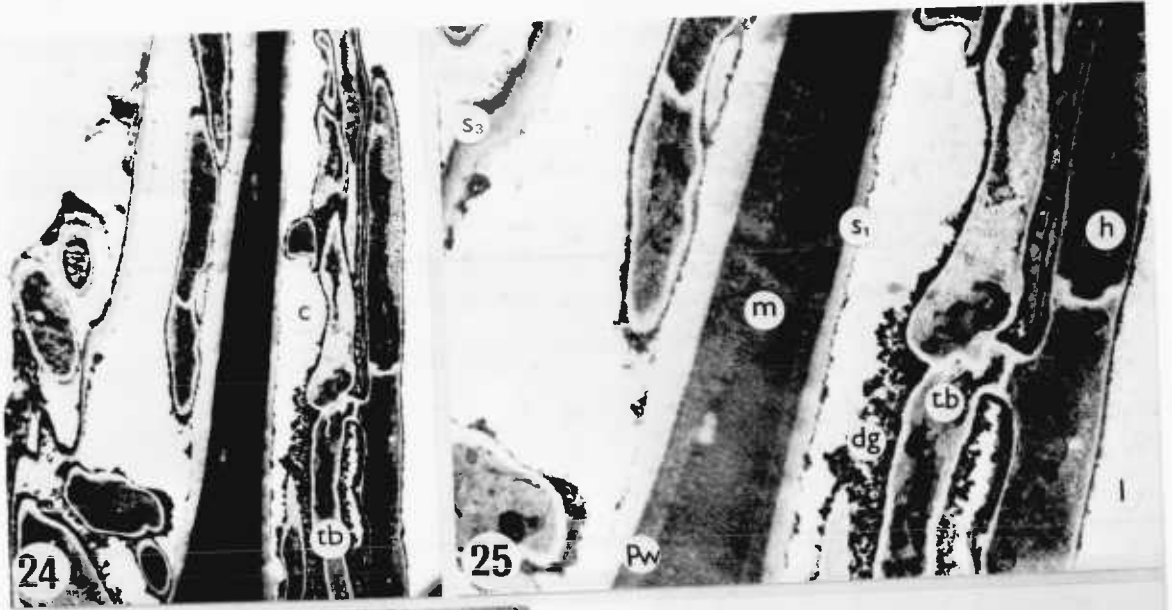
The penetration hypha after passing into the  $S_2$  layer at right angles to its orientation in the lumen either gives a T-branch (the initial stage of cavity formation) in the  $S_2$  layer of the first cell wall, or it proceeds through all the layers of the first cell wall until it reaches the  $S_2$  layer of the second cell and then forms a T-branch there. Both cases have been observed in the present work.

Fig. 24 (x 4,253) is a longitudinal section of a very late stage of a T-branch which produced an enormous cavity in the  $S_2$  layer of the first cell wall causing the disappearance of the whole layer (although the specimen was incubated for 1 week only). The interesting phenomenon of this micrograph is the high resistance of the  $S_1$  and the  $S_3$  layers.

Fig. 25 (x 7,521) is a high magnification of the T-branch area showing more clearly the middle lamella, primary wall,  $S_1$  layer, the whole of  $S_2$  which has been replaced by a cavity with the T-branch hypha, the  $S_3$  layer and the hypha lying in the lumen parallel and contiguous to the  $S_3$  layer. The T-branch hypha is surrounded by dark granular material, while the hypha in the lumen has a more amorphous surrounding. A septum has formed exactly at the point of penetration through the  $S_3$  layer.

Fig. 26 (x15,413) shows another T-branch within a cavity and also shows the resistant  $S_3$  and  $S_1$  layers and a septum formation at the former.

Fig. 27 (x16,656) shows a T-branch which has occurred in the  $S_2$  layer of the second cell after penetration through the first cell. The micrograph presents a hypha in the lumen which gave a branch which penetrated the cell wall layers of the first cell then penetrated the compound middle lamella with a constriction and then gave a T-branch in the  $S_2$  layer of the second cell wall resulting in cavity formation. Unfortunately it is apparent that the sectioning did not occur exactly in the median plane, therefore the penetration hypha is not apparent in the first cell wall except for a small part in the  $S_1$  layer.



(d) Cavity formation in the cell wall (Figs. 28 to 38)

Fig. 28 (x5,625) is a longitudinal section of two fibre cells with typical soft rot cavities in the  $S_2$  layer. The top cavity has a pointed end (arrowed), while in the lower cavity the hypha is constricted (arrowed) and a septum is present in the constricted area.

Fig. 29 (x9,614) shows the previous figure in a higher magnification.

Fig. 30 (x6,812) shows two fibres with very large soft rot cavities occupying the whole of the  $S_2$  layers. One of the cavities has a very sharp pointed end towards the  $S_1$  layer.

The  $S_1$  and the  $S_3$  layers are intact.

Fig. 31 (x13,650) shows the pointed end of the cavity shown in Fig. 30 which is ending exactly in the microfibrils of the transitional area between the  $S_1$  and  $S_2$  layers. The cavity has a sharp edge along which the microfibrils are cut very sharply. The dark granular material is surrounding the hypha in both cavities.

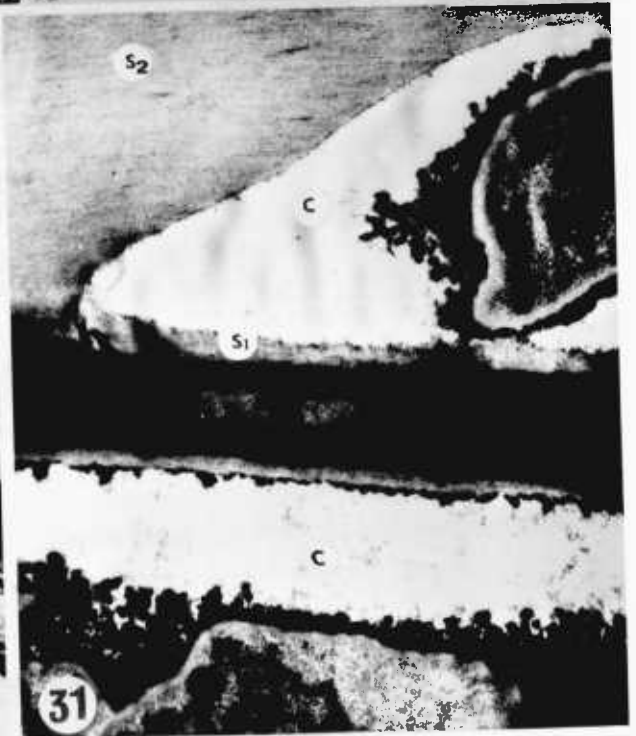
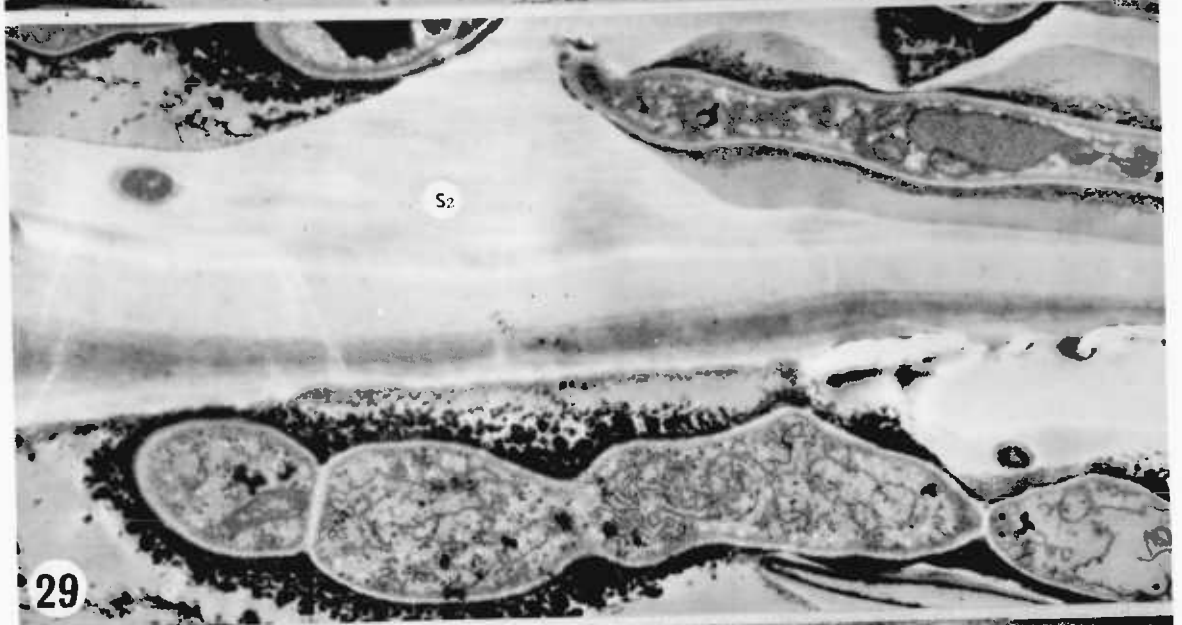


Fig. 32 (x11,852) shows two large cavities in two fibre cells, one with pointed end and the other with almost flat end. The interesting phenomenon in this micrograph is that there is a relatively large space between the end of the cavity and the hypha. One would assume that the end of the hypha does not represent the tip of it, probably because the whole fungal hypha was not on the same plane of sectioning which may also explain the absence of a pointed end to the cavity.

Fig. 33 (x28,869) is a high magnification of a part of Fig. 32 showing the orientation of the microfibrils in each layer of the secondary wall. The evidence of the section being non-median is conspicuous from the flat ended cavity which shows the thickness of the section.

Fig. 34 (x35,833) is a little out of focus but it shows the edge of the cavity clearly. The hyphal wall (arrowed) with its microfibrillar skeleton and the amorphous matrix are well defined in the cavity on the left hand side of the micrograph.

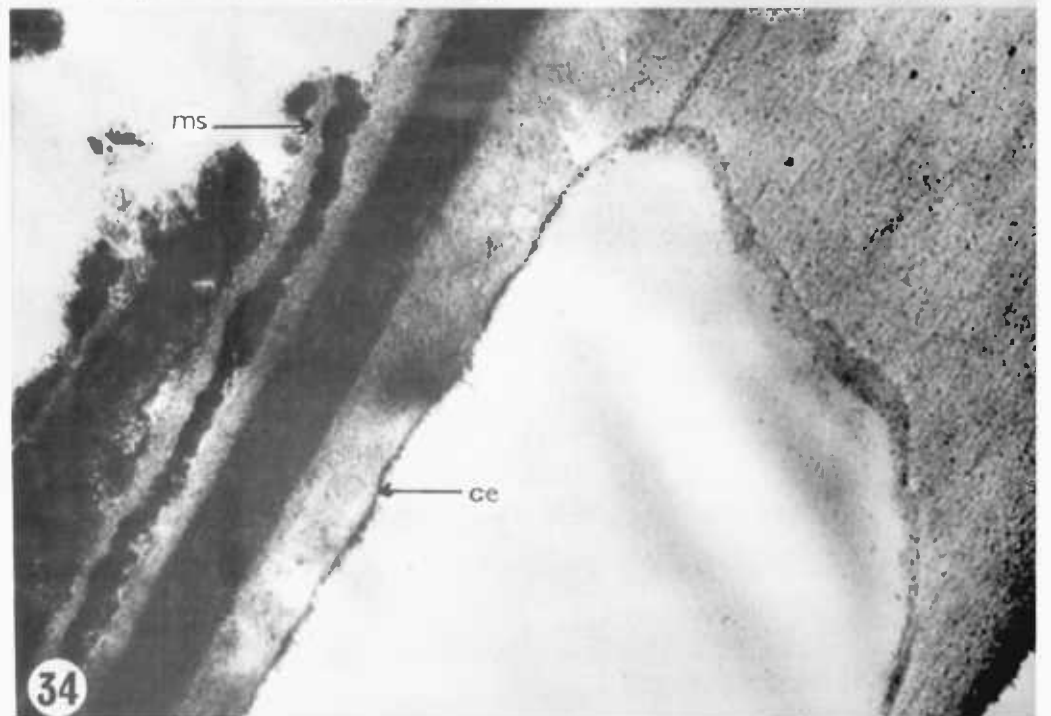
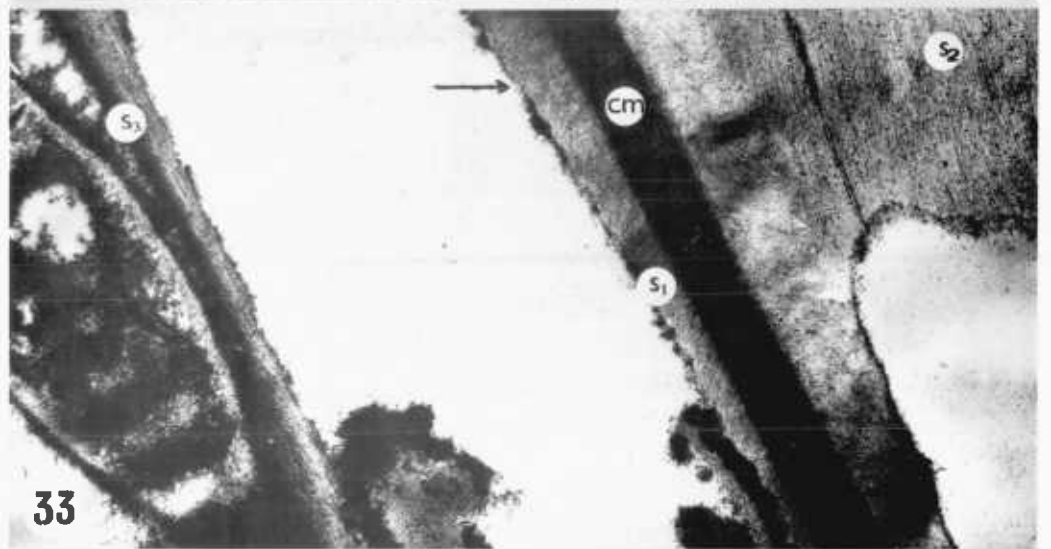
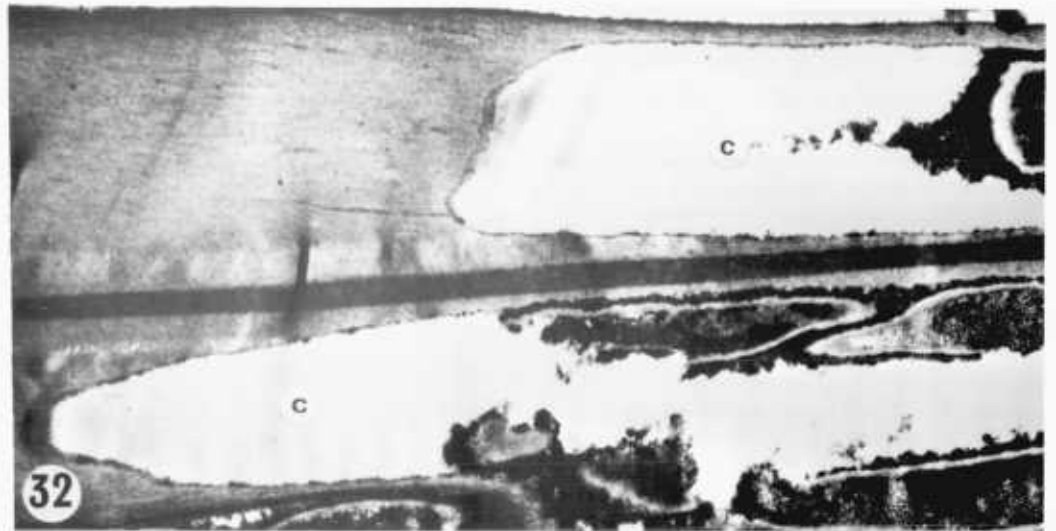


