

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

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

A thesis presented in partial fulfilment of
the requirements for the degree of Master
of Science in Biochemistry at
Massey University.

PETER JOHN BRUNT

1970

ACKNOWLEDGEMENTS

I would like to express my thanks to the following:

Dr. I.G. Andrew, my supervisor, for help and advice throughout the course of the work;

Dr. R.W. Bailey, Applied Biochemistry Division, D.S.I.R., Palmerston North, for samples of sugars and for his helpful comments;

Staff and friends within the Chemistry and Biochemistry Department and other departments at Massey University for comment and help;

The N.Z.F.S. for a research grant;

Forest Research Institute, Rotorua, staff and N.Z.F.S., Palmerston North, staff for collection and supply of cultures and materials;

My wife, Merrill, for her encouragement and help;

Mrs. V. Lauridsen for typing this thesis.

TABLE OF CONTENTS

	Page
Acknowledgements	ii
Table of contents	iii
List of Figures, Plates and Tables	.v
<u>CHAPTER</u> 1 BIOCHEMICAL PLANT PATHOLOGY	1
2 <u>DOTHISTROMA PINI</u>	12
3 THE ENZYMOLOGY OF PATHOGENICITY	25
4 POLYSACCHARIDES OF <u>PINUS RADIATA</u> NEEDLES	39
5 ISOLATION AND GROWTH OF <u>DOTHISTROMA PINI</u>	54
6 CARBOHYDRASES PRODUCED IN GLUCOSE-RAULIN'S SOLUTION	64
7 ENZYMES PRODUCED BY <u>D. PINI</u> GROWING ON POLYSACCHARIDES	69
8 FURTHER ENZYMES PRODUCED BY <u>D. PINI</u> , WITH PARTICULAR REFERENCE TO LIPASES AND PROTEASES	77
9 ENZYMES PRODUCED ON INFECTION OF <u>P. RADIATA</u> NEEDLES	89
10 DISCUSSION AND CONCLUSION	94
<u>SUMMARY</u>	100
<u>APPENDICES</u>	
1 List of Carbohydrate Structures, Equipment and Systematic Names of Enzymes	102
2 Preparation of Raulin's Solution	105
3 Preparation of 10% Malt Solution	106
4 Preparation of <u>Chlorella</u> agar	107
5 Citrate-Phosphate Buffer	108
6 Acetate Buffer	109
7 Chromatography of Carbohydrates	110
8 Cup Plate Diffusion Assay	113

9	Scheme for Isolation of Polysaccharides from Plant Material	114
10	3,5-dinitrosalicylate Reagent Preparation	119
11	Specific Enzyme Assays	120
	<u>REFERENCES</u>	127

LIST OF FIGURES, PLATES AND TABLES

FIGURE	Page	TABLE	Page
4.1	46	5.2	57
4.2	51	6.1	67
5.1	59	7.1	71
5.2	60	7.2	72
6.1	65	7.3	73
8.1	80	8.1	79
8.2	84	9.1	92
8.3	85	9.2	93
8.4	87		
		TABLE APPENDIX	
PLATE		7.1	111
2.1-5	14	7.2	112
2.6	21		
2.7	23	APPENDIX	
4.1	40	1	102
4.2	50	2	105
8.1	83	3	106
		4	107
TABLE		5	108
4.1	44	6	109
4.2	44	7	110
4.3	49	8	113
4.4	49	9	114
4.5	49	10	119
5.1	57	11	120

CHAPTER 1BIOCHEMICAL 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 tumefaciens 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 slash 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 Phytophthora 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 than 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 fusario

acid, α picolinic acid, novarubin, 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 colletotrin 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 (Huirra, 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 (Tomiya, 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 Kuč (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 resistant 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 (Zaunmeyer, 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 (Samborski, 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.