AN INVESTIGATION INTO THE EXTRACELLULAR ENZYMES PRODUCED BY *DOTHISTROMA PINI* AND THEIR POSSIBLE RELATION TO PATHOGENICITY

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CHAPTER 1

BIOCHEMICAL PLANT PATHOLOGY

The aim of this chapter is to provide a background against which the pathogenic action of *Dothistroma pini* can be discussed.

Plant Diseases.

There are three principal groups of organisms implicated as causal agents of diseases in plants; fungi, bacteria and viruses. These pathogens cause diseases which in general can be divided into three kinds: necrosis, hypertrophy and hypoplasia.

Necrosis

Necrotic diseases can be general or local in nature. General necrosis is called rotting or decay and is usually caused by fungi or bacteria. An example is *Rhizoctonia* disease of sugar beet. Local necrosis is more limited in its extent. Examples are leaf spots, fruit spots, anthracnoses and certain types of cankers e.g. the leaf spot of maize caused by *Cochliobolus carbonum* and red band blight of pines caused by *Dothistroma pini*.

Hypertrophy

Hypertrophic disease, or abnormal growth, is caused by many fungi and bacteria (e.g. the crown gall organism *Agrobacterium tumifaciens* and also by some viruses, nematodes and certain chemicals. Cells are stimulated to abnormal division with individual cell enlargement. Growth regulators can also cause overgrowths.

Hypoplasia

Hypoplastic disease results in dwarfing and stunting, and

is common in virus diseases where the cell metabolism is controlled by the virus e.g. virus yellows of sugar beet. Many fungi and bacteria also cause hypoplasia. For example leaf rust and stem rust of wheat may prevent normal formation of kernels.

These three general types of disease are all the result of invasion of the host tissue by the pathogen, and in each case the host's metabolism is affected in some way or another.

Mechanism of Invasion

Pathogenic, or disease-causing, fungi and bacteria invade plant tissue by various mechanisms.

Soil inhabitants often enter via wounds and root hairs, under the attractant stimulus of root exudates e.g. onion root exudates cause the sclerotia of Sclerotium cepivorum to germinate and grow towards the onion roots (Coley-Smith, 1959).

Air borne fungal and bacterial spores, carried by the wind, may enter through wounds e.g. Stereum sanguinolentum and Diplodia pinea which infect Pinus radiata slasher wounds (Zondag and Gilmour, 1963) or frost-damaged terminals (Gilmour, 1964). More often they are able to enter via the natural openings of the plant tissue viz. stomata, hydathodes and lenticels in leaf tissue.

Water-borne spores, carried in rain splashes, enter in the same way as air-borne spores. Dothistroma pini conidia are water-borne.

We shall take a closer look at the different modes of entry of air-borne and water-borne spores.

Spores of some species enter only through stomata or hydathodes. Examples are Cercospora beticola (beet leaf spot

causal organism) and Plasmopora viticola (downy mildew of grapes). Others can enter either through stomata or wounds, or by direct penetration through plant surfaces. An example is Phyto phthora infestans (potato blight organism). The bacterium causing cabbage black rot Xanthomonas campestris enters regularly through hydathodes or water pores. The fire blight organism, Erwinia amylovora gains entry through stomata, nectaries and various types of wounds.

In the case of fungi, penetration through stomata is gained by hyphae growing over the leaf surface from the germinated spore. In some cases the pathogen is attracted to the stomata by exudates. Recently Turner and Graniti (1969) have demonstrated that Fusicoccum amygdali (a pathogen of almonds and peach) produced a toxin called fusicoccin which affected stomata causing them to open and thereby allow the fungus entry into the host.

Organisms invading via lenticels can usually invade via wounds also e.g. common scab of potatoes caused by Streptomyces scabies.

Direct penetration is a common form of entry for plant pathogens. In species studied this is generally achieved by the formation of an appressorium, a swelling on a hypha, which becomes firmly attached to the cuticle by a gelatinous sheath. A small protuberance dents the cell wall and this is followed by formation of a small "infection plug" from the protuberance, which actually penetrates the cuticle. After this the "infection hypha" enlarges to form a haustorium.

Once penetration of the host is achieved, provided it is a susceptible host, then the pathogen begins to parasitise its host. Exotoxins may destroy living tissue or block the host's metabolism, enzymes may disrupt the tissue and provide nutrients for the pathogen, while some fungi and bacteria produce plant growth regulators e.g. auxins, cytokinins and gibberellins which cause the host to over-react to the invasion, resulting in massive overgrowths.

Toxins and plant growth regulators will be considered first. A discussion on enzymes involved in pathogenicity is treated briefly here but in more detail in chapter 3.

Toxins.

The toxin concept is one of particular interest to plant pathologists. Some toxins which have an antimetabolite action (Owens, 1969), influence to a greater or lesser extent the metabolism of particular tissues of the host. For example, a toxin of Pseudomonas tabaci (wild fire toxin) causes chlorosis in tobacco leaves in the form of a halo around the necrotic locus of infection (Braun, 1955).

In some diseases the whole plant is affected, as in the case of "Victoria blight" disease of victoria oats (Avena sativa var. victoriae). The disease is caused by Helminthosporium victoriae which produces a toxin, victorin, which was discovered by Meehan and Murphy (1947). Pringle and Braun (1958) isolated and partially characterized the toxin as a polypeptide derivative with a molecular weight of between 800 and 2000. This can be cleaved into a biologically inactive polypeptide and a nitrogen-containing sesquiterpene called victoxinine (Pringle and Scheffer, 1964). The latter is toxic to victoria oats but very much less then victorin.

The cleaved peptide moiety of victorin is non-toxic and, when added to solutions of the intact toxin, reduces its toxicity. This suggests that the peptide is competing for toxin receptor sites and therefore toxin activity is conferred by its peptide moiety (Scheffer and Pringle, 1964).

Although many secondary effects of the toxin from H. victoriae have been reported, the primary effect is now postulated to be an alteration in the permeability of the cell membrane. Wheeler and Black (1962, 1963) showed that toxin-treated or infected tissue from a susceptible oat variety rapidly begins to lose electrolytes into a bathing solution. Tissue from resistant varieties was not affected, which shows that these are host specific, and not general, effects.

Samaddar and Scheffer (1968), with plant cell protoplasts, obtained further support for the membrane alteration theory. Protoplasts from susceptible cells quickly stopped protoplasmic streaming and burst within one hour after toxin treatment. Protoplasts from resistant varieties were unaffected.

Fomannosin, a sesquiterpene produced by the root rot fungus Fomes annosus, can seriously affect the metabolism of, and even kill, Pinus taeda seedlings (Bassett et al, 1967). Fomannosin has no polypeptide fraction.

Because fusarial wilt is an economically important disease in many crops, it has been studied extensively. It appears to be the result of a complex interaction of several toxins, toxic enzymes and a pathogen-produced plant hormone. The syndrome usually includes epinasty, (i.e. more rapid growth of the upper side of a leaf), plugging and browning of xylem vessels, necrosis, wilt, and ultimately death. Toxins implicated in diseases of various Fusarium sp. include fusaric

acid, α picolinic acid, lycovarubin, phytonivein and lycomarasmin.

The fusarial toxins and many others are of relatively simple organic structure e.g. alternaric acid, α picolinic acid, and lycomarasmin (Owens, 1969). But as Owens (1969) points out in his review of toxins in plant disease, not all phytotoxic products produced by microorganisms are of similar simplicity in molecular structure e.g. victorin and colletotin a glycopeptide from Colletotrichum fuscum.

Other complex phytotoxins include polysaccharides and glycopeptides secreted by certain phytopathogenic bacteria. These polysaccharides may cause wilting of the host through physical obstruction of the xylem vessels. However, as in the case of victorin, recent evidence suggests that at least in some cases, their main action involves membrane changes and not simply plugging of the host's vascular tissue. Such an effect on the membrane has been postulated for glycopeptides produced by Corynebacterium michiganense, causal organism of bacterial canker of tomato, on the basis of electron microscopy and autoradiography of cells treated with labelled toxin (Rai and Strobel, 1969b).

Three glycopeptide fractions, all toxic, have been isolated from the crude toxin produced by this organism. They range in M.W. from 35,000 to 200,000 and contain residues of 4 or more amino acids together with fucose, galactose, glucose, mannose (Rai and Strobel, 1969a).

Other phytotoxic polysaccharides are produced by bacteria-causing crown gall, bean blight and soft rot (Feder and Ark, 1951, Leach et al, 1957).

Plant Growth Regulators.

Plant growth regulators act at very low concentrations in

the physiologically healthy plant and deviation from the normal intracellular level can result in striking abnormalities. In diseases such as Pseudomonas solanacearum infection of tobacco (Sequeira and Kelman; 1962; Sequeira and Williams, 1964), and that caused by the crown gall organism Agrobacterium tumifaciens (Kapper and Veldstra, 1958), the pathogens are known to secrete a plant growth regulator, indole acetic acid, at concentrations very much higher than that required for normal growth in healthy plants, thus greatly influencing the metabolism of the host.

In other diseases the host itself is induced to produce more auxin (Sequeira, 1965) and hence to stimulate its own over-growth.

Gibberelins are also implicated in plant disease. For example, in bakanae disease of rice caused by Gibberella fujikuroi (Fusarium moniliforme) the "toxic" product of the fungus is a naturally occurring and very potent plant growth regulator, gibberellic acid. Affected rice plants grow rapidly and are conspicuous by their unusual height hence the term "foolish seedling disease".

Cytokinins are growth regulators that are also implicated in some diseases. For example, they are involved in the rust disease of beans and broad beans, caused by Uromyces phaseoli and U. fabae respectively. Bioassay has shown that in diseased tissue there are elevated cytokinin activities (Király et al, 1966). Accumulation of nutrients in the infected tissue, abnormal phloem transport, arrest of secondary growth, and senescence can be stimulated in uninfected leaf tissue (Poysár and Király, 1966).

In virus diseases and in such diseases as crown gall the

cells are not destroyed but their metabolism is altered to cause, in the former case, yellowing, resulting in dwarfing of the host, and in the latter, the formation of neoplastic tissue, believed to be the result of toxin production. Indole acetic acid, an auxin, is produced by Agrobacterium tumifaciens in its attack on carrot cells. Host cell production of this auxin is increased also, resulting in tumour cell formation. However, I.A.A. on its own will not transform healthy cells to tumour cells and this has led to the idea of a tumour inducing principle being involved (Goodman, Király and Zaitlin, 1967).

Enzymes.

Many types of enzymes are secreted by various pathogens to degrade host tissue and thereby obtain nutrients for growth. Brown (1965) in his review differentiates between enzymes involved in tissue disorganisation i.e. "macerating enzymes" and those which, by apparently altering the permeability of the protoplast cell membrane i.e. the plasmalemma, cause the death of the cell i.e. "lethal enzymes". They are considered to be possibly of proteolytic or lipolytic activity as opposed to the macerating polysaccharidases (see chapter 3.)

It has been shown that enzymes are elaborated by a host in response to invasion as well as by the invading pathogen (Goodman, Király and Zaitlin, 1967).

Host Defences.

Phytoalexins. Ward (1905) and Bernard (1911) were among the first to demonstrate that plants are capable of producing compounds in response to pathogen invasion, resulting, in some cases, to the combatting of that invasion. The term phytoalexin was introduced to refer to such substances (Müller and Börger, 1940 in Cruickshank, 1963). A phytoalexin has been

defined as an antibiotic which is produced by the host, as a result of the disruption of its metabolism, due to infection by microorganisms pathogenic to plants (Müller, 1956 quoted by Cruickshank, 1963). Cruickshank and Perrin (1960) demonstrated that these phytoalexins were not produced all the time, but only in response to invasion, irrespective of the pathogen. Also the phytoalexin produced need not be able to combat the invader, i.e. its action is non-specific. Pisatin, a coumarin derivative found in peas Pisum sativum (Cruickshank and Perrin, 1960) and ipomeamarone and other furanoterpenoids in potato Solanum tuberosum (Huira, 1940; Akazawa, 1960) are examples of phytoalexins. Other examples include

(a) Phenolic compounds

Phenolic glucosides are among the most noticeable phytoalexins with respect to disease resistance. Many pathogens have been shown to have β-glucosidase activity (Tomiyama, 1963) and hence it is suggested that hydrolysis of phenolic glucosides, releasing aglycone, may play a role in the disease resistance (Holowcyak et al in Tomiyama, 1963).

Some consider that the probable effect of these phenolic substances on extracellular enzymes is to precipitate the proteins. It is thought significant that many of the instances cited refer to oxidised and polymerised forms of the phenolics. Noveroske, Williams and Kuć (1962) showed that host resistance to Venturia inequalis could be "broken" by a polyphenol oxidase inhibitor, 4-chlororesorcinol. Phenolic compounds based on a C_{15} monomer e.g. d-catechin are more effective inhibitors than those based on a C_9 structure e.g. chlorogenic acid. This suggests that molecular size, charge, and shape have an effect in the inactivating properties of phenolic compounds (Byrde,1963).

Cellulase and pectinase inhibition have also been ascribed to a class of polyphenols (tannins) (Porter and Schwartz, 1962).

Amylase inhibitors are known in sorghum extracts, but their chemical nature has not yet been reported (Miller and Kneen, 1947).

(b) Other inhibitory compounds.

Coumarins and their derivatives have been implicated in carrot resistance to disease (Condon and Kuć, 1960). Terpenoids and their derivatives have also been implicated as phytoalexins in potatoes, and steroid alkaloids in Irish potato tuber resistance (Allen and Kuć, 1964). The inhibitory effect of potato juice may also be due in part to its divalent cations, shown in vitro to inhibit pectinases.

Inhibition of enzyme biosynthesis as a factor in disease resistance.

Deese and Stahman (1962 a,b) have shown that levels of pectolytic and cellulolytic enzymes are lower in infected resistan hosts than in infected susceptible plants. This suggests that instead of disease resistance arising from the inactivation of extracellular fungal enzymes of vital importance to the pathogen, a similar effect, whereby enzyme formation is inhibited, may operate. Byrde (1963) reports that the amino acid L-canavanine, an antimetabolite of L-arginine, is effective in inhibiting the induced biosynthesis of polygalacturonase by Sclerotinia fructigena.

Control of Plant Disease.

Control of plant diseases can be divided into two aspects: chemical control, and breeding for resistance.

Fungitoxic chemicals, such as bordeaux mixture, antibiotics and organophosphorus compounds, are used to control plant

disease. Bordeaux, the first chemical mixture used to control disease viz. powdery mildew of grapes, is also effective in the control of bacterial diseases (Zaumeyer, 1956). Antibiotics have also been used with some effect. For example, streptomycin has been used in the control of some Fusarium species (Rhodes, 1962).

However, the best means of control would appear to lie in the production of disease resistant stock. Breeding programmes aimed at finding plants which are resistant to attack by a certain organism, are common. They depend on chemically inducing resistance (Samb-rski, 1963) or finding resistant stock in a heavily infected area and breeding clones from it. The latter is being employed in breeding programmes aimed at producing Pinus radiata stock resistant to Dothistroma pini.

CHAPTER 2

DOTHISTROMA PINI

Dothistroma pini Hulbary is a pathogenic fungus infecting members of the genus Pinus and producing a necrotic disease commonly known as red band blight of pines. Dothistroma pini was first identified by Hulbary (1941) as causative agent of a needle blight of the Austrian pine Pinus nigra var austriaca. In 1966 Funk and Parker described evidence showing D. pini to be the imperfect form of the ascomycete Scirrhia pini.

Three varieties of D. pini have been differentiated chiefly on the basis of spore dimensions. (Thyr and Shaw, 1964; Gibson, 1965 ; Ivory, 1967) viz.

D. pini var pini, central and eastern U.S.A., Chile, England and New Zealand.

D. pini var linearis, western U.S.A. and Canada.

D. pini var keniensis, Africa.

No physiological differences have been reported in these three forms; spore dimensions overlap between varieties, and the division of the species is not universally accepted (Gadgil, 1967).

Other regions where D. pini has been recorded are in parts of Europe, North and South America, India, and parts of Russia (Gilmour, per. communication). Most reports are comparatively recent, suggesting that the fungus is spreading, or that, with increasing attention being focused on the disease, more locations are being found.

D. pini was first identified in New Zealand on Pinus radiata D. Don in 1964 (Gilmour, 1965), from an initial locus of infection near Tokoroa. Infections have been recorded on

25 species and varieties of pine (Gilmour, 1967), and also on Douglas fir, Pseudostuga menziesii (Dubin and Walper, 1967), and larch, Larix decidua (Bassett, 1969).

Gilmour (1967) lists the known infected species in order of susceptibility. It would seem that most Pinus species are more or less susceptible. P. radiata, P. ponderosa and P. nigra var laricio are all grown extensively in N.Z. and are all highly susceptible. The disease is therefore of considerable economic importance to the New Zealand forest industry. Most of the more resistant species are grown in N.Z. on a small scale only. Certain species increase in resistance markedly at a certain age (P. radiata is susceptible up to about 20 years, P. muricata at 35-40 years, P. contorta at 30-40 years), but others, such as P. ponderosa remain highly susceptible for over 70 years. See plate 2.1 - 2.5, page 14.

Successful control of the disease was achieved in Kenya, through aerial spraying with aqueous suspensions of insoluble copper compounds, such as copper oxychloride and cuprous oxide (Gibson, 1964). This method is employed in New Zealand, where young forest is sprayed regularly. However the best means of controlling the disease lies in the production of resistant stock with which to re-establish milled forest. Occasionally, an apparently completely resistant pine tree is found in areas of high D. pini infection. Such trees, together with resistant stock imported from Kenya, are being used in programmes aimed at producing disease-free stock (Thulin, personal communication).

Little is known at present about the way in which D. pini is spread. The vegetative spores are believed to remain viable for a relatively short time (Bassett, personal communic-

1.

2.

3.

Photo 1. P.radiata killed by D.pini.

Photo 2. High infection area in Kai ngaroa S.F.

Photo 3. Same trees as in photo 2 1 month after spraying with Cuprous oxide.

Photo 4. Infected P.radiata twig

Photo 5. Close up of twig showing red banding and black stromata.

4.

5.

Photographs of Dothistroma pini infection.

ation) and hence their function would seem to be associated with the local spread of the fungus. This is thought to occur through rain splashing on infected forest floor litter, droplets with spores then being disseminated by wind currents. Distribution could also occur through water splashing on infected needles washing spores from mature stroma. The dramatic spread of the organism through New Zealand pine forests could be due to the occurrence of the sexual stage, Scirrhia pini, only once so far reported in New Zealand (Bassett, 1968). The ascospores of the perfect form would be readily dispersed by wind and would remain viable for much longer periods.

Mechanism of Infection by D. pini.

Gadgil (1967) reported details of the infection process of the fungus on P. radiata. He sprayed nine month old P. radiata seedlings with a conidial suspension of a two-week-old D. pini culture and maintained the seedlings under polythene with a supplementary light source. The humidity was 95-100% and the average temperature was 20°C. About 90% of the conidia germinated within 3 days of inoculation. Needles were removed at intervals, for examination. Gadgil demonstrated that, on germination, the hyphae spread across the needle surface to create, after six days, a dense mycelial mat which provided secondary conidia. He suggested that they may provide a further source of inoculum and may account for the rapid spread of the fungus. This surface mycelium had practically disappeared after 45 days.

Microscopic examination of transverse sections of ten-day-old inoculated material showed the formation of appressoria-like structures. These just covered the stomatal opening.

Hyphae showed no specific attraction to stomata.

Gadgil also sprayed nine-month-old seedlings with macerated mycelium and observed direct penetration of the epidermis. He did not observe this with hyphae from conidial suspensions. The mycelial fragments penetrated either the stomata or the epidermis directly, by sending a hyphal wedge through the epidermis into the mesophyll tissues, where it branched and permeated the mesophyll. Resin canals were penetrated and the hyphae were seen to be both intra and inter-cellular. Little mesophyll cell disintegration was evident up to 16 days after inoculation.

To observe lateral hyphal spread within the needle, Gadgil selected needles with red bands flanked on either side by green healthy-looking areas, and examined transverse sections. He noted in some cases that the change was sharp from green to red, while, in others, yellowish areas were interposed between the green areas and the red band. In each case examined there was no evidence of any hyphae in the green area, and disorganisation of mesophyll tissue was seen in advance of hyphal spread. Vital staining showed that the mesophyll cells adjacent to areas colonised by D. pini hyphae were dead. To account for mesophyll disorganisation, Gadgill suggests the possibility that the hyphae produce an extracellular enzyme system, or an exotoxin.

Stromata of D. pini appeared on needles of inoculated seedlings $3\frac{1}{2}$-$4\frac{1}{2}$ months after inoculation. The stroma arose at any point in the mesophyll, to erupt through the epidermis. Considerable disorganisation of mesophyll tissue was evident in areas near stroma. His evidence suggested that, after penetration of the host cell, the membranous tissue

is destroyed first, leaving a skeletal structure, thought to be carbohydrate.

The destruction of host membranous tissue may involve protease and lipase elaborated by the pathogen. Carbohydrases may also be required at this stage, but the survival of a cell-wall-skeleton noted by Gadgil suggests that carbohydrolytic activity is not complete in the early stages.

Further consideration of D. pini enzymology must be deferred until the production of extracellular enzymes by other plant pathogens has been discussed in chapter 3.

Physiology of the diseased host.

Autoradiographic techniques using P. radiata seedlings infected with D. pini which had been exposed to $^{14}CO_2$, have provided a picture of photosynthesis and translocation of ^{14}C-labelled assimilates within the infected needles. Rook (1969) has shown that D. pini does not appear to affect photosynthesis during the early infection period. Once the pathogen forms bands across the needle, however, the rate of photosynthesis of the part of the needle distal to the infected area is reduced, while that proximal to it appears unaffected. He reports that if infected seedlings labelled with $^{14}CO_2$ are autoradiographed to show where the assimilates have been translocated, ^{14}C accumulates principally on the basal side of the infeoted part of the needle, as if the ^{14}C-assimilates were being attracted to the infected area, or held there in some form which was preventing their translocation. Occasionally, resin plugs (shown by microscopic examination) appeared in association with D. pini infection and they seemed to prevent translocation of assimilates from the distal part of the needle past the infected area. Whereas there was no

significant accumulation of ^{14}C in the infected part of the needle during the incubation period, a marked increase of ^{32}P content was observed when a twig containing infected needles was placed in a solution of inorganic ^{32}P.

Thus we can say that the pathogenicity of this organism may be the result of

(a) enzymatic attack on host tissue

(b) resin plug formation and blockage of phloem transport

(c) specific exotoxin action on host metabolism.

The principal aim of this thesis is to establish the type of enzymes produced by D. pini and relate them to the relevant substrates in the pine needle.

Toxin production by D. pini is outside the immediate scope of this thesis, but will be discussed in some detail for the sake of completeness, as a possible contributing factor to pathogenesis.

Toxin production by D. pini.

Gadgil (1967) showed that in D. pini infection, host cells were killed some distance from the hyphae, and suggested that an exotoxin or extracellular enzyme may be responsible for cell death. The discolouration of the host tissue in the area of infection was consistent with some alteration in host metabolism. Rook (personal communication) has shown that phloem transport in the needle is hindered by the infection and this is thought to impair host metabolism, but such an alteration would be expected to have a more general effect on the needle, whereas cell death and discolouration are confined to a relatively narrow zone about the infection site.

Many fungal toxins are coloured, and it seemed of interest to determine whether the red pigment produced by D. pini both in pine needles and in culture, might bear any relation

to toxicity.

Brunt and Bassett, working at the Forest Pathology Laboratory of the New Zealand Forest Research Institute, in 1967, showed that a red pigment isolated from D. pini was indeed toxic to Chlorella pyrenoidesa. Their work is reported here.

Isolation of Pigments from D. pini Culture Medium.

Materials and methods are outlined in chapter 5 and appendices 2 and 3, pages 105 and 106 respectively.

During growth of D. pini (an F.R.I. isolate) on both 10% malt extract and Raulin's solution in shake culture, the medium gradually became red and finally black after 10-12 days, depending on the spore concentration. The pigments responsible for the colour change were found to be extractable to varying degrees with a number of organic solvents, including butanol, chloroform, ethyl acetate and hexane.

