ZYMOGRAMS AS CRITERIA IN THE TAXONOMY OF FUNGI

by

Ralph Hayes Cruickshank, B.Sc.(Hons.),Tas.

Submitted in fulfilment of the requirements for the degree of

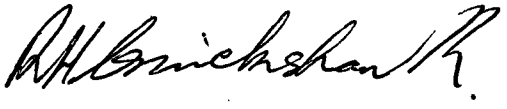
Doctor of Philosophy

University of Tasmania

Hobart

December 1988

The thesis contains no material which has been accepted for the award of any other higher degree or graduate diploma in any tertiary institution and, to the best of my knowledge, contains no material previously published or written by another person, except where due reference is made in the text of the thesis.



R.H. Cruickshank

University of Tasmania

Hobart

December 1988

TABLE OF CONTENTS

TABLE OF CONTENTS

(Continued)

TABLE OF CONTENTS

(Continued)

LIST OF FIGURES

ACKNOWLEDGEMENTS

I wish to thank Dr. T.A. McMeekin for his interest in this project and for encouraging its completion. I appreciate the interest in this project shown by Dr. M.A. Line and for his comments on literary style in presentation.

I am particularly indebted to Dr. M.W. Sweetingham, Department of Agriculture, South Perth, Western Australia, for his interest and discussions of electrophoresis, and collaboration in studies on the Rhizoctonia populations in Western Australia.

I thank Drs. A. Rovira, S.M. Neate and H.J. McDonald of the CSIRO Division of Soils, Urrbrae, South Australia, for the provision of Rhizoctonia cultures and collaboration in publication of results.

Particular thanks also go to Dr. J.I. Pitt, CSIRO Division of Food Research, North Ryde, New South Wales, for his collaboration in studies on Penicillium, and for proposing my membership of the Subcommission on Penicillium and Aspergillus Systematics, of the International Commission on the Taxonomy of Fungi.

I wish to thank the following for the donation of cultures which have helped to enrich these studies:

Dr. D. Backhouse, School of Botany, University of New South Wales, Kensington, New South Wales, Australia.

Dr. R.E. Beever, DSIR, Plant Diseases Division, Auckland, New Zealand.

Professor M. Bisiach, Instituto di Patologia Vegetale, Universita di Milano, Milan, Italy.

Dr. M. Christensen, DSIR Plant Diseases Division, Palmerston North, New Zealand.

Dr. J.R. Coley-Smith, Department of Plant Biology, University of Hull, Hull, England, United Kingdom.

Dr. J.G. Harrison, Scottish Crop Research Institute, Dundee, Scotland, United Kingdom.

Dr. A.D. Hocking, CSIRO Division of Food Research, North Ryde, New South Wales, Australia.

Dr. Y. Homma, National Institute of Agro-Environmental Sciences, Ibaraki, Japan.

Dr. W.R. Jarvis, Canada Department of Agriculture, Research Station, Harrow, Ontario, Canada.

Dr. D.H. Lorenz, Landes-Lehr-und Forschungsanstalt, Neustadt, German Federal Republic.

Mrs. D.M. Masaba, Pathology Section, Coffee Research Foundation, Ruiru, Kenya.

Professor A. Ogoshi, Faculty of Agriculture, Hokkaido University, Sapporo, Japan.

Professor P. Ribéreau-Gayon and Dr. B. Donéche, Institut d'Oenologie, Université de Bordeaux II, Talence, France.

Dr. K. Sivasithamparam, Department of Soil Science and Plant Nutrition, University of Western Australia, Nedlands, Western Australia, Australia.

Mr. J. Walker, Biological and Chemical Research Institute, Department of Agriculture, Rydalmere, New South Wales, Australia.

Dr. J.A-L. Wong, Department of Agriculture, New Town, Tasmania, Australia.

I sincerely thank Mrs. Sally Jones for the final presentation of this thesis.

Financial support was provided by the Rural Credits Development Fund of the Reserve Bank of Australia, and a Research Grant from the University of Tasmania.

SUMMARY

A sensitive and efficient electrophoretic technique for the examination of fungal pectic enzymes was developed. This involved electrophoresis of culture fluids in polyacrylamide slab gels containing pectin. Gels were soaked in malic acid solution which gradually reduced the gel pH from 8.7 to 3.0 and allowed the enzymes to act on the incorporated pectin when the pH was suitable. Ruthenium red treatment stained the unaltered pectin revealing unstained zones due to polygalacturonase activity and zones with intensified staining where pectinesterases had converted the pectin to pectic acid. Results were recorded as photograms. Other enzyme systems were investigated.

Zymogram clarity was improved by reduction of gel thickness from 3.5 to 2.0 mm and sample wells from 15 to 4.5 ul. Curvature of buffer fronts was eliminated by efficient gel cooling. Tailing was reduced and isozyme band clarity was improved by loading samples as a slurry with Sephadex G150-superfine. Thorough gel polymerization before gel moulds were opened was found to be essential for migration characteristics to be uniform through the depth of the gels.

The technique was applied to the enzymes of Botrytis and Penicillium species and the Rhizoctonia complex. The influences of culture medium composition were examined for each genus and media suitable for pectic enzyme production were developed. Potent yields of polygalacturonase were obtained from Botrytis species in the absence of pectin. Zymograms from these differed from those

produced from pectin media and both were used to advantage. Distinctive and characteristic zymograms were obtained from Botrytis species and provided an accurate means for their identification, and indications of synonymy. An extensive study of B. cinerea detected nine pectic enzyme genotypes. One of these was principally associated with Vitis and may constitute B. fuckeliana.

The use of zymograms as taxonomic criteria in Penicillium provided a means for the accurate identification of species in this notoriously difficult genus. The extensive synonymy in subgenus Penicillium was examined by zymograms and 80 taxa were assigned to 21 species, with significant changes in assignments to P. aurantiogriseum and P. commune. The latter was shown to be the previously unknown ancestor of the domesticated P. camembertii.

Zymogram studies revealed the presence of five zymogram groups in Thanatephorus, five in Ceratobasidium and one in Waitea, from Rhizoctonia isolates provided from the grain belt of Western Australia. A similar result was obtained from isolates provided from South Australia and a link was shown between grouping of isolates by anastomosis behaviour and by zymograms in the case of four anastomosis groups available from that State. Other anastomosis groups and species in the Ceratobasidiaceae were examined and the correlations found between zymogram patterns and anastomosis groups and subgroups gave promise of aiding the practical recognition of species in the complex.

I INTRODUCTION

There is a great need for a universally accepted taxonomy of fungi that reflects biological relatedness and has a maximum predictive value. Taxonomy based largely on morphology, has produced useful working systems but, due to the paucity of phenotypic characters available for examination, and to their plasticity in response to environments, this taxonomy frequently fails to meet these ideals. A number of approaches have been used aiming to rectify this situation through broadening the bases of comparison by studies of vital processes and their products. One such approach is the electrophoretic examination of enzymes produced by fungi, the subject of this dissertation.

II LITERATURE SURVEY

1. Fungal taxonomy

Taxonomy has been defined as classification, especially classification of animals and plants according to their natural relationships; from Greek taxis an arrangement, order, and -nomia from nomos law, denoting a system of laws governing a specified field or the sum of knowledge regarding them (Webster, 1945).

An effective taxonomy is essential for the storage, retrieval and exchange of information on organisms and the names used in the taxonomy must be unambiguous and universal. A practical guide to the principles and practices of naming in biology has been presented by Jeffrey (1973). The taxonomic concept of species has no absolute definition. It is broadly a series of recognisably similar individuals, recognisably distinct from other such series, among living organisms existing in a particular place at a particular time (Jeffrey, 1973). In sexually reproducing organisms, the members of a species are generally interfertile but reproductively isolated from other species. Sexual reproduction is often unknown or examined with great difficulty in microorganisms and Mayr (1964) suggested that for these, the category "species" would legitimately represent the concept "something clearly distinct", while being convenient, having maximal predictive value and being consistent with the greatest number of characters.

Traditional taxonomy has evolved from studies of morphology but these have practical limitations in organisms such as fungi where few morphological characters may be available, insufficient to distinguish organisms that are genetically distinct, where morphological simplicity may mask genetic diversity (Hall, 1973). As well as being scarce, morphological characters may show plasticity in expression in response to changes in the environment (Menzinger, 1966) and there may be overlap between species, for example, in the dimensions of conidia in Botrytis species (Shidla, 1972). Information to supplement morphology and aid the accuracy of delimitation and identification of species may be provided by any facet of the biology of the organisms, provided by studies in chemotaxonomy, physiology, cytology, ultrastructure, genetics, serology and ecology (Nelson, 1965; Gams and Jülich, 1984).

Studies on soluble proteins have been made on a great variety of organisms and have proven to be valuable aids in taxonomy. Gel electrophoresis has been the principal method used in these studies and has provided a sensitive method for the separation of proteins including enzymes, the primary products produced and orchestrated in response to the genome. Such studies include those on fungi (Hall, 1969; 1973).

2. The electrophoretic technique and its use in taxonomic studies of fungi

Gel electrophoresis separates proteins by means of differences in their mobility in an electric field, governed primarily by

differences in their net electrostatic charges, with some secondary influence from their size and configuration. The proteins separated by electrophoresis may be detected by means of general protein stains such as amido black or Coomassie brilliant blue. As an alternative, or in addition, specific classes of proteins may be detected by means of their enzymic activity on their respective substrates (Shields, Orton and Stuber, 1983; Micales, Bonde and Peterson, 1986).

Proteins may have multiple forms with a particular enzymic activity. These were named "isozymes" by Markert and Moller (1959). Some authors have placed limitations on the scope of this term, for example, Moss (1982) gave the definition of isozymes as multiple molecular forms of enzyme within a single species resulting from the presence of more than one structural gene, multiple gene loci or multiple alleles. In the last instance, a separate term "allozyme" has been proposed for variant enzymes produced by allelic forms of the same locus (Prakash, Lewontin and Hubby, 1969). In wide general usage the term isozyme is used for enzymes with the same catalytic activity which can be separated by suitable techniques such as electrophoresis although the reason for their multiplicity is not yet known (Jakubke and Jeschkeit, 1983).

Distinction between species of fungi by means of protein profiles has been attained in many studies involving a wide spectrum of fungi. A partial listing of examples is provided in Table 1. Success from this method has not always been achieved and inadequacy has been reported from studies on Aspergillus

TABLE 1.

Examples of the use of protein and enzyme electrophoresis in the taxonomy of fungi.

Genus	Authors	Species	Isolates	Medium[a]	Detection[b]	Distinction
Ascophaera	Maghrabi and Kish (1985)	3	57+	P. disc	12 enz.	species
Aspergillus	Nealson and Garber (1967)	15	32	starch	3 enz.	species
	Sorenson et al. (1971)	5	11	P. disc	prot.	none
	Kurzega and Garber (1973)	1	90	P. slab	1 enz.	species
	Nasuno (1974)	2	44	P. disc	3 enz.	species
Botrytis	Backhouse et al. (1984)	6	30	P. slab	prot., 4 enz.	species
Candida	Shechter et al. (1972)	6	31	P. disc	prot.	species
Ceratocystis	Stipes (1970)	4	13	P. disc	prot., 1 enz.	species
Dermatophytes	Jones and Noble (1982)	17	84	P. slab		species
Endothia	Stipes et al. (1982)	2	12	P. disc	prot., 2 enz.	species
Fusarium	Meyer et al. (1964)	2		starch	3 enz.	species
	Glynn and Reid (1969)	14	33	P. disc	prot.	none
	Meyer and Renard (1969)	2 f.sp.	9	P. disc	prot., 1 enz.	none
	Reddy and Stahmann (1972)	5	10	P. disc	13 enz.	species
	Matsuyama and Wakimoto (1977)	3	45	P. slab	2 enz.	species
	Scala et al. (1981)	6	26	P. disc	1 enz.	species
Humicola	Moorhouse and De Bertoldi (1975)	6	27		6 enz.	species
Monilinia	Penrose et al. (1976)	2	7	P. slab	prot. 4 enz.	species
Mucor	Stout and Shaw (1974)	20	28	starch	11 enz.	species
	Nguyen-The et al. (1984)	3	14	P. slab	2 enz.	species

Neurospora	Chang et al. (1962)	3	5	P. o starch	prot.	species
	Reddy (1973)	8	34	P.	1 enz.	none
Penicillium	Bent (1967)	3	3	P. disc	prot.	species
	Anné and Peberdy (1981)	2	13	P. disc	8 enz.	species
Phytophthora	Clare and Zentmyer (1966)	3	9	starch	prot., 3 enz.	species
	Gill and Powell (1968)	1	17	P. disc	prot.	species
	Hall et al. (1969)	3	43	P. slab	prot., 1 enz.	species
	Gill and Zentmyer (1978)	2	35	P. disc	prot.	species
	Kaosin and Zentmyer (1980)	3	26	P. disc	prot., 2 enz.	species
	Old et al. (1984)	1	183	starch	13 enz.	mating types
	Erselius and Shaw (1982)	2	31	P. slab	prot., 4 enz.	species
Perenosclerospora	Bonde et al. (1984)	3	10	starch	11 enz.	species
Pleurotus	Boisselier-Dubayle (1983)	3	24	P. disc	3 enz.	species
	May and Royse (1988)	9	60	starch	9 enz.	species
Puccinia	Shipton and Fleischmann (1969)	4	9	P. disc	prot.	none
	Burdon and Marshall (1981)	11		starch	prot.	species
Pyricularia	Matsuyama and Kosaga (1971)	1	132	prot., 2 enz.		subgroups
	Matsuyama et al. (1977)	1	17	P. slab	2 enz.	species
	Leung and Williams (1986)	1	335	starch	12 enz.	2 types
Pythium	Clare (1963)	6	11+	starch	prot.	species
	Clare et al. (1968)	11			prot., 1 enz.	species
Rhizoctonia	Matsuyama et al. (1978)	5	48	P. slab	1 enz.	groups
Rhizopus	Seviour et al. (1985)	8	20	P. disc	prot.	species
Sclerotinia	Wong and Willetts (1975)	3	47	P. slab	prot. 5 enz.	species
	Cruickshank (1983a)	3	20	P. slab	1 enz.	species
Septoria	Durbin (1966)	3	36	P. disc	prot.	species

Serratia	Goullet (1981)	3	42	P + agarose	1 enz.	species
Taphrina	Snider and Kramer (1974)	31	50	P. disc	prot.	species
Thamnidium	Stout and Shaw (1973)	2	7	starch	10 enz.	species
Trichoderma	Zamir and Chet (1985)	1	23	starch	7 enz.	types
Ustilago	Bradford *et al.* (1975)	14	164	P. slab	3 enz.	none
Verticillium	Milton *et al.* (1971)	5	16	P. disc	prot.	species
	Selvaraj and Meyer (1974)	2	18	P. disc	prot., 2 enz.	species
Wood rots	Schuster and Jung (1981)	8		agarose	prot., 3 enz.	species

[a]Medium employed in electrophoresis: P. disc (polyacrylamide disc system); P. slab (polyacrylamide slab gels); starch (starch gel).

[b]Detection: prot. (soluble proteins); enz. (enzymes).

(Sorenson, Larsh and Hamp, 1971), Fusarium (Glynn and Reid, 1969) and Puccinia (Shipton and Fleischmann, 1969). Protein profiles have provided evidence contrary to current classifications. For example, cluster analysis of protein profiles from the sporangiophores of Rhizopus isolates supported a reduction in the number of species (Seviour, Pethica, Soddell and Pitt, 1985).

The use of protein profiles to attain distinctions below species level to formae speciales, races or types has been generally unproductive. Reports of failure in this regard include those on Fusarium oxysporum f.sp. (Meyer and Renard, 1969; Glynn and Reid, 1969); Puccinia (Shipton and Fleischmann, 1969), and Phytophthora fragariae races (Gill and Powell, 1968). Success in subspecific grouping by means of protein profiles has been reported for Pyricularia oryzae (Matsuyama and Kosaga, 1971; Leung and Williams, 1986) and for Trichoderma harzianum (Zamir and Chet, 1985).

Many studies have made use of enzyme profiles, named zymograms by Hunter and Markert (1957). In most reported cases, consistent distinction between species has been attained in agreement with separations made by more traditional methods (Table 1). In some cases, the presence of additional species has been indicated as in cryptic or sibling species associated with Aspergillus nidulans (Kurzega and Garber, 1973), in Phytophthora palmivora (Kaoziri and Zentmyer, 1980) and in Mucor racemosus (Stout and Shaw, 1974).

The gel used in electrophoresis has been polyacrylamide in the majority of studies, although a considerable amount of work

has used starch gels (Table 1). A surprising number of workers have used disc electrophoresis involving a discontinuous system of buffers and acrylamide concentrations in vertical tubes (Ornstein, 1964; Davis, 1964). While this system can use relatively dilute samples of proteins and enzymes and gives good resolution of their components, samples are run in individual tubes which makes accurate comparisons difficult. This problem is overcome by the use of slab gels in which a number of samples can be resolved simultaneously in the same gel. The usefulness of such systems has been stressed by Matsuyama and Kozaka (1971).

Most electrophoretic studies have used soluble proteins including enzymes extracted from cytoplasm and hence access to the multiplicity of enzymes involved in metabolism. This involves a number of preparative steps between disruption of the living material, to use in electrophoresis. An alternative is to use the extracellular enzymes secreted by fungi. Both extracellular and intracellular enzymes were examined from wood rotting Basidiomycetes (Schuster and Jung, 1981) and from Rhizoctonia solani (Zuber and Manibhushanrao, 1982). Some isozymes were both intra- and extracellular, while others were obtained only from one or the other class.

Studies which have made use of extracellular enzymes include those of Kurzega and Garber (1973) examining amylase of Aspergillus nidulans; studies on Fusarium spp. by Meyer, Garber and Shaefer (1964) examining esterase, phosphatase and pectin methyl esterase, and by Matsuyama and Wakimoto (1977) examining esterase and catalase; also studies by Matsuyama, Kato and

Yamaguchi (1977) who examined esterase and peroxidase of Pyricularia strains. All of these studies obtained satisfactory distinction between species, or strains in the case of Pyricularia. Another group of extracellular enzymes that have been studied by electrophoresis are the pectic enzymes. The literature concerned with these is considered in Section II 3.

3. Electrophoretic techniques for the study of pectic enzymes

Pectins are linear polymers of galacturonic acid partially esterified with methanol and have neutral sugars as side chains. In the absence of esterification the material is known as pectic acid or, in its salt forms, as polypectate. Enzymes which degrade these materials by hydrolysis are commonly known as polygalacturonases (PG). These may attack the substrate either subterminally or at random sites and are described as exo-PG or endo-PG, respectively. Enzymes which cleave the polymers by a mechanism of beta-elimination which results in an unsaturated product are known as lyases or as transeliminases. The enzymes which catalyse the de-esterification of pectin are known as pectinesterases (PE). In some publications they are named pectin methylesterases (PME), but since their action is not restricted solely to methyl esters, the inclusion of 'methyl' in the designation is superfluous. This brief description of pectic substrates and enzymes is made in the terms usually encountered in electrophoretic and plant pathological works. A more complex

terminology, based on carbohydrate nomenclature, is used in studies directed at carbohydrate chemistry. The points above are elaborated and supported by references in an extensive review on pectic enzymes, their classification, action patterns, specificity, occurrence, formation, purification, assay and involvement in pathogenicity, provided by Rexová-Benková and Marcovic (1974).

Electrophoretic methods for the examination of pectic enzymes have involved separation in starch or polyacrylamide gels and there have been several approaches to the detection of the enzymes. Meyer, Garber and Schaeffer (1964) used starch gel electrophoresis to examine PE from culture filtrates of Fusarium species. The liberation of carboxyl groups by de-esterification was detected by two methods. In one, a pectin-in-agar overlay gel at pH 5.5 was applied to the starch gel after electrophoresis. The fall in pH at sites of PE activity was detected by a change from yellow to red in methyl red indicator included in the overlay gel. The other method was to segment the gel, freeze the segments, crush them in pectin solution at pH 5.5, incubate, then titrate with NaOH to pH 7.7.

Roeb and Stegemann (1975) described a method for the electrophoresis and detection of PE in polyacrylamide gels. Pectin was incorporated in the gel mixture prior to polymerization to form the gel. After electrophoresis the gel was incubated at pH 8.3 then stained with methylene blue. Sites of PE activity stained more deeply since the dye has a greater affinity for pectic acid than for pectin.

Höfelmann, Kittsteiner-Eberle and Schreier (1983) separated enzymes by isoelectric focusing in polyacrylamide gels then detected several enzymes by ultra-thin substrate-in-agar print techniques. For PE, pectin at pH 4.5 was the substrate and sites of PE activity were detected by their lack of staining with acidified ferric chloride following treatment of the substrate gel with alkaline hydroxylamine reagent.

Several methods have been used to detect PG following electrophoresis. Garber and Beraha (1966) separated the enzymes in starch gels then detected sites of PG activity by segmentation of the gel and reaction with pectin which was examined by viscometry and by paper chromatography of the reaction products. The substrate incorporation method has been used to detect PG in polyacrylamide gels. Stegemann (1967) used this method, incorporating pectic acid in the gel used for electrophoresis. Enzymic activity was allowed by acidification of the gel in acetic acid solution followed by treatment in ammonia solution. The gel was then stained with ruthenium red or methylene blue. These stained the unaltered pectic acid and revealed sites of PG activity by their lack of staining. Roeb (1974) also made use of pectic acid incorporation in the electrophoresis gel. In this case, PG detection made use of methylene blue background staining.

Pectic acid in solution has been used to steep polyacrylamide gels following electrophoresis, to introduce this substrate into the gel surface. Lisker and Retig (1974) detected PG activity by steeping gels in sodium polypectate solution

citrate buffered to pH 4.8. Enzymic activity was revealed by background staining of the unaltered substrate by ruthenium red. Magro, Di Lenna, Marciano and Pallavicini (1980) used a similar technique to reveal PG activity following isoelectric focusing in tube gels.

Pectin lyase activity has been detected in gels by the segmentation method (Nasuno, 1974) and by the steep method (Lisker and Retig, 1974). Segmentation was used following disc electrophoresis. The gels were frozen, sliced into 2mm discs, the enzymes extracted, reacted with pectin at pH 5.5 and activity was measured by change in extinction at 235 nm caused by formation of unsaturated products. The steep method was used following isoelectric focusing in polyacrylamide gels. The steeping solution contained pectin, tris-HCl buffered to pH 8.3 and containing calcium chloride, the latter since lyases active at high pH are activated by calcium ions. Activity was detected by ruthenium red staining. Reid and Collmer (1985) used techniques after the style of Höfelmann et al. (1983) in the use of ultra-thin polyacrylamide gel isoelectric focusing following by ultra-thin pectate-agarose overlays. The overlays were buffered suitably and contained either EDTA or calcium chloride to allow detection of PG or pectin lyase when the overlay was stained with ruthenium red.

Simultaneous detection of PG and PE was attained by the author when pectin was incorporated in polyacrylamide gels and enzymic activity was detected by staining with ruthenium red (Cruickshank and Wade, 1980). The development and refinement of this technique is described in Section III of this thesis.

4. Taxonomy of *Botrytis* species

To gain an appreciation of the genus *Botrytis* Pers. and the current status of its taxonomy, many facets of the extensive literature on the genus were considered. Much of this has been reviewed by Jarvis (1977) and in Coley-Smith, Verhoeff and Jarvis (1980).

The place of *Botrytis* amongst higher taxa was considered by Whetzel (1945) who proposed the encompassing family Sclerotiniaceae and teleomorphic state *Botryotinia*. In the critical revision of *Botrytis* and *Botrytis*-like genera, Hennebert (1973) selected 22 species of *Botrytis* to give a non-exhaustive list and moved some taxa previously ascribed to *Botrytis* to his new conidial state genera *Streptobotrys*, *Amphobotrys* and *Verrucobotrys*. These generic designations have been used in the present study, but the necessity for their separation from *Botrytis* has been questioned by Kendrick (1980). Von Arx (1974) considered there were about 30 *Botrytis* species with 200 described as herbarium specimens, and noted the urgent need for revision of the genus.

Serious problems in species identification are overlap in their characteristics and the plasticity of these characteristics in response to environments (Menzinger, 1966; Shidla, 1972). Despite these problems, consideration of all the characteristics available, heavily weighted by consideration of the host plant, has allowed identifications to be made, often with confidence.

There has been debate on the status of some taxa among those listed with authorities in the Appendix. For example, is B. byssoidea distinct from B. aclada? The differences used to separate these species were judged by Siemaszko (1929) to be too slight and not sufficiently convincing to justify separation. However, studies on the natural and mutagen-induced variability in these species lead Owen, Walker and Stahmann (1950) to the conclusion that there was no indication that either species could be regarded as a variant of the other.

In studies on anastomosis between species, Menzinger (1966) found B. byssoidea would only anastomose with B. allii (B. aclada). While Patterson and Grogan (1984) have questioned the validity of anastomosis as a criterion for speciation taking Sclerotinia minor as a model system, Menzinger's results would seem to indicate a close relationship between B. byssoidea and B. aclada. Both species were recognised by Hennebert (1973).

The validity of separating B. septospora with multicelled conidia, from B. aclada was doubted by Jarvis (1977) considering that Menzinger (1966) was able to induce the production of multicelled as well as unicelled conidia from eight of ten isolates of sporulating Botrytis species.

While Kelbahn (1930) and Brierley (1931) had doubts on the status of species in the collective species B. cinerea, including the microsclerotial taxa, Morgan (1971b) was able to distinguish between B. tulipae, B. hyacinthi and B. narcissicola by the use of numerical taxonomy applied to morphological, physiological and pathogenic characters. These species were recognised by Hennebert

(1973).

The endophyte taxa B. anthophila showing host specificity to Trifolium pratense (Silow, 1934), and B. spermophila with host specificity to Trifolium repens (Sampson and Western, 1954), were separated in the key-to-species provided by Morgan (1971b), although he suspected they were conspecific. Jarvis (1977) regarded them as probably valid species.

Another example in this series of doubts was the case of a pathogen of lily-of-the-valley (Convallaris majalis), identified by Pape and Hemer (1964) as B. cinerea f.sp. convallariae. The alternative was to place this pathogen as a distinct species, B. convallariae (Klebahn, 1930; Hennebert, 1971).

The ubiquitous grey mould fungus B. cinerea with its all-inclusive host range has attracted a great deal of attention in scientific studies. Various attempts at subdivision of the taxon have been made. Paul (1929) recognised three forms based on gross cultural appearance: sclerotial with a strongly marked tendency to form sclerotia; mycelial with a general tendency to form aerial mycelium, and the freely sporing form. These forms tended to be maintained on a variety of media. No evidence of selective parasitism was found and all had the same ability to penetrate formalised gelatine membranes.

From a numerical taxonomic study of B. cinerea, Morgan (1971a) found it to be a very complex entity with distinct cultural races, but found no justification for new taxa. The results allowed separation of the isolates to two groups: (A) producing grey cultures with good sporulation, lacking or with few

sclerotia, and (B) cream or white, sparsely sporulating, developing sclerotia. He considered that examination of additional isolates may have shown a bridging of A to B to form a continuum. A review of these and other attempts at subdivision of B. cinerea has been provided by Lorbeer (1980).

Formae speciales of B. cinerea have been described including f. sp. convallariae (mentioned previously), f. sp. lini van Beyma and f. sp. coffeae Hendrickx. The forma specialis lini was separated primarily on its ability to produce citric acid. Van der Spek (1965) concluded this was not a valid criterion since the ability was common in B. cinerea and other species. The f. sp. coffeae, isolated from coffee berries, was differentiated by conidial size and an assumption of host specificity.

A case of mistaken identification to genus has been pointed out by Walker and Minter (1981). When they examined the type culture of Gonatobotryum sclerotigenum van Warmelo, they found it had the characteristics of Botrytis. This conclusion had been reached at the Centraalbureau voor Schimmelcultures since G. sclerotigenum was included with B. cinerea in a photocopied portion of their culture catalogue supplied in 1981.

The debate on the connection between B. cinerea and the teleomorph Botryotinia fuckeliana (De Bary) Whetzel was reviewed by Buchwald (1949) who concluded that Botryotinia fuckeliana had a Botrytis cinerea type conidial state, but he linked the fungi involved exclusively to Vitis as host and proposed the name Botrytis fuckeliana for the conidial state. Groves and Loveland (1953) produced apothecia from matings between Swiss isolates from

Vitis and Canadian single ascospore cultures from other hosts. They concluded that the connection between Botryotinia fuckeliana and Botrytis cinerea had been established and that these were not restricted to Vitis as host.

The natural occurrence of Botryotinia fuckeliana in bean fields in New York was recorded by Polack and Abawi (1975). Single ascospore cultures yielded B. cinerea, and apothecia were produced in the laboratory from matings between these isolates. The occurrence of apothecia in the field was noted as providing inoculum and a source of genetic variation in the B. cinerea population.

The mating system involved in the production of Botryotinia fuckeliana was investigated by Groves and Loveland (1953) and was reported as bipolar, with self-sterility and intra-group sterility but with inter-group fertility. Lorbeer (1980) expected that self-fertility would be found within species of Botryotinia and this was shown for Botryotinia fuckeliana by Lorenz and Eichorn (1983). Working with cultures ex vitis from the Rhine Valley, they found of 56 wild strains, 22 were homothallic as were 5 of 6 cultures derived from single ascospores.

