ON THE EFFECT OF BLUE STAINING FUNGI ON THE PERMEABILITY OF SITKA SPRUCE

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A thesis submitted to the

UNIVERSITY OF ASTON IN BIRMINGHAM

for the Degree of MASTER OF SCIENCE

FEBRUARY 1972

SUMMARY

Some aspects of the ecology and physiology of blue-staining and mould fungi were investigated with particular reference to their wood permeability enhancing mechanisms.

Of the thirty three species tested, all were seen to be considerably amylolytic, but many species did not exhibit a similar intensity of cellulolytic or pectinolytic activity. Some species were seen to possess both active cellulase and pectinase systems, though the greater number of test species were seen to possess active cellulase systems only, under the test conditions.

The growth rates of the individual fungal species was seen to considerably influence their production of enzymes. Faster-growing species apparently produced greater quantities of enzymes than slower-growing species, and they similarly produced a more extensive degradation. However, the intensity of degradation produced by the slower-growing species, relative to that produced by the faster-growing species, was considered to be highly significant.

It was hypothesised that many of the blue-staining and mould fungi tested were primarily amylolytic but possessed secondary cellulase or pectinase systems. It was considered, however, that the interrelationships between the amylase, cellulase and pectinase systems of these species would require further more detailed investigation.

It was suggested that the celluloytic or pectinolytic activities of many of the species tested may would considerably influence their ability to enhance the permeability of wood.

ACKNOWLEDGEMENTS

I wish to thank the Institute for Industrial Research and Standards, Dublin, for their financial support and for their permission to publish the results of my research.

I wish to express my sincere thanks to Dr. H.O.W. Eggins for accepting me as a research student, for introducing me to biodeterioration research, and for his unfailing interest, encouragement, and guidance for the duration of my work.

I am grateful to my colleagues, Mr. T.G. Barnes and Dr. G. Canevascini, for their advice and helpful criticisms, and also to Miss D. Smith for her able technical assistance.

I am particularly grateful to my wife, without whose encouragement and patience this work would not have been undertaken, and without whose typing ability the present form of this thesis would not have been possible.

CHAPTER 1

I N T R O D U C T I O N

INTRODUCTION

The utilization of timber for exterior usage is largely dependent on the durability of the individual timber species. Durable timbers are resistant to attack by micro-organisms, whilst non-durable timbers must be treated with preservatives before they are used in locations where conditions favourable to decay prevail.

The production of coniferous forests has been a major part of the Irish Forestry program, and Sitka spruce (Picea sitchensis) is one of the commercially important species which are planted. Spruce is extremely suitable to use for structural wood, interior joinery and pulpwood, and due to its straight grain and strength properties (Randle 1957) is particularly suitable for use as transmission pole material.

Sitka spruce grows rapidly in Ireland, reaching maturity in forty to fifty years, and it contains large areas of sapwood. The sapwood of spruce is non-durable and cannot adequately be treated with preservatives due to the impermeable nature of the wood. Petty (1970) suggested that the utilization of Sitka spruce should be limited to areas in which preservative treatment of the wood would be unnecessary.

A country such as Ireland, with limited resources, must utilize these fully to achieve its maximal economic potential. As spruce is impermeable, the fullest use of this timber is unavoidably prevented since only slight impregnation of the wood with

preservatives is possible.

The proposed program by the Electricity and Transport authorities in Ireland to replace the present transmission pole system places a demand for a steady supply of transmission pole material. If permeability of native spruce could be improved by any economic means, a ready supply of suitable material would be available to meet this demand.

1.1. Spruce permeability

Liese & Bauch (1967) have reviewed the literature relating to anatomical features controlling spruce permeability. They noted from the work of Curtois (1964b) that axial permeability was related to the length of earlywood tracheids in spruce, greater permeability being found in wood with longer tracheids than in that with shorter tracheids. Frey-Wyssling & Muhlethaler (1965), Petty (1970), Stamm (1970) and Smith & Banks (1970) considered that axial permeability was greatly affected by the number of bordered pits per tracheid, the number of these pits remaining unaspirated, and the size and number of pit membrane pores. Petty (1970) showed that the latewood regions of spruce were sometimes considerably more permeable than the earlywood regions.

Liese & Hartmann Fahrenbrock (1953) revealed that the inner pit chamber of spruce was smooth, whereas a warty layer covered the inner pit surface of pine, a permeable species. It was suggested that the warty

layer prevented pit aspiration in pine whereas the smooth pit chamber of spruce contributed towards pit aspiration and sealing. Colmstock and Cote (1968) and Vakin et al. (1968) also agreed with this hypothesis. However, Banks (1970) showed that longitudinal and tangential flow paths of spruce were not blocked when the wood was in the green condition.

Radial permeability of spruce seems greatly associated with rays and ray structure. Koljo (1953) observed that the radial permeability of pine was ten times that of spruce, and Schultze (1960) noted that permeable wood had higher percentages of ray area than impermeable wood. Dunleavy & McGuire (1970) considered that radial preservative penetration of spruce was greatly impeded by ray structure, and Banks (1970) showed that the radial permeability of spruce was limited even when in the green state.

The ray structures of pine and spruce differ considerably. Ray parenchyma cells of pine are interconnected, and are connected with vertical tracheids by large windowloid pits. The ray parenchyma cells of spruce, however, are interconnected by small channels and are connected to vertical tracheids by small, half-bordered pits.

Huber (1949) noted that the ray tracheids above and below parenchyma rows of spruce rays were frequently discontinuous. Ray parenchyma cells were also frequently seen to be between ray tracheids at the

borders of annual rings. Ray tracheids of spruce possess unaspirated bordered pits even when air dried. When compared with ray parenchyma cells, ray tracheids were seen to be preferentially penetrated after preservative treatment, though this penetration may be seen to end at the borders of annual rings where parenchyma cell insertion had occurred.

Permeable ray tracheids are therefore of some importance in radial impregnation of spruce but in this respect spruce is seen to suffer from a lack of that tissue. Nyren & Bach (1960) showed that the ratio of ray tracheids to ray parenchyma in spruce was 1 : 2.7, and in pine was 2.8 : 1, while Liese and Bauch (1967) showed that ray tracheid - ray parenchyma ratio in spruce was 1 : 4 and in pine was 1 : 1. Liese & Bauch (1967) similarly found close correlation between the water absorption levels of both pine and spruce and their respective ray tracheid areas.

It therefore appears that the lack of permeability in spruce, relative to other species, can be attributed to the following anatomical features:

(1) The smooth inner pit chamber of the spruce bordered pit contributes towards pit aspiration when the wood dries. Pit aspiration seals connecting pathways between tracheids thus limiting axial and tangential impregnation.

(2) (i) The parenchyma cells of spruce possess pits of small size in relation to permeable species;

(ii) The ray tracheid system of spruce ray tissue is discontinuous;

(iii) Sprucewood possesses smaller proportions of ray area than permeable species;

(iv) The ray parenchyma - ray tracheid ratio of spruce is adverse in comparison with permeable species.

Features (i) and (ii) retard liquid penetration of individual rays and features (iii) and (iv) limit the overall impregnation capacity, the four features thus effectively preventing deep radial impregnation of the wood.

It is therefore indicated that any enchancement of spruce permeability must depend on overcoming the barriers to penetration presented by the anatomical features mentioned above. To overcome this problem, it is necessary that the impeding tissue must be disrupted or destroyed. It was therefore considered that biological methods of selective degradation of wood should be examined with particular reference to their application in enhancing spruce permeability.

1.2. Biologically induced enhanced permeability

Isle (1957), Stutz and Stout (1957), and Soulahti and Wallon (1958) all observed an increased permeability of wood after water storage. Forest Products Research

Laboratory Reports of 1959, 1960 and 1963 indicated a similar reaction, and an F.P.R.L. Report of 1960 referred to a progressive increase in permeability of Sitka spruce when logs of that species were pond stored for twenty months. Holmgren (1961), McPeak (1963) and Schulz (1968) observed enhanced permeability in wood after water storage, but McPeak did not detect permeability differences between wood stored in ponds, or wood sprayed with fresh water.

Enhanced wood permeability due to water storage has been attributed to bacterial activity, (Ellwood and Ecklund 1959; Cowling 1965; Vasilev 1965; Schulz 1968; Liese and Karnop 1968; Vakin et al 1968, and Dunleavy & McGuire 1970). Shigo (1965) and Lutz, Duncan & Scheffer (1966) isolated motile bacteria from such wood, Cowling (1965) isolated sulphur-reducing bacteria, whilst Boutelje and Keissling (1964) isolated gram-negative bacteria.

Blew (1952) observed that wood exhibiting blue stain and incipient decay showed significantly enhanced permeability in comparison with non-infected wood. Lindgren (1952) and Lindgren & Harvey (1952) observed an increased permeability of wood when infected with Trichoderma viride and an even greater permeability when logs treated with sodium flouride were colonised by that species. Lindgren & Wright (1954) however, considered that a mixed microfungal infection including species of Alternaria, Ophiostoma, and Trichoderma produced a greater enhanced permeability of wood than when wood was treated with sodium flouride and colonized

by T. viride alone. Graham (1954) and Perry (1955) also observed significant permeability increases in wood infected with mixed micro-fungal and blue-staining fungal species. Perry (1955) similarly considered that wood infected by a mixed group of micro organisms showed greater preservative absorption when attacked by these groups than when attacked by T. viride alone, while Schulz (1956) Panek (1957), and Verral (1957) noted significant permeability increases in wood when attacked by the single species T. viride.

Liese & Bauch (1967) considered that enhanced permeability of spruce due to infection by blue-staining and mould fungi was insufficient to enable adequate preservation, and also that the presence of basidiomycete fungi could not be precluded. However, Vakin et al. (1968) noted that permeability of both sapwood and heartwood of spruce was so enhanced after infection by mould and staining fungi that it was possible to use the impregnated logs for transmission pole purposes. Hulme & Shields (1970) suggested that wood permeability might effectively be enhanced by a number of microfungi, whilst Greaves and Barnacle (1970) considered that selective wood colonization by Trichoderma spp. and Gliocladium spp. should result in a required enhancement.

It can therefore be considered that certain biological activities, namely fungal or bacterial activity, will enhance the permeability of both permeable and impermeable woods. In the former, this can be a considerable disadvantage since fungal or bacterial activity of this

wood may result in "overloading" of the treated wood with preservatives, but in the case of impermeable species the enhanced permeability to preservatives will aid the more economic usage of the wood.

1.3. Colonization Patterns and Degradation Mechanisms of Organisms inducing Enhanced Permeability

The wood colonization patterns of both bacteria and fungi, resulting in enhanced wood permeability, appear to be very similar. The basic pattern would seem to be one in which initial colonization of ray tissue, followed by subsequent colonization of adjacent vertical tracheids by the infecting organisms, takes place.

1.3.1. Bacterial Colonization Pattern.

An alternation in the structure of ray tissue in association with starch depletion in ponded logs, as a result of bacterial activity, was first noted by Ellwood and Ecklund (1959). Knuth (1964) in laboratory wood colonization experiments, using Bacillus polymyxa and Bacillus subtilis, observed that both species attacked the pits of both pine and sweetgum, whilst Greaves (1965) also using B. polymyxa, observed a build-up of the bacterium in ray cells resulting in a slow breakdown of parenchyma walls and subsequent movement of the organism to vertical tracheids via the pits, but not degrading the latter. A number of workers have corroborated these findings, including Harmsen and Vincents-Nisson (1965); Lutz, Duncan and Scheffer (1966); Levy (1967); Boutelje and Bravery (1968);

Vakin et al. (1968); Greaves (1969); Highly and Lutz (1970); / Dunleavy and McGuire (1970). Greaves and Foster (1969) considered that the ray tissue acted as nutrient sites for the bacteria before pit colonization commenced.

1.3.2. Blue Staining and Mould Fungal Colonization Pattern.

Starch depletion and utilization in combination with colonization of ray tissue has similarly been associated with the growth of blue-staining and mould fungi (Savory 1954; Liese and Schmid 1962; Norkrans 1967; Hulme and Shields 1970). Colonization of ray tissue, containing much of the easily-metabolized carbohydrates of wood, is normally the first stage of infection by these fungi. From the ray tissue colonization spreads to the vertical elements in passive fashion. Schmid and Liese (1965) and Liese (1965) considered that some blue-staining fungi mechanically penetrated cell walls by special hyphal extensions called transpressoria. However, Corbett (1965) and Levy (1967) suggested that many soft-rot fungi, some of which are "mould fungi" (Merrill 1965, 1966), and others blue-staining fungi (Duncan and Eslyn 1966) colonize wood in an apparently similar fashion i.e. initial colonization of ray tissue followed by passive penetration into the lumina of adjacent tracheids via the pits.

It can therefore be seen that bacteria, and mould, blue-staining, and soft-rot fungi, have essentially similar colonization patterns and that these patterns correspond with those areas of spruce i.e. ray tissue and bordered pits, which require disruption or destruction before permeability is enhanced. Therefore, provided

that the infecting organisms have the necessary degradation mechanisms, enhanced permeability may be induced in those areas of wood colonized by them.

1.3.3. Biological Degradation Mechanisms Producing Enhanced Permeability.

Recent enzyme studies on enhanced permeability of wood have shown interesting results. Nicholas and Thomas (1968) using the enzymes pectinase, cellulase and hemicellulase, considered that pectinase was the most effective of the three in producing enhancement of permeability. Pectinase was seen to act by creating large openings in bordered pits and severe degradation of parenchyma cells. However, Liese (1970), comparing the effects of both cellulase and pectinase on wood permeability, considered that either enzyme created a similar permeability increase, cellulase by acting on the pit-margo microfibrils, and pectinase by acting on the tori of pits.

Permeability enhancement in vivo seems similarly associated with a cellulolytic and pectinolytic activity of the causative organisms. Lindgren (1952) ascribed enhanced permeability of wood to decreased pectin content as a result of microfungal activity. Similarly, Soulahti and Wallon (1958), observed that the pectin content of water-stored wood decreased considerably, presumably as a result of bacterial action. Ellwood and Ecklund (1959) noted that Bacillus polymyxa, apparently the causative organism of the enhanced permeability observed by them, was capable of decomposing hemiculluloses and pectins,

whilst Knuth and McKoy (1962) observed that four strains of B. polymyxa under test, all utilized cellobiose, hemicellulose, xylan, and pectin. Liese and Karnop (1968) observed that bacteria isolated from ponded Scots pine showed a pectinolytic activity, while Highly and Lutz (1970) attributed enhanced permeability of ponded pine and poplar to loss of pectins due to bacterial activity. Liese and Bauch (1967) and Vakin et al. (1968) considered that permeability enhancement in wood, after colonization by blue-staining and mould fungi, could be attributed to a cellulolytic activity of the colonizing species.

The literature also shows that the mechanical strength of wood may not greatly be affected as a result of biologically-induced enhanced permeability. Knuth (1964), Vakin et al. (1968), and Scheffer, Duncan and Wilkinson (1969) did not observe significant strength losses in bacterially attacked timber exhibiting enhanced permeability, and similarly, neither Vakin et al. (1968) nor Hulme and Shields (1970) detected strength losses in wood which was subject to blue-stain and mould-fungal colonization similarly resulting in enhanced permeability. However, some slight strength loss in impact resistance has been recorded for wood attacked by certain blue-staining fungal genera. (Cartwright and Findlay 1958; Campbell 1959).

This general lack of significant strength loss may be due to the fact that neither bacteria nor staining fungi seem capable of degrading lignified cellulose. Savory (1954) considered that soft-rot fungi did not

attack heavily-lignified cellulose, although Krapvina (1960) showed that lignified cellulose could be degraded by certain species of blue-staining or soft-rot fungi. Lutz, Duncan and Scheffer (1966) observed that bacterial attack did not result in degradation of heavily-lignified tissue while Henningson (1967) observed that Bacillus omelianscii, the causative organism of enhanced permeability of spruce in some German lakes (Liese and Karnop 1968), was cellulolytic, but was not, however, capable of degrading lignified tissue. Similarly, Levy (1967) showed that the noted soft-rot fungus, Chaetomium globsum, was unable to degrade the heavily-lignified latewood of softwoods.

This basic data on spruce permeability and biological methods of inducing enhanced permeability may therefore be summarised as follows.

1) Sprucewood, because of its structure, is resistant to preservative impregnation. Aspiration of bordered pits in tracheids, small volume of rays, and small pit sizes of individual ray cells, are all contributory factors.

2) Both individual blue-staining fungal genera and some bacterial strains may be able to enhance spruce permeability.

3) Enhanced permeability is primarily due to the colonization and penetration patterns of these organisms and their individual

degradation mechanisms.

4) The action of both bacteria and fungi may be considered not to affect significantly the mechanistic properties of the wood, apparently because of their non-lignolytic activity.

5) The cellulase and pectinase systems of the colonizing organisms may be considered to be the biological mechanisms most likely to enhance permeability.

1.4. Research Theme

The literature survey at this stage has shown that permeability of refractive spruce may be enhanced by the action of bacteria during ponding of spruce or alternatively by the action of blue-staining and mould fungi during forest storage of the wood.

As the Forestry Department in the Republic of Ireland was interested in maximal utilization of native sprucewood, it was considered that biological methods of inducing enhanced permeability in spruce should be investigated, the aspects of obvious interest being 1) bacterial activity, and 2) blue-staining fungal activity.

The Forest Products Department of the Institute for Industrial Research and Standards, Dublin, undertook research on the use of bacteria and log ponding in enhancing spruce permeability. It was decided that the activities of blue-staining fungi in the context of

enhanced wood permeability would be the object of the investigation undertaken at the Biodeterioration Information Centre in the Department of Biological Sciences in the University of Aston in Birmingham.

1.5. Blue Staining and Mould Fungi

Microfungi colonizing wood and causing discoloration may be described in the literature as "bluing fungi", "sapstain fungi", "blue stain fungi" and "mould fungi", sometimes with little differentiation between the terms used (Eslyn 1967). Microfungal colonization of wood resulting in its discoloration may be placed in three categories:

1) Wood colonization by fungal species, the dematiaceous hyphae of which within the lumina of tracheids and parenchyma cells, result in the appearance of staining in wood (Savory 1954, 1966, Butcher 1968).

2) Wood colonization by fungal species resulting in surface discoloration due to pigmented spores and hyaline hyphae (Norkrans 1967; F.P.R.L. Leaflet No. 12, 1969).

3) Colonization of wood by fungi with either hyaline or dematiaceous hyphae, by whose metabolism the sap of green wood is discoloured by iron reactions resulting in deep or surface staining of wood (Gadd 1965, Norkrans 1967).

Generally speaking, however, stained wood may exhibit

simultaneous colonization by species within these three groupings, and colonization is not necessarily limited to individual blue-staining fungal species, or any of the individual groupings outlined above (Kaarik 1968; Dowding 1970).

Karkanis (1966) showed that initial colonization of wood by blue-staining fungi occurred in ray tissue, and from there spread to adjacent vertical tissue. He considered blue-staining fungi to be intermediate between mould fungi which apparently only colonize the surface of wood and do not cause degradation, and the Basidiomycetes, which penetrate deeply into wood, and cause serious degradation.

However, differentiation between staining and mould fungi on the basis of their penetration of wood is difficult since many mould fungi e.g. Trichoderma spp. and Gliocladium spp. penetrate deeply into wood (Butcher 1968; Hulme and Shields 1970), and similarly many dematiaceous staining fungi e.g. Alternaria spp., Aureobasidium pullulans and Sclerophoma pithyophila only colonize the surface of wood (Dowding 1970).

Liese and Schmid (1964), Liese (1965) and Liese and Schmid (1966) have shown that the hyphae of the blue-staining fungi, Aureobasidium pullulans, Ceratocystis piceae, Ceratocystis pilifera and Phialocephala phycomyces, all exhibit transpressoria, by means of which lignified cell walls are penetrated. They suggested that cell wall penetration by these species was due to mechanical pressure exerted at the

transpressorium by the individual organisms, but similarly, they did not discount the possibility of local enzymatic activity. Butcher (1968) observed that the passage of blue-staining fungal penetration from cell to cell lay through the bordered pits or through bore holes produced by mechanical pressure. This penetration pattern is similar to that described by Corbett (1965) for passive penetration of soft-rot fungi as mentioned earlier. Savory (1954) defined a soft-rot fungus as a fungus producing cavities in secondary walls of wood tracheids. Krapvina (1960), when working with Ophiostoma picea, O. caerulescens, O. pini, Diplodia pinicola and Phialophora fastigiata, observed that all produced T branches, thus demonstrating that blue-staining fungi could also in fact be soft-rot fungi. Karkanis (1966) and Findlay (1970) have noted a similar effect for individual blue-staining fungal species including Ceratocystis pilifera.

A number of workers including Duncan (1960), Duncan and Eslyn (1963), Merril (1965) and Merril and French (1966) have similarly indicated a number of mould fungi which are similarly capable of cavity formation in wood cells, thus it appears that differentiation between mould and blue-staining fungi, on the basis of their respective soft-rotting activity, is also difficult.

Cartwright and Findlay (1958) considered that blue staining fungal colonization of wood resulted in insignificant losses of compression and bending strengths, but could considerably affect toughness or impact bending strength. This viewpoint was corroborated by Campbell

(1959), who showed that though Diplodia pinea did not cause strength losses, Diplodia natalensis decreased wood toughness by 17 per cent. He further observed that strength losses of up to 43 per cent were recorded for Obeche and Corsican pine after their colonization by Botriodiplodia theobromae, whilst alternatively, Vakin et al. (1968) did not observe significant mechanical deterioration of wood infected by a mixed group of mould and staining fungi.

"Blue-staining and mould fungi" can therefore be considered to encompass a number of organisms which include Ascomycetes and Fungi Imperfecti. It would further seem that they include both surface-colonizing and deeply-penetrating species, and species which have either dematiaceous or hyaline hyphae. Similarly, it would seem as if the group would include both actively and passively penetrating species.

The anomalous behaviour of blue-staining and mould fungi with reference to wood penetration and degradation may be explained by their physiological requirements, particularly with regard to their carbohydrate utilization.

Savory (1954) considered that blue-staining and mould fungi utilized mainly starches and sugars in sapwood cells. Liese and Schmid (1962) and Norkrans (1967) agreed with this, suggesting that only protoplasm of parenchyma cells was utilized and that blue-staining fungi did not produce cellulase or ligninase. Krapvina (1960), however, while agreeing that protoplast components were utilized by blue-staining fungi, considered that cell wall

degradation occurred, after depletion of cell contents and Olofinboba and Lawton (1968) demonstrated that Botriodiplodia theobromae, generally considered to be the most important fungal species involved in the staining of Antiaris africana, utilized a considerable range of carbohydrates. Umezurike (1969) also working with B. theobromae, considered that it could utilize cellulose and hemicellulose after depletion of starch reserves in wood, and further, while able to degrade the wood, could not in fact colonize wood unless the starches and cell contents were present at initial colonization.

Since blue-staining and mould fungi are not economically significant in terms of wood degradation, other than the usually serious aesthetic defects caused by their discoloration, little attention has been paid to their enzymatic activity. However, some recent work by Rosch, Liese and Berndt (1969) has shown that the staining fungi, Aureobasidium pullulans, Alternaria humicola, and Ceratocystis minor produced a range of enzymes including carboxymethylcellulase, polygalacturonase, pectinesterase and diphenyloxidase.

Staining and mould fungi could therefore be considered to occupy a similar ecological niche i.e. green wood in which starch reserves were present, and would similarly seem to be primarily starch-utilizing organisms. However, the laboratory evidence of cellulase production and cavity formation would indicate that some species were capable of timber degradation, as would their production of enhanced wood permeability.

axial and tangential penetration of preservatives. The colonization pattern of blue-staining fungi is such that ray tissue is preferentially colonized, with subsequent colonization of adjacent tracheids via pits and bore holes. However, degradation in association with this colonization must also be produced to enhance the permeability of spruce.

1.6. Outline of Research

It has been pointed out that the enzymes cellulase and pectinase were chiefly responsible for enhancing wood permeability, and that many of the bacterial species associated with enhanced permeability produced both these enzymes. However, similar evidence is not available for many blue-staining or mould fungi. As these systems may be critical in producing selective degradation, a first stage towards the use of these species is the verification of their possession of these degradation mechanisms.

The second point to be considered is the procedure used to infest green wood with organisms possessing suitable degradation mechanisms. If only some blue staining fungi possess the necessary selective degradation mechanisms, random infestation by blue-staining fungi, on exposure of the timber to forest fungal flora, need not therefore lead to any significant enhancement of spruce permeability. Alternatively, if all or many blue-staining and mould fungi possess the selective degradation mechanisms necessary to enhance spruce permeability, it is probable that the latter may be induced by random infestation. It is therefore necessary to determine the extent of possession of cellulose and pectinase systems within

the blue-staining and mould fungal grouping, since this would decide whether the organisms could be used in random fashion, or by mono or multi culture inoculation under controlled conditions.

The third point to be considered is the traditional attitude towards the role of staining fungi in the ecology of wood degradation. It has commonly been assumed that staining fungi only degraded starches and cell contents of wood, and did not degrade wood components. However, recent information indicates that some staining fungi may degrade wood. A comparative investigation of their degradation and non-degradation activities may therefore throw some light on this anomalous situation, and also on whether staining fungi would be likely to enhance permeability of wood.

The problem of enhanced spruce permeability due to the action of blue-staining and mould fungi can therefore be stated in the form of three questions:

1) Are blue-staining and mould fungi amylolytic, and do blue-staining fungi only utilize starches and cell components of green wood?

2) Can blue-staining and mould fungi degrade wood components along with wood cell contents?

3) Can blue-staining and mould fungi as a grouping undertake either or both of the above functions, or are they undertaken by individual blue-staining fungal species only?

The answers to these questions would indicate the

degradation capabilities of blue-staining and mould fungi, and therefore their potential usage in enhancing spruce permeability either by random infestation or by mono or multi culture inoculation under controlled conditions. The answers would also indicate the role of blue-staining and mould fungi in the ecology of the degradation offreshly-felled wood, and whether they were primarily amylolytic with ancillary degradation mechanisms, or were in fact wood degradation organisms.

The hypothesis that blue staining and mould fungi could enhance wood permeability was therefore examined with reference to these aspects. A number of test species were selected with reference to the literature on wood discoloration resulting from blue staining and mould fungal colonization, and these species were screened for their deterioration activities.

As a preliminary stage to these investigations, simple growth studies were undertaken on agar plates to determine differences between individual test species, and also to observe possible differences in their growth patterns on the carbon sources starch, cellulose and pectin.

Subsequent to these initial investigations, using starch and cellulose agars and pectate gels, relative amylolytic, cellulolytic and pectinolytic activities were assayed <u>in vivo</u>.

To qualify the results of the agar studies on cellulolytic activity, the latter was also examined using loss in tensile strength of fibrous cellulose

as a criterion. Pectinolytic activity assayed by the pectate gel technique was similarly assessed by a viscosimetric method. The latter also provided some initial data on the interrelationship of cellulolytic and pectinolytic activity.

A limited ecological survey was undertaken, concurrently with the degradation investigations, to verify the presence of the selected test species in Irish forests.

CHAPTER 2

TEST ORGANISMS

2. TEST ORGANISMS

2.1. Introduction

While some staining and mould fungi have been seen to enhance the permeability of spruce, it was not clear from the literature whether all or only some of these species possessed the degradation mechanisms necessary for wood permeability enhancement. This factor was considered to be of some importance as the use of those fungi to enhance spruce permeability could be undertaken in two ways: (a) controlled infestation, or (b) random infestation.

(a) Using a controlled infestation process, green wood would be inoculated with known species possessing the necessary cellulase or pectinase systems in such fashion that only growth of the inoculated species would take place; while

(b) using a random infestation process, the green wood requiring an enhanced permeability would merely be exposed to the naturally-occurring staining and mould fungal flora.

The former process would therefore entail the selective growth of particular species on the wood under controlled conditions, while the latter process would require neither selected species nor controlled conditions. Before either of these processes could be applied, however, and since basic data on the degradation mechanisms of commonly found blue staining and mould fungi in the context of enhanced permeability was scarce, it was decided that as a pre-requisite to the application of either system basic data

on degradation mechanisms of these fungi should be established.

2.2. Selection of Test Species

This approach to the study therefore raised the question of the means of selection of test species. It was obviously desirable that blue-staining and mould fungal species native to Irish forests should be used in the investigation. However, this was largely precluded since the duration of the project was limited to two years, and it was considered that the time necessary for a detailed ecological survey would be of a minimal 12-18 months from the beginning of the project.

It was therefore decided that the problem would be approached by acquiring a range of blue-staining and mould fungi commonly associated with staining of wood. This group of organisms was selected with reference to the literature on wood-staining fungi, and from literature referring to timber degradation by micro-fungi. The group included both Ascomycetes and Fungi Imperfecti, and fungal species with both hyaline and dematiaceous mycelial structure. A list of these organisms is presented in Table 2.1. The table includes, along with the names of the individual organisms, literature sources referring to the individual fungal species, acquisition source, data on wood degradation pattern, and some notes on the activities of the individual species in laboratory culture. These species were used for the determination of degradation activities of blue-staining fungi.

It was also decided that the determination of naturally-occurring species in Irish forests should not be ignored since both the genera of colonizing species and their growth patterns could considerably qualify any observations resulting from the laboratory studies on the degradation mechanisms of the selected test species. Consequently, a limited ecological investigation was undertaken, concurrent with the laboratory studies, the detail of which is outlined in Chapter 9.

Twenty-six fungal genera represented by thirty-three species were selected with reference to the literature, while twenty-five genera represented by thirty-one fungal species were isolated from green wood exposed to fungal infection at three Irish forests.

The isolated genera included three species of Aspergillus, three species of Chaetomium, two species of Paecilomyces, two species of Mucor and one species each of Rhizopus and Zygorhynchus. In comparison with these results the selected test species included one Aspergillus species, A. niger, one species of Paecilomyces, P. variotii, and one Phycomycete, Pythium aphanidermatum.