One litre of D. pini shake culture on 10% malt extract medium, buffered at pH4·8 with citrate/phospate buffer, was incubated for twelve days at ambient temperature on a shake machine. The mycelium was then filtered off. The blackish brown filtrate was extracted by shaking with an equal volume of ethyl acetate. The ethyl acetate phase was concentrated to 10 mls. A dark red/orange solution remained.

0·01 ml. of concentrated ethyl acetate extract was subjected to thin layer chromatography (Merck G and GF_{254} silica gel 0·25m.m. thick and developed for 45 minutes chloroform/ethyl acetate 4:6) Twelve coloured bands were observed, and a thirteenth band was detected by its fluorescence under ultraviolet light at 254mμ.

A control experiment, performed on an ethyl acetate extract of uninoculated 10% malt extract medium, revealed none of these bands. This suggested they were all of fungal origin.

The same thirteen bands were also observed after growth in Raulin's solution.

Bioassay of pigments for toxicity, using Chlorella Pyrenoidosa as test organism. (See plate 2.6, page 21).

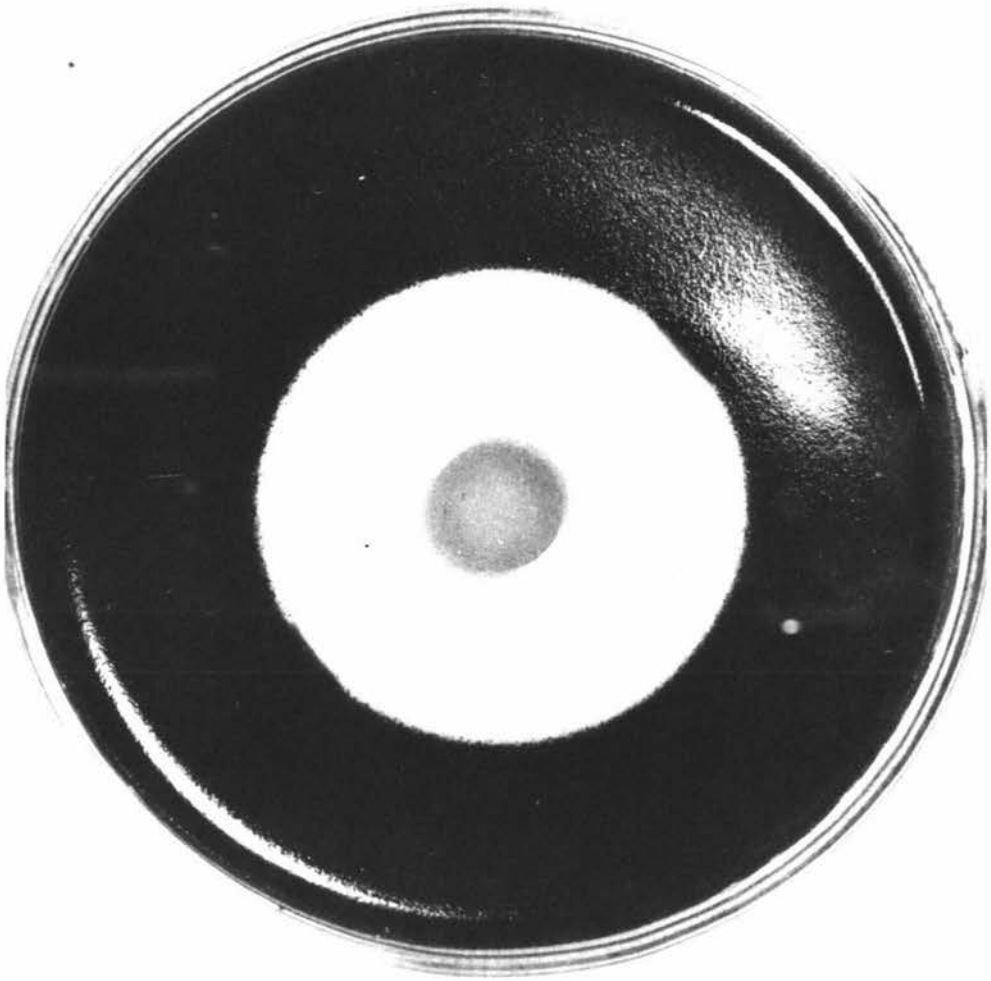
Each of the thirteen visible and U.V. flourescing bands separated by thin layer chromatography was carefully scraped off the plate and into 20 mls. of 95% ethanol. The suspension was shaken, then centrifuged, and the supernatant decanted and concentrated to ten mls.

Chlorella pyrenoidosa, a unicellular green alga, was used as the test organism according to the method of Warren and Winstead (1965). Chlorella plates were poured and antibiotic discs (1.27 c.m. diameter) were soaked in the concentrated ethanol fractions, dried thoroughly and placed in the centre of the Chlorella agar plate. Control discs of ethanol and plain paper were treated likewise. The plates were incubated under white light for 48 hours at ambient temperature. Where a disc contained a compound toxic to the alga then a zone of inhibition in which there was either diffuse, or partial, or no growth at all, was present over a certain area. A bright red pigment, the fifth band from the origin R_f 0.24, gave a zone of total inhibition 4.67 c.m. in diameter. The rest gave varying amount of inhibition from diffuse to nil. The red pigment was the predominant component. Because of these observations, effort was concentrated on the red pigment. The black aqueous phase, ethyl acetate and malt solution were used as controls which were negative with respect to the bioassay.

Purification.

The red pigment was purified using thin-layer chromatography. The plates were developed in chloroform: acetone 3:7.

PLATE 2.6



Chlorella pyrenoidosa bioassay for *D. nini* compound showing zone of inhibition.

The red band was scraped off, eluted in 20 mls. 95% ethanol centrifuged, concentrated to 10 mls. and rechromatogrammed in chloroform: acetone, 8:2. The red pigment was then the only pigment detectable visibly, under U.V. or with phosphomolybdic acid spray. One m.m. thick silica gel plates were then used to obtain quantities of the pigment for spectroscopic studies.

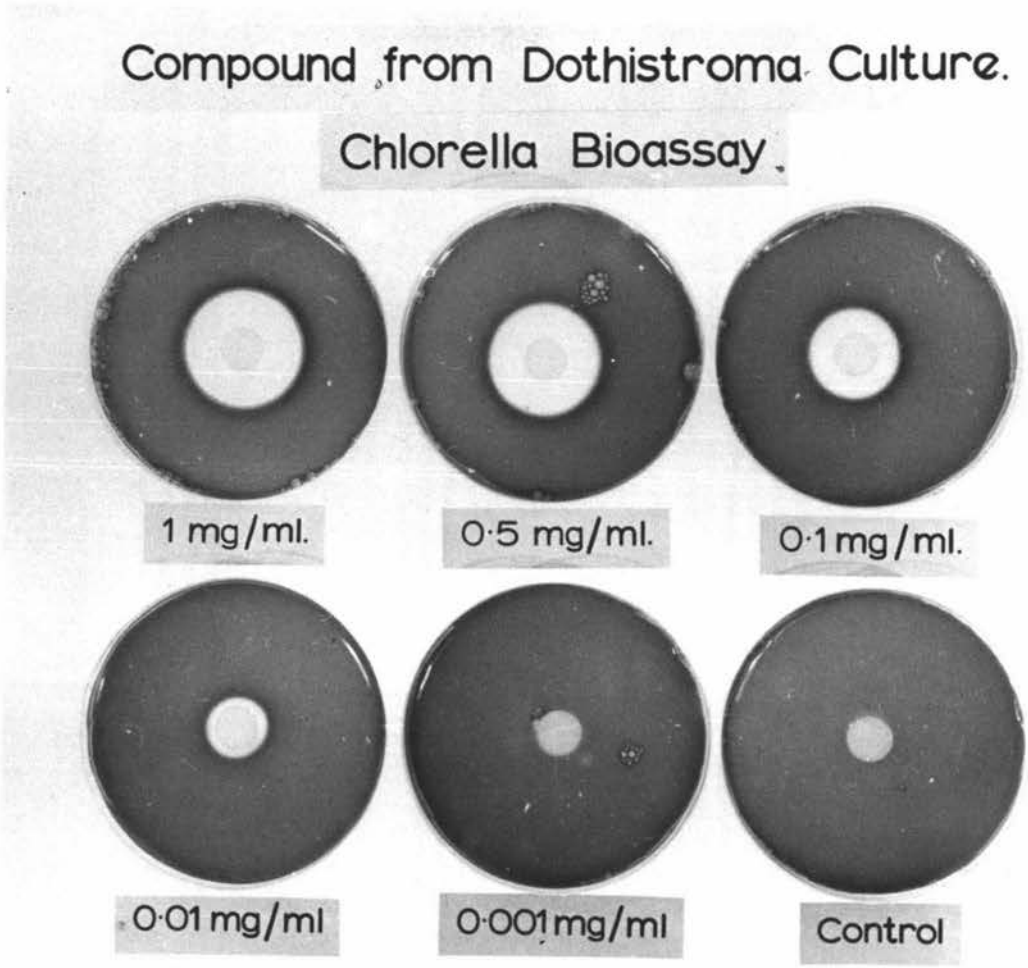
Isolation and Purification from P. radiata needles.

Necrotic regions around stroma were removed and eluted in ethyl acetate for three hours. An orange solution was obtained. The method outlined above for the isolation and purification of the pigment from shake culture was used to purify this pigment too. Both pigments had very similar infra red, visible and ultra-violet spectra, and toxicity to Chlorella pyrenoidosa.

Further work on the red pigment has been carried out at the Chemistry and Biochemistry Dept.. at Massey University by Gallagher and Hodges. They have determined the M.W. as 372, an empirical formula of $C_{18}H_{12}O_9$. A yellow pigment, M.W. 356 and $C_{18}H_{12}O_8$ exists in close association on thin layer plates. Both of these pigments have been shown to be substituted anthraquinones with a complex cyclic side chain. The side-chain structures are similar to the aflatoxins of Aspergillus flavus.

To date little is known about the toxicity of these pigments and others produced by D. pini. Buchanan and Bassett (personal communication) have shown that the bioassay is a rather insensitive test. (See plate 2.7, page 23).
They also point out that the compound toxic to Chlorella has yet to be conclusively shown to be harmful to pine needles. Part of the difficulty apparently lies in the development of a

PLATE 2.7



Compound from D. pini culture in Chlorella bioassay showing low toxicity of compound.

suitable test method although preliminary work by Brunt and Bassett suggested that the compound could cause metabolic changes in needles.

Bassett and Buchanan (1969) report that toxin production is not correlated with mycelial growth; by varying the medium it is possible to produce a high mycelial yield with almost no toxin, or simultaneously high yields of toxin and mycelium.

CHAPTER 3

THE ENZYMOLOGY OF PATHOGENICITY

Biochemical studies of pathogenic fungi have implicated a variety of degradative enzymes in the production of disease symptoms. Such studies are generally dated as beginning with de Bary's work (1886) on carrot roots infected with the fungus Sclerotinia libertiana, a pathogen of carrots. Using the expressed juice of the diseased carrot root, he demonstrated the presence of a thermolabile substance which was capable of disorganising healthy host tissue. He also demonstrated the lethal action of this component on carrot cells but was unable to determine the nature of the degrading substance. Ward (1888) demonstrated the presence of a similar substance in lilies infected with a Botrytis sp. and Jones (1909) showed that Bacillus carotovorus and other organisms produced a water-extractable component which could macerate plant tissue. Each of these studies demonstrated that the middle lamella of the plant tissue was destroyed, resulting in the characteristic rotting or maceration of the tissue, i.e. the cohesiveness of the tissue was destroyed.

The middle lamella of most plant tissues consists largely of pectin and although the enzyme nature of the active principle was not known to the earlier workers, the macerating principles of a variety of fungi eventually came to be generally identified with pectolytic enzymes i.e. enzymes specifically degrading the pectin material of the middle lamella (Bateman and Miller, 1966).

A number of pectolytic enzymes, or pectinases, of differing

specificities are now known. Other fungal enzymes, also implicated in the pathogenicity of some species, degrade a variety of other plant structural carbohydrates, as well as cell membranes. Brown (1965) has reviewed some of the enzymic activities and proposed criteria for distinguishing "macerating enzymes" from "lethal enzymes".

These polymer-degrading enzymes are all extracellular, in that they are secreted by the fungal cells into the surrounding tissues, where their action can be demonstrated. In artificial media, the enzymes are conveniently assayed in the culture filtrate..

The principal groups of enzymes, grouped according to substrate specificity, are as follows:-

Enzymes acting on pectin (Bateman and Miller, 1966).

Polygalacturonase (PG) - catalysing hydrolysis of pectin or polygalacturonic acid.

Pectin methylesterase (PE) - catalysing removal of methyl groups from pectin.

Pectin lyase (PL) - catalysing non-hydrolytic splitting of the pectin chain.

Enzymes acting on cellulose (Erikssen, 1967; Mandels and Reese, 1964; Norkrans, 1967).

Cellulase (C_1) - thought to act on insoluble cellulose. Quite distinct from

Cellulase (C_x) - hydrolyses soluble cellulose i.e. carboxy methyl cellulose, by action on terminal monose units of the polymer.

Enzymes acting on hemicellulose (Brown, 1965).

A variety of enzymes, generally fairly specific, catalysing hydrolysis of hemicelluloses; including arabanase, xylanase, etc.

Enzymes acting on non-carbohydrate substrates. (Brown, 1965).

Proteasea and lipases are also secreted by phytopathogenic fungi and have been implicated in pathogenecity (possibly as "lethal enzymes").

Other extracellular enzymes, such as nucleases, have no apparent role in pathogenesis, but may participate in the breakdown of host tissues and thera utilisation by the pathogen.

In considering the specificity and mode of action of extracellular enzymes of plant pathogens, a knowledge of the chemistry of the host cell, and in particular the host cell wall, is essential. The cell wall is principally polysaccharide in nature, with a classical division into pectin, cellulose, and hemicellulose. Lipid and protein predominate in the cell membrane, or plasmalemma, as well as the inner cell membranes.

Pectins.

The basic structure of the pectins of the higher plants is a linear, generally non-branched, polymer of D-galacturonic acid units in α-1,4 linkage. The following types are recognised:

(a) Polygalacturonic acid has a free carboxyl group at C-6 of the galacturonic acid.

(b) Pectic acid is high molecular weight polygalacturonic acid, generally of about 100 D-galacturonic acid residues.

(c) Pectinic acid is polygalacturonic acid with carboxyls randomly methylated and between 100 and 200 residues in length. Pectinic acids of low methoxy content have properties similar to pectic acids. Solubility increases with increasing methoxy content as does that of polyvalent cation pectinates.

(d) Pectin is pectinic acid of high methoxy content.

(e) Protopectin is a name commonly applied to all insoluble pectic substances.

The precise location of water soluble pectins is not known. Pectic acids have been reported in the vacuoles of plant cells and possibly in the cell wall as low molecular weight polymers. But it is generally believed that insoluble pectins are the primary components of the middle lamella of plant tissues, existing mainly as the calcium salt (Bateman and Miller, 1966).

The chemistry of their association in plant tissue with other cell wall polysaccharides and with lignin is not understood. Divalent cations contribute to the structural integrity of plant tissues by forming salt bridges between uronic acid carboxyls of adjacent uronide polymers and possibly between uronide polymers and proteins (Bateman and Miller, 1966).

Enzymes utilising pectin as substrate.

Polygalacturonase PG

PG enzymes attack the polygalacturonic acid molecule at the α-1,4 linkage to produce an increase in reducing sugar and shorter chain molecules. Two types of PG enzymes have been demonstrated. Exo-PG attacks the α-1,4 link at the non-reducing end of the molecule. Endo-PG randomly hydrolyses the molecule to produce an increase in short chains each with its reducing group. Galacturonic acid is the ultimate end product of both enzymes. Bateman (1968) has shown the macerating principle of Sclerotium rolfsii to be an endo-polygalacturonase.

Pectin methyl esterase PE

PE enzymes catalyse the hydrolysis of methyl esters of pectin and pectinic acid resulting in the formation of methanol and pectic acid. Complete de-esterification is rare, even under optimal conditions about 10% of the methoxy groups are

left. The enzyme is found in most higher plants and is produced by many micro-organisms, bacteria and fungi, saprophytes and pathogens alike. They are source-dependent with respect to effect of pH, salt-activation and inhibitors. There appears to be little dependence on the molecular weight of the substrate (Bateman and Miller, 1966).

Pectin lyase PL

This enzyme is also called pectin transeliminase because its action results in the formation of the $\Delta^{4:5}$ unsaturated uronide product. Both endo- and exo- forms exist (Bateman and Miller, 1966). Albersheim (1963) has shown this enzyme purified from a commercial preparation, Pectinol R-10, to be subject to product inhibition. Indole acetic acid enhances product inhibition of PL but is not inhibitory in the absence of products. This enzyme is produced by Aspergillus fonsecaeus (Edstrom and Phaff, 1964) and Rhizoctonia solani, Aspergillus niger and Botrytis cinerea (Sherwood, 1966).

"Depolymerase"

This term is not a precise one for it implies a single enzyme when a multi-enzyme system most likely operates. It usually refers to limited hydrolytic action usually between 5 and 10%. Little is known of its action (Bateman and Miller, 1966).

Protopectinase.

This refers to the enzymes which hydrolyse pectin in plant cell walls to produce soluble pectinic acid (Bateman and Miller, 1966).

These "pectinases" are employed by pathogens in attacking host tissue. Early studies established that destruction of certain constituents in the middle lamella of the plant cell wall was responsible for maceration but neither the type of enzyme nor the precise nature of the substrate was known.

Bateman (1968) points out the difficulty in defining on a

chemical basis the maceration of tissue. He attributes it in part to the lack of understanding of the chemical nature of the "intercellular cement" between plant cells, and also to the routine use of enzyme mixtures in studies on tissue maceration. Detailed studies have shown that cellulase makes little or no contribution to the tissue maceration process (Bateman, 1963; Spalding, 1963; McClendon, 1964; Zaitlin and Coltrin, 1964). Work by Ginzburg (1961) has produced evidence for the existence of a protein gel structure cross-linked by metal cations in the intercellular cement of young pea root tip cells. Kud (1962) showed, with crude enzyme preparations, that protease aided cucumber hypocotyl maceration in the presence of endo-PG. McClendon (1964) has shown that enzymes which attack proteins, galactomannan, xylan and cellulose do not macerate potato tuber tissue. In those cases where "macerating enzymes" have been characterised and identified, they have been identified as endo-forms of PG or Pectin lyase (Demain and Phaff, 1957; Bateman, 1966, 1968; McClendon, 1964; Zaitlin and Coltrin, 1964; Dean and Wood, 1967). A concept has emerged, therefore, which associates pectolytic enzymes with the process of tissue maceration, even though the idea that the "macerating enzyme" may not be one given enzyme type, or a given group of enzymes acting on a similar substrate, has received fairly wide consideration (Bateman and Miller, 1966).

However, there are reports of the separation of "macerating enzymes" from endo-PG or pectolytic enzymes (Kaji, 1958; Naef-Roth et al, 1961; Byrde and Fielding, 1962), but in none of these cases has the identity of the "macerating enzyme" been established.

Bateman (1968) in examining the " macerating enzyme" of

Sclerotium rolfsii concludes that enzymes attacking arabinic acid, araban, arabinogalactan, carboxymethyl cellulose, galactan, mannan and xylan do not contribute significantly to maceration of potato tuber tissue. He points out that the possibility that enzymes other than the endo-forms of PG and pectin lyases can contribute to maceration should not be eliminated, since it is known that the action of one type of enzyme may serve to unmask the substrate for the action of another enzyme upon the polysaccharides in plant cell wall material (Dehority et al, 1962). Bateman considers the possibility that cleavage of some chemical bond other than the α-1,4 bond of the pectin molecule results in tissue maceration. In 1965, Byrde and Fielding reported the assodiation of an arabanase with a maceration factor from Sclerotinia fructigena, but more recent studies have shown that the macerating factor is not an arabanase (Bateman, 1968).

Of significance is the report by Leal and Villeneuva (1962) that nonpathogenic strains of various Verticillium species all lacked pectolytic enzymes. They equated pathogenicity with the presence of pectolytic enzymes. They tested twenty varieties of various species viz. V. albo-atrum, V. dahliae V. tricorpus, V. nigrescens, V. lateritium, and in all the pathogenic species the pectolytic enzymes, PG, PE and depolymerase were produced, but the non-pathogenic species did not produce this group of enzymes at all. Much other work exists equating the role of the pathogens with the production of pectolytic enzymes (Waggoner and Dimond, 1955; Striden and Winstead, 1961).

Cellulose

There are various types of cellulose. In its pure

chemical state, the polymer is linear and composed of anhydro-glucose units linked in β-1,4 configuration. The basic unit is D-glucose. Native cellulose is an insoluble polymer.

Enzymes utilising cellulose as a substrate.

Enzymes which definitely belong to the cellulase system are cellulases and β-glucosidases. Cellulases hydrolyse β-1,4-glucosidic linkages between anhydroglucose, residues in cellulose, cellulose derivatives and cellodextrins to produce cellobiose or cellobiose derivatives. Cellulase is therefore the name for enzymes with the systematic name β-1,4-glucan glucanohydrolase.

Some cellulolytic micro-organisms can degrade cellulosic materials only after some kind of modification of the cellulose has occurred. Other organisms also degrade cellulose in native forms, such as cotton fibres. Only the latter organisms are regarded as truly cellulolytic.

In 1950, Reese et al introduced the "C_1,C_x" hypothesis. It was postulated that "truly cellulolytic micro-organisms" are equipped with both C_1 and C_x enzymes i.e. "C_1" converts cellulose to reactive or soluble cellulose and "C_x" reactive cellulose to cellobiose. Micro-organisms able to hydrolyse only modified cellulose lack the C_1 enzyme. Mandels and Reese (1964) have given the C_1,C_x concept the following schematic description.

Cellulose	⟶	Reactive cellulose	⟶	Cellobiose	⟶	Glucose
C_1			C_x		β-glucosidase	
?			hydrolytic		hydrolytic	

However, cell-free enzyme preparations from many micro-organisms, which according to the above statement are truly cellulolytic, fail to solubilise native cotton unless this is

first converted into a reactive form by swelling in acidic or basic solutions (Gascoigne and Gascoigne, 1960; Reese, 1963). Thus these organisms do not seem to produce a soluble C_1 enzyme.

The C_1-C_x concept has been extensively studied in Trichoderma viride and T. koningi. It was shown that cell-free culture solutions of these fungi were able to solubilise cotton fibres. The C_1 enzyme has been isolated from T. viride culture filtrates by Selby and Maitland (1967). They showed that the enzyme did not act on cellobiose or carboxy methyl cellulose and did not lose its ability to solubilise cotton in the absence of the C_x component. The mechanism of action of the C_1 enzyme is thus still obscure, although many different hypotheses have been presented (Norkrans, 1967; Eriksson, 1967).

The cellulolytic enzymes are nearly always induced, in contrast to pectinases, which are often constitutive (Eriksson, 1967). In some cases, in hydrolysis of cellulose, the products partially or totally inhibit enzyme secretion (Eriksson, 1967) they are thought to be significant in pathogenesis for they would not be secreted in the presence of other readily available carbon sources (Norkrans, 1967).

It is considered that they are unlikely to have any part in the early stages of pathogenicity in many diseases, for the cell wall remains essentially intact (Albersheim, Jones and English, 1969).

Hemicellulose

These polysaccharides include polymers such as arabino-galactan, xylan, arabinoxylan and araban (see chapter 4). They yield on total hydrolysis xylose, arabinose, galactose, mannose, glucose, rhamnose and uronic acids (Mirov, 1967).

They can be cold water soluble, but in general they are not readily extracted from lignified tissue (Hamilton and Thomson, 1959).

Enzymes utilising hemicellulose as substrates

Pathogens produce a wide range of these enzymes e.g. Byrde and Fielding (1965) showed that Sclerotinia fructigena produced an extracellular α-L-arabinofuranosidase. Also Rhizoctonia solani (Van Etten, Maxwell and Bateman, 1967), and Sclerotium rolfsii (Van Etten and Bateman in Albersheim et al, 1969; Bateman, 1968) grown on bean hypocotyls produce hemicellulose-hydrolysing enzymes.