Heterokaryosis and its influence on variation in B. cinerea was recognised by Hansen and Smith (1932), and it has been held to explain the variability within the species that was found in a number of investigations, for example, that of Menzinger (1966) on variation among single spore cultures from a single mycelium. Caten and Jinks (1966) held reservations on the frequency and importance of heterokaryosis and found the sharing of a number of

identical alleles was a prerequisite for heterokaryon formation. Although it is a possible outcome of heterokaryosis, parasexual behaviour in B. cinerea has apparently not yet been reported (Lorbeer, 1980).

Botrytis cinerea is capable of causing a subsurface-limited rot in grape berries, known as 'noble rot', which makes an important contribution to the quality of certain sweet wines. The factors that control the development of this particular type of rot are considered to be agropedological and climatic (Ribéreau-Gayon, Ribéreau-Gayon and Seguin, 1980). The possibility that noble rot production might be restricted to a particular type of B. cinerea does not appear to have been examined.

Benzimidazole fungicides have been very useful in controlling Botrytis infections in plants. Continued exposure of Botrytis populations to these fungicides has lead to the evolution of insensitive strains. These fungicide-resistant strains were not characterised by any particular features of their morphology (Richmond and Pring, 1980; Beever and Brien, 1983).

Apart from the traditional approaches to Botrytis taxonomy (making use of the information on host plant, cultural characteristics and micromorphology), limited use has been made of biochemical and serological methods (Jarvis, 1980). The use of electrophoretic examination of enzymes and soluble proteins in relation to taxonomy has been applied by a number of workers, typically on the scale of introductory experiments involving only several isolates. Multiple forms of the pectic enzyme polygalacturonase were shown to be formed by the few isolates of

B. cinerea examined by Magro, DeLenna, Marciano and Pallavicini (1980): DiLenna, Marciano and Magro (1981) and DiLenna and Fielding (1983). A more extensive survey of sclerotial proteins, arylesterase, acid and alkaline phosphatase and glucose-6-phosphate dehydrogenase from 30 isolates representing six species of Botrytis was made by Backhouse, Willetts and Adams (1984). They found from similarity matrices and computed dendrograms, that B. cinerea formed a single, well-defined cluster, clearly distinct from the rest of the isolates which formed five clusters corresponding to B. aclada, B. fabae, B. gladiolorum, B. tulipae and B. viciae. This confirmed the initial separation into species made by the use of traditional criteria. They concluded that electrophoresis of sclerotial proteins appeared to be a useful supplement to other criteria in the taxonomy of Botrytis.

5. Taxonomy of Penicillium species

The taxonomy of the ubiquitous fungi which constitute Penicillium Link is difficult and continues to evolve. Currently, the taxonomic systems of Raper and Thom (1949), Samson, Stolk and Hadlok (1976) and Pitt (1979) are in use. These systems all use the classical methods of observation of growth in culture and microscopy to observe micromorphology. A number of alternative approaches have been made, based on a variety of physiological and biochemical methods. One of these was the use of pyrolysis gas

chromatography by Kulik and Vincent (1973). From an examination of 27 isolates they concluded that examination of a large number of strains could provide a system amenable to computer analysis for species identification. Pyrolysis gas chromatography was also applied by Söderström and Frisvad (1984), to nine isolates from the P. crustosum complex. They found the method allowed the differentiation of groups with good but not perfect agreement with the grouping obtained by means of mycotoxins produced.

Other examples of the use of physiological and biochemical methods related to taxonomy include responses to selective media and the presence of various toxins in media (Frisvad, 1981). Extensions of this approach included such tests as pH limits for growth, the ability to hydrolyse a variety of substrates (Bridge, 1985), and the use of enzyme test strips to examine enzymes from conidia (Bridge and Hawksworth, 1984). Very limited experimental work employing serology has been useful in investigating relationships between taxa, for example, the work of Polonelli, Castagnola, D'Urso and Morace (1985) and Polonelli, Morace, Rosa, Castagnola and Frisvad (1987).

Many species of Penicillium produce mycotoxins, a property of considerable importance in consideration of human and animal health (Food and Agriculture Organisation, 1979). Studies relating the production of mycotoxins and other secondary metabolites to taxonomy in Penicillium have been particularly rewarding. Principal in these studies have been those of Ciegler, Fennell, Sansing, Detroy and Bennett (1973) on the P. viridicatum complex, carried further by Ciegler, Lee and Dunn (1981), and the

monumental studies of Jens Frisvad and co-workers, e.g. Frisvad and Filtenborg (1983), Frisvad (1985), Polonelli *et al.* *cum* Frisvad (1987). This approach can even be applied to the dried cultures preserved in herbaria (Paterson and Hawksworth, 1985).

An endeavour to integrate results from a number of approaches has been proposed by Onions, Bridge and Paterson (1984). The complexities involved have been pointed out by Bridge, Hudson, Kazakiewicz, Onions and Paterson (1987) who demonstrated that morphological and physiological characteristics, DNA content and secondary metabolite production may all vary significantly between single-conidium isolates of a single strain, so they may not be sound criteria for species delineation if treated in isolation.

The electrophoretic approach has been examined on a very limited scale. Garber and Beraha (1966) used electrophoresis to examine the pectic enzymes produced by *P. italicum*, *P. digitatum* and *P. expansum* both *in vitro* and *in vivo* and found that patterns of endo- and exo-polygalacturonase depended on species, virulence, carbon source and gel pH. Bent (1967) examined the proteins of three *Penicillium* species (*P. griseofulvum*, *P. chrysogenum* and *P. frequentans*) by means of electrophoresis. Although the patterns obtained varied greatly with culture age, each species could be distinguished by its protein pattern which was reproducible and characteristic at any stage of culture. Anné and Peberdy (1981) carried out iso-enzyme analysis of eight enzyme systems of *P. chrysogenum*, *P. roquefortii* and their inter-specific protoplast fusion hybrids. They found in most cases the enzyme patterns of

the species were distinct. They found parental isozymes and novel bands in hydrids. Further studies in hybridization between Penicillium species by means of protoplast fusion have been reported to provide indications of taxonomic relationships and/or genetic non-homology useful in taxonomy (Anné, 1985).

[An integrated approach aimed at solution of taxonomic problems in Penicillium (later including Aspergillus) was provided by the establishment in January 1986 of the International Penicillium Working Group (IPWG). This group consisted of traditional taxonomists Drs. A.D. Hocking, M.A. Klich, A.H.S. Onions, J.I. Pitt, R.A. Samson, and Mr. A.P. Williams, together with Dr. J.S. Frisvad providing expertise on mycotoxins and secondary metabolites, Dr. E. Mullaney providing expertise in molecular fungal genetics, and the author (R.H. Cruickshank) carrying out electrophoretic studies on enzymes from the fungi investigated. In September 1986, IPWG became a Subcommission of the International Commission on the Taxonomy of Fungi, known as the Subcommission on Penicillium and Aspergillus Systematics (SPAS).]

6. Taxonomy and identification of members of the Ceratobasidiaceae

The family Ceratobasidiaceae includes the teleomorphic genera Thanatephorus Donk, Ceratobasidium Rogers, Waitea Tu & Kimbrough, Agnathanatephorus Tu & Kimbrough and Uthatobasidium Donk (Tu and Kimbrough, 1978). These genera are included in the form genus Rhizoctonia DC. The development of Rhizoctonia taxonomy, evolution of anastomosis grouping as a practical taxonomy and the present status of research on anastomosis groups (AG) have been reviewed by Ogoshi (1985, 1987).

The teleomorphic genus Waitea and the species W. circinata were first described by Warcup and Talbot (1962). Among the characteristics of this species were the production of Rhizoctonia-like hyphae and pinkish to orange sclerotia tending to brown with age. In describing the new species Rhizoctonia oryzae, Ryker and Gooch (1938) mentioned the production of salmon sclerotia, and, in the description of the new species Rhizoctonia zeae, Voorhees (1934) described the production of salmon-pink mycelium which became red-brown with age. Since other members of the Rhizoctonia complex produce cream to brown colonies, the production of salmon-coloured mycelium in various forms by R. oryzae, R. zeae and W. circinata set them apart and they have been found to constitute a natural group. Windham and Lucas (1984) found the perfect stage of R. zeae to be Waitea. Martin, Lucas and Campbell (1984) suspected R. zeae and W. circinata to be related

from evidence of hyphal characteristics and tolerance to benomyl. Oniki, Ogoshi, Araki, Sakai and Tanaka (1985) found that both R. zeae and R. oryzeae had a W. circinata teleomorph but the two Rhizoctonia species constituted separate anastomosis groups WAG-Z and WAG-O, respectively.

While anastomosis grouping has provided the basis for a working taxonomy, it suffers from several deficiencies (apart from the difficulty and tedium of its performance). Isolates are encountered which fail to anastomose with any of the AG tester strains, indicating that a larger number of groups exist and await recognition. This process continues with the recognition of Ceratobasidium AG-P (Oniki et al., (1984) and AG-Q (Oniki, Kobayashi, Araki and Ogoshi, 1986), Thanatephorus AG-8 (Neate and Warcup, 1985) and AG-9 (Carling, Leiner and Kebler, 1986). Another deficiency is in the degree of identification attained by anastomosis grouping, since it has been shown that significant heterogeneity may exist within a single AG. An example is AG-4, regarded as so homogeneous as to warrant specific rank (Anderson, 1982), which was found to be heterogeneous when studied by DNA base sequence homology (Kuninaga and Yokosawa, 1984a). Investigation of DNA base sequence homology has also shown AG-6 to be an heterogeneous assemblage (Kuninaga and Yokosawa, 1984b).

Evidence in support of AGs as meaningful in taxonomy came from examination of the DNA base composition of members of AGs in Thanatephorus which indicated AG-1, AG-2-1, AG-2-2, AG-3, AG-4, AG-5, AG-6 and AG-BI were genetically independent units (Kuninaga and Yokosawa, 1980). From their series of studies on DNA base

sequence homology, Kuninaga and Yokosawa (1985a; 1985b) concluded that AG-1, AG-2 with AG-BI, AG-3, AG-4, AG-5, AG-6 and AG-7 were genetically isolated groups and that each was a biological species in the taxonomic species R. solani.

As mentioned in a review article (Ogoshi, 1985) electrophoresis of enzymes and proteins has been applied to R. solani in a few studies and has given promise of usefulness in distinguishing anastomosis groups. The author has used electrophoresis to examine extracellular enzymes from Thanatephorus, Ceratobasidium, Waitea and Aquathanatephorus isolates. Part of this study has been of cultures from the Western Australian wheat belt provided by Dr. M.W. Sweetingham. In a collaborative study, electrophoretic characterisation and grouping were performed by the author, while studies on the pathogenicity of the isolates were made by Dr. Sweetingham. Studies on the classical taxonomic characteristics of the isolates were made by Miss D.H. Wong. Results from these studies were collated and published (Sweetingham, Cruickshank and Wong, 1986). A copy of the paper is provided in Appendix 25.

This study demonstrated the value of pectic zymograms in allowing the grouping of isolates to zymogram groups (ZG) which had a useful biological information content. All members of a ZG had a similar cultural and morphological appearance, had the same nuclear state, the same teleomorph in cases where this was determined, and the same characteristics of pathogenicity and of disease symptoms produced. Five groups were detected among isolates allied to Thanatephorus and were coded ZG1 through to

ZG5. The five groups allied to Ceratobasidium were coded CZG1 through to CZG5, while the single Waitea group was coded WZG1.

An electrophoretic survey of Rhizoctonia isolates from agricultural soils in South Australia has also been made, with isolates provided by Drs. S.M. Neate, A.D. Rovira and H.J. McDonald. A range of groups was present, very similar to that found from Western Australia. Collaborative publications of this work have been made, detailing the groups detected, related to hosts and districts (Neate, Cruickshank and Rovira, 1988; Neate and Cruickshank, 1988) (Appendix 25). It was shown that isolates grouped by anastomosis behaviour were also placed in the same groupings by means of zymograms. This was shown for AG-2-1, AG-3 and AG-4. The new AG-8 (Neate and Warcup, 1985) was subdivided on zymogram terms into four groups which included ZG1 and ZG2 of Sweetingham et al. (1986).

III DEVELOPMENT AND REFINEMENT OF THE ELECTROPHORETIC TECHNIQUES USED IN THIS STUDY

1. Electrophoretic techniques and pectic zymograms

During studies which examined the induction, latency and outbreak of latent infections by Monilinia fructicola (Wint.) Honey in apricot fruit (Wade and Cruickshank, 1978), the need arose to examine the pectic enzymes produced by the fungus and to follow their behaviour during the development of methods to purify component enzymes. Since polyacrylamide slab gel electrophoresis was being used in the laboratory at the time to detect alpha-amylase activity by a substrate incorporation method (Boucher, 1975), this prompted the author to design a similar method for polygalacturonase. Pectin was incorporated in the gel, then after electrophoresis the gel was incubated in acetate buffer at pH 5.0 followed by staining with ruthenium red. A record of the results (in negative contrast) was produced by photographic printing. Working under a suitable safe-light, the stained gel was placed on bromide printing paper in a tray of water. An enlarger was used to give light exposure through the gel onto the paper. Use of the enlarger gave control of the light intensity and time. The developed paper gave a photogram of the gel (Figure 1).

In photograms, the unstained zones due to PG activity were recorded as black in the grey background. White zones were also present except from enzyme samples that lacked PE. These resulted from intensified staining and were assumed to be due to PE

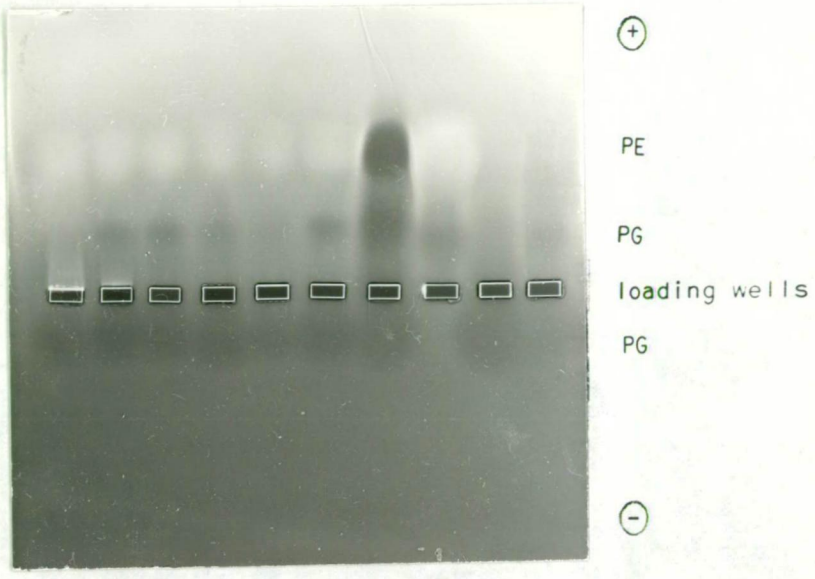


Figure 1 Zymograms of Monilinia fructicola pectic enzymes. Polyacrylamide gel 3.5 mm thick with loading wells centrally placed.

PE : pectinesterase (pale)

PG : polygalacturonase (dark)

The enzymes in wells 9 and 10 (at right) were partially purified and lacked PE.

activity. This assumption was supported by the finding of Sterling (1970), that ruthenium red staining of pectin is intensified on its de-esterification to pectic acid.

In Figure 1 the sample wells can be seen as the line of rectangles across the centre of the gel. This central loading position was used since the extent of cathodic migration of the enzymes at pH 8.7 used in electrophoresis was not known. From results as in Figure 1, cathodic migration of enzymes from M. fructicola at pH 8.7 was not extensive and consequently the loading well position was moved towards the cathode to a line 15 mm from the cathodic edge of the gels. In subsequent tests of the enzymes from a number of fungi, cathodic migration at pH 8.7 was not seen to exceed 10 mm under the conditions of electrophoresis terminated after bromophenol blue tracker dye migrated 50 mm from the sample wells towards the anode. This positioning of sample wells ensured that enzymes migrating towards the cathode were not lost from the gel as they are in systems employing vertical slab gels in which samples are loaded at the cathodic edge of the gels.

In attempts to improve the resolution and clarity of results, a large number of experiments were performed where electrophoretic conditions were varied. Satisfactory results were obtained for pectic enzymes in gels containing 10% acrylamide monomer and 0.25% bisacrylamide. No improvements were evident when this composition was varied in total acrylamide content or proportion of bisacrylamide. The discontinuous buffer system of Poulik (1957), used at half strength, gave satisfactory results and increase in concentration extended the time required for

electrophoresis without a gain in clarity of results. The rate of electrophoresis had limitations at both extremes. If slow, increased time of run allowed some diffusion of bands of enzymic activity. At high voltages, heat production in the system caused evaporation from the gels and problems in enzyme stability. A compromise was needed, and 7.5 V per cm initial potential difference with electrophoresis at constant current gave a reasonably short run time of 80 minutes without overheating.

Initially, electrophoresis was performed in a cold room at $4^{o}C$. This aided stability of enzymes and gave much better results than those obtained from electrophoresis at room temperature. However, gel cooling was not uniform, in evidence the final buffer front was curved, with less migration towards the sides of gels where cooling was more efficient (Figure 2). To overcome this problem, copper plates were used to support the gels during electrophoresis (in place of the original glass plates). These copper plates were cooled by a flow of water at $4^{o}C$ passed through copper tubing sweated to the lower face of the plates. Efficient and uniform cooling resulted in straight buffer fronts and made results from any positions across the gels comparable without need to calculate Rf values at each position.

Since evaporation from gels during electrophoresis may have reduced the water content at the surface and altered enzyme migration in this region, tests were made comparing results from gels with and without a 'Parafilm' covering during electrophoresis. No difference was detected, so this modification of the method was not used subsequently.

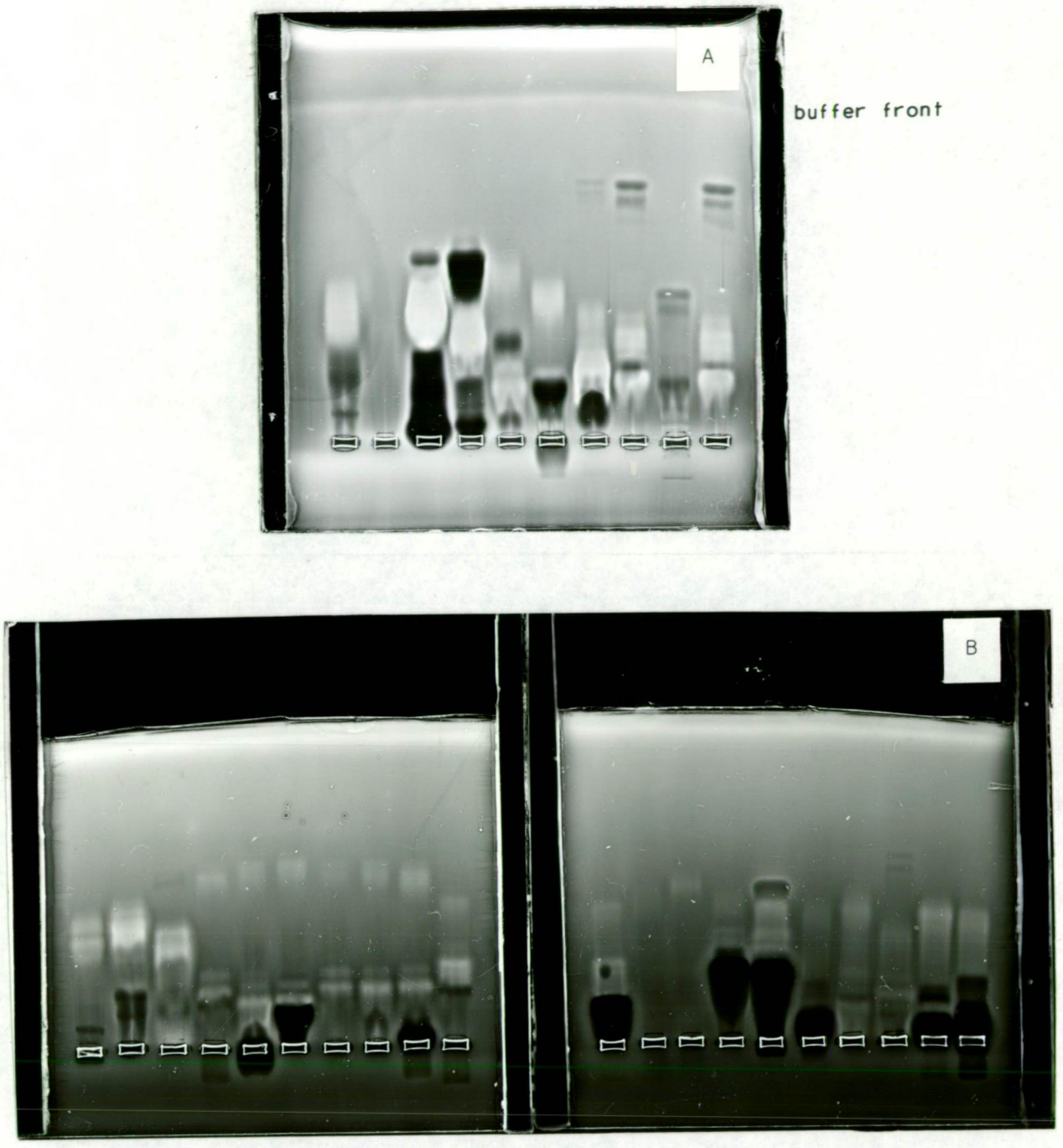


Figure 2 Curved buffer fronts resulting from inefficient cooling in (A) a single gel and (B) two gels run side by side. The gel above the buffer front was cut away in (B).

Observation of the loading wells during electrophoresis revealed that fluid was soon lost, resulting in obstructions to the uniformity of electrical flow in the gel. Tests were performed aiming to rectify this by maintaining filling in the wells, comparing results from inclusions of sucrose, filter paper, Sephadex G25-superfine and Sephadex G150-superfine. No improvement in results was obtained, except from the addition of Sephadex G150-SF which significantly reduced tailing effects evident in zymograms from some fungi and generally improved the clarity of results (Figure 3). In practical use, sufficient Sephadex G150-SF was used to give a pipettable slurry in admixture with the enzyme sample. This was peformed economically by the addition of 50 µl of enzyme solution to approximately 2.5 mg Sephadex. With practice the latter could be judged by its appearance on the tip of a fine scalpel blade. Mixing was performed on the surface of a plastic petri dish by drawing up and expulsion several times from the disposable tip of the automatic pipette used to add the enzyme sample in the first place. This technique was incorporated in the standard method and was used in all subsequent work.

The 10 well gels used initially were 3.5 mm thick and 82 mm square. The sample walls were 1.5 mm wide, 4.0 mm long and 2.5 mm deep. While this provided a sturdy system of gel moulds and resultant gels, it was extravagant in use of gel components, not designed to handle large numbers of enzyme samples, and there was a prospect for better resolution in a more delicate system employing thinner gels and smaller loading wells. On test, reduction of gel thickness to 2 mm did improve resolution.

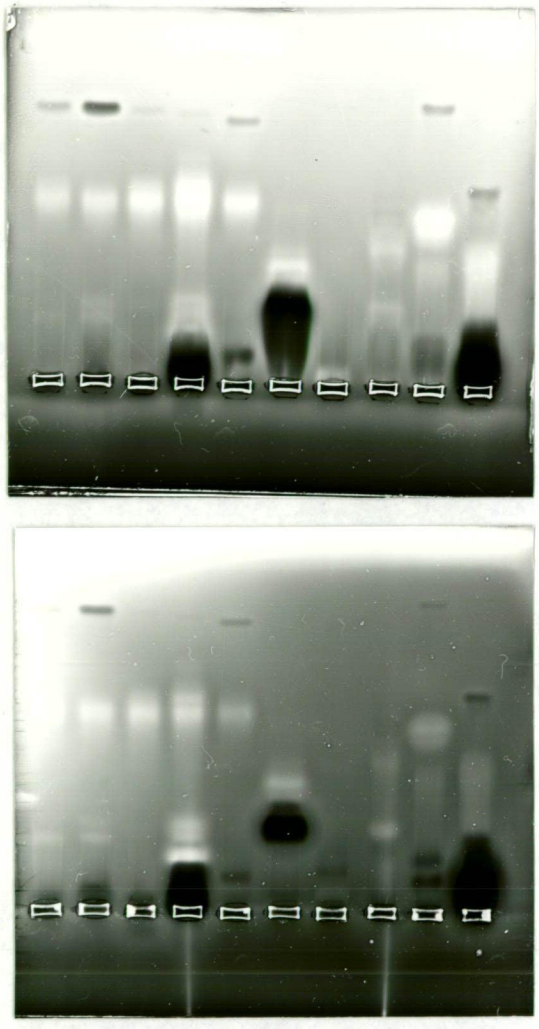


Figure 3 Improved resolution obtained by the addition of Sephadex G150-Superfine with enzyme samples (B), compared with results in its absence (A).

Reduction to 1 mm gel thickness did not give significant differences from results from 2 mm gels and introduced problems in recording results due to insufficient staining intensity. A gel thickness of 2 mm was introduced to the standard method.

A trial was made using wells 1 mm wide, 1.5 mm deep and lengths across the gel ranging from 2 mm to 4 mm. After electrophoresis and detection of enzymes (Figure 4), a well length of 3 mm and well separation also 3 mm was chosen for standard use, to provide best use of gel space without loss of clarity in the enzyme patterns. The changes in gel thickness and loading well details were incorporated in a system giving gels still 85 mm long but widened to 160 mm and having 26 loading wells. This system was used in all subsequent work.

In the preparation of gels, particularly with the 26 well slim gel format, problems were sometimes encountered when stripping apart the gel moulds after gel polymerization. The gel was required to remain in place on the glass mould when the perspex cover plate with loading well-forming pegs was removed. This was aided by lack of polymerization of a thin film of the gel mixture in contact with the perspex. With extended time to stripping, the gel tended to adhere to the perspex and render the gel unsuitable for use. It was found that gels stripped 5 minutes after evident polymerization usually gave the desired separation when the ambient temperature was 21^{o}C. Experience in laboratory temperatures ranging from 17^{o}C to 26^{o}C has shown that a suitable time for stripping (y) after the admixture of all gel components is given by $y = 39 - 1.2\ x$, where x is the ambient temperature.

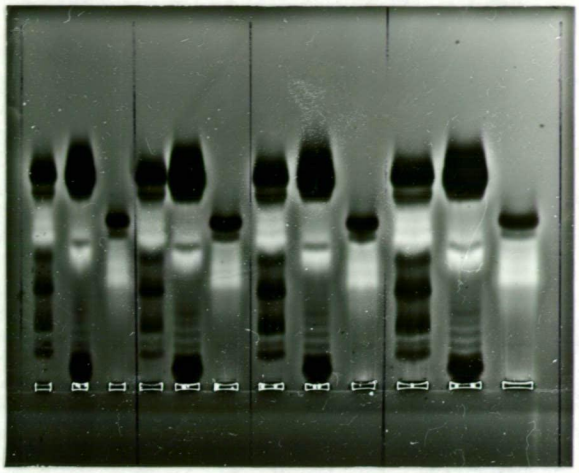


Figure 4 A comparison of results from loading wells of different lengths or spacing.

Lanes 1 to 3 : 2 mm pegs spaced 2.5 mm

Lanes 4 to 6 : 3 mm pegs spaced 2 mm

Lanes 7 to 9 : 3 mm pegs spaced 3 mm

Lanes 10 to 12 : 4 mm pegs spaced 3 mm

This is somewhat of a compromise since the crispest results in zymograms result in gels thoroughly polymerized before stripping. At the other extreme, in gels stripped immediately after the initial setting, isozyme bands were blurred and single isozymes appeared as twin bands (Figure 5). This was apparently due to inhibition of complete polymerization in the gel face exposed to atmospheric oxygen and resulted in isozymes in this region of the gel migrating more rapidly during electrophoresis.

Enzyme detection following electrophoresis at pH 8.7 required action of the enzymes on the incorporated pectin substrate at pH levels suitable for this activity. Fungal pectic enzyme activity has traditionally been examined in the vicinity of pH 5, and both PE and PG activity were detected in gels incubated at pH 5 before staining with ruthenium red. However, crisper results and additional isozymes of PG were detected if the final pH in the gels was lowered to the region of pH 2.5 to pH 3 (Figure 6). In tests of a range of acids used to produce pH reduction, citric acid resulted in poor results, apparently due to reduced ruthenium red staining; tartaric acid gave acceptable results, but the best results were obtained from the use of malic acid; the synthetic DL form was suitable. In the standard technique, each gel was bathed in 150 ml of 0.1 M DL-malic acid for 1 hour after electrophoresis.