Only one species of each of the genera, Ceratocystis, Phialophora, and Diplodia were isolated from wood samples while four Ceratocystis species, and three species each of Phialophora and Diplodia were selected with reference to the literature. Species of six of the isolated genera, Fusarium, Humicola, Papulospora, Penicillium, Phoma and Verticillium were not included among the selected test

organisms, while ten species representing the genera Botriodiplodia, Cytospora, Rhinocladiella, Myxotrichum, Helminthosporium, Trichocladium, Bisporomyces, Sclerophoma, Phialocephala and Discula, selected with reference to the literature were not isolated from the exposed wood.

Fifteen genera, represented by twenty two species, selected for degradation studies were similarly isolated from the wood exposed to forest conditions. Three of the remaining ten isolated species were Phycomycetes, and one of the isolated genera was Chaetomium, the noted soft-rot fungus (Savory, 1954). Ten species, not isolated from the wood, were substituted for the remaining six isolated species.

It can be seen from these results that the selection procedure in comparison with the isolation procedure was moderately successful, many of the test species selected with reference to the literature similarly being isolated from green wood stored in the forests in Ireland. It was therefore considered that since many of the test species could be found in Ireland, the results of this research were applicable to Irish forest conditions with reference to enhanced spruce permeability.

Wood Colonization or Degradation Pattern	Fungal Activity in Laboratory Culture	Acquisition Source
Permeability enhancement Active penetration	Carboxymethyl-cellulase and polygalacturon-ase production	Commonwealth Mycological Institute (C.M.I.)
-	Ballmilled cellulase production	Forest Products Research Laboratory (F.P.R.L.)
Passive penetration Active penetration	Carboxymethyl-cellulase production	C.M.I.
-	-	C.M.I.

Wood Colonization or Degradation Pattern	Fungal Activity in Laboratory Culture	Acquisition Source
Strength loss Active penetration	Carboxymethyl-cellulase production	C.M.I.
-	Cellulase and pectinase production	C.M.I.
-	Cellulase production	C.M.I.
Passive penetration	-	C.M.I.
Passive penetration Active penetration	-	C.M.I.
Passive penetration Active penetration	-	C.M.I.

Wood Colonization or Degradation Pattern	Fungal Activity in Laboratory Culture	Acquisition Source
-	-	C.M.I.
-	-	C.M.I.
-	-	C.M.I.
Strength loss	Cellulase production	F.P.R.L.
-	-	F.P.R.L.
-	-	C.M.I.
Permeability enhancement	-	F.P.R.L.

Wood Colonization or Degradation Pattern	Fungal Activity in Laboratory Culture	Acquisition Source
-	Ballmilled cellulase and carboxymethyl-cellulase production. Permeability enhancement	C.M.I.
-	-	B.I.C.
Soft-rot cavities	-	C.M.I.
Passive penetration	-	C.M.I.
-	-	C.M.I.

Wood Colonization or Degradation Pattern	Fungal Activity in Laboratory Culture	Acquisition Source
Active penetration	Cellulase, carboxy-methylcellulase, hemicellulase and pectinase production	C.M.I.
Passive penetration	-	C.M.I.
-	-	C.M.I.
Active penetration	-	C.M.I.
Weight loss	-	C.M.I.

Wood Colonization or Degradation Pattern	Fungal Activity in Laboratory Culture	Acquisition Source
-	Carboxymethyl-cellulase production	C.M.I.
Passive penetration	-	C.M.I.
-	Cellulase and ligninase production	C.M.I.
-	Antagonistic to basidiomycetes	C.M.I.
-	Antagonistic to basidiomycetes	C.M.I.

Wood Colonization or Degradation Pattern	Fungal Activity in Laboratory Culture	Acquisition Source
-	Cellulase and pectinase production	B.I.C.

CHAPTER 3

GROWTH RATE STUDIES

3. GROWTH RATE STUDIES

3.1. Introduction

Factors which can influence the extent of colonization and subsequent degradation of wood by fungi include the rate of growth of the species producing decay (Cartwright and Findlay 1934). Some fungi colonize wood more rapidly than others, and if the faster-growing fungi have similar degradation mechanisms to the slower-growing fungi, then it is likely that the extent of degradation produced by the faster-growing fungi will be greater than that produced by the slower-growing species (Hulme and Shields 1970).

As thirty three blue-staining fungal species were being screened for patterns of group activity, it was considered that basic differences between organisms might become apparent during simple growth studies. Lilly and Barnett (1951) reviewed methods of assessing fungal growth rate. Fungal growth rate may be measured both visually, using linear extension of fungal mycelium, and quantitatively, using the weight of mycelium produced on a particular substrate, as criteria of growth. Quantitative growth assessment would seem to be a better indication of substrate utilization than visual assessment (Walsh 1971), but since many blue-staining and mould fungi apparently colonize wood extensively without significantly utilizing the wood substrate, it was considered that growth rate studies using a visual assessment technique might be a simpler

gauge of colonization rate than biomass determination. The former technique has been used successfully by a number of workers to elucidate fungal nutritional and degradation patterns (Ryan et al. 1943; Brancarto and Goulding 1957; Butcher 1968; Evans 1969; Mills and Eggins 1971; and Walsh 1971).

It was therefore decided that similar studies might provide initial data on the colonization rates of blue-staining and mould fungi, whilst simultaneously, possibly indicating the nutritional preferences of the individual test species.

3.1. Materials and Methods

3.1.1. Test Organisms.

The test organisms used for this study included those species outlined in Table 2.1.

3.2.2. Materials.

Growth studies were undertaken on three selective media, which contained respectively starch, cellulose, and sodium pectate as sole carbon sources. Both cellulose and pectate were associated with those wood components, the degradation of which has been seen to result in permeability enhancement, while starch is considered to be the main constituent of cell contents of green timber.

Cellulose was in the form of cellulose powder CF 11 (Whatman), ball-milled for 72 hours, pectate was in the form of sodium polypectate Grade 11 No. P 1879 (Sigma), and starch was in the form of soluble starch

(May & Baker). The compositions of the three media are presented in Appendix I. The composition of cellulose agar was that of Eggins and Pugh (1962), while the composition of starch and pectin agars included the mineral components of that medium.

The pH of the three test media was 5.4., and growth studies were undertaken only at this pH level due to the large number of species under test, even though many of the species may have had pH optima for growth which did not correspond with this pH level. However, since many fungal species grow well at pH levels proximate to 5.4, it was considered that growth studies undertaken on the starch, cellulose and pectate media at the pH level might provide useful data on the comparative growth patterns of the test species.

3.2.3. Methods.

Using a 6-mm corkborer inocula were removed from actively-growing cultures of the thirty three test species. These cultures were maintained on Potato Dextrose Agar (Oxoid CM 139). Inocula were aseptically transferred to the centre of petri dishes containing 15-ml quantities of each of the three media. These subcultures were incubated at 25°C for fifteen days. Pechman (1965) considered that staining fungi could grow at a wide range of temperatures. However, he also considered that many staining fungi grow optimally between 20°C and 28°C, and many workers have considered that mesophilic fungi grow well at similar temperature ranges. As a considerable number of species were being tested on three media, it

was considered that an incubation temperature of 25°C was adequate to provide initial basic growth data for the test species.

Growth measurement of three replicates was made for each organism on each medium per measurement interval. The measurements taken were the diameters of the fungal colony taken at right angles to each other. These measurements were converted to a percentage (% surface colonization) calculated from the area of the colony in relation to the surface area of the medium from a standard curve. Measurments were made on the second, fourth, seventh, tenth and fifteenth day of incubation.

Species showing growth in excess of 50% of the surface area of the medium after the experiment duration were regarded as rapidly growing, species showing surface growth in excess of 25%, but not greater than 50% of the medium surface area were regarded as growing moderately, and species showing surface growth which did not exceed 25% of the surface area of the medium were considered to grow slowly.

3.3. Results

The relative growth rates of thirtythree blue staining fungal species were examined using the percentage surface colonization of three media by the individual species as an indication of growth rate. The influence of the carbon sources starch, cellulose and pectate, on the growth rate of the individual species was also examined. Results are presented in tabular and graphical form.

The relative growth rates of the test organisms revealed a wide range of variation ranging from 2.5% surface colonization of pectate agar by Bisporomyces chlamydosporis after fifteen days, to the 100% surface colonization of starch agar by Aspergillus niger after two days. Variation between the test species was consistent, the individual fungal species exhibiting growth rates which seemed independent of the carbon sources used as nutrients. Fungal species growing rapidly on one particular medium were generally seen to exhibit a similar rapidity of growth on the other two media. Slow-growing fungi were seen to exhibit a similar behavioural pattern. The growth rates of the individual organisms are presented graphically in Fig. 3.2.

It can be seen from the results that a rapid growth rate was exhibited by twenty one fungal species when grown on starch agar, by twenty one species when grown on pectate agar and by seventeen species when grown on cellulose agar. Moderate growth rates were exhibited by three species when grown on starch agar, by four species on pectate agar, and by two species on cellulose agar. Slow growth rates were exhibited by nine species on starch, by eight species on pectate agar and by fourteen species on cellulose agar. Tables 3.1.-3.3. indicate respectively those organisms showing rapid, moderate, and slow growth rates, on starch, pectate and cellulose agars, and organisms are presented in the tables in the descending order of their degree of surface colonization of the individual media.

It can be seen from Fig. 3.1 that the individual fungal species, both fast and slow-growing, exhibited marginal differences in growth rate on the individual media, and that, though the inherent growth rates of the individual species did not seem to be affected, preferential growth of one or other of the media was observed for most fungi. It was seen that the individual species exhibited, a maximal growth, a minimal growth, and an intermediate growth called median growth, on one or other of the media.

It was therefore seen that of the thirty three fungal species examined, fourteen exhibited maximal surface growth on starch agar, thirteen exhibited maximal surface growth on pectate agar, while six exhibited maximal growth on cellulose agar. Fifteen species showed median growth on starch agar, eight species showed median growth rates on pectate agar, while ten species showed median growth on cellulose agar. Four species showed minimal growth on starch, twelve species showed minimal growth on pectate, whilst seventeen species showed minimal growth on cellulose agar. These trends are diagrammatically presented in Fig. 3.2.

FIG. 3.1. HISTOGRAMS ILLUSTRATING MAXIMAL, MEDIAN, AND MINIMAL GROWTH OF FUNGAL SPECIES ON STARCH, PECTATE, AND CELLULOSE AGARS

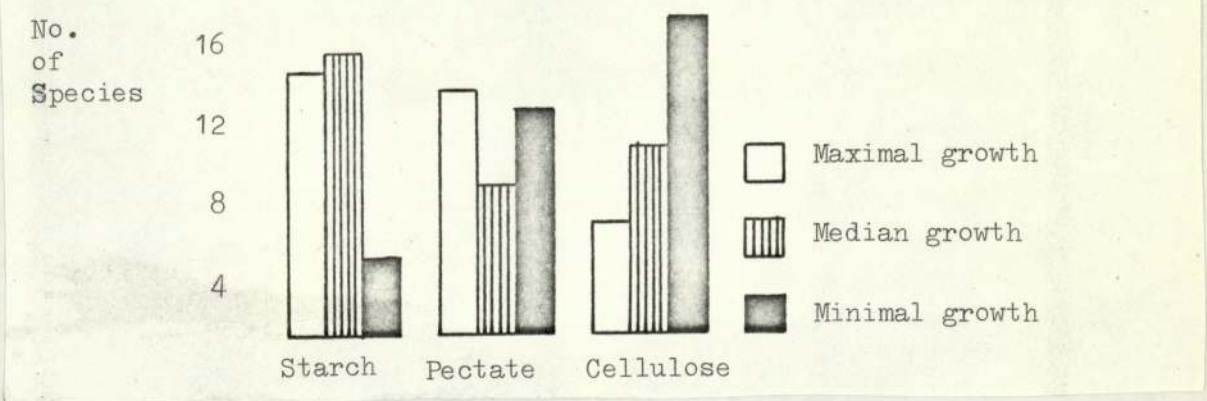
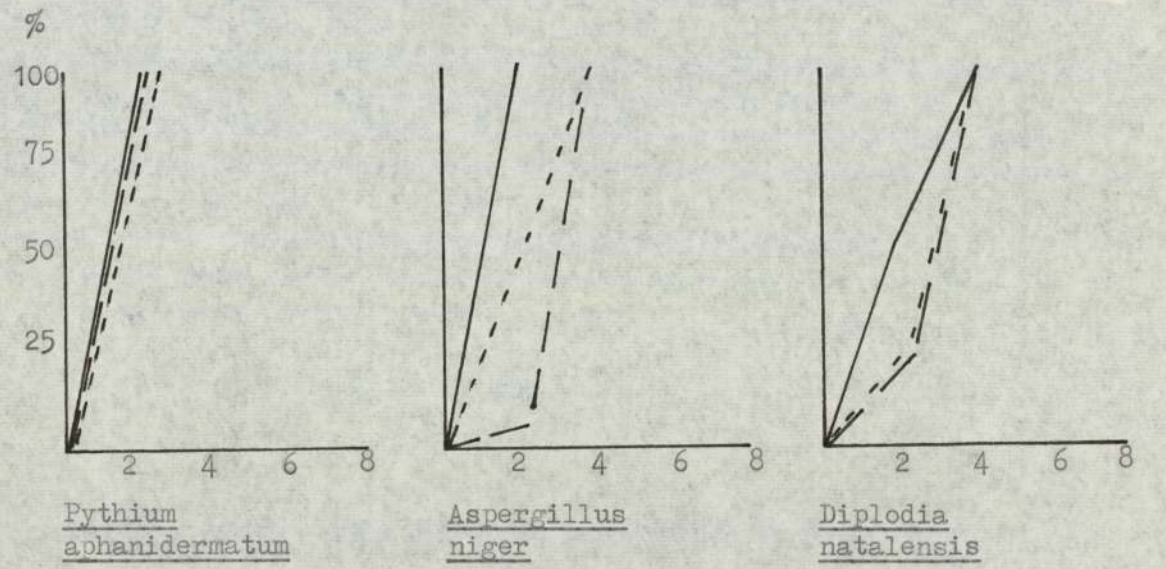
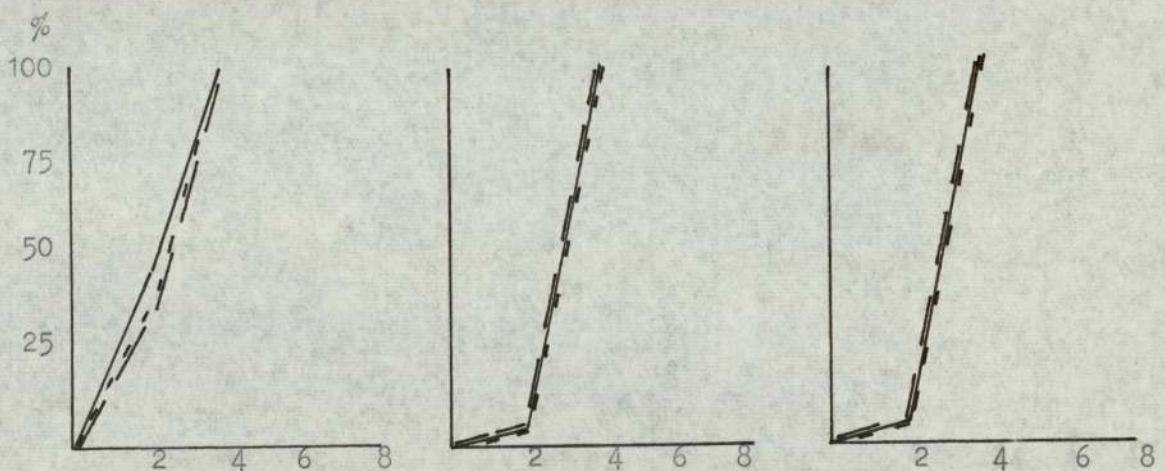


FIG. 3.1 Percentage surface colonization of Starch, Cellulose and Pectate agar plates by individual test species.



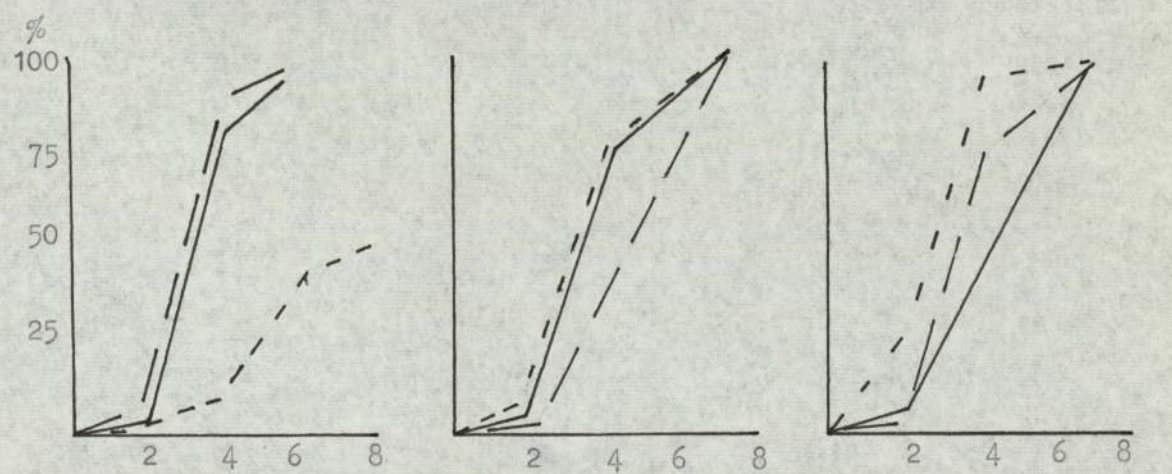
Pythium aphanidermatum | Aspergillus niger | Diplodia natalensis

Vertical axis : % Surface colonization
Horizontal axis : duration of colonization in days



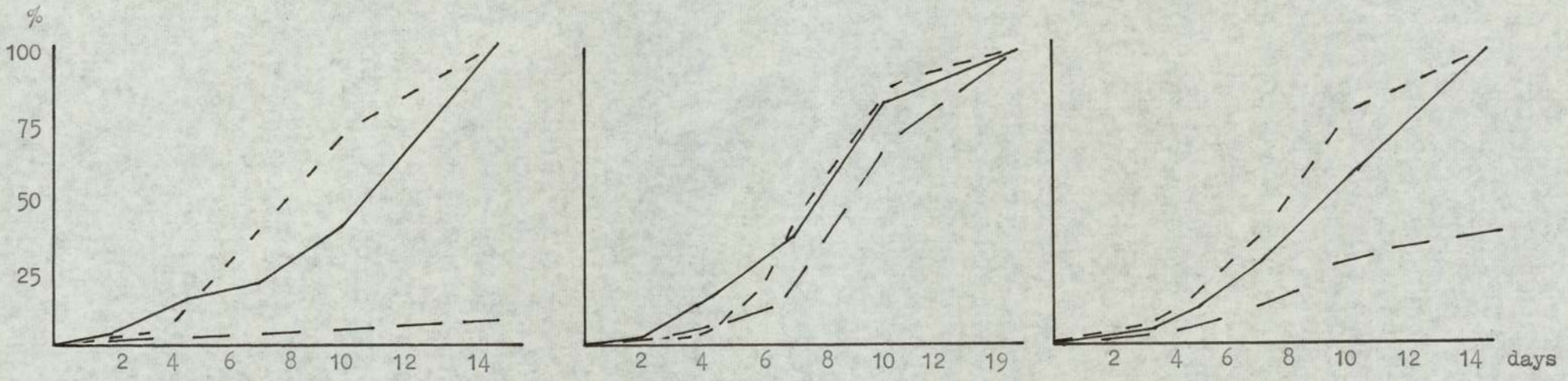
Trichoderma viride | Trichocladium canadense | Paecilomyces variotii

Starch agar
Cellulose agar
Pectate agar



Cytospora leucostoma | Botrytis cinerea | Botridiodiplodia theobromae

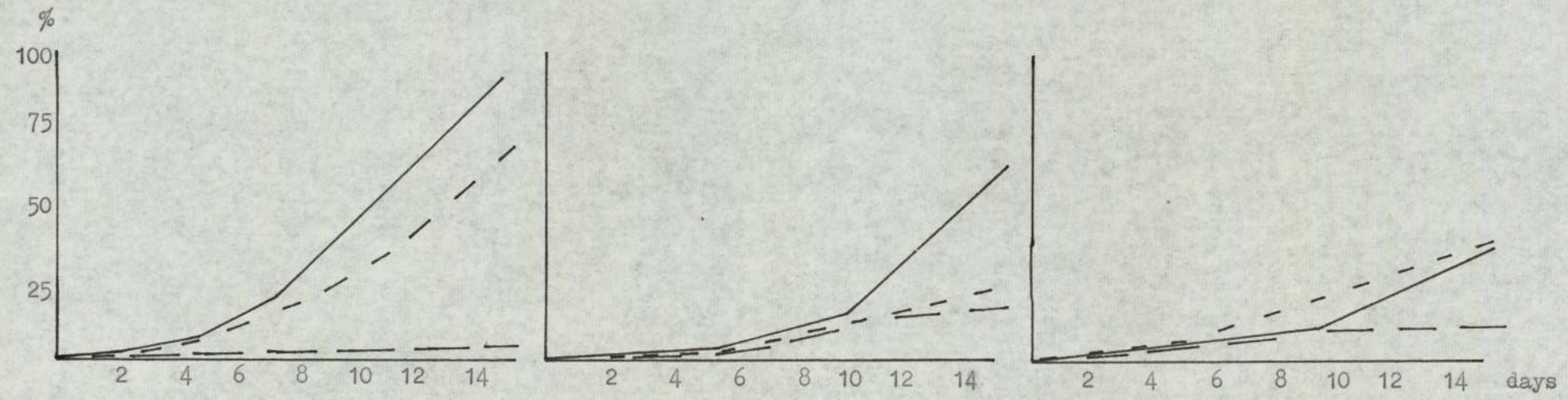
Fig. 3.1 - continued



Ceratocystis ulmi

Helminthosporium erythrospilum

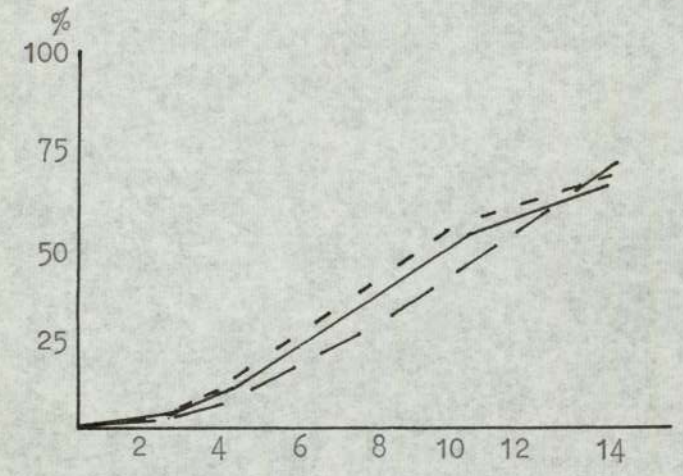
Sclerophoma pithyophila



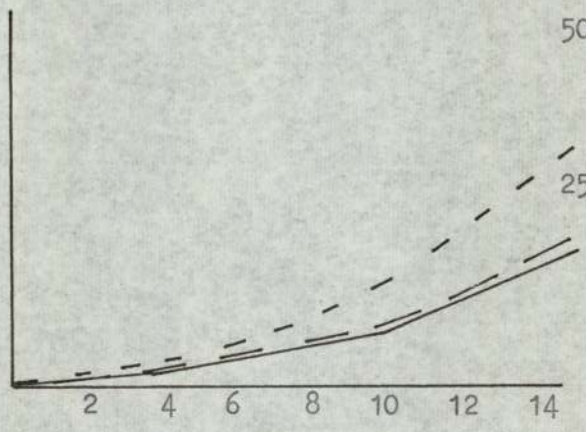
Ceratocystis picea

Ceratocystis coerulescens

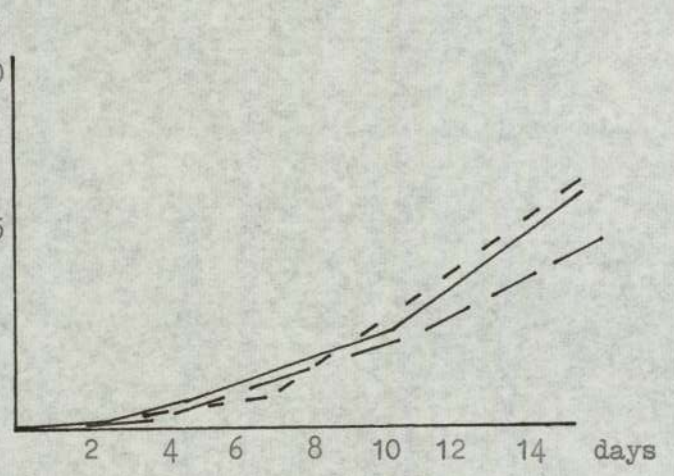
Graphium sp.



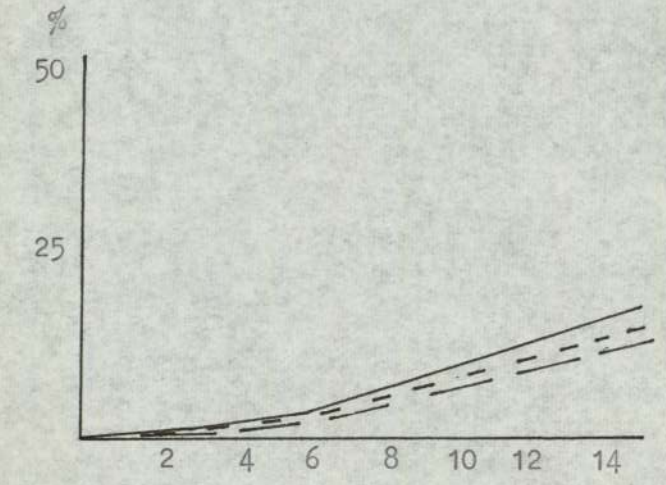
Discula brunneo-tingens



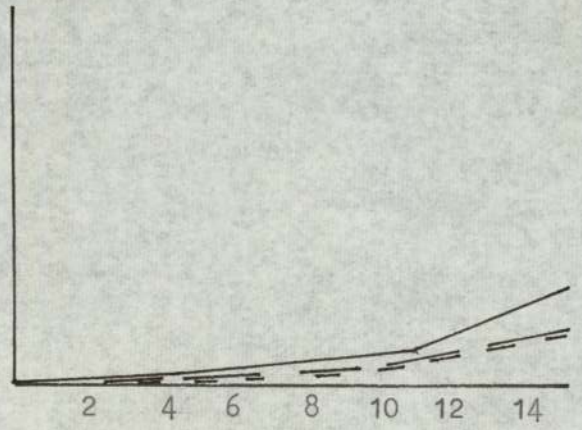
Gliocladium roseum



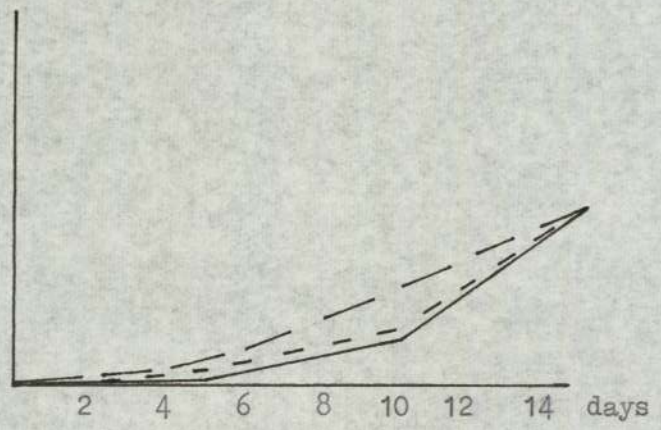
Cephalosporium acremonium



Phialophora melinii



Myxotrichum deflexum



Phialophora fastigiata

Fig. 3.2. - continued

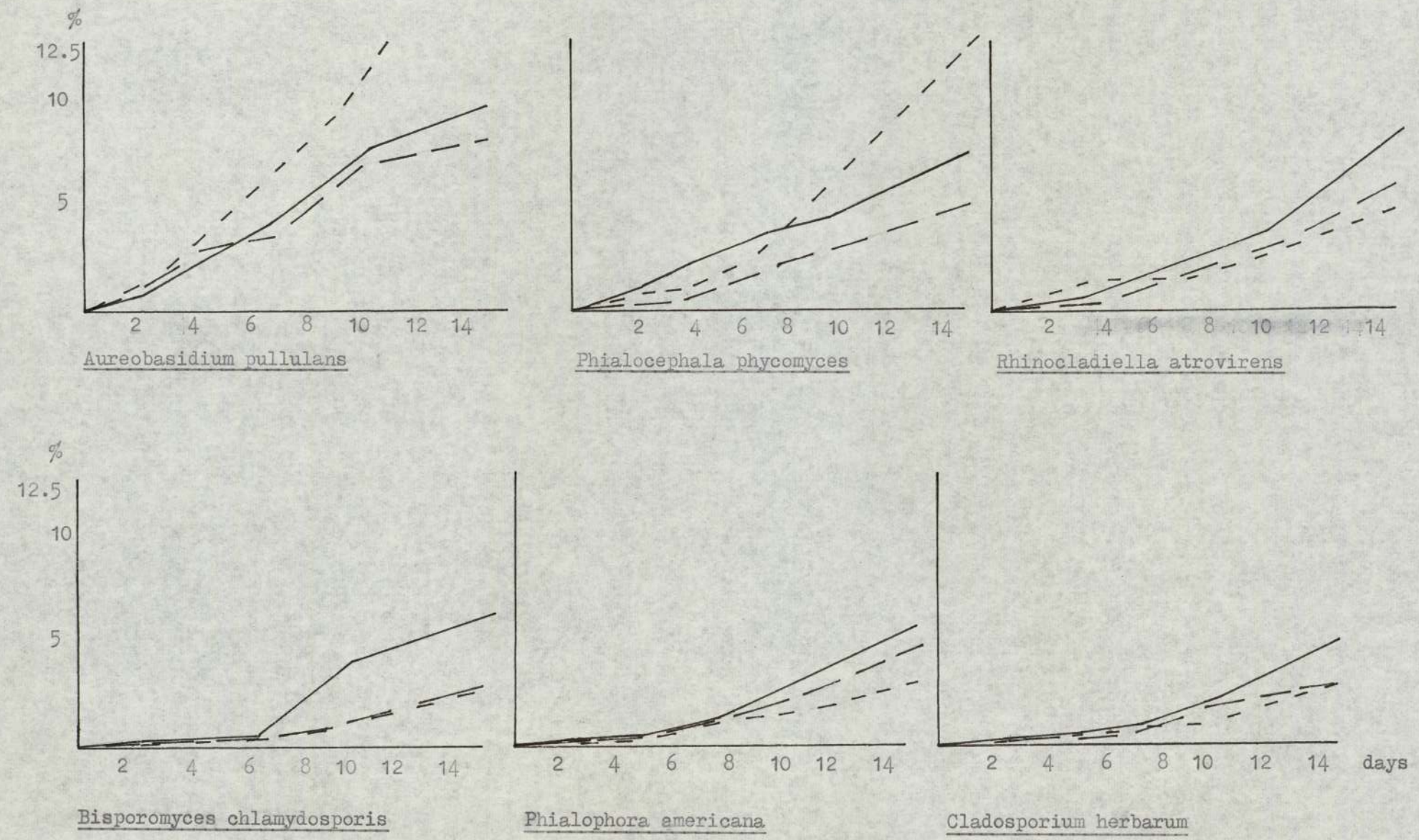


TABLE 3.1

FUNGI SHWOING RAPID GROWTH RATES ON STARCH PECTATE AND CELLULOSE AGARS

Starch	Pectate	Cellulose
Pythium aphanidermatum	Pythium aphanidermatum	Pythium aphanidermatum
Aspergillus niger	Trichoderma viride	Trichoderma viride
Trichoderma viride	Diplodia natalensis	Diplodia natalensis
Diplodia natalensis	Aspergillus niger	Aspergillus niger
Paecilomyces variotii	Paecilomyces variotii	Paecilomyces variotii
Trichocladium canadense	Trichocladium canadense	Trichocladium canadense
Cytospora leucostoma	Botriodiplodia theobromae	Cytospora leucostoma
Botrytis cinerea	Botrytis cinerea	Botriodiplodia theobromae
Botriodiplodia theobromae	Scytalidiium lignicola	Botrytis cinerea
Discula brunneo-tingens	Diplodia pinea	Scytalidium lignicola
Scytalidium lignicola	Diplodia sapinea	Discula brunneo-tingens
Leptographium lundbergii	Discula brunneo-tingens	Ceratocystis pilifera
Diplodia pinea	Leptographium lundbergii	Diplodia pinea
Ceratocystis pilifera	Ceratocystis pilifera	Alternaria tenuis
Helminthosporium erythrospilum	Helminthosporium erythrospilum	Helminthosporium erythrospilum
Alternaria tenuis	Sclerophoma pithyophila	Diplodia sapinea
Sclerophoma pithyophila	Alternaria tenuis	Leptographium lundbergii