Proteins

It is considered that large amounts of structural protein are present in the walls of growing cells (Priestly, 1943; Ginzburg, 1961). Lamport (1965) reports a protein containing hydroxyproline in the walls of callus cells of at least eleven plant species in amounts of more than 1% dry weight. In experiments, Lamport showed with hydroxyproline-rich and hydroxyproline-poor cell walls that the latter could be completely solubilised by cellulases. On the other hand, cellulase treatment of hydroxyproline-rich sycamore cells, although removing 70% of the wall, left a wall-shaped structure rich in protein, and a galacto-araban component. He suggests that the galacto-araban component covalently joins the protein and cellulose.

Cleland (1968), using reaction kinetics, showed that the protein component is probably not a cytoplasmic contaminant, but electron microscopy (Israel et al, 1968) of rapidly proliferating plant cells e.g. carrot cells which had incorporated labelled proline, led to the conclusion that no

cell wall protein existed.

Sadava and Chrispells (1969), using carrot phloem parenchyma cells plasmolysed after proline incorporation and before coating with emulsion, have obtained evidence of a protein moiety accumulating in plant cell walls.

Proteases

Protein degradation occurs in many diseases e.g. in the disease of cucumber, Cucumis sativus, caused by Pseudomonas lachrymans. The bacterium produces a protease which could hydrolyse casein, serum albumin and cucumber leaf proteins (Keen, Williams and Walker, 1967). The bacterium produced the enzyme in culture and during pathogenesis in cucumber angular leaf spot. In diseased leaves, concentrations of amino acids derived from cucumber proteins increased concommitantly with increases in bacterial numbers and bacterial protease.

Lipids and lipases

Significantly, this area of substrates utilised by pathogens has not been considered in very much detail, even though they are presumed major components of cell membranes (Weier and Benson, 1967).

Recently, however, Keen and Williams (1969) have investigated lipase and esterase and fatty acid synthetase activity in Plasmodiophora brassicae infected cabbage hypocotyls. They demonstrated that lipids did not accumulate in the host cytoplasm during disease development, and pronounced changes in the three enzyme activities did not occur. Specific activities of the fatty acid synthetase were 40-70 times higher in extracts of isolated P. brassicae plasmodia than in non-infected or infected tissue extracts. This could explain the accumulation of lipids in the parasite but not in the infected cell cytoplasm.

Fungal extracellular enzymes.

A number of enzymes have been localised on the outer surface of the cell membrane of many fungal cells (Rothsten, 1965; Bull and Chester, 1966). They include carbohydrases, proteases, lipases and phosphatases. They are hydrolysing enzymes that convert non-utilisable substances, into products readily utilised by the cell.

It appears that these enzymes are secreted through the spaces of the cell wall (Burger et al, 1961; Friis and Ottolenghii, 1959 a,b in Zalokar, 1965). Some of the enzymes are only loosely bound and can be obtained by simple washing of cells e.g. Neurospora crassa α-glucosidase (Eberhardt, 1961).

A further example: laminarinase is produced extracellularly in bacteria and fungi and is readily isolated from culture filtrates. It is actively secreted for it is produced extensively during logarithmic growth and not released suddenly at autolysis. It seems that it is synthesised at the cell wall or protoplasmic membrane. It is a constitutive enzyme in many fungi e.g. Aspergillus, Rhizopus and Penicillium. (Bull and Chester, 1966).

Recently Reese et al, (1968) compared exo-α-glucanases from Aspergillus niger and an Endomyces sp. with α-glucosidase from A. niger, Penicillium parvum and Paecilomyces varioti and exo-β-1,3-glucanases from Sporotrichum pruinosum and Basidiomycete sp. with β-glucosidases from A. niger, Penicillium melini and almond emulsin. They showed that dimers, trimers and tetramers of glucose were substrates for both exo-glucanases and glucosidases. Exo-glucanases acted much more rapidly on the longer oligomers. Exo-glucanases remove glucose (or dimer)

from the non-reducing ends of glucose polymers also. They act with inversion of configuration; glucosidases with retension.

Other extracellular enzymes include pentosanases in Aspergillus niger and Trichoderma viride (Simpson, 1959) and mannan- and galacto mannan- hydrolysing enzymes in Sclerotium rolfsii (Bateman, 1968). Whitney et al (1969) demonstrated extracellular cellulase C_x production by Verticillium albo-atrum in vitro and in lucerne. Cellulase, mannanase, xylanase, β-glucosidase, aryl-β-glucosidase, mannosidase and xylosidase have been obtained from culture filtrates of the fungi Stereum sanguinolentum, Fomes annosus and Chrysosporium lignorum and analysed by Ahlgren and Eriksson (1967). Proteases from Streptomyces fradiae and Aspergillus oryzae have recently been isolated and studied (Morihara and Tsuzuki, 1969).

Lyr (1959 a.b.c.d.e) examined extracellular carbohydrase production of ten species of wood-rotting fungi (brown rotters e.g. Fomes marginatus), and wood-inhabiting fungi (white rotters e.g. Trametes versicolor) on different media containing pectin, cellulose, amylose, xylan, glucose or a combination of all substrates as carbohydrate-source, and he determined the rate of production with time. He showed that generally mycelial formation and enzyme-production of the single species have no relation to each other. The typical wood-rotting fungi mostly have a high enzyme-production and a low mycelium formation. The brown-rotting fungi had the highest activity in the enzymes investigated.

Amylase, xylanase and pectinase were demonstrated in all culture-filtrates, but cellulase was secreted by most species only in the presence of cellulose. Considerable differences existed between species with regard to extent of the induction

of enzyme-formation by a specific substrate. Usually pectinase and cellulase synthesis was enhanced by the corresponding substrate. Amylase induction was very weak while xylanase was not induced at all. He considers the latter two enzymes to be constitutive.

The course of enzyme secretion showed an enzyme-specific dharacter. The xylanase-activity generally reached its maximum before the logarithmic phase was ended, but amylase and cellulase maxima usually lay in the "autolytic" range. Pectinase reached its maximum usually concurrently with mycelial production.

Although the species belonged to very different taxonomical and ecological types, no peculiarities specific for species or groups with regard to enzyme formation, (apart from quantitative differences) could be stated. Lyr supposed then that a uniform system for synthesis and regulation existed.

So,for an organism to be successful as a pathogen it must be capable of producing extracellular enzymes capable of degrading host material. If it can do this then the organism can act pathogenically.

CHAPTER 4

POLYSACCHARIDES OF PINUS RADIATA NEEDLES

A study of the relation of extracellular enzymes of D. pini to pathogenicity must be based on a knowledge of the substrates in the living host tissue of the pine needle. We have seen in chapter 2 that early pathogenesis may involve a combination of enzymic attack on the cell membranes and part of the cell walls of the mesophyll tissue, together with a disruption of host metabolism and possible blockage of phloem transport. The basic substrates for enzymic attack by the pathogens may therefore be the polysaccharides of the cell wall and the lipoprotein of the cell membrane or plasmalemma.

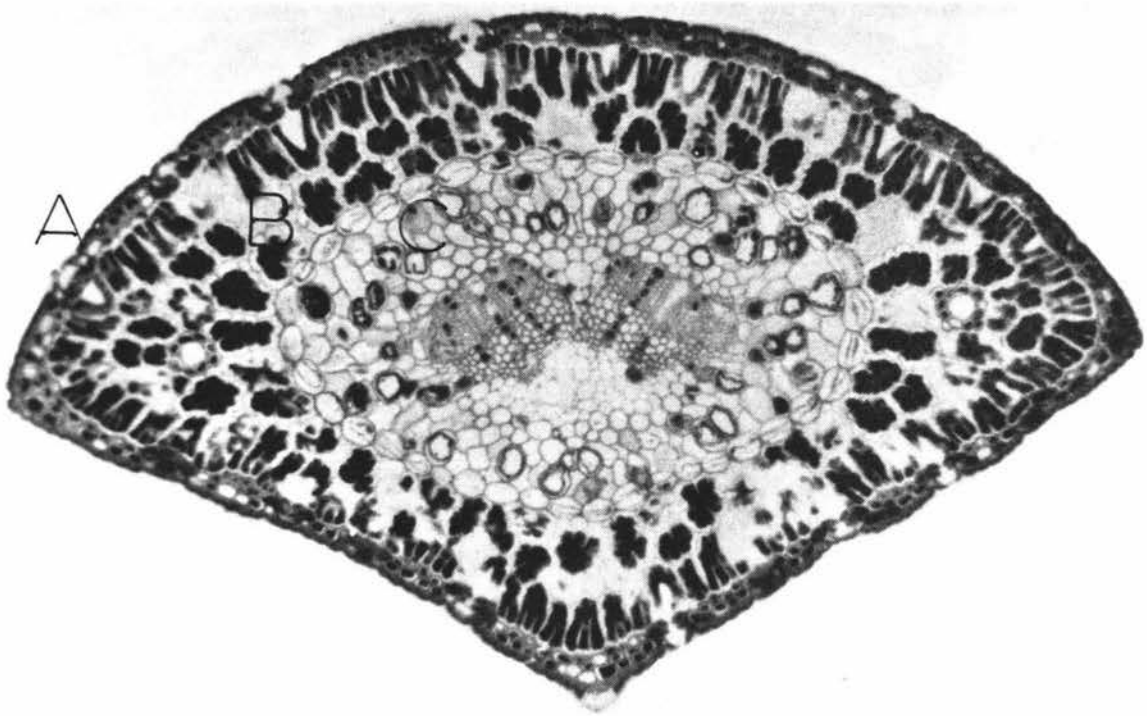
In this chapter the cell wall structure of P. radiata needles is studied in some detail to provide reference for the enzymological studies in chapters 6 to 9. Other chemical features are also discussed briefly in so far as they may bear on the disease caused by D. pini.

General structure and chemistry.

There are three distinct regions in the transverse section of the P. radiata needle; the outer dermal tissue, the middle mesophyll tissue and the inner vascular region (see plate 4.1, page 40).

The dermal tissue consists of thick-walled epidermal cells, whose outer walls are covered with a thick layer of cutin, a highly polymerised mixture of lipids and waxes. This cutin layer may be relatively impervious to D. pini, since penetration by freshly germinated hyphae is almost exclusively via the stomata. Direct penetration of the epidermis has, however,

PLATE 4.1



T.S. of P. radiata needle showing
A. outer dermal tissue
B. middle mesophyll tissue and
C. inner vascular region. (x 150)

been observed with fragmented older hyphae (see chapter 2). The cutin, which is apparent as a waxy bloom on needles and young shoots, consists principally of long chain polyesters of hydroxy acids, chief amongst which are the C_{16} acids, juniperic and salvinic.

The stomata of P. radiata needles are sunken well into the hypodermis or outer mesophyll, and located on all surfaces of the needle in a regular array. Subsidiary and guard cells surrounding the stomata are partially lignified. The inner mesophyll, or mesophyll proper is the main photosynthesising and starch- and fructosan- storage tissue (Mirov, 1967) consisting of undifferentiated parenchyma cells. These seem to be the first cells destroyed during D. pini infection.

The central vascular region of the needle is separated from the mesophyll by the endodermis. The vascular bundles (two in each P. radiata needle) are surrounded by transfusion tissue consisting of dead lignified tracheids and living parenchyma, and by an inner layer of sclerenchyma cells (Mirov, 1967).

The vascular bundles of the needles are not penetrated by D. pini during infection but phloem blockage, possibly associated with resin plugs, appears to be induced by the disease.

Two medial resin ducts are present in the mesophyll of P. radiata needles, and these conduct an oleoresin, rich in volatile oils. The composition of this oleoresin has been investigated in pine needles of other species, and is considered of chemotaxonomic significance. The chief constituents are terpenes, among which 1-α-pinene, 1-β-pinene and borneol are always present in varying amounts (Mirov, 1967).

Any role played by these oleoresins in D. pini pathogenesis

is, however, completely obscure. It is not known whether the plugs observed in the vascular bundles in diseased portions of needles are indeed oleoresin in nature (Rook, personal communication).

Needles of P. radiata and other Pinus spp. contain several other classes of secondary metabolites, including glucosidic flavonoids (e.g. quercetin and kaempferol), phenolic glycosides such as piceine, and unidentified alkaloids (Mirov, 1967). Compounds of these classes are known to play a part in the resistance of some plants to pathogens (see chapter 1).

The principal structural component of the needle is undoubtedly polysaccharide (see later this chapter), but soluble carbohydrates play an important part in the metabolism of the pine needle. Starch and fructosans are found in the soluble sugar fraction of pine needles. In some species seasonal variations have been noted in starch, simple sugars and lipid content. Raffinose content of P. pinaster needles has been correlated with cold hardiness. Simple sugars identified in P. radiata needles in the course of this work (see chapter 9), are glucose, fructose, sucrose, raffinose and galactose. Cyclitols, such as pinitol (5-methyl ester of d-inositol), are also present in the needles of many gymnosperms, including the genus Pinus (Mirov, 1967).

The Cell Wall.

Polysaccharide and lignin both contribute to the structure of the pine needles, and they are predominantly associated with the cell wall.

The polysaccharides shown to be present in pine needles include cellulose, hemicellulose A, branched and linear

hemicellulose B and pectin.

Cellulose

Cellulose was shown in this investigation to be the major insoluble polysaccharide of pine needles (see Table 4.1, .page 44).

Hemicellulose.

Several polysaccharides of this group have been identified in the wood of various members of the genus Pinus e.g. arabinogalactan has been found in P. palustris (Forman and Englis, 1931), P. banksiana (Bishop, 1957) and P. contorta (Laidlaw and Smith, 1962), and galactoglucomannan and a glucomannan in P. sylvestris (Timell, 1957).

Wadman et al (1954) have determined the structure of an arabinogalactan from P. jeffreyi, and shown it to be a highly branched molecule with L-arabinose residues in terminal positions.

Galactoglucomannans and glucomannans are major components of the hemicellulose fraction of pine wood. From P. strobus Timell (1957) isolated a galactomannan which was 10% of the total dried weight of neutral sugar residues.

The monose content of the polymers varies considerably (see Table 4.1, .page 44).

Corroborative work on similar polymers from other conifers, using methylation, periodate oxidation and partial hydrolysis, showed them to be composed of linear 1,4 linked β-D-glucopyranose and β-D-mannopyranose residues, with D-galactose residues constituting 1,6 linked branches (Timell, 1962).

Many carbohydrate workers now regard galactose-, glucose- and mannose- containing polysaccharides with a low ratio of galactose: glucose as glucomannans (Harwood personal communication) and in this light interpret Timell's (1957) P. sylvestris polysaccharide (see Table 4.1, page 44) as a glucomannan

TABLE 4.1

Monose content of galactoglucomannans from *Pinus* sp.

Source	galactose	glucose	mannose	reference
P.sylvestris wood	0.3	1.0	1.0	Timell, 1957.
P.strobus wood	1.0	1.0	1.8	Timell, 1957.
P.elliotii wood	0.9	1.0	2.9	Hamilton *et al* 1958.
P.palustris wood	1.0	1.2	3.0	Hamilton *et al* 1958.

TABLE 4.2

Results of extraction and hydrolysis of carbohydrates of needles.

200 gm freeze dried pine-needles extracted as in appendix 10.

Polysaccharide	wt. recovered (gms)	% dry wt. of total dry wt. recovered in cell wall	component sugar
cellulose	60	50	Ga : G : M
hemicellulose A	4	4	1.2 : 1.0 : 1.8
hemicellulose B	8	7	--------
pectin	9	8	galacturonic acid
lignin	36	31	--------
soluble polysaccharides			
starch and fructosans	37	--	--------
		% hemicellulose B.	Ga : G : M
linear hemicellulose B per gm hemicellulose B	0.4	40	1.0 : 1.0 : 3.0
			Ga : G : M
branched hemicellulose B	0.5	50	2.0 : 1.0 : 1.0
			A : X : R
per gm hemicellulose B			2.5 : 4.3 :< 2.0

Ga - galactose G - glucose
M - mannose A - arabinose
X - xylose R - rhamnose

with galactose artifact.

On the other hand Meier (1960), working on a glucomannan from spruce showed that there was random distribution of the hexose residues, most of which were in β-1,4 linkage. However, 6-O-α-D-galactopyranosyl-D-mannose Ga-α-1,6-M, and a trisaccharide Ga-α-1,6-M-β-1,4-M were isolated. Galactose was present as a minor component. Meier considered this to be either a galactomannan or a galactoglucomannan, in addition to the glucomannan. Timell (1962) suggests that this was in fact a galactoglucomannan.

Pectin.

There is apparently no literature on this polysaccharide in pine wood or needles.

Lignin.

The nature of lignin is very complex, most preparations are believed to be heterogeneous. The coniferyl alcohol glucoside, coniferin, is found in the cambial sap of pines (Mirov, 1967). Lignin is a major component of the cell walls of P. radiata needles (see Table 4.2, page 44).

Identification of P. radiata needle polysaccharides.

Methods and materials

Carbohydrate extraction

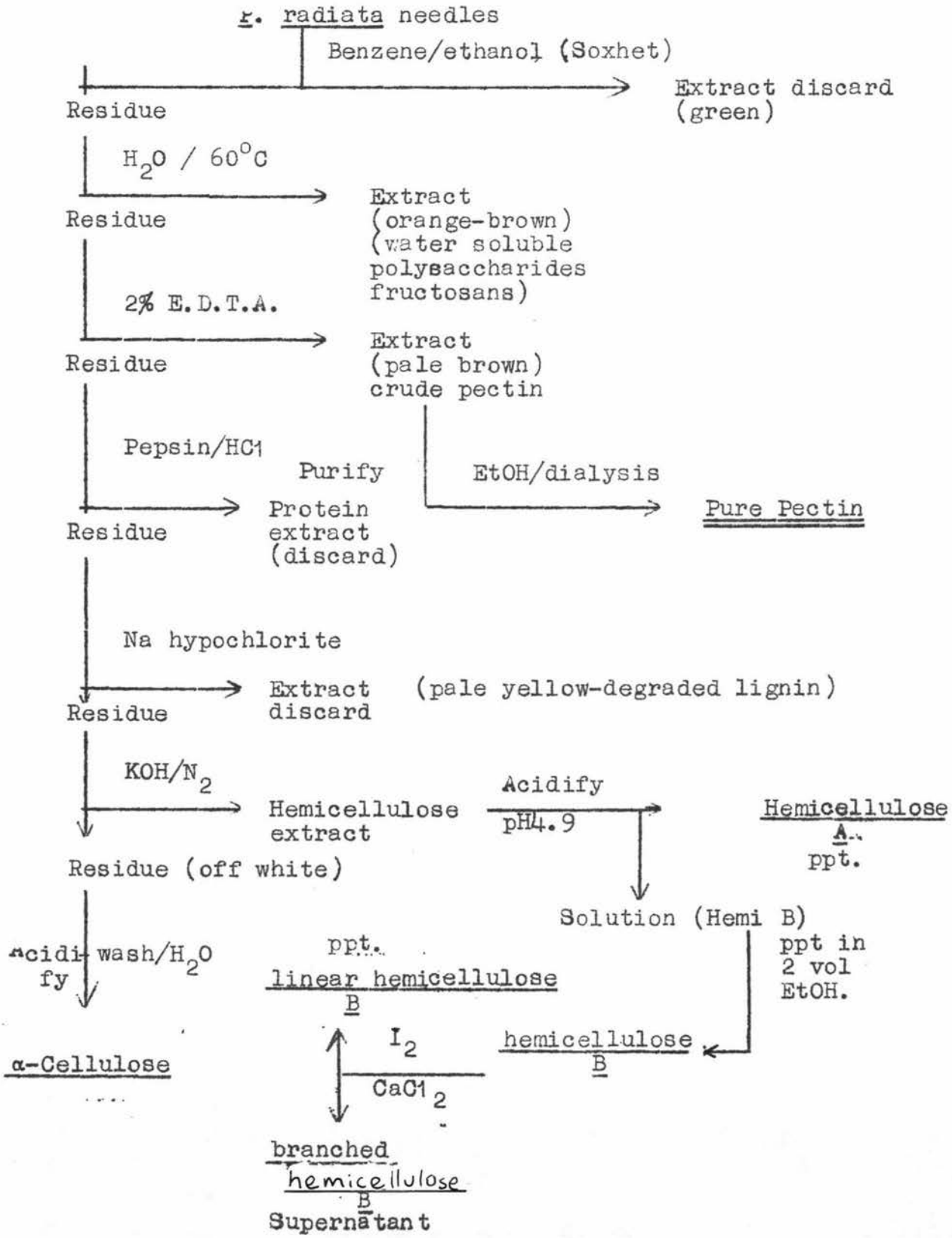
The polysaccharides from 200 gm. freeze dried P. radiata needles were separated by the method detailed in appendix 9,p.114. and outlined in figure 4.1, page 46. Cellulose, pectin, hemicellulose, and hemicellulose B, branched and linear, were isolated. The yield of each fraction is given in Table 4.2, page 44.

Total hydrolysis of polysaccharide fractions.

A portion of each polysaccharide fraction was hydrolysed

FIGURE 4.1

Scheme for fractionation of *P. radiata* needles into polysaccharide constituents.



with 1 ml of 72% sulphuric acid for two hours at ambient temperature, and incubated in a sealed tube at 110°C for 4 hours (R.W.Bailey personal communication). The solution was neutralised with barium carbonate and decanted from the precipitate then subjected to thin layer chromatography on cellulose (Wolfram et al, 1965).

Chromatography (see appendix 7, page 110).

Plates were developed in two dimensions. The sample was spotted at the origin (in one corner of the plate). Also a standard volume of glucose solution of known concentration was spotted on the plate (in the other corner). The plate was then run in the first solvent system. After 2 hours the plate was removed, dried thoroughly, turned through 90°, and a further volume of glucose solution applied in the third corner. Then the plate was run in the second solvent system. The plate was sprayed with aniline phthalate to locate the sugars which were then related to the internal standards of glucose (see appendix 7).

Quantitative estimation of component sugars.

A quantitative method for the estimation of the component sugars was developed, using a Photovolt colourimeter-densitometer to read the spot intensity on the developed plate. The aniline phthalate sprayed plate, with coloured sugar spots, was placed on the movable stage and run through the photometer set to read 454 mμ. The intensity of each spot was recorded on a trace as a peak. An integrator, incorporated into the system, allowed the accurate calculation of the area under the trace. The intensity of each hexose spot and each pentose was compared with a standard glucose or xylose spot respectively. Hence the concentration of the original solution in mg/ml could be

calculated. Rhamnose gives a grey colour with aniline phthalate spray. This colour does not absorb well at 454 mμ and hence its estimation was less reliable.

Individual polysaccharides.

Cellulose.

The cellulose from P. radiata needles was subjected to further analysis. An infra-red spectrum was compared with a known sample of cellulose. Spectra were taken using a Shimadzu infra-red spectrograph, model IR27G. The infra-red spectra of both compare favourably (see Table 4.3, page 49 and figure 4.2, page 51).

The X-ray powder diffraction pattern of both these samples was taken using a Phillips X-ray generator with a Debye-Scherrer powder camera (see appendix 1 page 102). Both the I.R. and X-ray work were kindly done by Dr. J. Kirkman, Soils Dept., Massey University.

Discussion.

Cellulose.

The values obtained in tables 4.2 and 4.3 are comparable for both the pure cellulose and the P. radiata samples. The reason for the high background in the infra-red spectrum of the pine sample is the fibrous nature of the material which prevented the obtaining of suitable spectra. The diffuse pattern obtained from the X-ray pattern of the pine sample can be explained similarly. The fibres of the pine sample were very much coarser than those of the standard cellulose sample.

Thus from these results and the chromatographic analysis, the sample from P. radiata needles is most likely cellulose.

Hemicellulose A.

The ratio of galactose: glucose: mannose is 1.2:1.0:1.8,

TABLE 4.3

Infra red spectra results of cellulose.

Standard cellulose sample		P. radiata cellulose sample.	
3350	cm^{-1}	3375	cm^{-1}
2875		2875	
1430	broad peak	1430	
1000		1000	
600		550-600	very broad peak.