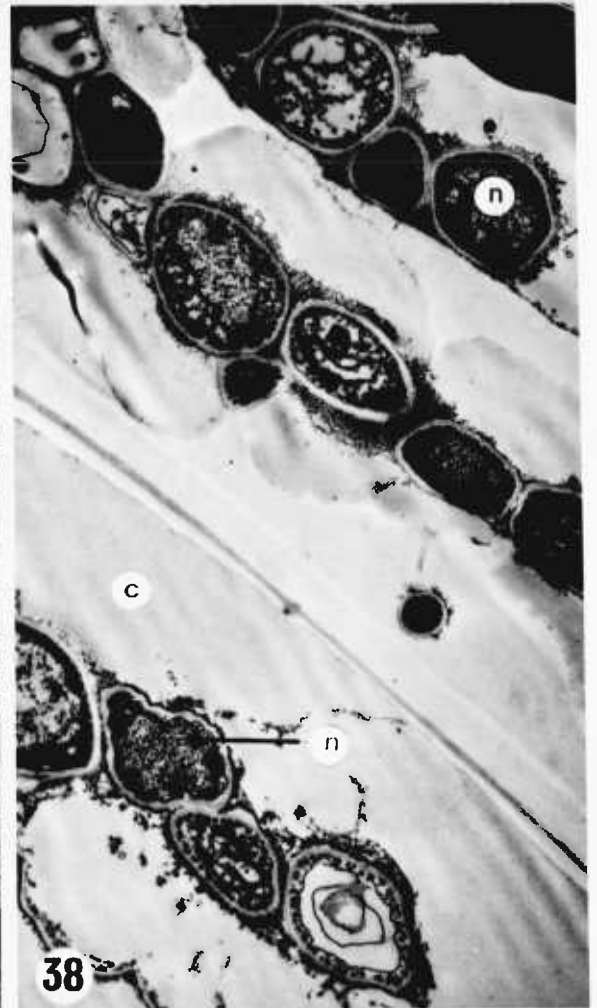
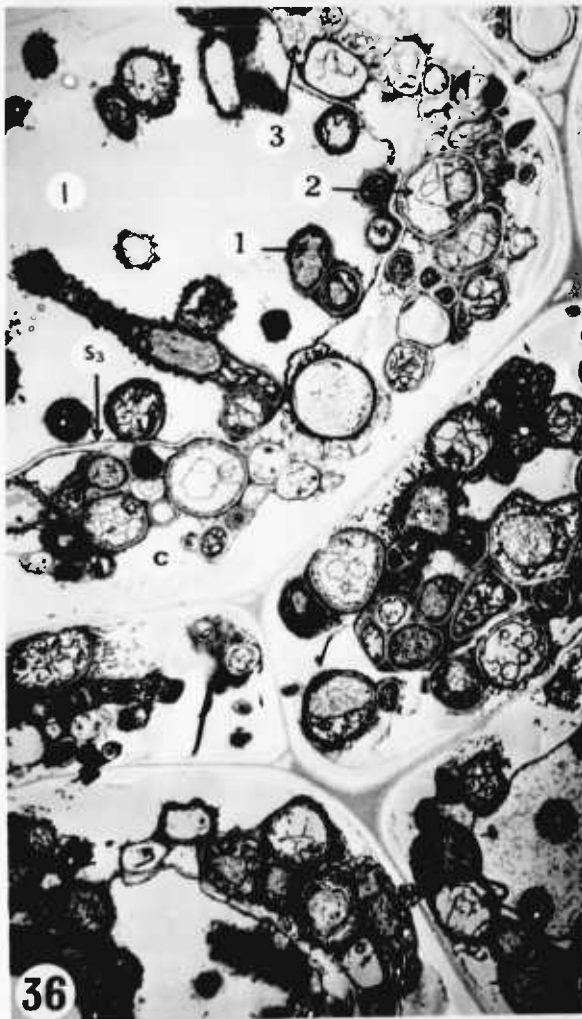
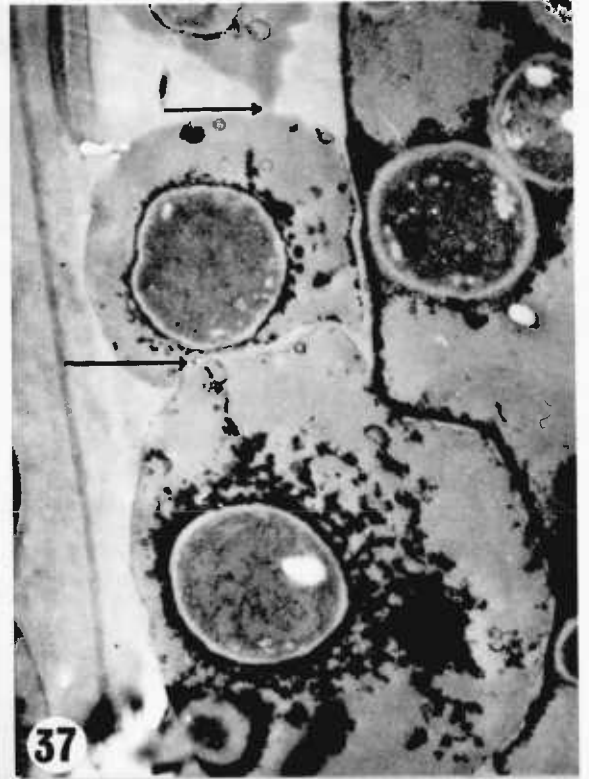
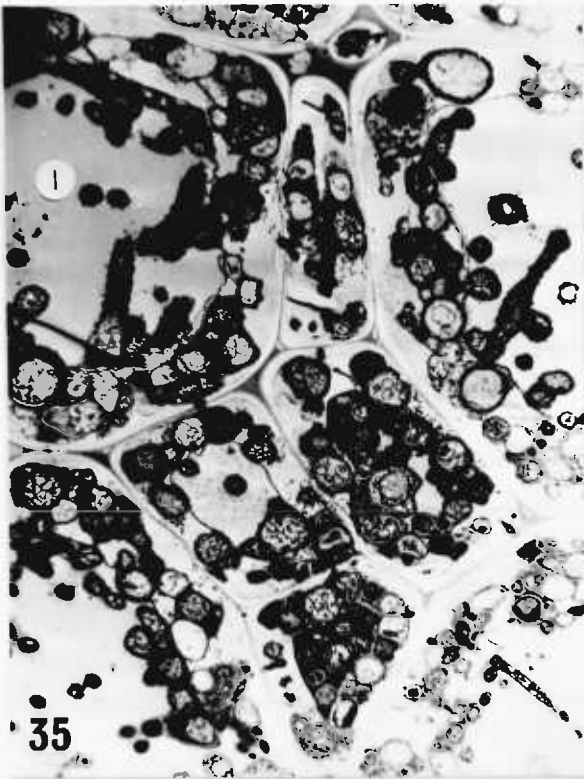
Fig. 35 (x2,360) is a transverse section of an area consisting of several fibres degraded severely. The compound middle lamella between the fibres is apparent as thin dark lines. The fungal hyphae are occupying the lumina and the  $S_2$  layer of the cell walls.

Fig. 36 (x4,135) is a higher magnification of a few fibres from Fig. 35 showing many cavities which have coalesced, producing larger cavities in the  $S_2$  layers of the fibres (compare with Figs. 32, 61 and 62 in Part III). These cavities and lumina of the fibres are occupied by very many fungal hyphae which can be classified as follows : (1) very young hyphae with dense cytoplasmic content. (2) highly vacuolated, probably senescent hyphae. (3) nearly lysed hyphae leaving debris behind.

Another interesting phenomenon which this micrograph presents is the high resistance of the  $S_3$  layer to the decay. Apparently this layer was affected by the looseness of the  $S_2$  layer after enormous cavity formation in the latter which produced a shapeless  $S_3$  layer (compare with Figs. 62 and 63 in Part III).

Fig. 37 (x3,795) is a transverse section of three cavities produced by three hyphae in the  $S_2$  layer of a fibre. The top cavity has already coalesced with the middle cavity by a small branch (arrowed), while there is still a narrow band of the  $S_2$  layer between the middle and the bottom cavities.

Fig. 38 (x12,979) shows the coalescence of cavities forming a long irregular shaped cavity in a transverse section. The sections of hyphae in the lumen and within cavities represent mostly young hyphae with nuclei. The amorphous surrounding of the hyphae on the right of the middle lamella suggests that these are secondary invading hyphae to an already existing cavity, Findlay (1970).



- (e) Examples of unexpected phenomenon which occurred during penetration, T-branching and cavity formation (Figs. 39 to 68)

1. Septum formation :

One of the most interesting phenomenon observed for the first time in the present work was the constant appearance of a septum at the point of penetration of the hyphae at the  $S_3$  layer and the middle lamella regions.

An examination of the electron micrographs taken by Findlay (1970), Unligil et al. (1974) and Liese et al. (1975), reveals the existence of the same phenomenon although it was not commented on, and its significance may therefore not have been appreciated.

1.a Septum formation in the penetration hypha at the  $S_3$  layer region

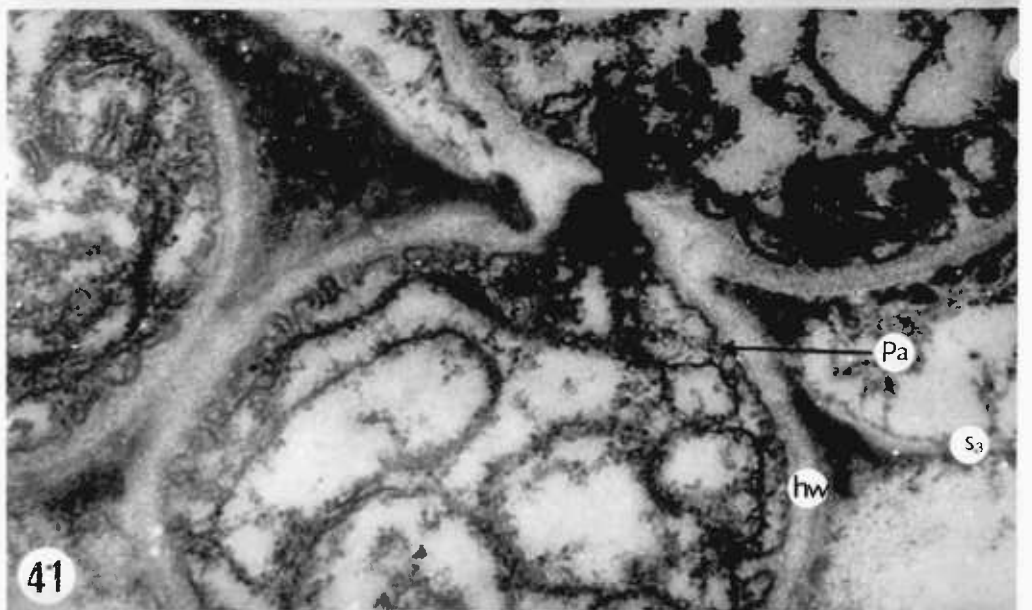
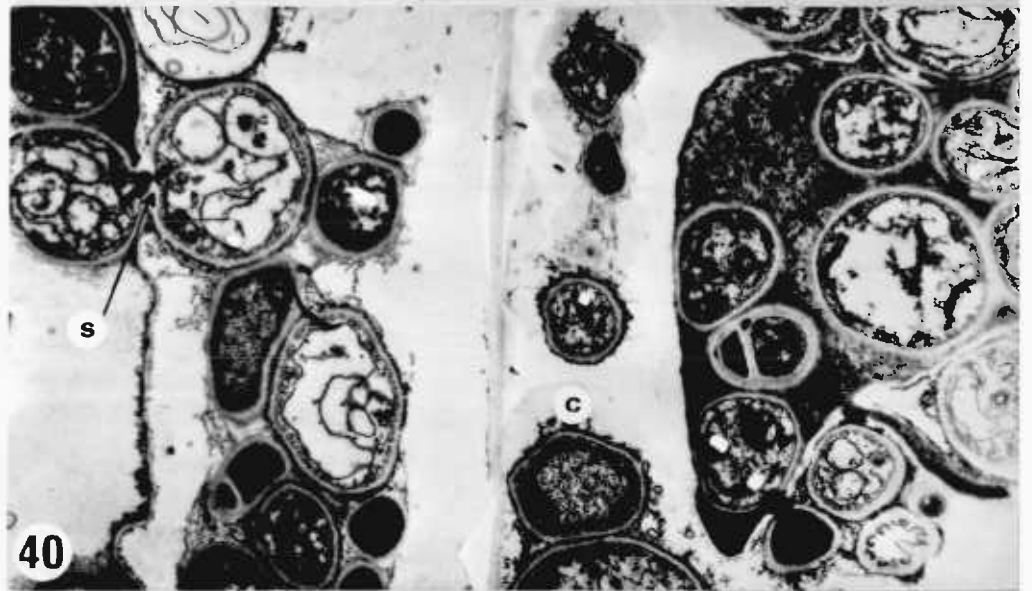
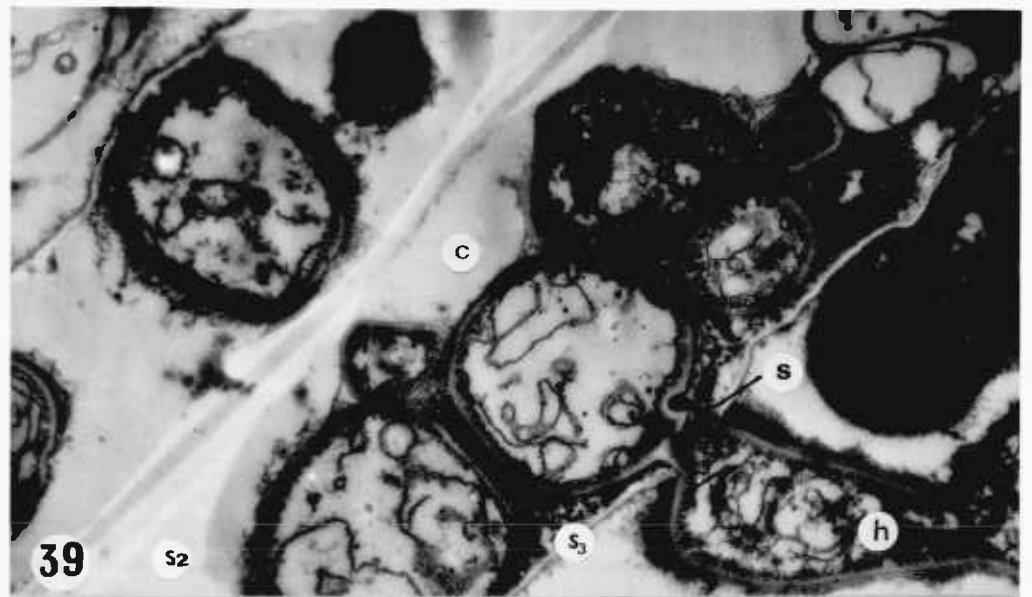
Figs. 24, 25 and 26 (p. 157) are longitudinal sections showing the septum formation in the penetration hyphae at the  $S_3$  layer level, while Figs. 39, 40 and 41 demonstrate the phenomenon in the transverse sections.