While 3.5 mm gels were still in use, changes in pH within gels in response to steeping in acid solutions was examined. Gels were subjected to electrophoresis until the buffer front at the citrate-borate discontinuity had moved past the loading wells,

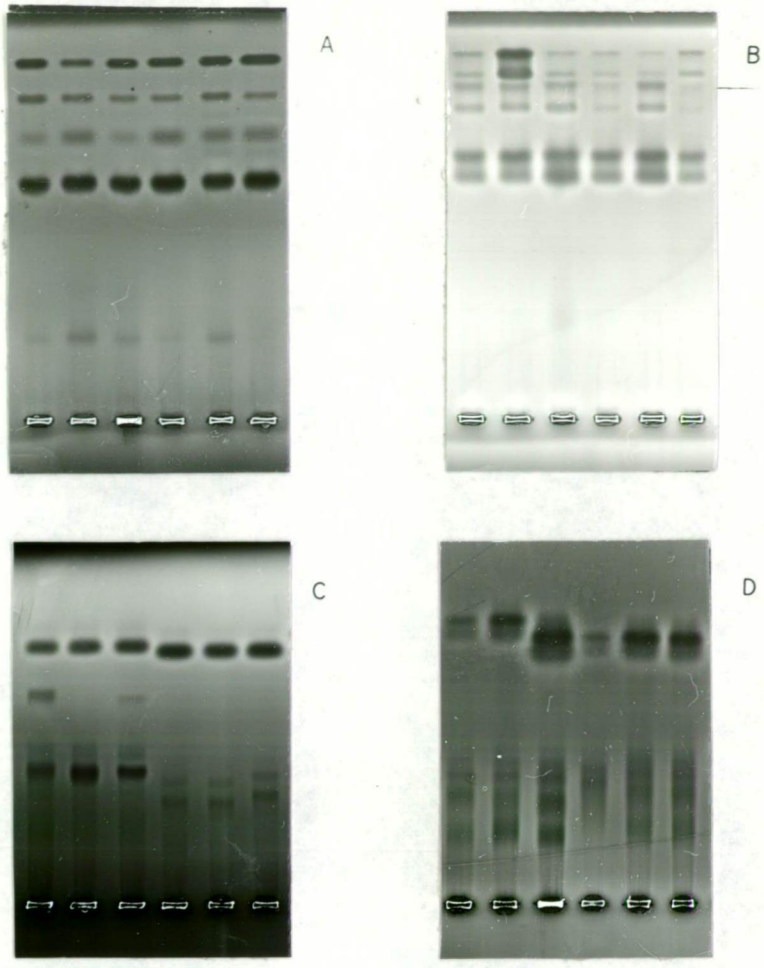


Figure 5 Spurious twin banding and diffuse bands from gels exposed to air before polymerization was complete (B, D), compared with results in more completely polymerized gels (A, C).

Aspergillus flavus enzymes

A, B pectin lyases

C, D ribonucleases

For comparison of band-form only; different isolates are represented in each gel.

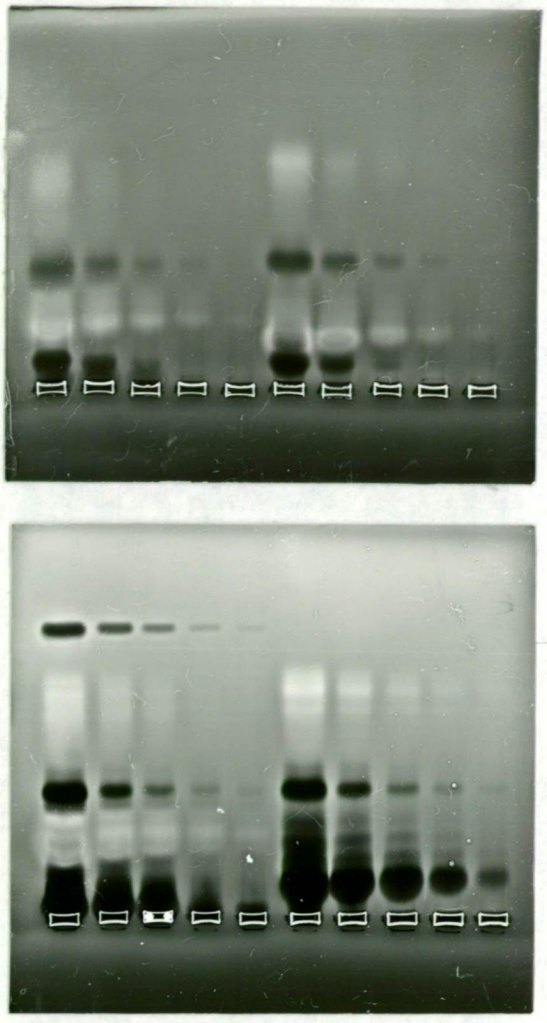


Figure 6 Identical gels incubated at pH 5.0 (A) and pH 3.0 (B).
Buffers : 0.1 M malate.
Dilution series of enzymes were loaded: undiluted culture fluid, then dilutions 1 : 3, 1 : 10, 1 : 30, 1 : 100 ; from two samples.

then indicator dyes responsive to changes within the range pH 2 to pH 8 were loaded into the sample wells then moved into the gel by electrophoresis. The gels were incubated in acidic solutions and pH changes over time were recorded, pH changes monitored by colour changes in the indicators. It had been expected that an acid front would move gradually through the gel depth, with the surface layers rapidly becoming acidic. However, this did not appear to happen, the gel responded as a unit and the pH of the entire gel gradually fell, giving a slightly sigmoidal curve as in acid-base titration when pH was plotted against time. This behaviour was advantageous for enzyme detection since it enabled each isozyme to act at its optimum pH during the incubation.

Since pectins from different plants, for example apples or citrus fruits, differ in their composition and may even show great differences in degree of esterification even from the same plant at different stages of development (Reeve, 1959; Morre, 1968), the influence of different grades of pectin on the pectic zymograms of fungi was investigated briefly. The sharpest results for PG were obtained when a batch of citrus pectin from Calbiochem was used. This pectin gave poor detection of PE isozymes. A batch of citrus pectin from Sigma, reported to contain 88.9% galacturonic acid and with 9.7% methoxyl content, gave clear detection of PE isozymes but less clarity in PG isozymes (Figure 7). To obtain qualities from both batches of pectic, a mixture was used made up of two parts Calbiochem pectin to one part Sigma pectin. The single batch of apple pectin that was tested gave reasonable results but not as clear as those from citrus pectins. A sample of food grade pectin,

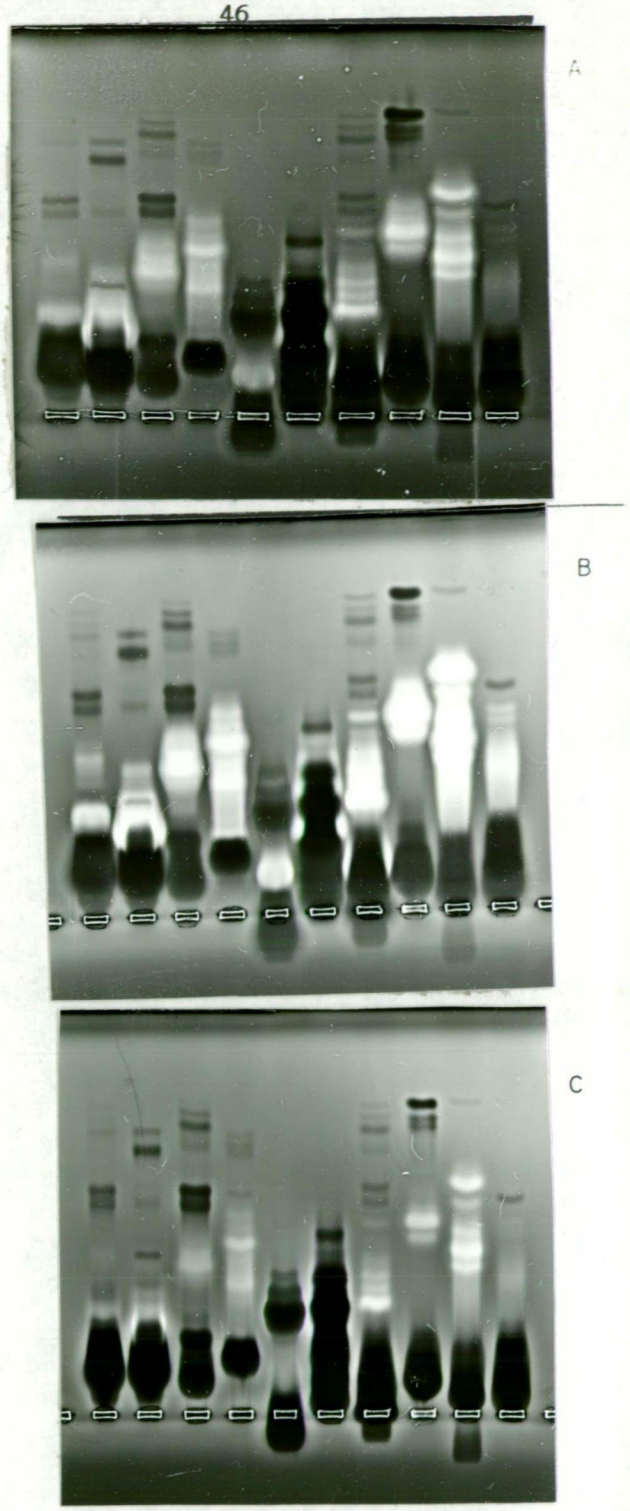


Figure 7 Influence of various pectin substrates in gels on the detection of PE and PG from 10 species of Botrytis.

A : Food grade pectin [4 mm loading wells]

B : Sigma pectin

C : Calbiochem pectin

sold for use in jam making, gave results that would be suitable for routine use, although not as sharp in detail as in the selected mixture of chemical grades (Figure 7).

It is customary in most electrophoretic studies to remove, by dialysis, any low molecular weight materials present in enzyme solutions which might interfere with electrophoresis. This was found to be unnecessary for use in the standard technique. Neither filtration, dialysis, nor the addition of acids or bases at reasonable physiological levels, nor additions of KCl, tested up to 0.1 M, made any detected difference to the results, so culture fluids were used directly without treatment.

In some zymograms, various isozymes appeared to be present in excessive concentration which prevented the detection of individual isozyme bands. Where necessary, this could be resolved by dilution of the enzyme samples (Figure 8). This was usually done with gel buffer, but no significant difference resulted from the use of distilled water.

Recording of results as photograms was used throughout these studies and was found to be suitable for any staining system involving red, yellow, brown or blue-black. Systems involving pale blue were not suitably recorded. The red staining of pectin gels by ruthenium red was suitable for this method, but contrast was enhanced giving blacker PG zones and whiter PE zones when the ruthenium red was oxidised to a yellow-brown form in the gels by steeping in 0.1% ammonium persulphate prior to the production of photograms. High contrast bromide paper was used in the early stages of this study, later changing to Ilfospeed 5.1 M which had

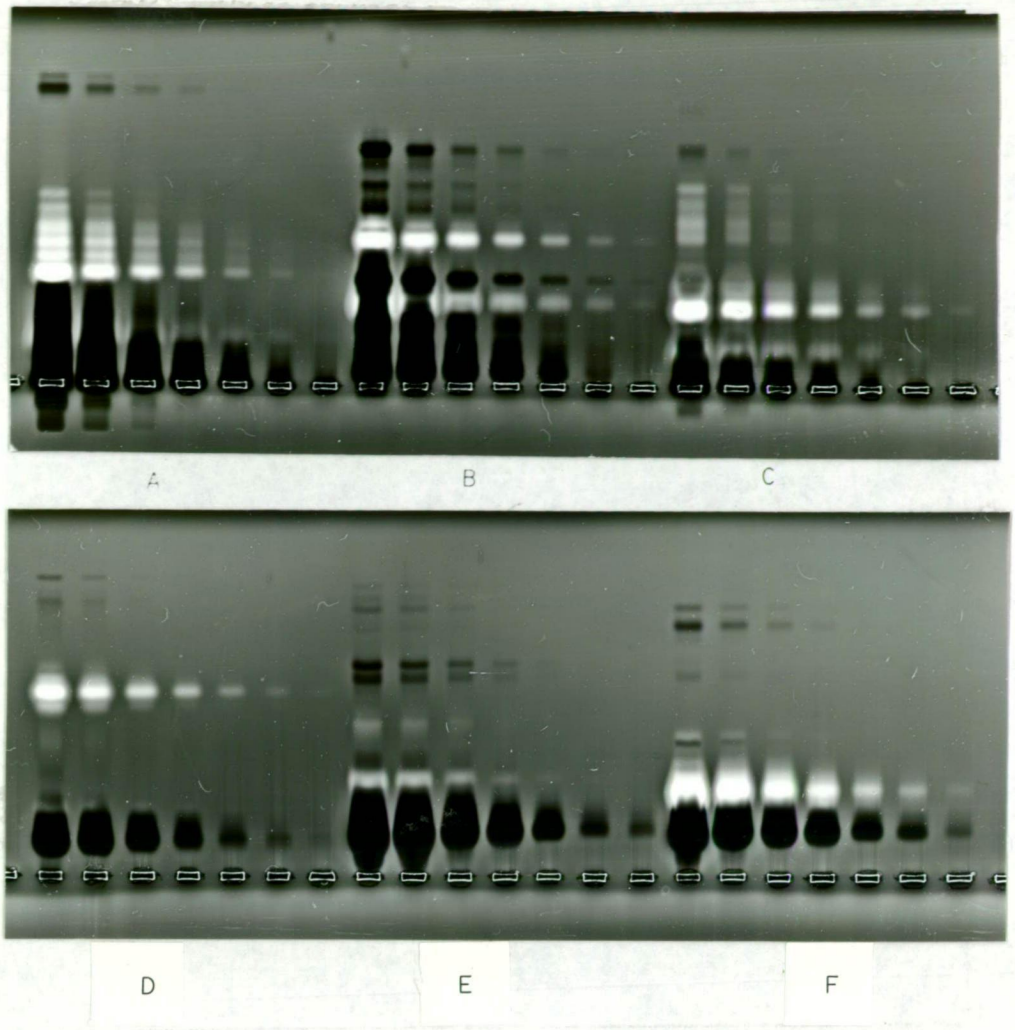


Figure 8 The use of dilution to resolve overloaded PG and PE zones.

For each example, culture fluids were loaded undiluted and diluted 1 : 3, 1 : 10, 1 : 30, 1 : 100, 1 : 300, 1 : 1000.

A : Botrytis narcissicola IMI 193610

B : B. hyacinthi CBS 128.37

C : B. galanthina CBS 327.78

D : B. tulipae IMI 143945

E : B. aclada DAR 28780

F : B. byssoidea CBS 104.23

the advantage of being self-glazing.

The enzymes produced by fungi varied in response to changes in composition of culture media and to some extent in response to temperature and time of incubation. There was a need to study these responses for each genus and to select cultural conditions appropriate to each. Details of such studies are provided in the Results sections for each genus or group in this thesis.

Distinctive pectic zymograms frequently allowed the direct placement of an isolate into a particular species or equivalent group. This tentative placement was substantiated by comparison of the migration distances of the component isozymes with those from authentic cultures. Parallel rulers were invaluable for this purpose. Comparisons of results from different gels were satisfactory in most cases, but if doubt persisted, comparisons were made on a single gel from adjacent wells or wells in close proximity.

2. On the choice of enzyme systems used in this study

The use of pectic enzymes in this study stemmed from attempts to find applications for a new and promising technique. Application to taxonomic studies followed when it was found that the same pectic zymogram was obtained when several isolates of a species were examined and that the pectic zymograms were clearly different between species. In addition, electrophoresis and detection methods for a number of enzyme systems were examined and tested for utility in aiding taxonomic studies. These included acid

phosphatase, amylase, cellulase, esterase, laccase, phosphatidase, protease and ribonuclease. Details of the methods involved are given in the Appendices 8 to 16.

In some instances, particularly in studies on several Penicillium species, results from pectic enzymes have been equivocal and in these instances amylase and ribonuclease zymograms have provided the necessary information to distinguish between the species. In studies on Botrytis species, all the enzyme systems mentioned above were examined. Cellulase and phosphatidase assays were relatively difficult to perform and gave diffuse results. Laccase assays required the use of a different buffer system since the enzymes were degraded above pH 7. The need to change tank buffers was inconvenient. Esterase zymograms were extremely variable from the same isolate.

In general, pectic zymograms were found to be of most value in this study; supporting information from amylase and ribunuclease zymograms was sometimes advantageous.

IV APPARATUS AND PROCEDURES FOR ELECTROPHORESIS

The electrophoresis apparatus used in this study was illustrated by Boucher (1975). An additional power supply was obtained from Middleton Instruments (a Division of Medos Co. Pty. Ltd., Melbourne). This was capable of providing constant voltage to 500 V or constant current to 125 mA. Three electrophoresis tanks were available and could be used simultaneously. They were housed, together with a cooling water reservoir and pump, in a top-loading deep-freeze cabinet modified to run at +4^{o}C.

The cooling plates for the support of gels during electrophoresis were made from flat 2 mm copper plate cut into rectangular form 120 mm by 170 mm. Copper tubing 6.6 mm o.d. was sweated to the lower surface of each plate as a broad S, providing three runs of tubing across each plate (Figure 9). This tubing carried water chilled to 4^{o}C. Electrical insulation of each plate was provided by polyethylene film and a working surface was provided by a sheet of glass 120 x 170 mm. Thermal conductivity was improved by the exclusion of air from between the copper, polyethylene and glass by paraffin oil.

Gel moulds (Figure 10) were constructed from glass and perspex sheets 166 mm x 84 mm. A 2 mm separation of the sheets was provided by cementing a glass strip 84 x 3 x 2 mm at each side of the glass plate. Trouble was experienced in obtaining durable adhesion of these strips, but this was overcome by the use of silicone sealant. All traces of this were removed from faces

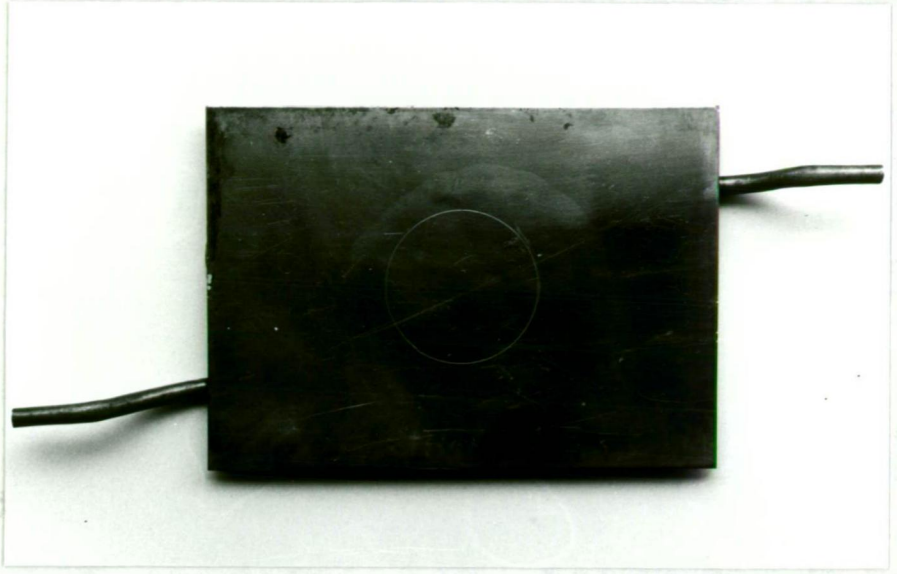
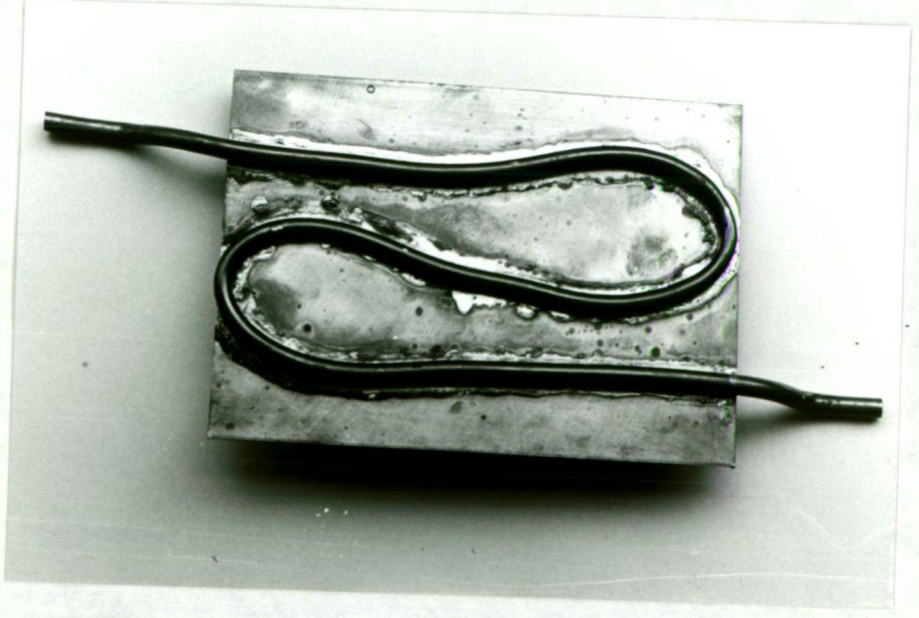


Figure 9 Cooling plates.

A : Top view

B : View from below



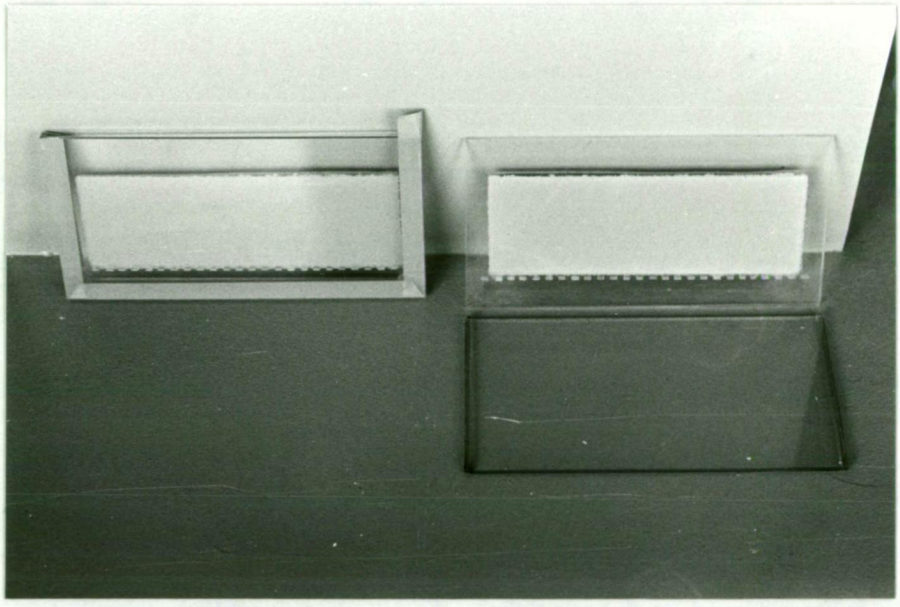


Figure 10 Gel moulds. Components, and a mould assembled for use.

exposed to the gel since its presence inhibited gel polymerization.

Sample wells were formed in gels by a line of perspex blocks 3 x 1 x 1.6 mm spaced 3 mm apart along a straight line 15 mm from one long edge of the perspex sheet. Since the perspex sheets tended to buckle from the heat generated by gel polymerization, they were reinforced by attachment of glass plates by means of double-sided adhesive tape, leaving 10 mm uncoated perspex margins (146 x 64 mm glass).

Gel moulds were assembled then sealed on three sides using Tesa polyester electrical tape 4107, 24 mm wide (BDF Australia Ltd., Boronia, Victoria), to form a trough with the well-forming blocks near its floor. Adhesion of the tape to the glass plate tended to be poor and was aided by warming over a bunsen flame. Tape folding and overlap at the corners of the assembly provided sites for leakage. This was prevented by sealing with molten dental wax.

After all other gel components were dissolved in gel buffer (Appendices 6 and 7), polymerization was initiated by the addition of ammonium persulphate. The mixture was added to the gel mould by means of a glass syringe fitted with a heavy gauge blunt-ended needle. This was rinsed out immediately after use. After gel polymerization, the tape was removed and the perspex plate was levered off, leaving the gel in place in the glass portion of the mould where it remained throughout all subsequent procedures.

After loading the enzyme samples slurried in Sephadex G150-superfine, the gel plate was placed on the cooling plate in

the electrophoresis tank, interposing a few drops of kerosine to displace air and prevent buffer seepage below the gel plate. A line of bromophenol blue solution tracker dye spots were applied to the gel between the cathodic edge and the loading wells. These spots eventually moved with the buffer front, verifying its linearity and providing the means for monitoring the progress of electrophoresis to the standard 50 mm dye migration beyond the loading wells. Electrical connection to the gel edges from the electrode tanks was made by double layers of hospital-grade lint, wet with tank buffer (Figure 11). Electrophoresis was performed with constant current set to give an initial potential difference of 70 V across the gel.

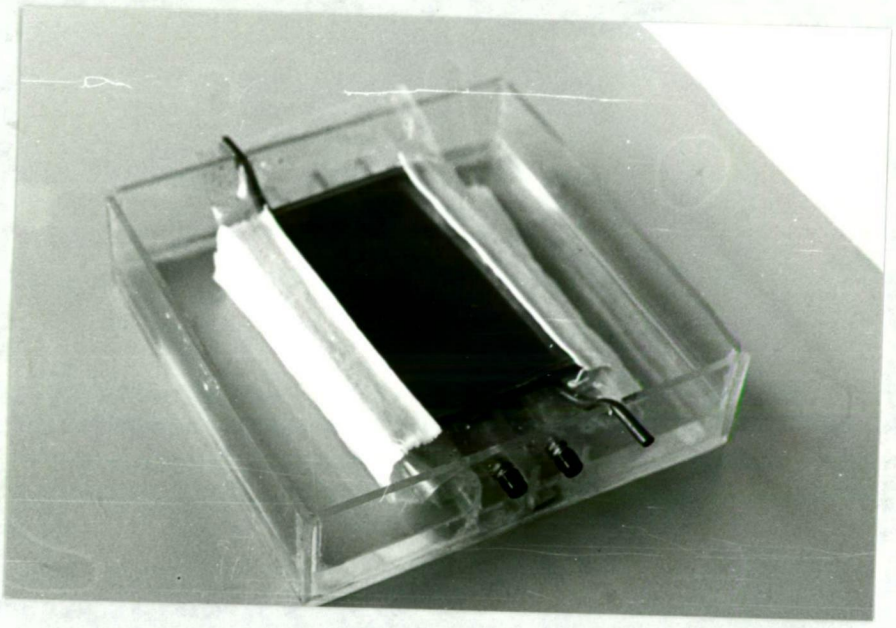


Figure 11 Gel in place in the electrophoresis tank, with electrode wicks applied to the gel.

V STUDIES ON BOTRYTIS SPECIES

1. Materials and Methods

1.1 Cultures examined

Cultures obtained from culture collections are listed in Appendix 17, giving species, authorities and culture collection numbers. The list includes the source of isolates and the host plants, where these have been specified. The number of isolates from other sources is noted.

Representative isolates of Botrytis cinerea from culture collections are listed in Appendix 18. The author's collection contained 231 isolates of this species.

1.2 Culture maintenance

Cultures were maintained on potato dextrose agar (PDA) slopes in bijou bottles, incubated at 22°C for 7 days after inoculation and then stored at 4°C. Subculturing was performed every 4 months.

1.3 Enzyme production

Most of the work on this genus made use of cultures in liquid media contained in 84 mm disposable Petri dishes, using 20 ml of medium per dish. A change to bijou bottles containing 2 ml of liquid medium was later found to give satisfactory results.

A wide variety of culture media for pectic enzyme production were devised and tested during the course of this study. Some

pertinent examples are given in Appendix 1. Cultures were inoculated by needle-point, preferably with conidia, and were incubated for 7 days at 22-25°C. Some isolates, including those of B. fabae, B. anthophila, B. spermophila, B. ranunculi and B. ficariarum, gave improved enzyme yields after incubation for 10-14 days.

Potato decoction was found to give suitable yields of ribonuclease.

1.4 Electrophoresis and detection of enzymes

Methods are given in the Appendices 8 to 16.

2. Results

Each isolate was found to have the potential to produce a range of isozymes of the pectic enzymes PG and PE. Examples are given in Figure 12 of enzyme production from one isolate of B. narcissicola and from one isolate of B. aclada (B. allii) grown in a variety of media. The presence or absence of pectin in the media had a marked effect on the PG isozymes produced. Lanes 1 to 9 were from media containing pectin and from each species there was a general similarity in PG isozymes, variation being mainly in intensity of the components. A different enzyme pattern was produced in media lacking pectin, best expressed from lanes 10 and 11 in Figure 12 A and B. Similar responses to media with and without pectin are shown in Figure 13; some PG isozymes appeared to be induced by pectin while others were repressed. The isozymes of PE were

Figure 12 The influence of culture media on pectic enzymes from (A) B. narcissicola IMI 193610, and (B) B. aclada DAR 28780.