TABLE 4.4

X-ray analysis of cellulose (see plate 4.1, page 40).

	Standard cellulose radius in m.m.	A°	P. radiata cellulose sample radius in m.m.	A°
A.	21.04	2.4914	20.61	2.5410
B.	14.23	3.6387	14.02	3.6926
C.	10.23	5.0510	10.20	5.0510

Dr. J. Kirkman (Soils Dept.) kindly performed this work.

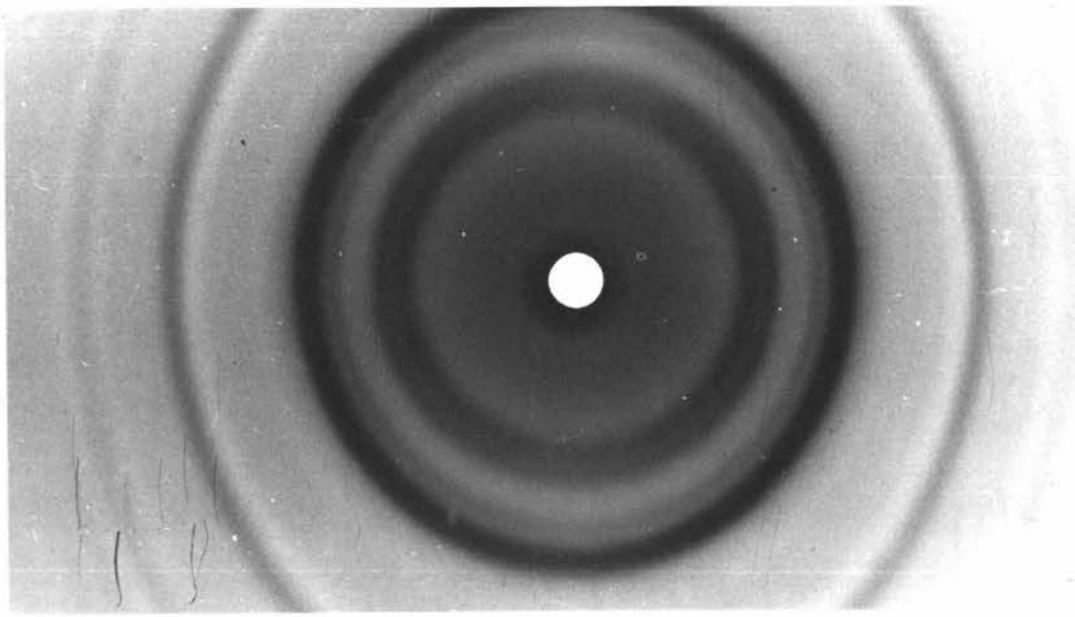
TABLE 4.5

Hemicellulose B (branched) hydrolysis products on incubation with various enzymic activities (cf Table 7.3, page 73).

Original substrate	hydrolysis products from hemicellulose B branched.
arabinoxylan	arabinose, xylose
arabinogalactan	arabinose, galactose
xylan	xylose

PLATE 4.2

a.

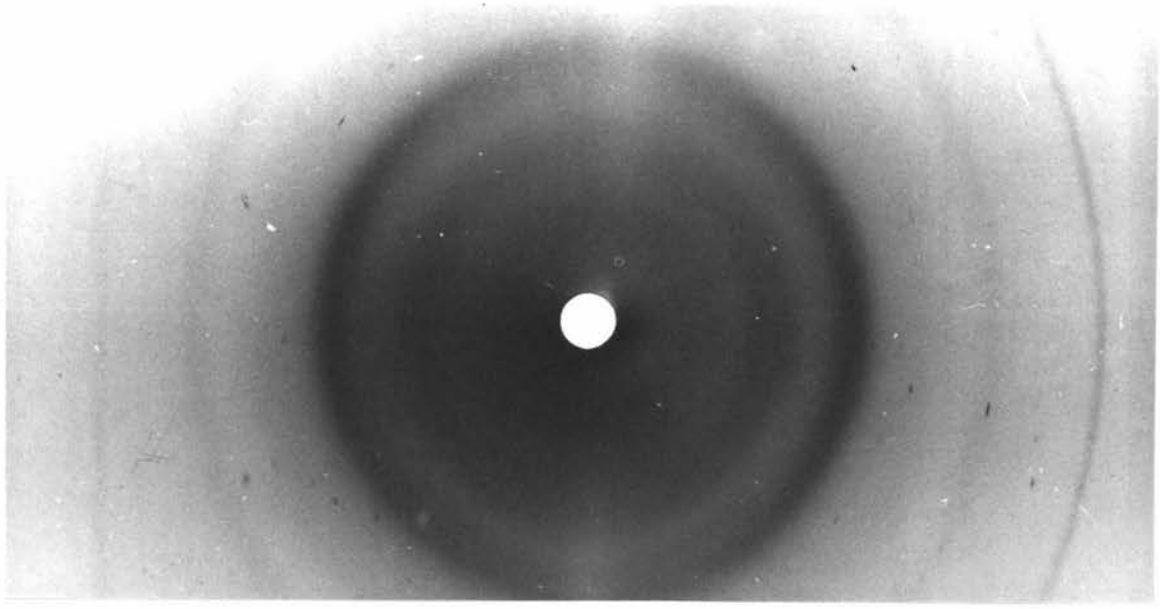


A

B

C

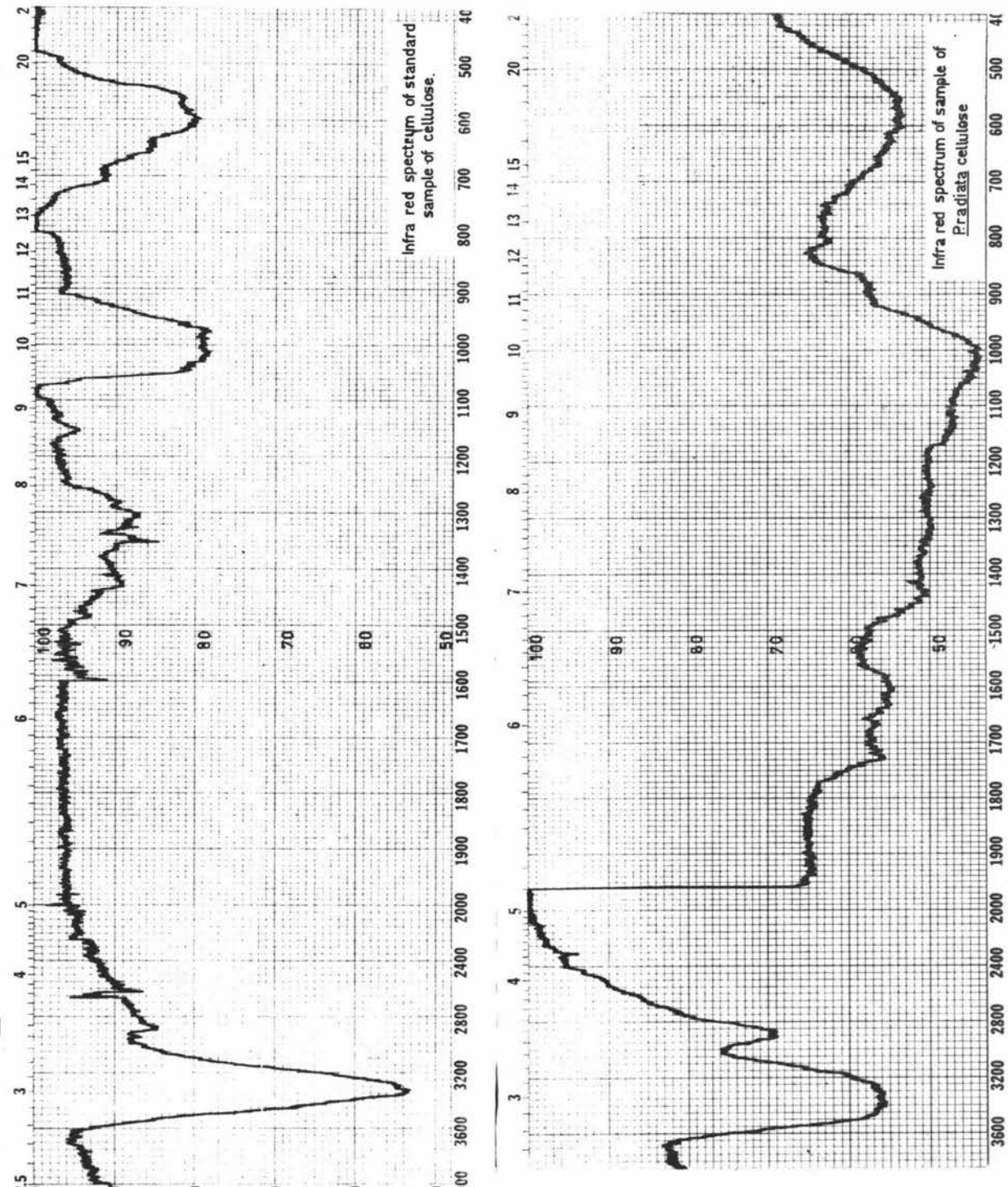
b.



a. X-ray powder diffraction pattern of standard Whatman cellulose sample. (x 5)

b. X-ray powder diffraction pattern of cellulose from *P. radiata* needles. (x 5)

Figure 4.2 Infra red spectra



This suggests a galactoglucomannan quite similar in composition to that in P. strobus wood (Timell, 1957). However, neither the P. strobus galactoglucomannan, nor any other of the polymers of Table 4.1 is described as a hemicellulose A or B component.

Hemicellulose B linear.

The ratio of the same three sugars is 1.0:1.0:3.0 also suggesting a glactoglucomannan quite similar in composition to those in P. elliotii and P. palustris wood. (Table 4.1, page 44).

Hemicellulose B branched.

Six monoses were detected in this polysaccharide fraction. While no evidence of the various polymers present was obtained from this analysis, evidence of likely polymers was obtained from enzymological work, discussed in chapter 7. (see Table 7.3, p.73). From the table it is seen that activities, produced by growth on arabinogalactan, arabinoxylan, or xylan hydrolyse the branched hemicellulose B to varying degrees. These activities each yielded the manose componentsof their respective polysaccharides on incubation with branched hemicellulose B (see Table 4.5, page 49). From this work it is probable that the branched hemicellulose B fraction contains an arabinogalactan and either an arabinoxylan or xylan, or both. The glucose and mannose in the branched hemicellulose B fraction may be present in an additional polymer, of glucomannan type.

Pectin.

The polysaccharide isolated in this step contained only D-galacturonic acid and hence it is probably pectin.

General Discussion.

Further work, either to separate the components of the polysaccharide fractions, in particular branched hemicellulose B, or to establish the homogeneity of the particular fractions, is required. Harwood (personal communication) at the New Zealand Forest Research Institute, Rotorua, has isolated a glucomannan with galactose, glucose and mannose in the ratio 0.1:1.0:3.0, and a galactoglucomannan with the sugars 1.0:1.0:3.0 from bisulphite treated P. radiata wood pulp. Probably these polysaccharides are similar to the components of the pine-needle hemicellulose fractions; the glucomannan in the branched hemicellulose B and the galactoglucomannan in the linear hemicellulose B fraction.

CHAPTER 5

ISOLATION AND GROWTH OF DOTHISTROMA PINI

Two isolates were used in this investigation, the first isolate was kindly provided by the Forest Pathology Laboratory, of the F.R.I. Rotorua, from their stock collection. The second was isolated from diseased P. radiata needles from Te Wera State Forest in Taranaki.

Method of Isolation.

Needles, with D. pini stroma visible within red bands, were placed on a microscope slide and incubated overnight at 30°C in a petri dish containing water in order to maintain the humidity near 100%. This resulted in conidia being extruded from the stroma. The conidia were removed with a sterile loop and transferred to drops of sterile water, identified microscopically as D. pini spores, and then streaked out on a 10% malt agar plate (see appendix 3).

After 10-12 days at 25°C, depending on the spore concentration in the inoculum, substantial mycelial growth had occurred and areas of spores were present. There was a low degree of bacterial contamination on some plates. Spores from uncontaminated areas (judged under the microscope) were streaked out on fresh malt plates, and after similar time and conditions substantial growth was again observed, this time with no evident contamination.

The Te Wera isolate produced large amounts of the red pigment on malt agar medium. The pigment was secreted into the medium and in some cases was present in concentrated spots parallel with the streaks of fungus on the plate. The F.R.I. isolate also produced a red pigment in liquid culture, but

this was not observed on malt agar plates of this strain (see chapter 2).

Growth of D. pini.

Growth of D. pini in culture was comparatively slow with hyphal extension occurring at approximately 5mm per week. The very dense nature of the mycelium, the slow growth, and the moist shiny appearance of masses of spores, liken the cultures on agar to bacterial cultures. Spore production on malt agar usually occurred 9-15 days after inoculation of the plates. The conidia formed were apparently secondary ones, very often concentrated in small areas, but apparently not in stromata.

Stock cultures were maintainted on 10% malt agar plates and slopes, and subcultured at two-monthly intervals. For toxin production (see chapter 2) the fungus was grown in 10% malt medium (see appendix 3) shake culture. For production of enzymes, the fungus was grown on glucose Raulin's medium (see appendix 2) in shake culture.

Sanders(1969) showed that D. pini in synthetic culture grew optimally at pH4.2 (range 2.2-7.8) with an optimum growth temperature of 22°C. Ivory (1967b) using a 3-4% malt extract medium (pH3.5, pH range pH2.2-5.5), found germination at 18°C, and optimum growth at 11°C. The differences in these two sets of results could be due to strain differences or to different varieties as claimed by Thyr and Shaw (1964) and Ivory (1967a).

For the experiments reported in this chapter, growth was followed at 20°C in a medium of initial pH4.0. For studies on enzyme production cultures were grown at pH4.0 and pH6.5, again at 20°C.

Growth of D. pini in simple synthetic medium.

(a) Raulin's solution, buffered at pH4.0 (citrate-phosphate) glucose as carbon source.

Two and a quarter litres of Raulin's solution were prepared with glucose as carbon source, and 11 x 200mls dispensed into eleven one litre erlenmeyer flasks and autoclaved (see appendix 2). A suspension of D. pini spores was prepared by flooding twenty plates of the sporing fungus each with 15mls of sterile water and scraping the spores off the mycelium with a sterile loop. The suspensions from all plates were decanted and pooled. The spore count, estimated using a haemocytometer, was 2×10^5 spores per ml. The eleven flasks were then inoculated each with 20mls of this spore suspension and shaken at 20°C. Flasks were harvested at 0, 2, 4, 7, 9, 12, 18, 24, 30, 36 and 42 days after inoculation; the mycelium was filtered off, dried and weighed (see Table 5.1), the pH of the filtrate measured, and the total protein in the supernatant estimated according to the method of Lowry et al (1951).

Results.

See Table 5.1, figures 5.1, 5.2, pages 57, 59, 60 respectively.

Discussion

From figures 5.1 and 5.2 no lag was evident in the onset of exponential growth which continues for the first 9-10 days. After this a linear rate of growth appears to be followed for 12 days, and after 24 days the growth decreases, finally ceasing at about 30 days.

The total protein concentration (figure 5.1, page 59) in the culture filtrate increased roughly in parallel with mycelial weight, but there was no logarithmic phase, the rate being more or less linear for the first 18 days, and slowly dropping off till cessation of growth about the thirtieth day (except for an initial phase where protein concentration appears to occur at a more rapid rate).

TABLE 5.1

Table showing average mycelial mat weight, average pH and average protein concentration for D. pini grown on glucose-Raulin's solution pH4.0.

day	mycelial mat weight	pH	protein concentration mg/ml.
0	0,0001	4.000	0
2	0.0095	4.124	0.71
4	0.0239	4.190	0.82
7	0.1098	4.490	1.19
9	0.2419	4.535	1.58
12	0.5490	4.325	2.01
18	1.1573	3.865	2.93
24	1.6387	3.856	3.27
30	1.7531	3.846	3.56
36	1.7647	3.853	3.65
42	1.7653	3.863	3.70

TABLE 5.2

Table of growth, pH and protein concentration from D. pini culture.

Polysaccharide	Incubation period (weeks)	final pH	Growth	Protein concentration mgm/ml original culture filtrate
agar	16	2.6	1	0.45
amylopectin	3	3.2	5	2.10
amylase	3	3.2	5	2.20
araban	5	3.05	3	1.10
arabinogalactan	15	3.88	2	0.58
arabinoxylan	14	3.9	3	0.82
carboxymethyl cellulose	10	5.42	4	1.36
cellulose (P. radiata needles)	16	4.22	2	0.72
cellulose (cotton wool)	16	4.28	2	0.74
cellulose acetate	16	4.00	0	0

Polysaccharide	Incubation period (weeks)	final pH	Growth	Protein concentration mgm/ml original culture filtrate
chitin	16	4.00	0	0
chitosan	16	4.00	0	0
dextran	7	3.10	5	2.51
galactan	16	4.48	1	0.12
glucomannan	16	4.00	0	0
β-glucan	7	3.2	5	2.62
hemicellulose A (*P. radiata* needles)	10	5.1	3	1.24
hemicellulose B (*P. radiata* needles)	10	5.3	3	1.21
hemicellulose B branched (*P. radiata* needles)	10	5.3	3	1.15
inulin	16	5.9	4	1.71
laminarin	8	5.10	5	2.61
lichenin	16	3.82	0	0
mannan	16	4.22	1	0.41
methyl cellulose	16	4.00	0	0
nigeran	16	4.00	0	0
pectin (citrus)	8	2.91	3	0.97
pectin (*P. radiata* needles)	8	2.84	3	0.79
polygalacturonic acid	9	2.67	2	0.70
xylan	10	4.92	3	0.99

Scale of growth (visual estimate).

0 - nil
1 - poor
2 - moderate
3 - average
4 - good
5 - excellent

The fungus failed to grow on those polysaccharides represented by a zero, indicating that both citric and tartaric acids in the growth medium were unsuitable as growth substrates.

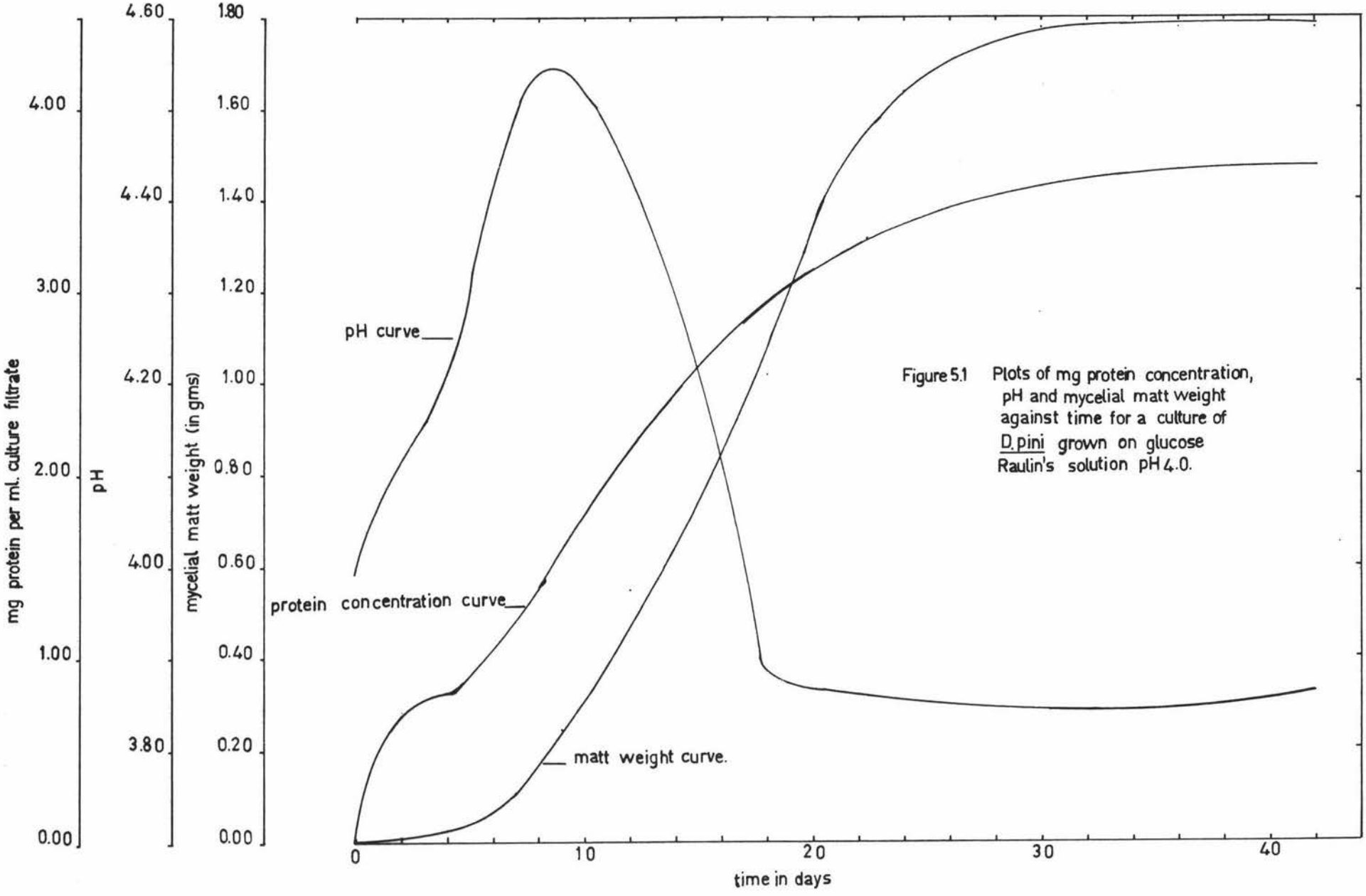


Figure 5.1 Plots of mg protein concentration, pH and mycelial matt weight against time for a culture of D. pini grown on glucose Raulin's solution pH 4.0.

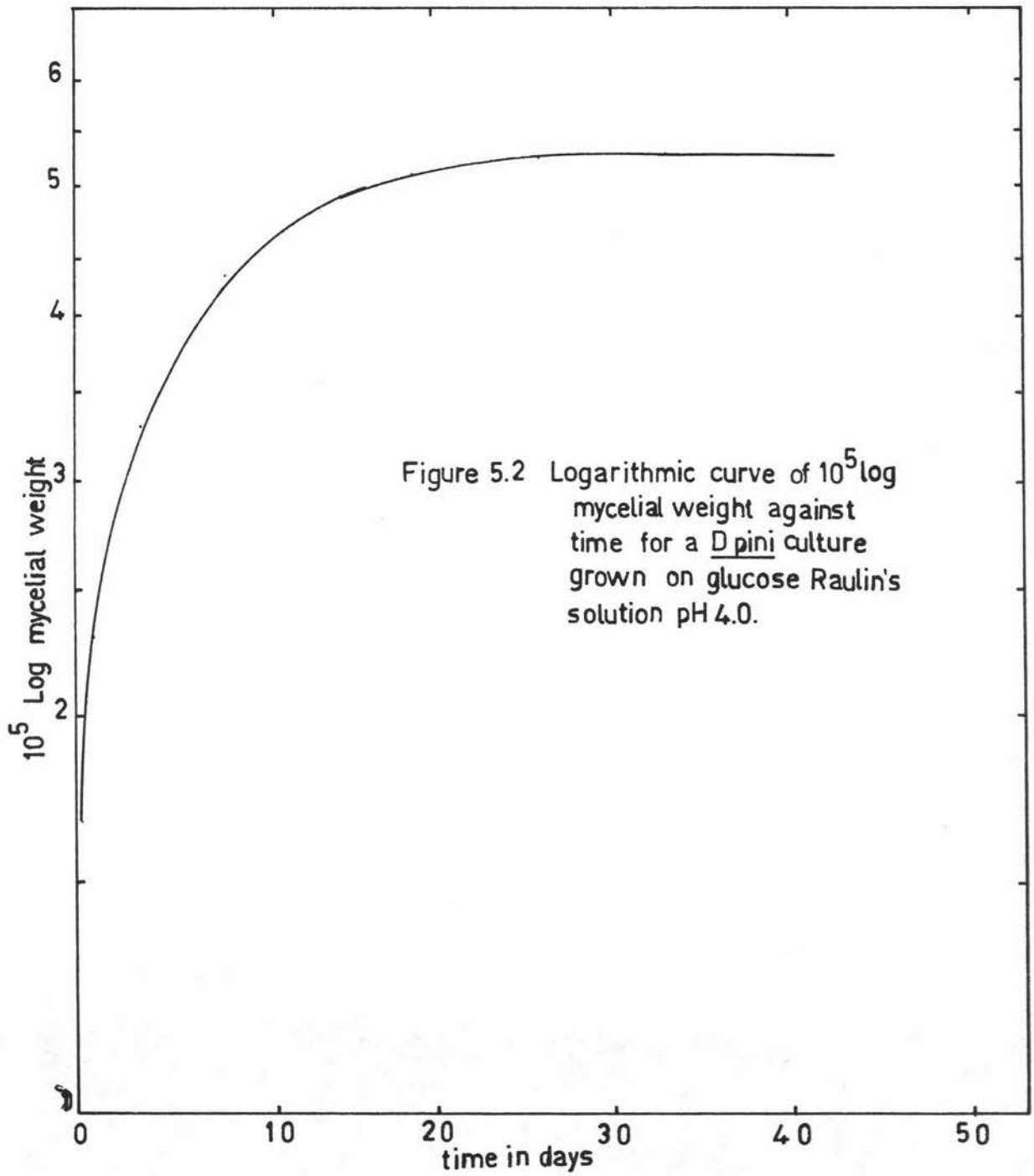


Figure 5.2 Logarithmic curve of 10^5log mycelial weight against time for a D pini culture grown on glucose Raulin's solution pH 4.0.