Fig. 39 (x14,806) shows an old fungal hypha (h) which has penetrated from the lumen into the  $S_2$  layer of the secondary cell wall through the  $S_3$  layer with a constriction. At this point a septum is formed in the constricted hypha at the level of

$S_3$  layer but because of non-median sectioning, the septum is not shown exactly in the plane. There is also a hypha in the cavity in the  $S_2$  layer of the adjacent fibre.

Fig. 40 (x12,004) is another feature of septum formation in the  $S_3$  layer with a very highly constricted penetration hypha. The highly vacuolated appearance of this hypha suggests it may be a relatively senescent hypha.

Fig. 41 (x47,277) shows the area of penetration in Fig. 40 in higher magnification. The continuation of the hyphal wall with plasmalemma through the  $S_3$  layer, and the amorphous matrix of the hyphal wall and lomasomes are conspicuous.



1.b      Septum formation in the penetration hypha at the level  
            of middle lamella

Fig. 42 (x13,460) shows a transverse section of several fibres degraded by soft rot cavities. Apparently the fungal hypha (h) within a cavity in one cell reduced its diameter remarkably at the point of leaving the cavity edge. It then penetrated through the  $S_1$  layer of the first cell wall, the compound middle lamella, the  $S_1$  layer of the second cell wall and then reached the  $S_2$  layer (which is replaced by a cavity) of the second cell wall.

Fig. 43 (x7,403) is a serial section of the previous figure demonstrating very clearly that misinterpretation of electron micrograph could very easily occur. For instance the middle hypha (which is penetrating through the compound middle lamella) appears to be a separate hypha from the large hypha behind it, but in fact both belong to one hypha which is apparent in Fig. 42. Therefore some of the transverse sections of hypha in the cell wall or in the lumen might belong to one hypha or to several hyphae.

This micrograph shows another penetration hypha which is most probably originating from the cell wall on the right hand side of the micrograph. The hypha with a reduced diameter at the point of leaving the cavity has penetrated the  $S_1$  layer, the intercellular space, the  $S_1$  layer of the second cell wall and then the cavity in the  $S_2$  layer of the second cell wall. The hypha expanded on entering the cavity (which is replacing the degraded  $S_2$  layer) and also has formed a septum in the intercellular space.

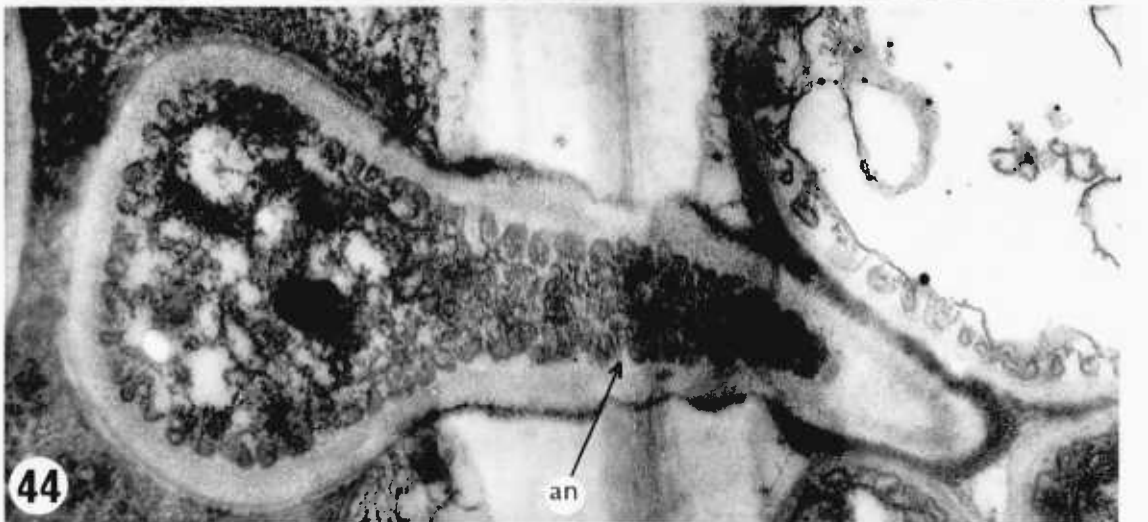
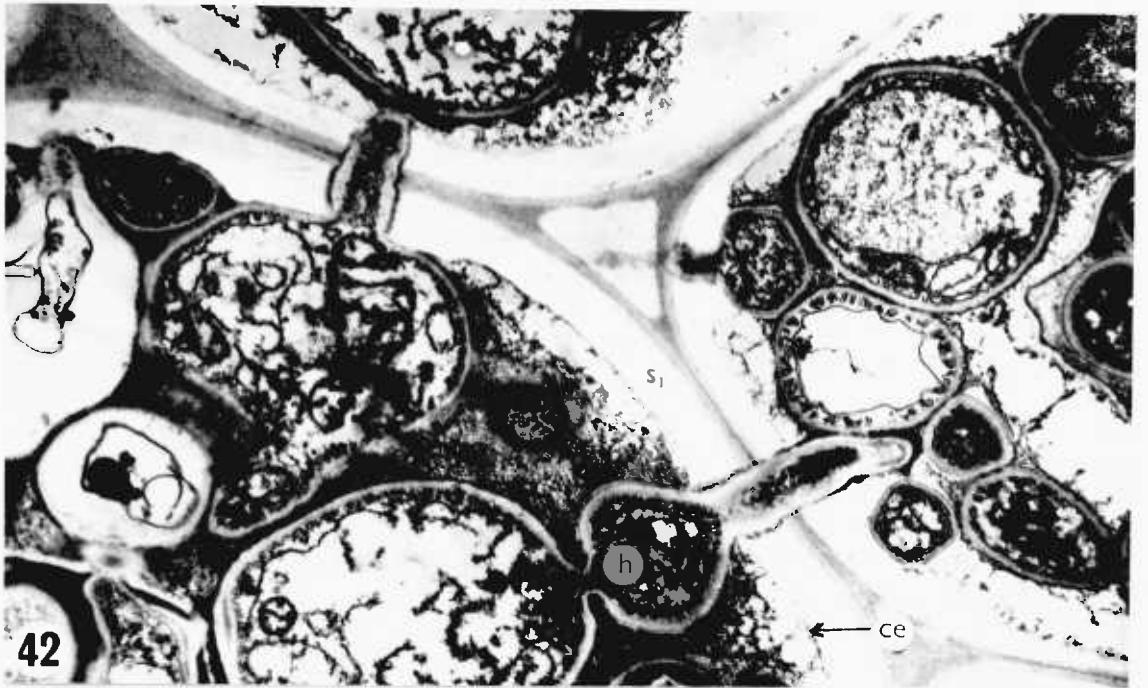


Fig. 44 (x39,827) is a higher magnification of the penetration hypha showing the constriction of the hyphal wall at the middle lamella level. The initial stage of the septum formation (arrowed) is apparent as a small annulus in the hyphal wall from inside, Gay and Martin (1971), and also the hyphal apex (Gay, personal communication) in the right of the micrograph is surrounded by electron dense material.

Fig. 45 (x11,526) shows the most beautiful and striking penetration hypha. The hypha in the lumen penetrated at a right angle through the  $S_3$  layer by reducing its diameter then advanced towards the middle lamella and crossed it, then penetrated (with a little increase in the diameter) into the  $S_2$  layer of the second cell wall with very dense material surrounding the hyphal apex. Apparently the hyphal apex ends in a cavity with several other hyphae, therefore the initiating hypha of the cavity is obscured.

Fig. 46 (x19,500) shows the penetration hypha from the previous figure in a higher magnification, and shows the large nucleus with its double membrane inside the hypha in the lumen.

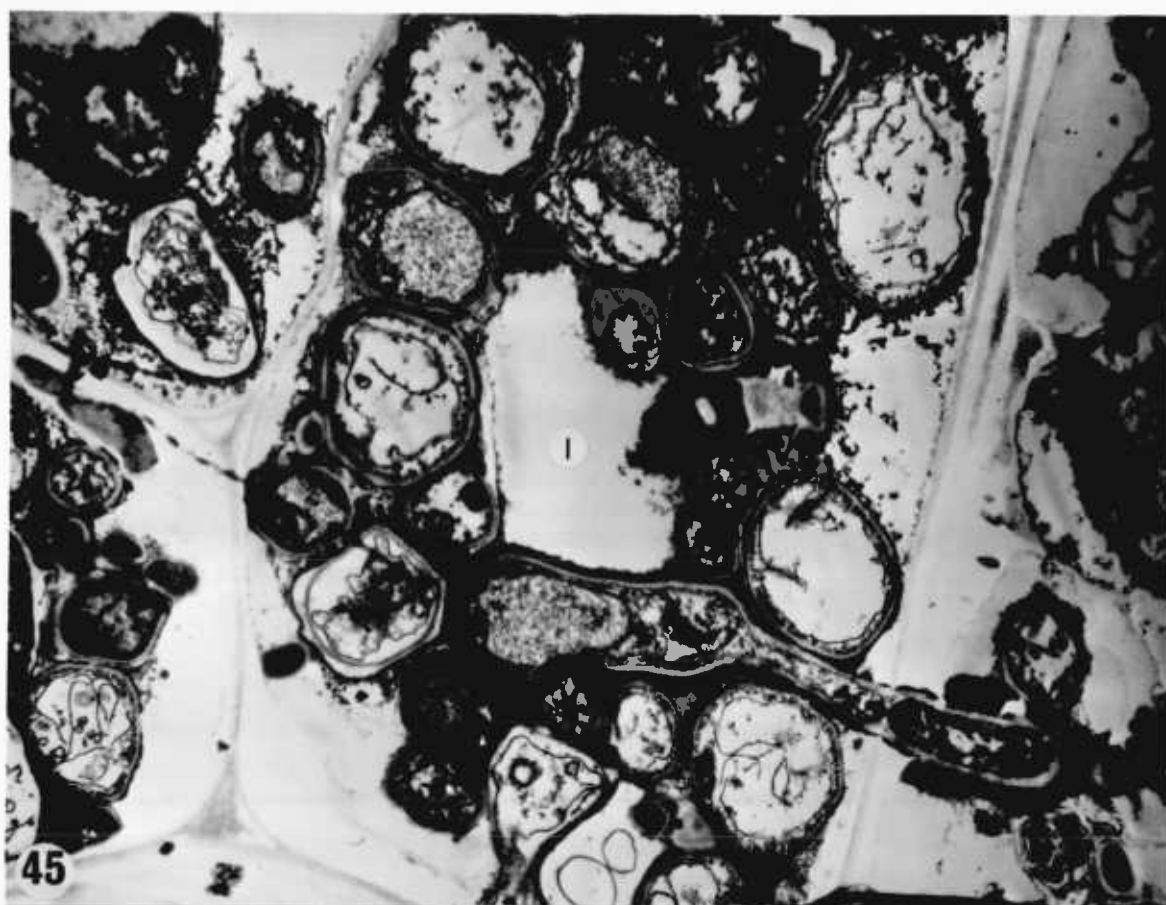


Fig. 47 (x70,652) is a higher magnification of the penetration hypha from the previous figure showing a septum formation exactly at the level of the compound middle lamella. The figure is illustrated by a diagram showing that the septal pore is located in the centre of the septum and formed by a tapering of the septum towards the pore. The pore is plugged with electron dense plugging followed by Woronin bodies one from each side. The septal plate is starting from a "wedge shaped ring" in the fungal wall, Gay and Martin (1971). The presence of Woronin bodies and the septal plate confirms strongly that this septum is a true septum, Reichle and Alexander (1965). The continuation of plasmalemma is conspicuous around the tapered end of the septum.

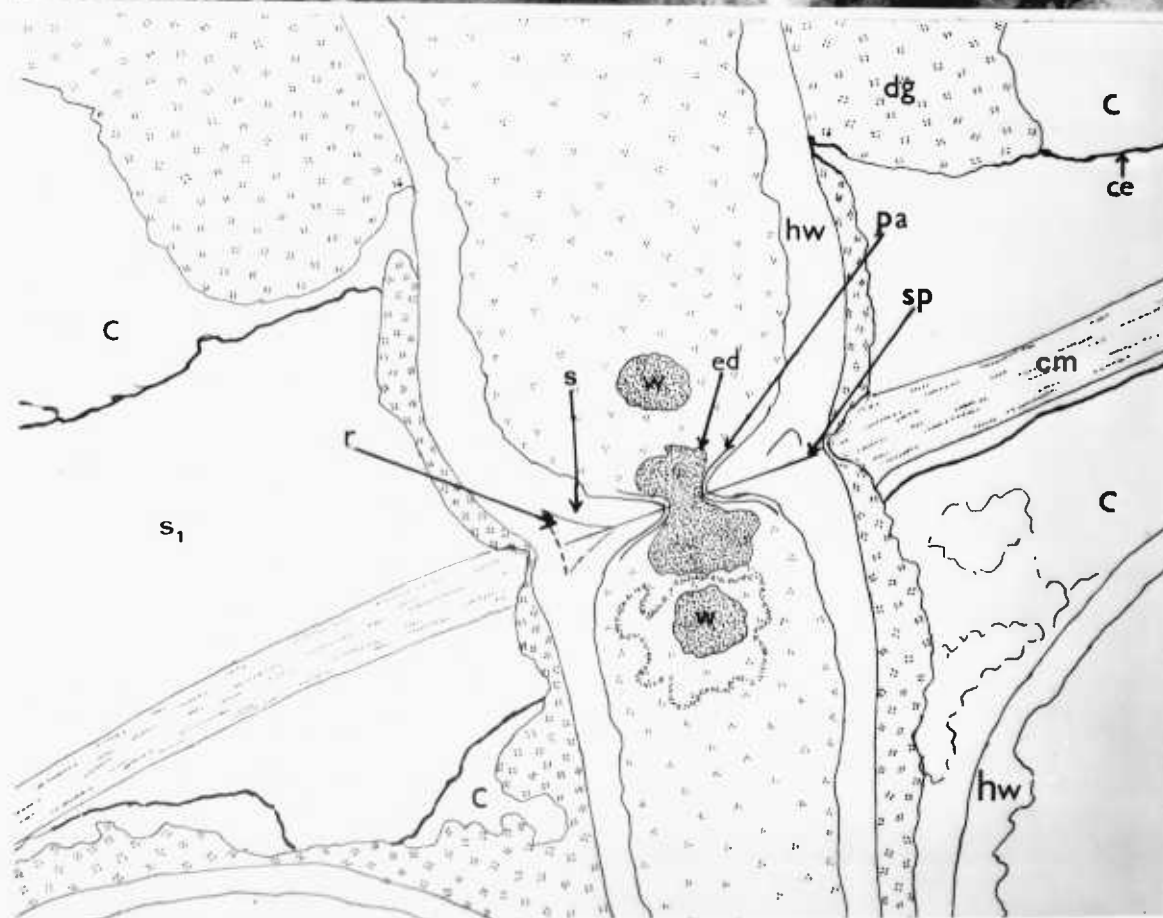
The cavity edge in the  $S_2$  layers of both cells is apparent and can be seen easily, surrounding the dark accumulation on the hyphal apex wall on the top of the micrograph.