Media:

1. 0.5% alcohol-insoluble residue of onion.
2. 0.5% alcohol-insoluble residue of pumpkin
3. 0.5% alcohol-insoluble residue of apricot
4.* Pectin, ammonium tartrate
5.* Pectin, NH_4NO_3, buffered to pH 4 by malate
6.* Pectin, $(NH_4)_2SO_4$
7. Pectin, Czapek salts
8. Pectin, Czapek salts, yeast extract
9.* Pectin, sucrose, NH_4NO_3
10.* Sucrose, NH_4NO_3, sodium malate, pH 6
11. 3% sucrose, Czapek, yeast extract
12.* Sucrose, $(NH_4)_2SO_4$
13.* Glucose, ammonium tartrate
14.* Soluble starch, NH_4NO_3

*Containing 1.0 g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$, 0.5 g Oxoid yeast extract, 2.0 g nitrogen source, 10.0 g carbon source per litre.

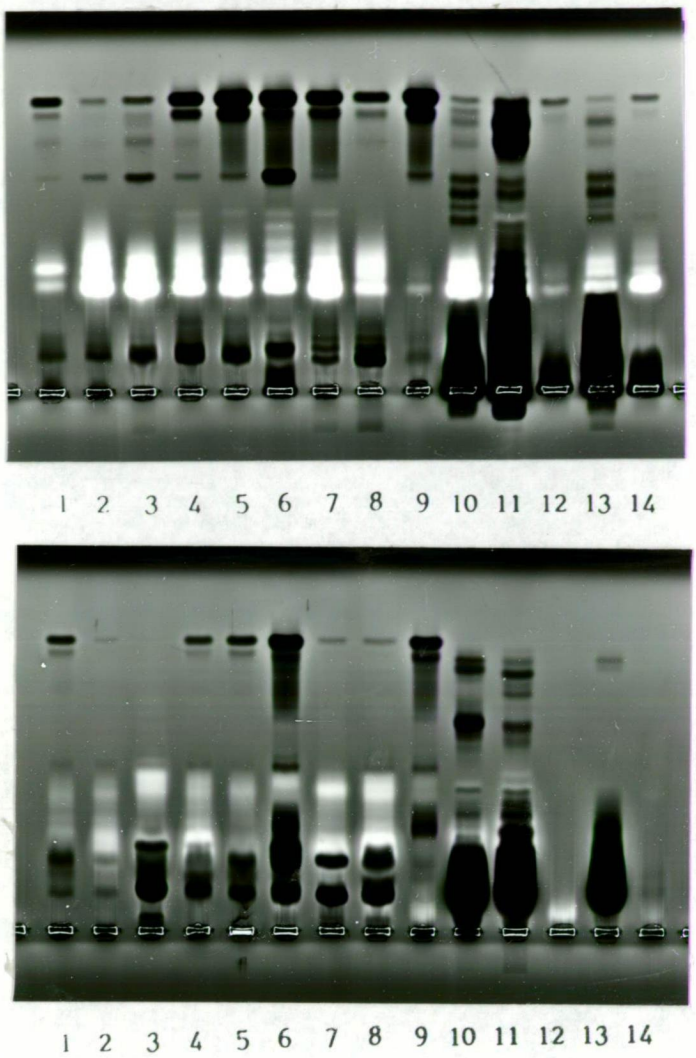
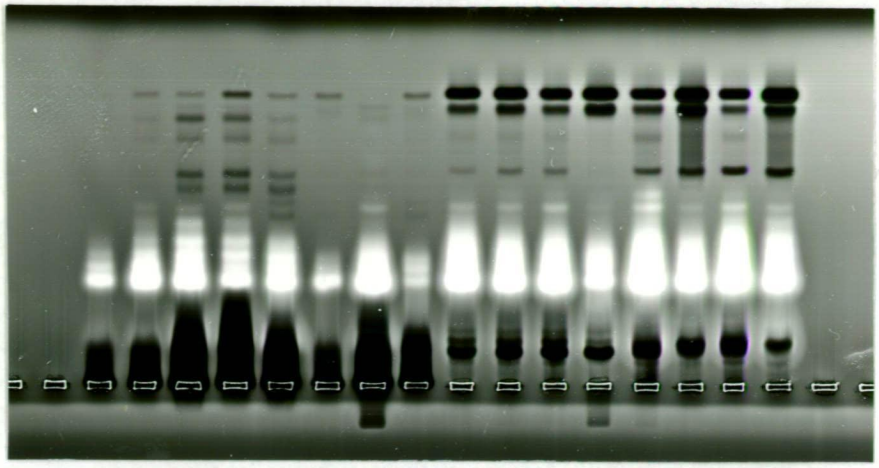


Figure 12



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 13 Influence of culture media on pectin enzyme production by B. narcissicola IMI 193610.

Media for lanes 1-8 contained 1% sucrose; for lanes 9-16, 1% citrus pectin.

Lanes	Salts	N Source[a]	Additional
1, 9	Czapek	$NaNO_3$	
2, 10	Czapek	$NaNO_3$	yeast extract
3, 11	standard[b]	$NaNO_3$	
4, 12	standard	asparagine	
5, 13	standard	NH_4 tartrate	
6, 14	standard	NH_4NO_3	
7, 15	standard	NH_4NO_3	0.15% malic acid, pH 6.0 by NaOH
8, 16	standard	$(NH_4)_2SO_4$	

[a]Nitrogen sources at concentrations to give a nitrogen content equal to that in Czapek medium (0.494 g/L).

[b]"Standard salts" : 1.0 g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$, 0.5 g Oxoid yeast extract per litre.

Excepting 7 and 15, media were adjusted to pH 4.5 before autoclaving.

produced constitutively in media containing either pectin or sugars. To obtain most information from each isolate, results were examined from both pectin and sugar-based media.

Pectic zymograms prepared from a number of isolates of a species were found to have a general uniformity of pattern and correspondence in the Rf of the component bonds. This is illustrated in Figure 14 for nine isolates of B. tulipae. These show very close similarities, except in PG zones near the loading wells which vary greatly in intensity. Similarities within a large sample of a species are shown for 77 isolates of B. cinerea and taxa allied with this species by their zymogram characteristics in Figure 15. Variations in the components of these zymograms will be considered later in the major section dealing with B. cinerea (Section VI).

Zymograms from isolates of B. aclada grown in three media are presented in Figure 16. An isolate of B. septospora was included (lane 1 in each gel) as were isolates of B. byssoidea (lanes 23 and 24 in each). General similarities were present within B. aclada grown in each culture medium. Very close similarity in each gel supported synonymy of B. septospora with B. aclada and very close relationship to B. byssoidea.

Zymograms from isolates representative of species (Table 2) are given in Figure 17, illustrating their distinctive characteristics. These show a clear difference between B. hyacinthi (lane 13) and B. tulipae (lane 22) for example. Similarly, zymogram evidence distinguished between B. anthophila and B. spermophila (Figure 18). As well as providing evidence to

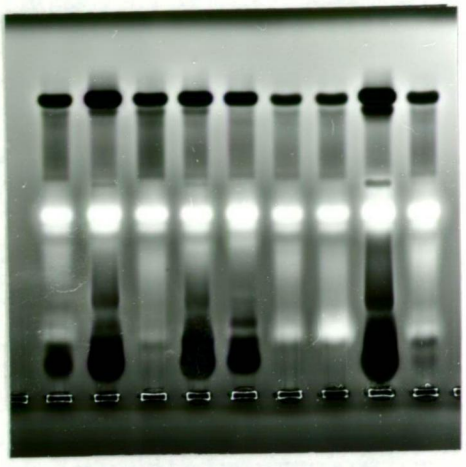


Figure 14 Pectic zymograms from nine isolates of B. tulipae, illustrating their general similarity.

Figure 15 Zymogram similarity in the B. cinerea complex. 77 isolates. "P,$(NH_4)_2SO_4$" medium.

a : B. cinerea f.sp. lini CBS 131.28

b : B. convallariae CBS 179.63

c : B. cinerea f.sp. coffeae IMI 100942

d : Gonatobotryum sclerotigenum CBS 261.71

e : B. palargonii CBS 497.50

All others, B. cinerea.

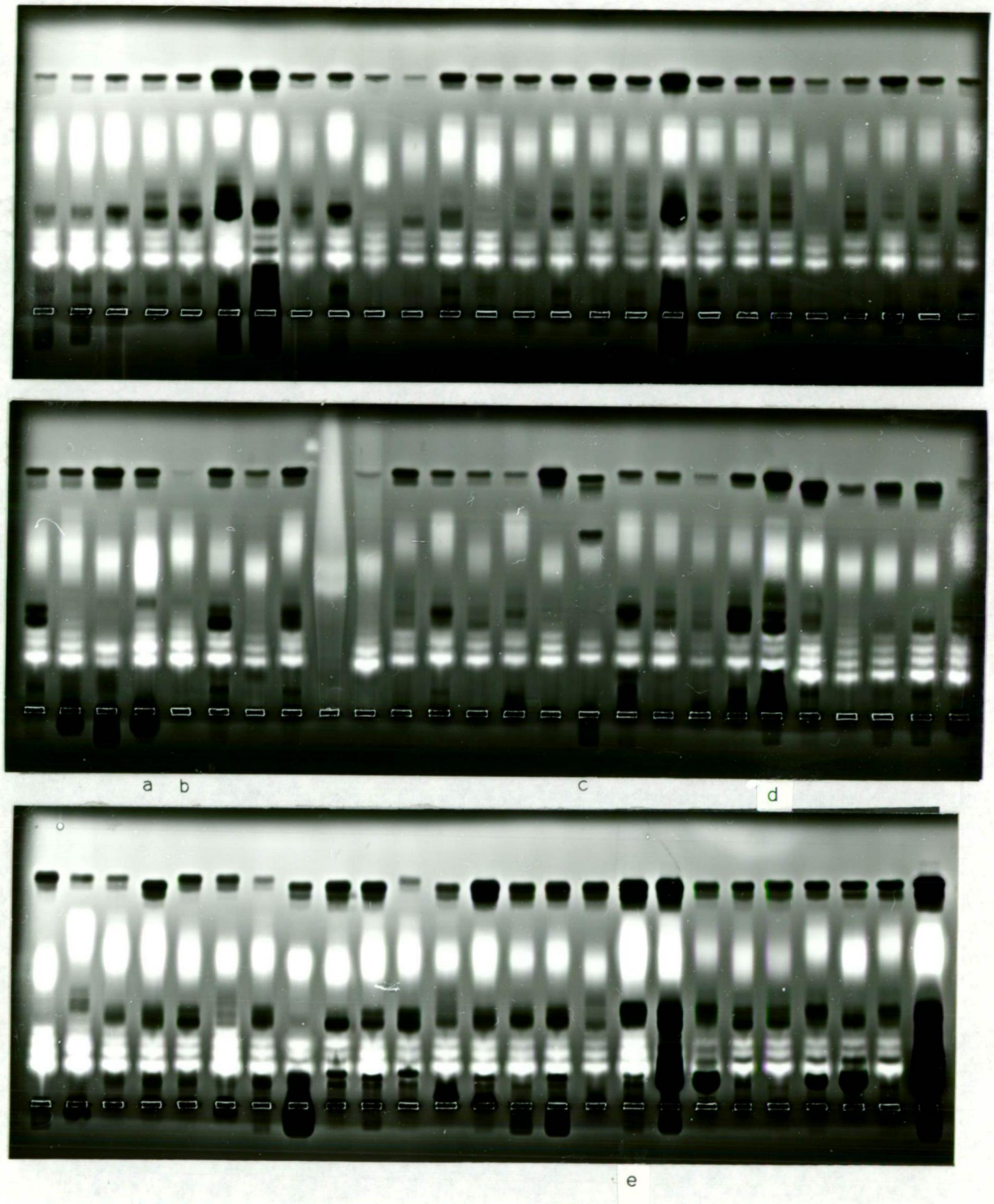


Figure 15

Figure 16 Zymogram similarity from B. aclada and allied species grown in three media:

A : sucrose

B : pectin

C : P, $(NH_4)_2SO_4$

The same loading order was used in each gel, A, B and C.

1. B. septospora IMI 79154 ex type
2. B. aclada IMI 15276
3. B. aclada No. 13, W.R. Jarvis
4. B. aclada DAR 33222
5. B. aclada DAR 28780
6. B. aclada DAR 27004
7. B. aclada B11, D. Backhouse

8–22. B. aclada from onions, Tasmania

23. B. byssoidea CBS 104.23 ex type
24. B. byssoidea PDCC 5601, R. Beever

Lanes 21 to 24 were from isolates producing byssoid growth on PDA plates.

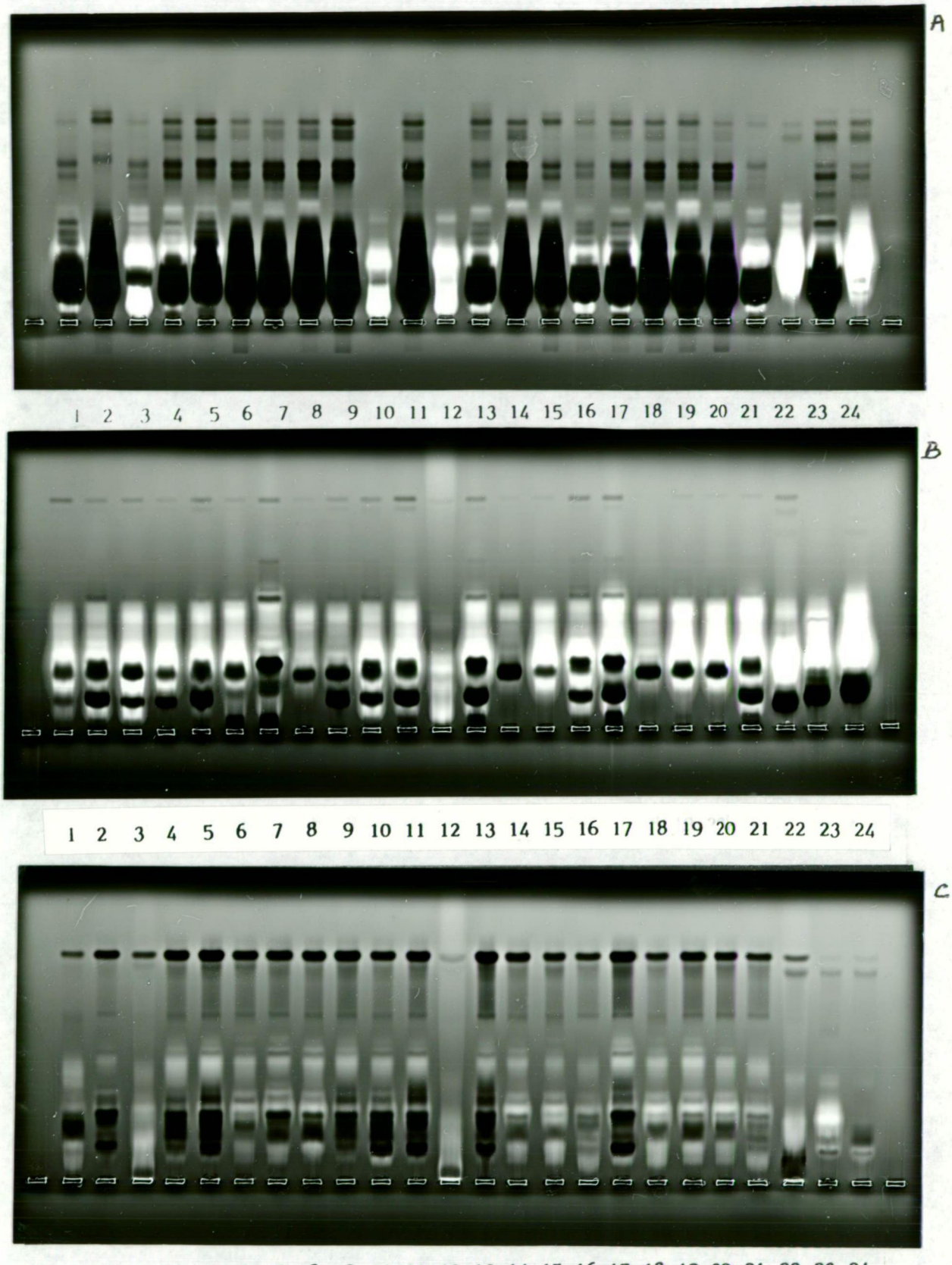

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Figure 16

Table 2. Accepted species of Botrytis and allied genera.

Botrytis aclada Fresen. = B. allii Munn.

B. byssoidea Walker[a]

B. septospora El Helaly et al.

B. anthophila Bondarzew

B. calthae Hennebert

B. cinerea Pers.

[See following Section VI]

B. convoluta Whetzel & Drayton

B. elliptica (Berk.) Cooke

B. fabae Sárdiña

B. ficariarum Hennebert

B. galanthina (Berk. & Br.) Sacc.

B. gladiolorum Timm.

B. globosa Raabe

B. sphaerosperma Buchw.

B. hyacinthi Westerd. and Beyma

B. narcissicola Kleb. ex Westerd. & Beyma

B. paeoniae Oud.

B. polyblastis Dowson

B. porri Buchw.

B. ranunculi Hennebert

B. spermophila Noble

B. squamosa Walker

B. tulipae Lind.

B. viciae Greene

Streptobotrys arisaemae Hennebert

Amphobotrys ricini (Buchw.) Hennebert = *B. ricini* Buchw.

B. bifurcata Miller, Giddens & Foster

Verrucobotrys geranii (Seaver) Hennebert

To be excluded:

B. cryptomeriae Kitajima = *Sclerotinia sclerotiorum* (Lib.) de Bary

---

[a]Taxa indented following a species were grouped with that species by zymograms.

Figure 17 Zymograms from species of Botrytis and allied genera. (A) "S6" medium, (B) "SP" medium.

1. B. aclada DAR 28780
2. B. anthophila CBS 122.26
3. B. byssoidea CBS 104.23 (T)
4. B. calthae CBS 175.63
5. B. cinerea B6 Tas.
6. B. convoluta CBS 285.38
7. B. elliptica IMI 145552
8. B. fabae IMI 225851
9. B. ficariarum CBS 176.63 (T)
10. B. galanthina CBS 127.37
11. B. gladiolorum CBS 144.41
12. B. globosa CBS 388.52
13. B. hyacinthi CBS 128.37
14. B. narcissicola IMI 193610
15. B. paeoniae CBS 112.47
16. B. polyblastis CBS 377.63
17. B. porri CBS 190.26 (T)
18. B. ranunculi CBS 178.63 (T)
19. B. spermophila CBS 219.46
20. B. sphaerosperma CBS 381.63
21. B. squamosa CBS 145.54
22. B. tulipae IMI 147187
23. B. viciae DAR 31932
24. Steptobotrys arisaemae CBS 111.47
25. Amphobotrys ricini CBS 352.36
26. Verrucobotrys geranii CBS 168.24

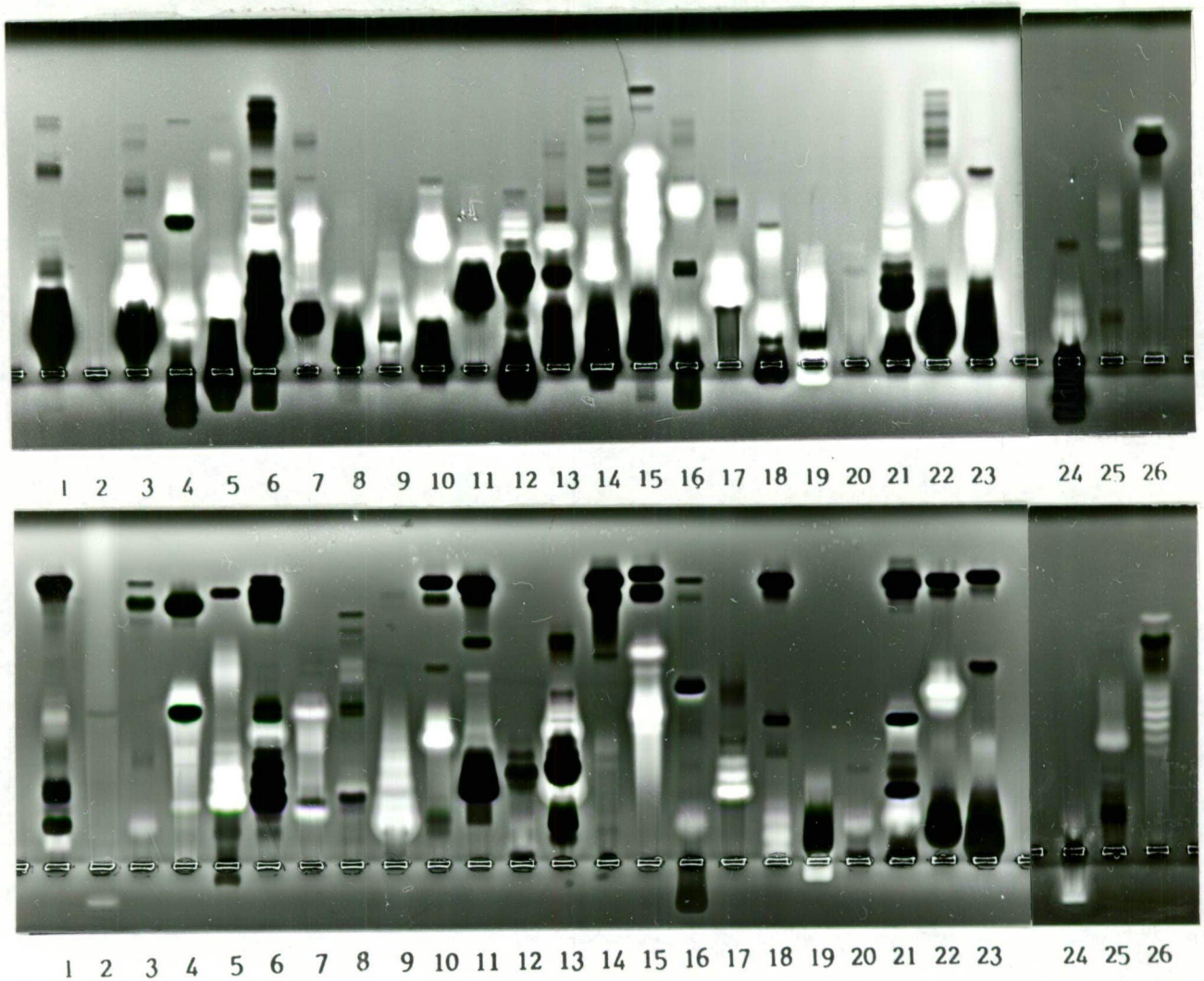

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

Figure 17

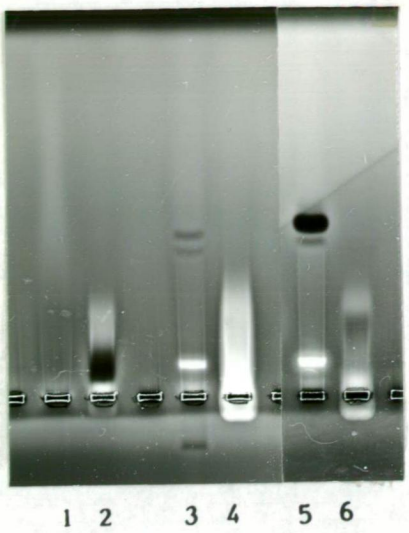


Figure 18 Lack of similarity in the zymograms of B. anthophila and B. spermophila. Grown in three media:

1, 2 "SP" medium

3, 4 "P6" medium

5, 6 "PT" medium

1, 3, 5 B. anthophila CBS 122.26

2, 4, 6 B. spermophila CBS 219.46

separate species, zymograms have provided supportive evidence or disclosure of close relationships. Examples have been provided in Figure 15 of taxa allied to B. cinerea and in Figure 16 of those allied to B. aclada. Evidence from pectic enzymes and from ribonucleases has disclosed a very close relationship between B. globosa and B. sphaerosperma (Figure 19). Representatives of the synonymous taxa B. bifurcata and Amphobotrys ricini gave very similar zymograms (Figure 20).

Pectic, amylase and ribonuclease zymograms all supported the placement of B. cryptomeriae in Sclerotinia sclerotiorum. Pectic zymogram evidence has exposed other misidentifications and cases where the original cultures have been replaced by contaminant B. cinerea (Table 3).

3. Discussion

All members of a species were found to have the ability to yield very similar pectic zymograms, not only in pictorial effect but also in the precise Rf values of their PE and PG components. In most cases, the zymograms of a taxon were clearly distinct from those of all other taxa, but in a few cases different taxa gave zymograms with a significant proportion of PE and PG components in common, providing evidence of close relationship. This was so for B. septospora and B. aclada from results obtained during the testing of media and in the examples presented in Figure 16. This supports conspecificity of these taxa as suspected by Jarvis (1977).

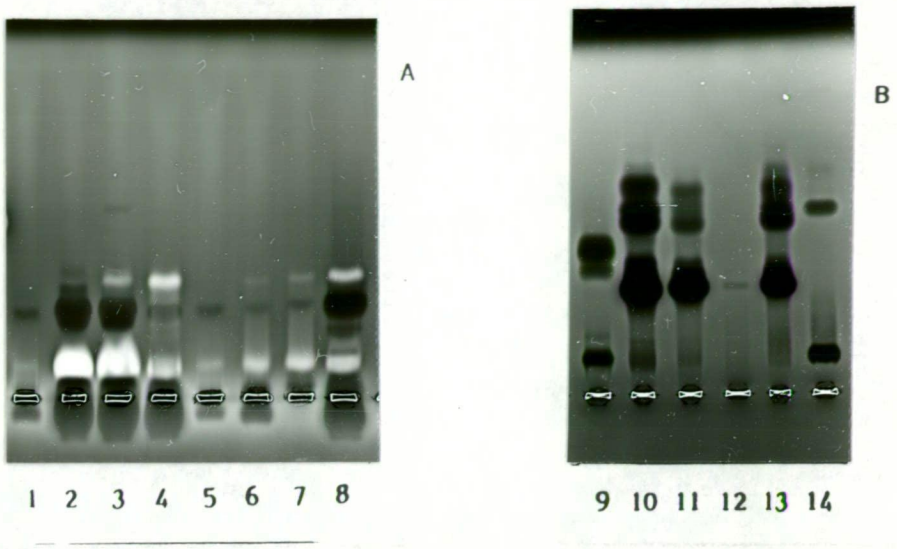


Figure 19 Zymogram evidence linking B. globosa with B. sphaerosperma.

A. Pectic zymograms

1-4. B. globosa CBS 388.52

5-8. B. sphaerosperma CBS 381.63

Sucrose NH_4NO_3 medium, citrate-phosphate buffered to pH 3, 4, 5 or 6, left to right, for each species.

B. Ribonuclease zymograms. Potato decoction.

9. B. porri CBS 379.63

10. B. globosa CBS 388.52

11. B. globosa CBS 375.63

12. B. globosa CBS 333.52

13. B. sphaerosperma CBS 381.63

14. B. tulipae IMI 143945

Figure 20 Similarity of zymograms from B. bifurcata and Amphobotrys ricini. Grown in five media:

A. "P4" medium

B. "P6" medium

C. "PT" medium

D. "SP" medium

E. "S6" medium

Left in each case : B. bifurcata IMI 100717

Right in each case : A. ricini CBS 352.36

Table 3. Examples of misidentification, according to pectic zymogram evidence.

Culture	Supplied as	Zymograms of
CBS632.77	B. narcissicola	B. hyacinthi
CBS327.78	B. galanthina	B. hyacinthi
CBS132.53	B. paeoniae	B. convoluta
CBS382.63[a]	B. squamosa	B. cinerea
CBS346.80	B. byssoidea	B. porri
CBS127.58	B. paeoniae	B. cinerea
ATCC12482[b]	B. paeoniae	B. cinerea
CBS119.20	B. ricini	B. cinerea[c]
CBS380.63	B. ricini	B. cinerea[c]

[a]This culture produced globose conidia on short conidiophores, atypical of either B. squamosa or B. cinerea.

[b]ATCC12482 = CBS127.58. Both strains were examined and found to give identical B. cinerea zymograms.

[c]Examples where the original cultures had been replaced by contaminants. When this was reported to CBS they confirmed that this was so in their holdings of these cultures.

The relationship between B. byssoidea and B. aclada has been debated over a considerable period. For example, Siemasko (1929) regarded them as conspecific, but Owen, Walker and Stahmann (1950) regarded them as distinct species as did Hennebert (1973). Zymograms from three byssoid isolates ex onion and those ex type of B. byssoidea were compared with those from 30 isolates of B. aclada and showed a high degree of similarity as in the examples presented in Figure 16. This evidence pointed towards synonymy but could warrant further comparisons at an institution with copious supplies of isolates attributed to the two species.

A single culture of B. sphaerosperma was examined. Although this lacked vigour and tended to give poor enzyme yields, its zymograms indicated a close relationship with B. globosa and it is suspected that they are conspecific.

Hennebert (1973) gave B. bifurcata as a synonym of A. ricini. This was supported by zymogram evidence. Brierley (1931) considered B. tulipae as a collective of microsclerotial races including B. hyacinthi, B. narcissicola and B. galanthina. Zymogram evidence has set each of these apart from the others in support of Hennebert (1973) who accepted them as distinct species.