During the exponential phase of growth the pH of the culture medium (figure 5.1) rises from pH4.00 to 4.52 but falls again during linear growth, till a level is reached at about pH3.88 after 18 days. The initial pH rise may be due to citrate utilisation (resulting in lowered buffer capacity), while the subsequent pH drop possibly reflects production of an organic acid, resulting from glucose metabolism. The source of these pH changes was not investigated.

The linear rather than exponential growth may be explained by the fact that hyphae elongate linearly and do not divide exponentially, as do yeasts and bacteria.

(b) Unbuffered Raulin's solution; glucose and polysaccharide as carbon source.

No systematic study of growth rate and pH changes was made, except with the buffered glucose-Raulin's solution reported above. With other carbohydrate substrates, pH changes were close to those observed with glucose. However, when citrate-phosphate buffer was omitted from the culture medium, pH changes were the same as those recorded in the presence of buffer for the first 22 days, after which a rapid rise to pH6.5 - 6.8 occurred. Mycelial weight continued to increase.

(c) Buffered Raulin's solution; polysaccharides as carbon source.

Most of the polysaccharides considered were not hydrolysed when incubated with the various fractions of D. pini grown on Raulin's solution (see chapter 6). Because many of these polysaccharides are considered important structural components of plant tissue, it was necessary to determine whether they would support growth of D. pini when they were the sole

carbohydrate source.

Method.

200mls Raulin's salt solution buffered at pH4.0 was used with the polysaccharides listed in table 5.2 (page 57) as carbon source at 1% concentration. A stock spore solution was prepared (see above) at a concentration of 7×10^5 spores/ml and 10mls inoculated into each flask. The flasks were incubated at 20°C with shaking. One ml samples were taken at 5 day intervals (20 day intervals for those carbohydrates which supported poor growth) and both the pH and protein concentration (Lowry et al, 1951) measured and thin layer chromatography carried out to determine hydrolysis products. Samples were examined microscopically at intervals to check for contamination.

After a period of 3 to 16 weeks, depending on the rate of growth of D. pini on the particular polysaccharide used as carbon source, the flask was harvested and mycelium and insoluble carbohydrate removed by centrifugation at 5000 x g for 2 minutes. The culture filtrate of 120-180mls was then concentrated overnight by vacuum dialysis (ultrafiltration) (Smith, 1958) and assayed for enzyme activity. (see chapter 7)

Results

See Table 5.2, page 57.

Discussion.

There were several polysaccharides that failed to support any detectable growth of D. pini viz. cellulose and its derivatives (other than carboxy methyl cellulose), chitin, chitosan, glucomannan (ex P. radiata wood) lichenin and nigeran. No hydrolysis products were detected chromatographically in the concentrated culture filtrate, and no increase in concentration of protein in the culture medium occurred during

sixteen weeks incubation.

However, with the other polysaccharides, (Table 5.2) significant growth was achieved after three to fourteen weeks. The P. radiata polysaccharide fractions each supported D. pini growth moderately well, though the glucomannan from P. radiata wood pulp did not support detectable growth.

CHAPTER 6

CARBOHYDRASES PRODUCED IN GLUCOSE-RAULIN'S SOLUTION.

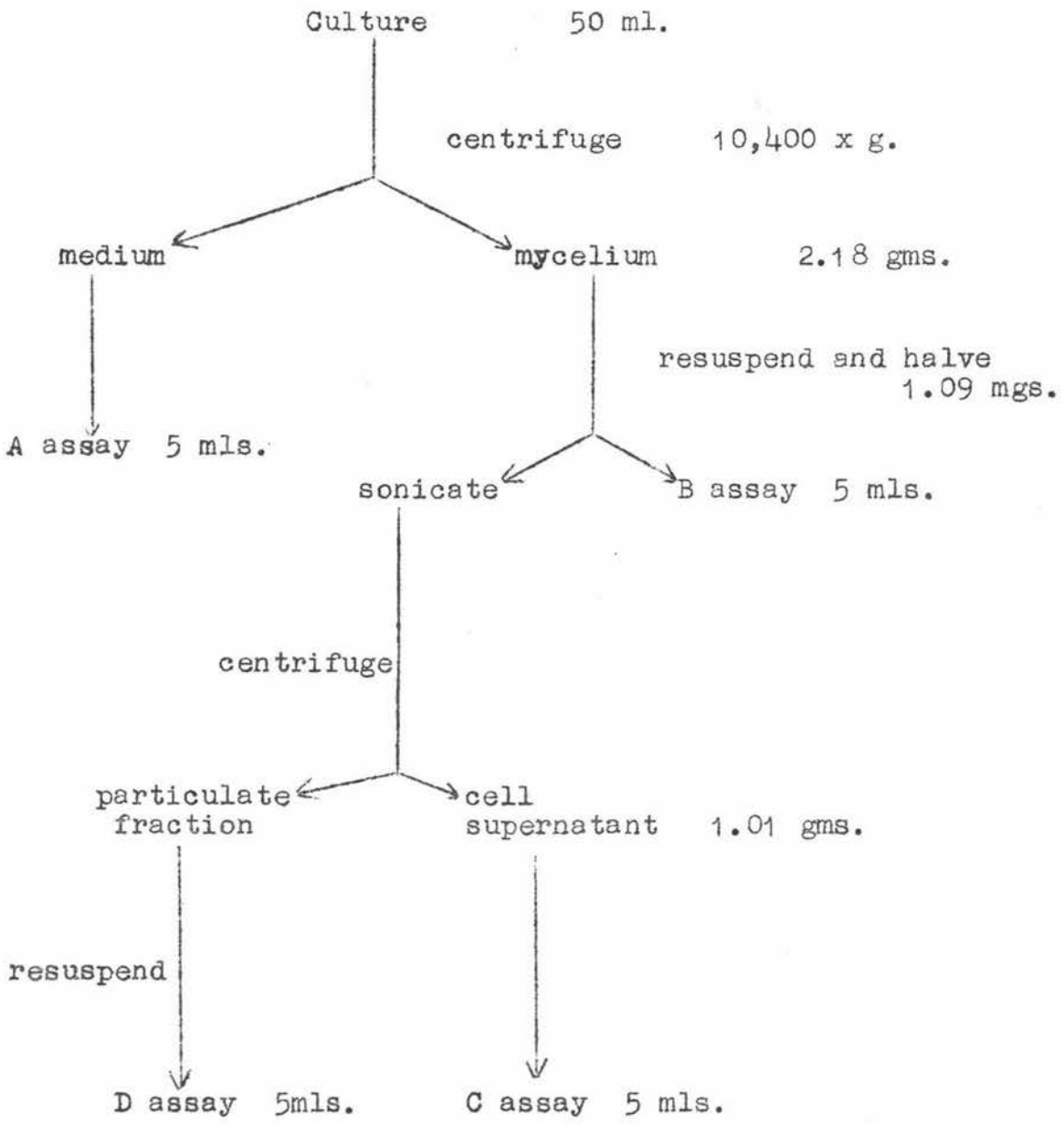
Twenty-two day old cultures in 200ml glucose-Raulin's solution pH4.0 in 500ml flasks (see chapter 5 and appendix 2, page 105) were harvested by centrifugation, 5 min at 4900 x g in a Sorval RCB2 refrigerated centrifuge. The culture filtrate was decanted, and 4gms of the mycelial precipitate resuspended in 50mls of citrate-phosphate buffer pH4.0. The culture filtrate of 200mls was concentrated overnight to 20mls by vacuum dialysis, and protein determined by the method of Lowry et al (1951). The resuspended mycelium was halved; one half was retained intact while the other was sonicated in a MSE sonicator, model L666-L667, until no whole cells were evident. The sonicated fraction was then centrifuged for 5 minutes at 10,400 x g. The cell free extract was decanted and the particulate fraction resuspended in citrate-phosphate buffer pH4.0. This procedure resulted in four fractions all at pH4.0. Two controls were also prepared. See figure 6.1

A. culture filtrate
B. resuspended intact mycelium
C. cell supernatant (cell free extract)
D. resuspended particulate fraction
E. control - citrate/phosphate buffer pH4.0
F. control of boiled concentrated culture filtrate.

Carbohydrase activities were detected by incubation of the fractions with selected substrates at 25°C under tol ene (to prevent micro-organism fermentation) followed by chromatographic identification of hydrolysis products. Aliquots of 5µl were taken at 0 hrs and 24 hrs and spotted on a thin layer chromatogram of cellulose. Details of method, including solvent systems, location sprays and quantitation of results,

FIGURE 6.1

Illustrating fractionation of D. pini liquid culture.



Controls.

E citrate/phosphate buffer pH4.0.

F boiled concentrated culture filtrate.

are described in appendix 7.

Results

See Table 6.1 page 67.

Discussion.

From Table 6.1 it is evident that D. pini produces in glucose-Raulin's solution α-1,1, β-1,3, α-1,4, β-1,4, α- and β-1,6 glucosidase activities, α-1,3 and β-1,2 fructofuranosidase activities, α-1,6 activity, together with amylase and laminarinase activities. No further separation or purification of these activities was followed.

The experiment was semiquantitated by comparing spot size and colour intensity with a standard plate of glucose standards, ranging from 2-30 µg of glucose. Except for the pentosanases, all enzyme activities detected were found in all four fractions. So, although a particular activity may be predominantly intracellular or surface bound, the fact that it is secreted is probably significant in the nutrition of the organism.

TABLE 6.1

Hydrolysis of carbohydrates by fractions of D. pini cultures grown on glucose Raulin's solution pH4.0. (See appendix 1, page 102 for structures).

Oligosaccharide	Hydrolysed	Degree of hydrolysis spot intensity + fraction number *			
		A	B	C	D
cellobiose	+ **	2	2	2	3
gentiobiose	+	2	2	2	3
isomaltose	+	2	2	2	2
lactose	-	0	0	0	0
laminaribiose	+	2	2	3	2
maltose	+	2	3	2	2
α-D-melibiose	+	2	2	2	2
β-D-melibiose	-	0	0	0	0
sophorose	-	0	0	0	0
sucrose	+	2	3	2	3
α α-trehalose	+	2	3	2	3
turanose	+	2	2	2	1
raffinose	+	2	2	2	2
Polysaccharide					
Pentosans					
araban	-	0	0	2	1
arabinogalactan	-	0	0	0	0
arabinoxylan	-	0	0	0	0
xylan	-	0	0	0	2
hemicellulose A (P. radiata needle)	-	0	0	0	0
hemicellulose B linear (P. radiata needle)	-	0	0	0	0
hemicellulose B branched (P. radiata needle)	-	0	0	0	0
Hexosans					
agar	-	0	0	0	0
amylopectin	+	2	2	2	1
amylose	+	2	2	2	1
cellulose (P. radiata)	-	0	0	0	0
cellulose (cotton wool)	-	0	0	0	0
dextran	-	0	0	0	0

Polysaccharide	Hydrolysed	Degree of hydrolysis spot intensity + fraction number *			
		A	B	C	D
galactan	-	0	0	0	0
β-glucan	+	1	1	2	1
inulin	-	0	0	0	0
laminarin	+	2	2	2	2
lichenin	-	0	0	0	0
mannan	-	0	0	0	0
nigeran	-	0	0	0	0
pectin (citrus)	-	0	0	0	0
pectin (*P. radiata* needle)	-	0	0	0	0
polygalacturonic acid	-	0	0	0	0
substituted hexosans					
carboxymethyl cellulose	-	0	0	0	0
chitin	-	0	0	0	0
chitosan	-	0	0	0	0
methyl cellulose	-	0	0	0	0
cellulose acetate	-	0	0	0	0

* Fraction number as described in text and in figure 6.1

** + hydrolysis achieved with component monose sugars being detected in each case.

\+ 0 less than 2µg sugar produced on hydrolysis

1 equivalent to 2-9.9µg sugar produced on hydrolysis

2 equivalent to 10-19.9µg sugar produced on hydrolysis

3 equivalent to 20-30µg sugar produced on hydrolysis.

CHAPTER 7

ENZYME PRODUCTION BY D. PINI GROWING ON POLYSACCHARIDES

This chapter reports an investigation to determine (a) which polysaccharides support growth for D. pini, and (b) which polysaccharide - degrading enzymes are produced in culture, with purified P. radiata polysaccharides or polysaccharides from other sources as sole growth substrate. Pectinases and cellulases have received the predominant attention of pathologist investigating the enzymes of plant pathogens (see chapter 3) and they are therefore considered in more detail in this chapter.

Methods and materials.

Raulin's solution, without glucose, buffered at pH4.0, was used with the carbohydrates in Table 7.1 as the sole growth substrate at 1% concentration. Flasks were prepared as in appendix 2, page 105.

A stock spore suspension was prepared (see chapter 5), at a concentration of 7×10^5 spores per ml. and 10mls were inoculated into each flask. The flasks were incubated at 20°C with shaking. One ml samples were taken at 5 day intervals. Both the pH and protein concentration were measured (Lowry et al, 1951), and portions were subjected to thin layer chromatography to determine the free sugars produced during culture.

Flasks were examined microscopically at intervals to check for contamination.

After a period of three to sixteen weeks, depending on the rate of growth of D. pini on the particular polysaccharide

used, the flask was harvested and mycelium and insoluble carbohydrate removed by centrifugation as in chapter 6. The culture filtrate (about 95mls) was concentrated to 10mls by vacuum dialysis. Enzyme activities in each concentrated culture filtrate were assayed semiquantitatively (see chapter 6), pH was measured, and total protein concentration estimated (Lowry et al, 1951). In addition individual assays were performed for particular polysaccharidase activities.

Results.

The results of the assays are reported in Tables, 7.1, 7.2, 7.3, and 5.2, pages 71,72,73,57, respectively.

Discussion.

Of the 29 polysaccharides used in this experiment, methyl and acetyl cellulose, chitin and chitosan, glucomannan, lichenan and nigeran all failed to support growth of the fungus. No hydrolysis products were detected chromatographically in the culture filtrate nor did increase in concentration of protein in the culture medium occur during sixteen weeks incubation (Table 5.2). The polysaccharides which were hydrolysed yielded their component monosaccharides, e.g. P. radiata hemicellulose A gave galactose, glucose and mannose.

The scale of growth was based on a visual estimate of the amount of mycelium.

As seen in Table 7.1, those glucose polymers with α-1,4, α-1,6, β-1,3 and β-1,4 bonds support the best growth. Each pine needle polysaccharide supported moderate growth of the fungus.

Specific assays (see appendix 11) were carried out for several enzyme activities and the results are presented in Table 7.2. The viscometric and titrimetric assays for poly-

TABLE 7.1

Table of polysaccharides and identified hydrolysis products. *D. pini* was grown on the selected polysaccharide, and the culture filtrate, after harvesting, was tested for hydrolytic activity towards that same polysaccharide, as listed below.

Polysaccharide	hydrolysis product intensity							
	G	Ga	F	M	A	X	Gu	R
agar	1	1	0	0	0	0	0	1
amylopectin	3	0	0	0	0	0	0	0
amylose	3	0	0	0	0	0	0	0
araban	0	0	0	0	2	0	0	0
arabinogalactan	0	2	0	0	1	0	0	0
arabinoxylan	0	0	0	0	1	2	0	0
carboxymethyl cellulose	3	0	0	0	0	0	0	0
cellulose (cotton wool)	2	0	0	0	0	0	0	0
cellulose acetate	0	0	0	0	0	0	0	0
cellulose (*P. radiata* needle)	2	0	0	0	0	0	0	0
chitin	0	0	0	0	0	0	0	0
chitosan	0	0	0	0	0	0	0	0
dextran	3	0	0	0	0	0	0	0
galactan	0	1	0	0	0	0	0	0
glucomannan (*P. radiata* wood)	0	0	0	0	0	0	0	0
β-glucan	3	0	0	0	0	0	0	0
hemicellulose A (*P. radiata* needle)	1	1	0	1	0	0	0	0
hemicellulose B linear (*P. radiata* needle)	1	1	0	1	0	0	0	0
hemicellulose B branched (*P. radiata* needle)	2	2	0	2	1	1	0	1
inulin	0	0	3	0	0	0	0	0
laminarin	3	0	0	0	0	0	0	0
lichenin	0	0	0	0	0	0	0	0
mannan	0	0	0	1	0	0	0	0
methyl cellulose	0	0	0	0	0	0	0	0
nigeran	0	0	0	0	0	0	0	0
pectin (citrus)	0	0	0	0	0	0	2	0
pectin (*P. radiata* needle)	0	0	0	0	0	0	2	0
polygalacturonic acid	0	0	0	0	0	0	2	0
xylan	0	0	0	0	0	2	0	0

0 equivalent to less than 2µg sugar produced on hydrolysis

1 equivalent to 2-9.9µg sugar produced on hydrolysis

2 equivalent to 10-19.9µg sugar produced on hydrolysis

3 equivalent to 20-30µg sugar produced on hydrolysis

2µg monose was the limit of resolution with the technique used.

G	-	glucose	A	-	arabanose
Ga	-	galactose	X	-	xylose
F	-	fructose	Gu	-	galacturonic acid
M	-	mannose	R	-	rhamnose

TABLE 7.2

Table of enzymes assayed from culture of *D. pini* grown on various polysaccharides.

Growth substrate	Enzyme assayed	assay type *	activity + (mg/min/ml)	reference No to assay in appendix 11.
carboxymethyl-cellulose	cellulase Cx	a)	2.6	8 i)
		b)	350	8 ii)
		c)	0.67	8 iii)
cellulose (cotton wool)	cellulase Cx	a)	0	8 i)
		b)	0	8 ii)
		c)	0.1	8 iii)
cellulose (*P. radiata* needle)	cellulase Cx	a)	0	8 i)
		b)	0	8 ii)
		c)	0.2	8 iii)
dextran	dextranase	a)	2000	1
β-glucan (barley flour)	β-glucanase	a)	14.5	2
	laminarinase	a)	6.6	3
laminarin	laminarinase	a)	8.3	3
	β-glucanase	a)	3.8	2
inulin	inulinase	a)	0.2	4
pectin (citrus)	1) pectin esterase PE	d)	25	5
	2) pectin lyase PL	a)		7
	3) polygalacturonase PG	b)		6 ii)
		c)	0.1	6 iii)
		d)		6 i)

Growth substrate	Enzyme assayed	assay type *	activity + (mg/min/ml)	reference No. to assay in appendix 11.
pectin (P. radiata needles)	1 P E	d)	30.2	5
	2 P L	a)		7
	3 P G	b)		6 ii)
		c)	0.2	6 iii)
		d)		6 i)
polygalactu-onic acid	1 P E	d)	26	5
	2 P L	a)		7
	3 P G	b)		6 ii)
		c)	0.1	6 iii)
		d)		6 i)

* Assay type

a) colourimetric assay c) chromatographic assay

b) viscometric assay d) titrimetric assay.

\+ PG and Cx enzyme activities expressed semiquantitatively as the mg uronic acid or glucose (respectively) released / ml / minute. The other enzyme expressed as mg/min/ml.

TABLE 7.3

Substrate for enzyme assay. Pinus radiata needle.

Growth substrate	Cellulose	pectin	hemicell-ulose A	hemicell-ulose B linear	hemicell-ulose B branched
araban	0	0	0	0	1
arabinogalactan	0	1	0	0	3
arabinoxylan	0	0	0	1	2
carboxymethyl-cellulose	3		0	0	0
dextran	1	0	1	1	0
β-glucan	1	0	1	1	0
inulin	0	0	0	0	0
mannan	0	0	1	0	0
pectin (citrus)	0	3	0	0	0
xylan	0	0	0	1	3

Scale semiquantitative estimation (see appendix 7)

0 less than 2µg sugar produced on hydrolysis.

1 2-9.9µg sugar produced on hydrolysis

2 10-19.9µg sugar produced on hydrolysis

3 20-30µg sugar produced on hydrolysis.

Value refers to total sugar concentration in aliquot chromatogrammed. 2µg monose was the limit of resolution of this assay.

galacturonase, PG, were both negative, but the chromatographic assay was positive. All three assays gave good results for a standard enzyme preparation. But because of the longer time involved in the hydrolysis of the substrate and the small aliquot assayed the chromatographic assay is more sensitive than the other two for the D. pini enzyme.

The fungus grew moderately well both on insoluble cellulose (purified cotton wool) (Corbett, 1965), and on pine needle cellulose. Neither viscometric nor colourimetric evidence was obtained for a cellulase enzyme of either type for D. pini grown on either cellulose; but glucose was produced in the medium during growth, and was detected chromatographically. The failure of the viscometric and colourimetric assays with the concentrated culture filtrate, could be explained in terms of the C_1-C_x hypothesis of Reese et al (1950).

From Table 7.3 it is evident that, in general, the enzymes produced in in vitro culture can hydrolyse most of the polysaccharides of pine needles to their component monosaccharides. (Therefore it is possible that the in vitro substrates and the corresponding P. radiata substrates have similar structures). Hemicellulose A and linear hemicellulose B are different structures because of the different action on them of in vitro enzymes (Table 7.3). From this table it is evident that activities, produced by growth on arabinogalactan, arabinoxylan, or xylan hydrolyse the branched hemicellulose B to varying degrees. These activities each yielded the monose components of their respective polysaccharides on incubation with the branched hemicellulose B (see Table 4.5, page 49). From this then it is probable that the branched hemicellulose B contains an arabinogalactan and either an arabinoxylan or xylan, or both.

The enzyme(s) produced by *D. pini* on citrus pectin hydrolysed the *P. radiata* needle pectin.

Therefore it is possible that the *in vitro* substrates and the corresponding *P. radiata* substrates have similar structures.

CHAPTER 8

FURTHER ENZYMES PRODUCED BY D. PINI, WITH PARTICULAR REFERENCE TO LIPASES AND PROTEASES.

Methods and materials.

Flasks of glucose-Raulin's solution were prepared as in appendix 2, page 105 at pH4.0 and pH6.5. Each flask was inoculated with 10mls of D. pini spores at a concentration of 5×10^5 spores/ml, and shaken for 20 days.

Lipase.

8.1 Determination of production of a constitutive soluble lipase by D. pini.

One culture at each pH was harvested and prepared, as in chapter 6, to provide two groups of four fractions each (see figure 6.1).