Table 3.1 - continued

Starch	Pectate	Cellulose
Diplodia sapinea	Ceratocystis ulmi	-
Ceratocystis picea	Cytospora leucostoma	-
Ceratocystis ulmi	Ceratocystis picea	-
Ceratocystis coerulescens	Gliocladium roseum	-

TABLE 3.2

FUNGI SHOWING MODERATE GROWTH RATES ON STARCH PECTATE AND CELLULOSE AGARS

Starch	Pectate	Cellulose
Graphium sp.	Graphium sp.	Gliocladium roseum
Gliocladium roseum	Cephalosporium acremonium	Sclerophoma pithyophila
Cephalosporium acremonium	Ceratocystis coerulescens	-
-	Aureobasidium pullulans	-

TABLE 3.3

FUNGI SHOWING SLOW GROWTH RATES ON STARCH PECTATE AND CELLULOSE AGARS

Starch	Pectate	Cellulose
Phialophora melinii	Phialophora melinii	Cephalosporium acremonium
Myxotrichum deflexum	Phialocephala phycomyces	Ceratocystis coerulescens
Phialophora fastigiata	Phialophora fastigiata	Phialophora melinii
Aureobasidium pullulans	Myxotrichum deflexum	Graphium picea
Rhinocladiella atrovirens	Rhinocladiella atrovirens	Phialophora fastigiata
Phialocephala phycomyces	-	Ceratocystis ulmi
-	Phialophora americana	Myxotrichum deflexum
Bisporomyces chlamydosporis	Cladosporium herbarum	Aureobasidium pullulans
Phialophora americana	Bisporomyces chlamydosporis	Rhinocladiella atrovirens
Cladosporium herbarum	-	-
-	-	Phialocephala phycomyces
-	-	Phialophora americana
-	-	Bisporomyces chlamydosporis
-	-	Cladosporium herbarum
-	-	Ceratocystis picea

3.4. Discussion and Conclusions

It can be seen from the results that the test species could be placed into two major and one minor grouping, i.e. respectively, rapid or slow-growing species, and a small group of species showing moderate growth rates. Most of the species demonstrated a consistent growth rate which did not seem to be unduly influenced by the carbon sources of the growth media. Cytospora leucostoma grew extremely rapidly on both starch and cellulose agars, but did not show an equivalent growth rate when grown on pectate agar. Yet this significantly poorer relative growth rate on pectate agar still resulted in a surface colonization of the medium which exceeded 50 per cent of the available area, thus indicating the inherent relative rapid colonization rate of the species.

It was interesting that the results of the comparative growth studies corroborated the observations of other workers on wood colonization. Graphium sp., assessed as a moderate or slow-growing species in this study, was seen to colonize wood only to a limited extent due to its slow growth rate (Butcher 1966), and Diplodia pinea, assessed as a rapidly growing species in this study, was the species most frequently isolated by Butcher (1966) from blue-stained Pinus radiata posts. He considered that this species extensively colonized the wood, presumably due to its rapid growth rate.

Botrytis cinerea and Trichoderma viride were considered to show rapid growth rates in this study and were similarly found to grow rapidly in wood by Butcher (1968). He noted

that Cladosporium herbarum and Cephalosporium acremonium when colonizing wood were slow-growing species, and they were similarly found to be slow growing in the comparative study undertaken. Botriodiplodia theobromae, which demonstrated rapid growth rates on the three media, has been similarly seen to colonize both Theobroma cacao and and Ulmus americana extremely rapidly (Norris 1965), whilst Desai and Shields (1971) considered that the faster growth of some blue-staining fungi when colonizing wood chips after exposure to u.v. light, precluded the isolation of slower-growing Ceratocystis spp.

It therefore seems feasible that a positive relationship may exist between fungal growth rate, as exhibited on agar media, and the growth rate of similar fungal species when colonizing wood. However, the results also show that the carbon sources of the individual media, influenced the extent of growth of the individual fungal species, although not enough to affect their inherent growth rates, and that the individual fungal species exhibited preferential growths on the individual carbon sources.

This reaction of fungal species to agar media has similarly been noted by Brancarto and Golding (1953). Shrimpton and Whitney (1968) when observing the influence of wood extractives on Europhium sp. included surface growth rate as a measure of inhibition, and Olofinboba (1969) examined the influence of xylem extractives of Antiaris africana on B. theobromae by using the fluctuation in rate of hyphal extension as a criterion of reaction.

Mandels, Weher and Parizek (1971) observed that the surface growth rate of T. viride, when grown on cellulose agar, was slower than when the species was grown on starch agar and that minimal cellulolytic activity was exhibited on the latter, and that maximal activity was exhibited on the former.

In this context it is therefore worth reconsidering the results of the growth rate studies with particular reference to the growth of the test organisms on the individual media. Of the fourteen species showing maximal growth on starch agar, seven species exhibited median growth on pectate agar, and the remaining seven species showed median growth on cellulose agar. Of the thirteen species exhibiting maximal growth on pectate agar, ten produced median growth on starch, whilst three produced median growth on cellulose agar. Of the six species showing maximal growth on cellulose, one showed median growth on pectate, and five showed median growth on starch agar. It can therefore be seen that of the thirty three species examined, twenty nine exhibited maximal or median growth on starch agar, twenty one showed maximal or median growth on pectate agar, while sixteen exhibited maximal growth on cellulose agar. It is therefore apparent that approximately 42 per cent of the blue-staining and mould fungal species tested exhibited maximal growth on starch agar, and that approximately 88 per cent of the species included starch as a primary or secondary nutritional preference.

It can also be seen that only approximately 18 per cent of the test species showed maximal growth on cellulose agar, and that only approximately 48 per cent of the test species included cellulose as a primary or secondary nutritional preference.

It can therefore be seen that twenty nine species apparently included starch as either a first or second nutritional preference in combination either with cellulose or pectate, and only four species included either cellulose or pectate as apparent primary or secondary nutritional preferences and showed minimal growth on starch agar.

This small group included both B. theobromae and P. fastigiata. While preferential surface growth of a test species on one particular medium need not necessarily indicate a preferential utilization of that medium, it is coincidental that B. theobromae and P. fastigiata, both known for their degradation and soft-rotting activities, (Krapvina 1960; Karkanis 1966; and Umezurike 1969), should both be seen to exhibit maximal surface growth rates on cellulose or pectate agars. There may well be a connection between this latter growth pattern, and the fact that some wood-decaying fungi grow more slowly on media containing simple sugars as carbon sources, than on media containing cellulose as carbon source (Hulme and Shields 1970).

Since some staining and mould fungi are known to possess wood-degradation mechanisms, and that the extent of degradation produced by these species may well be directly related to their colonization rates, it can be

suggested that growth rate may be one of the factors controlling the extent of wood degradation produced by these fungi, e.g. it is feasible that C. acremonium, a slow-growing species, (Butcher 1968) which produces soft-rot attack of wood (Duncan and Eslyn 1966), would produce significantly less degradation than B. theobromae, which is rapidly growing (Norris 1965) and which also produces soft-rot attack of wood (Umezurike 1969).

The thirty three species examined represent a good cross-section of the genera of commonly-found blue-staining and mould fungi, the traditional picture of which is one which indicates that they attack the starches and cell contents of the sapwood of green timber. Recent information, however, reveals that some species may produce soft-rot cavities, and wood-degradating enzymes in liquid culture and also both loss in weight and strength of wood.

The pattern which emerges from this initial study indicates that the blue-staining and mould fungi tested may have a range of colonization and penetration rates. If significance can be attributed to the relative growth rates of the individual species on starch, cellulose and pectate agars, i.e. the exhibited maximal growth on starch agar, and minimal growth on cellulose agar by most of the test species, then the traditional picture of blue-staining and mould fungal activity in wood is corroborated.

However, if the preferential growth on starch agar exhibited by many of the test species is considered to

be indicative of the saprophytic role of blue-staining fungi in nature, then the equally rapid growth rates of some of the species on either cellulose or pectate agar could well indicate a potential ability of these species to degrade cellulose or pectate in wood. It is not suggested that these abilities are as potentially dangerous as those of the wood-rotting or higher fungi but it is certainly feasible that they might possess the enzyme mechanisms necessary for cell pentration.

It is possible that, while the cell contents are still present in sapwood, little or no degradation might occur. On depletion of cell contents, however, which may be preferentially utilized, the degradation mechanisms may then be seen to be evident, and that the extent of degradation produced by these mechanisms may be directly related to growth rate.

CHAPTER 4

RELATIVE AMYLOLYTIC ACTIVITY

4. RELATIVE AMYLOLYTIC ACTIVITY

4.1. Introduction

Fergus (1969), reviewing literature on amylolytic activity of thermophilic or mesophilic fungal species, noted that many could not produce amylase, although Cochrane (1958) considered that amylase production was virtually universal for fungi. Lily and Barnett (1951) considered that starch utilization by fungi was common but by no means universal.

Blue-staining and mould fungi are generally regarded as utilizing starches and cell contents of green sapwood although Krapvina (1960) has suggested that woody tissue can be degraded after depletion of cell contents. Savory (1954), Cartwright and Findlay (1958), Liese (1967), Norkrans (1967) and F.P.R.L. Leaflet No. 12 (1969) all indicate that the starches in wood are utilized by blue staining and mould fungi. Olofinboba and Lawton (1968) indicate the wide range of non-structural carbohydrates, including starch, utilized by Botriodiplodia theobromae, while Umezurike (1969) has indicated that B. theobromae can utilize cellulose and hemi cellulose in wood after depletion of starch reserves. Olofinboba and Lawton (1968) further suggest that B. theobromae cannot colonize or degrade wood if the starch reserves are removed, and that the presence of starch in Antiaris africana is necessary for initial colonization of wood by B. theobromae.

However, for all the information relating to starch utilization by blue-staining and mould fungi, little

factual data was found in the literature on actual amylolytic activity of blue-staining and mould fungi, or on the relative amylolytic activity of individual blue-staining or mould fungal species.

As growth studies indicated that the greater number of test organisms grew preferentially on starch agar, but that the inherent growth rates of the individual organisms generally influenced their degree of surface colonization, it was considered that investigations to determine the relative amylolytic activity of the test organisms might provide data relating both to amylolytic activity of blue-staining and mould fungi, and to relationships between growth rate and amylolytic activity of the individual fungal species.

Assessment of starch degradation can be undertaken by determination of reducing sugars in culture fluids containing starch as the sole carbon source. Data on relative amylolytic activity can then be obtained by comparison of levels of reducing sugars in the culture fluids of the test organisms being compared.

A simple method for determination of amylolytic activity is the reaction of iodine with starch. When iodine is added to starch solution, a deep blue-black coloration results. The hydrolysis of starch results in a lack of coloration in hydrolized areas of solid substrates containing starch e.g. starch agar, or a lessening in the intensity of coloration of liquid substrates containing starch when treated with iodine. This technique of assaying amylolytic activity has been used by Ellwood and Ecklund (1959), Seely and Vandeemark (1962), King (1967),

Fergus (1969) and Walsh (1971).

4.2. Materials and Methods

4.2.1. Test Organisms.

The test organisms used in this study were those included in Table 2.1.

4.2.2. Materials.

The starch agar used for amylolytic assays was similar to that used for growth studies, the composition of which is outlined in Appendix I. The iodine used for staining was incorporated in a 1% w/v solution of Potassium iodide. Iodine concentration was approximately 0.1% w/v.

4.2.3. Methods.

Using a sterile six-mm corkborer, inocula were removed from actively-growing cultures of the individual blue staining and mould fungal species, maintained on Potato Dextrose Agar (Oxoid 139), and transferred to the centres of petri dishes which contained 15-ml quantities of the starch agar. The inocula were transferred to the media in such fashion that the inoculum surface bearing fungal growth, was in contact with the medium surface. The subcultures were incubated at 25°C for ten days.

Duplicate petri dishes for each organism were tested at intervals of three, five, seven and ten days. The rate of growth of each organism was assessed using the method outlined in 3.2, and the inoculum and surface mycelium of the organisms was gently removed. The starch agar was

then flooded with the ~~saturated~~ iodine-potassium iodide solution, was allowed to stand for about 30 seconds, the solution decanted, and the hydrolysis area measured. Non-hydrolized starch was indicated by its deep blue-black coloration, while hydrolyzed starch was indicated by the unstained areas.

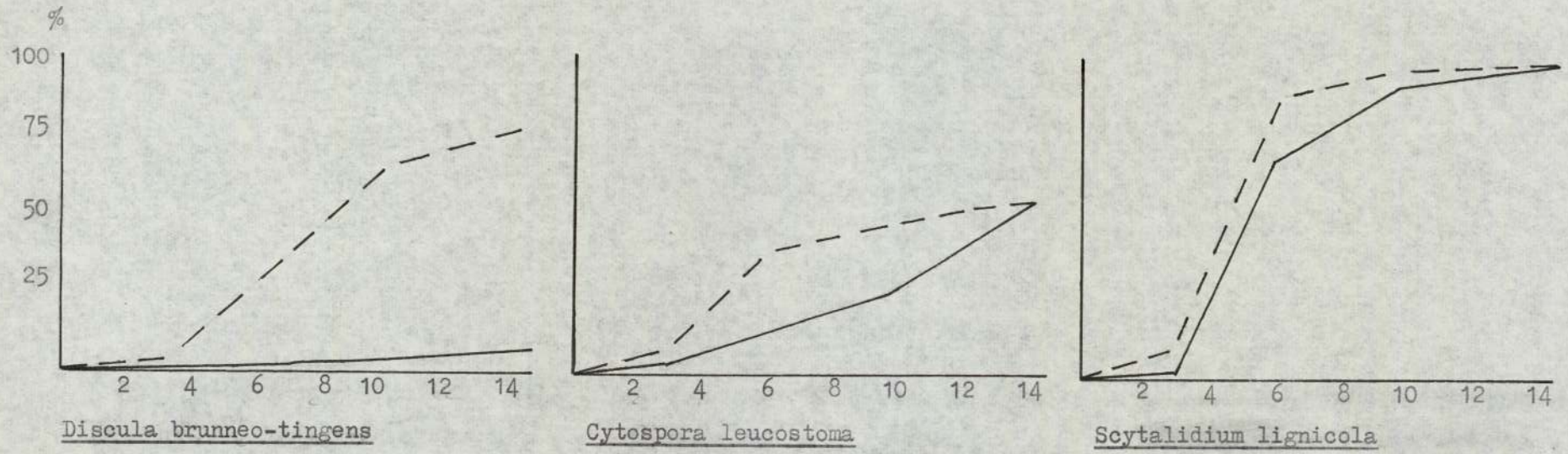
Relative amylolytic activity was indicated by the area of cleared unstained agar in the petri dishes. Fungal colonies exhibiting clear unstained zones in excess of 50 per cent of the medium were considered to show good amylolytic activity, hydrolysis zones in excess of 25 per cent but not greater than 50 per cent of the surface area of the medium were considered indicative of moderate amylolytic activity, while hydrolysis zones not exceeding 25 per cent of the surface area of the medium were considered indicative of fair amylolytic activity.

4.3. Results

Thirty three blue-staining and mould fungi when tested for amylolytic activity on starch agar were found to produce varying degrees of starch hydrolysis.

The relative hydrolysis rates of the test species revealed a wide range of variation, ranging from 100 per cent hydrolysis produced by Trichoderma viride in two days to 2 per cent hydrolysis produced by Discula brunneotingens in ten days. Most species showed growth rates to which amylolytic activity seemed closely related. Results for the individual species showing both rates of hydrolysis and rates of growth are presented graphically

FIG. 4.1. Relative amylolytic activity of the individual test species expressed in terms of % starch hydrolysis in relation to % surface colonization of starch agar plates.



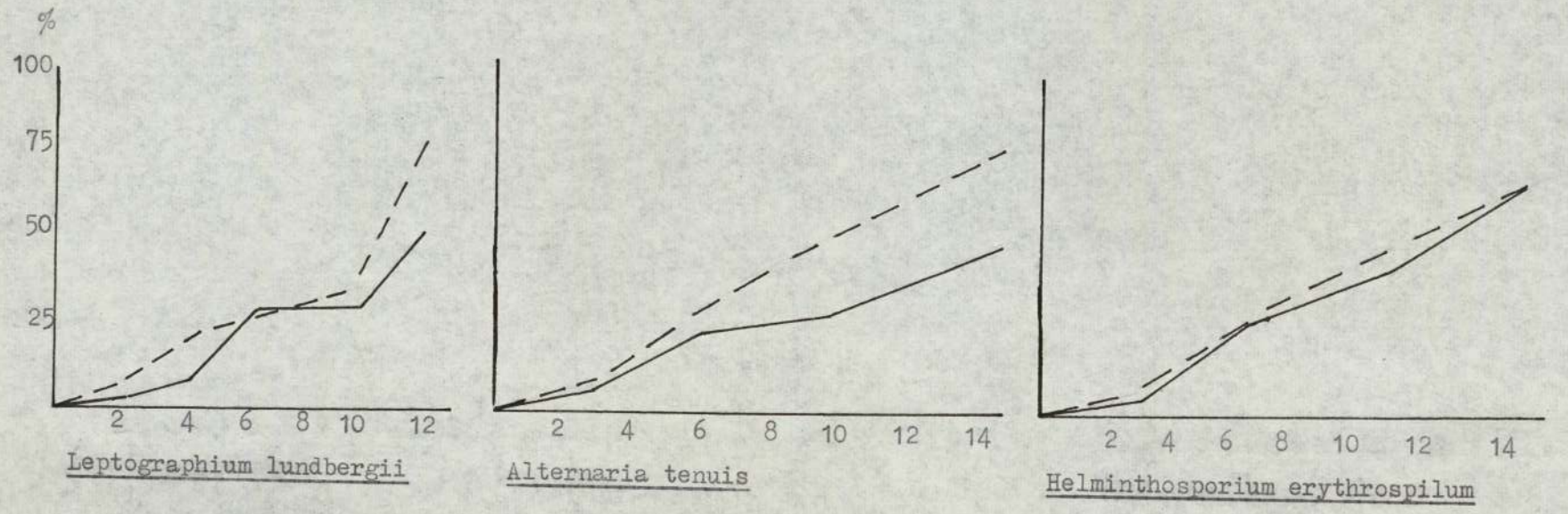


Fig. 4.1 - continued

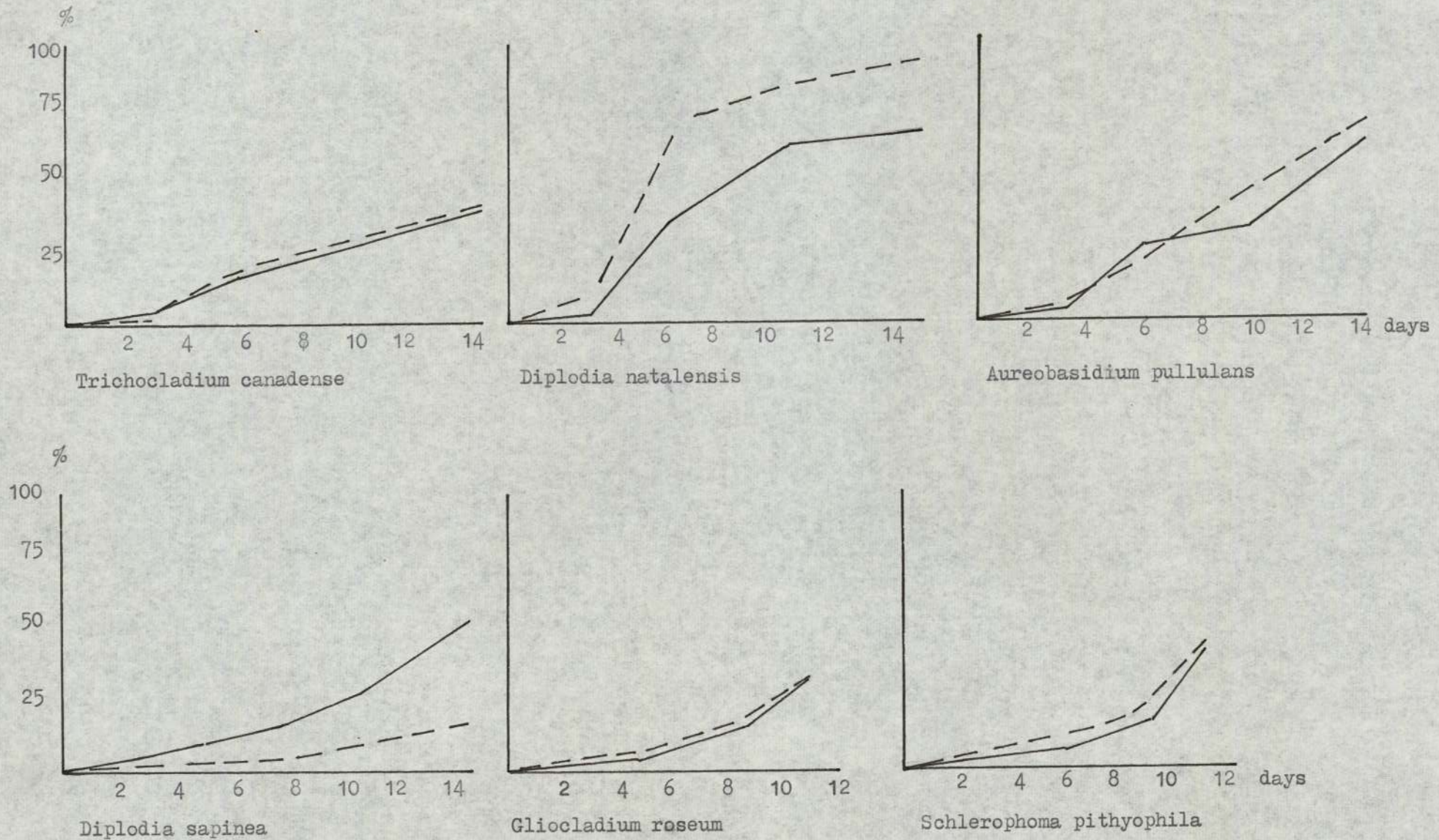
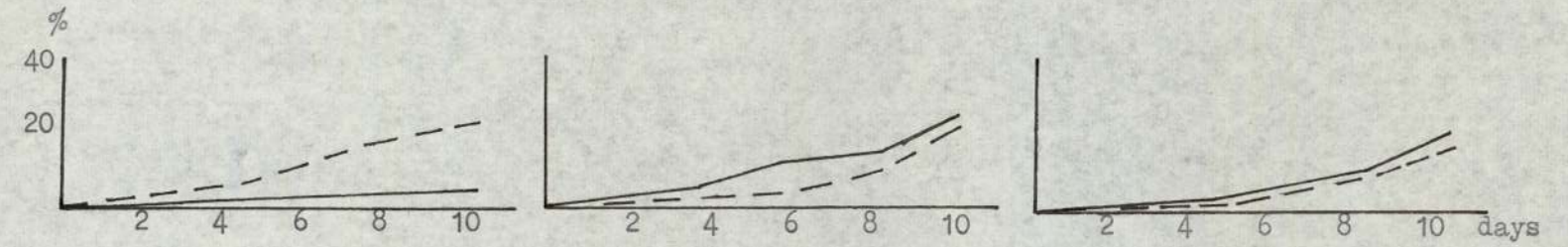
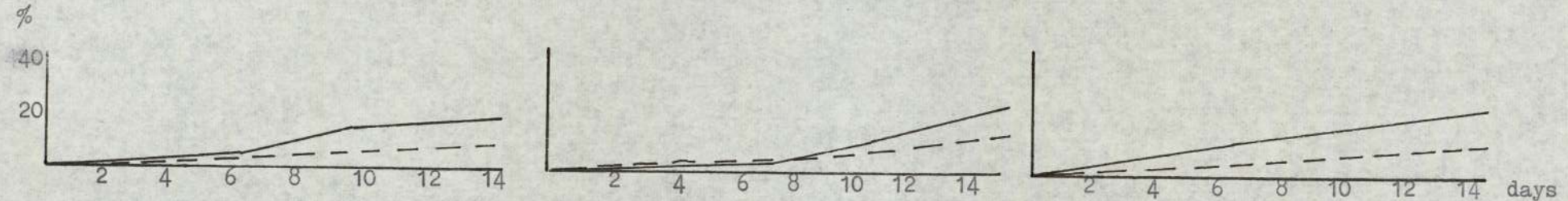


Fig. 4.1 - continued

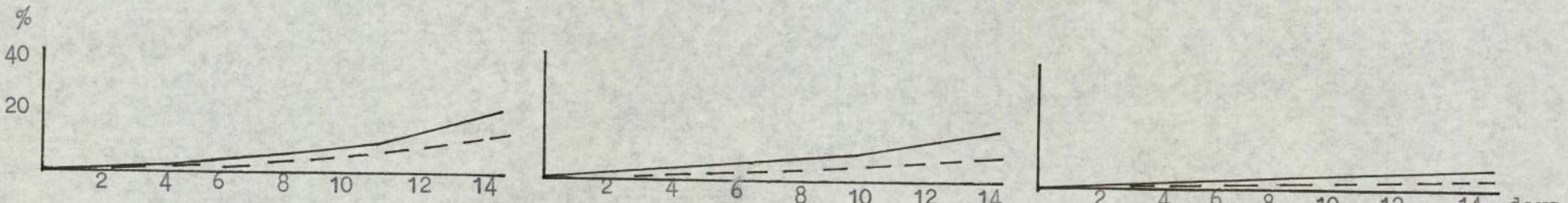
Fig. 4.1 - continued



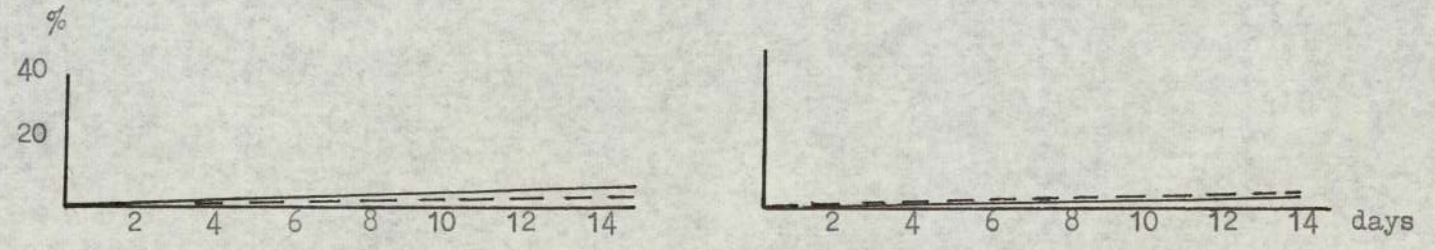
Ceratocystis ulmi — Phialocephala phycomyces — Phialophora melinii



Phialophora americana — Bisporomyces chlamydosporis — Phialophora fastigiata



Myxotrichum deflexum — Graphium sp. — Ceratocystis coerulescens



Cladosporium herbarum — Rhinocladiella atrovirens

TABLE 4.1

ORGANISMS SHOWING GOOD MODERATE AND FAIR AMYLOLYTIC ACTIVITY

Good Amylolytic Activity	Moderate Amylolytic Activity	Fair Amylolytic Activity
Trichoderma viride	Ceratocystis picea	Phialocephala phycomyces
Pythium aphanidermatum	Helminthosporium erythrospilum	Phialophora melinii
Aspergillus niger	Leptographium lundbergii	Phialophora fastigiata
Paecilomyces variotii	Aureobasidium pullulans	Bisporomyces chlamydosporis
Ceratocystis pilifera	Alternaria tenuis	Phialophora americana
Botriodiplodia theobromae	Cephalosporium acremonium	Myxotrichum deflexum
Botrytis cinerea	Sclerophoma pithyophila	Graphium sp. picea
Diplodia natalensis	Diplodia sapinea	Ceratocystis ulmi
Scytalidium lignicola	Gliocladium roseum	Ceratocystis coerulescens
Diplodia pinea	Cytospora leucostoma	Cladosporium herbarum
	Trichocladium canadense	Rhinocladiella atrovirens
		Discula brunneo-tingens

in Fig. 4.1. It can be seen from Fig. 4.1 that twenty species produced hydrolysis zones of lesser area than the area of their colonies, two species produced hydrolysis zones equal in area to the area of their colonies, while eleven species displayed hydrolysis zones greater in area than the area of their colonies.