Jarvis (1977) suspected that B. anthophila and B. spermophila might be conspecific. Zymograms of enzymes produced by these species in several culture media appeared quite distinct, with no indications of close relationship.

Apart from the cases of relationship discussed above, all other species examined gave distinctive zymograms which supported their validity. The method provides a practical means for species

identification and is effective even for isolates long maintained in culture. The list of isolates examined (Appendix 17) shows a number of examples of aged cultures, indicated in the isolate codes, for example CBS105.23 indicates culture 105 deposited in 1923.

An aside to this study of enzymes in relation to taxonomy was the finding that B. convoluta had the capacity to produce potent pectic enzymes (lanes 6 in Figure 17), so the unusually firm rot it produced in Iris rhizomes (Maas and Powelson, 1972) was not due to a lack of this capacity. An unusual feature of Botrytis species was their production of PG in the absence of pectin as an inducer and in the presence of sugars such as glucose, sucrose and maltose. This was in direct contrast to the reports of Berg and Yang (1969) who found that pectolytic enzymes of B. cinerea and Sclerotinia sclerotiorum were produced only when readily metabolizable energy sources (ethanol, carbohydrates) were not available. This is a generally accepted response and the suppression of PG production by sugars has been reported from several studies (Bateman and Millar, 1966; Keen and Horton, 1966; More, 1968). Only traces of PG were produced by Fusarium cultures when glucose was the sole carbon source (Baldwin and Corden, 1969). The ability of Botrytis spp. to produce PG in sugar media (even early in growth with rich supplies of sugar available) may be significant in pathogenesis and in the rot of plant tissues rich in sugars.

VI STUDIES ON THE *BOTRYTIS CINEREA* COMPLEX

1. Materials and Methods

See Section V, Studies on *Botrytis* species.

2. Results

Pectic zymograms from representatives of the *B. cinerea* complex were illustrated in Figure 15. The primary feature which distinguished all members of the *B. cinerea* complex from other species (Figure 17) was their pattern of PE. This consisted of two groups of isozymes within Rf 0.7-0.4 and 0.3-0.1 relative to bromophenol blue migration as 1.0. From their estimated isoelectric points (Cruickshank and Wade, 1980), these groups are described as acidic and basic, respectively. The isozymes of the acidic PE group were usually poorly resolved and their mean Rf varied with culture age (Figure 21). It appeared that a series of labile isozymes were formed, beginning with those of lower Rf. Mixed fluids from four- and seven-day cultures gave zymograms that were the sum of those from the two components.

The PE isozymes of the basic group appeared as three to seven clearly defined bands with the lowest band widest and most intense. Two types were evident by the Rf of this lowest major band, type A at Rf 0.16 and type B at Rf 0.12 (Figure 22A). Production of both acidic and basic PE appeared to be constitutive

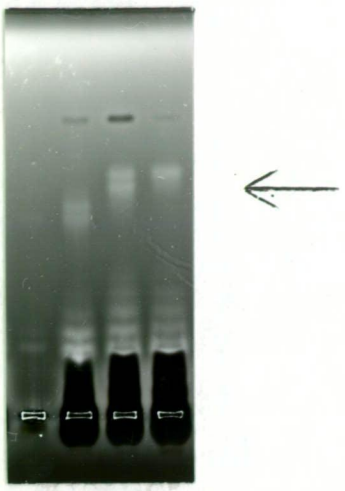


Figure 21 Variation in Rf of acidic PE of B. cinerea with time-since-inoculation of a culture.

2, 4, 7 and 10 days, left to right.

Figure 22 Pectic zymograms from the B. cinerea complex, (A) P,$(NH_4)_2SO_4$ medium; (B) PT medium; (C) GT medium, showing PE and PG characteristics used in grouping. Types represented in each gel:

1.	1A5	7.	B. cinerea f.sp. coffeae
2.	1A4	8.	B. convallariae
3.	1A3	9.	B. cinerea f.sp. lini
4.	1B5	10.	G. sclerotigenum
5.	2B4	11.	B. palargonii
6.	2B2		

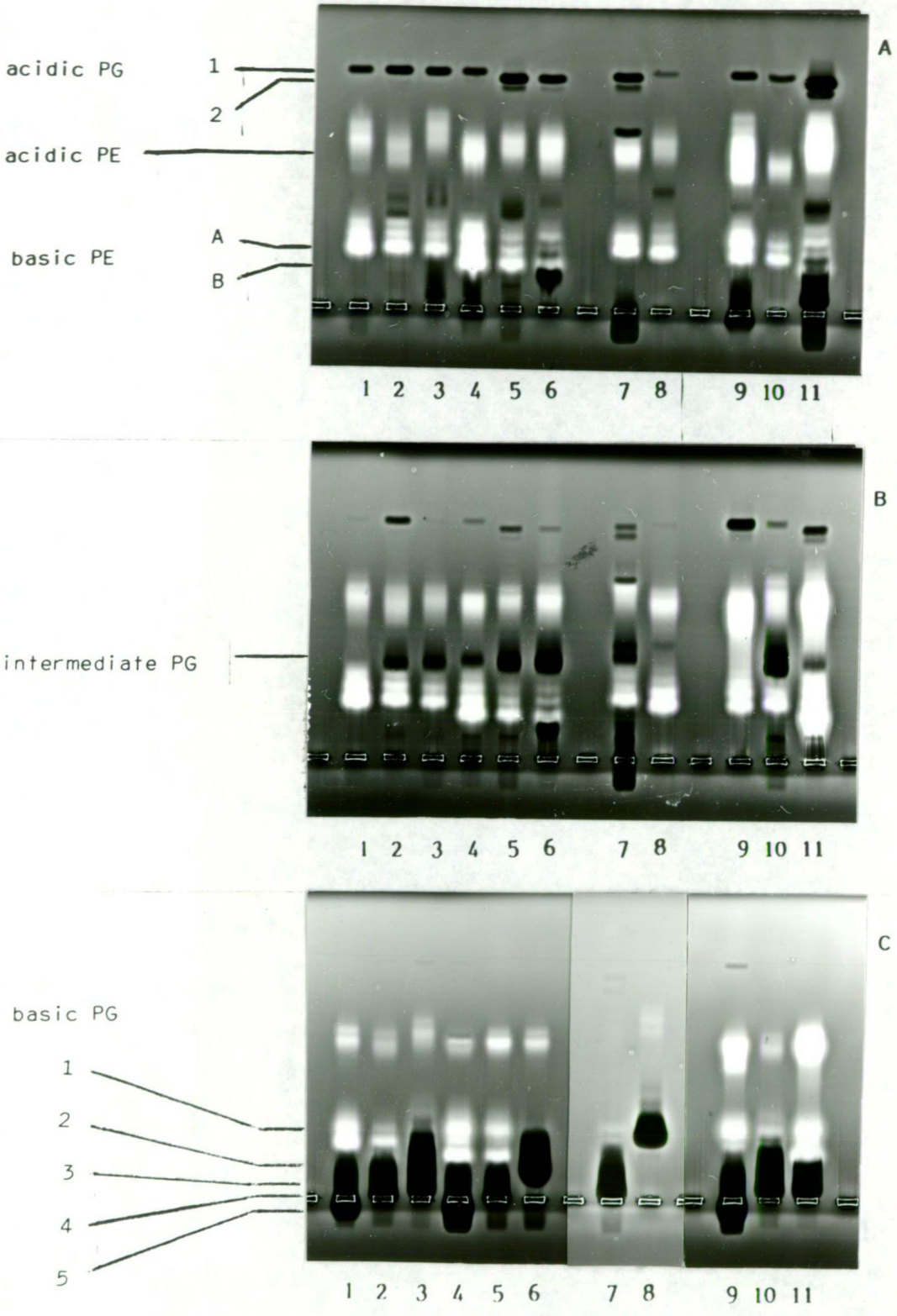

acidic PG
1
2
acidic PE
basic PE
A
B
A
1 2 3 4 5 6 7 8 9 10 11
intermediate PG
B
1 2 3 4 5 6 7 8 9 10 11
basic PG
1
2
3
4
5
C
1 2 3 4 5 6 7 8 9 10 11

Figure 22

since they were produced in the presence or absence of pectin in all media tested.

The production of PG by members of the B. cinerea complex was influenced by the composition of the culture medium and in total, showed as three isozyme groups, acidic at Rf 0.77 to 0.71, intermediate at Rf 0.42 to 0.28 (between the PE groups), and basic at Rf 0.24 through to -0.1 (cathodic migration noted as negative). The isozymes in the acidic PG group gave a major band closely followed by a minor band. Although all isolates were found to be capable of giving the minor band, it was most evident when conditions favoured strong yields of this PG group. They were produced in media containing either sugar or pectin, although in greater potency from the latter. When the Rfs of the major bands were examined, two types were found to be present, type 1 at Rf 0.74 and type 2 at Rf 0.70. Since these values tended to vary slightly between gels, known standards were included in all gels when this characteristic of the isolate was being assessed.

The enzymes in the intermediate PG group were formed in media containing pectin. They were produced in a variable and unreliable fashion and were not examined in detail.

The enzymes in the basic PG group were produced strongly in media containing glucose, maltose or sucrose, in the absence of pectin (Figure 22C, Figure 23). These enzymes were detected as single bands or as a series of isozymes with activity decreasing with increasing Rf. All isolates with the exception of B. convallariae were capable of yielding a rectangular zone of PG which extended cathodically, below the loading wells. This was

Figure 23 Zymograms from the B. cinerea complex. Maltose medium. Isolates and loading order were the same as in Figure 15.

a, B. cinerea f.sp. lini

b, B. convallariae

c, B. cinerea f.sp. coffeae

d, G. sclerotigenum

e, B. pelargonii

All others, B. cinerea

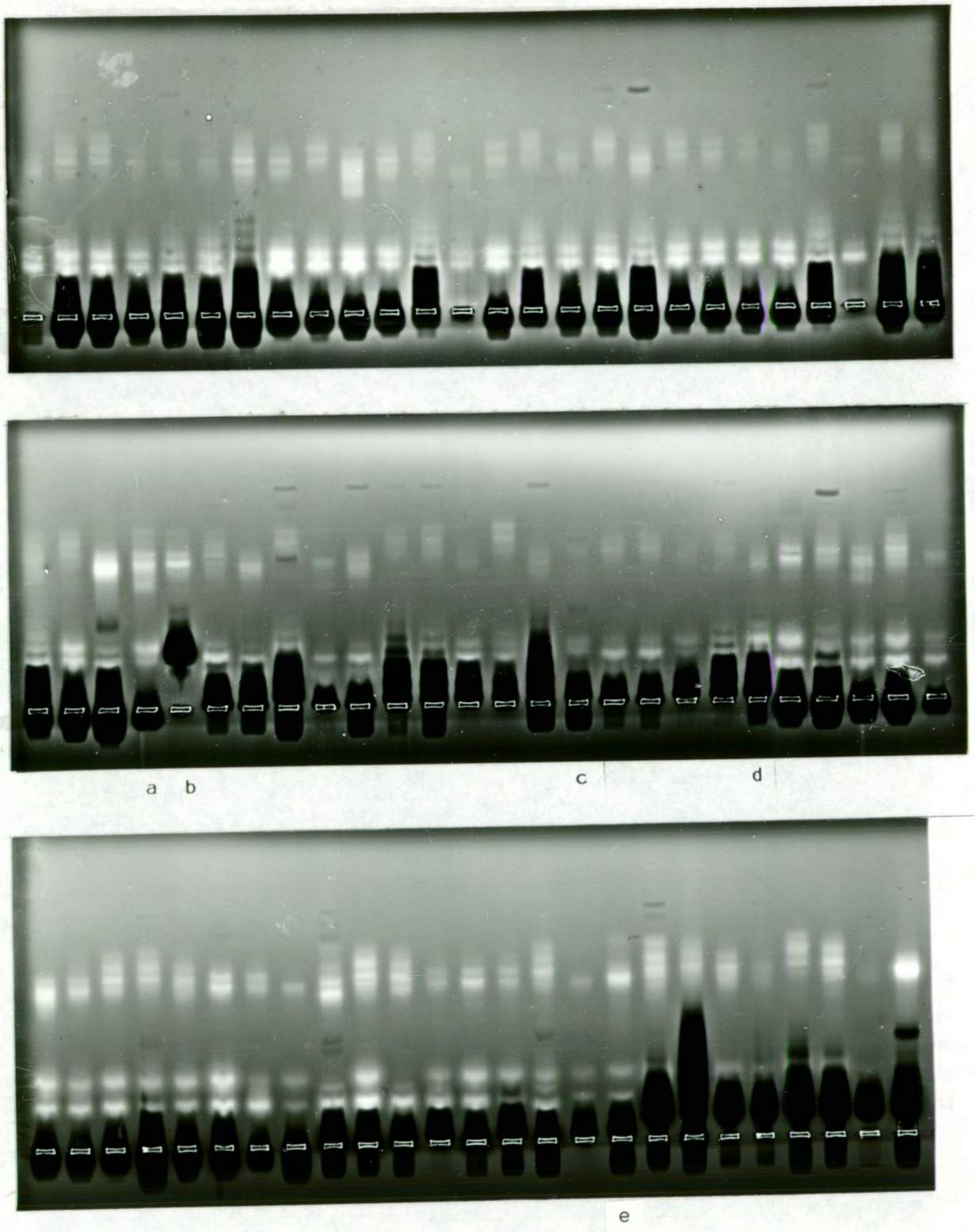


Figure 23

produced in both sugar and pectin media and was the first enzyme detected from conidia germinating in the presence of pectin. This zone was disregarded when assessing the five types of basic PG present.

Acidic and basic PG differed in the cultural conditions favouring their production and in the pH favourable for their detection. Incubation of gels at pH 4.5 after electrophoresis allowed the detection of basic PGs but detection of the acidic PGs was favoured by incubation at pH 3. Because of these differences and the considerable differences in their Rf, acidic and basic PGs were regarded as separate genetic series and were coded by separate number series.

Basic PG type 1 was superimposed on the basic PE group at Rf 0.19 and was only produced by B. convallariae (Figure 22C, Figure 23). Type 2 often obliterated the basic PE zone by a series of isozymes with the major band at Rf 0.09 separated from the loading well. Type 3 gave a series of bands extending into the basic PE zone, with its major band at Rf 0.02, typically extending from the upper edge of the sample well. Type 4 gave a major band centred on the upper edge of the sample well and thus had n Rf very close to zero. Type 5 had a major band that migrated cathodically, with Rf -0.02, distinct from the common rectangular zone mentioned above.

Each isolate could be placed in a pectic zymogram or genotype group coded either 1 or 2 for acidic PG, A or B for basic PE, and one of 1 to 5 for basic PG, in that order. An exception was found in the case of isolates from coffee berries in Kenya.

These gave distinctive zymograms close to 1A3, but their acidic PG was mid-way between 1 and 2, and an extra PG band was present in the upper region of the acidic PE zone, at Rf 0.56 (Fig. 22). This extra PG band was produced in both sugar and pectin media and an additional PG band at Rf 0.54 was produced on some occasions. Additional PG bands in this region were not produced in pectic media by other isolates of B. cinerea but traces at Rf 0.5 were occasionally found from cultures grown in sugar media.

The genotype combinations detected in close scrutiny of 232 isolates producing pectic zymograms of the B. cinerea type were 1A1 (B. convallariae only), 1A3, 1A4, 1A5, 1B4, 1B5, 2B2, 2B3, 2B4 and 2B5. Isolates obtained from culture collections and characteristic of these genotype groups are listed in Appendix 18. This includes taxa with pectic zymograms which lacked features distinguishing them from the ambit of B. cinerea. These were B. cinerea f.sp. lini von Beyma, which was a member of the 1A5 group; B. pelargonii Roeed, a member of the 2B3 group, and Gonatobotryum sclerotigenum van Warmelo, a member of the 1A3 group (Figure 22).

Isolates of B. cinerea were obtained from a wide variety of plants. These, together with the frequencies of the genotypes involved, are given in Appendix 19. Only one or a few isolates were available from most host plants, so conclusions on most favourable fungal genotype-host plant interactions could not be drawn. The major exception was the case of isolates from grapes (Vitis vinifera and Vitis lebrusca hybrids). In this case, 85 isolates were examined and 54% of these were of genotype 2B2.

The frequencies of detection of the various pectic enzyme

genotypes in samples from regions round the world are presented in Table 4.

Cultures grown on PDA in Petri dishes were examined for cultural characteristics. No consistent relationship was found between pectic enzyme genotype and the size of sclerotia or the habits of being predominantly sclerotial or conidial.

A survey was made in New Zealand of resistance in B. cinerea to the dicarboximide fungicide iprodione and to the benzimidazole fungicide carbendazim (Beever and Brien, 1983). The author was provided with 69 isolates from this and continuing studies, selected to represent normal and fungicide-resistant phenotypes from a range of hosts and localities. These were assorted to pectic enzyme genotype groups (Appendix 20). There was no obvious association between the different fungicide-resistant groups and particular pectic enzyme genotypes.

3. Discussion

Pectic zymogram evidence clearly supported the recognition of B. cinerea as a separate species in Botrytis. Since many isolates were available for study, useful results were obtained in studies of variation within the species. The characteristics of the enzymes that were coded for use in sub-group recognition were suspected to show the presence of two alleles at the locus controlling acidic PG production, two at the basic PE locus, and five at the basic PG locus. In consequence, the code combinations were described as genotypes.

Table 4. Frequency of detection of pectic enzyme genotypes in the Botrytis cinerea complex, from various regions.

Hosts and Locality	Genotype 1A3	1A4	1A5	1B4	1B5	2B2	2B3	2B4	2B5	Total
A. Other than Vitis										
Canada	0	1	0	1	2	4	0	0	1	9
Europe	2	1	4	0	2	3	1	2	0	15
New Zealand	0	2	3	5	2	8	19	5	1	45
N.S.W., Victoria	1	2	6	0	1	1	1	3	1	16
Tasmania	7	3	28	1	10	4	3	3	2	61
B. Vitis (total)										
Europe	3	0	0	0	0	27	0	0	0	30
New Zealand	0	0	0	0	2	16	6	0	0	24
Tasmania	3	1	6	0	3	2	0	7	8	30
Victoria	0	0	0	0	0	1	0	0	0	1

C. Vitis (Noble Rot)

Europe	0	0	0	0	0	6	0	0	0	6
New Zealand	0	0	0	0	2	4	0	0	0	6
Tasmania	2	0	5	0	1	1	0	5	8	22

In addition : 1A1 ex Convallaria majalis, Europe, 1.

f. sp. coffeae ex Coffea arabica, Kenya, 3.

The frequency of detection of each genotype from hosts other than Vitis is given in Table 4A, with results from major sampling areas presented separately. This table shows that the various genotypes have wide distributions and their relative abundance may vary between countries. For example, 2B3 was prevalent in New Zealand, whereas the genotype most commonly found in Tasmania was 1A5. Isolates from 43 genera of plants were examined. The number from each host genus was generally low, but particular association between host and fungal genotype was not evident in the cases where more were examined. For example, various genotypes were represented, a total of six in each case, in 21 isolates from Actinidia chinensis, 8 isolates from Allium cepa, 15 isolates from Fragaria x ananassa, and 14 isolates from Lycopersicon lycopersicum.

Table 4 B is concerned with isolates from Vitis vinifera except six of the isolates from New Zealand which were V. vinifera x V. lebrusca (3 isolates of 2B2 and 3 of 2B3 were found from the latter). This table shows a strong association between Vitis and the genotype 2B2, particularly in the samples from Europe and New Zealand. Part of the European material from V. vinifera was a sample from the Rhine Valley, provided by Dr. D.H. Lorez, which consisted of three wild strains and six monoascosporial cultures from Botryotinia fuckeliana (de Bary) Whetzel. All of these were found to have the genotype 2B2.

Buchwald (1949) regarded the fungus which attacked vines in central and southern Europe as a species within the collective B. cinerea and named it Botrytis fuckeliana Buchw. It was found here

that, while isolates with genotype 2B2 were not restricted to Vitis as host, 27 of 30 isolated from Vitis in central and southern Europe had this genotype and were probably representative of B. fuckeliana Buchw. This can now be recognised by its pectic zymograms either as a forma specialis of B. cinerea or as a distinct species should it prove to be genetically isolated from the rest of the B. cinerea complex.

The successful mating by Groves and Loveland (1953) of isolates obtained in Canada from apple, potato and celery with isolates from grape vines in the Rhine Valley, does not prove conspecificity between B. cinerea and B. fuckeliana since the isolates may all have been B. fuckeliana. This possibility was shown here when nine isolates obtained in Ontario, Canada, from hosts other than Vitis, were examined and four were found to have the genotype 2B2.

Noble rot of grapes involves a distinctive form of B. cinerea infection in which invasion is confined to the surface layers of the berries (Ribéreau-Gayon et al., 1980). The fungal genotypes involved in this were first investigated using 21 isolates from noble rot in grapes grown in Tasmania. Genotype 2B2 was not among them, and this was suspected to be significant until samples from Bordeaux, France, and Te Kauwhata, New Zealand, were examined and found to be all and mainly 2B2, respectively (Table 4 C). The 2B2 genotype was eventually detected from noble rot in Tasmania.

In the total B. cinerea complex, genotype 1B3 and combinations beginning with 2A were not detected and the possible

presence of some system governing compatibility in parasexual or sexual behaviour was considered. A mating matrix was prepared, assuming free recombination of alleles. Matings that would yield only detected genotypes were regarded as compatible and, conversely, any mating that could yield undetected genotypes was regarded as not occurring in Nature from being incompatible. The matrix indicated the relationship given in Table 5.

If this hypothesis is sound, while groups are isolated from direct interaction with some others, stepwise exchange of genes is possible throughout the entire population which can thus remain morphologically homogeneous. This result is in keeping with the conclusion that B. cinerea is a complex of races with none justifying placement in a new taxon (Morgan, 1971a).

It is difficult to reconcile such a compatibility system with theories concerning variation in B. cinerea reviewed by Lorbeer (1980). Heterokaryosis is widely accepted to explain the variability of Fungi Imperfecti and is necessary if different genotypes are to result from parasexual behaviour. However, Caten and Jinks (1966) held reservations on the frequency and importance of heterokaryosis in natural populations, as distinct from systems used for its demonstration in culture, involving forced heterokaryon formation between nutritionally-deficient mutants.

Considering enzymic evidence of heterokaryosis, the enzymes of hybrids result from the expression of all relevant alleles and are not influenced by dominant and recessive states (Moss, 1982). It is to be expected that the same should apply to heterokaryons. Should the nuclei involved differ in enzyme genotype, then the

Table 5. Hypothetical compatibility groups (CG) and their interrelationships.

Genotypes	Group	Compatibility[a]				
		CG1	CG2	CG3	CG4	CG5
1A3,	CG1	+	+	-	-	-
1A4, 1A5	CG2	+	+	+	-	-
1B4, 1B5	CG3	-	+	+	+	-
2B4, 2B5	CG4	-	-	+	+	+
2B3, 2B2	CG5	-	-	-	+	+

[a] + : compatible

\- : incompatible

zymogram from the heterokaryon should be a composite from all the alleles present. For example, the heterokaryon of 1B4 plus 2B5 would be expected to give a pectic zymogram with both types of acidic PG and both types of basic PG. No such composite zymograms were detected. This does not preclude the presence of heterokaryons but indicates that if they are present, then the component nuclei have the same pectic enzyme genotype. Behaviour of this type was reported by Caten and Jinks (1966) who found that the sharing of a number of identical alleles was a prerequisite for heterokaryon formation.

It is not certain that the compatibility group hypothesis could function in the sexual process. The mating system involved in the production of the Botryotinia fuckeliana teleomorphic stage of Botrytis cinerea was held by Groves and Loveland (1953) to be bipolar, with intragroup sterility, intergroup fertility and individual self-sterility. Lorbeer (1980) expected self-fertility would be found within species of Botryotinia, and Lorenz and Eichorn (1983) succeeded in this for B. fuckeliana when 22 of 56 wild strains isolated from V. vinifera were found to be homothallic as were five of six monoascosporial progenies. The six monoascosporial cultures were examined here and all were 2B2, so homothallic and heterothallic behaviour has been demonstrated for this genotype, but no definite information on mating systems in the rest of the B. cinerea complex is available.

Returning from these conjectures on compatibility grouping to matters with visible evidence, the pectic zymograms of B. convallariae indicated a close relationship to B. cinerea but the

distinctive nature of its basic PG was evidence of a degree of divergence. This supported its placement as B. cinerea f. sp. convallariae by Pape and Hemer (1964) cited by Hódosy (1964). Perhaps less significant evidence supported the placement of isolates ex Coffea arabica in B. cinerea f. sp. coffeae Hendrickx as a divergence from the general complex.

The cultures ex type of some taxa lacked distinctive zymogram features that would support their separation from the general complex. One of these was B. cinerea f. sp. lini van Beyma. The validity of this forma specialis was also doubted by Spek (1965) since it was erected primarily on the ability to form citric acid and this ability was common in B. cinerea 2B3. Another case was that of B. pelargonii which gave the pectic zymograms of B. cinerea 2B3. A third case was that of Gonatobotryum sclerotigenum van Warmelo which was placed in Botrytis by Walker and Minter (1981) and placed here by means of its pectic zymograms in B. cinerea 1A3.

The utility of the pectic zymogram technique for species recognition and the exposure of false identifications has been considered in Section V. An example of its use in the identification of a mutant form was the case of CBS283.63 supplied as B. squamosa ex Allium cepa. This culture was barren on PDA but sporulated when it was used to infect onion bulb tissue, producing globular conidia on short sporophores. This was atypical of both B. squamosa and B. cinerea which produce obovoid conidia on long sporophores, yet zymograms from both the original culture and cultures from the globular conidia were typical of B. cinerea 1A5.

In the absence of pectic zymogram results, the possibility of the culture being B. cinerea would not have been considered.

VII STUDIES ON *PENICILLIUM* SPECIES

1. Materials and Methods

1.1 Cultures examined

Cultures were provided by the Curator, Dr. Ailsa D. Hocking, from the CSIRO Food Research Culture Collection at North Ryde, N.S.W. An alphabetical list of the species examined, with authorities and culture collection numbers, is provided in Appendix 21. In all, 233 isolates were studied.

1.2 Culture maintenance

Cultures were maintained on Czapek malt maintenance medium (Pitt, 1979) on slopes of 2 ml in bijou bottles, incubated at 22°C for 7 days after inoculation and then stored at 4°C. Subculturing was performed every four months.

1.3 Enzyme production

Cultures were grown in loosely capped bijou bottles, each containing 2 ml of culture medium, autoclaved at 121°C for 15 minutes. These were inoculated by needle point from maintenance slopes and were incubated at 22°C.

A wide variety of culture media were devised and tested for their influence on the diversity and yield of pectic enzymes and their clarity in zymograms. A medium containing citrus pectin and buffered initially to pH 6 by monobasic and dibasic ammonium phosphates was found to be generally useful for members of the

subgenus Penicillium. Its composition is given in Appendix 3. Cultures in this medium were incubated for 7 days at 22°C.

For amylase and ribonuclease production, cultures were grown in potato decoction as used in PDA or else in a medium consisting of five wheat grains in 2 ml distilled water in each bottle. Cultures were incubated at 22°C for 10 days.

1.4 Electrophoresis and detection of enzymes

Methods for the study of pectic enzymes, amylase and ribonuclease are given in Appendices 8, 9 and 10, respectively.

2. Results

Results obtained during a study of 181 isolates from the Penicillium subgenus Penicillium have been published:

Cruickshank and Pitt (1987a; 1987b)

Pitt, Cruickshank and Leistner (1986)

Reprints of these papers are provided in Appendix 25.

The study has been continued in an examination of an additional 43 taxa regarded as synonyms by Pitt (1979). A total of 80 taxa were examined and assigned to 22 species; one of these [Penicillium sp. in Cruickshank and Pitt (1987a)] has not been characterised by morphology. This disposition of taxa is presented in Table 6. Agreement with Pitt (1979) in placement of taxa as distinct species or as synonyms was found in 46 cases and these are marked with an asterisk in Table 6. The different placements of the remaining 31 cases are given in Table 7.

TABLE 6

Species accepted and synonymy[a] in Penicillium subgenus Penicillium

---

P. arenicola*

P. canadense*

P. atramentosum*

P. aurantiogriseum*

P. aurantiocandidum*

P. brunneoviolaceum*

P. corneolutescens

P. cyclopium*

P. martensii*

P. porraceum

P. puberulum

P. viridicyclopium*

P. brevicompactum*

P. brunneostoloniferum

P. hagemii*

P. lanosum

P. olsonii

P. patris-mei*

P. stoloniferum*

P. volgaense*

TABLE 6

continued

P. chrysogenum*

P. aromaticum f. microsporum*

P. camerunense*

P. chlorophaeum*

P. flavidomarginatum*

P. griseoroseum

P. harmonense*

P. meleagrinum*

P. notatum*

P. rubens*

P. commune

P. camembertii

P. aurantiogriseum var. poznoniense

P. australicum

P. biforme

P. candidum

P. flavoglaucum

P. lanoso-coeruleum

P. lanosogriseum

P. lanosoviride

P. ochraceum var. macrosporum

P. palitans

P. roquefortii var. punctatum

P. concentricum

TABLE 6

continued

P. crustosum*

P. farinosum*

P. pseudocasei*

P. terrestre*

P. digitatum*

P. echinulatum*

P. palitans var. echinoconidium*

P. expansum*

P. aurantiovirens

P. resticulosum

P. fennelliae*

P. granulatum*

P. griseofulvum*

P. flexuosum*

P. griseofulvum var. dipodomyicola[b]

P. patulum*

P. urticae*

P. hirsutum*

P. hordei

P. italicum*

P. japonicum

P. roquefortii*

P. conservandi*

P. gorgonzolae*

P. roquefortii var. viride*

TABLE 6

continued

P. solitum

P. casei var. compactum

P. mali[b]

P. psittacinum

P. verrucosum var. melanochlorum

P. verrucosum*

P. crustosum var. spinulosporum

P. viridicatum*

P. aurantiogriseum var. neoechinulatum[b]

P. olivicolor

P. olivinoviride*

---

[a]Authorities provided in Appendix 21.