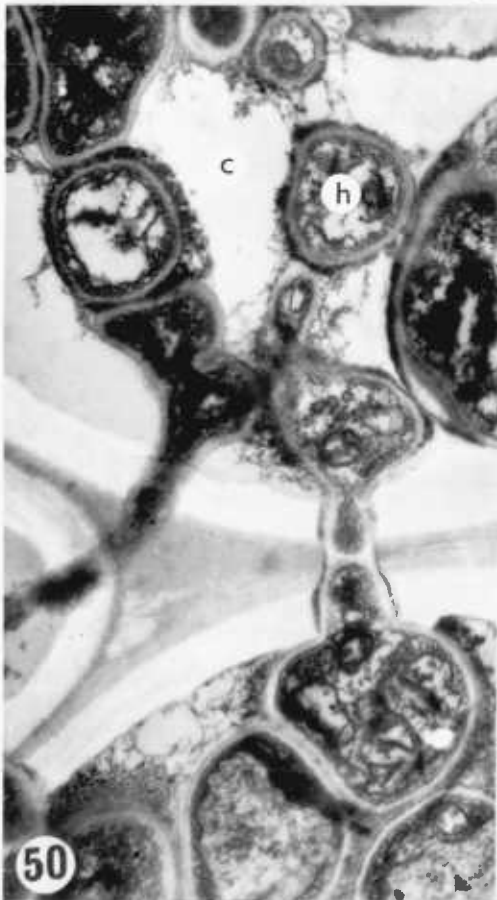
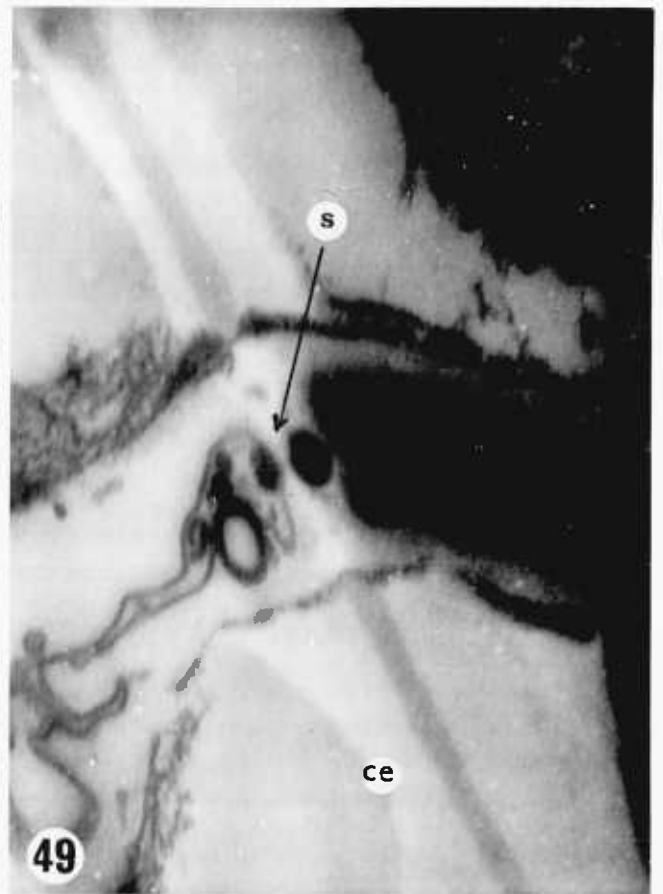
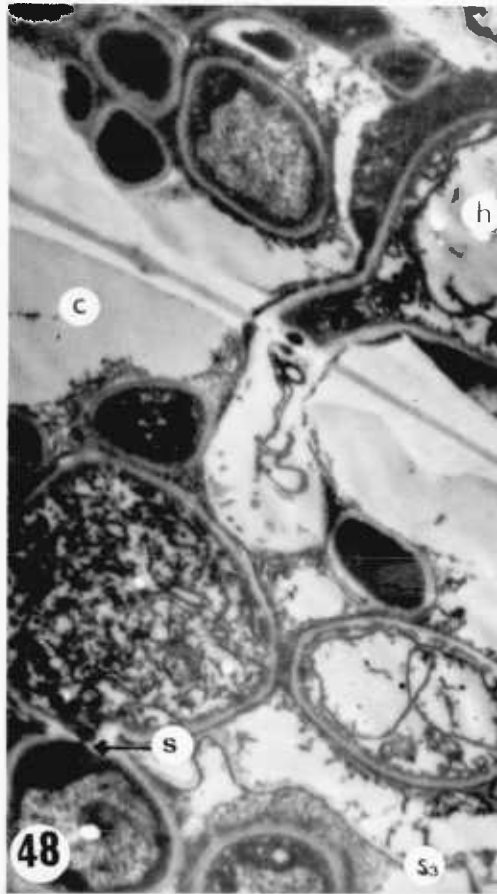
The striking phenomenon of this micrograph is the constriction of the hypha at the compound middle lamella level, and there is clear evidence of alteration of the wood cell wall contiguous with the fungal wall (especially in the primary wall and the  $S_1$  layer levels) (see Figs. 22, 27, 44, 49, 51, 53, 55, 56, and 63). This alteration is apparent as erosion of the wood cell wall shown by the dense electron material on both sides of the hyphal wall.

Fig. 48 (x15,142) shows a complete septum formed in the penetration hypha at the middle lamella level. Another septum with pore (arrowed) is present in a hypha penetrating the  $S_3$  layer.

Fig. 49 (x44,608) is a higher magnification of Fig. 48 showing a fully formed septum at the middle lamella level with a constriction in the hyphal wall.

Figs. 50 (x14,820) and 51 (x29,076) show another septum formation feature in a different penetration hypha.





2. Other unexpected phenomenon in the fungal behaviour

Figs. 52 (x15,062) and 53 (x12,237) are serial sections showing a secondary infection of an existing cavity by hyphae originating from a cavity in the  $S_2$  layer of another cell wall. Two penetration hyphae are very close to each other and penetrating almost from the same area. Both hyphae are constricted at the penetration points at the middle lamella level with a septum formation in the senescent hypha. In Fig. 53, the hypha (a) is relatively young (note the surrounding wall and the cytoplasmic content), this hypha formed a branch which penetrated into the cell wall and invaded an existing cavity (c) which was probably formed by hyphae (b) which is surrounded by dark granular material.

Figs. 54 (x11,104) and 55 (x26,807) show a hypha (h) in the  $S_2$  layer with cavities formed at the ends of the hypha. This hypha has formed two separate branches each of which pass into the neighbouring cell wall. This hypha could well represent a T-branch, if this is so, it is very interesting to observe that the cavities initiate from both ends of the T-branch but not along it.

The lumen of both wood cells are full of fibrous substance which could well be debris of lysed hyphae.

Fig. 56 (x14,768) shows another specimen with two penetration hyphae almost from the same area. Apparently one of the hypha originated from the lumen of the cell and formed a septum, which is not located at the middle lamella region because of the non-median sectioning; while the other penetration hypha is younger without a septum and originated from a hypha in the cavity.

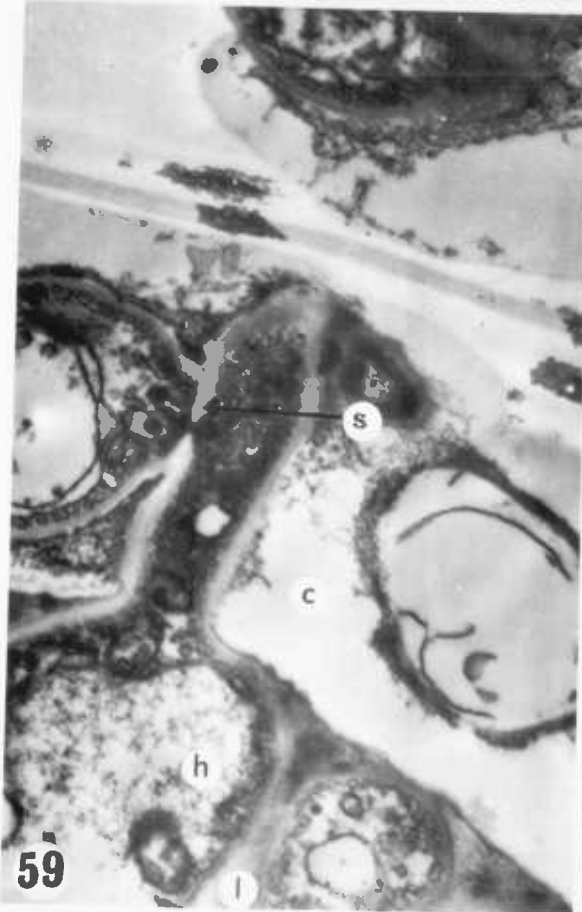
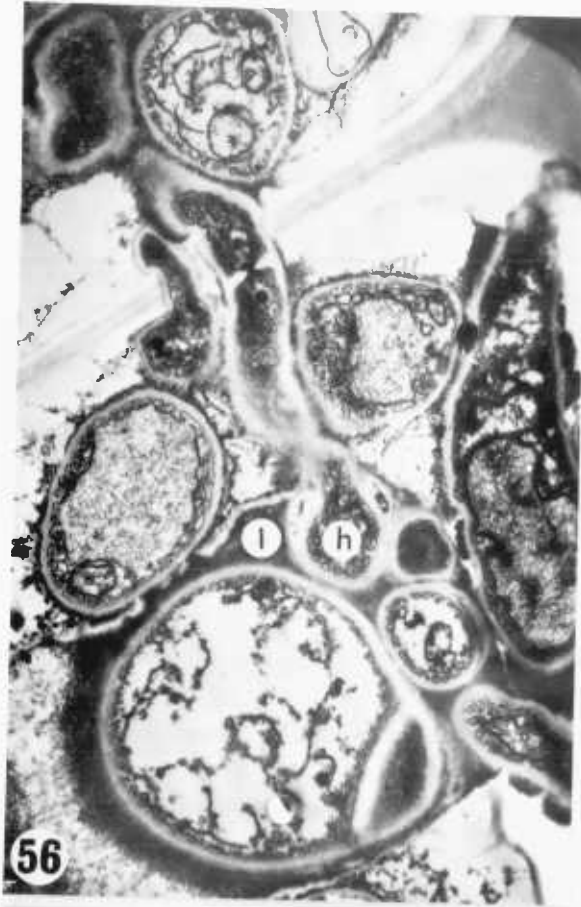


Figs. 57 (x6,623) and 58 (x12,311) show a cavity formation in the intercellular space. A hypha from this cavity formed a penetration hypha which is trying to penetrate through the cell wall to invade the neighbouring cell. The phenomenon of cavity formation in the intercellular space is interesting. Was the cavity formed in the same pattern as cavity formation in the  $S_2$  layer, and is there any particular orientation to follow in this area ?

Fig. 59 (x23,166) shows a hypha in the lumen which has produced a penetration hypha and penetrated into the  $S_3$  layer and formed a branch in the  $S_2$  layer with a constriction and then passed on towards the middle lamella. A septum is formed between the penetration hypha and the branch in the constricted area in the  $S_2$  layer. Could this septum be similar to the septum observed and described by Levi (1965) and Findlay (1970) in the constricted areas of a hypha forming a chain of cavities ?

Fig. 60 (x14,155) presents another peculiar penetration. The hypha penetrated from the lumen through the  $S_3$  layer into a cavity in the  $S_2$  layer. This hypha gave a branch (arrowed) and then proceeded to penetrate all the remaining layers of the cell wall until it reached a cavity in the  $S_2$  layer of the neighbouring cell.