	Fraction No.
A) culture filtrate (concentrated).	1
B) resuspended intact mycelium.	2
C) cell supernatant (cell free extract).	3
D) resuspended particulate fraction.	4
E) control of appropriate buffer	5
F) control of boiled concentrated culture filtrate.	6

5 mls of each fraction was dispensed into flasks and 10µl of 3-^{14}C triolein (34.5µc/µM) was added to the ten flasks which were then incubated with shaking for 45 minutes in a water bath at 37°C. After this, 5 mls of 95% ethanol was added to inactivate any enzyme present, and the flask shaken for 5 minutes. The mixture was washed into a separatory funnel, shaken with 15 mls of chloroform and allowed to separate. The chloroform fraction was then separated and the procedure repeated twice. The combined extracts were then evaporated to dryness and the

concentrate dissolved in one ml of hexane. Aliquots of each of the twelve concentrates were spotted onto silica gel plates (Merck G, prepared at 0.25 mm thickness and dried for 2 hrs at 120°C). Fatty acid, mono-, di-, and triglyceride markers were also applied, and the plates were then developed, for 30 minutes in benzene : diethyl ether : acetic acid 70:30:1.

Radioactive spots were located and their radioactivity determined by passing the plate through a Packard radiochromatogram scanner model 7200 with recording ratemeter model 385. The amount of radioactivity was determined by measuring the area under the tracing so obtained. A planimeter was used for this measurement. The plate was then sprayed with dichlorofluorescein and examined under U.V. light (λ=254mμ) to determine theposition of the markers. Radioactive spots could then be identified by correspondence of their R_F valves with those of the markers (Clarke and Hawke, personal communication).

The complete experiment was performed in duplicate.

Results.

See Table 8.1 page 79 and figure 8.1 page 80.

Radioactivity in each compound was determined by measurement of the area under the tracing, and expressed as a percentage of the total in the fraction. Total hydrolysis, i.e. percentage loss of radioactive triolein, was then expressed in terms of μ mole triolein hydrolysed per minute per ml of original culture.

Discussion.

In all pH4.0 fractions i.e. those from the culture grown at pH4.0, no evidence of hydrolysis of 3-^{14}C-triolein was found; even when the thin layer plates were overloaded no radioactivity above the levels in the two pH4.0 controls was encountered with any fraction.

TABLE 8.1

Radioactive assay for lipase at pH6.5

Fraction No.	Average % of total 3-^{14}C-triolein				10^{-3} µmoles/min/ml.
	TG	FA	DG	MG	
1	70.2	17.3	6.9	5.6	6.3
2	80.5	6.5	4.1	8.9	1.0
3	58.8	31.4	4.6	5.2	8.6
4	52.9	16.9	9.3	21.7	3.6
5	99.8	0.1	0.0	0.1	0.01
6	99.7	0.1	0.1	0.1	0.01

TG - triglyceride, FA - fatty acid

DG - diglyceride MG - monoglyceride

For experimental details see text.

Radioactivity in each compound was determined by measurement of the area under the tracing, and expressed as a percentage of the total in the fraction. Total hydrolysis i.e. percentage loss of radioactive triolein, was then expressed in terms of µmole triolein hydrolysed per minute per ml of the original culture.

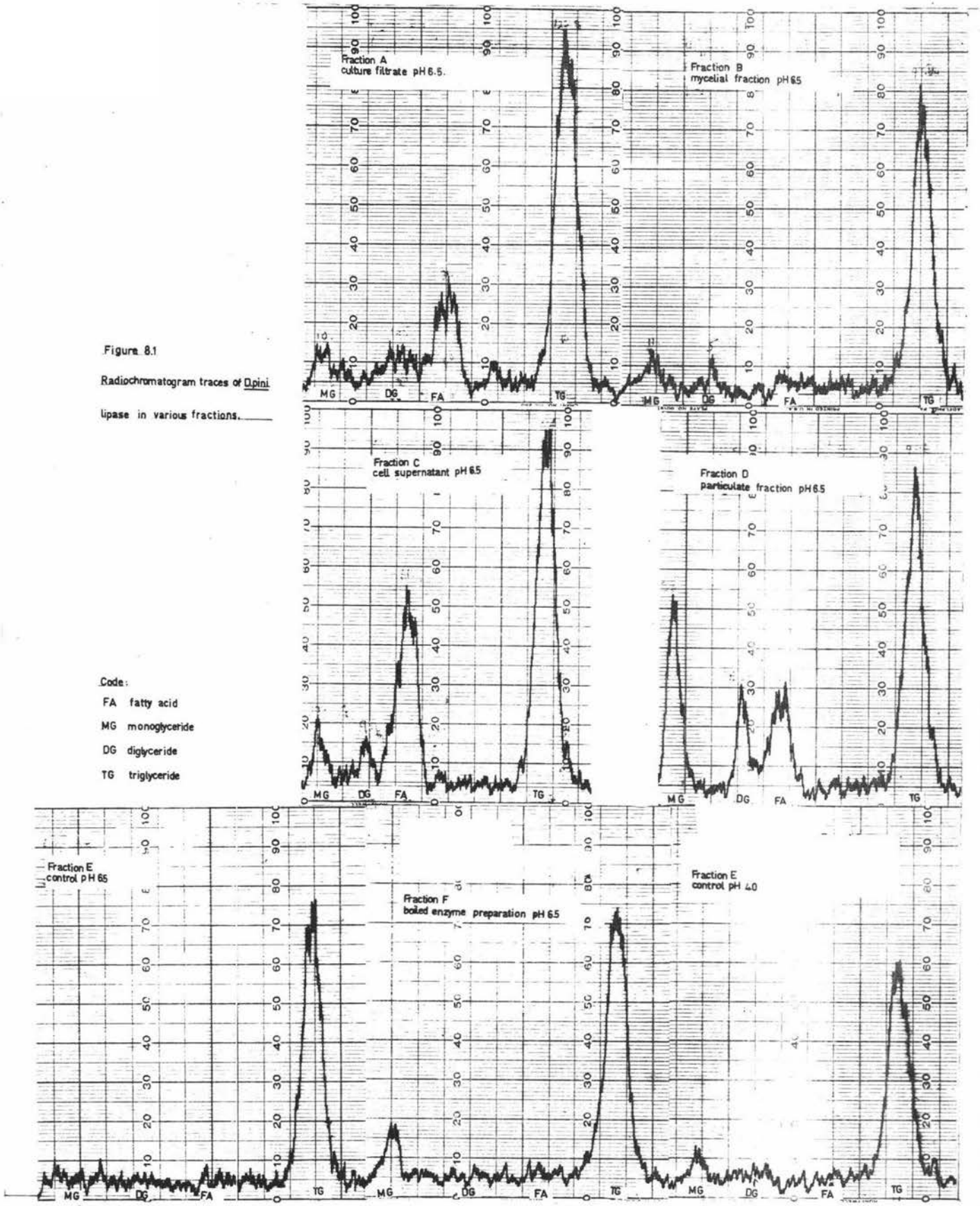


Figure 8.1

Radiochromatogram traces of O.pini lipase in various fractions.

Code:

FA fatty acid

MG monoglyceride

DG diglyceride

TG triglyceride

At pH6.5, hydrolysis of 3-^{14}C-triolein was observed in all four fractions at levels greater than that observed for the appropriate controls (see figure 8.1, page 80). The quantitative values obtained in all four fractions are of the same order of magnitude. Therefore these results show that while little or no hydrolysis is observed in the controls (fractions 5 and 6) there is considerable activity in the culture medium (fraction 1) and a low activity in the mycelium (fraction 2) which is greatly increased on sonicating (fractions 3 and 4).

Conclusion.

The D. pini culture medium buffered at pH6.5 contains a moderately active soluble lipase. The mycelium has a lipase which is either surface bound or intracellular. If it is surface bound it would appear to be weakly so, because of the appreciable activity in the culture medium. Sonicating results in increased activity of both soluble and particulate fractions.

The soluble enzyme(s) (fractions 1 and 3) appear to differ in specificity from the particle bound enzyme(s) (fractions 2 and 4). The soluble activity results in relatively more free fatty acid and less monoglyceride product than the particulate activity. Further work would be needed to confirm these apparent differences. However, the most relevant observation is that D. pini does produce an active extracellular lipase, at least when cultured in Raulin's solution at pH6.5.

8.2 Lipase determination using the cup plate diffusion assay technique.

Confirmation of the lipase activity of D. pini in artificial culture was obtained by using this alternative assay. This assay is described in detail in appendix 9 and the apparatus

illustrated in plate 8.1 page 83.

Materials and method.

The substrate Tween 20 (polyoxyethylene sorbitan monolaurate) was dissolved in citrate phosphate buffer at the appropriate pH. (see appendix 5). Pancreatic lipase solution (3.05mg/ml) was used as a standard.

For the experiment at pH6.5, duplicate D. pini culture media from 20 day old Raulin's grown cultures were diluted and placed in cups flush with the top. Controls consisted of both buffered boiled D. pini culture medium and citrate phosphate buffer at pH6.5. For determination of activity at other pH values, the culture filtrate was first dialysed against citrate-phosphate buffer at the appropriate pH; and controls at the same pH were used.

After 18 hrs incubation at 37°C each plate was developed using a 10% (w/v) solution of copper sulphate. Where hydrolysis had occurred cloudy zones were produced on a clear background.

Results and Discussion.

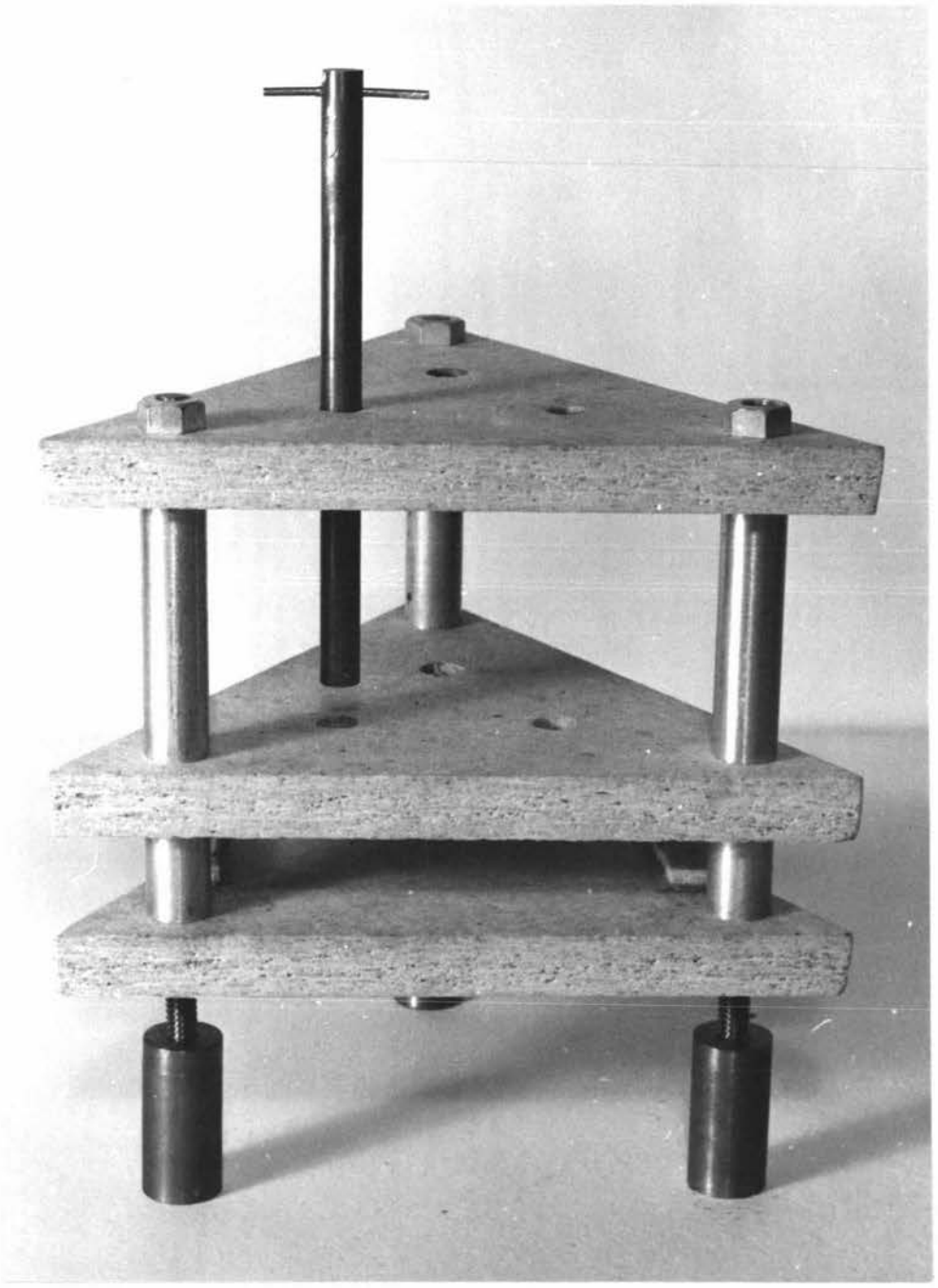
The results of this assay are presented in figures 8.2 and 8.3, pages 84 and 85 respectively.

From figure 8.2 it can be seen that the lipase activity of D. pini culture medium at pH6.5 is equivalent to 9.4 mgm of standard per ml.

Dingle et al (1953) report that the pH of the gel has a marked effect on the size of the zone hydrolysed. They equate maximum zone size with optimum pH for the enzyme.

A plot of zone diameter against pH, averaged over two duplicate experiments, is shown in figure 8.3. This plot shows two regions of maximum activity with regard to pH for the crude Dothistroma lipase at pH5.1 and pH6.5. Thus, either there is

PLATE 8.1



Apparatus for preparing cup plate.

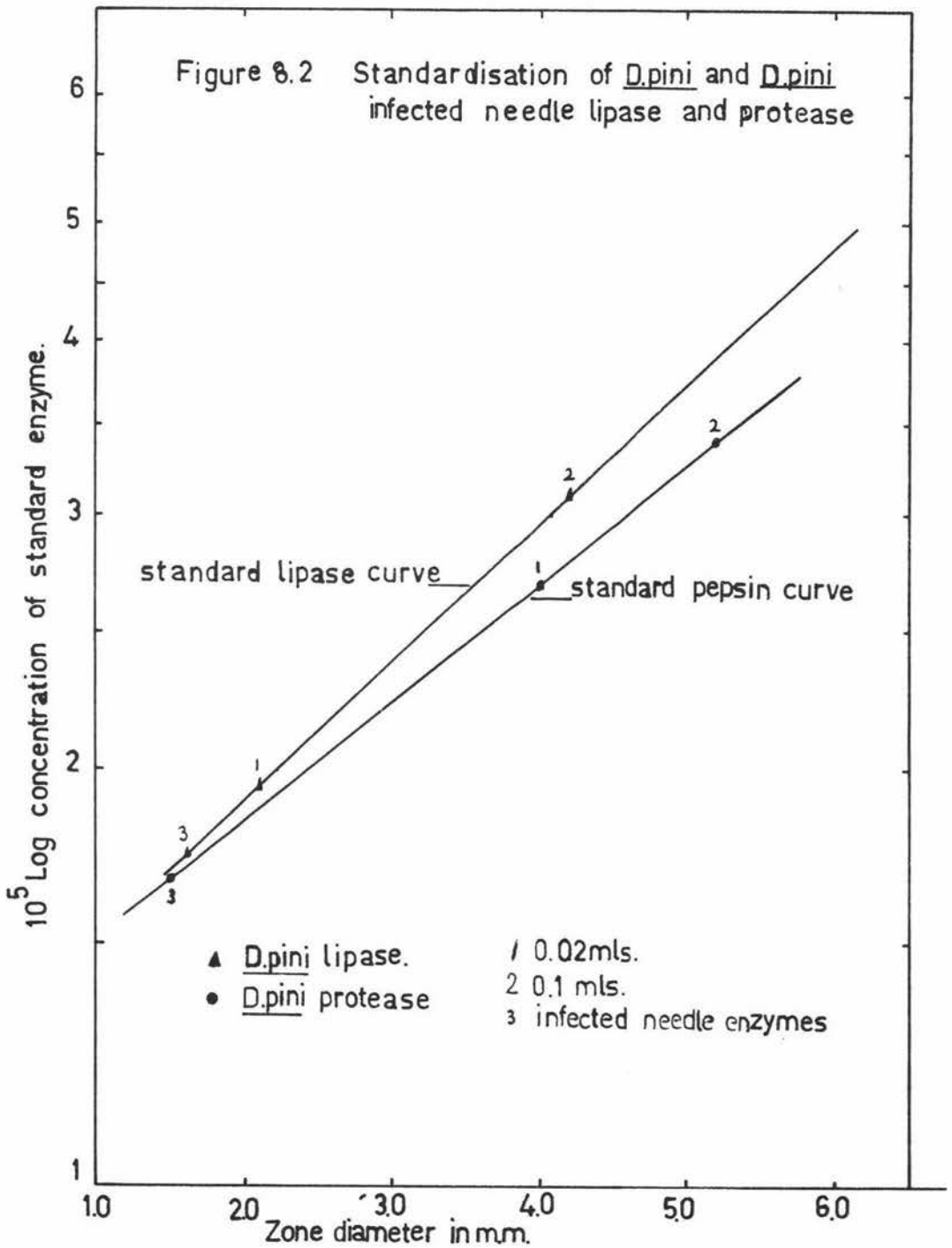


Figure 8.2 Standardisation of D.pini and D.pini infected needle lipase and protease
standard lipase curve
standard pepsin curve
▲ D.pini lipase.
● D.pini protease
1 0.02mls.
2 0.1 mls.
3 infected needle enzymes
10^5 Log concentration of standard enzyme.
Zone diameter in m.m.
1
2
3
4
5
6
1.0
2.0
3.0
4.0
5.0
6.0

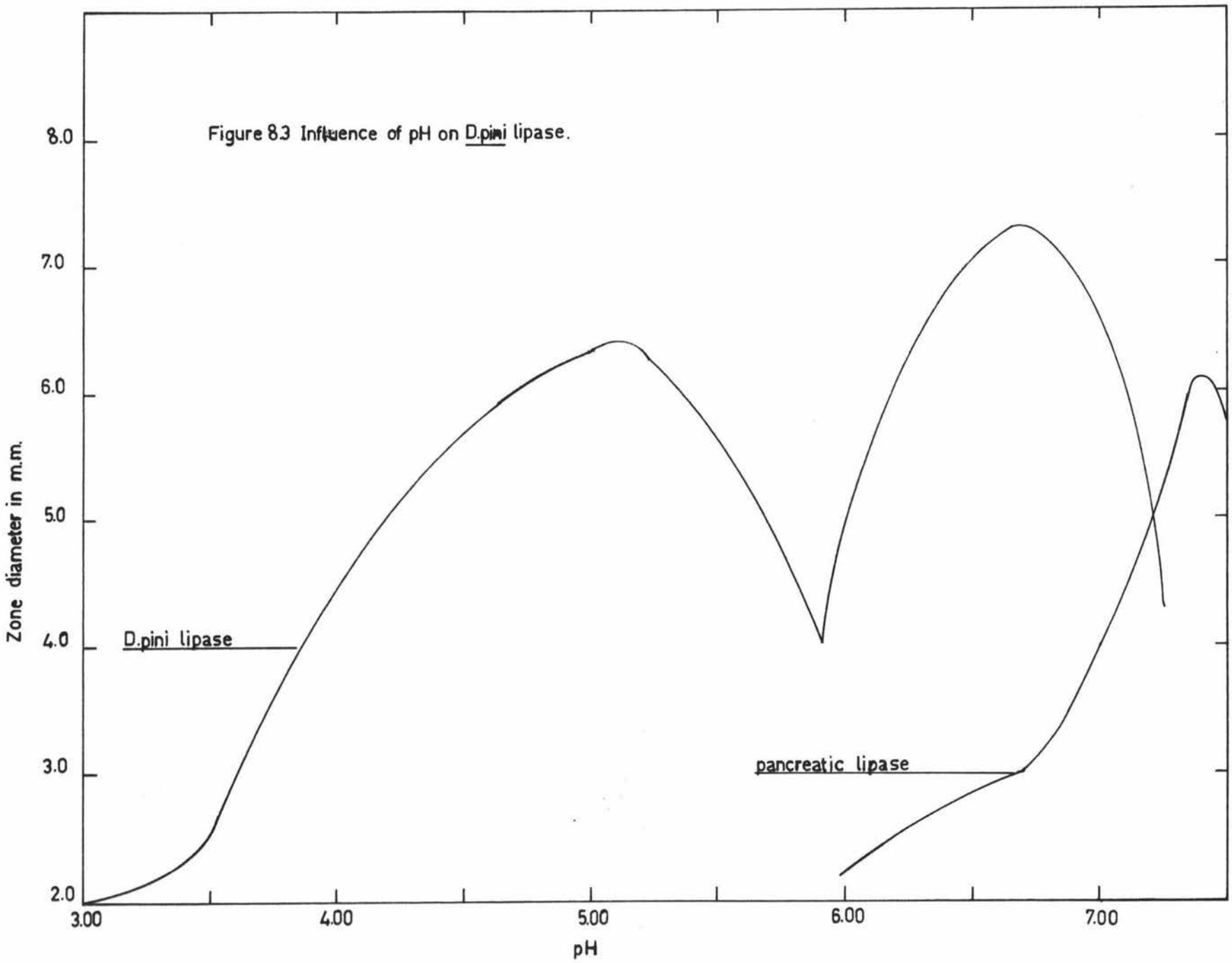


Figure 8.3 Influence of pH on D.pini lipase.

more than one enzyme of lipolytic activity present or the lipase present has two pH optima. The former conclusion supports the work on radiochromatography but further corroborative evidence is required.

Protease.

8.3 Production of a constitutive soluble proteolytic enzyme by D. pini.

The same basic cup-plate assay procedure was used as for lipase assay in section 8.2. A plot of zone diameter versus pH was also prepared as in section 8.2, fractions tested were culture medium, particulate fraction, cell supernatant all at pH6.5, prepared in a similar manner to those in section 8.1. Pepsin (4.25mg/ml) was used as the standard protease. Controls consisted of both boiled buffered D. pini culture medium and citrate-phosphate buffer at the appropriate pH. A 5% solution (w/v) of sulphosalicylic acid applied after incubation revealed the hydrolysed gelatin as clear zones on an opaque background.

From these results, in figures 8.1 and 8.4, page 80 and page 87 , it is evident that D. pini produces a proteolytic enzyme, with an activity, at pH6.5, equivalent to that of about 2.5 mg pepsin per ml at pH2.6. The influence of pH on D. pini indicates a pH optimum of pH6.0 for the cell supernatant and the particulate fraction and an optimum of pH6.2 for the protease of the culture medium.

Lipoxidase.