Taxa indented following a species were grouped with that species by zymograms and were suspected to be synonymous.

*In agreement with Pitt (1979) in placement as a distinct species or a synonym.

[b]Not examined by Pitt (1979).

TABLE 7

Cases where taxa were assigned differently by zymogram characteristics and by Pitt (1979).

Taxon	By zymograms	By Pitt (1979)
P. carneolutescens	P. aurantiogriseum	P. hirsutum
P. porraceum	P. aurantiogriseum	P. puberulum
P. puberulum	P. aurantiogriseum	Distinct species
P. brunneostoloniferum	P. brevicompactum	P. olivicolor
P. lanosum	P. brevicompactum	P. puberulum
P. olsonii	P. brevicompactum	Distinct species
P. flavidomarginatum	P. chrysogenum	P. griseoroseum
P. commune	Distinct species	P. puberulum
P. camembertii	P. commune	Distinct species
P. aurantiogriseum var. poznoniense	P. commune	P. crustosum
P. australicum	P. commune	P. crustosum
P. biforme	P. commune	P. camembertii
P. candidum	P. commune	P. camembertii
P. flavoglaucum	P. commune	P. verrucosum
P. lanoso-coeruleum	P. commune	P. aurantiogriseum
P. lanosogriseum	P. commune	P. puberulum
P. lanosoviride	P. commune	P. viridicatum

TABLE 7

continued

P. ochraceum var. *macrosporum*	*P. commune*	*P. olivicolor*
P. palitans	*P. commune*	*P. viridicatum*
P. roquefortii var. *punctatum*	*P. commune*	*P. verrucosum*
P. concentricum	Distinct species	*P. italicum*
P. aurantiovirens	*P. expansum*	*P. aurantiogriseum*
P. resticulosum	*P. expansum*	Distinct species
P. hordei	Distinct species	*P. hirsutum*
P. japonicum	*P. italicum*	*P. resticulosum*
P. solitum	Distinct species	*P. aurantiogriseum*
P. casei var. *compactum*	*P. solitum*	*P. aurantiogriseum*
P. psittacinum	*P. solitum*	*P. viridicatum*
P. verrucosum var. *melanochlorum*	*P. solitum*	*P. crustosum*
P. crustosum var. *spinulosporum*	*P. verrucosum*	*P. crustosum*
P. olivicolor	*P. viridicatum*	Distinct species

Of the 43 taxa examined since publication, only one was found to represent a distinct species. This was P. concentricum, regarded as a synonym of P. italicum by Pitt (1979), but distinct by zymogram evidence as in the examples given in Figure 24. Examples of pectic zymograms in support of Table 6 are provided in Figures 25 to 29.

Figure 24 Zymograms of Penicillium concentricum and P. italicum, (A) amylase; (B) ribonuclease; (C) pectic enzymes.

1. P. concentricum FRR 1715 ex type
2. P. concentricum FRR 1716
3. P. concentricum FRR 3217
4. P. concentricum FRR 3222
5. P. concentricum FRR 3228
6. P. italicum FRR 983 ex type
7. P. italicum 0109(b)
8. P. italicum P49 (Tasmania)
9. P. japonicum (=P. italicum) FRR 3431 ex type

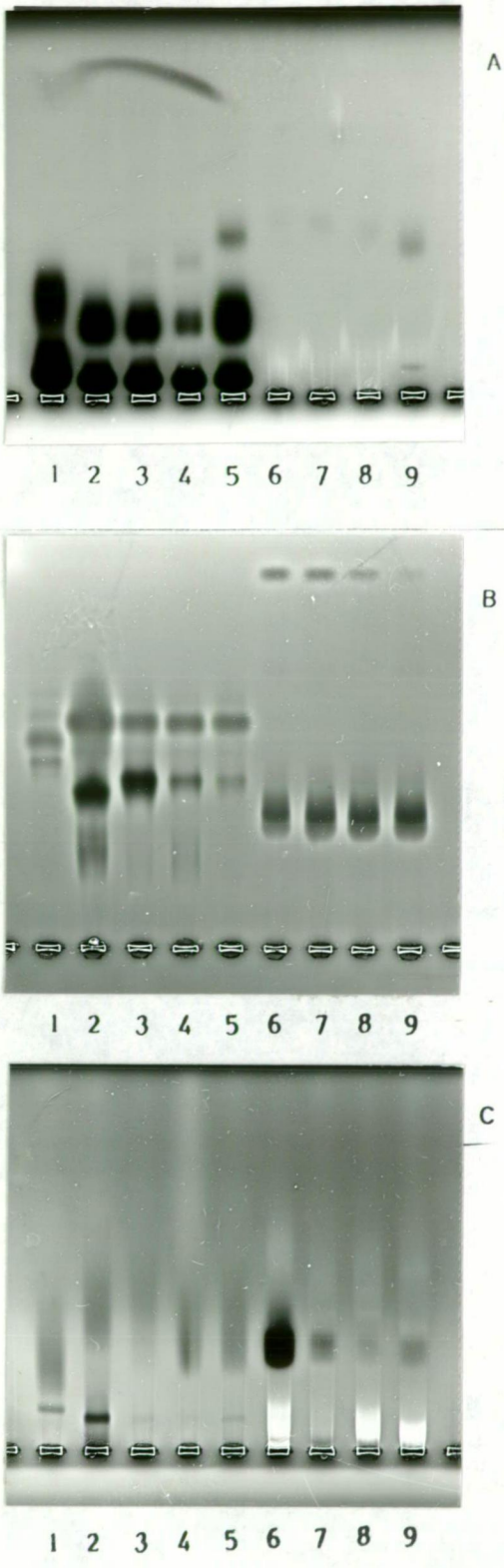


Figure 24

Figure 25 Pectic zymogram evidence for grouping taxa with *P. aurantiogriseum* (1-11) and *P. brevicompactum* (12-22).

1. *P. aurantiogriseum* FRR 971 (NT)
2. *P. brunneoviolaceum* FRR 2137 (T)
3. *P. cyclopium* FRR 1888 (T)
4. *P. porraceum* FRR 970 (T)
5. *P. puberulum* FRR 2040 (NT)
6. '*P. viridicatum*' FRR 1642 Ciegler's Group III
7. '*P. viridicatum*' FRR 1637
8. *P. aurantiocandidum* FRR 884 (NT)
9. *P. viridicyclopium* FRR 1364a (T)
10. *P. carneolutescens* FRR 2035 (T)
11. *P. martensii* FRR 2029
12. *P. brevicompactum* FRR 862 (NT)
13. *P. brevicompactum* 0044 (b)
14. *P. olsonii* FRR 432 (NT)
15. *P. olsonii* FRR 3165
16. *P. brunneostoloniferum* FRR 1363 (T)
17. *P. hagemii* FRR 866 (T)
18. *P. lanosum* FRR 2009 (T)
19. *P. stoloniferum* FRR 3573 (T)
20. *P. stoloniferum* FRR 859 (T)
21. *P. volgaense* FRR 3576 (T)
22. *P. patris-mei* FRR 3575 (T)

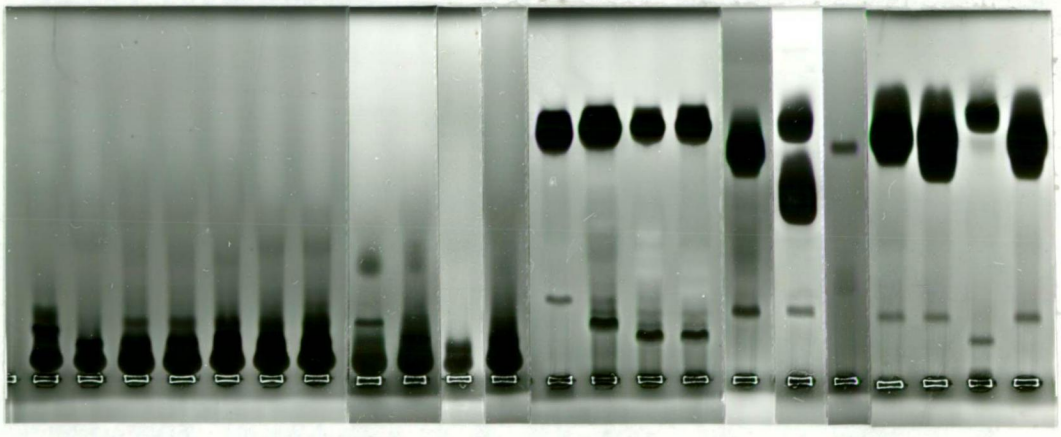


Figure 25

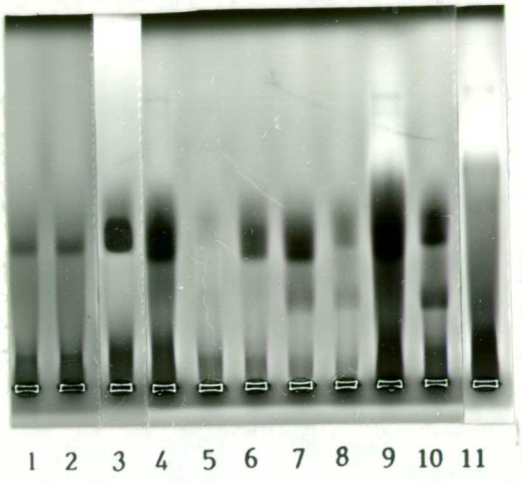


Figure 26 Pectic zymogram evidence for grouping taxa with P. chrysogenum.

1. P. griseoroseum FRR 820 (T)
2. P. chrysogenum FRR 807 (T)
3. P. aromaticum var. microsporum FRR 1362 (T)
4. P. harmonense FRR 512 (T)
5. P. rubens FRR 792 (T)
6. P. chlorophaeum FRR 817 (T)
7. P. notatum FRR 821 (T)
8. P. meleagrinum FRR 836
9. P. meleagrinum FRR 2136
10. P. camerunense FRR 3401 (T)
11. P. flavidomarginatum FRR 3569 (T)

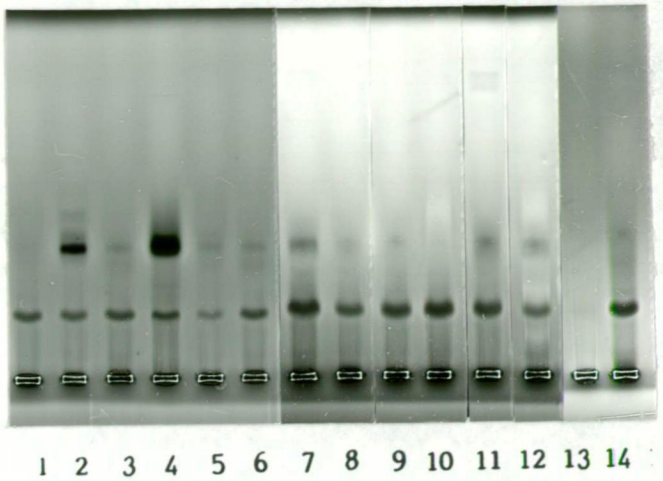


Figure 27 Pectic zymogram evidence for grouping taxa with *P. commune*.

1. *P. camembertii* FRR 877 (T)
2. *P. commune* NRRL 890a (T)
3. *P. palitans* FRR 2033 (T)
4. *P. lanoso-coeruleum* FRR 888 (T)
5. *P. lanosogriseum* FRR 894 (T)
6. *P. ochraceum* var. *macrosporum* FRR 873 (T)
7. *P. aurantiogriseum* var. *poznoniense* FRR 972 (T)
8. *P. australicum* FRR 935 (T)
9. *P. lanosoviride* FRR 879 (T)
10. *P. commune* Leistner Sp. 119
11. *P. flavoglaucum* FRR 948 (T)
12. *P. roquefortii* var. *punctatum* FRR 1364a (T)
13. *P. candidum* FRR 876 (T)
14. *P. biforme* FRR 885 (T)

Figure 28 Pectic zymogram evidence for grouping taxa with *P. crustosum* (1-6), *P. echinulatum* (7-8) and *P. griseofulvum* (13-20).

1. *P. crustosum* R35
2. *P. farinosum* FRR 1478 (T)
3. *P. crustosum* R35
4. *P. pseudocasei* FRR 3457a (T)
5. *P. terrestre* FRR 933
6. *P. terrestre* FRR 934
7. *P. echinulatum* FRR 1151 (IT)
8. *P. palitans* var. *echinoconidium* FRR 637 (T)
9. *P. expansum* FRR 976 (NT)
10. *P. expansum* DAR 33866
11. *P. resticulosum* FRR 2021 (T)
12. *P. aurantiovirens* FRR 2138 (T)
13. *P. griseofulvum* FRR 3571 (T)
14. *P. griseofulvum* FRR 1414
15. *P. griseofulvum* *P. griseofulvum* III of Frisvad
16. *P. griseofulvum* var. *dipodomyicola* FRR 3580 (T)
17. *P. griseofulvum* FRR 1414
18. *P. urticae* FRR 989 (T)
19. *P. flexuosum* FRR 992 (T)
20. *P. patulum* FRR 994 (T)

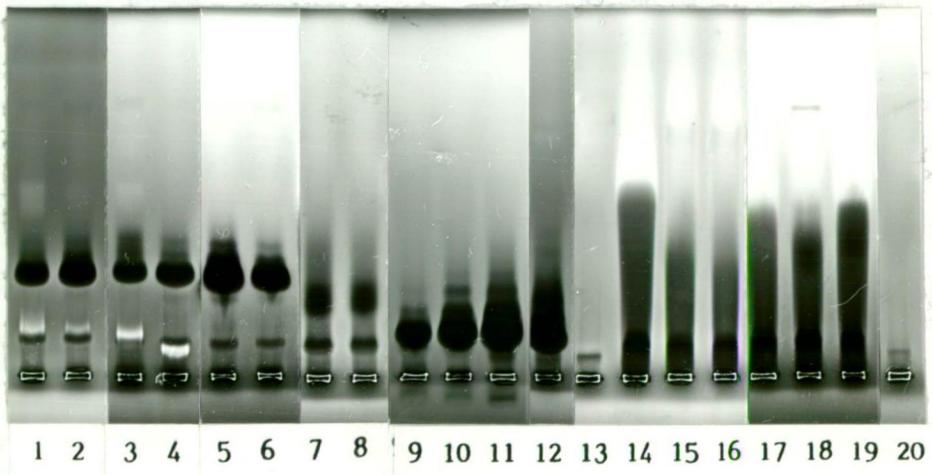


Figure 28

Figure 29 Pectic zymogram evidence for grouping taxa with *P. roquefortii* (1-4), *P. solitum* (5-9), *P. verrucosum* (10-12) and *P. viridicatum* (13-20).

1. *P. roquefortii* FRR 849 (T)
2. *P. conservondi* FRR 1480 (T)
3. *P. gorgonzolae* FRR 857 (T)
4. *P. roquefortii* var. *viride* FRR 1775 (T)
5. *P. psittacinum* FRR 932 (T)
6. *P. solitum* FRR 937 (T)
7. *P. casei* var. *compactum* FRR 732a (T)
8. *P. mali* FRR 3121
9. *P. verrucosum* var. *melanochlorum* FRR 2152 (T)
10. *P. verrucosum* FRR 965 (T)
11. *P. verrucosum* FRR 1639
12. *P. crustosum* var. *spinulosporum* FRR 1621 (T)
13. *P. viridicatum* FRR 963 (T)
14. *P. viridicatum* L2, F. Leistner
15. *P. viridicatum* J. Frisvad
16. *P. olivicolor* FRR 882
17. *P. viridicatum* FRR 963 (NT)
18. *P. olivicolor* FRR 3572 (T)
19. *P. aurantiogriseum* var. *neoechinulatum* FRR 3589 (T)
20. *P olivinoviride* FRR 2028 (T)

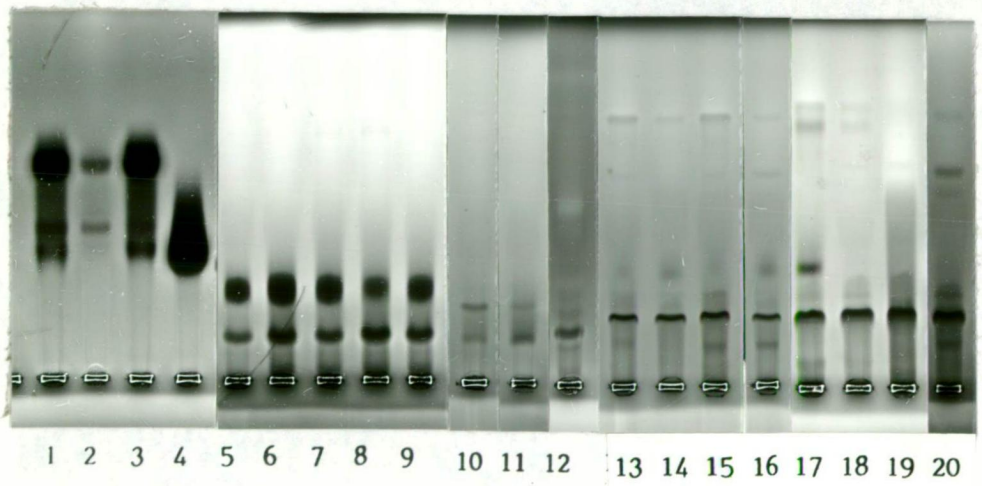


Figure 29

3. Discussion

Penicillium isolates from the subgenus Penicillium were readily assigned to groups by means of close similarity of pectic zymograms within each group. Where the pectic zymogram results were equivocal, particularly from isolates of P. chrysogenum, P. griseofulvum and their synonyms, amylase and ribonuclease zymograms provided the means for differentiation. In the majority of cases, isolates assigned to a species by traditional means were found to belong in the same zymogram group, and it seemed probable that the members of a zymogram group were conspecific (Cruickshank and Pitt, 1987a; 1987b).

There was extensive synonymy in the subgenus (Table 6), in agreement with Pitt (1979) in the majority of cases. Some exceptions (in Table 7) have been discussed (Cruickshank and Pitt, 1987a). A change in conclusion is the case of P. olsonii and P. brevicompactum, regarded as closely related but now, from further examination of the species including synonymous taxa (Table 6), regarded as synonymous, in support of Stolk and Samson (1985). P. brevicompactum is the accepted species since it has priority in publication, 1901 compared with 1912. The placement of P. concentricum (= P. coprophilum) as a distinct species agreed with the opinions of Stolk and Samson (1985).

The disposition of taxa that has resulted from studies by others on mycotoxins and secondary metabolites has shown a correlation with results obtained from zymograms. Examples are given in Table 8, where cultures with the same derivation have been examined by both methods. There was general agreement in the

TABLE 8

Grouping by zymograms compared with grouping by mycotoxins and secondary metabolites[a-d] when cultures with the same derivation were examined.

Species	Cultures for: Zymograms	Metabolites	Grouping by metabolites
P. aurantiogriseum zymogram group			
P. aurantiogriseum NT	FRR 971	= NRRL 971	*P. aurantiogriseum* I[c]
P. brunneoviolaceum T	FRR 2317	= IMI 92199	*P. aurantiogriseum* II[c]
P. cyclopium T	FRR 1888	= NRRL 1888	*P. aurantiogriseum* II[a, c]
P. puberulum NT	FRR 2040	= NRRL 1889	*P. aurantiogiseum* II[c]
P. viridicatum (III)	FRR 1641	= NRRL 5573	*P. viridicatum* III[a]
P. viridicatum (III)	FRR 1642	= NRRL 5574	*P. viridicatum* III[a]

TABLE 8

continued

P. commune zymogram group				
P. commune T	FRR	890	= NRRL 890	P. camembertii II[b c] [c], P. commune[d]
P. camembertii T	FRR	877	= CBS 299.48	P. camembertii I[a]
P. camembertii	FRR	2160	= FRR 2160	P. camembertii II[b, c] P. commune[d]
P. australicum T	FRR	935		P. commune[d]
P. biforme T	FRR	885	= NRRL 885	P. camembertii III[c]
P. flavoglaucum T	FRR	948	= NRRL 948	P. camembertii II[a], III[c], P. palitans[c]
P. lanosoviride T	FRR	879	= NRRL 879	P. camembertii II[c], P. commune[d]
P. ochraceum var. macrosporum T	FRR	873	= NRRL 873	P. camembertii II[c] P. commune[e]

TABLE 8

continued

P. palitans T	FRR 2033	= NRRL 2033	*P. camembertii* II[b], III[c], *P. palitans*[d]
P. roquefortii var. *P. punctatum* T	FRR 772	= IMI 68234	*P. camembertii* III[c], *P. palitans*[d]
P. crustosum zymogram group			
P. crustosum T	FRR 1669	= IMI 91917	*P. crustosum*[c]
P. farinosum T	FRR 1478	= IMI 174717	*P. crustosum*[c]
P. echinulatum zymogram group			
P echinulatum IT	FRR 1151	= NRRL 1151	*P. echinulatum*[a, c]
P palitans var. *echinoconidium* T	FRR 637	= CBS 337.59	*P. echinulatum*[c]

TABLE 8

continued

P. expansum zymogram group			
P. expansum NT	FRR 976	= NRRL 976	P. expansum[a, c]
P. resticulosum T	FRR 2021	= NRRL 2021	P. expansum[c]
P. hirsutum	FRR 48	= FRR 48	P. expansum[c]
P. solitum zymogram group			
P. solitum T	FRR 937	= NRRL 937	P. camembertii III[c], P. solitum[a]
P. psittacinum T	FRR 932	= NRRL 932	P. camembertii III[c], P. palitans[d]
P. verrucosum var. melanochlorum T	FRR 2152	= CBS 487.75	P. mali[a], P. solitum[d]

TABLE 8

continued

P. verrucosum zymogram group			
P. verrucosum NT	FRR 965	= NRRL 965	P. viridicatum III[a, c]
P. crustosum var. spinulosporum T	FRR 1621	= FRR 1621	P. echinulatum[c]
P. viridicatum zymogram group			
P. viridicatum NT	FRR 963	= NRRL 963	P. viridicatum I[a, c]
P. viridicatum (I)	FRR 1636	= NRRL 5569	P. viridicatum I[a, c]
P. olivicolor	FRR 870	= NRRL 870	P. viridicatum I[c]
P. olivicolor	FRR 882	= NRRL 882	P. viridicatum I[c]
P. olivinoviride T	FRR 2028	= NRRL 2028	P. viridicatum I[a, c]

[a]Frisvad and Filtenborg (1983); [b]Söderström and Frisvad (1984); [c]Frisvad (1985); [d]Polonelli et al. (1987)

grouping of taxa as P. commune from zymogram results and as subgroups of P. camembertii by metabolites. The terminology for the latter has evolved to recognise P. camembertii for P. camembertii I, P. commune for P. camembertii II and P. palitans for P. camembertii III (in the main). Zymograms did not separate these taxa. Independent studies (Pitt, Cruickshank and Leistner, 1986; Polonelli et al., 1987) found the domesticated species P. camembertii was derived from P. commune.

There was agreement in conclusions from zymograms and from metabolites in taxa linked with P. crustosum, P. echinulatum, P. expansum, P. viridicatum and in the main for P. aurantiogriseum and P. solitum. Different conclusions were reached for the single-culture species P. psittacinum, placed with P. solitum by its zymograms and with P. palitans by its secondary metabolites. Also, different conclusions were reached for the green cultures FRR 1641 and FRR 1642. These gave zymograms of P. aurantiogriseum (Cruickshank and Pitt, 1987a), but were placed in P. viridicatum III by metabolite characteristics (Frisvad and Filtenborg, 1983), a group which included the culture ex type of P. verrucosum. Resolution of these differences may result from further studies when zymogram grouping is taken into account as a significant characteristic.

VIII STUDIES ON THE CERATOBASIDIACEAE

1. Materials and Methods

1.1 Cultures examined

These included:

cultures from the Western Australian wheat belt (Sweetingham, Cruickshank and Wong, 1986);

cultures from agricultural soils in South Australia (Neate, Cruickshank and Rovira, 1988; Neate and Cruickshank, 1988);

cultures of Aquathanatephorus, Ceratobasidium, Rhizoctonia and Waitea, together with anastomosis- grouped cultures, listed in Appendix 22;

Rhizoctonia cultures from the BCRI collection, Appendix 23;

Rhizoctonia cultures from DSIR, New Zealand, listed in Appendix 24.

1.2 Culture maintenance

Cultures were maintained on 2 ml slopes of PDA in bijou bottles, colonised at 22°C then stored at 4°C. Subcultures were prepared quarterly.

1.3 Enzyme production

Extracellular pectic enzymes were produced in 2 ml cultures of the liquid medium described in Appendix 4. Inoculum for each culture was a block 3 x 1 x 1 mm with active growth from a PDA culture.

Cultures were incubated at 22°C for 9-10 days.

1.4 Electrophoresis and detection of enzymes

Pectic enzymes in culture fluids were examined by electrophoresis, using the method described in Appendix 8.

2. Results

2.1 Introductory Studies

During the development of the culture medium specified, the influence of variation in composition on the yield and diversity of enzymes was examined. The medium of Steinberg (1950) that was optimised for the growth of Rhizoctonia was taken as a basis. The sucrose in this medium was replaced by citrus pectin. Ammonium sulphate gave superior results when compared with NH_4NO_3, $NaNO_3$ or asparagine at a nitrogen content equal to that of NH_4NO_3 used by Steinberg. Addition of trace elements or thiamine was found to be unnecessary and the addition of yeast extract (0.05%) inhibited enzyme yields.

The initial pH of the medium was found to be suitable if it was within the broad range 3.5 to 8.0 (Figure 30). Growth and enzyme yields were scant to zero in the unadjusted medium (pH 3.2). Enzyme yields were poor from cultures with initial pH 9.0, despite reasonable growth of mycelium.

The zymogram of each isolate was found to be effectively constant after the gradual production of enzymes during three to

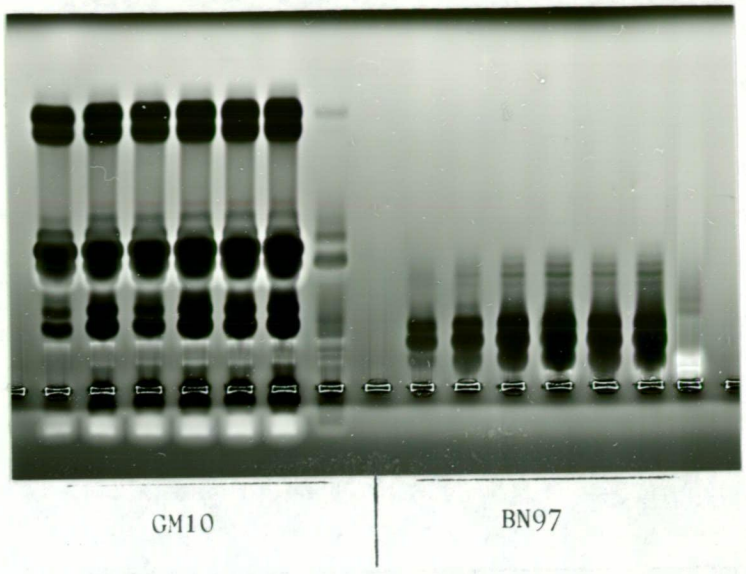


Figure 30 Variation in pectic zymograms in response to the initial pH of the culture medium, pH 3.5, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, left to right, from an isolate of Thanatephorus (GM10) and of Ceratobasidium (BN97).

six days, with yields from some isolates waning at 18 days. Optimum activity was reached in 9 to 10 days (Figure 31). Exceptions to constancy were the loss of the most electrophoretically mobile polygalacturonase (PG) isozymes after extended incubation of members of each subgroup of AG-1 (e.g. CS-2 in Figure 31), from AG-6 and from Ceratobasidium AG-E; also, the major PG group was absent and pectin esterase (PE) strongly developed from CAG-1 (BN97 in Figure 31) at 18 days. Minor cathodically migrating PG of some isolates of Ceratobasidium were evident at day six then reduced or absent by day nine.

Constancy in the zymogram given by numerous isolates collected across a continent is illustrated in Figure 32. The cultures were obtained from widely separated districts in both Western Australia and South Australia and included an example from New South Wales. These isolates belong in ZG1 (Sweetingham et al., 1986) and in the ZG1-1 component of Thanatephorus AG-8 (Neate et al., 1988).