Ten species were considered to show a good amylolytic activity, eleven species were considered to show a moderate amylolytic activity, and twelve species were considered to show a fair amylolytic activity. These species are indicated in Table 4.1.

4.4. Discussion

It can be seen from the results that all of the blue-staining and mould fungi tested were amylolytic, and that generally, those species exhibiting more rapid growth rates similarly produced the larger areas of starch hydrolysis. However, direct correlation between starch, hydrolysis and growth rate was not altogether apparent, i.e. rapid growth rate of an individual species did not necessarily indicate good amylolytic activity, and similarly the colony area was not always equal to the starch hydrolysis area.

Fifteen species were considered to show rapid growth rates, i.e. colony areas in excess of 50 per cent of the surface area of the medium, but only ten of these exhibited good amylolytic activity, i.e. hydrolysis areas in excess of 50 per cent of the medium surface area. Of these ten species, *B. theobromae*, *Diplodia pinea* and *T. viride* are known rapidly to colonize wood

substrates (Norris 1966; Butcher 1966), and B. theobromae has similarly been shown to require starch reserves in wood before wood colonization began (Umezurike 1969). T. viride has been shown to grow preferentially on starch media by Mandels, Weher and Parizek (1971).

Of the remaining five species showing rapid growth rates, Ceratocystis picea, Helminthosporium erythrospilum, Lectographium lundbergii, and Alternaria tenuis showed moderate amylolytic activity, and D. brunneotingens showed fair amylolytic activity. Of these species, A. tenuis has been shown to be a primary colonizer of the untreated sapwood of Pinus radiata (Butcher 1968).

Of the seven species showing moderate growth rates, all but one exhibited moderate hydrolysis, the exception, Ceratocystis ulmi, produced fair hydrolysis, and of the eleven species showing slow growth rates, all but one produced fair hydrolysis, the exception, Diplodia sapinea, produced moderate hydrolysis.

Of the species showing good amylolytic activity, D. pinea and T. viride were considered by Butcher (1968) to be primary wood colonizers, while Aureobasidium pullulans and A. tenuis, both of which showed moderate amylolytic activity, and Cladisporium herbarum, which showed fair hydrolysis, were similarly considered by him to be primary wood colonizers. Paecilomyces and Botrytis, species of which when tested showed good amylolytic activity, were considered by Butcher to be secondary wood colonizers, and both were considered to

be cellulolytic by Merrill (1965) and Domsch and Gams (1968) respectively. Species of Helminthosporium, Gliocladium, and Cephalosporium were similarly seen to be secondary wood colonizers (Butcher 1966) and also cellulolytic (Siu and Reese 1953; Duncan and Eslyn 1963).

The results also show that the rate of starch hydrolysis in relation to growth rate varied. Of the fifteen species showing rapid growth rates, all but one showed surface growth areas which exceeded the areas of starch hydrolysis. The exception, P. varioti, showed a hydrolysis area greater than the area of the fungal colony. All but one of the species showing moderate growth rates showed surface colonization rates which exceeded hydrolysis rates, the exception, Trichocladium canadense, showed a hydrolysis rate equal to its colonization rate. Alternatively, however, of the eleven slow-growing species, all except one showed hydrolysis rates which exceeded their colonization rates, the exception Rhinocladiella atrovirens, showed a hydrolysis rate equal to its colonization rate.

A pattern can therefore be seen which indicates an indirect correlation between growth rate and amylolytic activity. Generally, those species which showed rapid growth rates similarly showed higher relative amylolytic activity than the slower-growing species. However, whereas the more rapidly-growing species produced more extensive hydrolysis areas then

the slower-growing species, the hydrolysis rates of the slower-growing species generally exceeded their growth rates, whereas the hydrolysis rates of the faster-growing species was less than their growth rates. Thus it seems that species such as D. sapinea, the colony area of which was 10.5 per cent, and the hydrolysis area of which was twenty eight per cent of the surface area of the medium, could be considered to be more amylolytic than species such as D. brunneo-tingens, the colony area of which covered sixty four per cent of the surface area of the medium and the hydrolysis area of which covered two per cent of the medium surface. It could also be inferred that many of the slower-growing fungi, in relation to apparent growth rate, were more amylolytic than the faster-growing fungi.

The technique used to determine amylolytic activity suffered in that starch hydrolysis was directly related to the apparent rate of growth of the test organisms. Better techniques for assaying amylolytic activity include determination of reducing sugars, or using liquid culture filtrate assays. However, reducing sugars determinations are complex when used to study a fungal grouping and the use of liquid culture filtrates does not take the surface colonization rate of the organism into account since many species grow atypically in liquid culture. Fergus (1969) considered the method used in this experiment to be very pertinent for the screening of fungal groupings.

It can therefore be seen that all of the species tested were distinctly amylolytic in relation to growth. The implications of this in relation to wood colonization are interesting. If the amylolytic activities of blue staining and mould fungi in wood are related to growth rate, then the faster-growing fungi will produce starch depletion in green sapwood more rapidly than slower growing fungi, while slower-growing fungi may well produce intensive starch depletion of the areas of wood colonized by them. Olofinboba and Lawton (1968) have pointed out the extensive starch depletion by B. theobromae in Antiaris africana, and the results of this experiment similarly indicate the rapid growth rate and starch hydrolysis of that organism. Similarly, Phialophora fastigiata produced intensive starch hydrolysis in relation to its growth. Enzyme production at these levels in green sapwood could well result in complete depletion of starches in the areas colonized, and if these species possess adaptive enzyme systems, their growth could well result in the soft-rot cavities produced by them in wood (Krapvina 1960; Karkanis 1966; Umezurike 1969).

Knuth and McKoy (1962) and Knuth, McKoy and Duncan (1965) have pointed out the extensive starch utilization of bacteria bringing about increased permeability of ponded wood, and Greaves and Foster (1969) have indicated that ray cell contents including starches act as nutritional reserves for bacterial degradation of wood-cell walls resulting in enhanced permeability.

It has been shown for T. viride and B. theobromae that both preferentially grow on starch media but that both can, if necessary, also utilize cellulose. In this context, dependent on growth rate and rate of starch utilization, if blue-staining and mould fungi possess a cellulolytic activity, upon starch depletion in wood, it may therefore be possible for them to produce a limited structural degradation in wood tissue.

CHAPTER 5

RELATIVE CELLULOLYTIC ACTIVITY

5. RELATIVE CELLULOLYTIC ACTIVITY

5.1. Introduction

Data on the cellulolytic activity of blue-staining and mould fungi is both slight and conflicting. As outlined in the Introduction (1.5), evidence is presented relating both to blue-staining and mould fungal utilization of cell contents and also to blue-staining and mould fungal degradation of wood tissue.

These fungi are usually not considered to affect wood strength, although Cartwright and Findlay (1958) noted that impact bending or toughness was sometimes considerably affected by their growth. Campbell (1959) noted that Diplodia pinea did not cause strength loss in wood, Diplodia natalensis decreased toughness by seventeen per cent and Botiodiplodia theobromae was seen to decrease the strength of Obeche (Triplochiton scleroxylon), and Corsican pine (Pinus nigra) by 43 per cent. Merrill (1965) observed severe weight loss in wood blocks when attacked by microfungi including Phialophora melinii. Liese (1970) observed that Aureobasidium pullulans produced carbohydrate losses in wood.

It is therefore apparent that individual blue-staining and mould fungal species have been seen either to degrade wood or to show degradation mechanisms in laboratory culture, aspects which in relation to the degradation mechanisms of other groups of fungi, may seem to be insignificant. However, with the recognition of soft rot production by microfungi in wood (Savory 1954; 1955;

Corbett 1965; Levy 1967), it now seems possible that some blue-staining and mould fungi may produce soft-rot cavities after starches in wood have been utilized (Krapvina 1960; Scheffer and Cowling 1966). It further appears that some staining and mould fungi have been seen to demonstrate a cellulolytic activity in wood resulting in an enhanced permeability of spruce (Vakin et al. 1968). All of these facts indicate that the cellulolytic activity of blue-staining and mould fungi merit investigation.

Techniques used to study the cellulolytic activity of microfungi include in vivo and in vitro studies. The latter include enzyme assays, assessment of reducing sugar production, and viscosity losses in cellulosic substrates, while the former include weight loss assessments, strength loss assessments and agar culture methods.

Eggins and Pugh (1962) used suspensions of ball-milled cellulose incorporated into mineral agar to isolate and detect cellulose decomposing fungi. Cellulolytic activity was indicated by the appearance of clear areas in the opaque medium due to dissolution of the crystalline cellulose particles. Comparison of the extent of these areas produced by individual fungal species has been used by other workers to indicate their relative cellulolytic activity. Bravery (1968) considered that D. L. Asparagine and yeast extract inhibited the production of cellulase by fungi when grown on the medium of Eggins and Pugh while Savory, Mather, Maitland and Selby (1967)

considered that the growth of dematiaceous fungi frequently obscured the clearing produced by them in cellulose media and suggested that sodium azide should be incorporated into the medium to inhibit fungal growth and thus aid detection of clearing.

Rautella and Cowling (1966) used the depth of clearing produced in columns of cellulose agar by fungi as an indication of their relative cellulolytic activity. They found good correlation between relative cellulolytic activity assessment undertaken by that method and relative cellulolytic activity by the same organisms when weight loss of cotton duck was used as an assessment basis. Walsh and Stewart (1969) considered that the methods of Savory et al. (1967) and those of Rautella and Cowling (1966) to be inadequate and instead used a count of cellulose particles before and after growth of the test organisms as a measure of relative cellulolytic activity. Significant correlation was found by them to exist between this method and relative cellulolytic activity indicated by tensile strength losses in cotton yarn.

It therefore appears that variations both in technique and composition in the use of cellulose agars, have been used to determine cellulolytic activity by fungi, and that some of these methods have been correlated with strength and weight loss testing. From the viewpoint of accuracy and simplicity, the method of Rautella and Cowling (1966) and Walsh and Stewart (1969) seemed particularly useful for the screening of

test organisms and the results of both type of test correlate well with other methods of assaying celluloytic activity. Of the two, however, the method of Rautella and Cowling is the simpler and has been correlated with a greater number of alternative methods of assessing relative celluloytic activity. This method has been used by Sharp and Eggins (1970), Sharp (1970), Malik (1970) and Mills (1971).

5.2. Materials and Methods

5.2.1. Test Organisms.

The test organisms used in this study were those outlined in Table 2.1.

5.2.2. Materials.

The substrate used for the determination of relative cellulolytic activity was the cellulolse medium of Eggins and Pugh (1962) with the cellulose concentration at a level of 0.25 per cent. The technique used was that of Rautella and Cowling (1966).

5.2.3. Method.

Columns of agar were prepared by transferring molten cellulose agar into sterile 18-mm diameter boiling tubes to a depth of 6 cm approximately. The temperature of pouring was approximately 40°C, and the medium was poured in such fashion that the molten agar did not touch the side of the tubes and that the cellulose was maximally dispersed in the medium so that uniform opacity of cellulose agar columns was obtained. The tubes were immediately

plunged into cold water after pouring to solidify the agar and to prevent precipitation of the cellulose.

One sq. cm inocula were removed from actively-growing cultures of the test species, which were maintained on P. D. A. (Oxoid 139), and transferred to the surface of the cellulose agar columns in such fashion that the surface of the agar inocula bearing mycelium was in contact with the surface of the test medium. Triplicate test specimens were prepared for each test species and all test specimens were incubated at 25°C for 28 days. Measurement of clearing was undertaken at seven-day intervals.

The height of the agar column was marked on the side of each test tube at the time of inoculation. Measurement of depth of clearing, transpiration and evaporation were taken from this marked point. Control samples were included, from which evaporation levels were calculated over the test period, and from which results were adjusted.

Test species which produced clearing were considered to be cellulolytic.

5.3. Results

Growth of the thirty three test species took place, resulting in a range of reactions with the cellulose agar columns. Eleven species produced the clearing described earlier, the variation of which ranged between organisms from 19 mm to 3 mm over the test period. Control columns showed 3 mm dehydration over the test

period.

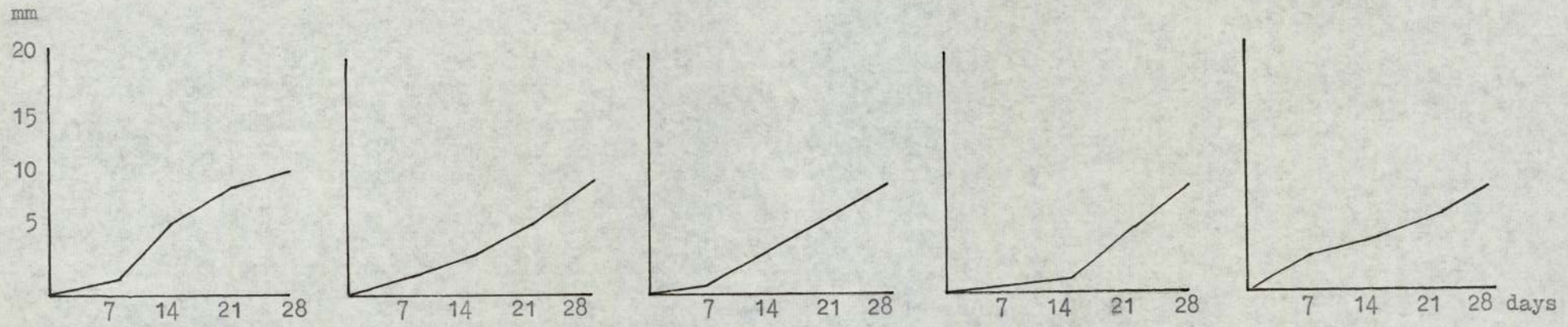
Sixteen species which did not produce clearing over the test period, instead produced an excessive dehydration combined in some cases with a slight or barely-perceptible clearing underneath the fungal colony. The extent of this excessive dehydration ranged from 10 mm to 1 mm over the test period.

Six species demonstrated neither the clearing nor the excessive dehydration mentioned above, but instead produced a deep penetration of dematiaceous hyphae into the medium. This hyphal penetration was sometimes accompanied either by faint clearing or shrinkage. Hyphal penetration ranged from 6 mm to 10 mm over the test period.

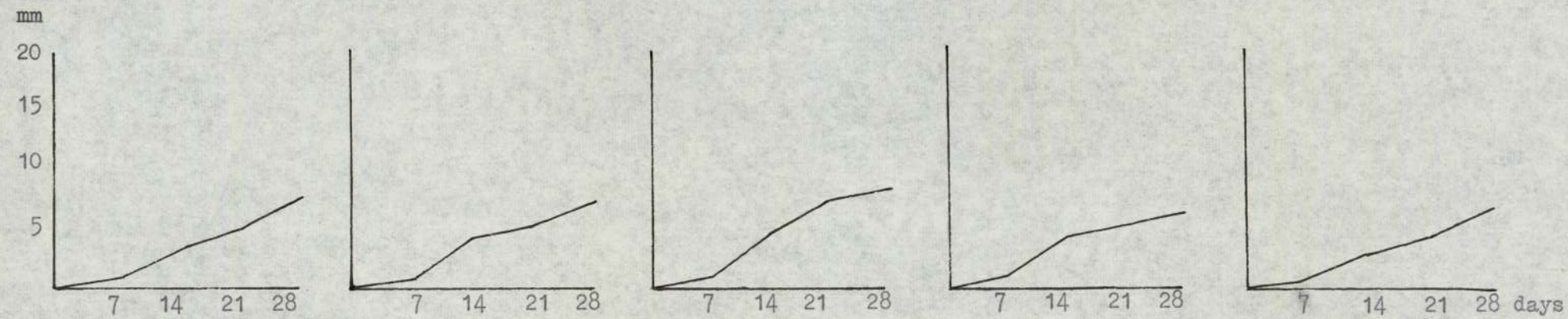
Species were described by the domination of one form of reaction, i.e. a species producing faint clearing e.g. 0.5 mm, and 5 mm excessive dehydration was considered to produce shrinkage while a species producing 1 mm shrinkage and 5 mm hyphal penetration was considered to produce hyphal penetration.

Results are presented in tabular and graphical form. The reaction level in mm of the individual fungal species with the cellulose agar columns are presented in Fig. 5.1. Table 5.1. indicates those species producing clearing, excessive dehydration, hereafter referred to as shrinkage, and hyphal penetration.

FIG. 5.1 Reaction level in cellulose agar columns produced by the individual test species measured in mm.



Bisporomyces chlamydosporis | Helminthosporium erythrospilum | Trichocladium canadense | Phialophora americana | Diplodia sapinea



Phialophora melinii | Ceratocystis pilifera | Graphium sp. | Diplodia pinea | Ceratocystis picea

Fig. 5.1 - continued

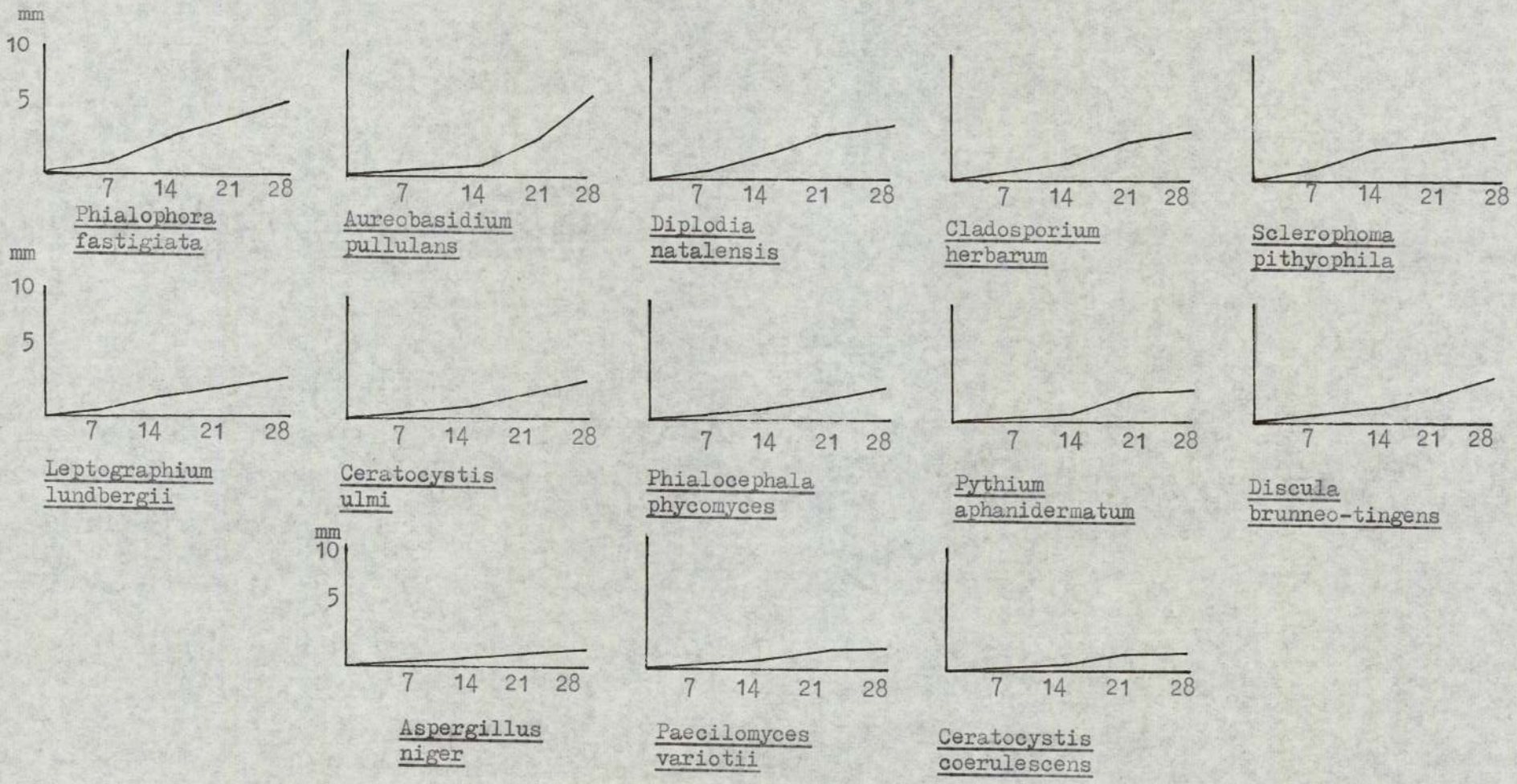


Fig. 5.1 - continued

TABLE 5.1

REACTION TYPES OF SOME BLUE-STAINING FUNGI

Clearing	Shrinkage	Hyphal penetration
Botriodiplodia theobromae	*Myxotrichum deflexum*	*Rhinocladiella atrovirens*
Cytospora leucostoma	*Alternaria tenuis*	*Trichocladium canadense*
Botrytis cinerea	*Helinthosporium erythrospilum*	*Phialophora americana*
Gliocladium roseum	*Diplodia sapinea*	*Phialophora melinii*
Cephalosporium acremonium	*Ceratocystis picea*	*Diplodia pinea*
Trichoderma viride	*Phialophora fastigiata*	*Aureobasidium pullulans*
Scytalidium lignicola	*Diplodia natalensis*	
Bisporomyces chlamydosporis	*Cladosporium herbarum*	
Ceratocystis pilifera	*Sclerophoma pithyophila*	
Graphium sp.	*Ceratocystis ulmi*	
Leptographium lundbergii	*Phialocephala phycomyces*	
	Pythium aphanidermatum	
	Paecilomyces variotii	
	Ceratocystis coerulescens	
	Aspergillus niger	
	Discula brunneo-tingens	

5.4. Discussion

It can be seen from the results that the blue-staining and mould fungi tested displayed both a range of reaction levels, ranging from 19 mm to 3.0 mm, and reaction forms with cellulose agar columns. Initial cellulolytic assays were undertaken on a medium containing 1 per cent ball-milled cellulose, but as the degree of reaction of the test species with this medium was generally slight, results are presented for assays undertaken on a medium containing 0.25 per cent ball-milled cellulose.

Since the presence of clearing in media containing ball-milled cellulose is indicative of a cellulolytic activity of the organism growing therein (Eggins and Pugh 1962), and the clearing of ball-milled cellulose is indicative of the activity of the C_1 cellulase component as crystalline cellulose is degraded (King 1966; Liu and King 1967), those blue-staining fungi producing clearing were considered to be cellulolytic.

Of the eleven species producing clearing, eight species, B. theobromae, C. leucostoma, B. cinerea, T. viride, S. lignicola, L. lundbergii, G. roseum and C. pilifera all showed rapid or moderate growth rates on cellulose agar plates, whilst three species, C. acremonium, B. chlamydosporis and Graphium sp. showed slow growth rates. The eight species showing rapid or moderate growth rates all showed good or moderate amylolytic activity, while of the three species showing slow growth rates, two showed fair amylolytic activity,

and C. acremonium showed moderate amylolytic activity.

B. theobromae, L. lundbergii and C. pilifera, have been shown to produce soft-rot cavities in wood (Krapvina 1960; Umezuricke 1969; and Findlay 1970). B. cinerea, G. roseum, and T. viride have been shown to produce carboxymethylcellulase in liquid culture (Domsch and Gams 1969) and a species of Cephalosporium has been shown to produce severe weight loss in wood (Butcher 1968).

Of the sixteen species producing shrinkage of cellulose agar columns, nine, including A. tenuis, H. erythrospilum, D. sapinea, D. natalensis, D. brunneo-tingens, A. niger, S. pithyophila, P. aphanidermatum and P. varioti showed rapid or moderate growth rates and all but one of which showed good or moderate amylolytic activity, the exception, D. brunneo-tingens, showing fair amylolytic activity. The remaining seven species, M. deflexum, C. picea, P. fastigiata, C. herbarum, C. ulmi, P. phycomyces and C. coerulescens all showed slow growth rates on cellulose agar plates and all but one of which showed fair amylolytic activity, the exception, C. picea, showed moderate amylolytic activity.

P. varioti, P. fastigiata and C. coerulescens have been shown to produce soft-rot cavities in wood (Karkanis 1966; Krapvina 1960); A. tenuis, A. niger and P. aphanidermatum have been shown to produce carboxymethyl-cellulase in liquid culture, Domsch and Gams (1969) and Siu and Reese (1953) considered C. herbarum to be slightly celluloltyic.

Of the six species producing hyphal penetration, two species, T. canadense and D. pinea both showed rapid growth rates and good or moderate amylolytic activity, while the remaining four species showed slow growth rates. One of these, A. pullulans, was shown to be moderately amylolytic, and the remaining three species, R. atrovirens, P. americana, and P. melinii all showed fair amylolytic activity.

T. canadense has been associated with butt rot in wood (Shigo 1965), D. pinea has been shown to produce carboxymethylcellulase in liquid culture (Pathak and Prasid 1969) and both P. americana and P. melinii have been associated with weight loss in wood (Merrill 1965).

It can be seen from the presented results that species not producing clearing produced marked and defined alternative reactions with the cellulose agar columns. Many of the fungi not producing clearing, showed rapid growth rates on cellulose agar plates, and similarly showed good or moderate amylolytic activity. The degree of these alternative reactions was significant in relation to the species producing clearing, e.g. H. erythrospilum, and R. atrovirens produced significantly higher reaction levels of shrinkage and hyphal penetration than L. lundbergii did of clearing, and similarly, some of the species not producing clearing have been seen to produce structural degradation in wood.

However, if it is considered that agar columns

supporting growth of fungal species with high transpiration rates might decrease in volume, and that this volume will be lost from the area proximate to the fungal colony, then, in the case of cellulose agar columns, that portion of the column showing clearing is the area from which transpiration is most likely to take place. It was similarly considered that a deep penetration and ramification of fungal mycelium into an agar column in comination with a slow rate of enzyme production, would significantly obscure the medium to make clearing undetectable. It was therefore considered that it was not so much that species producing shrinkage and hyphal penetration did not produce clearing or degrade cellulose, but that clearing was not seen to be produced by them due to the extent of their other reactions.

Savory et al. (1967) and Walsh and Stewart (1969) both found difficulty assaying cellulolytic activity on cellulose agar plates due to fungal growth, either covering or obscuring cleared areas. To remedy this, Savory et al. (1967) assayed cellulase production and inhibited fungal growth, while Walsh and Stewart (1969) counted cellulose particles after removal of the fungal colony. The experiments with blue-staining and mould fungi undertaken were similarly affected since hyphal penetration or transpiration were considered to obscure actual cellulolytic activity as indicated by the technique of Rautella and Cowling (1966).

To take this into account, particularly since some of the test species were known to produce wood degradation,

it was therefore considered that relative cellulolytic activity of the test species could be graded on the degree of reaction with cellulose agar columns along with grading them on their production or non-production of clearing in those columns. Species producing reaction levels in excess of 13 mm were considered to show a good cellulolytic reaction, species producing reaction levels in excess of 5 mm but less than 13 mm were considered to be moderately cellulolytic, while species showing reaction levels of less than 5 mm were considered to be slightly cellulolytic.

It can therefore be seen that seven species produced good reaction levels, all of clearing, and 15 species produced moderate reaction levels, three of which were of clearing, six of which were of shrinkage and six of which were of hyphal penetration. Eleven species might be considered to be slightly cellulolytic, ten of these produced shrinkage and the remaining species produced clearing. These results are presented in Table 5.2.

All but one of the species producing good reaction levels on cellulose agar columns showed either rapid or moderate growth rates and either good or moderate amylolytic activity. The exception, C. acremonium, showed a slow growth rate on cellulose agar plates and a moderate amylolytic activity.

Of the fifteen species producing moderate reaction levels, A. tenuis, H. erythrospilum, T. canadense, D. sapinea, C. pilifera and D. pinea all showed rapid growth rates on cellulose agar plates, and all similarly

TABLE 5.2

ORGANISMS SHOWING GOOD MODERATE AND SLIGHT REACTION LEVELS ON CELLULOSE AGAR COLUMNS

Good Reaction	Moderate Reaction	Slight Reacion
Botriodiplodia theobromae	Bisporomyces chlamydosporis	Diplodia natalensis
Cytospora leucostoma	Myxotrichum deflexum	Discula brunneo-tingens
Botrytis cinerea	Rhinocladiella atrovirens	Cladosporium herbarum
Gliocladium roseum	Alternaria tenuis	Aspergillus niger
Cephalosporium acremonium	Helminthosporium erythrospilum	Sclerophoma pithyophila
Trichoderma viride	Trichocladium canadense	Leptographium lundbergii
Scytalidium lignicola	Phialophora americana	Ceratocystis ulmi
	Diplodia sapinea	Phialocephala phycomyces
	Ceratocystis pilifera	Pythium aphanidermatum
	Phialophora melinii	Paecilomyces variotii
	Graphium sp.	Ceratocystis coerulescens
	Ceratocystis picea	
	Aureobasidium pullulans	
	Phialophora fastigiata	
	Diplodia pinea	

demonstrated good or moderate amylolytic activity. The remaining nine species showed slow growth rates, and all but two showed fair amylolytic activity, the exceptions, A. pullulans and C. picea, showed moderate amylolytic activity.