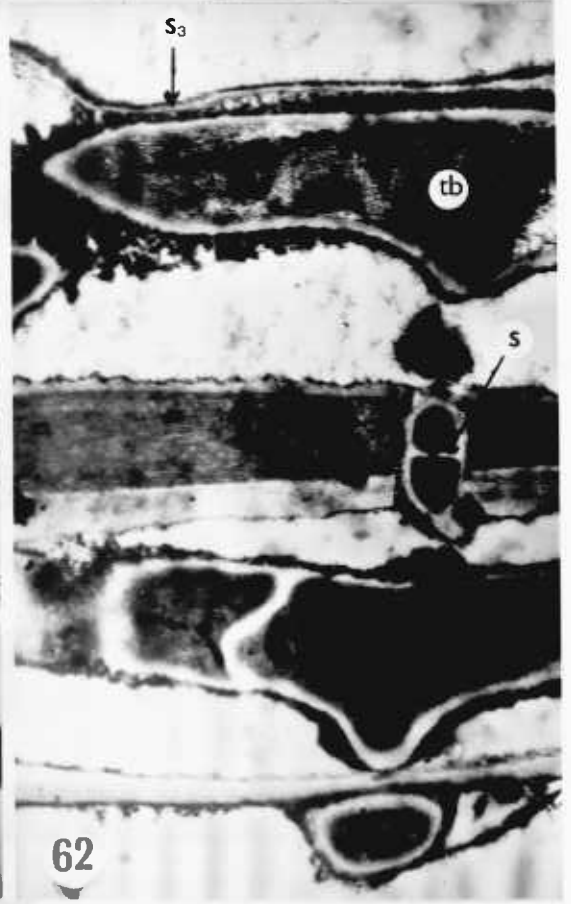
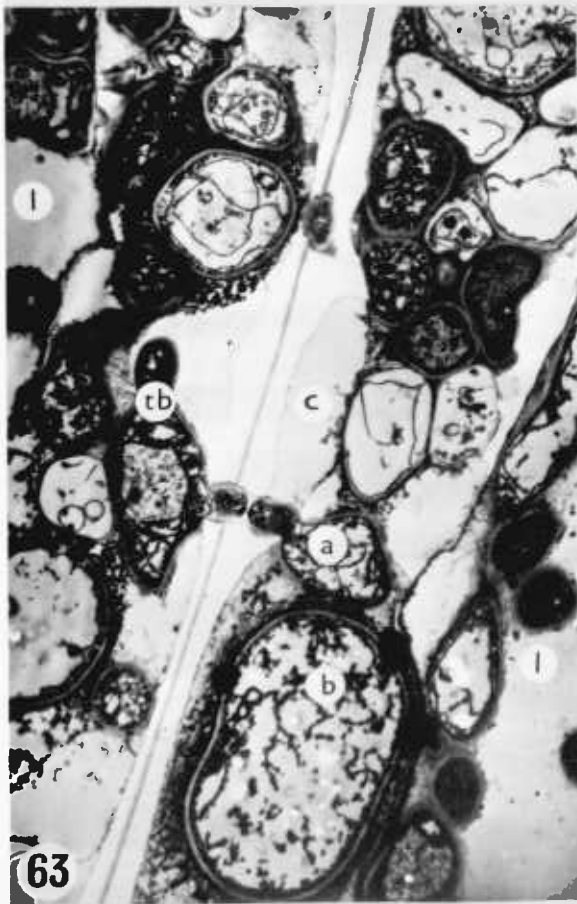
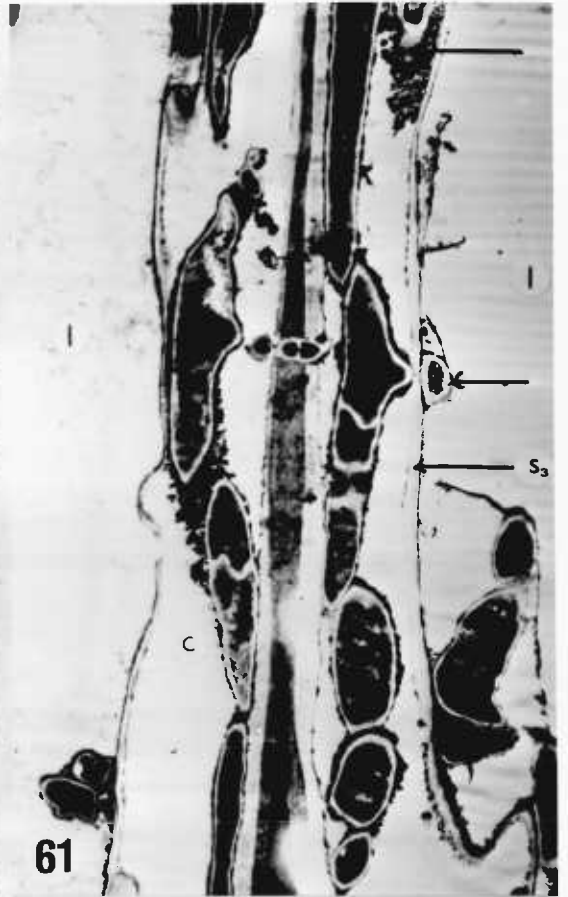
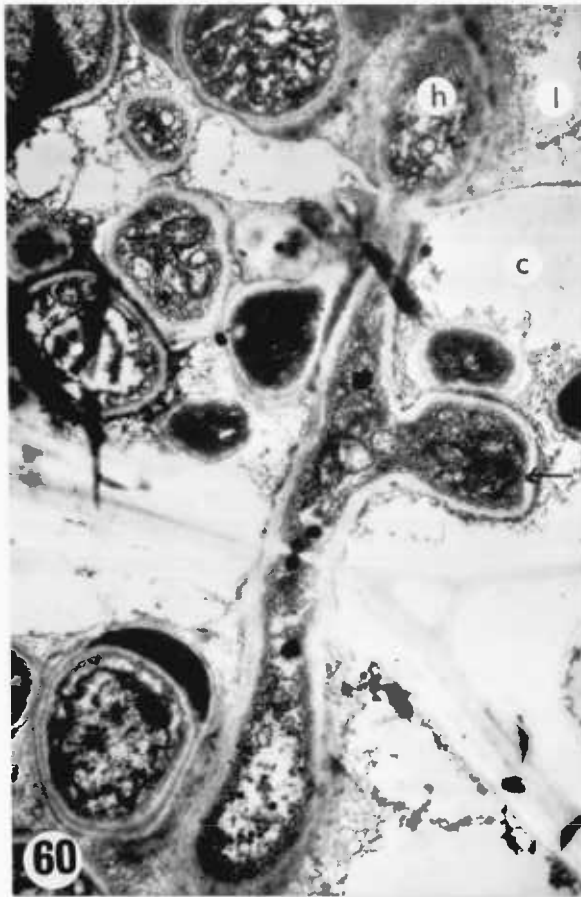
Figs. 61 (x5,353) and 62 (x15,703) show a very peculiar penetration and a T-branch, but unfortunately the section is non-median and therefore the continuation of the hypha is not apparent. Most likely the hypha (middle arrow) touched the  $S_3$  layer and penetrated through it giving a T-branch in an existing cavity which was probably formed by a hypha (top arrow). This T-branch did not find cellulose to degrade and may possibly, therefore, have formed

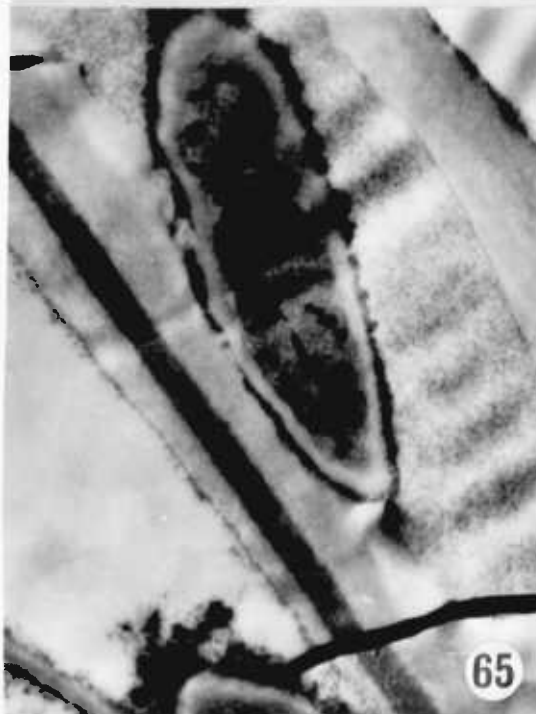
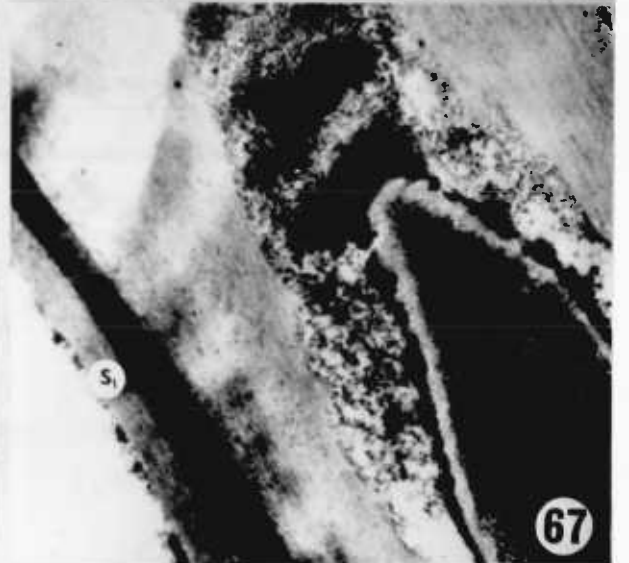
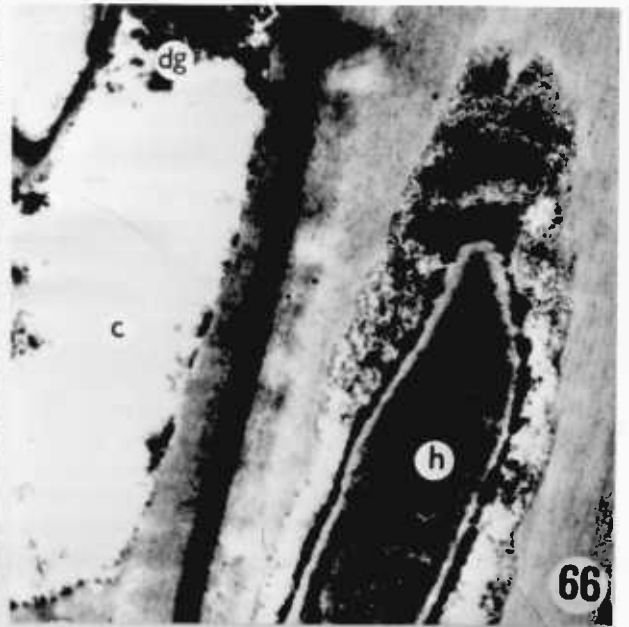


a penetration hypha which penetrated the remaining cell wall into the  $S_2$  layer of the second cell wall, where it formed a T-branch causing a cavity formation.

Fig. 63 (x8,071) shows the hypha (a) occupying a part of an existing cavity (c) which was most probably formed by the hypha (b) or by another hypha which has left debris after lysis. The hypha (a) produced a penetration hypha which crossed the remaining wall layers and then reinfected a cavity by producing almost a T-branch. This micrograph shows many reinfections of cavities by young hyphae.

Figs. 64 (x18,171), 65(x20,850), 66(x16,683), 67(x24,741) and 68 (x12,690) show cavity formation in the  $S_2$  layer of two fibres. The cavity on the right hand side of each of the micrographs is full of an unknown substance which is not present in the cavity on the left hand side of each of the micrographs. Could this material be debris of lysed hyphae? Is it an artefact formed during the specimen preparation? Is it lignin residue which has not yet accumulated around the hyphal wall? Is it cellulose microfibrils which are not completely digested? The answers to these questions are discussed in detail (see p. 206 ).





3.4.3 Scots pine attacked by Phialophora fastigiata S6A  
cultured on 2.5% malt agar medium (Figs. 69 to 73)

The figures show the stainer and soft rot behaviour of the fungus in softwood. These sections also show that the early stages of the present work carried out in this field were affected by a lack of experience resulting in poor fixation (Sorensen's buffer with osmium), infiltration of resin and embedding. Furthermore there is stain contamination appearing on the specimens as dark particles.

Fig. 69 (x2,294) shows typical stainer behaviour of the fungus. The hypha lying in the lumen of the tracheid (1) penetrated into the cell wall at right angles by reducing its diameter, and crossed the first and the second tracheid walls. The hypha then proceeded horizontally in the lumen of the second tracheid until it touched the cell wall of the next cell, reduced its diameter and penetrated into the cell wall.

Fig. 70 (x6000) is a high magnification of the previous figure showing the hypha has constricted even more in the middle lamella region.

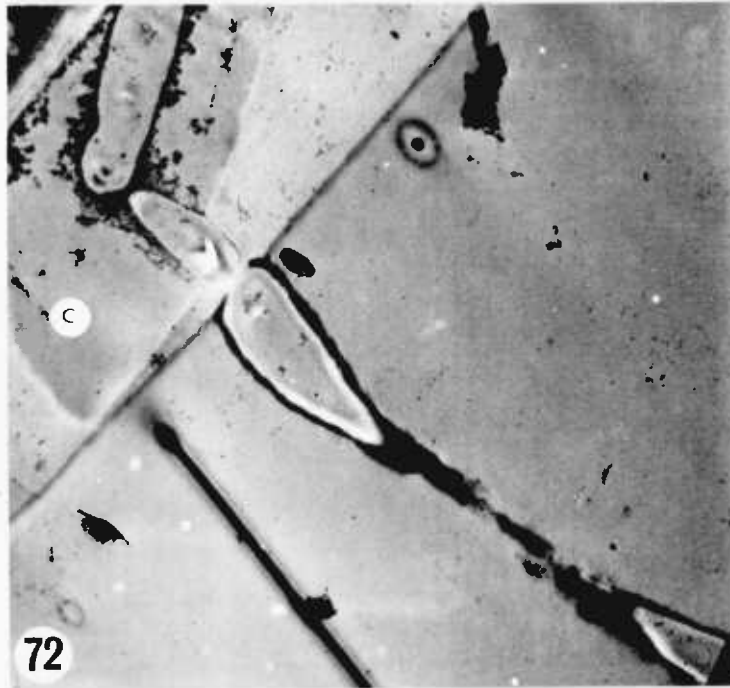
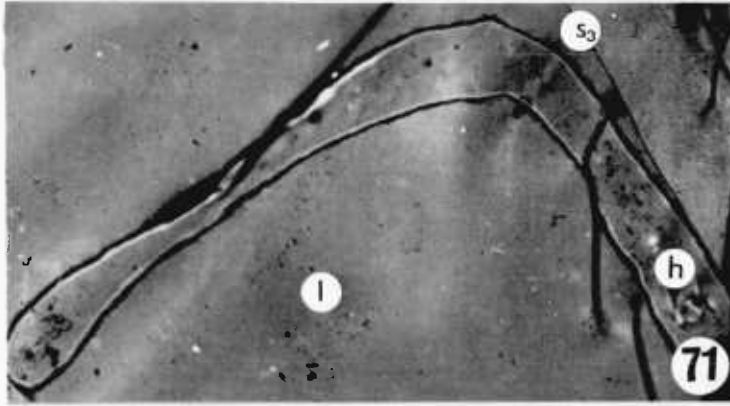
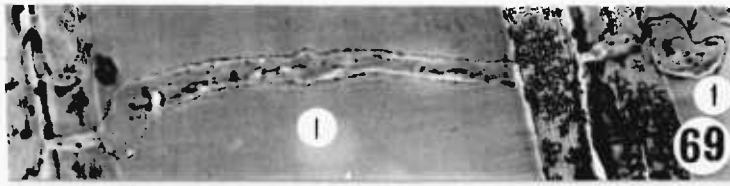
In a few specimens the fungus showed soft rot behaviour by producing pointed-end cavities.

Fig. 71 (x 5,540) shows the hypha in the lumen contiguous and parallel to the  $S_3$  layer, turning to the left and crossing the lumen horizontally approaching the opposite cell wall.

Fig. 72 (x 5,814) is a serial section of the previous figure showing that the penetration hypha has entered a cavity which might have been produced by the hypha which is surrounded with dark granular material.

The striking phenomenon of Figs. 69, 70, 71 and 72 is that the hyphae in the lumina which is contiguous with the  $S_3$  layer has a swollen end adpressed to this layer. This end has produced a very fine penetration hypha which has penetrated through the cell wall by constricting more in the middle lamella region. Could these swollen parts of the hyphae represent transpressoria ?

Fig. 73 (x6,120) shows a long cavity formed in the  $S_2$  layer of a tracheid.



### 3.5 Delignified wood attacked by fungi (Figs. 74 to 93)

The extreme difficulty of fixing and embedding the delignified and decayed wood specimens produced sections that were not of the same standard as those of normal wood.