8.4 Determination of lipozidase production.

The cup-plate method of Blain and Todd (1958) was followed. Commercial lipoxidase from soya beans was used as the standard enzyme preparation. The substrate was sodium linoleate and

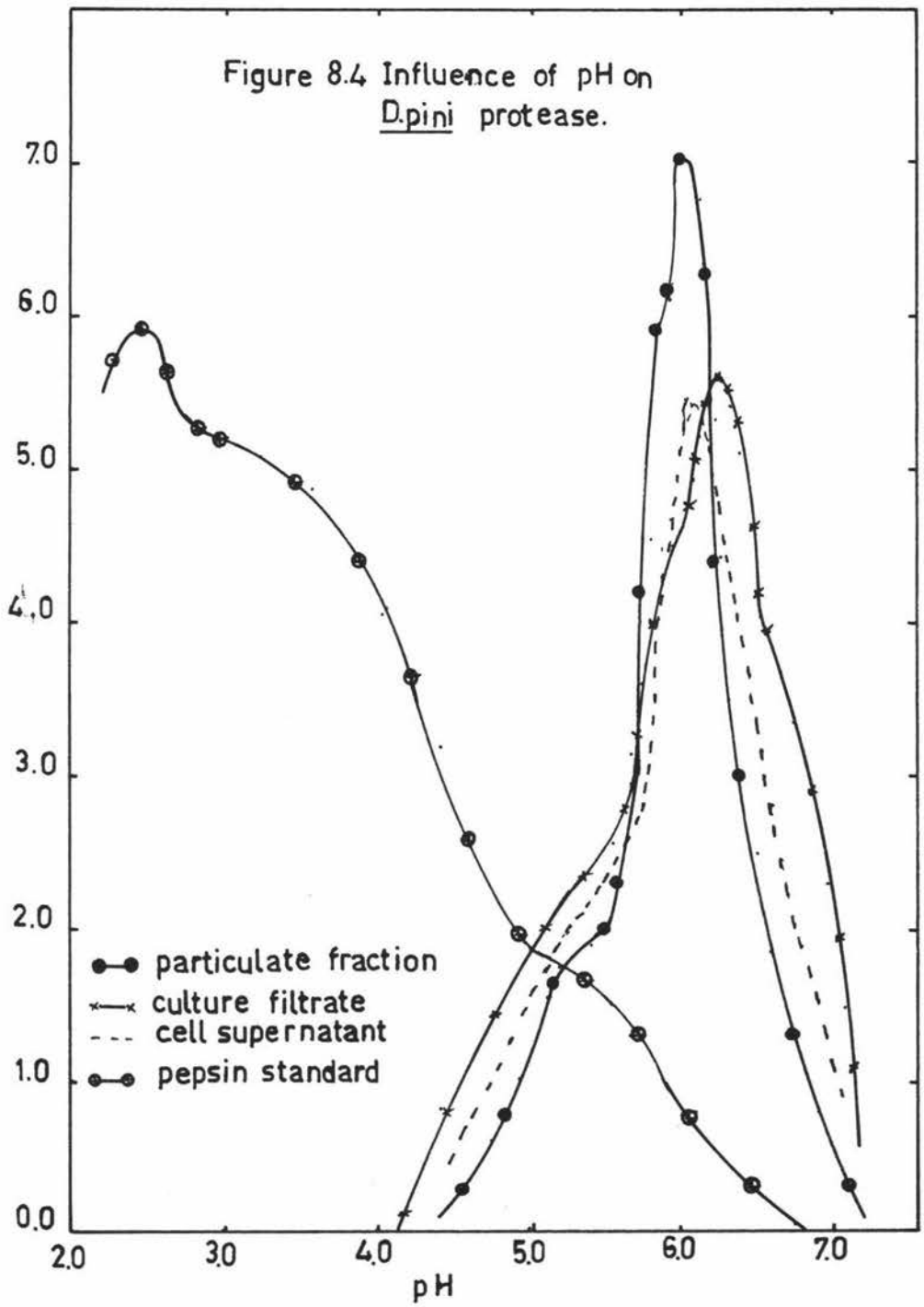


Figure 8.4 Influence of pH on D.pini protease.

carotene was used as an internal indicator. The appropriately buffered *D. pini* culture medium was placed in the cups of the prepared plates buffered at pH4.0 and pH6.5 and incubated at 37°C for 18 hrs.

No evidence for lipoxidase activity (judged by zones of hydrolysis) was obtained at either pH value.

Thus in addition to the carbohydrases enumerated in chapters 6 and 7, lipases and proteases have been demonstrated in the culture filtrates of *D. pini* cultures. Tests for lipoxidase and certain carbohydrases have proved negative.

CHAPTER 9

ENZYMES PRODUCED ON INFECTION OF P. RADIATA NEEDLES.

In the preceding chapters, enzymes have been studied in artificial cultures of D. pini. To determine which, if any, of these enzymes have a role in pathogenesis in the pine needles, it would be desirable to follow the course of their production in needles after artificial inoculation. Attempts to infect P. radiata seedlings artificially by spraying with a spore suspension according to the method of Gadgil (1967) were unsuccessful. Naturally-infected needles were therefore chosen for comparison with uninfected needles from a disease-free area. The course of enzyme production during the early stages of infection could not be followed, since infection cannot be ascertained, except by microscopic dissection, until the late stages when discoloured bands appear on the pine needles. However, significant differences in enzymic activity were observed when late stage infected needles were compared with healthy needles.

Methods.

Protein extraction.

50 gms each of frozen heavily infected stroma-bearing needles from Te Wera S.F., Taranaki and of frozen uninfected needles from Santoft S.F. were cut up in one-inch lengths and then treated in identical preparations. Each was added to 200 mls of citrate/phosphate buffer pH4.0 and homogenised in a Waring blender for 10 minutes at 0°C. This was repeated at regular intervals until no whole needles were evident. After centrifuging in a Sorvall RCB2 refrigerated centrifuge at 10,400g for 10 minutes at 0°C, the supernatant was decanted and the macerated needles rehomogenised in 200 mls of the same buffer for the same length of time. This process

was repeated a third time. After each preparation, the protein concentration in the supernatant was estimated (Lowry et al, 1951). The protein concentration after the third extraction was sufficiently low not to warrant a fourth homogenisation. The respective fractions were pooled and concentrated for 48 hrs, by vacuum dialysis at $0^{o}C$, to a volume of 60 mls, representing a 10-fold concentration. The infected-needle extract contained 2.75 mg protein per ml, and the uninfected needle-extract, with 3.10 mg protein per ml, was diluted to give the same protein concentration as the infected-needle extract.

Carbohydrase determination.

0.5 ml aliquots of each extract were incubated for 24 hrs with the carbohydrates as listed in Table 5.2 to determine the carbohydrase activities as in chapter 7.

Quantitative assays were carried out for pectin methyl esterase, polygalacturonase, pectin lyase, cellulase, carboxy methyl cellulase, protease and lipase. Experimental details are as in chapters 7 and 8 and appendices 8 and 10.

The filtrate from vacuum dialysis was analysed for component sugars (see appendix 7).

Results.

a) Analysis of the vacuum dialysis filtrate showed raffinose, glucose and sucrose to be present with small amounts of galactose and fructose. The level of the predominant sugars was higher by approximately 10% in infected needles.

b) Chromatographic enzyme assays.

With the extract of uninfected needles, sucrose and raffinose were the only oligosaccharides hydrolysed and no polysaccharides were hydrolysed.

With the extract of infected needles raffinose, sucrose, α-D-melibiose and laminaribiose were hydrolysed as were arabinoxylan, arabinogalactan, amylopectin, amylose and carboxymethyl cellulose. (See Table 9.1 page 92).

c) Specific enzymes.

For the uninfected needle extracts no protease or lipase activities were detectable. However, both were shown to be present in the infected needle extract. (See Table 9.2, page 93).

Pectin esterase (see appendix 1, assay 5, page 121) was present in both uninfected and infected needles but the activity of the former was only 10% of the latter. (See Table 9.2, page 93).

Polygalacturonase and pectin lyase: no evidence for either of these two enzymes was obtained by methods described in appendix 9.

Carboxymethyl cellulose (see appendix 9) was present only in the infected needles. (Table 9.2, page 93).

Discussion.

The fact that not one P. radiata needle polysaccharide was hydrolysed by the extract from infected needles would seem to suggest that the activity of some enzymes, if present, was too low to detect at the concentration used for assay.

Both infected and uninfected needles were taken from living trees (about 10-15 years old), at the same time of the year, and it seems probable that the difference in enzymic activities reported above were the result of infection of one tree and not the other by Dothistroma pini. The enzymes demonstrated in the extract of infected needles were also present in artificial D. pini cultures, with a variety of

polysaccharides as carbon source for growth (see chapter 7). It seems likely, therefore, that these enzymes are produced in the infected needles by the fungus, and not by the host in response to invasion.

TABLE 9.1

Hydrolysis of carbohydrates by infected and uninfected needle extracts.

The following carbohydrates were the only ones from Table 6.1 which were hydrolysed by either uninfected or infected *P. radiata* needle extracts.

Carbohydrate	Spot intensity	
	uninfected needles	infected needles
laminaribiose	0	1
α-D-melibiose	0	2
sucrose	1	2
raffinose	1	2
arabinogalactan	0	1
arabinoxylan	0	2
hemicellulose B branched (*P. radiata* needles)	0	1 (arabinose+xylose) 1 (arabinose+galactose)

0 less than 2µg sugar produced on hydrolysis

1 equivalent to 2 - 9.9 µg sugar produced on hydrolysis

2. equivalent to 10 - 19.9 µg sugar produced on hydrolysis

3 equivalent to 20 - 30 µg sugar produced on hydrolysis.

TABLE 9.2

Table of specific enzyme activities.

Uninfected needles	enzyme assayed	assay* type	activity	reference to assay in appendix 11.
	protease	e)		app 8
	lipase	e)		app 8
	pectin esterase	d)	1.9	5
	polygalacturonase	b)c)d)		6
	pectin lyase	a)		7
	cellulase Cx	a)b)c)		8
Infected needles				
	protease	e)	0.3mg/ml	app 8
	lipase	e)	0.2mg/ml	app 8
	pectin esterase	d)	20.2	5
	polygalacturonase	b)c)d)		6
	pectin lyase	a)		7
	cellulase Cx	a)	0.4	8
		b)		6
		c)	0.7	app 7

Cx enzymic activities expressed semiquantitatively as the mg glucose released / ml / minute. Protease and lipase expressed as concentration gm/ml, while PE is mg/min/ml.

* a) colourimetric assay
b) viscometric assay
c) chromatographic assay
d) titrimetric assay
e) cup plate assay.

CHAPTER 10

DISCUSSION AND CONCLUSION.

Pinus radiata needles have as structural polysaccharides, cellulose, pectin, two galactoglucomannans and a complex branched hemicellulose B containing principally arabinose, xylose and galactose.

Dothistroma pini in culture can grow on any of the pine needle polysaccharides as sole carbon source, and can be induced, by appropriate substrates, to form the enzymes for their complete degradation. Enzymes are induced which are specific for the hydrolysis of the polysaccharide growth substrate. Thus pectinase (polygalacturonase) is induced during growth on citrus pectin or P. radiata needle pectin, but not during growth on glucose, cellulose, β-glucan, inulin, xylan, arabinoxylan or arabinogalactan; cellulase is induced during growth on carboxymethyl cellulose, cotton wool cellulose or pine needle cellulose, but not during growth on glucose, pectin, xylan, inulin, arabinoxylan, etc.

Pine needle hemicellulose A and linear hemicellulose B have similar composition, and both may be galactoglucomannans. No purified galactoglucomannan was available for comparison, but glucomannan failed to support growth and mannan and galactan both supported poor growth only, whereas both hemicellulose A and linear hemicellulose B supported moderate growth, in each case producing enzymes for hydrolysis of the growth substrate.

Enzymes for hydrolysis of hemicellulose A and linear hemicellulose B were produced at low level also during growth on β-glucan and dextran, but not on arabinogalactan. The

hydrolysis products were glucose, galactose and mannose. Growth on mannan led to induced synthesis of the enzyme for hemicellulose A hydrolysis but not for linear hemicellulose B hydrolysis. This, together with their differing solubilities in acetic acid, (used as the basis for their separation, appendix 9, page 116) suggests that they are structurally dissimilar.

Branched hemicellulose B was of more complex structure, and was evidently heterogeneous. Galactose, arabinose and xylose were the main monose units present in branched hemicellulose B, with smaller amounts of glucose, mannose and rhamnose. On the basis of enzymological studies it is likely that it contains similar linkages to those present in arabinogalactan, arabinoxylan and xylan. Enzymes for its hydrolysis were produced during growth on arabinogalactan, arabinoxylan and xylan, but not on β-glucan or dextran. Enzymic activities produced on arabinogalactan, arabinoxylan and xylan hydrolysed different fractions within the branched hemicellulose B. The activity produced from growth on arabinogalactan produced arabinose and galactose as branched hemicellulose B hydrolysis products; that from arabinoxylan produced arabinose and xylose; and that from xylan, xylose only. This suggests the presence in branched hemicellulose B of at least two polysaccharides, an arabinogalactan and an arabinoxylan, together with at least three enzyme activities; an arabinofuranosidase (which would remove the arabinose side-chains from arabinogalactan and/or arabinoxylan), a xylan hydrolase (splitting the backbone of arabinoxylan), and a galactan hydrolase or arabinogalactan hydrolase (acting on arabinogalactan, but not on arabinoxylan). A third polysaccharide may account for the glucose, mannose and rhamnose units in branched hemicellulose B.

Uninfected pine needles have enzymes for hydrolysis of sucrose and raffinose, plus low pectin esterase activity. Enzymes capable of catalysing the degradation of cellulose, and arabinogalactan and arabinoxylan (components of branched hemicellulose B) have been demonstrated in infected needles. Presumably these enzymes are induced by the branched hemicellulose B substrates in the pine needle. Pectin methyl esterase was also demonstrated but not polygalacturonase although this could be induced in artificial culture.

Thus D. pini can produce, in vivo and in vitro, extra cellular enzymes which catalyse the degradation of pine needle structural polysaccharides. These enzymes are all induced by the substrate, either in the artificial growth medium or in the pine needle.

D. pini also produces, in vivo and in vitro, several other extra cellular enzymes which appear to be constitutive, and may be useful to the fungus in utilising the host tissue. These include a protease, a lipase, an amylase, a β-1,3-glucanase, and a range of oligosaccharidase activities including α- and β-glucosidases, α-galactosidases, and α- and β- fructofuranosidases. The full range of in vitro activities found, with respect to carbohydrate substrates, as given in chapter 6.

Most of the activities detected in infected needles are presumably of fungal rather than host origin, since they were not detected in uninfected needles (chapter 9), and they are produced in in vitro D. pini cultures.

Other extracellular activities are induced during growth of D. pini on different polysaccharide substrates. In each case, enzymes are induced specific for the hydrolysis of the

specific growth substrate, e.g. inulinase is produced with inulin as substrate. Certain polysaccharides failed to support growth e.g. chitin, chitosan, lichenin, glucomannan and nigeran. (A full range of activities found is discussed in chapter 7.)

The range of constitutive extracellular carbohydrases produced by D. pini is similar to that found in other organisms. Thus amylase, β-1,3-glucanase, and many oligosaccharidase activities are generally found to be constitutive, whereas β-1,4-glucanase or cellulase, pectic enzymes, xylanase, and arabanase are frequently induced (see chapter 3).

D. pini appears to have low xylanase and arabanase activities when grown on glucose, but these are not secreted into the culture medium. All the extracellular activities detected have also been detected in both soluble and particulate cell fractions from washed mycelia. In two cases viz., protease and lipase, evidence was obtained that the particulate activity differs somewhat from the soluble activity, possibly representing a different enzyme species.

The role of the D. pini extracellular enzymes in pathogenesis has not been determined. But the constitutive protease and lipase, and the inducible pectinases, cellulase, and hemicellulases could act together to bring about the complete degradation of host tissue. Also, oligosaccharidases could allow the fungus to utilise the raffinose and sucrose of pine needles in its initial establishment within the needle.

The pine needle cell wall contains (on a dry weight basis) about 50% cellulose, 30% lignin, 10% pectin, 10% hemicelluloses. Lignin decomposition by D. pini was not investigated, but the polysaccharides (70% of the cell wall) are all degraded by

induced enzymes.

Gadgil showed that cell death preceded tissue disorganisation, the latter being a comparatively late stage in the disease. Thus visible breakdown of the cell wall is not necessary for growth of the fungus in the early stages. Cell death may result from disruption of the plasmalemma by the action of the constitutive protease and lipase; either of which could be a "lethal enzyme" (cf Brown, 1965) (see chapter 3), or it may result from the specific action of a toxin elaborated by the fungus (see chapter 2).

The delay in visible breakdown of the host cell wall during infection suggests some regulatory mechanism, which may be of host or fungal origin. However, to investigate this it would be necessary to study in more detail the time course of production of extracellular enzymes during the infection.

Gadgil showed that the mesophyll cells of the host are the first to be destroyed. These serve in part as storage cells for fructosans and amylose (see chapter 4). The fungus secretes a constitutive amylase and an inducible inulinase and so destruction of the mesophyll tissue would provide a valuable carbohydrate reserve for fungal growth.

The healthy pine needle also produces carbohydrases, although only sucrose and raffinose were hydrolysed by cell-free extracts in this study. These are two important reserve oligosaccharides of pine needles. D. pini grown in culture also elaborates enzymes which catalyse the hydrolysis of sucrose and raffinose. Thus the enzymes found in diseased needles could have been of host or fungal origin, or both.

It is possible that other carbohydrases are also present in healthy needles, but in a concentration low enough to have

escaped detection in the present study. Pectin methyl esterase was present at low level in healthy needles; the ten-fold increase in its level of activity in diseased needles could be due either to over-production of host enzymes in response to infection, or to fungal enzyme production. Since D. pini is known to produce in culture all of the enzyme activities observed in diseased needles, the latter seems at least superficially more likely (chapter 7). However, to confirm the source of the enzymes in the infected needles it would be desirable to characterise them further e.g. by electrophoresis and immunochemistry, and compare them with the enzymes produced in culture.

The interrelationships of different observed manifestations of D. pini infection - red pigment production and its possible toxicity to pine needle tissue; blockage of phloem transport and its possible association with resin plug formation; secretion of "lethal enzymes" and cell membrane destruction; the destruction of cell walls by induced polysaccharidases; host resistance, phytoalexin production and increasing resistance of the host with age - may be elucidated by studies on artificially infected pine seedlings.

SUMMARY

The extracellular enzymes of Dothistroma pini Hulbary, causal organism of red band blight of pines, have been investigated.

Pinus radiata needles have, as structural polysaccharides, cellulose, pectin, two galactoglucomannans, and a complex branched hemicellulose B containing principally galactose, arabinose and xylose units.

D. pini in culture can grow on any of the pine needle polysaccharides as sole carbon source, and can be induced by appropriate substrates to form the enzymes responsible for their complete degradation.

Enzymes capable of catalysing the degradation of cellulose, and arabinoxylan and arabinogalactan (components of branched hemicellulose B) have been demonstrated in infected needles. Presumably the enzymes hydrolysing the latter two polymers are induced by the branched hemicellulose B substrates in the pine needle.

Thus D. pini can produce, in vivo and in vitro, extracellular enzymes which catalyse the degradation of pine needle structural polysaccharides. These enzymes are all induced by the substrate in the artificial growth medium or in the pine needle.

D. pini also produces, in vivo and in vitro, several other extracellular enzymes which appear to be constitutive, and may be useful to the fungus in utilising the host tissue. These include a protease, a lipase, an amylase, a β-1,3-glucanase, and a range of oligosaccharidase activities.

Other extracellular enzymes are induced during growth of

D. pini on different polysaccharide substrates. In each case, enzymes are induced specific for the hydrolysis of each growth substrate.

The range of constitutive extracellular carbohydrases produced by *D. pini* is similar to that found in other organisms. Enzymes which are inducible in *D. pini* are generally inducible in other fungal species.

All the extracellular activities detected were present also in both soluble and particulate cell fractions from washed mycelia.

The role of the *D. pini* extracellular enzymes in pathogenesis is discussed.

APPENDIX 1.

List of carbohydrate structures, equipment and systematic names of enzymes.

Carbohydrate structures.

oligosaccharides

cellobiose	(β-G-1,4-G)
gentiobiose	(β-G-1,6-G)
isomaltose	(α-G-1,6-G)
lactose	(β-Gal-1,4-G)
laminaribiose	(β-G-1,3-G)
maltose	(α-G-1,4-G)
α-D-melibiose	(α-Gal-1,6-G)
β-D-melibiose	(β-Gal-1,6-G)
sophorose	(β-G-1,2-G)
sucrose	(α-G-1,2-β-Fru)
α,α trehalose	(α-G-1,1-G)
turanose	(α-G-1,3-Fru)
raffinose	(α-Gal-1,6-α-G-1,2-β-Fru)

polysaccharides

pentosans

araban	(α-A-1,5-A and α-A-1,3-A)
arabinogalactan	A : Gal 1:4.6 (wt basis) Larix occidentalis
arabinoxylan	β-A-1,5-X and β-X-1,4-X ex wheat flour (Perlin,
xylan	$(\beta\text{-X-1,4-X})_n$ ex corn cob (Whistler et al, 1951) 1947.)

hexosans

agar	very complex structure
amylopectin	α-G-1,4-G and α-G-1,6-G ex potato starch
amylose	$(\alpha\text{-G-1,4-G})_n$ ex potato starch
cellulose	$(\beta\text{-G-1,4-G})_n$ ex cotton wool (Corbett, 1963).
dextran	α-G-1,6-G, α-G-1,4-G, α-G-1,3-G ex Leuconostoc mesenteroides
galactan	$(\beta\text{-Gal-1,4-Gal})_n$ ex Lupinus albinus
β-glucan	$(\beta\text{-G-1,3-G})_n$ ex Barley flour (Parrish et al 1960).
inulin	$\beta\text{-Fru-2-(1-}\beta\text{-Fru-2)}_n\text{-1-}\beta\text{-Fru-2-1-}\alpha\text{-G}$ ex Helianthus tuberosa
laminarin	$(\beta\text{-G-1,3-G})_n$ ex Laminarai hyperborea
lichenin	β-G-1,3-G and β-G-1,4-G ex Cetraria islandica

mannan	α-M-1,2-M,α-M-1,3-M and α-M-1,6M ex yeast
nigeran	$(\alpha\text{-G-1,3-}\alpha\text{-G-1,4-G})_n$ ex Aspergillus niger
pectin	$(\beta\text{-Gm-1,4-Gm})_n$ citrus pectin
polygalacturonic acid	$(\beta\text{-Galu-1,4-Galu})_n$

substituted hexosans

carboxymethylcellulose	$(\beta\text{-Glu-1,4-Glu})_n$
chitin	$(\beta\text{-Gmn-1,4-Gmn})_n$ crustacean
chitosan	approx 97% deaminated chitin
methyl cellulose	$(\beta\text{-Gme-1,4-Gme})_n$
cellulose acetate	$(\beta\text{-Ga-1,4Ga})_n$

G	D-glucose	Ga	6-O-acetyl-D-glucose
Gal	D-galactose	Galu	D-galacturonic acid
F	D-fructose	Glu	6-O-methyl-D-glucuronic acid
A	D-arabinose	Gm	6-O-methyl-D-galacturonic acid
X	D-xylose	Gme	6-O-methyl-D-glucose
		Gmn	D-glucosamine

Simple sugars polysaccharides and other chemicals were obtained through the normal commercial sources. Samples of turanose and sophorose were kindly given by Dr. R.W. Bailey.

Source list of unusual chemicals.

dessicated malt extract	A. Wander and Co., Chch.
Chlorella agar	Difco, U.S.A.
Bacto agar	Difco, U.S.A.
3-^{14}C-triolein	Radiochemical Centre, U.K.
peetinol-R.10	Rohm and Haas Co. Ltd., U.S.A.

Equipment

The shaker used throughout this investigation was a New Brunswick model with a variety of platforms for different flask sizes.

The X-ray apparatus used was a Phillips X-ray generator EW1011 with a Debye-Scherrer 57mm. powder camera. The specimen was oriented to the X-ray beam. The apparatus was run at 50 kv, 20ma for 30 minutes. The wavelength used was $K_{\alpha 1}$ λ Co 1.78890.

Systematic names of enzymes used in the text.

E.C.N.°	trivial name	systematic name
3.1.1.3	lipase	glycerol ester hydrolase
3.1.1.11	pectin esterase	pectin pectyl-hydrolase
3.2.1.1	α-amylase	α-1,4-glucan4-glucanohydrolase
3.2.1.2	β-amylase	β-1,4-glucan maltohydrolase
3.2.1.4	cellulase Cx	β-1,4-glucan4-glucanohydrolase
3.2.1.6	laminarinase	β-1,3(4)-glucan glucanohydrolase
3.2.1.7	inulase	inulin 1- fructanhydrolase
3.2.1.8	xylanase	xylan 4-xylanohydrolase
	arabanase	araban 3,5-arabanohydrolase
3.2.1.a	1,3-xylanase	xylan 3-xylanohydrolase
3.2.1.11	dextranase	α-1,6-glucan6-glucanohydrolase
3.2.1.15	polygalacturonase (pectinase)	poly-α-1,4galacturonide glycanohydrolase
3.2.1.20	α-glucosidase (isomaltase,maltase)	α-D-glucoside glucohydrolase
3.2.1.21	β-glucosidase (cellobiase,gentiobiase laminaribiose)	β-D-glucoside glucohydrolase
3.2.1.22	α-galactosidase (melibiase)	α-D-galactoside galactohydrolase
3.2.1.26	β-fructofuranosidase	β-D-fructofuranoside fructohydrolase
3.2.1.28	trehalase	trehalose 1-glucohydrolase
3.4.4.1	pepsin	
4.2.99.3	pectin lyase pectin transeliminase	poly-α-1,4-D-galacturonide lyase

APPENDIX 2.