Of the eleven species showing slight reaction levels, seven showed rapid or moderate growth rates on cellulose agar plates, and all but one showed good or moderate amylolytic activity, the exception, D. brunneo-tingens, showed fair amylolytic activity. The remaining four species, C. herbarum, C. ulmi, C. coerulescens and P. phycomyces, showed both slow growth rates and fair amylolytic activity.

It can therefore be seen that, though nineteen species produced rapid or moderate growth rates on cellulose agar plates (see Tables 3.2-3.4), only eight species produced clearing in cellulose agar columns, while of the fourteen species showing slow growth rates, three produced clearing of cellulose. These species can, by definition, be considered cellulolytic.

However, the species producing reaction forms other than clearing included P. fastigiata and P. melinii, the former demonstrating shrinkage and the latter hyphal penetration of cellulose agar columns. Krapvina (1960) and Karkanis (1966) observed the cavity formation in wood, produced by P. fastigiata, and Merrill (1965) noted the severe weight losses in wood produced by P. melinii. These species may possibly also be considered

cellulolytic, even though they did not produce clearing of cellulose agar. If the alternative reaction forms are considered to indicate cellulolytic activity, then, of the nineteen species showing rapid growth rates, thirteen species showed good or moderate reaction levels in cellulose agar columns, and of the fourteen species showing slow growth rates, ten showed moderate reaction levels while four showed slight reaction levels. It can therefore be seen that if the clearing of cellulose agar columns is the only indication of cellulolytic activity, then only eleven of the thirty three blue-staining and mould fungal species tested could be considered cellulolytic, whereas if the degree of reaction of the test organisms with the medium is a criterion of cellulolytic activity, then at least twenty three species might be considered to be actively cellulolytic.

A pattern can be seen to emerge in which none of the species showing slow growth rates on cellulose agar plates produce a high reaction level in cellulose agar columns or showed good amylolytic activity. However, all of the species producing high reaction levels on cellulose agar columns similarly showed good or moderate amylolytic activity and all but one, C. acremonium, showed a rapid or moderate growth rate.

It is therefore evident that, whereas there was a general correlation between growth rate and amylolytic activity for the test species, this same correlation was not so apparent for cellulolytic activity, even though

the growth rates of the individual species did not vary greatly on the two media. However, all species showing good reaction levels on cellulose agar columns, similarly showed good or moderate amylolytic activity, and all but one showed a rapid or moderate growth rate.

This could possibly infer that cellulase production by cellulolytic blue-staining and mould fungi could be related to their growth rates, i.e. a certain defined quantity of growth would have to take place before the activity of the liberated cellulase was detectable, and this factor might not be taken into account with the test method used.

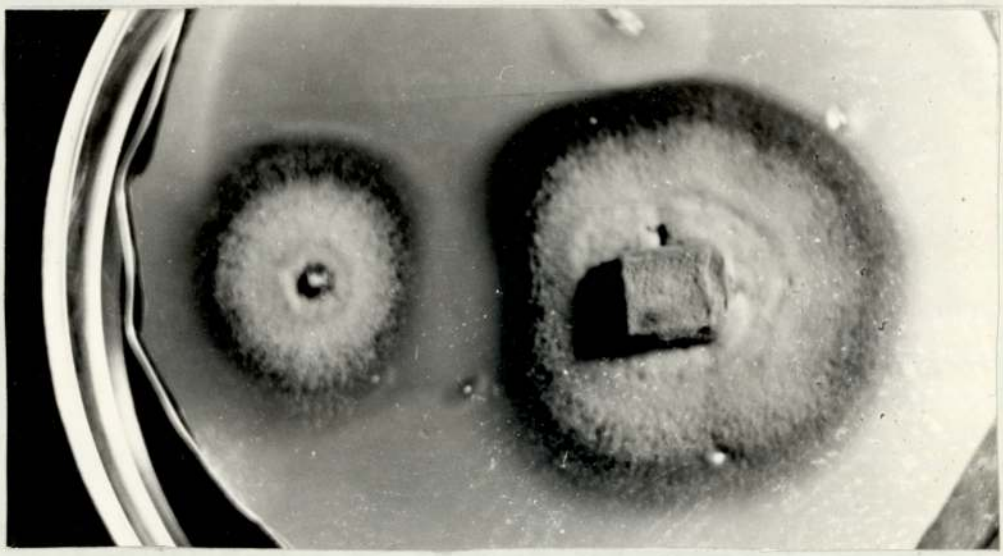
It is not difficult to conclude that few of the test species exhibited a marked cellulolytic activity, particularly since the cellulose concentration of the medium was considerably reduced to make production of clearing more evident. This lack of significant cellulolytic activity was particularly noticeable in comparison with the marked amylolytic activity of all of the test species. This would seem to corroborate the traditional viewpoint on blue-staining and mould fungi, i.e. that they are primarily starch-degrading species and not cellulose degrading. However, the slight cellulolytic activity of some of the species evidenced by either clearing or other reaction form, would indicate a need for further investigation before finite conclusions were drawn.

Cellulolytic activity is therefore further examined in Chapters 7 and 8, using strength loss and viscosimetric techniques.

CHAPTER 6

RELATIVE PECTINOLYTIC ACTIVITY

PLATE I PECTATE GEL DEGRADATION



a. Liquifaction zone produced by Myxotrichum deflexum

b. Gel collapse produced by Cephalosporium acremonium

RELATIVE PECTINOLYTIC ACTIVITY

6.1. Introduction

Permeability increases in wood seem strongly associated with loss in pectin content. Lindgren (1952) ascribed increased permeability of wood to preservatives after microfungal attack, to decreased pectin content. Soulahti and Wallon (1958) noted that the pectin content of water-stored wood decreased considerably, and Ellwood and Ecklund (1959) noted that Bacillus polymyxa, the causative organism of increased permeability of ponded pine, was capable of decomposing pectins, as did Knuth and McKoy (1962). Nagel and Vaughan (1961) have shown that B. polymyxa in fact produces pectin transeliminase. Soulahti (1961) noted that a combination of pectinase and ammonium oxalate significantly affected wood permeability. Liese and Karnop (1968) noted a pectinolytic activity of bacterial isolates from ponded pine and spruce, and Highly and Lutz (1970) attributed increased permeability of ponded pine and poplar to a pectinolytic activity of bacteria. Nicholas and Thomas (1968) noted that pectinase produced large openings in bordered pits and severe degradation of ray parenchyma cells, while Liese (1970) noted that pectinase enhanced wood permeability by acting on the tori of bordered pits.

It is therefore apparent that organisms naturally colonizing wood tissue, if found to possess a pectinolytic activity, may significantly affect wood permeability. Little is known of the pectinolytic activity of wood-

inhabiting fungi although Soulahti (1961) has shown that the wood-rotting fungi Coniophora cerebella and Poria vaporia have marked pectinolytic activity. He quotes Bashenov (1956), who attributed increased permeability of wood due to the action of blue-staining fungi to pit penetration, while Lindgren (1952) infers that fungi found to have increased wood permeability have digested pectins. Domsch and Gamm (1968) noted that many soil fungi found to colonize freshly-felled timber and causing blue staining (Dowding 1970) possessed a pectinolytic activity, and Rosch, Liese and Berndt (1969) have pointed out the polygalacturonase production by the blue-staining fungi Aureobasidium pullulans, Alternaria humicola and Ceratocystis minor.

There is therefore evidence to show that some individual blue-staining fungal species may have pectinolytic activity. However, the definition of pectinolytic activity itself needs some discussion. Deuel and Stutz (1958), Wood (1960) and Bateman and Miller (1966) have reviewed breakdown of pectins by pectic enzymes. Pectinases may be divided into two main groupings, pectinesterases and polygalacturonases, the latter which hydrolize the methyl ester groups of pectinic acids, whilst the former break the polygalacturonide chains to shorter chains and reducing sugars. Polygalacturonases have been further classified into endo and exo types. Deuel and Stutz (1958) considered that optimal pH for activity of polygalacturonases of bacteria and Phycomycetes was in the region of 7, and for

Fungi Imperfectii and Ascomycetes, in the region of 4. Bateman and Miller (1966) have divided pectinases into eight categories dependent on the mechanism by which the -1,4 glycosidic linkage is split, by the enzyme's preference for substrate, and by the position in the pectin chain at which clearage occurs.

It therefore appeared that a range of pectic substrates and a range of pectic enzymes were involved in the assessment of pectin degradation, and that to give a true assessment of pectinolytic activity of the individual test organisms, tests would have to be undertaken individually for each of the pectic enzymes, the methods used for which were normally in vitro studies involving viscometric and reducing sugar determinations.

However, for screening purposes it was considered that comparative results might be obtained from microbiological test methods, undertaken in vivo, similar to studies carried out for amylolytic and cellulolytic activity. Luckhurst (1953) used pectate gels as a substrate for qualitative determination of pectinolytic activity, and Eggins (1956) used a similar method for the determination of pectinolytic activity of algae, activity being indicated by a breakdown of pectin in the gel resulting in its dissolution and liquifaction. A recent paper by Jasankar and Graham (1970) has indicated that cetyl trimethyl ammonium bromide (a polysaccharide precipitant) may be used for qualitative and quantitative assessment of pectinolytic activity. For the purposes of establishing basic data on pectinolytic activity of

blue-staining fungi, a modified medium similar to that of Luckhurst (1953) and Eggins (1956) was used.

6.2. Materials and Methods.

6.2.1. Test organisms.

The test organisms used in this study were those outlined in Table 2.1.

6.2.2. Materials.

The substrate used for determining relative pectinolytic activity was a modification of the calcium pectate gel (Luckhurst 1953; Eggins 1956). The preparation of this gel is outlined in Appendix II.

6.2.3. Method.

One sq. cm inocula were removed from actively growing cultures of the selected test organisms, which were maintained on P.D.A. (Oxoid 139) and transferred to the surface of the pectate gels in such fashion that the surface of the agar inoculum bearing mycelium was in contact with the surface of the gel. Triplicate test specimens were prepared for each fungal species, and all test specimens were incubated for 21 days at 25°C.

Assessment of relative pectinolytic activity was on the basis of the area of gel liquified by the individual fungal species. Species producing liquifaction of over 50 per cent of the gel area were considered to show good pectinolytic activity; species producing liquifaction of over 25 per cent but less than

50 per cent of the gel area, were considered to be moderately pectinolytic; and species showing liquefaction of less than 25 per cent of the gel area were considered to be fairly pectinolytic. Species producing gel degradation, but not in a measurable fashion, were considered to be slightly pectinolytic.

6.3. Results

Thirty three blue-staining fungi were tested for pectinolytic activity using liquifaction of pectate gels as a criterion of pectin degradation. Two forms of gel dissolution were noted, liquifaction and gel collapse. Liquifaction was generally seen when the test organism had reached the limit of its lateral growth, or else liquified areas were seen to surround slow-growing fungal colonies. Gel collpase was seen in the form of deep depressions formed in gels by the action of the growth of fungi, resulting in the fungal colony resting on the permeable membrane supporting the gel. Gel collapse areas were seen to increase with the growth of the species exhibiting this effect. Control plates exhibited neither gel collapse nor liquifaction. Some organisms exhibiting liquifaction exhibited gel collapse at the early stages of growth. Gel collapse was generally seen to proceed from the inoculum towards the periphery of the colony, the colony area generally exceeding the collapsed area. Both gel collapse and gel liquifaction were considered to be indicative of pectin degradation.

Grades of pectin degradation were also apparent.

Some species grew slowly, e.g. Myxotrichum deflexum, and produced distinct liquifaction areas surrounding the colony, while others rapidly colonized the entire gel area, producing gel collapse and liquifaction, e.g. Botriodiplodia theobromae. Yet other species grew rapidly e.g. Trichoderma viride, or slowly e.g. Phialophora melinii, but did not produce measurable gel degradation. Instead, these species produced a "dappled" effect on the surface of the gels, comprised of minute liquifaction areas. These areas were considered to indicate a slight pectinolytic activity.

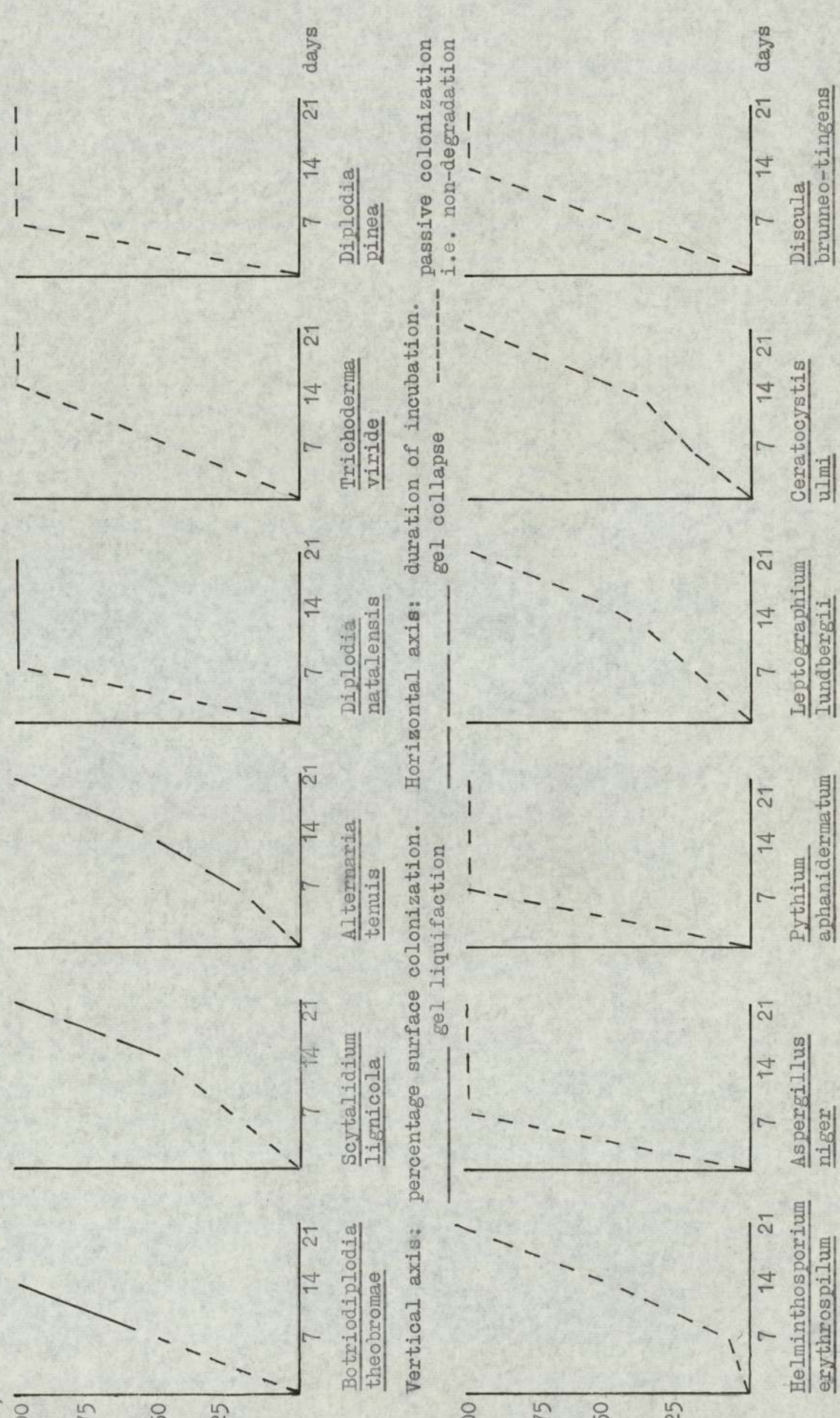
Of the thirty three blue-staining and mould fungi tested, only twelve showed a visible and measurable gel degradation. Four of these species produced good liquifaction, four produced moderate liquifaction, and four produced fair liquefaction. These species are indicated in Table 6.1. One species, Pythium aphanidermatum, did not show gel degradation, while the remaining twenty species showed visible but not measurable gel degradation. Degree of gel degradation and growth rate are related and shown graphically for the individual species in Fig. 6.1.

TABLE 6.1

Blue-staining fungi showing good, moderate and fair pectinolytic activity

Good Activity	Moderate Activity	Fair Activity
B. theobromae	G. roseum	D. sapinea
A. tenuis	B. cinerea	C. pilifera
D. natalensis	C. acromonium	T. canadense
S. lignicola	G. picea	M. deflexum

FIG. 6.1 Percentage colonization of pectate gels by the individual test species. Form of reaction with gels is also indicated.



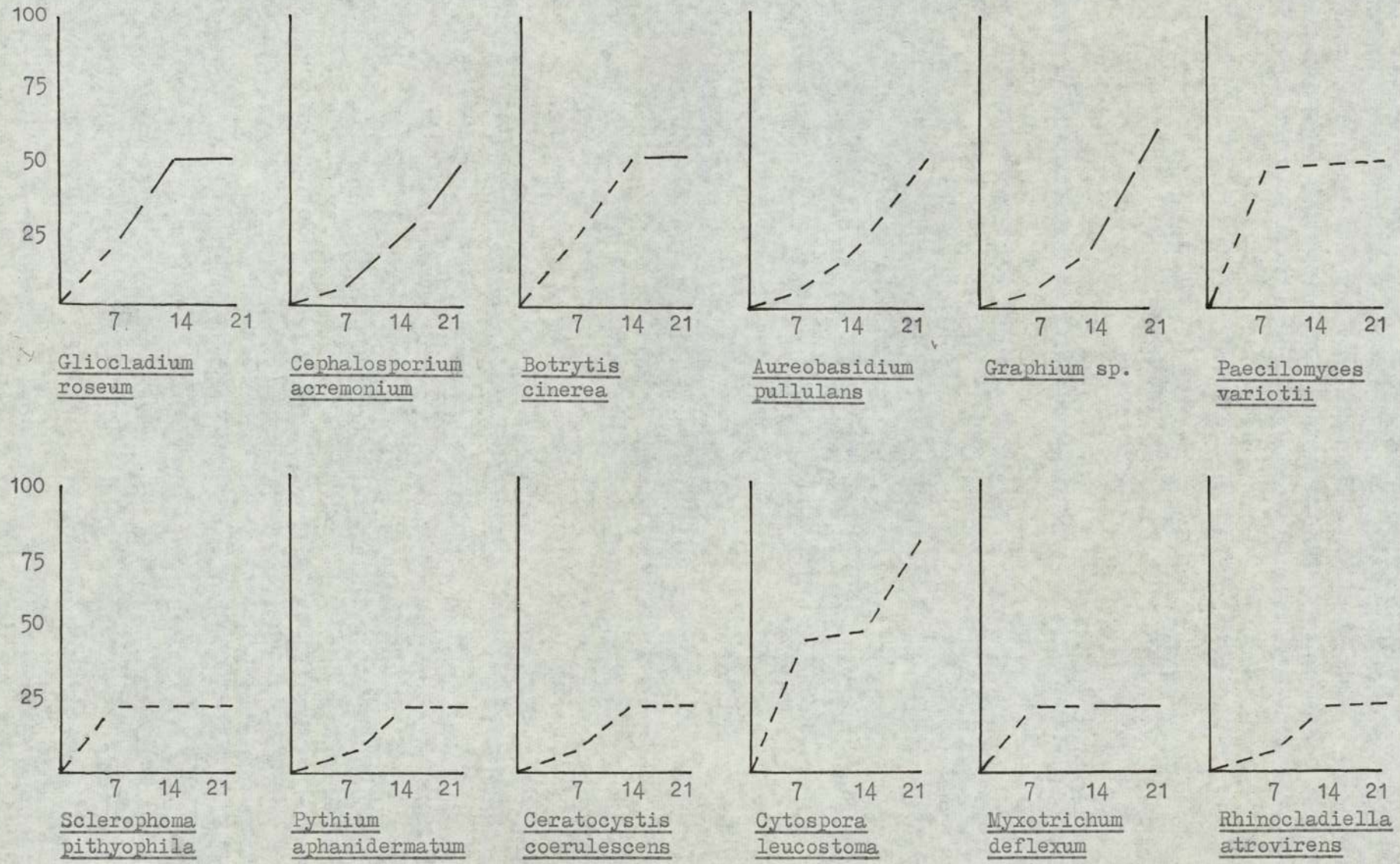
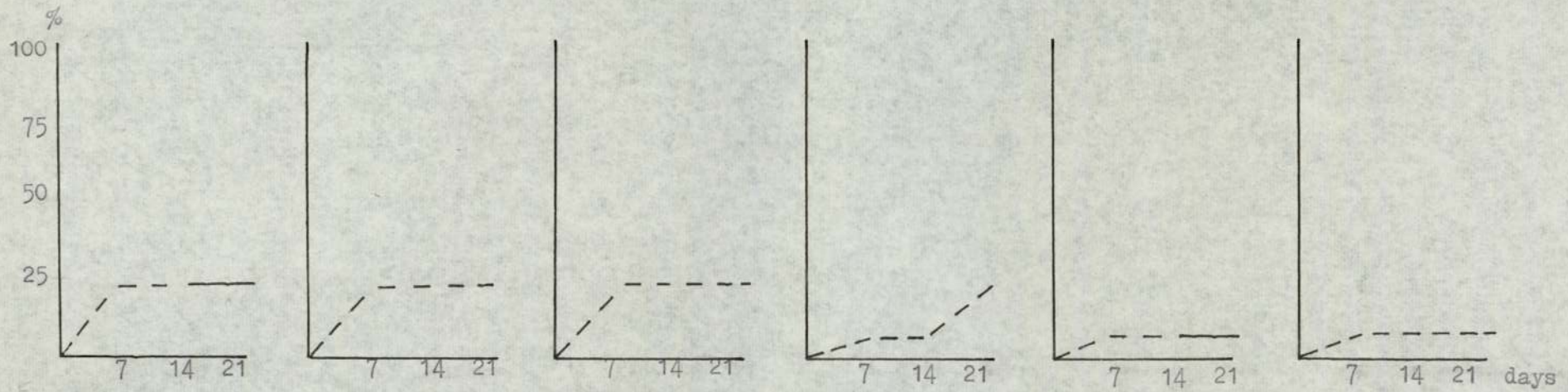


Fig. 6.1 - continued



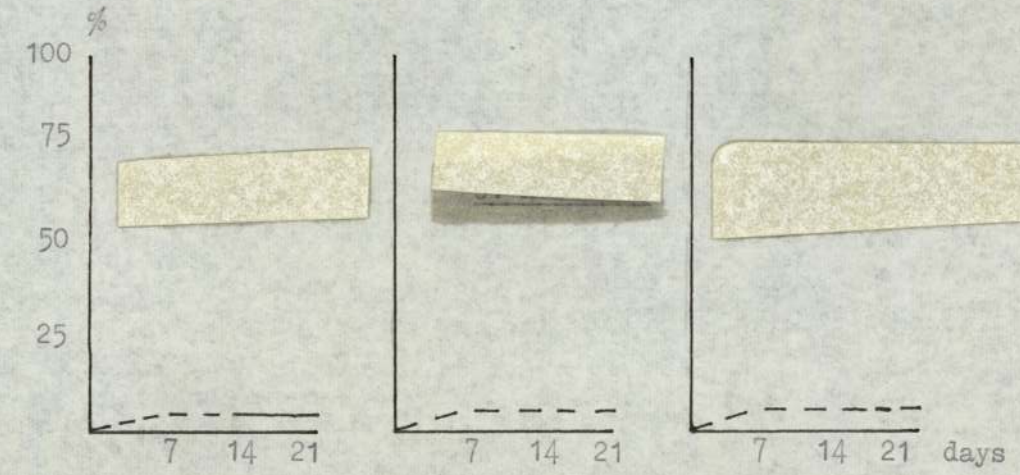


Fig. 6.1 - continued

6.4. Discussion.

All of the blue-staining and mould fungi, excepting Pythium aphanidermatum, were seen to produce some form of degradation of pectate gels. However, only twelve species produced a measurable gel degradation. The latter were considered to be pectinolytic.

Rate of gel degradation varied, ranging from 100 per cent degradation by Botriodiplodia theobromae to 6 per cent degradation by Diplodia sapinea over the twenty-one day test period. All but one of the species showing measurable pectin degradation grew rapidly or moderately on pectin agar (See Tables 3.1-3.3); the exception, Myxotrichum deflexum, grew slowly on that agar. All but two of these organisms showed good or moderate amylolytic activity; the two exceptions, M. deflexum and Graphium sp., showed fair amylolytic activity (see Table 4.1), and all but one of these species showed good or moderate reaction levels with cellulose agar columns; the exception, Diplodia natalensis, showed a slight reaction level (See Table 5.2).

Rate of growth did not correlate well with gel degradation. Of the twenty five species showing rapid or moderate growth rates on pectin agars, only eleven showed an ability to measurably degrade pectate gels. However, of the nine species demonstrating slow growth rates on pectin agar, only one, M. deflexum, produced a measurable degradation of pectate gels.

Rosch, Liese and Berndt (1969) found that the staining

fungi Ceratocystis minor, Alternaria humicola, and Aureobasidium pullulans, produced polygalacturonase in liquid culture. When four species of Ceratocystis were tested for pectinolytic activity on pectate gels, only one, C. pilifera, was found to be measurably pectinolytic. A species of the Alternaria tenuis group was similarly found to be pectinolytic, but a strain of A. pullulans, when tested, did not reveal measurable pectinolytic activity. Botrytis cinerea, known to produce pectinases (Wood 1960; Bateman and Miller 1966; and Van den Berg and Yang 1969), was found to be distinctly pectinolytic on pectate gels, as were Gliocladium roseum, Cephalosporium acremonium, and Trichocladium canadense. However, Ceratocystis ulmi and Aspergillus niger and species of Cladosporium, Pythium, and Trichoderma, noted for their pectinolytic activity (Deuel and Stutz 1958, and Domsch and Gams 1969), were not found to be pectinolytic on pectate gels.

It is therefore apparent that only twelve of the test species showed a measurable pectinolytic activity, and that species of six of these have been shown to be pectinolytic using ~~xxxx~~ viscosimetric methods by other workers. Data was not found on pectinolytic activity of the remaining six species. All but one of the pectinolytic species, M. deflexum, showed good or moderate growth rates on pectin agar and all were amylolytic. If clearing of cellulose agar is indicative of cellulolytic activity, then seven cellulolytic species, B. theobromae,

Scytalidium lignicola, G. roseum, B. cinerea, C. pilifera, C. acremonium, and Graphium sp. were also pectinolytic. If, however, reaction level with cellulose agar columns is indicate of cellulolytic activity, then all but one of the pectinolytic species showed good or moderate cellulolytic activity, the exception, D. natalensis, showed a slight cellulolytic activity.

It is therefore seen that, whereas a rapid surface growth rate of the individual species when grown on agar media did not indicate a high relative amylolytic, cellulolytic, or pectinolytic activity, it is apparent from the results of the tests undertaken, that very few species with slow growth rates exhibited marked amylolytic, cellulolytic or pectinolytic activities relative to the rapidly-growing fungal species when tested in vivo.

The use of pectate gels for determination of pectinolytic activity does not define the type of pectin breakdown undertaken by the organisms whose growth it supports i.e. whether the breakdown was due to acid hydrolysis or enzymatic hydrolysis. The types of enzyme produced by the test organisms are also not determined. P. aphanidermatum was not seen to be pectinolytic in this experiment yet was seen to produce pectin transeliminase by Turner and Bateman (1968). The calcium present in pectate gels would inhibit the activity of pectin transeliminase (Bateman and Miller 1966), and therefore gel liquefaction would not result from the growth of P. aphanidermatum even if this enzyme was produced. However, the optimal pH for polygalacturonase activity would seem to be in the

region of pH 4, and for transeliminase activity, in the region of pH 7 (Deuel and Stutz 1958). These pH levels are also optimal for the production of the respective enzymes (Bateman and Miller 1966). The pH of green spruce is approximately 5.4, and when attacked by microfungi within the approximate range 5.4-4.7 (Hatton 1970). The pH of the gel was 5.5 after autoclaving. It is therefore suggested that, with reference to the gel PH and to the pH of green spruce, the medium was appropriate for the detection of polygalacturonases at the pH ranges at which they would be likely to be produced in nature.

It was therefore concluded that pectinase production, similar to cellulase production, was not nearly as significant as amylase production under the test conditions by the group of blue-staining and mould fungi under test. However, some species did produce gel liquefaction, and many of these similarly produced by reaction levels in cellulose agar columns. Alternatively, however, none of the "non-cellulolytic" species produced gel liquefaction and there therefore may be a connection between cellulase and pectinase production. Similarly, there may be a relationship between the rate of enzyme production and growth rate, since few of the slow-growing species were seen to produce sufficient enzymes to produce gel liquefaction, although this need not imply a lack of pectinase production on the part of the latter species.

These hypotheses are examined further in Chapter 8, using viscosimetric techniques.

CHAPTER 7

DEGRADATION OF FIBROUS CELLULOSE

PLATE II

A bank of perfusion devices

7. DEGRADATION OF FIBROUS CELLULOSE

7.1. Introduction

The cellulolytic activity studies undertaken using cellulose agar columns (Rautella and Cowling 1966) showed that only eleven of the thirty three species under test produced clearing of cellulose agar. The remaining twenty two species did not produce clearing but instead produced shrinkage of the columns, or deep hyphal penetration within columns. Since some of the species not producing clearing were noted from the literature to produce wood degradation, evidenced by strength loss, weight loss, or cavity formation within wood-cell walls, it was considered that a more sensitive test might be required to determine the possession or lack of possession of a cellulase system by a group of species traditionally regarded as non-cellulolytic.