#### 3.5.1 Delignified birch attacked by Phialophora fastigiata

S6A cultured on 2.5% malt agar medium (Figs. 74 and 75)

Fig. 74 (x10,168) is a longitudinal section showing several cavities formed in the  $S_2$  layer of one of the fibres, while the  $S_2$  layer (indicated) of the second fibre is intact. It is difficult to interpret and suggest a particular shape for the cavity. Undoubtedly more than one cavity is formed by fungal hyphae in the long axis of the  $S_2$  layer, leaving a narrow band of unattacked microfibrils (arrowed) in between. The presence of dark granular material surrounding the hypha is surprising, and one would ask whether any lignin has been left after delignification which would be left as debris after the fungal degradation.

Fig. 75 (x9,635) is a continuation of the attacked area in the previous figure showing two hyphae (arrowed) in the  $S_2$  layer very close to middle lamella, but without a sign of cavity or black debris around them.

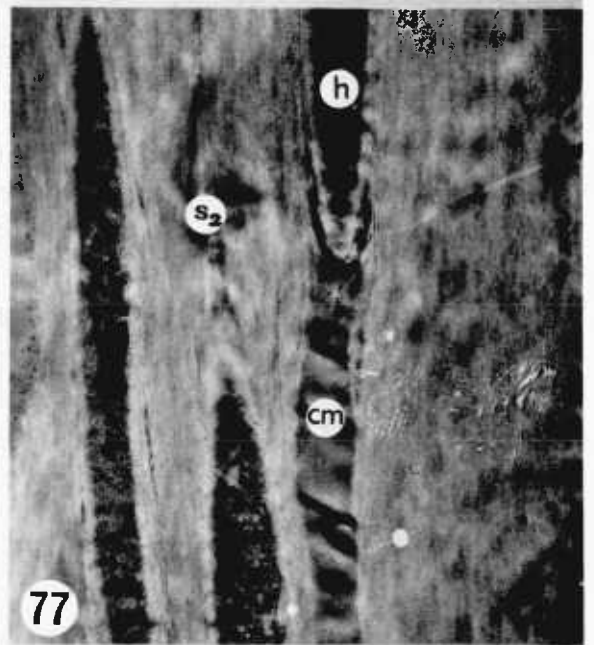
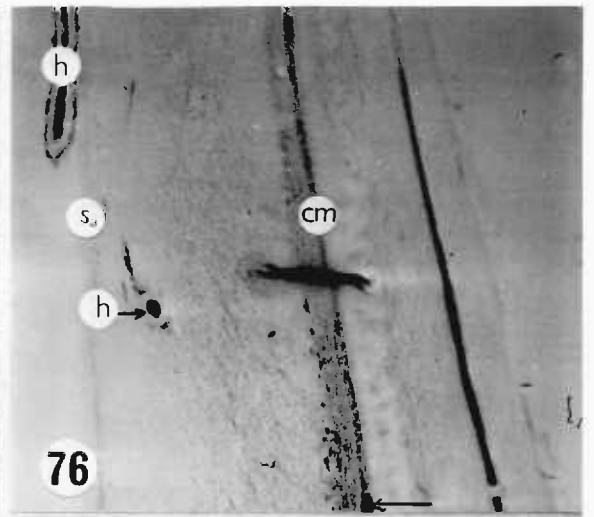
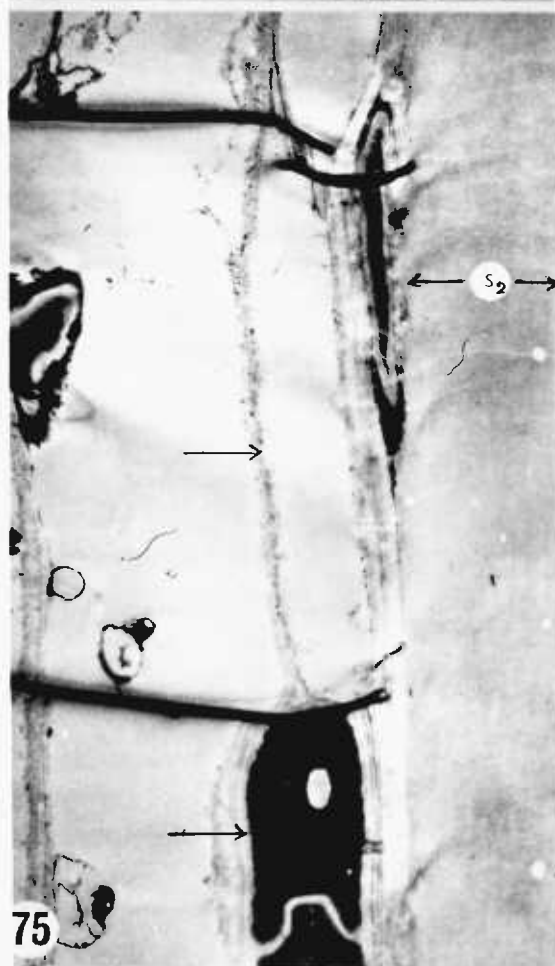
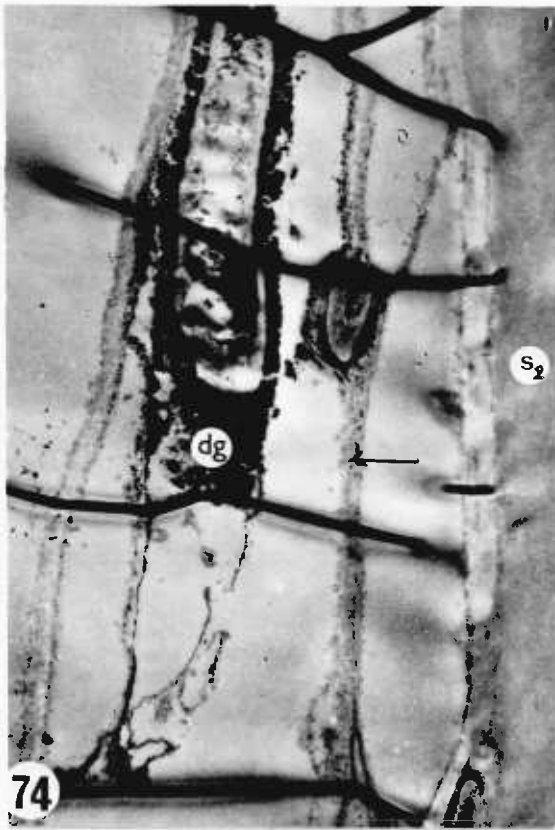
#### 3.5.2 Delignified Scots pine attacked by Phialophora fastigiata

731-1-3b cultured on 2.5% malt agar medium (Figs. 76 to 78)

Fig. 76 (x5,731) shows the hypha lying in the lumen contiguous and parallel to the  $S_3$  layer. Furthermore there are hyphae (arrowed) in the compound middle lamella and the  $S_2$  layer.

Fig. 77 ( $\times 16,800$ ) shows the hyphae attacking the compound middle lamella and the  $S_2$  layer and extending along the axis of the cell in these layers. Obviously the hyphae are invading these layers but without cavity formation (see Figs. 78 to 83 in Part III).

Fig. 78 ( $\times 21,491$ ) shows an oblique section of fungal hyphae in the cell wall.



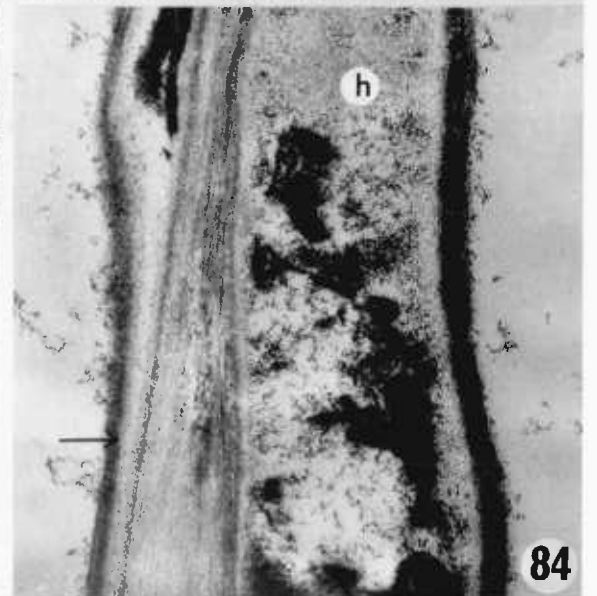
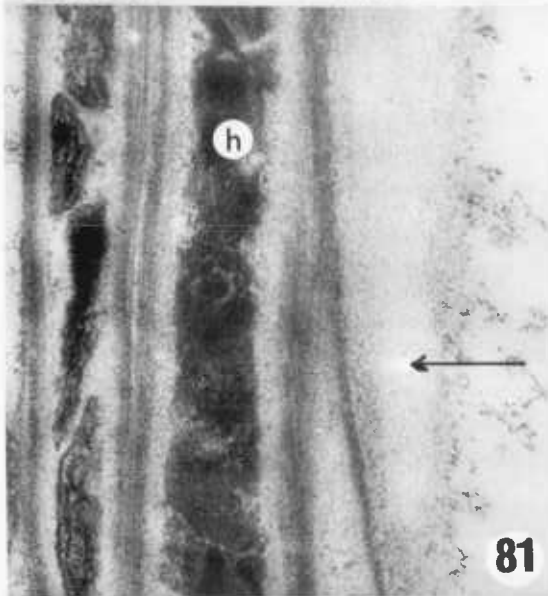
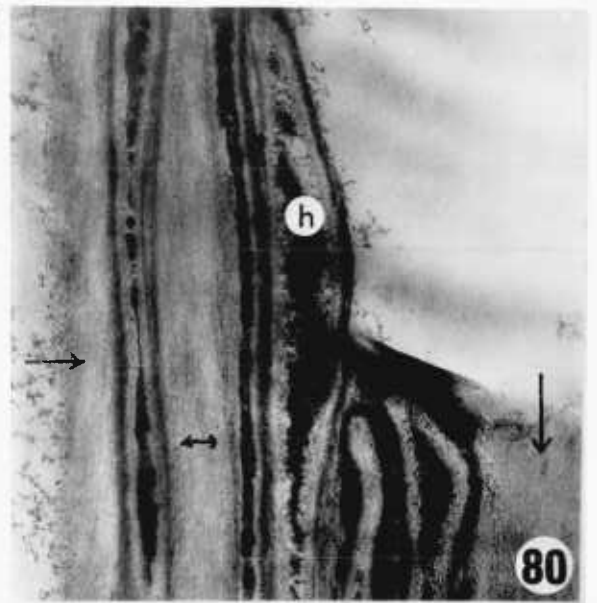
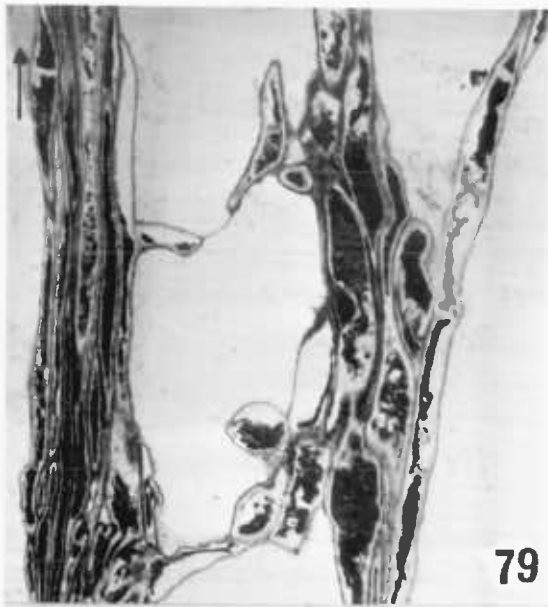
3.5.3 Delignified Scots pine attacked by Phialophora fastigiata S6A cultured on cellulose agar medium (Figs. 79 to 93)

Fig. 79 (x5,220) shows a general view of the delignified and decayed Scots pine wood specimens with some of the cell wall layers remaining (arrowed). Undoubtedly the separation in the cell wall layers is due to the specimen preparation for TEM rather than delignification. Obviously the delignification enabled the fungal hyphae to penetrate easily into the cell wall and to attack its layers aggressively without differentiation. This is conspicuous in Figs. 80 (x25,071), 81 (x48,355) and 82 (x45,500).

In the previous figures there is little of the cell wall layers remaining (arrowed), and these probably belong to the  $S_2$  layer in which the fungal hyphae are following the microfibrillar orientation.

Figs. 83 (x10,455) and 84 (x34,944) may present an interesting feature. Could the arrowed layer be the  $S_3$  layer? If it is, the fungal hypha had started to break down the  $S_2$  layer from the microfibrils of the transitional area between the  $S_3$  and  $S_2$  layers, but without cavity formation. Obviously another hypha (immediately on the right of the previous hypha) attacked and degraded the rest of the  $S_2$  layer, with no cavity formation.

None of the previous figures in this section show any dark granular material surrounding the fungal hyphae in the cell wall, but a few sections of another specimen which were delignified, decayed and prepared for TEM in the same way as for the previous figures, showed very aggressive decay and heavy granular material



surrounding the hyphal wall. These sections are shown in Figs. 85 to 93.

Figs. 85 (x4,138) and 86(x7,872) present a general view of the severe cell wall degradation.

Figs. 87 (x5,905) and 88 (x12,300) show the degradation of the cell walls without being able to differentiate the wall layers. On the left hand side of the micrograph there is a group of transverse sections of fungal hyphae, presumably they were scattered during the specimen preparation.

The area in the top square shows a protuberance which might represent a penetration. The square near the middle in Fig. 87 (the serial section of which is shown in Figs. 89 (x8,120) and 90 (x12,618)) probably indicates another penetration or a T-branch.

In the same area in another serial section, another indication of penetration was observed as represented by Figs. 91 (x6,141) and 92 (x15,142).

Fig. 93 (x11,353) shows another indication of penetration (arrowed) and heavy degradation of the cell wall.

Undoubtedly all the sections presented by previous figures were non-median, otherwise it would have been very interesting to observe the behaviour of fungal hyphae in delignified wood. The following questions arise :

Is the penetration real ?

Is there septum formation in the  $S_3$  layer and middle lamella region ?

Is the penetration followed by T-branching ?

And if so, why was cavity formation not observed ? Is it because of the lignin loss or because of easy enzyme diffusion  
..... etc. ?

The answers to these questions are discussed in detail on  
page 206 .