Results from enzymes examined in separate gels run simultaneously or at different times were sufficiently constant for inter-gel comparisons to be meaningful and for results to be presented as composites from several gels. This has been done in Figures 33 to 36.

2.2 Studies on cultures from the Western Australian wheat belt

See: Sweetingham, Cruickshank and Wong (1986).

A reprint of this paper is provided in Appendix 25.

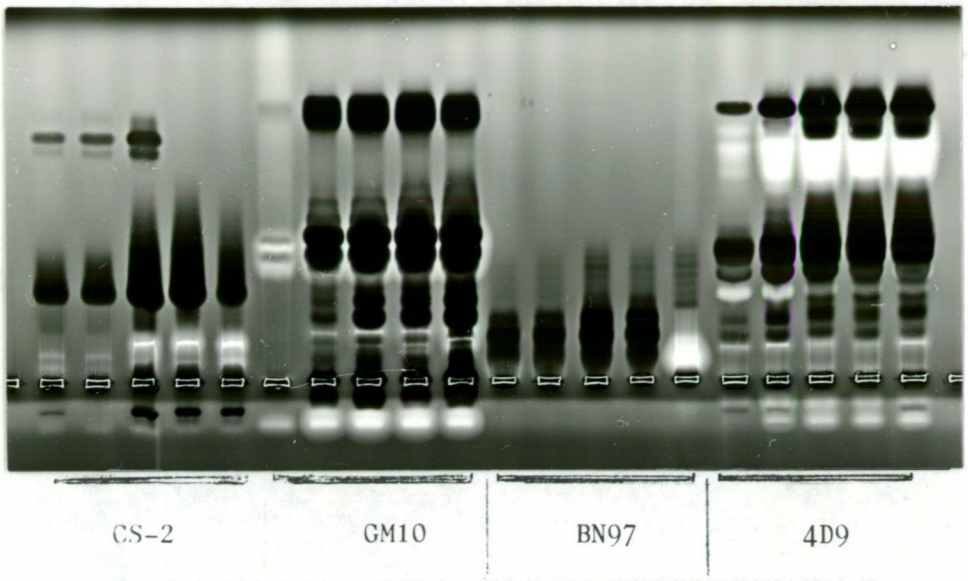


Figure 31 Influence of culture age on the pectic zymograms of respresentative isolates of Thanatephorus (CS-2; GM10) and Ceratobasidium (BN97; 4D9). In each case zymograms were from cultures incubated 3, 6, 9, 12 and 18 days, left to right.

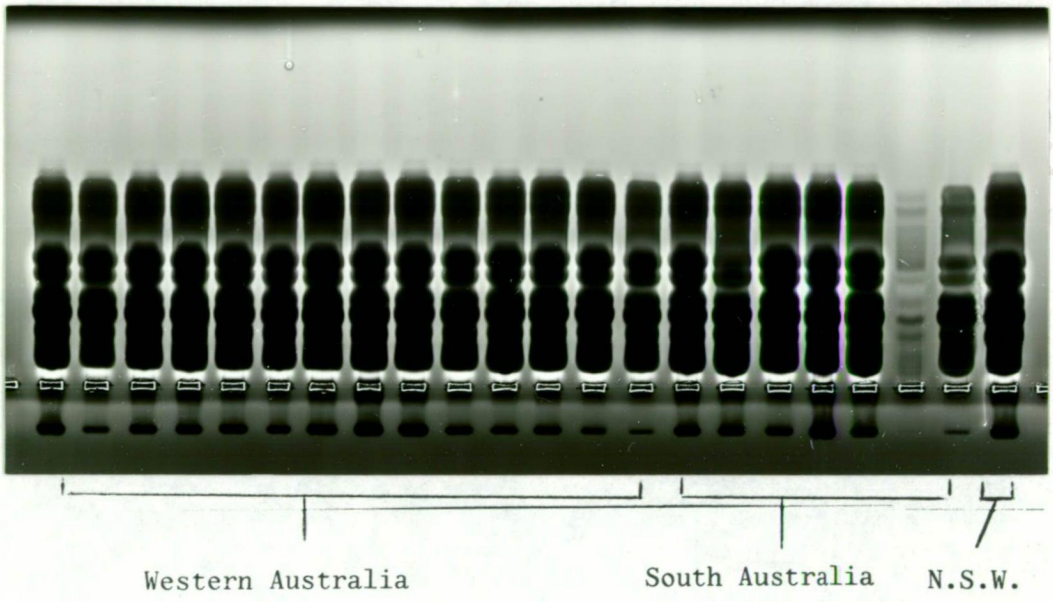


Figure 32 Pectic zymograms from 22 members of a zymogram group (ZG1) showing constancy of isozyme pattern from cultures isolated across the continent of Australia. One isolate gave a low enzyme yield but sufficient for placement in this group.

The regions where the cultures originated are marked below the figure.

2.3 Studies on cultures from agricultural areas in South Australia

See: Neate, Cruickshank and Rovira (1988); Neate and Cruickshank (1988).

Reprints of these papers are provided in Appendix 25.

2.4 Studies on Thanatephorus zymograms

Zymograms from anastomosis grouped cultures of Thanatephorus are illustrated in Figure 33 and Figure 34. A diversity of zymograms was obtained and where similarities in these allowed grouping, the groups corresponded with AGs or recognised subdivisions of these. Zymograms of AG-1 (1A), AG-1(1B) and AG-1(1C), shown in lanes 1 to 3 in Figure 33, were distinct from each other and from those of all other groups. Cultures provided simply as AG-1 or fusion group (FG)-A could be linked to subgroups by their zymograms, for example, lane 5 and lane 7 to AG-1(1B) in lane 2.

Zymograms gave evidence of considerable heterogeneity in AG-2. Lanes 8 and 9 were from AG-2-1 cultures and showed the general characteristics of ZG5. The AG-2 cultures examined in lanes 10 to 13 and FG-D cultures in lanes 14 and 15 showed these characteristics and it was expected that they also belong in AG-2-1. Zymograms from representatives of AG-2-2(IIIB) in lane 16, AG-2-2(IV) in lane 18 and AG-2-3 in lane 20 differed from each other and from those of AG-2-1.

The anastomosis subgroup AG-2-3 of W. Loerakker and J.A. Stalpers was isolated from bulbs of Monocotyledons from cold glasshouses in the Netherlands (J.A. Stalpers, personal

Figure 33 Pectic zymograms from Thanatephorus cultures in AG-1 to AG-3.

1.	AG-1 (1A)	CS-2
2.	AG-1 (1B)	B-39
3.	AG-1 (1C)	F-2
4.	AG-1	CBS 323.84
5.	AG-1	CBS 324.84
6.	AG-1	CBS 325.84
7.	FG-A	CBS 360.82
8.	AG-2-1	TG1
9.	AG-2-1	PS4
10.	AG-2	CBS 172.83
11.	AG-2	CBS 326.84
12.	AG-2	CBS 327.84
13.	AG-2	CBS 328.84
14.	FG-D	CBS 361.82
15.	FG-D	CBS 362.82
16.	AG-2-2 (IIIB)	C-330
17.	AG-2	CBS 174.83
18.	AG-2-2 (IV)	R1-64
19.	AG-2-2	C127
20.	AG-2-3	CBS 343.82
21.	FG-F	CBS 363.82
22.	AG-3	RS-30
23.	AG-3	CBS 163.83
24.	AG-3	CBS 168.83

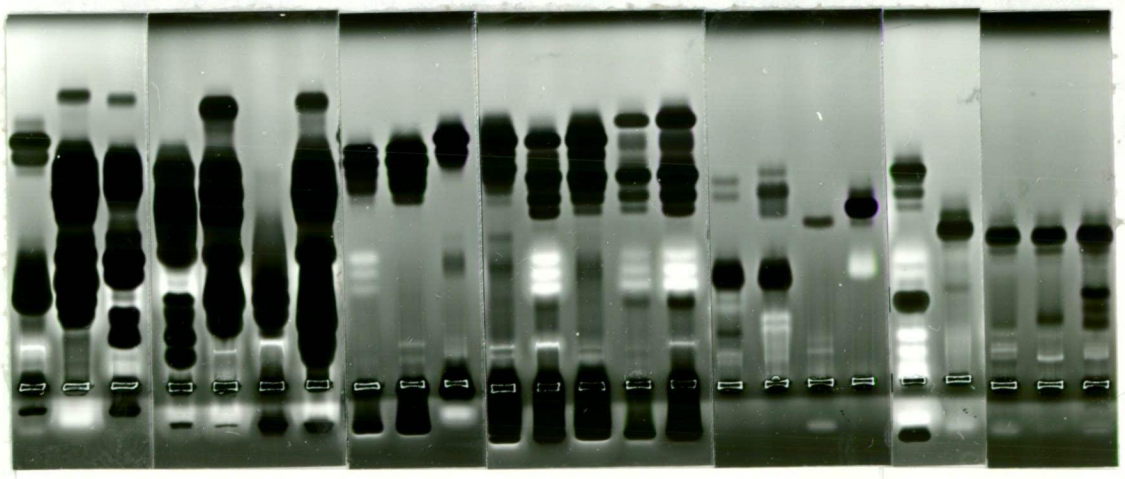


Figure 33

Figure 34 Pectic zymograms from Thanatephorus cultures in AG-4 to AG-7, and AG-BI.

1.	AG-4	SN1
2.	AG-4	CBS 164.83
3.	AG-4	CBS 171.83
4.	AG-4	CBS 333.84
5.	AG-4	CBS 334.84
6.	AG-4	CBS 335.84
7.	AG-4	CBS 336.84
8.	GF-C	CBS 364.82
9.	AG-5	CBS 337.84
10.	AG-5	CBS 338.84
11.	AG-5	CBS 339.84
12.	AG-5	CBS 160.83
13.	AG-5	CBS 161.83
14.	AG-5	GM1
15.	AG-5	GM10
16.	AG-5	CBS 160.83
17.	AG-6	AO1-6
18.	AG-7	535
19.	AG-7	1556
20.	AG-BI	CA2-1

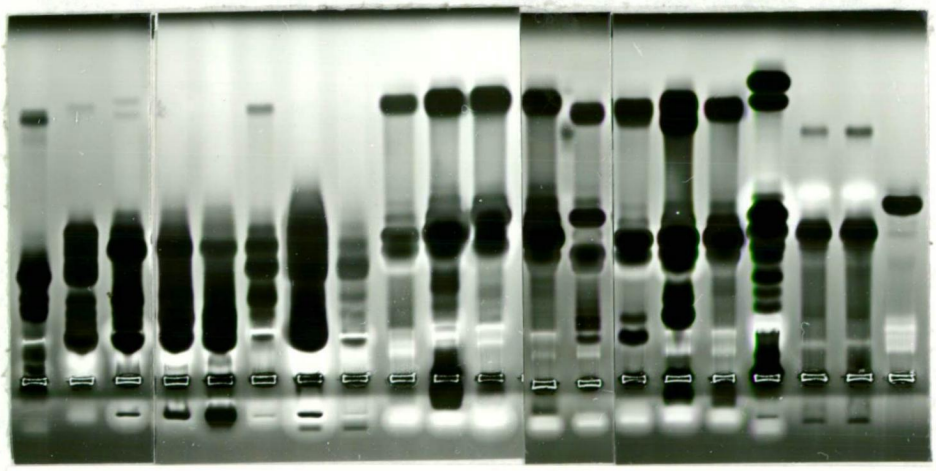


Figure 34

communication, 1985). During a study here of 12 Thanatephorus cultures from New Zealand isolated from Brassica oleracea (Appendix 24), two cultures were found to give the zymogram of AG-2-3 while the rest gave that of AG-2-1.

Zymograms from cultures provided as AG-3 and FG-F were all very similar and characteristics of ZG7, lanes 21 to 23, Figure 33. This zymogram was given by 30 of the 35 isolates from potatoes, including material from Australia (Appendix 23), Europe and Japan. The remaining five included one isolate of ZG5 and four as yet unplaced. Zymograms from AG-4, lanes 1 to 7 of Figure 34, showed similarities in general form but there was evidence for subgrouping from details in their patterns. The zymogram in lane 8 was from a culture of FG-C deposited by R. Schneider. This showed relationship to AG-4, particularly to CBS355.84 in lane 6.

All the representatives of AG-5 shared a distinctive zymogram pattern, shown in lanes 9 to 16 in Figure 34, with minor variations in some details. A single example of AG-6 was examined and its zymogram, lane 17 in Figure 34 was distinct from all others. The two isolates of AG-7 gave identical distinctive zymograms, in lanes 18 and 19 of Figure 34. The single isolate of AG-BI also gave a distinctive zymogram, in lane 20 of Figure 34.

2.5 Studies on Ceratobasidium zymograms

Zymograms from representatives of Ceratobasidium anastomosis groups are shown in Figure 35. From these and other zymograms, similarities were found between AG-D in lane 5, CAG1 in lanes 14 and 15 and R. cerealis in lanes 11 to 13. Some similarity was

Figure 35 Pectic zymograms from *Ceratobasidium* cultures.

1.	AG-A	SH-6
2.	AG-Ba	C-484
3.	AG-Bb	C-348
4.	AG-C	54 D 25
5.	AG-D	C-57
6.	AG-E	F-18
7.	AG-F	SIR-1
8.	AG-G	4D9
9.	AG-I	AV-2
10.	AG-K	AC-1
11.	*R. cerealis* CBS 236.77 (T)	
12.	*R. cerealis* CBS 558.77	
13.	*R. cerealis* CBS 559.77	
14.	CAG-1	BN97
15.	CAG-1	N132
16.	CAG-2	*
17.	CAG-3	*
18.	CAG-4	*
19.	CAG-5	BN37
20.	CAG-6	BN74
21.	CAG-7	
22.	FG-E	CBS 347.84
23.	FG-E	CBS 340.84
24.	FG-E	CBS 341.84
25.	FG-E	CBS 342.84

*Isolate codes were not provided by the donor.

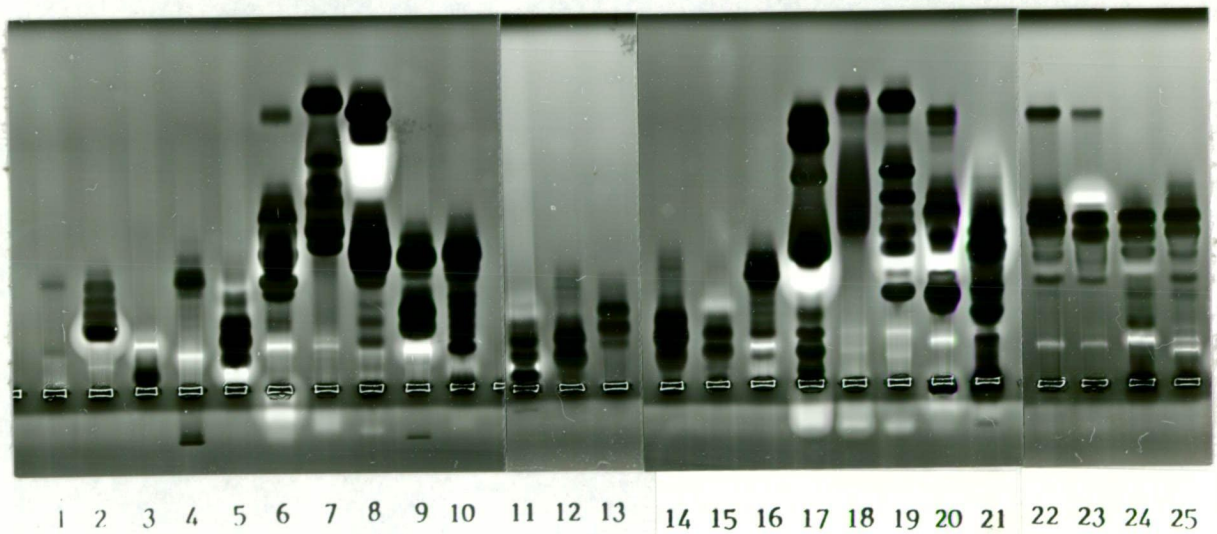


Figure 35

found between AG-F in lane 7 and CAG4 in lane 18; also between AG-E in lane 6, CAG6 in lane 20, FG-E in lanes 22 and 23 of Figure 35 and R. muneratii in lane 12 of Figure 36. When Ceratobasidium AGs and CAGs were compared with Australian CZGs illustrated in Sweetingham et al. (1986), there was an evident relationship between CZG5 and AG-K. While common in Australia (Sweetingham et al., 1986; Neate and Cruickshank, 1988; Roberts and Sivasithamparam, 1986), this type was predominant in a sample of binucleate isolates from pasture species and Brassica oleracea from New Zealand (Appendix 24).

The examples of binucleate species illustrated in lanes 4 to 13 in Figure 36 were distinctive and not obviously related to accepted AGs or CAGs except for the relationship between R. muneratii, FG-EAG-E and CAG6 mentioned previously. The multinucleate species R. mucoroides was represented by the culture ex type in lane 14 of Figure 36 and CBS338.36 in the adjacent lane 15. These did not appear to be related to each other or to the AG representatives that were examined in this study.

2.6 Studies on Waitea zymograms

Zymograms from the group with affinities to W. circinata are shown in lanes 16 to 19 in Figure 36. The culture ex type of R. zeae in lane 18 appeared to be closely related to W. circinata in lane 19. A culture provided as R. oryzae, lane 17, appeared distinct from the culture ex type of this species in lane 16. All were distinct from WZG1 of Sweetingham et al. (1986).

Figure 36 Pectic zymograms from *Aquathanatephorus*, *Pseudopapulaspora*, *Ceratobasidium* species, *Rhizoctonia* species and *Waitea circinata*.

1. *Aquathanatephorus pendulus* CBS 700.82 (T)
2. *Pseudopapulaspora kendrickii* CBS 702.82 (T)
3. *Ps. kendrickii* CBS 566.83
4. *Ceratobasidium angustisporum* CBS 568.83
5. *C. globisporum* CBS 569.83
6. *C. papillatum* CBS 570.83
7. *C. stevensii* CBS 477.82
8. *C. cornigerum* CBS 148.54
9. *C. anceps* CBS 152.32
10. *Rhizoctonia ramicola* CBS 400.51 (T)
11. *R. fraxini* CBS 311.35 (T)
12. *R. muneratii* CBS 298.37 (T)
13. *R. pini-insignis* CBS 312.35 (T)
14. *R. mucoroides* CBS 126.08
15. *R. mucoroides* CBS 338.36 (T)
16. *R. oryzae* CBS 273.38 (T)
17. *R. oryzae* CBS 472.82
18. *R. zeae* CBS 384.34 (T)
19. *Waitea circinata* CBS 180.82
20. *R. carotae* CBS 464.48 (T)

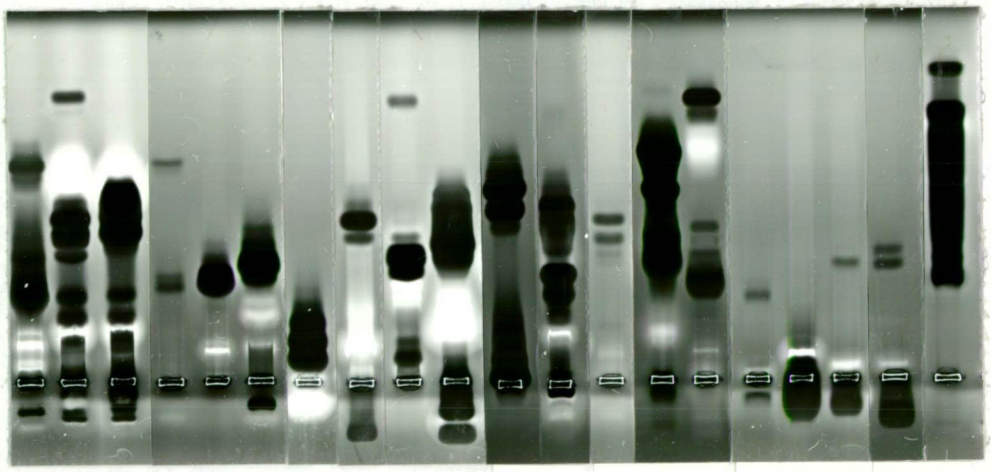


Figure 36

2.7 Studies on Aquathanatephorus and others

The culture ex type of A. pendulus, lane 1 in Figure 36, gave a pectic zymogram with similarities to that of AG-1 (1A), lane 1 in Figure 33. Although there was a difference in the Rf of their fast-migrating PG isozymes, there was correspondence in this in the case of the AG-1 isolate in lane 6 of Figure 33.

Pseudopapulaspora kendrickii had the characteristics of Rhizoctonia but its relationships in the complex were not elucidated from its distinctive zymogram, lanes 2 and 3 in Figure 33.

The production of clamp connections by R. carotae (Burton and Coley-Smith, 1985) is atypical of the Ceratobasidiaceae. This species gave a distinctive zymogram in lane 20 of Figure 36.

2.8 Examination of cultures from New South Wales (Appendix 23) and New Zealand (Appendix 24)

The identifications made by means of pectic zymograms are provided in these Appendices.

3. Discussion

Practical requirements for pectic enzyme production from members of the Ceratobasiaceae were found to be simple, there was no need for critical adjustment of initial pH or for accurate timing of incubation of the cultures. The latter was found to be necessary for the production of reproducible results for esterases (Matsuyama, Moromizato, Ogoshi and Wakimoto, 1978) and for soluble proteins (Reynolds, Weinhold and Morris, 1983). The pectic

zymogram technique provided a ready means for the characterisation of large numbers of isolates from Rhizoctonia populations and provided a permanent record of distinctive characteristics from all isolates. This was not dependent on possession of AG tester strains or relationships of the isolates to known AGs. Inability to identify some isolates to AGs is well known (Parmeter and Whitney, 1970; Roberts and Sivasithamparam, 1986).

As the number of cultures examined has increased, it has become apparent that a single zymogram can be representative of many isolates collected over a wide geographic area. This has been seen from ZG1 for example (Figure 32) and from other groups prevalent in the collection, such as ZG5 (AG-2-1), ZG7 (AG-3) and CZG5 (AG-K). The placement of an isolate in a particular zymogram group was found to have predictive value (Sweetingham et al., 1986), and in some cases gave relationship to AGs (Neate, Cruickshank and Rovira, 1988), as summarized in the Literature Review and presented in the actual papers in Appendix 25.

In studies on the cultures listed in Appendix 22, considering anastomosis grouped cultures of Thanatephorus, the results supported the conclusions of Matsuyama et al. (1978) from examination of the extracellular non-specific esterase zymograms of such cultures. They found that zymogram similarities roughly agreed with AGs but sometimes were more closely correlated with ecological types. The latter are now recognised as subgroups of AGs.

As far as tested, agreement was found with the conclusions of Kuninaga and Yokosawa (1980, 1982a, 1982b, 1983, 1984a, 1984b)

on the genetic homogeneity or divergence within AGs from evidence of DNA base composition and sequence homology. In this regard, zymograms showed AG-1(1A) to be distinct from AG-1(1B); AG-2-1, AG-2-2(IIIB) and AG-2-2(IV) as distinct groups; AG-3 as a distinct and homogeneous group; AG-4 as a distinct and heterogeneous group; AG-5 as distinct and homogeneous; AG-6, AG-7 and AG-BI as distinct groups. Kuninaga and Yokosawa found AG-7 and AG-BI to be homogeneous groups, while AG-6 was heterogeneous.

There is a growing body of evidence in support of the hypothesis that all the major AGs in Thanatephorus constitute species, for example, Adams and Butler (1979) from serological studies, and Reynolds et al. (1983) from electrophoretic examination of soluble proteins. In the case of AG-4, Anderson (1982) described this group as a cosmopolitan interbreeding population and a good biological species, T. praticola. Kuninaga and Yokosawa (1985a, 1985b) have added to the body of evidence by studying DNA base sequence homology, concluding that AG-1, AG-2 with AG-BI, AG-3, AG-4, AG-5, AG-6 and AG-7 were genetically isolated groups and that each was a biological species in the taxonomic species R. solani. They retained subgroup [sub-species] status for the subgroups in AG-1 and AG-2 as well as for those revealed by their studies, in AG-4 and AG-6. This contrasted in part with their previous findings from DNA base composition (Kuninaga and Yokosawa, 1980) that AG-1, AG-2-2, AG-2-2, AG-3, AG-4, AG-5, AG-6 and AG-BI were genetically independent units.

As pectic zymogram evidence has been assembled it has supported the distinction between AGs but it has also

distinguished between AG subdivisions, AG-2-1 from the rest of AG-2, for example. In studies on Sclerotinia (Cruickshank, 1983a), Botrytis (Cruickshank, 1983b) and Penicillium (Cruickshank and Pitt, 1987a) it was found that each species gave its own characteristic pectic zymogram. By comparison, the AG subdivisions in Thanatephorus would be regarded as separate species. The relationship of subgroups indicated by anastomosis behaviour may be that of relatively close phylogenic derivation, with broadening of such relationships shown by links as in the anastomosis of AG-BI with AG-2, AG-3 and AG-6.

While the zymogram evidence of relationship between Aquathanatephorus pendulus and AG-1(1A) may prove to be fortuitous, it is an interesting association of taxa that are pathogenic in aquatic environments, A. pendulus to water hyacinth and AG-1(1A) to rice. However, the teleomorph of AG-1(1A) has been shown to be T. cucumeris (Oniki, Ogoshi and Araki, 1986).

Zymogram evidence supported the conclusions of Parmeter, Sherwood and Platt (1969) on the relationship between AGs and the fusion groups of Richter and Schneider (1953). This evidence also supported the relationship found by Ogoshi, Oniki and Araki (1983) between the Japanese AGs of Ceratobasidium and the CAGs found in North America by Burpee, Sanders, Cole and Sherwood (1980). However, while Ogoshi et al. (1983) found a relationship of AG-E to CAG3, and of some members of CAG3 to CAG6, pectic zymograms only showed similarity of AG-E to CAG6. Results supported the alliance of this group, typified by FG-E, to R. muneratii (Burpee et al., 1980). Allowing for these associations of groups, the

distinctive nature of their zymograms indicated the presence of 14 species among the Ceratobasidium AG and CAG isolates examined and a further 10 species in cultures of binucleate Rhizoctonia species and Ceratobasidium species. Undoubtedly more are yet to be recognized.

IX GENERAL DISCUSSION

Taxonomic studies based on the morphology of fungi, have provided useful working systems, but further intensive studies have often raised doubts and led to continuing debate. Phenotypic comparisons have been made in ever-increasing detail and yet remained inconclusive. Practical application of any taxonomic scheme can be fairly straightforward for the majority of cultures, but clairvoyance has even been mentioned as a useful attribute for those attempting the identification of some isolates of Penicillium (Pitt, 1979, p. 520)!

While morphology, the study of the fabric of life, is at present essential as a basis for taxonomy, form, size and cultural characteristics are probably influenced by the interaction of a multitude of genes, with the possibility of saltation from mutation at a single locus. Convergent evolution to particularly favourable phenotypes can be expected to have blurred species-distinctive characteristics of morphology.

Comparison of the total genome would provide the ultimate evidence of relationships but this is not a practical method. However, enzymes, the tools of life, can be examined by electrophoresis to provide zymograms as tangible direct evidence of discreet parts of the genome of the organisms under study. Zymograms can be examined for coincident similarities in overall pattern (Ferguson, 1980) to provide evidence of close relationship or indications of synonymy. While providing evidence for these,

zymograms (even with broader evidence from several enzyme systems) only examine part of the genome and may be insufficient evidence, taken alone, to delimit species on the basis of the total genome (Cruickshank and Pitt, 1987a). The alternative consideration, evidence for lack of close relationship is generally provided clearly by zymograms.

The investigations reported in this thesis were based primarily on pectic zymograms. The technique involved in their production was developed and gradually improved to a state that provided high sensitivity and adequate resolution of PG and PE isozymes, with these enzymes detected simultaneously. Simple means were developed for the production of enzymes which were secreted by the fungi into liquid media, making use of economic micro-cultures. Fluids from these cultures were immediately suitable for electrophoresis. The recording of zymograms as photograms was a very simple and quick method which gave sharply defined results.

Pectic zymograms were found to be species-distinctive for the Sclerotinia species, S. sclerotiorum, S. trifoliolorum and S. minor (Cruickshank, 1983a). This non-subjective identification could be made rapidly by the use of enzymes extracted by diffusion from PDA cultures of the fungi.

Zymogram studies on Botrytis were fruitful in solving problems in taxonomy and in providing the means for species identification. The pectic zymogram method was useful for characterising old or degenerate cultures not only in Botrytis but also in Penicillium (Cruickshank and Pitt, 1987a) and Rhizoctonia (Neate, Cruickshank and Rovira, 1988).

Studies on Penicillium species in the subgenus Penicillium gave very useful results and reinforced the notion of species-specific pectic zymograms. The species concepts of Pitt (1979), based on morphological and broadly physiological grounds, were strongly supported and some probable errors and misconceptions in Pitt (1979) were revealed.

Pectic zymograms have been put to practical use in the identification of zymogram groups in the Rhizoctonia complex. These have predictive value in plant pathology and provide a form of taxonomy not dependent on the possession of live AG tester strains. The method proved to be more sensitive than anastomosis grouping in revealing groups with the characteristics of species.