Hartley (1958) considered that destructive and non-destructive tests were both useful in assaying fungal degradation of wood. He observed that bending strength and toughness could be utilized to evaluate rapidly wood degradation, and that toughness of wood was sometimes significantly affected by incipient decay. Armstrong and Savory (1959), working with Chaetomium globosum, found that toughness of beechwood was considerably reduced before significant weight losses were apparent, but that gradual reductions in bending strength were not significant until definite weight losses were evident. However, Duncan (1960) considered that weight loss could be

correlated with bending strength provided that the test material was carefully selected.

Weight loss of wood has been used as a criterion of fungal decay by a considerable number of workers (Armstrong and Savory 1959; Cowling 1961; Merrill 1965; Merrill and French 1966; Kerner Gang and Becker 1968; and Levi 1969). Scheffer and Cowling (1966) observed that mould and staining fungi could cause soft rot and consequently weight loss in wood, but only under prolonged exposure to highly favourable conditions, and Duncan (1963) considered that mould and staining fungi were the least important of the wood-decaying micro-organisms. It was therefore considered that many of the tests normally used for assaying the degradation activities of wood-decaying fungi would therefore not be useful except under long-term experimental conditions.

It was therefore decided that some of the micro-destructive techniques might be of some use in evaluating fungal cellulolytic activity, e.g. weight loss of filter paper or cotton. However, initial tests undertaken with these techniques showed that any weight losses produced by the test species was in many cases adequately recompensed by the luxuriant growth of the inocula.

Eggins, Malik and Sharp (1968) used perfused model substrates which included filter paper as a carbon source, for the isolation of cellulolytic soil microfungi. Sharp and Eggins (1970) and Sharp (1970) modified this technique to include either filter paper strips or veneer test specimens as isolation substrates, with the added

advantage that strips could subsequently be tested for strength loss. Pettifor and Findlay (1946)Ghosh, Bose and Basu (1968) and Stewart and Walsh (1969) all used tensile strength methods to determine the cellulolytic activity of both blue-staining and soil fungi using cotton fibre or wood veneers as test specimens. Using tensile strength as a criterion of fungal degradation, significant comparative results were obtained for weakly cellulolytic species such as Aspergillus niger.

It was therefore decided that a technique including tensile strength as a measure of cellulolytic activity, in combination with the perfusion culture system of Eggins, Malik and Sharp (1968), might provide demonstrative significant evidence of a cellulolytic activity of the blue-staining and mould fungi under investigation.

7.2. Materials and Methods

7.2.1. Test Organisms.

The test organisms used in this study were those outlined in Table 2.1.

7.2.2. Test Substrate.

The substrate used for strength loss testing was chromatography paper (Whatman No. 3). Strips of this paper were cut using a small hand-press cutter (manufactured by H.W. Wallace, Croydon) in such fashion that the fibre alignment in the paper was parallel with the length of the strip. The strips were eighty mm long and 3 mm wide . The exact size and shape of a strip is shown in Diagram 7.1.

Diagram 7.1

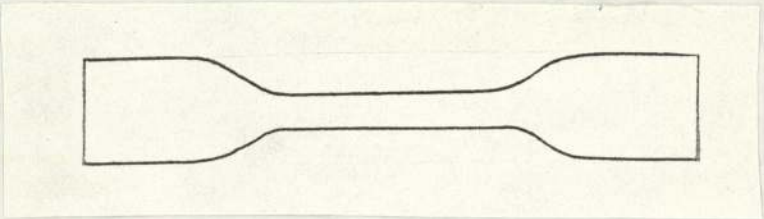


Diagram of strip of chromatography paper used for tensile strength testing (actual size).

7.2.3. Preparation of Test Specimens.

The paper strips were placed in petri dishes and were perfused with the mineral salts solution of Eggins and Pugh (1962) using the perfusion technique of Eggins Malik and Sharp (1968). The strips were covered by glass microscope slides, which in turn were attached to the lower valve of the petri dish by adhesive glass fibre tape. This procedure prevented distortion of the paper strips during autoclaving, which was at 15 lbs for 15 minutes.

The perfusion system used was such that banks of 5 petri dishes were supplied with nutrients by a common nutrient reservoir. Six of these banks were prepared for each test species, each bank containing four inoculated strips, and one uninoculated controlled strip. Using a 6-mm corkborer, inocula were removed from actively-growing cultures of the thirty three test species, which were maintained on Potato Dextrose Agar, and transferred aseptically to the centres of the sterile paper strips. The

inoculated banks were incubated at 25°C.

7.2.4. Test Method.

Two banks of petri dishes of each test species were sacrificed at 7-day intervals over a twenty-one day test period. The paper strips were removed from the perfusion devices and the inoculum and surplus mycelium was removed from each strip. After soaking each strip in distilled water for approximately fifteen seconds, the strips were tested for tensile strength using a tensometer designed for this experiment which is described in Appendix III.

Five inoculated strips and two control strips were tested for each test species at each test period. Tensile strength was measured in grams, and evaluation of tensile strength loss was calculated from the strength of control strips simultaneously perfused and incubated with inoculated strips.

Test species producing strength losses in excess of 50 per cent over the three-week test period were considered to be highly cellulolytic, species producing strength losses in excess of 25 per cent but not greater than 50 per cent were considered to be moderately cellulolytic, while species producing strength losses of less than 25 per cent were considered to be only very slightly cellulolytic.

7.3. Results

Many of the thirty three species tested produced considerable tensile strength losses in the fibrous cellulose strips. These strength losses were seen to commence during the first week of incubation, and were

seen progressively to increase during the test period. All species produced some strength loss, ranging from 4 per cent produced by Phialocephala phycomyces to 100 per cent produced by Graphium sp., over the 21 days of incubation. Species not producing clearing of cellulose agar in columns, but which instead produced shrinkage or hyphal penetration, were seen to produce extensive degradation of the paper strips. In some cases, the degree of reaction produced by these species in cellulose agar columns, was a better indication of cellulolytic activity than clearing, particularly for those species of slight or only suspect cellulolytic activity.

Of the thirty three species tested, strength losses of over 50 per cent were produced by 19 species, moderate strength losses were recorded for 7 species, and strength losses of less than 25 per cent were produced by seven species. Species producing respectively high, moderate and slight stength losses are presented in Table 7.1. The pattern of strength loss produced by the individual test species is illustrated graphically in Fig. 7.1.

7.4. Discussion

It can be seen from the results that all of the tested species produced strength losses in fibrous cellulose thus indicating a cellulolytic activity of a considerable number of fungal species associated with staining of wood.

All but two of the species which prduced clearing in cellulose agar columns similarly produced high strength

TABLE 7.1

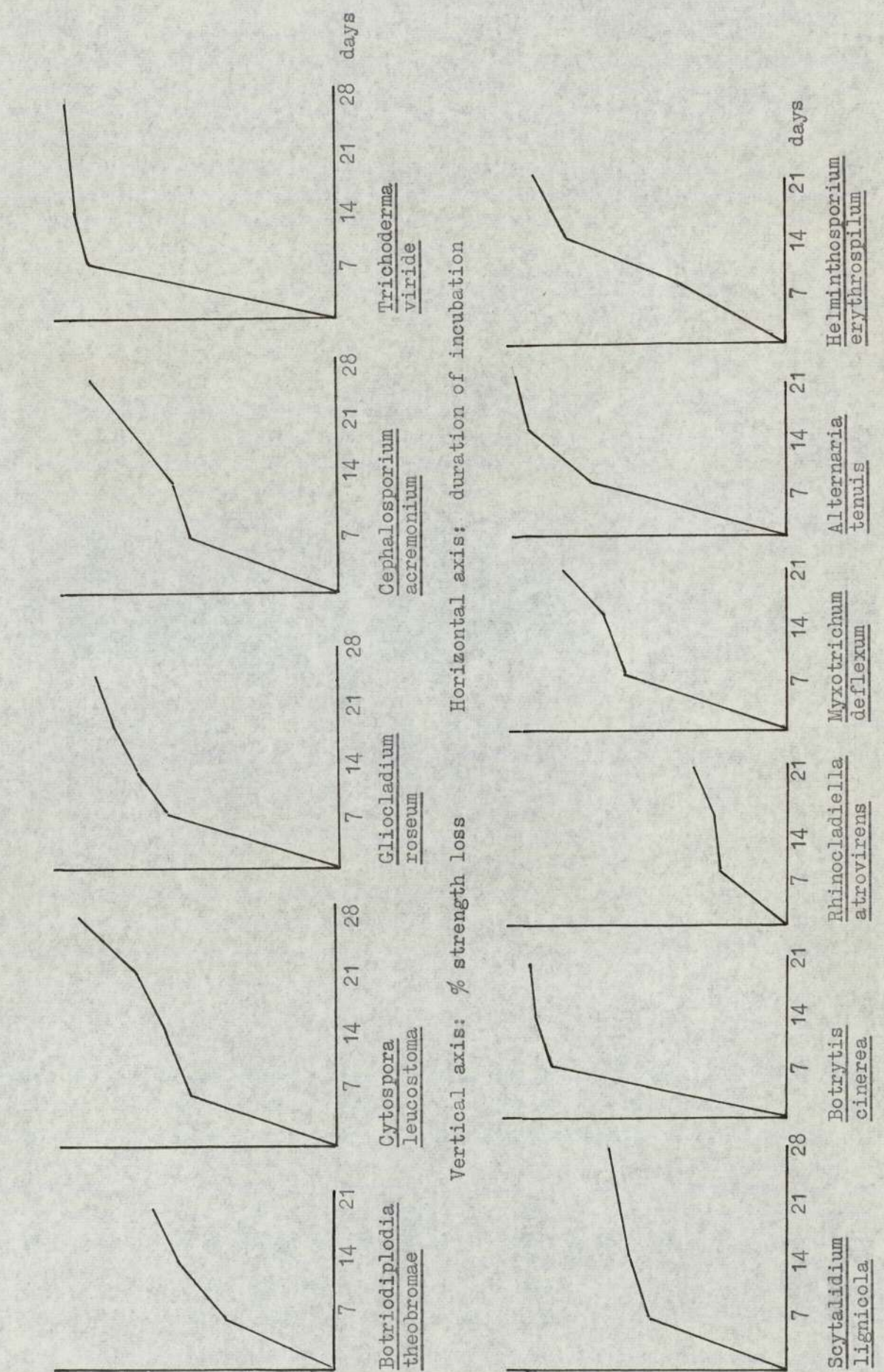
SPECIES PRODUCING HIGH MODERATE AND SLIGHT TENSILE STRENGTH LOSSES IN FIBROUS CELLULOSE STRIPS

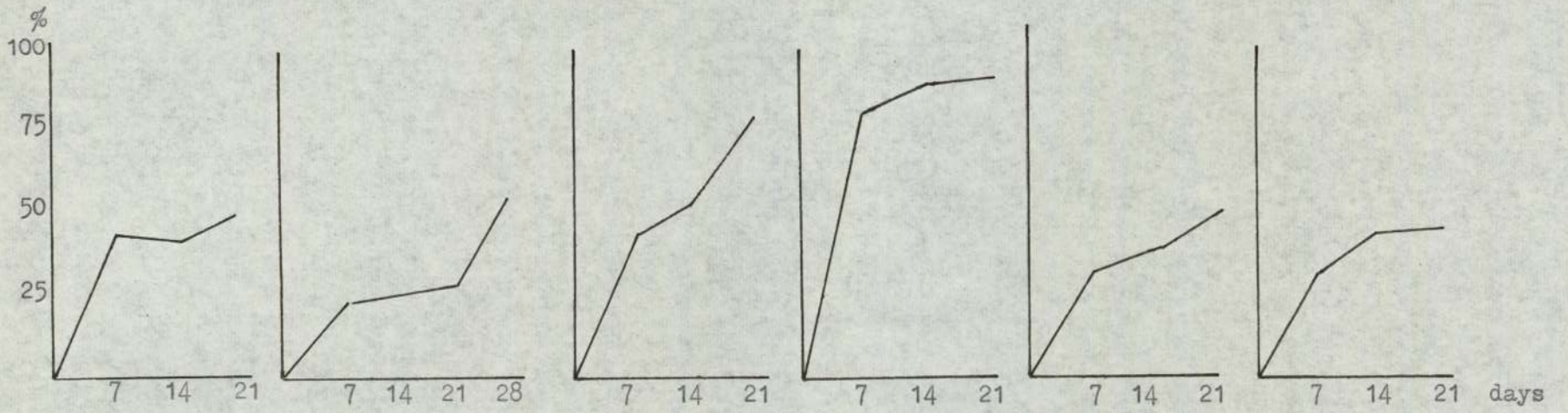
High Strength Loss >50%	Moderate Strength Loss >25% but <50%	Slight Strength Loss <25%
Graphium sp.	Phialophora americana	Ceratocystis coerulescens
Alternaria tenuis	Aureobasidium pullulans	Cladosporium herbarum
Trichoderma viride	Bisporomyces chlamydosporis	Phialocephala phycomyces
Cephalosporium acremonium	Sclerophoma pithyophila	Pythium aphanidermatum
Gliocladium roseum	Rhinocladiella atrovirens	Paecilomyces varioti
Cytospora leucostoma	Leptographium lundbergii	Aspergillus niger
Botrytis cinerea	Ceratocystis ulmi	Discula brunneo-tingens
Ceratocystis pilifera		
Helminthosporium erythrospilum		
Diplodia sapinea		
Phialophora melinii		
Phialophora fastigiata		
Diplodia natalensis		
Ceratocystis picea		

Table 7.1 - continued

High Strength Loss 50%	Moderate Strength Loss 25% but 50%	Slight Strength Loss 25%
Myxotrichum deflexum	-	-
Botriodiplodia theobromae		
Scytalidium lignicola		
Trichocladium canadense		
Diplodia pinea		

FIG. 7.1. Percentage strength loss in fibrous cellulose strips produced by the individual test species.





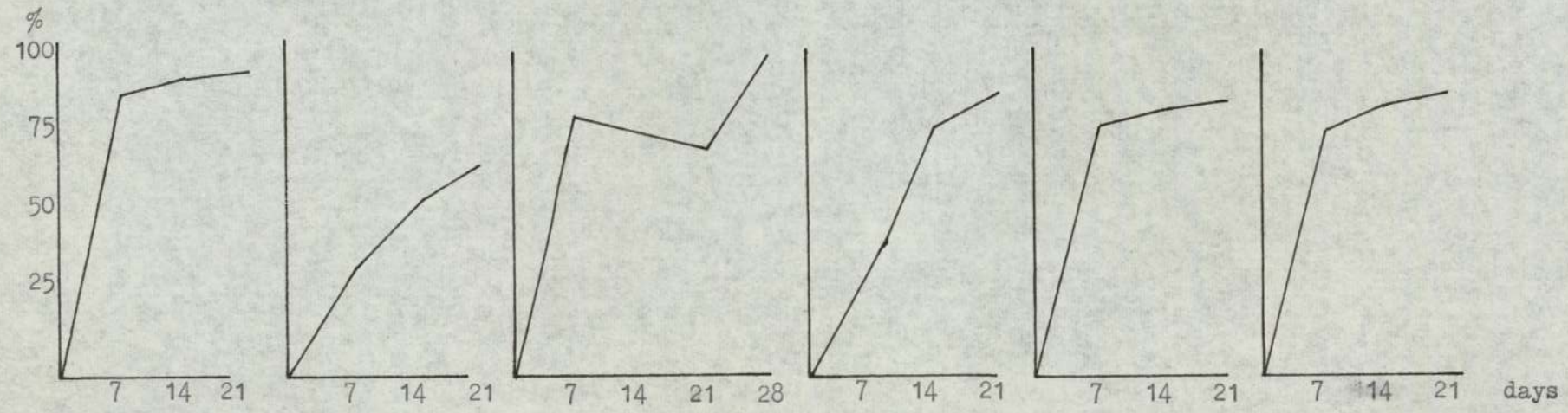
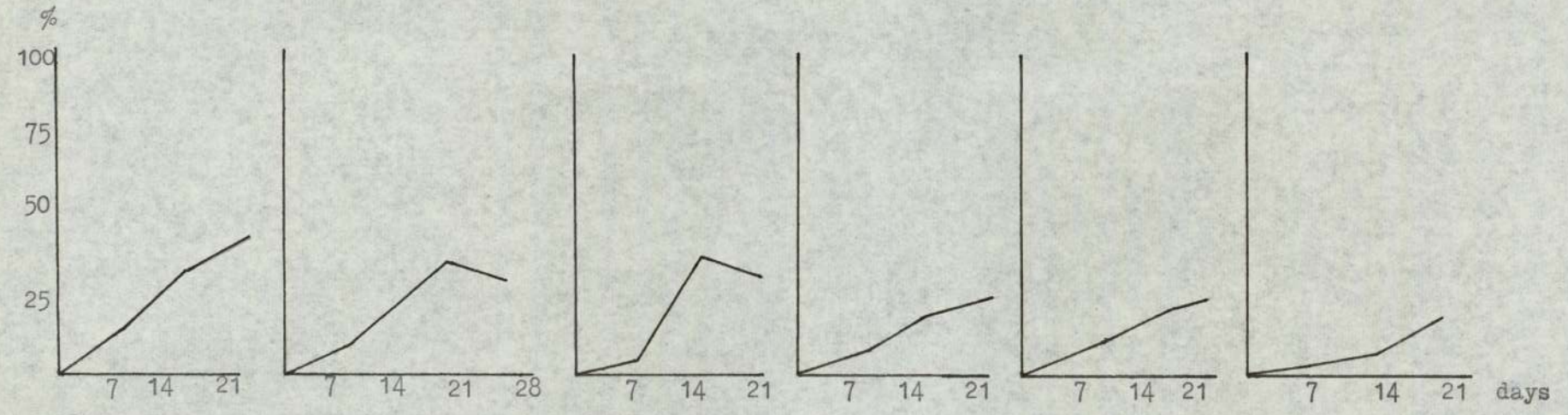


Fig. 7.1 - continued



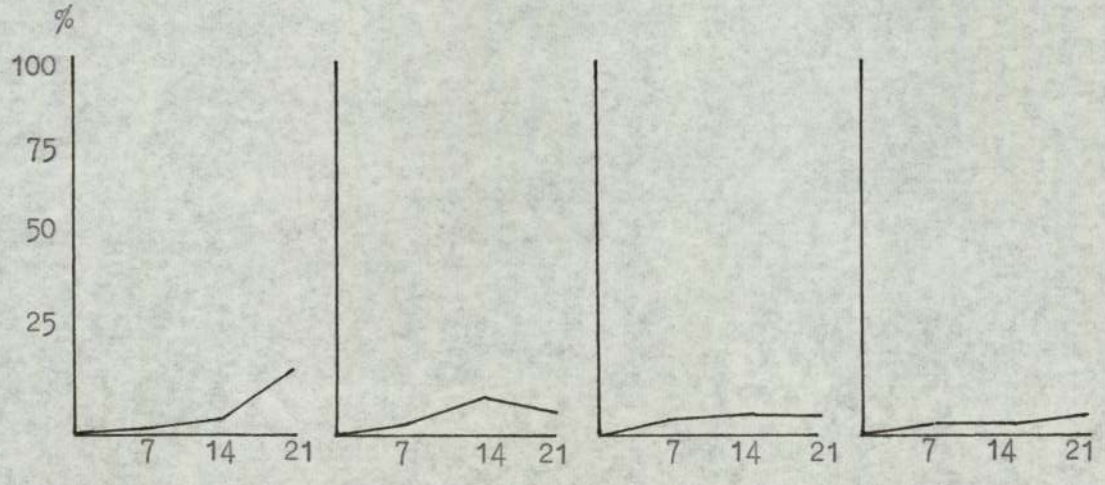


Fig. 7.1 - continued

losses. The two exceptions, Bisporomyces chlamydosporis and Leptographium lundbergii, both produced tensile strength losses of 29 per cent which was considered to be moderate. Of the twenty two species which produced good or moderate reaction levels in cellulose agar columns, all similarly produced high or moderate strength losses in the paper strips. Of the eleven species which produced slight reaction levels in cellulose agar columns, two species, L. lundbergii and Sclerophoma pithyophila, produced moderate strength losses, while one species, Diplodia natalensis, produced high strength losses. The remaining eight species, however, produced strength losses of less than 25 per cent.

These findings complement observations by other authors on the activities of blue-staining and mould fungi. Campbell (1969) observed from the work of Pettifor and Findlay (1946) that wood-strength was significantly affected by the activities of Botriodiplodia theobromae and D. natalensis, the former affecting wood strength to a greater degree than the latter. The experiments with cellulose agar columns demonstrated a much greater cellulolytic activity by B. theobromae than D. natalensis but alternatively the tensile strength tests undertaken showed that D. natalensis was apparently a more active deteriogen than B. theobromae.

Siu and Reese (1953) considered that B. theobromae was not a cellulolytic microfungus, thought Umezuricke (1969) disagreed with this showing that the organism produced soft-rot cavities in Antiaris africana.

Similarly, Siu and Reese (1953) considered Paecilomyces varioti, Aureobasidium pullulans, and Aspergillus niger, to be non-cellulolytic, Gliocladium roseum and Cladosporium herbarum to be cellulolytic, and Trichoderma viride to be highly cellulolytic. Alternatively, however, P. varioti was found to be considerably more cellulolytic than T. viride by Liese and Ammer (1964), and A. niger was seen to be considerably more active as a deteriogen than T. viride in the deterioration of fibre board (Merrill 1965), whilst Merrill, French and Hosfield (1965) demonstrated that Cladosporium sp. did not produce degradation of fibre board, all observations assessed by weight loss methods. The results presented in Table 7.1showthat C. herbarum was only slighly cellulolytic as was A. niger and P. varioti, and that these fungi were considerably less cellulolytic than T. viride, thus corroborating the work of Siu and Reese (1953). However, the differences in degradation patterns between these species when grown on cellulose and lignified cellulose would seem to necessitate further investigation.

Crossley (1956) observed that Alternaria tenuis and L. lundbergii both caused a uniform grey stain in wood, but that A. tenuis significantly reduced toughness whereas L. lundbergii did not. Both the cellulose agar and tensile strength loss experiments corroborated this evidence, showing that A. tenuis produced moderate reaction levels in agar columns and high tensile strength loss, whereas L. lundgergii produced slight reaction levels in agar columns and only twenty nine per cent strength loss.

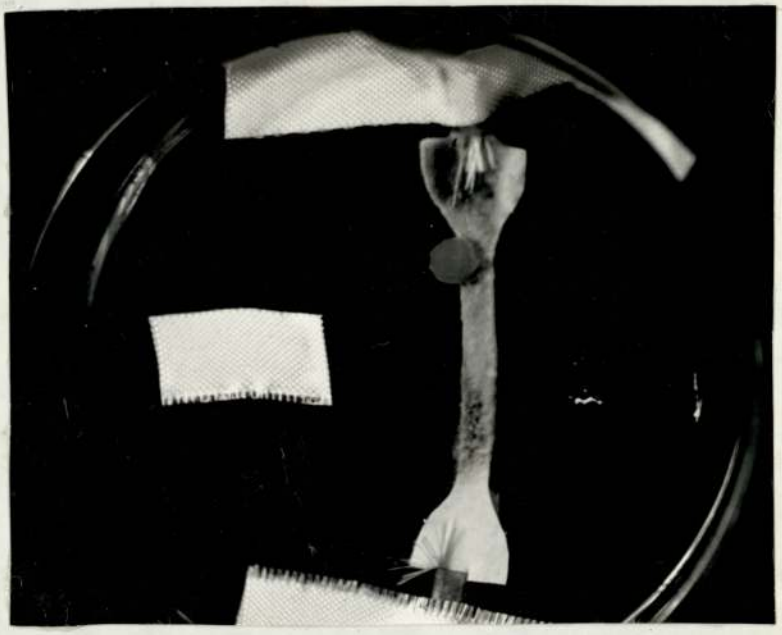
Merrill and French (1964) suggested that Alternaria spp. might be capable of wood-rotting activities.

Phialophora fastigiata has been consistently associated with both soft-rotting and wood-rotting activities (Krapvina 1960; Boutelje and Kiessling 1964; Karkanis 1966; Byler and True 1966; Shigo 1965; and Butcher 1968). This species was not seen to produce clearing of cellulose agar but produced very high tensile strength losses in the paper strips. Ceratocystis coerulescens, Ceratocystis pilifera, L. lundbergii, A. pullulans, and Rhinocladiella atrovirens were considered by Levy (1967) to passively penetrate wood tissue, though Findlay (1970) showed that C. pilifera could produce soft-rot cavities in wood-cell walls. Significantly, only one of these species, C. pilifera, was seen to produce high tensile strength losses in the fibrous cellulose strips.

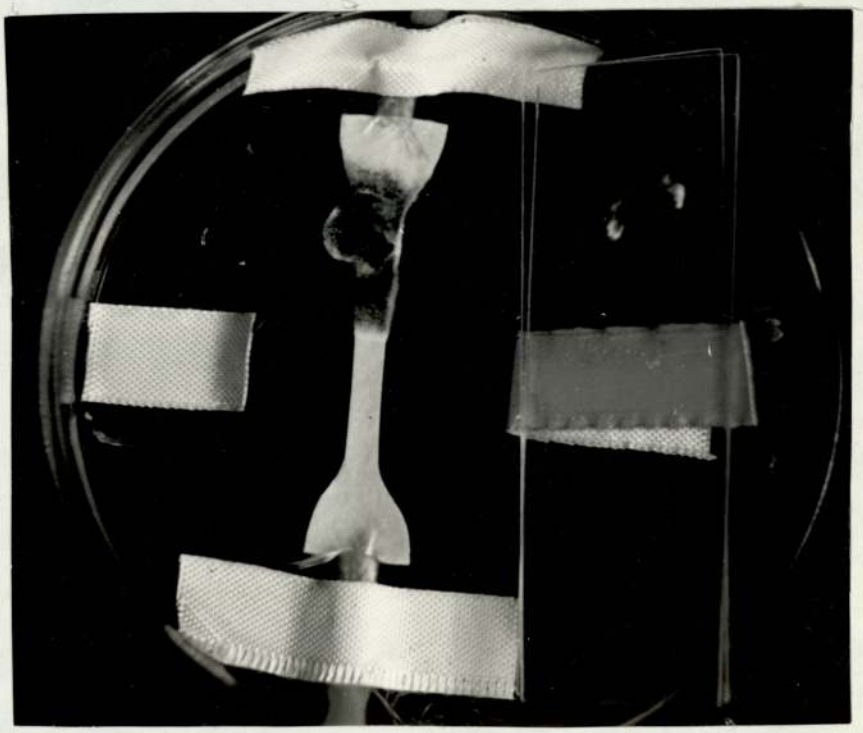
A significant correlation can therefore be seen between both soft-rotting and toughness reduction in wood, and the loss in tensile strength of paper, produced by the same test species. Similarly, while lack of clearing in cellulose agar columns would seem to indicate a lack of cellulase production, those fungi which produced high reaction levels in cellulose agar, not necessarily clearing, were similarly shown to produce high strength losses.

The breaking pattern of strips was also seen to be interesting. Control strips were seen to defibrate

PLATE III

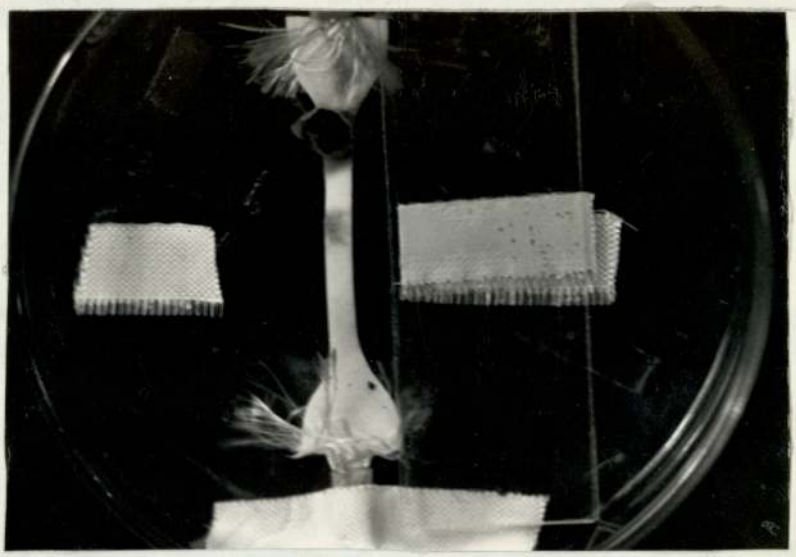


a. A perfused strength test strip exhibiting extensive colonisation by the staining fungus Botriodiplodia theobromae



b. A perfused strength test strip showing moderate colonisation by Diplodia natalensis

PLATE III (contd.)



c. A perfused strength test strip exhibiting limited colonisation by the slow growing species Phialophora melinii

d. Paper strips tested for tensile strength showing location and nature of break areas. From left, a control strip, and strips showing colonisation by fungal species with respectively, rapid, slow, and moderate growth rates.

at the break area when under tensile stress, while degraded strips were frequently found to break cleanly. Similarly, strips inoculated with species which showed slow growth rates were seen to break in the vicinity of the inoculum, while strips inoculated with rapidly growing species were seen to break at random along their length, provided that colonization of those species had occurred over the entire length of the strip.

As break areas of inoculated strips were seen to occur within colonized areas, and as the tensile strength test detected the weakest point of the strips, some slow-growing species were seen to be as cellulolytic as faster growing species. B. theobromae was considered to be cellulolytic because of the clearing produced by it in cellulose agar columns, whereas Philophora melinii did not produce clearing. Similarly, B. theobromae demonstrated extremely rapid growth rates, while P. melinii showed slow growth rates. However, though B. theobromae colonized the entire length of the strips, while P. melinii colonized only two mm on either side of the inoculum, the strength loss produced by P. melinii was 83 per cent whereas the strength loss produced by B. theobromae was 66 per cent, thus indicating that P. melinii was highly cellulolytic in relation to growth.

The starch hydrolysis studies showed that the hydrolysis areas produced by the faster-growing species were generally considerably greater than those produced by the slower growing species. In relation to growth rate, however, many of the slower-growing species were considered to be

highly amylolytic. Similarly, this can be seen to apply to cellulolytic activity, where the intensity of cellulolytic activity of a slow-growing species e.g. P. melinii, may be equal to or greater than the intensity of cellulolytic activity of a faster-growing species, e.g. B. theobromae, though the extent of degradation produced by these two species may be entirely different.