Preparation of Raulins solution.

The following were dissolved in 4 litres of warm distilled water:

D-glucose	23.3g.
D-(+)-tartaric acid	13.3g.
KH_2PO_4	2.0g.
NH_4NO_3	13.5g.
K_2CO_3	2.0g.
$MgCO_3$	1.3g.
$CaSO_4$ (anhydrous)	0.8g.
$ZnSO_4 \cdot 7H_2O$	0.3g.
$FeSO_4 \cdot 7H_2O$	0.3g.
thiamine-HCl	0.0025g.

Each was added and dissolved individually.

One litre of citrate phosphate buffer pH 4.0 was prepared according to appendix 5 and added to the above solution. The pH was adjusted to pH 4.0 with sodium hydroxide solution.

The solution was then dispensed into Erlenmeyer flasks, stoppered with cotton wool bungs and autoclaved for 15 min. at 15p.s.i. and 121 ^{o}C.

APPENDIX 3.

Preparation of 10% malt solution.

100 gms dessicated malt extract.
10 gms yeast
20 mg cholesterol
2.5 mg thiamine - HCl

were dissolved in one litre of distilled water and 250 mls of the solution dispensed into 4 x 1 litre flasks and treated as the solution in appendix 2.

To prepare 10% malt agar 25gms of agar, was added to the above solution and dispensed into 100 ml screw capped bottles and treated as above. Agar plates were poured after cooling to less than 40°C.

APPENDIX 4.

Preparation of Chlorella agar.

Chlorella pyrenoidosa was maintained in liquid culture of 5% Difco chlorella medium.

Chlorella medium	35 gms.
Bacto agar	5 gms.

To prepare plates for toxin assay the above mixtures were suspended in one litre of distilled water and dispensed into 10 x 100 ml corked bottles and autoclaved for 15 min at 15p.s.i. and 121°C. 10 mls of a 4 day old liquid culture of C. pyrenoidosa were thoroughly dispersed in 100 mls of cooled (35-40°C) sterile Difco chlorella agar and the suspension plated out into sterile petri dishes 20 ml per plate. Plates were then allowed to develop for 48 hrs under artificial illumination, after placing an antibiotic disc, with fraction to be tested, in the centre of the plate.

APPENDIX 5.

Citrate Phosphate Buffer. (McIlvaines buffer)

Stock solutions.

A: 0.1M solution of citric acid (19.21 g in 1000 ml)

B: 0.2M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in 1000 ml)

x ml of A + y ml of B, diulted to a total of 100 ml.

x	y	pH
44.6	5.4	2.6
42.2	7.8	2.8
39.8	10.2	3.0
37.7	12.3	3.2
35.9	14.1	3.4
33.9	16.1	3.6
32.3	17.7	3.8
30.7	19.3	4.0
29.4	20.6	4.2
27.8	22.2	4.4
26.7	23.3	4.6
25.2	24.8	4.8
24.3	25.7	5.0
23.3	26.7	5.2
22.2	27.8	5.4
21.0	29.0	5.6
19.7	30.3	5.8
17.9	32.1	6.0
16.9	33.1	6.2
15.4	34.6	6.4
13.6	36.4	6.6
9.1	40.9	6.8
6.5	43.6	7.0

APPENDIX 6.

Acetate Buffer. (Walpoles buffer)

A: 0.2M solution of acetic acid (11.55 ml in 1000 ml)

B: 0.2M solution of sodium acetate (16.4 g of CH_3COONa or 27.2 g of $CH_3COONa.3H_2O$ in 1000 ml).

x ml of A + y ml of B, diluted to a total of 1000 ml.

x	y	pH
46.3	3.7	3.6
44.0	6.0	3.8
41.0	9.0	4.0
36.8	13.2	4.2
30.5	19.5	4.4
25.5	24.5	4.6
20.0	30.0	4.8
14.8	35.2	5.0
10.5	39.5	5.2
8.8	41.2	5.4
4.8	45.2	5.6

Pancreatic lipase activity per mg approx 65 μ equivalents of acid produced with olive oil at pH 7.4 and 37°C.

APPENDIX 7.

Chromatography of carbohydrates.

Thin layer plates of cellulose MN 300 were used throughout this investigation.

Preparation of thin layer plates.

15 gms of cellulose (without binder) were homogenised in 90 mls of distilled water in a Waring blender for 1 minute. Plates were wiped with acetone and chloroform consecutively to clean them. The cellulose was applied using a Desaga applicator at a thickness of 0.25 mm. The plates were air dried for 48 hrs and a very firm surface (suitable for writing on) was formed. (Wolfram et al, 1965).

Preparation of sugar standards.

Approximately 5 mg of each standard sugar was weighed accurately and dissolved in 1 ml of distilled water. 0.1 ml of a 2% mercuric chloride solution was added to prevent microbial growth.

5 µl of each was applied to a thin layer plate, which was then developed in one of the solvent systems (Table ap 7.1, page 111).

Solvent systems.

6 solvent systems were used. Solvent 4 was used routinely and 6 and 4 were used consecutively in the 2-D chromatography discussed in chapter 4. All systems gave reasonable separation (see Table ap 7.2, page 112).

TABLE APPENDIX 7.1

No.	Solvents and ratio	reference
1.	Butanol:acetic acid:water 3:1:1	Wolfram et al, 1965
2.	Formic acid:ethylmethylketone: t-butyl alcohol:water 3:6:8:3	Fink et al, 1953.
3.	Pyridine:ethylacetate:acetic acid:water 5:5:1:3	Fischer and Nobel, 1955.
4.	Ethyl acetate:pyridine:water (top layer) 2:1:2	Jermyn and Isherwood, 1949.
5.	Ethyl acetate:pyridine:water 12:5:1	R.W. Bailey, personal communication.
6.	Ethyl acetate:acetic acid: formic acid:water 9.0:1.5:1.0:2.0	

Assay of carbohydrase activities.

0.5 mg of the carbohydrates in Table 6.1 page 67 were weighed into small test tubes. 0.5 ml of the appropriate D. pini culture preparation was added and the first aliquot of 5 µl taken at 0 hrs and applied to a thin layer plate. 0.2 ml of toluene was added to the test tube to prevent growth of micro-organisms and the tube stoppered. A further 5 µl was taken after 24 hrs and spotted. The plates were then developed in solvent 4.

R_G values and quantitation of results.

These were related to an internal standard of glucose, R_G. The spot size and colour after spraying with aniline phthalate were related to a series of standard glucose spots for hexoses and xylose spots for pentoses for a semi quantitative estimation of component sugars. The limits of resolution of this assay were 2 µg sugar.

Spray reagents.

Each plate was duplicated; one was sprayed with aniline

phthalate so as to identify the sugar type by its colour (Partridge, 1949) and the other was sprayed first with $AgNO_3$ in acetone (0.1 ml saturated $AgNO_3$ per 20 ml acetone), then with 0.5N sodium hydroxide in methanol and finally a 3% aqueous sodium thiosulphite solution as a fixer (Trevelyan et al, 1950). This latter spray reagent was used for routine location of sugars.

TABLE APPENDIX 7.2

R_G values of simple sugars in six solvent systems.

Carbohydrate	Solvent system R_G x 100.					
	1	2	3	4	5	6
D-glucose	100	100	100	100	100	100
D-arabinose	121	139	105	122	138	151
D-ribose	146	154	121	156	206	208
D-xylose	150	159	110	135	166	176
D-fructose	116	121	106	118	130	146
D-galactose	93	84	92	89	85	90
D-mannose	116	92	107	120		124
L-rhamnose				170	179	240
D-galacturonic acid	75	89	59	22	10	103
D-glucuronic acid	77	96	56	34	11	
α-D-glucosamine-HCl	84	92	83	53	44	
cellobiose	53	38	79	63	35	
gentiobiose	42		73	56		
isomaltose				39		
lactose	49	32	64	49	26	
laminaribiose				68		
maltose	60	41	82	59	46	
α-D-melibiose	46	32	66	49		
β-D-melibiose	46	31	66	49		
sophorose				42		
sucrose				82	56	
α α trehalose	58	51	78	44	30	
turanose				42		
raffinose			65	44	17	

These values are the average of 3 replicates.

APPENDIX 8.

Cup plate diffusion assay.

Introduction:-

The cup plate diffusion assay for antibiotics has been applied to assaying for certain enzyme activities. It was shown by Dingle, Solomon and Reid (1953) that various enzymes gave zones of activity when inserted into cups cut into an agar substrate gel. With all the enzymes studied by Dingle et al. the relationship between diameter of the zone of enzyme activity and log (amount of enzyme) was linear over a wide range. This therefore gives a quantitative assay for activity and is now used for routine estimation of several enzymes viz. amylase, polygalacturonase, protease, cellulase and pectin esterase. Other enzymes assayed by this method include arabanase, xylanase and lipase (Dingle et al, 1953), urease (Blain and Caskie, 1965) and lipoxidase (Blain and Todd, 1958; Blain and Shearer, 1962).

Methods and materials. General procedure.

1% of the appropriate substrate was incorporated into a 2% buffered agar solution containing 0.01% salicylanilide as a fungicide. Gels were poured into 9.0 cm plastic Petri dishes (20ml aliquots) to give a standard depth of 5 m.m. Cups 7 m.m. in diameter, were cut using a specially made cork borer centred in a device to ensure vertical walls to the sides of the cup (see plate 8.1 page 83). Dilutions of a standard enzyme preparation were included on every plate. The dish was covered to prevent evaporation, incubated at 37°C for 18 hrs and then sprayed with the appropriate developing agent. The zone diameter was measured in m.m. and the log of the concentration calculated from the standards.

APPENDIX 9.

Scheme for isolation of polysaccharides from plant material.

This method utilises the procedures of various workers and is a modification of the method of Molloy and Richards (personal communication) see figure 4.1, page 46.

Procedure.

Fresh P.radiata needles, in which no D.pini lesions were visible, were freeze dried and then ground to pass through a 1 m.m. seive. The product was stored over silica gel.

Extraction.

1. Ether soluble material (lipids,chlorophylls etc.)

200 gms of freeze dried needles were extracted with 4 l of benzene:ethanol (2:1) solution in a macro-soxhlet for 20 hrs (20 ml/gm). The brown residue fraction A was dried overnight at 50°C in a warming oven and weighed. The filtrate was discarded.

Wt of fraction A = 170 gm.

2. Water soluble polysaccharides (starch and fructosans).

Fraction A was extracted with 3 l distilled water at 60°C for 30 min. with stirring. After filtration of the extract the insoluble fraction B was dried overnight at 50°C and weighed. The filtrate was discarded.

Wt of fraction B = 133 gm.

3. Pectin extraction (Polygalacturonic acid).

Pectin wasextracted by dissolving out the calcium of the calcium pectate of the cell walls with 2% E.D.T.A. (1g/20ml $Na^{\oplus}$form adjusted to pH 6.7 with sodium hydroxide) at 70°C for 2 hrs. This procedure was repeated twice. Constant stirring was necessary. The 3 E.D.T.A. extracts were pooled and the residue fraction C washed several times with distilled water,

dried and weighed.

Wt fraction C = 112 gm.

The washings were discarded.

4. Purification of pectin extract.

The pectin solution was dialysed several times against distilled water, filtered and poured into 1 volume of Ethanol. The precipitate was impure pectin. After flocculation and settling the supernatant was poured off, the precipitate collected on nylon gauze and redissolved in hot deionised water to approximately a 1% solution. The solution was cooled and made to 0.05N (pH1.5) with concentrated hydrochloric acid. The solution was then dialysed against deionised water.

The solution was concentrated to 50% of the dialysed volume, reacidified and poured into 2 volume ethanol, resulting in the formation of a colourless precipitate with some flocculent precipitate.

The precipitate was centrifuged down (7,000 x gfor 15 min) and soaked in unacidified ethanol overnight. It was then washed twice with 95% ethanol and the washings tested for chloride with silver nitrate solution. When no more chloride was detected, the precipitate was redissolved in hot water, frozen and freeze dried overnight to give fraction D, pure pectin.

Wt of pectin = 9 gm.

4. Deproteinisation.

The depectinised fraction C was extracted with pepsin in 0.1N HCl (1g. residue per 40ml of 0.5% pepsin BDH 1:2500) for 20 hrs at 46°C. The extract was discarded and the residue washed with water until the supernatant was clear. The fraction was dried and weighed.

Wt of fraction E = 109 gm.

5. Delignification.

The method chosen was the hypochlorite method of Whistler et al (1948).

Initial weight of fraction E = 109 gm.

Wt of F = 72 gm. (delignified)

6. Hemicellulose extraction.

All of fraction F was extracted with 10% KOH in deionised water at 20 ml/g of residue (under nitrogen to prevent oxidation) with stirring for 15 hours at 25°C. The nitrogen was deoxygenated by bubbling it through Feiser's solution.

20g KOH

2g sodium anthraquinone-2-sulphonate

15g sodium hyposulphite (commercial grade)

These were dissolved in 100 ml distilled water. (The solution was exhausted when blood red, dull brown or brown).

Treatment of extract.

The insoluble fraction G was filtered off and washed with deionised water. The filtrate and washings were combined.

Hemicellulose A.

The combined extract was acidified to pH2.5 with glacial acetic acid, covered, and allowed to stand for 7 days at room temperature. The precipitate was removed by centrifuging, washed, freeze dried and weighed.

Wt of hemicellulose A = 4 gm.

Hemicellulose B.

The acidified supernatant was poured into 2 volumes of 95% ethanol and allowed to stand until the precipitate caked. This was then filtered off on nylon gauze. The hemicellulose B was dissolved in a minimum volume of water, frozen and freeze dried.

Wt of hemicellulose B = 8 gm.

7. Fractionation of pure hemicellulose B. (after Gaillard, 1965).

1 gm of the hemicellulose B mixture was dissolved in 100 ml of $CaCl_2$ solution (S.G. 1.3) and clarified by brief centrifugation at 20,000 g. 15 ml of an aqueous solution of iodine (3%) and potassium iodide (4%) was added to this. The dark blue precipitate was left to settle for 2 hrs. The clear brown supernatant was neutralised with sodium thiosulphate and poured, with stirring, into 5 vol of ethanol, to precipitate the branched polymer. To remove calcium ions, the branched polymer was dissolved in 5 ml of 0.1N HCl and reprecipitated with 25 ml of ethanol. The polysaccharide was filtered off, washed with ethanol and ether, and finally dried over calcium chloride in a vacuum desiccator.

Wt of branched hemicellulose B = 0.5 gm.

The dark blue precipitate containing the linear polymer was washed with calcium chloride solution containing I_2/KI (15%). The washed precipitate was dissolved in 100 ml of hot water, the iodine neutralised with sodium thiosulphate, and the polymer re-precipitated by pouring the solution into 5 vol of ethanol. The calcium ions were removed by dissolving the precipitate in a minimum volume of N-KOH under nitrogen, neutralised with N-HCl and again precipitated in 5 vol of ethanol. The precipitate was washed, dried and collected as described for the branched polymer.

Wt of linear hemicellulose B = 0.4 gm.

8. Cellulose residue.

Fraction F was washed with N HCl until neutralised and then the excess HCl removed by washing with distilled water until the effluent was above pH5.0.

The light yellow material was collected on nylon gauze,

squeezed dry, frozen, freeze dried and weighed.

Wt of cellulose = 60 gm.

APPENDIX 10.

3,5-dinitrosalicylate reagent preparation.

DNS reagent is prepared by dissolving a mixture of

3,5-dinitrosalicylate	40 g
phenol	8 g
sodium sulphite	2 g
Rochelle salts	800 g

in 2 l. of 2% sodium hydroxide and diluting to 4 l.

APPENDIX 11.

SPECIFIC ENZYME ASSAYS

1. Dextranase assay.

The method is based on that of Janson and Porath (1966) and the analysis depends on reducing sugar formation. No standard enzyme preparations were available so controls consisted of a) no substrate, no enzyme

b) no enzyme

c) boiled enzyme.

The absorbance was read on a Hitachi colourimeter model No. 101 at 640 mμ.

Reagents.

Dextran 1% (w/v) solution in

0.1M K_3PO_4 pH3.5.

0.1% merthiolate was added as a preservative . Solution was then autoclaved.

3,5-dinitrosalicylate (DNS) reagent (see appendix 10).

Procedure.

2 mls of dextran solution were incubated with 10-100μl of enzyme solution for exactly 30 minutes in a water bath at 45°C. The reducing sugars formed were estimated using the DNS method (Miller et al, 1960).

Definition of Unit.

The unit is arbitrarily defined. Janson and Porath define it as the amount of enzyme necessary to liberate reducing groups corresponding to the formation of 2.0 mg of glucose per minute. A graph of a standard glucose solution (5.0mg/ml) was prepared to calibrate the solution.

2. β-glucanase assay.

The barley flour β-glucan is a polymer containing β-1,4

and β-1,6 glucosidic bonds (Parrish et al, 1960).

The assay method is that of Reese and Mandels (1966). All glucanases can be assayed in this way

0.5 ml β-glucan (5mg/ml in 0.05M citrate pH4.5)

0.5 ml enzyme solution (appropriate dilution)

were incubated for 1 hour at 50°C. This was followed by a reducing sugar determination (Nelson, 1954).

3. Laminarinase assay.

This assay was the same as for β-glucanase. Laminarin was substituted for barley glucan. The same standard glucose solution as that for β-glucanase assay was used to calibrate the enzyme solution (Reese and Mandels, 1966).

4. Inulin hydrolase assay.

The increment of reducing power is measured by the colourimetric determination of reducing sugar.

Reagents

0.05M acetate buffer pH5.0.

5% inulin.

Somogyi's copper reagent.

Nelson's arsenomolybdate reagent.

Method.

The method of Avigad and Bauer (1966) was followed. The solutions were incubated at 25°C for 30 minutes.

Definition of unit.

One unit of inulin hydrolase is defined as that amount of enzyme which causes the appearance of one micromole fructose equivalent per minute under the described assay conditions.

Pectic enzymes (Kertesz, 1958).

5. Pectin esterase PE assay.

$$\text{Pectin} + H_2O = \text{Pectic acid} + CH_3OH.$$

The method involves the determination of the increase in free carboxyl groups, followed titrimetrically while a constant pH in the reaction mixture is maintained.

Reagents

1% pectin solution containing o.1M NaCl. The mixture was prepared from stock solution of 2% pectin and 1.5M NaCl.

0.02N NaOH.

Enzyme solution from D. pini concentrated culture filtrate.

Procedure.

The assay was carried out using a Radiometer pH meter 26 with a radiometer automatic titrator model 11.

20 ml of 1% pectin solution containing 0.1M NaCl was adjusted to pH7.5 and placed in a constant temperature bath maintained at 30°C. The enzyme solution (0.1-5.0ml) was then added, the pH immediately adjusted to pH7.5 and the time noted. The alkali was added automatically over a 10 minute period, whenever the pH dropped below pH7.5. The quantity of enzyme was adjusted so that the pH did not drift below pH7.0 before readjustment to pH7.5.

Definition of unit.

The activity was expressed in mg of CH_3OH liberated in 30 minutes per ml of enzyme (PMUml)

6. Polygalacturonase assay PG.

The assay does not differentiate between exo- and endo-forms of the enzyme. Three assay methods were used.

i) titrimetric assay, Kertesz (1958)

ii) viscometric assay, Sherwood and Kelman (1964)

iii) chromatographic assay.

Assay i) titrimetric assay.

Reagents 0.5% pectin solution

1M Na_2CO_3 solution

0.1N I_2 solution

2M H_2SO_4

0.05N $Na_2S_2O_3$ solution

galacturonic acid monohydrate.

Procedure.

1 ml of enzyme solution was added to 99 ml 0.5% pectin solution adjusted to pH4.0 at 25°C; the time was noted and 5 ml aliquots were removed at 3 minute intervals and added to 0.9 ml Na_2CO_3 solution. 5 ml of I_2 solution was added, thoroughly mixed and the solution left to stand for 20 minutes exactly; then 2ml H_2SO_4 was added and the excess iodine titrated with the $Na_2S_2O_3$ solution. The galacturonic acid monohydrate solution was used to prepare a calibration curve. Under these conditions 1 m eq of reduced iodine corresponds to 0.513 millimoles of aldose liberated. Controls with boiled enzymes and without enzyme were run.

Definition of unit.

The activity was expressed as PGu/ml indicating millimoles reducing groups liberated per minute per ml enzyme.

Assay ii) Viscometric assay for polygalacturonase and cellulase Cx.

The method employed was that of Sherwood and Kelman (1964) using a Brookfield spindle viscometer model IVT with an ultra low adaptor.

Method.

To measure enzyme activity with the spindle viscometer, dial readings corresponding to 0% and 100% loss of viscosity

of the reaction mixture were first determined. The dial reading for substrate-buffer stock solution, mixed with an appropriate amount of boiled enzyme or water, substituted for enzyme solution corresponded to 0% viscosity loss i.e. the initial viscosity of the reaction mixture. The dial reading for water corresponded to 100% loss of viscosity. From these two values, newly determined for each batch of substrate, the dial reading corresponding to any given percentage viscosity loss could be calculated. Enzyme activity was measured as the elapsed time, in seconds, from initiation of the enzyme reaction until a given percentage loss of viscosity was achieved. With the viscometer the only measurement required during the reaction was the time elapsed until the pointer reached the calculated reading. The spindle speed was 30 r.p.m. Relative enzyme activity was expressed as 1000 x reciprocal time in seconds required to reach a certain percentage viscosity loss. The concentration of the enzyme was given as mls of culture filtrate per 6 mls of reaction mixture.

Materials.

1% pectin or carboxy methyl cellulose solution in 0.05M potassium citrate at pH4.6.

Assay iii) Chromatographic assay described in appendix 7 page 7. Pectin lyase assay (Albersheim, 1966).

This method is based on the rate of formation of the $\Delta^{4:5}$ unsaturated uronide product. Conjugation of the double bond with the C-6 carboxyl results in absorbance of U.V. maximally at 235mμ.

Reagents.

Buffer: 0.1MKH_2PO_4 and 0.1M citric acid at pH5.2.

Substrate: 0.5% pectin dissolved in the buffer.

Enzyme: 1.0 - 10.00 units/ml.

Standard enzyme: Pectinol R-10 preparation.

Procedure.

0.1 ml of enzyme solution was added at 2.0 ml of substrate dissolved in buffer. Absorbance is measured at 235mμ for 1-2 minutes.

Definition of unit.

One unit of enzyme is defined as that amount which catalyses an increase in a 1 cm cell of 0.555 absorbance units at 235mμ during a 1 minute period at pH5.2 and 25°C. The extinction coefficient of the unsaturated product is 5.55×10^3.

8. Cellulase assay.

i) colourimetric assay

ii) viscometric assay (see assay 6 ii))

iii) chromatographic assay (see assay 6 iii) this appendix and appendix 7.)

i) Colourimetric assay.

The assay is based upon the increase in reducing groups following the incubation of carboxymethy cellulose CMC with the enzyme. It is an adaptation of Pettersson and Porath (1966).

Reagents.

Sodium acetate buffer 0.1M pH4.5. The CMC solution was prepared by suspending 10 g of CMC in 900 ml of the acetate buffer. 10 mg of merthiolate was added, and the volume adjusted to 1 l with buffer. The solution was stored in the refrigerator.

D.N.S. - reagent. See appendix 10 page 119.

Procedure.

Tubes containing mixtures of substrates and enzyme solutions, 2 ml and 0.2 ml respectively were incubated in a water bath at 40°C for 10 minutes. 3 mls D.N.S. - reagent was added to each

tube. The tubes, together with a control containing no enzyme, were heated on a boiling water bath for 15 minutes. After the tubes were cooled to room temperature absorbancy measurements were made at 640mμ in a Hitachi spectrophotometer.

Definition of unit.

CMC is only a substitute for an ideal substrate. Therefore it is impossible to define a generally acceptable unit for cellulolytic activity. However an arbitrary unit is sufficient, viz. one unit is defined as the activity necessary to produce an increase in the reducing power corresponding to 1.00 unit of absorbancy at 640mμ.

All activities in this investigation are expressed in μg/min/ml unless otherwise stated.

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