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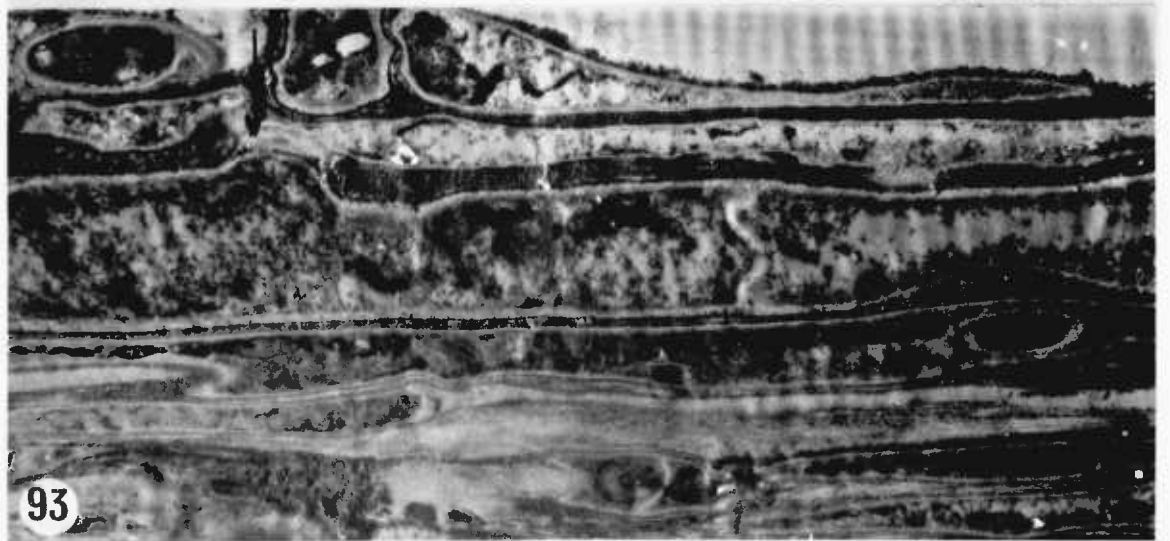
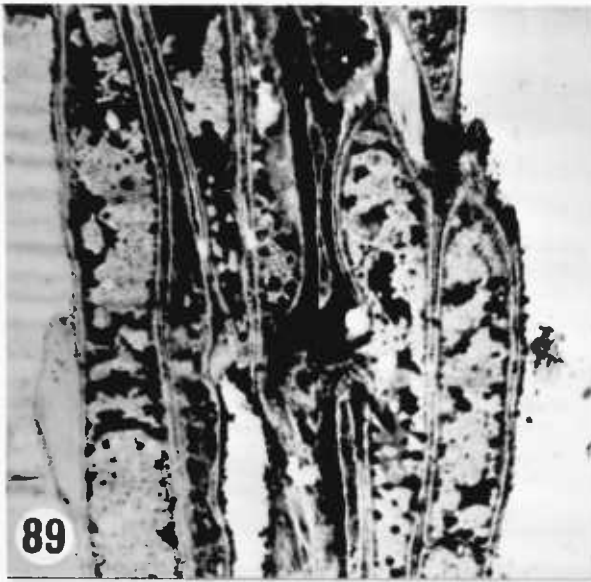
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#### 4. DISCUSSION

##### <sup>Fig</sup> ~~Fixation~~ and embedding the wood specimens

One of the first aims of the present work was to find the right fixative for the wood cell wall, to enable investigation of the orientation of microfibrils in each layer of the cell wall, especially at the points where the fungal hypha penetrates into the cell wall.

Potassium permanganate proved to be the best fixative to differentiate the microfibrillar orientation in very thin sections of sound and delignified decayed wood specimens. The obtaining of greater clarity of microfibrils is not dependent on the chemical changes produced in the wood cell wall by fungal enzyme attack or on the mild delignification as was postulated by Chou (1971). On the contrary the clarity is very much dependent on the type of fixative, stain and embedding resin used. All normal and delignified wood specimens which were fixed with potassium permanganate showed very clear microfibrillar orientation, except in a few figures 26, 74, 75 and 76. This might be due to the poor penetration of the fixative or the stain into that particular area of the specimen.

The micrographs of SEM and TEM, showed that orientation of microfibrils in each layer of the wood cell wall are in line with expectations. The rich lignin layers (compound middle lamella and  $S_3$  layer) have shown dense contrast, especially the former one. The primary cell wall is easily distinguished from the middle lamella in the areas near the intercellular space, otherwise it is difficult to differentiate. It was found that it was not easy to determine the exact orientation of microfibrils unless the section was longitudinal and median. The interesting feature appeared to be that

the transitional microfibrils "intermediate" between the  $S_1$  and  $S_2$  or between the  $S_2$  and  $S_3$  layers were the weakest (Harada, 1965; Heyn, 1969; and Preston, 1974) since these microfibrils were degraded frequently during the fungal attack on the  $S_2$  layer. This weakness might be due to the gradual change in the orientation of microfibrils from the  $S_1$  to the  $S_2$  and from the  $S_2$  to the  $S_3$  layers. The transitional microfibrils are seen more clearly between the  $S_1$  and the  $S_2$  layers than between the  $S_2$  and the  $S_3$  layers.

The orientation of microfibrils in delignified Scots pine (Figs. 4 - 6 Part III and 8 - 10 Part IV) did not show any conspicuous differences from normal wood microfibrillar orientation (Figs. 1 - 3 Part III and 5 - 7 Part IV), except in the compound middle lamella which was slightly separated. This is not surprising because of the high lignin content in this region by comparison with any other cell wall layer. The delignified wood specimen was less resistant to the electron bombardment in SEM, which was probably due to the weakness of the microfibrils when the lignin encrustation was decreased.

The second fixative used was glutaraldehyde which showed neither the wood cell wall nor the fungal cytoplasm in any detail, although this fixative is considered to be one of the best fixatives for cytoplasm. This could be due to the location of fungal hyphae in the wood cell wall, or to the lack of experience in the microscopy field of the author at that period. The latter is more likely, as this fixative gave satisfactory results with the fungal cytoplasm, but not the wood cell wall, when it was used by Findlay (1970) and Chou (1971).

Sorensen's buffer with osmium as a fixative gave excellent detail of the fungal cytoplasm with its surrounding wall. Some of the figures showed the fungal cell wall to consist of an inner lamellated layer and microfibrillar skeleton which is embedded in amorphous matrix. This fibrous (amorphous) surrounding is clear when the hypha is in the media, in the lumen of the wood cell or within the wood cell wall. The density of these fibrils is greater when the specimen is fixed with potassium permanganate, which is surprising, as this fixative causes dense staining of lignin in the cell wall (Hepler, Fosket and Newcomb, 1970). If this is so, one would assume that "lignin-like" substances are one of the fungal wall constituents, Ainsworth and Sussman (1965). This amorphous matrix is almost unrecognisable from the dark granular material which is supposed to be a product of breakdown of  $S_2$  layer of the wood cell wall by fungal enzyme, Figs. 25, 26, 27, 31 and 32.

The nucleus with its double membrane, plasmalemma, lomasomes, mitochondria, microtubules and septum formation are very conspicuous in most of the figures in which the specimens were fixed with Sorensen's buffered osmium; whilst these constituents were almost destroyed when potassium permanganate was used. This was expected since the fixative is known to be destructive of cytoplasmic constituents.

Therefore, by using potassium permanganate the difficulty of not being able to differentiate the microfibrils was overcome; and by using Sorensen's buffer with osmium, the clearness of fungal cytoplasm was achieved.

Methacrylate as an embedding medium has several disadvantages (see p.125) which cause misinterpretation, and for this reason epon resin was used extensively. Epon resin could give unsatisfactory results because of its poor infiltration and consequent distortion of the specimen, but the present work proved that this resin is an excellent embedding medium for wood specimens if it is infiltrated through the cell wall evenly. This was achieved by gradual and slow infiltration by using vacuum and cold temperature.

#### Attacked wood

The micrographs of invasion and colonization of birch fibres and ray cells by a soft rot fungus (Figs. 15 - 18) confirmed the observation of Corbett (1965) and Levy (1965a, b) by light microscopy, and the present observations by SEM. The penetration occurred between vessel, ray cells and fibres, but it should be kept in mind that it is extremely difficult with TEM micrographs to obtain confirmation of the direction of the penetration, unless the apex of the hypha is recognisable. Therefore misinterpretation of transmission electron micrographs could occur easily unless serial sections are available (Figs. 42, 43, 52 and 53).

The behaviour of Phialophora fastigiata in Scots pine as a stainer and soft rot fungus is confirmed by Figs. 69 - 73. The stainer behaviour is represented as a transpressorium formation with its swollen head and fine penetration hypha. Liese and Schmid (1961 and 1964) observed the transpressorium with its fine penetration hypha by using replica technique, while Findlay (1970) was not able to observe the fine penetration hypha of transpressorium in ultra-

thin sections. Findlay supported the mechanical penetration hypothesis of Liese and Schmid, he also postulated that the ability of Ph. fastigiata to form transpressorium and behave as a stainer fungus without cavity formation confirmed the mechanical penetration hypothesis. This postulation is arguable because of the observation of cavities in Scots pine in the present work. Fig. 72 shows a transpressorium which formed a fine hypha that penetrated the tracheid wall in which there is a cavity. This cavity might have been formed by the other hypha which is surrounded by the dark debris, or this hypha might represent one of the arms of a T-branch produced by the penetrating hypha. If one accepts the first possibility, the explanation of transpressorium formation in this particular area will simply be coincidence. The second possibility suggests that the transpressorium could produce a cavity, especially as the diameter of the penetration hypha of the transpressorium is almost the same as when the hypha is behaving as a soft rot fungus. Undoubtedly it is dangerous to draw a conclusion from these limited micrographs of the stainer behaviour of the fungus. If future work confirms the similarity in diameter of penetration hyphae in both types of behaviour of the fungus, it will mean that the fungus could act as a stainer in the lumen and could act (for one reason or another) as a soft rot fungus in the cell wall. The present work could not confirm either the mechanical or the enzymic penetration of the hypha when it was acting as a stainer, because of the lack of clarity of the microfibrils. However the presence of a slightly denser staining surrounding the penetration hyphae may indicate enzymic activity. Therefore it would be worthwhile carrying out an investigation in

this field to gain more information about the variation in the behaviour of the fungus.

One of the rare cases observed was the penetration of fungal hypha through plasmodesmata in a pit pair in ray parenchyma (Figs. 11 and 12). The penetration through plasmodesmata is most likely to have occurred randomly, while most of the micrographs of penetration do not show any evidence of this phenomenon. These results would suggest strongly that neither plasmodesmata nor any other cell wall capillaries are normally involved in the penetration.

As was mentioned before luck must play its part in obtaining sections of the desired area. The aim of this work was to investigate the manner of the very early penetration by the fungal hypha into the wood cell wall. Figs. 21 - 23 are representing this stage but unfortunately the specimens were fixed with Sorensen's buffered osmium, so the orientation of microfibrils at the penetration points were not apparent and it is not possible to observe the type of alteration that has occurred in this area. There is a dense colouration surrounding the penetration hypha which suggests that an enzymic dissolution occurred in this region. Figs. 19 and 20 are difficult to interpret because the protuberance might represent an initial stage of penetration when the hypha contiguous with the  $S_3$  layer was stimulated to produce a fine hypha to penetrate the wood cell wall. This protuberance probably represents a part of a penetration hypha which has already formed a cavity in the  $S_2$  layer, a part of which is apparent in the micrograph, but the non-median sectioning has caused a discontinuity of the branched hypha. The mottled appearance of the microfibrils in the  $S_2$  layer of the fibre wall is less likely to be

the result of enzyme diffusion, as the soft rot fungi are characterized by their limited enzyme secretion and diffusion, and also this has not occurred in the other cell wall where a contiguous hypha is present. Therefore it is probable that this thinning of the wall is due to the uneven infiltration of epon resin and to the uneven sectioning which caused the foldings (black lines) in the cell wall.

The observations made by Chou (1971) regarding bore hole formation by Poria vaillantii 16C in Scots pine showed the clarification of the microfibrils of the tracheid wall, which he suggested was due to enzyme diffusion. He also suggested that the S<sub>3</sub> layer was breached chemically but not mechanically. His micrographs showed a void space ahead of the hyphal tip. His interpretation of this space was that either it was produced as an artefact with which the present work entirely agrees, or it was originally representing the undigested lignin which was destroyed during specimen preparation. This is highly unlikely to occur because the undigested lignin will not dissolve in any of the chemicals which he used. It would be interesting to determine what has occurred to the lignin of the cell wall during bore hole formation.

Levi (1964) and Findlay (1970) observed and postulated that the lignin was left as granular material in the breakdown of the cell wall attacked by soft rot fungus. Therefore one would expect to observe a similar residue in Chou's micrographs because the brown rot fungi are known not to break down the lignin. The absence of this residue would suggest : (a) The residue was too small to appear in the micrograph, although this seems unlikely in view of Figs. 21, 23 and 27 in the present work. (b) The boring hypha produced limited

lignase which acted only on the high lignin content area ( $S_3$  layer) without leaving any residue. This is again unlikely to occur in view of the figures showing hypha penetrating through the middle lamella and  $S_1$  layer where it is surrounded by a densely stained area which could represent methoxyl groups produced after the enzymic activity on the cell wall during penetration (see diagram on page 172). (c) The lignin in the cell wall around the boring hypha was modified with "cellulose freeing" or "lignin-modifying" enzyme, Levi and Preston (1965). This process limited the breakdown of cellulose around the boring hypha which resulted in the non-enlargement of the size of the bore hole. If this is so, one would expect a constriction of the penetration hypha through the high lignin content areas. This assumption seems a more acceptable explanation for constriction of penetration hypha at the level of the  $S_3$  layer and compound middle lamella region in every figure of this part and Fig. 11 of part III.

The interesting phenomenon observed in the present work is the appearance of a septum in the penetration hypha at the levels of the  $S_3$  layer and middle lamella. The appearance of a septum is difficult to understand, but its constant occurrence suggests strongly that it is not formed randomly and there must be an important reason for its formation. A search of the literature on septum formation, revealed no explanation for the phenomenon. It is possible that the mechanical restraint imposed by the middle lamella and the  $S_3$  layer upon the hyphal wall at these points, by comparison with the lack of restraint in the  $S_2$  layer where cavities were freely formed, could produce a response by the hyphae which would react by the formation of septum to support the cell wall and prevent its collapse. It will be inter-

esting to carry out further investigations on this subject, which might also give an explanation why septum formation also occurs in the constricted region of the cavity in which the hypha lies.

The hypha in the lumen with a diameter  $2.26\mu$  approx. had reduced its diameter to  $0.58\mu$  approx. while penetrating the  $S_3$  layer and also in the  $S_1$  layer and compound middle lamella. The penetration "perforation" hypha produced a T-branch in the  $S_2$  layer along its microfibrillar orientation. Unfortunately a section of an early stage of T-branch was not obtained, almost all the T-branches had already formed large cavities. Many unsatisfactory hypotheses have been suggested to explain the T-branch formation in the  $S_2$  layer. The results of the present observations do not support the hypotheses of Levy (1965a) and Levi and Preston (1965), because no evidence of plasmodesmata or any "weak microfibrils" were observed to be involved in the phenomenon. The present observations confirmed the alignment of the T-branch along the microfibrils in the  $S_2$  layer in all longitudinal sections (Figs. 24 - 27, 61 and 62). The unexpected phenomenon of the penetration hypha producing a branch perpendicular to the  $S_2$  layer microfibrils (Figs. 54, 55, 59, 60) might support the haustorium hypothesis of Levy and Stevens (1966), and also suggests that the branch could grow against the  $S_2$  layer microfibrillar orientation. It is possible that the branching in Figs. 59 and 60 occurred when the hypha penetrated into an existing cavity in which there was sufficient space to form branches, but Figs. 54 and 55 support the proposition that the hypha is growing perpendicular to the microfibrillar orientation in the  $S_2$  layer. Recently Unligil et al (1974) published similar results and concluded that T-branching

could be formed perpendicularly to the microfibrillar orientation in the  $S_2$  layer. Here it should be said that this phenomenon is not frequent, but it would be interesting to carry out further investigations in this subject using transverse sections which might confirm that the T-branch could act as haustorium.