Some interesting patterns are therefore evident between strength loss figures and growth rate figures for the individual test species. Of the nineteen species which showed rapid or moderate growth rates on cellulose agar plates, all but four produced high or moderate strength losses in fibrous cellulose. These latter species included two heavily-sporing species, A. niger and P. varioti, a phycomycete, Pythium aphanidermatum, and Discula brunneo-tingens, the only rapidly-growing species which showed slight amylolytic activity. These four species similarly produced low reaction levels both in cellulose agar columns and on pectate gels.

Of the fourteen species demonstrating slow growth rates on cellulose agar plates, six produced high strength losses, five produced moderate strength losses and three species produced slight strength losses in fibrous cellulose strips. Of the six species producing high strength losses, Cephalosporium acremonium, and Graphium sp. produced clearing in cellulose agar columns, while the remaining four species, P. melinii, P. fastigiata, Ceratocystis picea, and Myxotrichum deflexum produced either shrinkage or hyphal penetration. Only one of these

species, C. acremonium, produced high reaction levels in cellulose agar columns. Of the five species producing moderate strength losses, B. chlamydosporis produced clearing in cellulose agar columns, while the remaining four species, Phialophora americana, A. pullulans, R. atrovirens and C. ulmi either produced hyphal penetration or shrinkage and all five species showed moderate reaction levels in cellulose agar columns. The remaining three species, C. coerulescens, C. herbarum, and P. phycomyces all produced slight strength losses and slight reaction levels, in the form of shrinkage, in cellulose agar columns.

It is therefore evident that, whereas all of the slow-growing species were only slightly amylolytic relative to the rapidly-growing species, eleven species, all of which were slow-growing, produced high or moderate strength losses in fibrous cellulose strips. Eight of these were not seen to produce clearing in cellulose agar, and presumably they would not be seen to be cellulolytic using such techniques. However, the technique used determined cellulolytic activity of the test species relative to growth, showing that many of the slow-growing species were intensely cellulolytic over their limited areas of colonization, and further, that most of the blue-staining and mould fungi tested were capable of degrading fibrous cellulose. The significance of this ability in relation to wood degradation resulting in enhanced permeability is discussed in Chapter 10.

CHAPTER 8

INTERRELATIONSHIP OF CELLULOLYTIC AND PECTINOLYTIC ACTIVITY

8. Interrelationship of Cellulolytic and Pectinolytic Activity

8.1. Introduction

The studies so far have revealed that all of the species tested were amylolytic, most were capable of cellulose degradation, while twelve species were capable of pectate gel liquifaction.

The amylolytic activity assays showed that the test species produced amylase, and the cellulose agar column and tensile strength loss tests indicated a cellulase production. The pectate gel results, however, were difficult to interpret. Liquifaction of gels was produced by eleven species, whilst the remaining twenty two species produced surface degradation of the gel. Gel liquifaction can be produced, either by enzymatic hydrolysis, or by acid hydrolysis, and the gel liquifaction studies did not indicate whether the test species produced oxalic or citric acid which would result in gel liquifaction (Soulahti, 1961) or whether gel degradation was due to liberation of pectinase by the test species.

Further, since none of the species which produced slight reaction levels in cellulose agar columns produced measurable liquifaction of pectate gels, and all of the species which produced gel liquifaction similarly produced good or moderate reaction levels in cellulose agar columns, along with high strength loss, it was considered that a relationship could possibly exist between cellulolytic and pectinolytic activity. Similarly, while pectate and cellulose were seen to be degraded in separate experiments,

it was not evident from the results whether the species were adaptive, or whether they simultaneously produced both pectinase and cellulase.

Little information was evident from the literature on pectinolytic activity of wood-inhabiting microfungi, although King (1966) showed that Coniophora cerebella produced polygalacturonase in liquid culture as did Bhat, Jayasankar, Agate and Balimoria (1965).

Deuel and Stutz (1958) considered that pectic enzymes could be divided into two groups, pectinesterases and polygalacturonases, the former removing the methyl-ester groups from pectic substances, while the latter hydrolysed the - 1, 4'- glycosidic linkages. They also considered that the activity of pectinase were pH dependent, polygalacturonases of bacterial or phycomycete origin having optimal activity near pH 7, while poly-galacturonases of ascomycete origin have optimal activity near pH 4. This was corroborated by Chatterji and Basu (1960) for some Penicillium species.

Wood (1960) observed that polygalacturonases could be divided into two types, an endo-type by which glycosidic linkages were broken at random, and the exo-type, by which end linkages were preferentially attacked. He also observed that pectinases could sometimes be produced when pectic substances were absent from the growth medium. Bateman (1966) considered that production of individual pectic enzymes was dependent on the substrate pH, and on the form of the pectic substrate, while Bateman and Miller (1966) classified

pectic enzymes on three criteria:

1) the mechanism by which the 1-4' glycasidic linkage was split i.e. transeliminations or hydrolytic clearage;
2) enzyme preference for substrate i.e. pectin or pectic acid;
3) the position in the pectic chain at which clearage occurred, i.e. random or terminal point of attack.

Codner (1971) concurred with this outline of the classification of pectic enzymes, as did Byrde and Fielding (1968).

As it seemed that some relationship might exist between cellulolytic and pectinolytic activity, it was desirable that this aspect should be investigated. However, such an investigation, involving all of the test organisms, could be of lengthy duration if production of all the suggested pectic enzymes was assessed. In designing an experiment to elucidate this relationship, the following facts were therefore taken into account.

1) The enzyme polygalacturonase in its transeliminative and endo and exo forms is responsible for pectin degradation, i.e. clearage of the pectin chain (Deuel and Stutz 1958; Wood 1960; Bateman and Miller 1966; Codner 1971).
2) Pectic enzyme production is related both to the pH of the growth substrate, and to

the type of pectic content of that substrate (Bateman 1966: Bateman and Miller 1966).

3) The pH of wood in the green state ranges from pH 5.0 to pH 5.6 (Hatton 1970; Highly and Lutz 1970).

4) Optimal polygalacturonase activity of cell-free culture filtrate would seem to be in the region of pH 3.5 - pH 5.0 (Brown 1965; Sherwood 1966; Ayers, Paparizas, and Dunn 1966; Hasegaura and Nagel 1967; Curren 1969; and Cole 1970).

5) Optimal cellulase activity of cell-free culture filtrates would seem to be in the region of pH 4 to pH 5 (King 1966; Bemillar, Teglmeier and Pappelis 1968; Garegg and Han 1969; Bateman 1969; and Wood 1971).

It can therefore be seen that the assessment of pectinolytic activity of blue-staining fungi must take both the pH of optimal activity of the pectic enzyme and the pH of its natural growth medium into account. Similarly, it is not unreasonable to suggest that if fungal species producing both cellulase and pectinase are growing in the same medium, e.g. wood, the pectinase system, to be efficient, must be operable in the

same conditions as the cellulase system and vice versa, i.e. governed by the pH of the wood substrate.

The major emphasis of this work was on the activities of blue-staining fungi, and consequently the production of pectin degrading enzymes rather than the types of pectic enzyme produced by them was used as a criterion of pectinolytic activity. However, it is evident that in screening a group of blue-staining fungi for pectinolytic activity with reference to their effective role in enhancing wood-permeability, some criteria must be used.

It was therefore decided that an assessment of both polygalacturonase and cellulase production by the test species would be undertaken, to establish the production of the former by blue-staining fungi, and to determine relationships, if any, between that production and cellulase production under pH conditions which approximated to those of green spruce.

8.2. Materials and Methods

8.2.1. Preparation of Cell-free Extracts.

Three liquid media were used to support the growth of the test species. These media contained respectively:

(i) Cellulose as sole carbon source;

(ii) Both cellulose and sodium polypectate as carbon sources;

(iii) Sodium polypectate as sole carbon source.

The exact formulation of these media is outlined in

Appendix IV.

Six ml aliquots of each medium were transferred to 100-ml erlynmeyer flasks, which were then plugged with cotton wool. After autoclaving (15 lbs/15 minutes), five flasks of each medium were inoculated with each test species, inocula being removed from actively-growing cultures maintained on Potato Dextrose agar with a 6-mm corkborer. The flasks were incubated at 25°C.

One flask of each medium inoculated with each species was removed on the fifth day of incubation, and two flasks were similarly removed on the sixth and seventh day of incubation. The flask contents were centrifuged in a Gallenkamp Jumer Centrifuge (setting 5) for ten minutes, and the cell-free extract decanted in such fashion that 30 mls of cell-free liquid was available for each species on each medium. This fluid was then freeze-dried and stored in a deep-freeze until tested.

8.2.2. Test Method.

Using a viscosimetric technique, the relative cellulolytic and pectinolytic activities of the test species was determined. Two solutions were used for viscosimetric determinations; these were a 0.1% solution of carboxymethylcellulose (Hercules 7HF) in acetate buffer at pH 4.4, and 0.2% sodium polypectate solution (Sigma Grade 11 No. P.1879) in acetate buffer at pH 4.0.

Each cell-free extract was made up to a volume of 2.5 mls in distilled water. From this solution 1.25 mls

was removed and made up to 2.5 mls with acetate buffer at pH 4.4. This solution was used for the determination of cellulolytic activity.

The remaining 1.25 mls of cell-free extract was made up to 2.5 mls with an Ammonium oxalate solution of 4 ppm. concentration. This procedure precipitated the calcium traces in the cell-free extract, which remained from the original culture medium. These were then removed by filtration. The traces, which were present particularly in extracts of slow-growing species, were seen to increase the viscosity of the sodium polypectate solution in the viscometer, thus making pectinolytic activity assessment particularly subject to error in initial experiments. This remaining aliquot was used for the determination of pectinolytic activity.

The viscometer used was of the reverse flow type (Gallenkamp Cat. No.VS340Size B) and tests were carried out at 40°C $\pm$ 0.5°C. Tests were carried out in duplicate for each organism for each medium generally at two dilutions. These dilutions were:

1) 9.75 mls of substrate/0.25 mls of enzyme soln.;

2) 9.5 mls of substrate/0.5 mls of enzyme soln.

Some enzyme solutions which were of particularly low activity were tested at the following dilution:

9.0 mls of substrate/1.0 mls of enzyme soln.

Results were recorded as the reciprocal of the time in minutes for 50 per cent loss in viscosity of the reaction mixture, expressed as relative viscosity units

(Bateman 1966; 1969).

Results are therefore presented for:

1) relative cellulolytic activity of the individual test species when grown on (i) cellulose, (ii) sodium polypectate, and (iii) cellulose and sodium polypectate, as carbon sources;

2) relative pectinolytic activity of the individual test species when grown on (i) cellulose, (ii) sodium polypectate, and (iii) cellulose and sodium polypectate, as carbon sources.

8.3. Results

The celluloytic and pectinolytic activities of thirty three blue-staining fungal species, expressed in relative viscosity units, are presented in Tables 8.1 and 8.2.

Of the thirty three species tested, only six species produced measurable pectinase when grown on the medium containing sodium pectate as sole carbon source. These species were Botriodiplodia theobromae, Scytalidium lignicola, Botrytis cinerea, Helminthosporium erythrospilum, Ceratocystis pilifera, and Graphium sp. Four of these species, B. theobromae, S. lignicola, Botrytis cinerea, and Helminthosporium erythrospilum, similarly produced measurable pectinase when grown on the medium containing cellulose and sodium polypectate as carbon sources, as also did Gliocladium roseum, Trichoderma viride, Alternaria tenuis,

Aureobasidium pullulans, and Discula brunneo-tingens. Three of these species, T. viride, A. tenuis, and D. brunneo-tingens, similarly produced measurable pectinase when grown on the medium containing cellulose as sole carbon source.

Twenty species,when grown on the medium containing sodium polypectate as sole carbon source, produced distinct but not measurable viscosity losses in the sodium polypectate solution, fifteen species produced distinct but not measurable viscosity losses in the polypectate solution when grown on the medium containing both cellulose and pectin as carbon sources, whilst twelve species produced distinct but not measurable viscosity losses in the polypectate solution when grown on the cellulose medium.

Similarly, seven pieces when grown on the polypectate medium, nine species when grown on the cellulose-sodium polypectate medium, and eighteen species when grown on the cellulose medium, all failed to produce any viscosity losses in the sodium polypectate solution. These species are indicated in Table 8.1.

Of the thirty three species tested, twenty five species produced measurable viscosity losses in the carboxymethyl-cellulose solution when grown on the cellulose medium. One species, Myxotrichum deflexium, failed to produce a viscosity reduction, and seven species, namely Phialophora melinii, Phialophora fastigiata, Cladosporium herbarum, Sclerophoma pityophila, Leptographium lundbergii,

Phialocephala phycomyces and Paecilomyces varioti, all produced distinct but not measurable viscosity losses in the carboxymethylcellulose solution, having been grown in the cellulose medium.

One species, Phialophora americana, which produced a measurable viscosity reduction when grown on the cellulose medium, failed to reduce the viscosity of the carboxymethylcellulose solution when grown on the cellulose sodium polypectate medium. Myxotrichum deflexum, which failed to reduce the viscosity of carboxymethylcellulose solution when grown on the cellulose medium, produced a distinct but not measurable viscosity reduction when grown on the cellulose-sodium polypectate medium, whilst five species which produced measurable viscosity reductions when grown on the cellulose medium did not produce measurable viscosity reductions when grown on the cellulose pectin medium. These species were G. roseum, B. cinerea, A. pullulans, Pythium aphanidermatum and Aspergillus niger.

Four species, P. melinii, P. americana, L. lundbergii, and P. phycomyces, all failed to reduce the viscosity of the carboxymethylcellulose solution when grown on the sodium polypectate medium. The remaining twenty nine species produced viscosity reductions in the carboxymethylcellulose solution, and ten of the latter produced measurable viscosity reductions. These results are indicated in Table 8.2.

TABLE 8.1

RELATIVE VISCOSITY UNITS OF PECTINASE PRODUCED BY THE TEST ORGANISMS WHEN GROWN ON CELLULOSE CELLULOSE-SODIUM POLYPECTATE AND SODIUM POLYPECTATE MEDIA

Test Organisms	Pectinase		
	C	CP	P
Botriodiplodia theobromae	x	0.1	0.3
Botrytis cinerea	x	0.2	0.5
Trichoderma viride	0.3	11.1	x
Ceratocystis pilifera	0	x	0.3
Scytalidium lignicola	0	0.6	0.4
Leptographium lundbergii	x	x	x
Diplodia natalensis	x	x	x
Diplodia pinea	0	x	0
Cytospora leucostoma	0	x	x
Trichocladium canadense	0	0	x
Diplodia sapinea	x	x	x
Alternaria tenuis	0.9	0.6	0
Helminthosporium erythrospilum	x	1.3	0.8
Pythium aphanidermatum	0	0	x
Aspergillus niger	0	0	x
Paecilomyces variotii	x	0	0
Ceratocystis picea	0	0	0
Aureobasidium pullulans	0	0.1	0
Discula brunneo-tingens	0.8	0.4	x

/over

Table 8.1 - continued

Test Organisms	Pectinase		
	C	CP	P
Cephalosporium acremonium	x	x	x
Sclerophoma pithyophila	0	x	x
Gliocladium roseum	0	0.1	x
Phialophora melinii	0	x	x
Phialophora fastigiata	0	0	x
Graphium sp.	x	0	0.1
Bisporomyces chlamydosporis	0	x	x
Myxotrichum deflexum	0	x	0
Phialophora americana	0	x	0
Rhinocladiella atrovirens	x	0	x
Ceratocystis ulmi	0	0	x
Phialocephala phycomyces	0	x	0
Cladosporium herbarum	x	0	x
Ceratocystis coerulescens	0	x	x

x Indicates a distinct but not measurable loss in viscosity

C Indicates cellulose medium

CP Indicates cellulose sodium polypectate medium

P Indicates sodium polypectate medium

TABLE 8.2

RELATIVE VISCOSITY UNITS OF CELLULASE PRODUCED BY THE TEST ORGANISMS WHEN GROWN ON CELLULOSE CELLULOSE-SODIUM POLYPECTATE AND SODIUM POLYPECTATE MEDIA

Test Organisms	Cellulase		
	C	CP	P
Botriodiplodia theobromae	26.7	44.4	3.7
Botrytis cinerea	0.9	x	x
Trichoderma viride	8.0	25.0	x
Ceratocystis pilifera	2.0	40.0	3.7
Scytalidium lignicola	1.3	0.7	x
Leptographium lundbergii	x	0	0
Diplodia natalensis	39.1	40.0	3.8
Diplodia pinea	0.5	0.6	0.2
Cytospora leucostoma	36.4	13.6	x
Trichocladium canadense	0.4	0.1	x
Diplodia sapinea	0.7	2.4	x
Alternaria tenuis	8.5	5.9	x
Helminthosporium erythrospilum	111.0	55.6	3.8
Pythium aphanidermatum	0.1	x	x
Aspergillus niger	0.1	x	x
Paecilomyces variotii	x	x	x
Ceratocystis picea	2.0	0.3	0.2
Aureobasidium pullulans	0.9	x	x
Discula brunneo-tingens	5.2	4.7	0.7

Table 8.2 - continued

Test Organisms	Cellulase		
	C	CP	P
Cephalosporium acremonium	0.7	0.2	x
Sclerophoma pithyophila	x	x	x
Gliocladium roseum	5.7	x	x
Phialophora melinii	x	x	0
Phialophora fastigiata	x	x	x
Graphium sp.	1.4	2.5	0.6
Bisporomyces chlamydosporis	0.6	1.1	1.1
Myxotrichum deflexum	0	x	x
Phialophora americana	0.6	0	0
Rhinocladiella atrovirens	1.1	0.3	x
Ceratocystis ulmi	0.8	0.3	x
Phialocephala phycomyces	x	x	0
Cladosporium herbarum	x	x	x
Ceratocystis coerulescens	9.5	16.0	1.3

x Indicates a distinct but not measurable loss in viscosity

C Indicates cellulose medium

CP Indicates cellulose sodium polypectate medium

P Indicates sodium polypectate medium

8.4. Discussion

It can be seen from the results that, of thirty three species tested, a total of eleven blue-staining fungal species produced measurable viscosity losses in sodium polypectate solution, and that a total of twenty five species produced measurable viscosity losses in the carboxymethylcellulose solution.

8.4.1. Pectinolytic Activity.

Six species showed measurable pectinolytic activity when grown on the sodium polypectate medium, ten species showed measurable pectinolytic activity when grown on the cellulose-sodium polypectate medium, while three species were seen to be pectinolytic after growth on cellulose media. Of the species which produced pectinase, *B. theobromae*, *B. cinerea*, *C. pilifera*, and *Graphium* sp. showed greatest pectinase production when grown on the sodium polypectate medium. Two species, *S. lignicola* and *H. erythrospilum*, which produced measurable pectinase when grown on the sodium polypectate medium, showed greatest pectinase production when grown on the medium containing both cellulose and polypectate as carbon sources.

Greatest pectinase production was noted for *T. viride* when it was grown on the cellulose-polypectate medium, but this species similarly produced pectinase when grown on the medium containing cellulose as sole carbon source. The remaining pectinolytic species, *A. tenuis*, and *D. brunneo-tingens*, while producing measurable pectinase

when grown on the cellulose-polypectate medium, were seen to demonstrate maximal pectinase production when grown on the medium containing cellulose as sole carbon source.

It was also seen that two species, C. pilifera and Graphium sp., only produced pectinase when grown on the polypectate medium, and similarly, a further two species only produced pectinase when grown on the cellulose-sodium polypectate medium. These species were G. roseum and A. pullulans.

It can therefore be seen that six species, B. theobromae, S. lignicola, B. cinerea, H. erythrospilum, C. pilifera, and Graphium sp., all only produced measurable pectinase when they were growing on a medium which included sodium polypectate as carbon source. Alternatively, five species, G. roseum, T. viride, A. tenuis, A. pullulans and D. brunneo-tingens, were seen to produce pectinase only when cellulose was included as a carbon source in the growth medium.

8.4.2. Cellulolytic Activity.

The results showed that twenty five species produced measurable cellulase when grown on the medium containing cellulose as sole carbon source. Six of these, however, failed to produce measurable cellulase when grown on the medium containing both cellulose and sodium polypectate. Three of these species, G. roseum, B. cinerea, and A. pullulans instead produced measurable viscosity losses in the polypectate solution, P. americana produced a

distinct but not measurable viscosity reduction in the polypectate solution, while the remaining two species, P. aphanidermatum and A. niger, failed to reduce the viscosity of the latter. Ten of the remaining nineteen species, when grown on cellulose-sodium polypectate medium, showed a decreased production of cellulase, whilst nine species showed an increased cellulase production. These species are indicated in Table 8.3.

When the test species were grown on the polypectate medium, ten species were found to produce cellulase. Two of these species were seen to produce greater amounts of cellulase when grown on that medium than when grown on the medium containing cellulose as sole carbon source. One of these species, C. pilifera, however, showed maximal cellulase production when grown on cellulose-sodium polypectate medium, while the other species, Bisporomyces chlamydosporis, showed equal cellulase production when grown on both cellulose and cellulose-sodium polypectate media. Five of the remaining eight species, however, produced maximal cellulase when grown on the cellulase polypectate medium, whilst the remaining three showed maximal cellulase production when grown on the cellulose medium. These species were respectively, B. theobromae, Graphium sp., Diplodia pinea, Diplodia natalensis, Ceratocystis coerulescens, and H. erythrospilum, Ceratocystis picea and D. brunneo-tingens.

The viscosimetric determinations undertaken have corroborated work by other authors. Domsch and Gams (1968)

TABLE 8.3

A list of species indicating (a) those species which showed a decreased cellulase production when grown on cellulose-sodium polypectate medium, and (b) those species which showed an increased cellulase production when grown on cellulose-sodium polypectate medium.

(a)	(b)
Cytospora leucostoma	*Trichoderma viride*
Cephalosporium acremonium	*Diplodia sapinea*
Scytalidium lignicola	*Bisporomyces chlamydosporis*
Rhinocladiella atrovirens	*Ceratocystis pilifera*
Alternaria tenuis	*Graphium* sp.
Helminthosporium erythrospilum	*Diplodia pinea*
Trichocladium canadense	*Diplodia natalensis*
Ceratocystis picea	*Ceratocystis coerulescens*
Ceratocystis ulmi	*Botriodiplodia theobromae*
Discula brunneo-tingens	

showed that a considerable number of soil fungi were pectinolytic, and these included B. cinerea, G. roseum, Cladosporium herborum, Pythium sp., Cephalosporum sp., Trichcladium canadense and T. viride. Brown (1965) and Van den Berg and Young (1969) all considered B. cinerea to be pectinolytic, although Brown (1965) suggested that Pythium spp. would be found to be highly pectinolytic in practice but not in culture. Similarly, Rosch, Liese and Berndt (1969) showed that A. tenuis and A. pullulans were both cellulolytic and pectinolytic. Umezurike (1969) demonstrated the production of carboxymethylcellulase by B. theobromae, and Pothak and Prasid (1969) showed that D. natalensis produced cellulase.

The results presented have shown that B. theobromae, D. natalensis, A. pullulans and A. tenuis all produced cellulase and similarly showed that B. cinerea, G. roseum and T. viride all produced pectinase. However, C. herborum, T. canadense, Cephalosporium sp. and P. aphanidermatum were not seen to produce measurable pectinase although all species produced distinct but not measurable viscosity losses in the sodium polypectate solution. It was therefore considered that either the strains tested were only slightly pectinolytic or the pH at which the tests were carried out was unsuitable. Slezarik and Roxana (1967), however, considered that optimal activity of endopolygalacturonase occurred between pH 3.4 and pH 4.6, and Turner and Bateman (1968) showed that P. aphanidermatum did not produce a

pectinolytic activity at pH 4.5 but only at optima around pH 8.0. It is therefore possible that, while many of the species were not seen to produce measurable pectinase which was active at the pH regions for stored wood, many blue-staining fungi, which are also soil fungi (Siu and Reece 1953, Merrill and French 1966), may in fact produce pectinases which are active under more alkaline conditions.

It was evident from the results that all of the species examined showed maximal cellulase production when cellulose was included in the growth medium as a carbon source, and similarly all but two of the pectinolytic species showed maximal pectinase production when sodium polypectate was included in the growth medium as a carbon source. One of the two exceptions, A. tenuis, did not produce pectinase when grown on the polypectate medium, while both species, A. tenuis and D. brunneo-tingens, showed maximal pectinase production when grown on the cellulose medium.

This latter factor may be explained by the fact that the technique used, measured only excess enzyme production, i.e. since the fungal colony and the growth substrate were centrifuged from the liquid culture, enzymes in combination with the growth substrate would not be detected in the cell-free extract. Rosch, Liese and Berndt (1969) considered that maximal cellulase production by A. tenuis occurred when that species was grown on glucose media, and similarly, the presented results showed that A. tenuis produced maximal pectinase when grown on the cellulose medium. This phenomenon may

be explained if it is considered that the activity of only "excess" enzymes were measured. If a species producing both pectinase and cellulase is grown on a cellulose medium, it can be assumed that the pectinase produced will not be found to be in combination with the cellulase, and consequently a certain pectinolytic activity may be detected in cell-free extracts.

If pectin is included with cellulose in the growth medium, it is likely that some of the pectinase produced will combine with the pectin in that medium. Thus, depending on duration of culture and the activity of the species, varying quantities of pectinase may be detected. It is therefore suggested that A. tenuis and D. brunneo-tingens, both apparently producing maximal pectinase when grown on cellulose, in fact simultaneously produce both pectinase and cellulase, and that the apparent drop in relative activity when the species were grown on the cellulose-sodium polypectate medium was due to the combination of some of the pectinase produced with the growth substrate.

The production of cellulase and pectinase by the test species seemed to be considerably affected by the presence of both cellulose and sodium polypectate in the growth medium. Wood (1960) considered that the presence of pectin in a growth medium could considerably inhibit the production of pectinase. However, in the experiment undertaken, the presence of pectate apparently inhibited the cellulase production of sixteen species and enhanced the cellulase production of nine species. Five of the

seventeen species with inhibited cellulolytic activity were, however, seen to demonstrate an enhanced pectinolytic activity, and four of the nine species showing an enhanced cellulolytic activity similarly showed an enhanced pectinolytic activity. The only conclusion which could be drawn from this, was that the presence of both carbon sources in the same medium either inhibited or enhanced the enzyme production of the individual test species. It was considered that this aspect might merit further more detailed investigation.

The results also showed that only seven slow-growing species produced measurable cellulose, when in fact fourteen slow-growing species were seen to produce significant strength losses in fibrous cellulose. It is suggested that slow-growing species, while cellulolytic relative to growth rate, did not appear to be cellulolytic relative to the faster-growing test species. This could possibly be due to a slow rate of enzyme release compatible with their growth rate. This hypothesis is borne out by the tensile strength studies. Similarly, it was found that none of the slow-growing species were measurably pectinolytic, although some did produce distinct but not measurable viscosity losses in the sodium polypectate solution.

Wood (1971) considered that enzymes acting on cellulose in a random manner produced greater decreased viscosity in a polymer solution than enzymes acting on the end of a chain. P. melinii and P. fastigiata were two slow-growing species which produced distinct but not measurable viscosity reductions in the carboxymethylcellulose solution. The graphs of these slight viscosity losses were linear,

however, indicating an exo-cellulase reaction. These species similarly produced significant tensile strength losses in fibrous cellulose, thus indicating their active cellulolytic nature. It is suggested that this exo-cellulase activity would merit investigation, particularly with reference to its cavity formation in wood cells.

It would appear that the cellulase system of the test species was the dominant enzyme system, since while many of the celluloytic species did not produce pectinase under the test conditions, all of the pecintolytic species were highly celluloytic, rapidly growing, highly amylolytic and produced significant strength losses in fibrous cellulose. Similarly, it was noted that while none of the cellulolytic species required the presence of polypectate before measurable cellulase production was detected, a significant number of the pectinolytic species (45 per cent) only produced pectinase in the presence of cellulose.

Of the species producing both cellulase and pectinase, all but three were indicated as having an adaptive nature, i.e. were capable of breaking down both cellulose and pectin, but did not simultaneously produce both cellulase and pectinase. It is suggested that, for those three species, T. viride, A. tenuis and D. brunneo-tingens, the pectinase system was therefore constitutive. The latter organism along with nine other species, similarly produced measurable viscosity reductions in the carboxymethylcellulose solution when grown on the polypectate medium. This would suggest that, for those species, the cellulase

system was constitutive.

It is therefore possible to conclude that many of the blue-staining fungal species tested were capable of cellulase (carboxymethylcellulase) production under the test conditions, and that many of the more highly cellulolytic species were similarly capable of pectinase (polygalacturonase) production. It was also indicated that many species which produced both cellulase and pectinase did so in an adaptive fashion, and that for some species either the cellulase or pectinase system was constitutive.

CHAPTER 9

ECOLOGICAL SURVEY

9. ECOLOGICAL SURVEY

9.1. Introduction

As pointed out in Chapter 2, due to the limited duration of the research program, it was found necessary to undertake degradation studies with species selected with reference to the literature. However, to verify the selection procedure, and to qualify the results of the degradation studies, a limited ecological survey was also undertaken.

In recent years it has become evident that an increased interest has been taken in the activities of those microfungi which colonized wood. Duncan and Eslyn (1966) outlined a list of 67 microfungal strains associated with soft-rotting of wood, and this list included some species associated with wood staining. Butcher and Howard (1968) indicated 42 microfungal species associated with "moulded blue stained and soft rotted" wood chips, whilst Kaarik (1968) isolated 49 microfungal species from freshly-felled spruce which had been buried in soil for eighteen months.

It was suggested in the introduction that little differentiation was sometimes evident between the terms used in describing fungi associated with staining of wood. Shigo (1965) considered that six microfungal species including Phialophora melinii, Trichocladium canadense, Cytospora decipiens, and Hypoxylon spp. to be instrumental in both the staining and degradation of the living wood of American beech (Fagus grandifolia).