Cavity formation by soft rot fungi is complicated and there is no satisfactory explanation for the process, although several hypotheses have been suggested:-

(a) Frey-Wyssling (1938) proposed three planes for cellulose decomposition, suggested by measuring the angles at the tip of the cavities. Here one could ask why the decomposition of cellulose should have occurred in those three planes and not randomly. Unless the crystalline regions of neighbouring microfibrils are orientated with respect to one another in a constant fashion.

(b) Roelofsen (1956) assumed that the rate of decomposition was governed by cellulose and that cellulase can diffuse only when there is simultaneous dissolution of cellulose, therefore he could not obtain cavities in pure cellulose. Nilsson (1974a) refuted Roelofsen's hypothesis because he obtained cavities in cotton. According to Roelofsen's hypothesis one would expect to observe stepped edges to the cavity and these were not apparent in the present work.

(c) Levi and Preston (1965) assumed that the lignin was a governing factor (this was supported by Findlay, 1970), and the present work supports this hypothesis, but not entirely. If the lignin was responsible for cavity formation one would expect this to occur more frequently in Scots pine attacked by Ph. fastigiata, also

one would not expect to observe cavities in lignin free material. Also this hypothesis was not supported by Nilsson (1974a) when he observed cavities in lignin free material.

None of these hypotheses agree entirely with the present observations, and so a new explanation for cavity formation is required. It is known that the cavity is initiated from a "proboscis hypha" (Corbett 1963). Proboscis hyphae have not been observed with SEM or TEM in the present work, but were recently observed by Crossley (1975). The interesting phenomenon in Crossley's SEM micrograph is that the hypha within the cavity in the  $S_2$  layer formed "proboscis hyphae" from both ends extending along the line of the microfibrillar orientation in this layer but in a much finer form and with a very small diameter space between the microfibrils (see Fig. A p.28). The "proboscis hyphae" do not show the rough surface of the original hypha in the cavity. Therefore one would assume that these fine hyphae have penetrated between the microfibrils mechanically, especially as the microfibrils are relatively widely separated (Figs. 1 and 2 in Part III). The length of the "proboscis hypha" could vary according to the length of the void between the microfibrils; and also according to the ability of the original hypha to produce a fine hypha. It can be postulated that the "proboscis hypha" starts secreting a "lignin-modifying" enzyme (Levi and Preston 1965) which frees the cellulose, and also secretes cellulase to decompose the freed cellulose. Both enzymes have the ability to diffuse longitudinally between the microfibrils, and transversely across them, which results in the decomposition of cellulose around the "proboscis hypha" and gives rise to a space around it. This hypha enlarges and resumes its

original diameter. Meanwhile the diffusion of enzymes continues to decompose more cellulose and this results in the formation of a pointed end to the cavity. The accumulation of enzymes near the pointed tip of the hypha might help to erode a very fine pathway, leading to the formation of a new "proboscis hypha" which will penetrate mechanically to fill the gap and then start the formation of a new cavity, with a constriction which will remain between the newly formed cavity and the one before.

If this is the true growth form, then the constriction of the hypha suggests that the growth form in the wall is : (1) extension along the line of the microfibrils followed by (2) a swelling of the hypha and enlargement of the cavity. If this is so, a septum formed when the hypha is of small diameter makes it difficult for the hypha to swell at this point and a constriction of the hypha causes a constriction of cavity formation. In other words the apparent chains of cavity are due to morphological development of the fungal hyphae in the wood cell wall.

Undoubtedly this postulation needs further research to investigate the mechanisms of "proboscis hypha" in serial transverse and longitudinal sections, which might support and explain more clearly the previous hypothesis or refute it.

A flattened cross section of cavities as suggested by Bailey and Vestal (1937) was observed in Figs. 39 and 54. The enlargement of the diameter of a cavity was very conspicuous with TEM results. This enlargement caused the coalescence of several neighbouring cavities which almost caused the disappearance of the  $S_2$  layer, while the  $S_3$  layer was very resistant and was never observed

to be decomposed.

The granular material surrounding the hypha within the cavity has been seen by Barghoorn (1949), Levi (1964) and Findlay (1970), and they suggested that it was a lignin residue. The micrographs of the present investigation indicate similar results but with relatively larger granules, especially when the specimens were fixed with potassium permanganate. The granular material is not surrounding the fungal hypha when it is invading an existing cavity or when it is in the lumen (except in Fig. 4 which is not surprising because the medium is belonging to birch sawdust). The absence of this material around the hypha in both these places would suggest that the material is a product of the enzymic hydrolysis of the S<sub>2</sub> layer. Fuller (1970) showed that Ph. fastigiata can secrete extracellular cellulases, so that the granular material could be an accumulation of residual lignin. Fig. 33 reveals dense dots (arrowed) in the cavities which are similar to the ones at the edges of cavities. These dots seem to be freed from the microfibrils which might suggest that they are the modified lignin particles which have not accumulated to form larger granules. It will be interesting in the future to delignify decayed wood specimens to investigate the presence or absence of granular material, its absence would confirm that it is lignin residue.

Many unusual and unexpected phenomenon were observed by using TEM many of which are difficult to interpret. One of these phenomenon is the invasion of an existing cavity (Findlay, 1970), which occurred in heavily degraded areas. This could be due to the starvation of the fungus for cellulose which was consumed almost entirely by the early hyphae, leaving the later hyphae to cross from

one cell to another by penetration "perforation" hyphae in search of cellulose and mostly encountering existing cavities.

Figs. 64 - 68 reveal two types of cavities, one with a hypha in an empty space and the other surrounded with fibrous or nearly granular substances. There is no doubt that it is not an artefact, but produced during the breakdown of the  $S_2$  and could well be the early stages of microfibrillar breakdown or an early stage of modified lignin.

As was mentioned before there was no visible alteration in the microfibrillar orientation of delignified wood specimens. The behaviour of the fungus was quite surprising in delignified wood specimens because of its heavy invasion of the cell walls (Figs. 78 - 83 part III and 74 - 93 part IV).

Cavity formation by Ph. fastigiata in delignified wood specimens was not observed, except in delignified birch (Figs. 74 and 75). This is difficult to interpret, especially when the hypha is surrounded by dark granular material which possibly suggests that the lignin was not lost sufficiently during the delignification.

Findlay (1970) and Nilsson (1974c) described the decay that occurred in delignified wood specimens as an erosion (from the lumen) decay. The observations of the present work do not support those of the previous workers because :

- (a) Their microscopical observations were not clear enough.
- (b) The decay produced in delignified Scots pine in the present work indicates that the fungus penetrated and occupied the cell wall layers, including the middle lamella.

By comparing microscopically the decay patterns produced by

the same fungus in normal and delignified Scots pine, under similar ecological conditions, a great difference appeared. In normal Scots pine the fungus was hardly degrading the cell wall at all but was behaving as a stainer; while in delignified Scots pine after similar exposure to the fungus, almost all the cell wall was degraded, causing a very high weight loss (see part II). Nilsson (1974c) observed no or very few cavities in pulped wood, and concluded that high lignin increases type 1 decay while delignification increases type 2. He attributed the sparse cavity formation in delignified wood to the disorganisation of microfibrils produced during delignification. The present work does not entirely support this conclusion, as the microfibrillar orientation in delignified wood is very similar to that in normal wood except for a slight separation in the middle lamella region.

The appearance of a T-branch without cavity formation might suggest that this stage could be considered as an initial stage of cavity formation in normal wood but not in delignified wood specimens. The absence of cavities in delignified Scots pine could be interpreted as follows :

(a) The delignification process stopped the  $S_3$  layer acting as a physical barrier to the fungal hypha, which penetrated easily and aligned itself along the microfibrillar orientation in the  $S_2$  layer.

(b) Delignification might have acted as a lignin-modifying enzyme which removed the necessity for this to be secreted by the fungus. This postulation could be supported by the results of Wardrop (1975) when he treated fibres and parenchyma cells with cellulase, the parenchyma cell microfibrils were

scattered and separated, while microfibrils in the fibres remained intact.

(c) The treatment might have opened up the cellulose structure, which resulted in more space between the microfibrils and therefore the penetration of whole hypha without forming "proboscis hypha" was possible. This direct penetration helped in direct diffusion of cellulase from the fungal hypha to breakdown the cellulose without cavity formation.

Micrographs of delignified attacked wood specimens revealed the absence of dark granular material around the fungal hypha, except in a few micrographs (Figs. 87 - 93). In these few micrographs the granular material is finer than in normal wood, which suggests either that lignin was left in that area and not washed away after delignification, or that cell wall constituents other than lignin were present in that residue. The variation which appeared in delignified birch and Scots pine makes it difficult to draw a very satisfactory conclusion applicable to all kinds of wood species. It may be concluded that the removal of lignin from the other cell wall constituents of Scots pine has supplied the fungus and its enzyme with a space in which to move freely through the cell wall; or else gives the enzyme clear access to the sites where the hemicelluloses or the cellulose can be broken down.

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## PART V

### GENERAL DISCUSSION AND CONCLUSIONS

Until the beginning of this decade, very little detailed information was available on soft rot decay and cavity formation in wood. Using both SEM and TEM Findlay (1970) obtained excellent TEM micrographs for soft rot cavities in birch caused by Phialophora fastigiata S6A, but he was unfortunate in not showing clearly the orientation of microfibrils in each layer of the secondary cell wall. He was no more successful with the delignified Scots pine attacked by the previous fungus. The present work followed-up Findlay's ideas with more intensive investigation of the effect of lignin on the behaviour of Phialophora fastigiata in normal and delignified Scots pine. Furthermore the effect of different media on the behaviour of Phialophora fastigiata in Scots pine was studied, using the SEM only. The results have been discussed in three different parts : II - Physiological background, III - Scanning electron microscopy, and IV - Transmission electron microscopy. From this current information several general conclusions can be drawn.

1. The fungus Phialophora fastigiata can behave as a stainer fungus and can cause soft rot decay (cavity formation) in Scots pine (softwood) as well as in birch (hardwood).
2. The high lignin content in Scots pine is a delaying factor for the cavity formation but not a prohibiting one. It can, therefore, be inferred from Sinner et al (1975) that hemicellulose has possibly a significant role in soft rot decay.
3. The delignification does not affect the orientation of the microfibrils in the secondary cell wall whatsoever, but some effect was slightly apparent in the middle lamella. It is possible that the delignification has removed a part of the hemicellulose which

has opened up the cellulose structure and enabled the fungal enzymes to move more freely through the wall or have access to the cellulose.

4. The delignification helped the fungal hypha to penetrate through the barrier  $S_3$  layer and align itself along the microfibrillar orientation in the  $S_2$  layer.

5. The delignification may have been acting as a lignin-modifying enzyme or in other words demethoxylating the lignin.

6. The decay pattern in delignified Scots pine caused by the fungus is neither erosion type nor cavity formation, because the fungal hyphae can invade the secondary wall aggressively causing remarkable weight loss which is almost 70% in cellulose medium and 50% in malt agar medium after 6 weeks attack.

7. The weight loss test is not very reliable as a measure of the decay capacity of a fungus, which can vary according to the size of the wood blocks, substrate, the amount of each of the chemical constituents of the substrate, pH, temperature and the wood specimen.

8. By culturing different strains of Phialophora fastigiata on different media it is possible to produce a great variation in the type of decay in Scots pine. This variation represents soft rot decay and a decay which is different from the type 2 decay (Corbett, 1965), because of the high resistance of the  $S_3$  layer. The decay pattern in the secondary cell wall has a porous (perforated) appearance which most probably occurs by enzyme diffusion from the fungal hypha in the lumen of the cell through the  $S_3$  layer without any conspicuous change in the latter. This type of decay could be called Trans- $S_3$  erosion. Here one would suggest further investigation on the physiology and the enzyme system of the fungus.

9. Potassium permanganate can be considered the best fixative to show the orientation of microfibrils in the secondary cell wall in both normal and delignified wood specimens, by comparison with osmic acid fixation which shows up the hyphal cell content.
10. Epon resin is an excellent embedding medium for wood specimens when the resin is infiltrated through the cell wall evenly.
11. The electron dense material surrounding the fungus at the apex of the penetration hypha probably supports the view that the early penetration of the fungal hypha into the cell wall is enzymatic.
12. It is argued from examination of lignified and delignified wood that the granular material surrounding the hypha within the cavity supports the view that this is lignin residue.
13. The penetration hypha is highly constricted at the middle lamella and the  $S_3$  layer levels. The appearance of septa in the penetration hyphae at these points seems to be a constant feature. Possibly the mechanical restraint imposed by these regions on the penetration hyphal wall is causing this septum to form to prevent its cell from collapsing.
14. The  $S_3$  layer is very resistant to the decay in normal wood and is seen time and again to be in an unbroken state.
15. Cavity formation possibly occurs by a combination of mechanical and chemical dissolution of the  $S_2$  layer originating from the proboscis hypha.

#### ACKNOWLEDGEMENTS

It is with a deep sense of gratitude that I acknowledge the encouragement and financial support which I have received from His Highness Shaikh Jaber Al-Ahmed, the Prime Minister of the State of Kuwait, throughout the period I have been engaged in this work.

I also acknowledge the financial assistance I have received from Mr. Abdul-Aziz Al-Ali Al-Mutawah, for which I am most grateful.

I acknowledge the assistance of the University of Kuwait in giving me leave of absence for four years.

For their friendship, help and co-operation during my time with them at Imperial College, my thanks to Professor Rutter the Head of the Botany Department, the members of the Electron Microscopy Section, Dr. Anthony Dickerson of the Biochemistry Department, my colleagues in the Timber Technology Section and the Technicians in the Botany Department. My thanks to Mr. Roy Adams for his helpfulness.

I am grateful to Dr. Thomas Nilsson of Sweden for his collaboration, and also to Dr. Susan Jutte for her invaluable guidance.

I give my thanks to Miss Ann Soley for her assistance in photography; and to Mrs. June Cheston for typing this thesis.

I am happy to acknowledge the indispensable assistance of Mrs. Padrina Dsouza, the devoted nanny of my three children Jehann Danna and Nawaf; and to express my gratitude and admiration for the forbearance she and they have shown during our separation.

Lastly I acknowledge the patient encouragement and the sympathetic guidance of my Supervisor, Dr. John Levy, who with his great kindness and understanding helped and lent moral support to me through all my problems, from my ignorance of microfibrils to my homesickness. My grateful appreciation to him.

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