Butcher (1968) considered that Ceratocystis picea was the most important fungal species involved in the staining of green Red beech (Nathogafus fusca), whilst Phialophora fastigiata was the most important species involved in the staining of boron treated Red beech but that Cladosporium herbarum and Alternaria tenuis were of secondary importance. Butcher (1968) working with Pinus radiata posts considered that Diplodia pinea was the species most frequently isolated from stained areas, but also isolated Alternaria tenuis, C. pinea, Leptographium sp., C. herbarum and Graphium sp. from stained areas.

Many of these species were included in the lists outlined earlier (Duncan and Eslyn 1966; Butcher and Howard 1968; Kaarik 1968) as mould fungi, or soft-rot fungi (Merrill 1965), or as soil fungi (Siu and Reese 1953; Domsch and Gams 1968). Dowding (1970) considered that the blue-staining fungi isolated by him be divided into two groups, those which only caused surface colonization of green wood, e.g. Aureobasidium sp., Alternaria spp. and Cladosporium spp., and those which caused deep discoloration of wood e.g. Ceratocystis spp., Graphium spp. and Leptographium spp. However, he also included the hyaline microfungi Trichoderma sp., Fusarium spp., and Penicillium spp. in his isolations. Butcher (1968) considered that these latter species were "mould fungi", and that Trichoderma viride was almost universally present in round timber. Kaarik (1968) however, did not differentiate between deep and surface-colonizing blue-staining fungi, nor did he differentiate between hyaline or dematiaceous species but considered them only

as Ascomycetes or Fungi Imperfecti.

It therefore appears that the point of demarcation between mould, blue-staining, dematiaceous and hyaline fungal species in microfungal colonization of green wood is difficult to assess, particularly when the degradation capability of the individual fungal species is taken into account. It was therefore considered that apart from verifying the selection procedure, the ecological survey might provide information on microfungal colonization of green spruce, irrespective of staining but with particular reference to fungal penetration into wood.

9.2. Materials and Methods

Three young spruce trees (about 20 years old) were felled at different forest sites and were converted to bolts of 80 cm in length. The bark on these was slashed and the bolts were then close piled and stored on the forest floor for three months. These bolts were thus subject to airborne spore infection and beetle infestation for the months June, July and August, 1970. At the end of this storage period discs, of nine inches deep, were removed from the upper and lower bolt of each tree.

Discs were sampled in such fashion that two radial sections were removed from each disc generally in the form of a diameter. Sawdust samples were removed aseptically from every fifth growth ring measured from the disc periphery to the pith using a hacksaw technique similar to that of Grant and Savory (1965). Similarly, sawdust samples were removed in a cross-sectional cut

through each disc, and each disc was also examined in the longitudinal plane for discolored areas, and wood samples removed from discolored areas thus discovered.

Microbiological examination of two butt discs and two upper discs was undertaken. The media used for isolation were the Starch, Cellulose and Pectin agars used in the growth studies the composition of which is outlined in Appendix 1. Three petri dishes of each medium were inoculated with the sawdust from each fifth annual ring of each disc, using the technique of Waksman (1917). Sawdust samples from the cross-sectional cuts were treated in a similar fashion, whilst wood samples (chips) removed from discolored areas were partially embedded in the agar in petri dishes. All samples were incubated at 25°C. The samples were examined at intervals of four and seven days. Isolates growing from the inocula were subcultured on to the three media for identification. The isolation of species on any of the media was considered to indicate its presence in the wood. Identifications were carried out with reference to the manuals of Gilman (1957) Barnett (1962), Ames (1963) Barron (1968), and Smith (1968).

9.3. Results

The genera of fungal species isolated from disc cross sections are indicated in Table 9.1. The results show that only marginal differences were evident in fungal flora isolated on the three media. The species of *Chaetomium* were only seen to occur on cellulose or pectin agars whereas *Zygorhynchus* sp. and *Rhizopus* sp.

were only isolated on Starch media. The species most widely occurring were those of Mucor sp. and T. viride. These species, due to their very fast growth rates, rapidly covered agar surfaces possibly precluding the isolation of slower-growing species. Other genera occurring frequently were Gliocladium, Fusarium and Aspergillus.

A greater number of species was isolated from the individual growth rings, the majority of these species being isolated from peripheral rings, with fewer species isolated from pith regions, particularly in butt discs. T. viride, Scytalidium sp., Penicillium sp., Mucor spp. Gliocladium sp., and Fusarium sp., isolated from inner growth rings, were isolated from throughout the sapwood regions. Similarly T. viride, Mucor spp., Gliocladium sp. and Fusarium sp. were seen to dominate most isolation plates. Cladosporium sp., Graphium sp. and Cephalosporium sp. were generally isolated from the inter rings of both upper and butt discs. The incidence of Alternaria sp. Aureobasidium pullulans, Botrytis cinerea, Ceratocystis sp., Papulospora sp., Phialophora sp. and Phoma sp. was low. These results are presented in Table 9.2.

Isolates from discoloured areas, were generally either Leptographium or an unidentified species tentatively suggested to be Diplodia sp.

9.4. Discussion

The results showed that thirty one microfungal species were isolated from green spruce belts which had been stored

TABLE 9.1

FUNGAL SPECIES ISOLATED FROM SAWDUST SAMPLES FROM CROSS-SECTIONAL CUTS THROUGH DISCS ON STARCH PECTATE AND CELLULOSE AGARS

Organisms	Starch	Cellulose	Pectin
Aspergillus niger		x	
Aspergillus sp.	xx	x	x
Cepholosporium sp.		x	
Cladosporium cladosporioides	x		
Chaetomium globosum		x	
Chaetomium erraticum			x
Chaetomium sp.		x	x
Fusarium sp.	xx	xx	x
Gliocladium sp.	x	xx	x
Humicola sp.	x		
Mucor sp.	xx	xxx	xxxx
Penicillium sp.	x	x	x
Paecilomyces variotii		x	
Phialophora sp.	x		x
Rhizopus sp.	x		
Trichoderma viride	xxxx	xxxx	xxxx
Zygorhynchus sp.	xx		

x Indicates number of discs from which the species was isolated.

TABLE 9.2

LIST OF ISOLATES SHOWING GROWTH RING FROM WHICH EACH WAS ISOLATED

Isolate	Peripheral Ring	5	10	15
Alternaria sp.	x			
Aspergillus sp.	x	xx		
Aspergillus fumigatus	xxx	xx	x	
Aspergillus niger	xxx	xxx		
Aureobasidium pullulans	x			
Botrytis cinerea	x			
Cephalosoporium sp.	xx	x		
Ceratocystis sp.		xx		
Chaetomium sp.				
Chaetomium globosum	x		x	
Chaetomium erraticum		x		
Cladosporium cladosporioides		x		
Fusarium sp.	xxxx	xxxx	x	x
Gliocladium sp.	xxx	xxxx	x	
Graphium sp.		x		
Leptographium sp.	x	x		
Humicola sp.	x			
Mucor sp.	xxxx	xxxx	xx	x
Mucor spinosum	x	x		
Paecilomyces sp.	x			
Paecilomyces variotii	x			
Papulospora sp.		x		

...contd./

Table 9.2 - continued

Isolate	Peripheral Ring	5	10	15
Phialophora sp.		x		
Penicillium sp.	xxxx	xxx	xx	
Phoma sp.	x			
Rhizopus sp.		x		
Scytalidium sp.	xxx	xxx	xx	
Trichoderma viride	xxxx	xxxx	xxx	x
Unidentified species similar to Diplodia sp.	xx	x		
Verticillium sp.	x			
Zygorhynchus sp.	xxx	xx		

x Indicates the number of discs from which each isolate was isolated

on the forest floor for three months, but that some fungi commonly associated with wood staining, e.g. Ceratocystis spp., Diplodia spp., Leptographium spp. were in little evidence. When the discs were split longitudinally, it was apparent that little staining of the wood was present, and that the few stained areas were limited to short streaks approximately two cm long in the longitudinal direction.

The isolated genera included three species of Aspergillus, three species of Chaetomium, two species of Paecilomyces, two species of Mucor and one species each of Rhizopus and Zyrgorhynchus. In comparison with these results, the selected test species included one Aspergillus species, A. niger, one species of Paecilomyces, P. variotii, and one Phycomycete, Pythium aphanidermatum. A Chaetomium species was not included among the selected test organisms because of the intense soft-rotting activity of that genus (Savory 1954), whilst a greater number of Phycomycetes was not included due to the general lack of wood-degrading ability of that grouping. However, the extensive colonization by Zygorhynchus sp. and the Mucor spp. might merit further investigation with reference to their role as primary colonizers of wood (Butcher 1968) and also to their role as nitrogen sources for the subsequent colonization of higher fungi.

Only one species of each of the genera Ceratocystis, Phialophora, and Diplodia (tentative identification) were isolated from the samples. However, in recognition

of the work of other authors, it was considered that their role in blue-staining of wood was more prominent than the frequency of their isolation indicated. Consequently, four Ceratocystis species, three Phialophora species, and three Diplodia species, were included among the selected test species.

Species of six of the isolated genera, Fusarium, Humicola, Papulospora, Penicillium, Phoma and Verticillium were not included among the selected test organisms, whilst species of the remaining ten isolated genera, Alternaria, Aureobasidium, Botrytis, Cephalosporium, Cladosporium, Gliocladium, Graphium, Leptographium Scytalidium and Trichoderma were included among the selected test organisms.

Of the twenty five genera isolated from the forest stored spruce, fifteen genera, represented by twenty two species were selected for degradation studies, while three phycomycete genera isolated were represented by one species, Pythium aphanidermatum. One isolated genus, Chaetomium, was not included in the test species because of its intense soft-rotting activity whilst the remaining six genera were not represented in the selected test organisms. However, ten commonly-found staining fungi representing the genera Botriodiplodia, Cytospora, Rhinocladiella, Myxotrichum, Helminthosporium, Trichocladium, Bisporomyces, Sclerophoma, Phialocephala and Discula were selected, and it was considered that the significance of these species in the staining of wood

was, if anything, greater than the untested isolates. As the function of the ecological survey was primarily to verify the procedure used to select the test organisms used for the duration of this research, the experimental results were therefore considered to be moderately successful.

Dowding (1970) showed that the incidence of bark beetle attack of green wood was greatest between the months of June, July, August and September, and that severity of staining could be associated with beetle infestation at that time. He also noted that greatest staining occurred at the exposed ends of bolts and at areas where blazes, comprised of wood on which bark was removed, were subject to aerobic contamination. Fungal infection was greatly diminished in areas where stages were protected.

Since the spruce bolts were subject to forest incubation during the most suitable time period for blue-staining and mould fungal infection, the lack of isolation of species of Diplodia, ~~xxxxx~~ Ceratocytis and Leptographium can only be due to three reasons:

i) the forests did not have a flora of these staining fungal species;

ii) the test discs were removed from the centres of bolts, thus they were not positioned in these areas, i.e. exposed bolt ends, where maximal infestation could be expected;

iii) the bark of bolts was slashed, but not blazed, thus simulating "protected blazes" and inhibiting aerobic infection.

Since the above species were seen to be dominant in some isolation experiments involving staining fungi (Butcher 1968; Dowding 1970), it was considerd that their lack of consistent isolation may have been due to the method of preparation and sampling of bolts.

It was shown in the results that the greater number of isolates were isolated from the peripheral rings and that fewer species were isolated from the inner rings. Included in the former group were Alternaria sp., Phialophora sp., A. pullulans and B. cinerea. Duncan (1963) considered that A. pullulans was a soft-rotting fungus that only penetrated slightly into wood and Butcher (1968) considered that B. cinerea was a secondary rather than a primary coloniser of green timber. Similarly, Dowding (1970) considered that Alternaria spp. and Phialophora spp. were only surface colonisers of wood. However, Banerjee and Levy (1971) considered that T. viride and Gliocladium spp. could greatly inhibit wood colonization by Phialophora spp., and Hulme and Shields (1970) considered that T. viride could inhibit a wide number of fungi including basidiomycetes, whilst Ricard and Bollen (1967) and Shields and Unligil (1968) observed an antagonistic behaviour of Scytalidium sp.

It therefore appears that this greater number of organisms isolated from the outer layers of wood may

be explained by:

(a) secondary colonization of wood by species with cellulose degradation activity, or

(b) antagonistic reactions between groups of organisms colonizing the same wood substrate.

However, a third viewpoint is also evident. Sharp and Eggins (1970) considered that the penetration rates of individual fungal species through beech veneers seemed to be a factor of their linear growth along these veneers in a longitudinal direction, and many of the isolated species from spruce discs e.g. Trichoderma Sp. and Fusarium sp., when similarly isolated from beechwood inoculated withsoil by Sharp (1970), were considered by him to show rapid growth rates. Butcher (1968) attributed the extensive colonization and staining of Pinus radiata posts by Diplodia pinea to its rapid growth rate, and similarly he attributed the relatively poor colonization of the same posts by Graphium sp. to the slow growth rate of the latter.

Species of Gliocladium, Trichoderma, and Fusarium, which were constantly isolated from throughout the spruce discs were seen by Sharp (1970) to be those most rapidly penetrating beech veneer blocks, and he also considered that some microfungi penetrated the entire area conducive to their growth before causing significant degradation. Similarly, species of Mucor, Rhizopus, Zygrohynchus, and Scytalidium, were seen to colonize extensively green sapwood of spruce, apparently conducive to their growth, and were seen to show extremely

rapid growth rates on the isolation media. However, Scytalidium lignicola, Gliocladium roseum, and Trichoderma viride, were also shown to be capable of cellulose and pectin degradation in the earlier chapters.

It therefore appears that a positive relationship may exist between growth rate i.e. rate of hyphal extension and rate of wood penetration. Many of the species isolated from the peripheral growth rings e.g. Cephalosporium sp. and Phialophora sp. were seen to have slow growth rates relative to the growth rates of the other test organisms e.g. Scytalidium lignicola and T. viride, and similarly the degree of wood penetration of the former as evidenced by isolation, was considerably less than that of the latter. It would therefore appear that, not alone did both fungal antagonisms, and nutritional sequence effect the genera of penetrating species, but it is also considered that the inherent growth rates of the organisms might considerably influence their degree of penetration and possible degradation. It is considered that this latter aspect may complement the results of the degradation studies, where the degree of growth of the individual fungal species seemed to considerably influence their degradation activity.

CHAPTER 10

DISCUSSION

10. DISCUSSION

The purpose of this research program was an initial investigation into those activities of blue-staining and mould fungi, which might enable them to enhance the permeability of Sitka spruce.

Liese (1970) pointed out the lack of clarity of present knowledge of enzymatic activity of staining fungi, particularly in relation to their penetration of lignified cell walls. He concluded that "the term 'blue-staining fungi' comprises a large number of Ascomycetes and Fungi Imperfecti with wide physiological and ecological variation. Some of the blue-staining fungi have to be regarded as capable of decomposing cell wall substances to some extent; further work on the group will lead to better differentiation. Several fungal species have so long been allocated to the blue stain group may have to be transferred to the soft-rot group".

Research has therefore been concentrated on the determination of blue-staining and mould fungal activities which

(a) enabled them to degrade wood; and

(b) reconciled the apparent amylolytic role of blue-staining fungi with their possible degradation mechanisms.

Thirty three test organisms representing twenty six genera of blue-staining and mould fungi were examined, and from the resulting data a pattern of activity can be seen which throws some light on their role as

deteriogens.

Comparison of the growth rates of the individual species on starch, cellulose and pectin agars indicated a first pattern. It was seen that the test species could be divided into two main groupings, i.e. fast and slow-growing species, and that the inherent growth rate of these groups generally controlled the degree of growth by their individual species on the media, irrespective of carbon source. In general, however, individual species were seen to produce marginally better growth when grown on starch media, than when grown on the cellulose or pectin media.

Fergus (1969) considered that little quantitative data on amylolytic activity was available for many mesophilic and thermophilic fungi. The starch hydrolysis experiments therefore revealed a further pattern, when all of the test species were seen to be amylolytic. Starch hydrolysis was directly related to growth rate, the faster-growing fungi producing distinctly larger hydrolysis areas than the slower-growing species. This is not to say that the intensity of amylolytic activity was lesser in the slower-growing species, but that the extent of starch degradation produced by both the rapidly-growing species and the slow growing species was greatly associated with growth rate.

In comparison with amylolytic activity, none of the test species produced an extensive clearing of cellulose agar in petri dishes that could in any way be equated with the clearing of starch agar produced by them in an equivalent time period. Similarly, only a limited number of species seemed capable of cellulose degradation in

cellulose

cellulose agar columns, and these included many of the more rapidly-growing species. Phialophora melinii and Phialophora fastigiata were two slow-growing species which did not register clearing of cellulose agar columns and which produced only slight decomposition of carboxy-methyl cellulose. However, both these species produced extremely high strength losses in fibrous cellulose over their limited area of colonization when tensile strength tests were undertaken.

It would therefore appear that, similar to amylolytic activity, some of the slower-growing species were as intensely cellulolytic as the faster-growing species over their limited area of colonization. Alternatively, however, the extent of degradation produced by the faster growing species, e.g. Botriodiplodia theobromae, was much greater since degradation could be produced over a greater area due to their more rapid growth rates.

Only twelve fungal species showed an ability to produce pectate gel liquefaction, and in comparison with amylolytic and cellulolytic activity, few of the species were considered to be measurably pectinolytic. A pattern thus seemed to emerge in which all of the species were amylolytic, many of them were cellulolytic while a few of the more highly cellulolytic species were also pectinolytic.

This pattern was substantiated by the viscosimetric studies, where it was seen that whereas twenty five species produced cellulase, only eleven of these species produced pectinase, and similarly that whereas many of the test species produced cellulase but not pectinase, none of the species produced pectinase but not cellulase.

This suggested that the pectinase system of the blue-staining fungi tested was cellulase-dependent, under the test conditions.

It is suggested that many of the species which produced both cellulase and pectinase were adaptive i.e. pectinase was not produced when the species were grown on cellulose, and similarly cellulase was not produced when the species were grown on pectin. However, it was also evident that ten of the species produced cellulase when grown on pectin, and that three species produced pectinase when grown on cellulose. This would suggest that the cellulase and pectinase systems of the species thus producing those enzymes were constitutive.

The growth rate factor seemed to considerably influence the relative amylolytic, pectinolytic and cellulolytic activities of the test species. When both amylolytic and cellulolytic activity were assayed, it was found that many of the slower-growing species were as intensely amylolytic and cellulolytic as the faster-growing species, the difference between the fast and slow-growing species, however, being the extent of degradation produced by them. It was therefore considered that the rate of growth of individual species could be a major factor in the ecology of wood degradation. The evidence of the ecological survey corroborated this when it was found that many of the slow-growing species tested were isolated only from the peripheral regions of the wood whereas faster-growing species were isolated from throughout the sapwood regions.

It can therefore be seen that colonization of wood by rapidly-growing cellulolytic species should probably result in a more extensive degradation of the wood than

if the wood is colonized by slow-growing cellulolytic species, even though the intensity of degradation by both groups of species may be similar with reference to their colonization areas.

The results showed that the test species produced a range of activities other than the starch degradation traditionally associated with their growth. If enzymatic degradation of crystalline cellulose is attributed to the activity of the C_1 component of cellulase (Liu and King 1967), then many of the test species, in degrading fibrous cellulase, could be said to produce that cellulase component. Similarly, if production of carboxymethylcellulase by blue-staining fungi is considered to be indicative of their production of the C_x component of cellulase (Liese 1970; Rosch, Liese and Berndt 1969), then a considerable number of the genera of blue-staining and mould fungi tested may produce both the C_1 and C_x components of cellulase. The production of these enzymes along with polygalacturonase and amylase should enable blue-staining fungi to produce at least a limited form of timber degradation.

It can be seen from the work of Krapvina (1960) and Levy (1967) that blue-staining and soft-rot fungi have similar patterns of gross penetration of wood. However, Krapvina observed that some blue-staining fungi were capable of wood degradation after starches and cell contents had been metabolized. This was substantiated

by Umezurike (1969), who showed that not only did B. theobromae produce soft-rot cavities in wood after depletion of starch reserves, but that the species was unable to colonize wood unless those materials were present at the time of colonization.

The growth studies showed that many test species exhibited a more rapid colonization of the starch agar than of the other two media. Similarly, the rate of starch degradation as evidenced by clearing was significantly more rapid for all species, than their rates of clearing of cellulose agar or production of gel liquefaction. This would indicate that the test species were easily capable of starch degradation, but that comparatively, their cellulolytic and pectinolytic systems were more limited. It is therefore suggested that many blue-staining and mould fungi may be primarily amylolytic but may also possess cellulase and in some cases, pectinase, systems.

The nature of this study was essentially comparative, and the results presented are therefore only relative to the species under test. It is therefore suggested that the amylase, cellulase and pectinase systems of blue-staining and mould fungi, and the interrelationship of these systems, would merit further more detailed investigation.

The implications of this study with reference to enhanced spruce permeability as a result of blue-staining and mould-fungal colonization are interesting.

It was pointed out in the Introduction that the enzymes cellulase and pectinase were most active in inducing enhanced wood permeability (Soulahti and Wallon 1958; Nicholas and Thomas 1968; and Bauch, Liese and Berndt 1970), and similarly, that organisms producing these enzymes and with suitable colonization patterns could be instrumental in enhancing permeability.

Since many of the test species produce both cellulase and pectinase and are known to produce extensive colonization of green timber, it is not unreasonable to suggest that, provided that the timber retains a suitable degree of moisture content, a limited degradation of the wood will occur on depletion of starch reserves, if colonization by those species takes place. It is suggested that it is this limited degradation which should result in enhanced permeability.

Similar to enzymatic activity, little data was seen in the literature on the nitrogen requirements of blue-staining and mould fungi deteriorating wood. Merrill and Cowling (1965) considered that basidiomycetes deteriorating wood were capable of conserving the meagre amount of nitrogen present by a process of autolysis and re-use of nitrogen in their own hyphae, and were similarly capable of translocating nitrogen from sources other than the wood colonized by them. Similarly, basidiomycetes are capable of adapting the amount of nitrogen in their mycelium to the amount of nitrogen in the wood (Merrill and Cowling 1965), and they may also be more capable of colonization and utilization of

substrates with low nitrogen contents than micro-fungi (Levi and Cowling 1969).

It can therefore be seen that even if blue-staining and mould fungi have the enzyme mechanisms necessary for wood-degradation, in comparison with basidiomycetes they may be restricted in their potential degradation capability due to the low nitrogen content of wood. It may be for this reason that only limited in depth degradation is produced by them, possibly resulting in enhanced permeability but not significant degradation of wood.

This study was intended as an initial investigation into the activities of blue-staining fungi, and as such it cannot be considered to encompass many aspects of their physiology and ecology. It is suggested, however, that further, more detailed investigation into enzymological and ecological aspects of blue-staining fungal colonization, in combination with field studies, should contribute to their fullest utilization in wood biodegradation.

A P P E N D I C E S

Appendix I

Composition of Starch Cellulose and Pectate Agars used in Growth Rate Studies

Materials	Starch Agar	Cellulose Agar	Pectate Agar
Potassium dihydrogen phosphate	1.0g	1.0g	1.0g
Ammonium sulphate	0.5g	0.5g	0.5g
Potassium chloride	0.5g	0.5g	0.5g
D.L. Asparagine	0.5g	0.5g	0.5g
Yeast Extract	0.5g	0.5g	0.5g
Magnesium sulphate	0.2g	0.2g	0.2g
Calcium chloride	0.1g	0.1g	0.1g
Agar	20.0g	20.0g	20.0g
Distilled water	1 litre	1 litre	1 litre
Starch	10.0g	-	-
Sodium pectate	-	-	10.0g
4% suspension of ball-milled cellulose	-	250 ml	-

Appendix II

Composition of Pectate Gels

Gels were prepared by aseptically pouring 10-ml quantities of the Calcium Chloride Agar into sterile petri dishes. When this agar was set, sterile permeable cellophone discs were transferred to the agar surfaces, upon which 10-ml quantities of the sodium pectate solution were poured.

These two-layer plates were allowed to stand in the laboratory until the gels were formed. The Calcium pectate gels were then transferred to further petri dishes by means of the permeable cellophane discs. These gel plates were then ready for inoculation. The compositions of the Calcium Chloride Agar, and of the Sodium Polypectate solution are outlined below.

Calcium Chloride Agar

Calcium Chloride	0.75g
Oxoid Agar No. 3	15.0g
Distilled Water	1 litre

Sodium Polypectate Solution

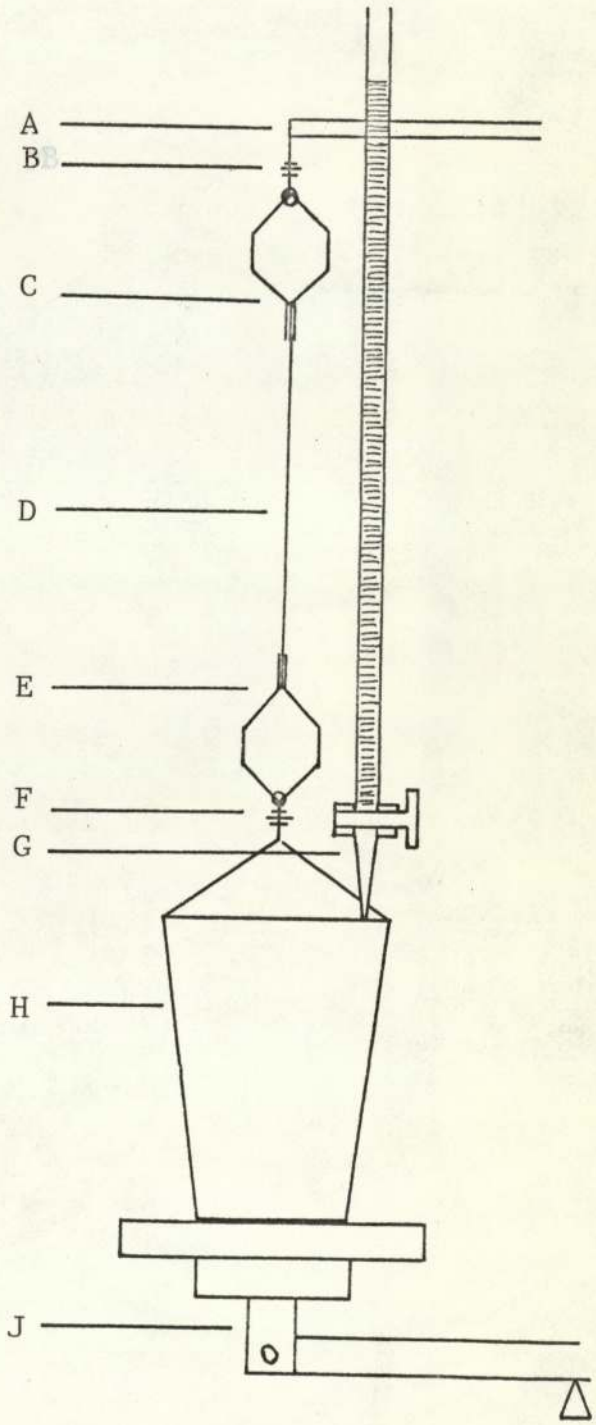
Sodium Polypectate	7.5g	D-Glucose	0.25g
Potassium dihydrogen phosphate	1.0g	Ammonium sulphate	0.5g
Potassium chloride	0.5g	L-Asparagine	0.5g
Yeast Extract	0.5g	Magnesium sulphate	0.2g
Distilled Water	1 litre		

Appendix III

Construction of Tensile Strength Testing Device

The device used for tensile strength testing is illustrated below.

A. Upper support
B. Swivel
C. Upper spring loaded jaw
D. Paper test strip
E. Lower spring loaded jaw
F. Lower swivel
G. Water delivery unit
H. Water container
J. Balance



This device consisted of two sets of jaws mounted in a vertical plane between which paper test specimens were mounted. The upper jaw was attached to a rigid support via a swivel, whilst the lower jaw was attached to a similar swivel mounted rigidly to a container. The function of the swivels was to prevent the distortion of test specimens during their positioning between the jaws.

The container was mounted on a counterpoise balance in such fashion that the distance between the upper edge of the upper jaw and the lower edge of the lower jaw equalled the length of the test specimen when the balance was adjusted to zero. Thus, when the apparatus was fully set up, the test specimen acted as a bridge between upper and lower jaws but with no forces acting upon it.

The operation of this device was simple. Water was delivered from a reservoir through the delivery unit to the container at a constant flow rate. This slowly-filling container acting downwards deflected the counterpoise balance, consequently moving the lower jaw away from the upper jaw, thus exerting tensile stress on the test specimen. This slowly-increasing stress eventually broke the strip upon which water delivery was terminated. The weight of water required to break each strip was calculated by returning the balance to zero.

Using this technique it was found that 82 per cent of a sample of 100 control specimens showed weight readings of within $\pm$ 3% of the average weight reading

for the sample, and that 96% of that sample showed weight readings of within $\pm$ 5% of that average figure.

As the technique was to be used as a screening procedure for cellulolytic activity, it was considered that this variation was not significant in terms of the criteria used in assessing strength loss production by the test species.

Appendix IV

Composition of Liquid Culture Media

Materials	Cellulose Medium	Cellulose Polypectate Medium	Polypectate Medium
Potassium dihydrogen phosphate	1.0g	1.0g	1.0g
Ammonium sulphate	0.5g	0.5g	0.5g
Potassium chloride	0.5g	0.5g	0.5g
D.L. Asparagine	0.5g	0.5g	0.5g
Yeast Extract	0.5g	0.5g	0.5g
Magnesium sulphate	0.2g	0.2g	0.2g
Calcium chloride	0.1g	0.1g	0.1g
Sodium polypectate	-	0.5g	0.5g
Ball-milled cellulose (4% suspension)	175 ml	162.5g	-
Fibrous cellulose Avicel - RC (Honeywell and Stein Ltd.)	3.0g	3.0g	-
Distilled Water	1 litre	1 litre	1 litre

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