The pectic zymogram approach to taxonomy can provide an excellent basis for studies by other means. The value of such an approach was realised by May and Royse (1988). They suggested that allozyme analysis could be used to separate isolates into putative specific classes and that these classes could then be subjected to more rigorous morphological, breeding and physiological analysis to determine a coherent systematic classification within a genus.

X BIBLIOGRAPHY

Adams, G.C. Jr. and Butler, E.E. (1979). Serological relationships among anastomosis groups of Rhizoctonia solani. Phytopathology **69**: 629-633.

Anderson, N.A. (1982). The genetics and pathology of Rhizoctonia solani. Annual Review of Phytopathology **20**: 329-347.

Anné, J. (1985). Taxonomic implications of hybridization of Penicillium protoplasts. In "Advances in Penicillium and Aspergillus Systematics" (edited by R.A. Samson and J.I. Pitt), pp. 337-350. Plenum Press, New York, N.Y.

Anné, J. and Peberdy, J.F. (1981). Characterisation of inter-specific hybrids between Penicillium chrysogenum and P. roqueforti by iso-enzyme analysis. Transactions of the British Mycological Society **77**: 401-408.

Arx, J.A. von (1974). "The Genera of Fungi Sporulating in Pure Culture", 2nd Edition. Cramer, Lehre, Germany.

Backhouse, D., Willetts, H.J. and Adam, P. (1984). Electrophoretic studies of Botrytis species. Transactions of the British Mycological Society **82**: 625-630.

Baldwin, C.H. Jr. and Corden, M.E. (1969). Influence of carbon source on production of pectolytic enzymes by Fusarium oxysporum f.sp. lycopersici. Phytopathology **59**: 1017 abst.

Bateman, D.F. and Millar, R.L. (1966). Pectic enzymes in tissue degradation. Annual Review of Phytopathology **4**: 119-146.

Beever, R.E. and Brien, H.M.R. (1983). A survey of resistance to the dicarboximide fungicides in Botrytis cinerea. New Zealand Journal of Agricultural Research **26**: 391-400.

Béguin, P. (1983). Detection of cellulase activity in polyacrylamide gels using congo red-stained agar replicas. Analytical Biochemistry **131**: 333-336.

Bent, K.J. (1967). Electrophoresis of proteins of 3 Penicillium species on acrylamide gels. Journal of general Microbiology **49**: 195-200.

Berg, L. and Yang, S.M. (1969). Effects of relative humidity on production of extracellular pectolytic enzymes of Botrytis cinerea and Sclerotinia sclerotiorum. Canadian Journal of Botany **47**: 1007-1010.

Bittner, M., Kupferer, P. and Morris, C.F. (1980). Electrophoretic transfer of proteins and nucleic acids from slab gels to diazobenzyloxymethyl cellulose or nitrocellulose sheets. Analytical Biochemistry **102**: 459-471.

Boisselier-Dubayle, M.-C. (1983). Taxonomic significance of enzyme polymorphism among isolates of Pleurotus (Basidiomycetes) from Umbellifers. Transactions of the British Mycological Society **81**: 121-127.

Bonde, M.R., Peterson, G.L., Dowler, W.M. and May, B. (1984). Isozyme analysis to differentiate species of Peronosclerospora causing downy mildew of maize. Phytopathology **74**: 1278-1283.

Boucher, W.D. (1975). "Structural, biochemical and physiological aspects of a systemic virus-host interaction". Doctoral Thesis, Agricultural Science, University of Tasmania.

Bradford, L.S., Jones, R.J. and Garber, E.D. (1975). An electrophoretic survey of fourteen species of the fungal genus Ustilago. Botanical Gazette (Chicago) **136**: 109-115.

Bridge, P.D. (1985). An evaluation of some physiological and biochemical methods as an aid to the characterisation of species of Penicillium subsection Fasciculata. Journal of general Microbiology **131**: 1887-1895.

Bridge, P.D. and Hawksworth, D.L. (1984). The APIZYM enzyme testing system as an aid to the identification of Penicillium isolates. Microbiological Sciences **1**: 232-234.

Bridge, P.D., Hudson, L., Kozakiewicz, S., Onions, A.H.S. and Paterson, R.R.M. (1987). Investigation of variation in phenotype and DNA content between single-conidium isolates of single Penicillium strains. Journal of general Microbiology **133**: 995-1004.

Brierley, W.B. (1931). Biological races in fungi and their significance in evolution. Annals of Applied Biology **18**: 420-434.

Buchwald, N.F. (1949). Studies in the Sclerotiniaceae. I. Taxonomy in the Sclerotiniaceae. Kongelige Veterinaer-og Landbohojskole Arsskrift **1949**: 74-191. Cited in Review of Applied Mycology **28**: 546-547.

Burdon, J.J. and Marshall, D.R. (1981). Isozyme variation between species and formae speciales of the genus Puccinia. Canadian Journal of Botany **59**: 2628-2634.

Burpee, L.L., Sanders, P.L., Cole, H. Jr. and Sherwood, R.T. (1980). Anastomosis groups among isolates of Ceratobasidium cornigerum and related fungi. Mycologia **72**: 689-701.

Burton, R.J. and Coley-Smith, J.R. (1985). Antibiotics in sclerotia and mycelium of Rhizoctonia species. Transactions of the British Mycological Society **85**: 447-453.

Carling, D.E., Leiner, R.H. and Kebler, K.M. (1986). Characterization of Rhizoctonia solani and binucleate rhizoctonia-like fungi collected from Alaskan soils with various histories. Canadian Journal of Plant Pathology **8**: 305-310.

Caten, C.E. and Jinks, J.L. (1966). Heterokaryosis: its significance in wild homothallic Ascomycetes and Fungi Imperfecti. Transactions of the British Mycological Society **49**: 81-93.

Chang, L.O., Srb, A.M. and Steward, F.C. (1962). Electrophoretic separations of the soluble proteins of Neurospora. Nature **193**: 756-759.

Ciegler, A., Fennell, D.I., Sansing, G.A., Detroy, R.W. and Bennett, G.A. (1973). Mycotoxin-producing strains of Penicillium viridicatum: classification into subgroups. Applied Microbiology **26**: 271-278.

Ciegler, A., Lee, L.S. and Dunn, J.J. (1981). Production of naphthoquinone mycotoxins and taxonomy of Penicillium viridicatum. Applied and Environmental Microbiology **42**: 446-449.

Clare, B.G., Flentje, N.T. and Atkinson, M.R. (1968). Electrophoretic patterns of oxidoreductases and other proteins as criteria in fungal taxonomy. Australian Journal of Biological Science **21**: 275-295.

Clare, B.G. and Zentmyer, G.A. (1966). Starch gel electrophoresis of proteins from species of Phytophthora. Phytopathology **56**: 1334-1335.

Clarke, B.G. (1963). Starch-gel electrophoresis of proteins as an aid to identifying fungi. Nature **200**: 803-804.

Coley-Smith, J.R., Verhoeff, K. and Jarvis, W.R. (eds.) (1980). The Biology of Botrytis. Academic Press, New York, London.

Crowle, A.J. and Cline, L.J. (1977). An improved stain for immunodiffusion tests. Journal of Immunological Methods **17**: 379-381.

Cruickshank, R.H. (1983a). Distinction between Sclerotinia species by their pectic zymograms. Transactions of the British Mycological Society **80**: 117-119.

Cruickshank, R.H. (1983b). Pectic zymograms and taxonomy of Botrytis. Fourth International Congress of Plant Pathology (Melbourne), Abstract 708.

Cruickshank, R.H. and Pitt, J.I. (1987a). Identification of species in Penicillium subgenus Penicillium by enzyme electrophoresis. Mycologia **79**: 614-620.

Cruickshank, R.H. and Pitt, J.I. (1987b). The zymogram technique: isoenzyme patterns as an aid in Penicillium classification. Microbiological Sciences **4**: 14-17.

Cruickshank, R.H. and Wade, G.C. (1980). Detection of pectic enzymes in pectin-acrylamide gels. Analytical Biochemistry **107**: 177-181.

Davis, B.J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. Annals of the New York Academy of Science **121**: 404-442.

DiLenna, P. and Fielding, A.H. (1983). Multiple forms of polygalacturonase in apple and carrot tissue infected by isolates of Botrytis cinerea. Journal of general Microbiology **129**: 3015-3018.

DiLenna, P., Marciano, P. and Magro, P. (1981). Comparative investigation on morphological and physiological features of three isolates of Botrytis cinerea. Phytopathologische Zeitschrift **100**: 203-211.

Durbin, R.D. (1966). Comparative gel-electrophoretic investigation of the protein patterns of Septoria species. Nature **210**: 1186-1187.

Erselius, L.J. and Shaw, D.S. (1982). Protein and enzyme differences between Phytophthora palmivora and P. megakarya: evidence for self fertilization in pairings of the two species. Transactions of the British Mycological Society **78**: 227-238.

Ferguson, A. (1980). "Biochemical Systematics and Evolution". Wiley and Sons, New York, N.Y.

Food and Agriculture Organisation (1979). Perspectives on mycotoxins. FAO Food and Nutrition Paper 13. (Joint FAO/WHO/UEP Conference on Mycotoxins, Nairobi, 1977.)

Frisvad, J.C. (1981). Physiological criteria and mycotoxin production as aids in identification of common asymmetric penicillia. Applied and Environmental Microbiology **41**: 568-579.

Frisvad, J.C. (1985). Profiles of primary and secondary metabolites of value in classification of Penicillium viridicatum and related species. In "Advances in Pencillium and Aspergillus Systematics" (e.g. R.A. Samson and J.I. Pitt), pp. 311-325. Plenum Press, New York, N.Y.

Frisvad, J.C. and Filtenborg, O. (1983). Classification of terverticillate Penicillia based on profiles of mycotoxins and other secondary metabolites. Applied and Environmental Microbiology **46**: 1301-1310.

Gams, W. and Jülich, W. (1984). Taxonomy and phylogeny of fungi. Progress in Botany **46**: 274-296.

Garber, E.D. and Beraha, L. (1966). Genetics of phytopathogenic fungi. XVI. Pectolytic enzymes of virulent and avirulent strains of three phytopathogenic penicillia. Canadian Journal of Botany **44**, 1645-1650.

Gill, H.S. and Powell, D. (1968). Polyacrylamide gel (disc) electrophoresis of physiological races A-1 to A-8 of Phytophthora fragariae. Phytopathology **58**, 722-723.

Gill, H.A. and Zentmyer, G.A. (1978). Identification of Phytophthora species by disc electrophoresis. Phytopathology **68**, 163-167.

Glynn, A.N. and Reid, J. (1969). Electrophoretic patterns of soluble fungal proteins and their possible use as taxonomic criteria in the genus Fusarium. Canadian Journal of Botany **47,** 1823-1831.

Goullet, Ph. (1981). Characterization of Serratia odorifera, S. fonticola and S. ficaria by electrophoretic patterns of their esterases. Journal of general Microbiology **127,** 161-167.

Groves, J.W. and Loveland, C.A. (1953). The connection between Botryotinia fuckeliana and Botrytis cinerea. Mycologia **45,** 415-425.

Hall, R. (1969). Molecular approaches to the taxonomy of fungi. Botanical Review **35,** 285-304.

Hall, R. (1973). Electrophoretic protein profiles as criteria in taxonomy of fungi and algae. Bulletin of the Torrey Botanical Club **100,** 253-259.

Hall, R., Zentmyer, G.A. and Erwin, D.C. (1969). Approach to taxonomy of Phytophthora through acrylamide gel-electrophoresis of proteins. Phytopathology **59,** 770-774.

Hansen, H.N. and Smith, R.E. (1932). The mechanism of variation in imperfect fungi: Botrytis cinerea. Phytopathology **22,** 953-964.

Hennebert, G.L. (1971). The principles of taxonomy of the form genus Botrytis in relation to its biology. Commun. to 1st Int. Mycol. Congr., **1971.** Cited in Jarvis (1977), p. 63.

Hennebert, G.L. (1973). Botrytis and Botrytis-like genera. Persoonia 7, 183-204.

Hódosy, S. (1964). [Incidence of Botrytis disease of lily of the valley in Hungary.] Növénytermelés **13,** 269-276. Cited in Review of Applied Mycology **44,** 1119 (1965).

Hofelmann, M., Kittsteiner-Eberle, R. and Schreier, P. (1983). Ultrathin-layer agar gels: a novel print technique for ultrathin-layer isoelectric focusing of enzymes. Analytical Biochemistry **128,** 217-222.

Hunter, R.L. and Markert, C.L. (1957). Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. Science **125,** 1294-1295.

Jakubke, H.-D. and Jeschkeit, H. eds. (1983). Concise Encyclopedia of Biochemistry. Walter de Gruyter, Berlin, New York.

Jarvis, W.R. (1977). Botryotinia and Botrytis species: taxonomy, physiology and pathogenicity. Research Branch, Canada Department of Agriculture, Ottawa, Monograph **15.**

Jarvis, W.R. (1980). Taxonomy. In The Biology of Botrytis (ed. J.R. Coley-Smith, K. Verhoeff and W.R. Jarvis). pp. 1-18. Academic Press, London, New York.

Jeffrey, C. (1973). Biological Nomenclature. Edward Arnold, London, U.K.

Jones, M.G. and Noble, W.C. (1982). An electrophoretic study of enzymes as a tool in the taxonomy of the Dermatophytes. Journal of general Microbiology **128,** 1101-1107.

Kaosiri, T. and Zentmyer, G.A. (1980). Protein, esterase and peroxidase patterns in the Phytophthora palmivora complex from cacao. Mycologia **72,** 988-1000.

Keen, N.T. and Horton, J.C. (1966). Induction and repression of endopolygalacturonase synthesis by Pyrenochaeta terrestris. Canadian Journal of Microbiology **12**, 443-453.

Kendrick, B. (1980). The generic concept in Hyphomycetes - a reappraisal. Mycotaxon **11**, 339-364.

Klebahn, H. (1930). [Contribution to the knowledge of some Botrytis forms of the type of Botrytis cinerea.] Zeitschrift fuer Botanik **23**, 251-272. Cited in Review of Applied Mycology **10**, 274 (1931).

Kulik, M.M. and Vincent, P.G. (1973). Pyrolysis-gas-liquid chromatography of fungi: observations on variability among nine Penicillium species of the section Asymmetrica, subsection Fasciculata. Mycopathologia et Mycologia Applicata **51**, 1-18.

Kuninaga, S. and Yokosawa, R. (1980). A comparison of DNA base composition among anastomosis groups in Rhizoctonia solani Kühn. Annals of the Phytopathological Society of Japan **46**, 150-158.

Kuninaga, S. and Yokosawa, R. (1982a). DNA base sequence homology in Rhizoctonia solani Kühn. I. Genetic relatedness within anastomosis group 1. Annals of the Phytopathological Society of Japan **48**, 659-667.

Kuninaga, S. and Yokosawa, R. (1982b). DNA base sequence homology in Rhizoctonia solani Kühn. II. Genetic relatedness within anastomosis group 2. Annals of the Phytopathological Society of Japan **48**, 668-673.

Kuninaga, S. and Yokosawa, R. (1983). DNA base sequence homology in Rhizoctonia solani Kühn. III. Genetic relatedness within AG-3, AG-5, AG-7 and AG-BI. Annals of the Phytopathological Society of Japan **49**, 647-652.

Kuninaga, S. and Yokosawa, R. (1984a). DNA base sequence homology in Rhizoctonia solani Kühn. IV. Genetic relatedness within AG-4. Annals of the Phytopathological Society of Japan **50**, 322-330.

Kuninaga, S. and Yokosawa, R. (1984b). DNA base sequence homology in Rhizoctonia solani Kühn. V. Genetic relatedness within AG-6. Annals of the Phytopathological Society of Japan **50**, 346-352.

Kuninaga, S. and Yokosawa, R. (1985a). DNA base sequence homology in Rhizoctonia solani Kühn. VI. Genetic relatedness among seven anastomosis groups. Annals of the Phytopathological Society of Japan **51**, 127-132.

Kuninaga, S. and Yokosawa, R. (1985b). DNA base sequence homology in Rhizoctonia solani Kühn. VII. Genetic relatedness between AG-BI and other anastomosis groups. Annals of the Phytopathological Society of Japan **51**, 133-138.

Kurzega, K.C. and Garber, E.D. (1973). A genetic study of electrophoretically variant extracellular amylolytic enzymes of wild-type strains of Aspergillus nidulans. Canadian Journal of Genetics and Cytology **15**, 275-287.

Leung, H. and Williams, P.H. (1986). Enzyme polymorphism and genetic differentiation among geographic isolates of the rice blast fungus. Phytopathology **76**, 778-783.

Lisker, N. and Retig, N. (1974). Detection of polygalacturonase and pectin lyase isoenzymes in polyacrylamide gels. Journal of Chromatography **96,** 245-249.

Lorbeer, J.W. (1980). Variations in Botrytis and Botryotinia. In The Biology of Botrytis (ed. J.R. Coley-Smith, K. Verhoeff and W.R. Jarvis). pp. 19-39. Academic Press, London, U.K.

Lorenz, D.H. and Eichorn, K.W. (1983). [Investigations on Botryotinia fuckeliana Whetz., the perfect stage of Botrytis cinerea Pers.] Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz **90,** 1-11.

Maas, J.L. and Powelson, R.L. (1972). Growth and sporulation of Botrytis convoluta with various carbon and nitrogen sources. Mycologia **64,** 897-903.

Maghrabi, H.A. and Kish, L.P. (1985). Isozyme characterisation of Ascophaerales associated with bees. I. Ascophaera apis, Ascophaera proliperda and Ascophaera aggregata. Mycologia **77,** 358-365.

Magro, P., DiLenna, P., Marciano, P. and Pallavicini, C. (1980). Variability of polygalacturonase and protein isoelectric focussing patterns in Botrytis cinerea isolates. Journal of general Microbiology **120,** 105-109.

Markert, C.L. and Møller, F. (1959). Multiple forms of enzymes: tissue ontogenetic and species specific patterns. Proceedings of the National Academy of Science, U.S.A. **45,** 753-763.

Martin, S.B., Lucas, L.T. and Campbell, C.L. (1984). Comparative sensitivity of Rhizoctonia solani and Rhizoctonia-like fungi to selected fungicides in vitro. Phytopathology **74,** 778-781.

Matsuyama, N., Kato, H. and Kayaguchi, T. (1977). Comparison of isoenzyme patterns of extracellular enzymes in Pyricularia strains from Gramineous and Zingiberaceous plants. Annals of the Phytopathological Society of Japan **43,** 419-425.

Matsuyama, N., and Kozaka, T. (1971). Comparative gel electrophoresis of soluble proteins and enzymes of rice blast fungus. Pyricularia oryzae. Annals of the Phytopathological Society of Japan **37,** 259-265.

Matsuyama, N., Moromizato, Z., Ogoshi, A. and Wakimoto, S. (1978). Grouping Rhizoctonia solani Kühn with non-specific esterase zymogram. Annals of the Phytopathological Society of Japan **44,** 652-658.

Matsuyama, N. and Wakimoto, S. (1977). A comparison of esterase and catalase zymograms of Fusarium species with special reference to the classification of a causal fungus of Fusarium leaf spot of rice. Annals of the Phytopathological Society of Japan **43,** 462-470.

May, B. and Royse, D.J. (1988). Interspecific allozyme variation within the fungal genus Pleurotus. Transactions of the British Mycological Society **90,** 29-36.

Mayr, E.M. (1964). The new systematics. In Taxonomic Biochemistry and Serology. (ed. C.A. Leone). pp. 13-32. The Ronald Press Co., New York, N.Y.

Menzinger, W. (1966). [On variability and taxonomy of species and forms of the genus Botrytis Mich. I. Studies on culture-guided variations in the morphological properties of forms of the genus Botrytis.] Zentralblatt fuer Bakteriologie, Parasitenkunde. Abt. 2. **120,** 141-178. Cited in Review of Applied Mycology **46,** 899 (1967).

Meyer, J.A., Garber, E.D. and Schaeffer, S.G. (1964). Genetics of phytopathogenic fungi. XII. Detection of esterases and phosphatases in culture filtrates of Fusarium oxysporum and F. xylaroides by starch gel electrophoresis. Botanical Gazette **125,** 298-300.

Meyer, J.A. and Renard, J.L. (1969). Protein and esterase patterns of two formae speciales of Fusarium oxysporum. Phytopathology **59,** 1409-1411.

Micales, J.A., Bonde, M.R. and Peterson, G.L. (1986). The use of isozyme analysis in fungal taxonomy and genetics. Mycotaxon **27,** 405-449.

Milton, J.M., Rogers, W.G. and Isaac, I. (1971). Application of acrylamide gel electrophoresis of soluble fungal proteins to taxonomy of Verticillium species. Transactions of the British Mycological Society **56,** 61-65.

Moorhouse, J. and De Bertoldi, H. (1975). Electrophoretic characteristics of enzymes as taxonomic criteria in the genus Humicola. Mycotaxon **3,** 109-118.

Morgan, D.J. (1971a). Numerical taxonomic studies of the genus Botrytis. I. The B. cinerea complex. Transactions of the British Mycological Society **56,** 319-325.

Morgan, D.J. (1971b). Numerical taxonomic studies of the genus Botrytis. II. Other Botrytis taxa. Transactions of the British Mycological Society **56,** 327-335.

Morre, D.J. (1968). Cell wall dissolution and enzyme secretion during leaf abscission. Plant Physiology **43,** 1546-1559.

Moss, D.W. (1982). Isoenzymes. Chapman and Hall, London, New York.

Nasuno, S. (1974). Further evidence on differentiation of Aspergillus sojae and Aspergillus oryzae by electrophoretic patterns of cellulase, pectin lyase and acid proteinase. Canadian Journal of Microbiology **20,** 413-416.

Nealson, K.H. and Garber, E.D. (1967). An electrophoretic survey of esterases, phosphatases and leucine amino peptidases in mycelial extracts of species of Aspergillus. Mycologia **59,** 330-336.

Neate, S.M. and Cruickshank, R.H. (1988). Pectic enzyme patterns of Ceratobasidium spp. and Rhizoctonia associated with sharp eye spot-like lesions on cereals in South Australia. Transactions of the British Mycological Society: **91:** 267-272.

Neate, S.M., Cruickshank, R.H. and Rovira, A.D. (1988). Pectic enzyme patterns of Rhizoctonia solani isolates from agricultural soils in South Australia. Transactions of the British Mycological Society **90,** 37-42.

Neate, S.M. and Warcup, J.H. (1985). Anastomosis grouping of some isolates of Thanatephorus cucumeris from agricultural soils in South Australia. Transactions of the British Mycological Society **85,** 615-620.

Nelson, R.R. (1965). Assessing biological relationships in the fungi. Phytopathology **55,** 823-826.

Nguyen-The, C., Bertheau, Y. and Coleno, A. (1984). [Studies on the polygalacturonase isoenzymes, endoglucanases of Rhizopus spp. and Mucor spp. and differentiation of isolates in the south-east of France.] Canadian Journal of Botany **62,** 2670-2676.

Ogoshi, A. (1985). Anastomosis and intraspecific groups of Rhizoctonia solani and binucleate Rhizoctonia. Fitopatologia Brasileira **10,** 371-390.

Ogoshi, A. (1987). Ecology and pathogenicity of anastomosis and intraspecific groups of Rhizoctonia solani Kühn. Annual Review of Phytopathology **25,** 125-143.

Ogoshi, A., Oniki, M. and Araki, T. (1983). Studies on the anastomosis groups of binucleate Rhizoctonia and their perfect stages. Journal of the Faculty of Agriculture, Hokkaido University **61,** 244-260. Cited in Review of Plant Pathology **62,** 3787 (1983).

Old, K.M., Moran, G.F. and Bell, J.C. (1984). Isozyme variability among isolates of Phytophthora cinnamomi from Australia and Papua New Guinea. Canadian Journal of Botany **62,** 2016-2022.

Oniki, M., Araki, T., Ogoshi, A., Kasai, K., Hamaya, E. and Ando, Y. (1984). [Some properties and taxonomic assessment of the tea black rot fungus.] Chagyó Gijutsu Kenkyū **66,** 7-14.

Oniki,M., Kobayashi, K., Araki, T. and Ogoshi, A. (1986). [A new disease of turf-grass caused by binucleate Rhizoctonia AG-Q.] Annals of the Phytopathological Society of Japan **52,** 850-853. Cited in Review of Plant Pathology **66,** 4335 (1987).

Oniki, M., Ogoshi, A. and Araki, T. (1986). Development of the perfect state of Rhizoctonia solani Kühn AG-1. Annals of the Phytopathological Society of Japan **52,** 169-174.

Oniki, M., Ogoshi, A., Araki, T., Sakai, R. and Tanaka, S. (1985). [The perfect state of Rhizoctonia oryzae and R. zeae and the anastomosis groups of Waitea circinata]. Transactions of the Mycological Society of Japan **26,** 189-198. Cited in Review of Applied Mycology **65,** 587 (1986).

Onions, A.H.S., Bridge, P.D. and Paterson, R.R. (1984). Problems and prospects for the taxonomy of Penicillium. Microbiological Scienes **1,** 185-189.

Ornstein, L. (1964). Disc electrophoresis. I. Background and theory. Annals of the New York Academy of Science **121,** 321-349.

Owen, J.H., Walker, J.C. and Stahmann, M.A. (1950). Variability in onion neck-rot fungi. Phytopathology **40,** 749-768.

Pape, H. and Hemer, M. (1964). [Diseases and pests of ornamentals and their control.] Paul Parey, Berlin/Hamburg, Germany. Cited by Hódosy (1964).

Parmeter, J.R. Jr., Sherwood, R.T. and Platt, W.D. (1969). Anastomosis grouping among isolates of Thanatephorus cucumeris. Phytopathology **59,** 1270-1278.

Parmeter, J.R. Jr. and Whitney, H.S. (1970). Taxonomy and nomenclature of the imperfect state. In Rhizoctonia solani, Biology and Pathology (ed. J.R. Parmeter Jr.) pp. 7-31. University of California Press, Los Angeles and London.

Paterson, R.R.M. and Hawksworth, D.L. (1985). Detection of secondary metabolites in dried cultures of Penicillium preserved in herbaria. Transactions of the British Mycological Society **85,** 95-100.

Patterson, C.L. and Grogan, R.G. (1984). Hyphal interactions among single sclerotial isolates of Sclerotinia minor. Phytopathology **74,** 834, Abstract A 361.

Paul, W.R.C. (1929). A comparative morphological and physiological study of a number of strains of Botrytis cinerea Pers., with special reference to their virulence. Transactions of the British Mycological Society **15,** 118-135. Cited in Review of Applied Mycology **8,** 528 (1929).

Penrose, L.J., Tarran, J. and Wong, A.-L. (1976). First record of Sclerotinia laxa Aderh. and Ruhl. in New South Wales: differentiation from S. fructicola (Wint.) Rehm. by cultural characteristics and electrophoresis. Australian Journal of Agricultural Research **27,** 547-556.

Pitt, J.I. (1979). The Genus Penicillium and its Teleomorphic States Eupenicillium and Talaromyces. Academic Press, London, New York.

Pitt, J.I., Cruickshank, R.H. and Leistner, L. (1986). Penicillium commune, P. camembertii, the origin of white cheese moulds and the production of cyclopiazonic acid. Food Microbiology **3**, 363-371.

Polack, F.J. and Abawi, G.S. (1975). The occurrence and biology of Botryotinia fuckeliana on beans in New York. Phytopathology **65**, 657-660.

Polonelli, L., Castognola, M., D'Urso, C. and Morace, G. (1985). Serological aproaches for identification of Aspergillus and Penicillium species. In Advances in Penicillium and Aspergillus Systematics. (ed. R.A. Samson and J.I. Pitt). pp. 267-280. Plenum Press, New York, London.

Polonelli, L., Morace, G., Rosa, R., Castagnola, M. and Frisvad, J.C. (1987). Antigenic characterisation of Penicillium camembertii and related common cheese contaminants. Applied and Environmental Microbiology **53**, 872-878.

Poulik, M.D. (1957). Starch gel electrophoresis in a discontinuous system of buffers. Nature **180**, 1477-1479.

Prakash, S., Lewontin, R.C. and Hubby, J.L. (1969). A molecular approach to the study of genetic heterozygosity in natural populations. IV. Patterns of genetic variation in central, marginal and isolated populations of Drosophila pseudoobscura. Genetics **61**, 841-858.

Raper, K.B. and Thom, C.A. (1949). A Manual of the Penicillia. The Williams and Wilkins Co., Baltimore, Md., U.S.A.

Reddy, M.N. (1973). Taxonomic applications of isozyme electrophoresis in Neurospora. Bulletin of the Torrey Botanical Club **100,** 304-308.

Reddy, M.N. and Stahmann, M.A. (1972). Isozyme patterns of Fusarium species and their significance in taxonomy. Phygytopathologische Zeitschrift **74,** 115-124.

Reeve, R.M. (1959). Histological and histochemical changes in developing and ripening peaches. II. The cell wall and pectins. American Journal of Botany **46,** 241-248.

Rexová-Benková, L. and Markovič, O. (1974). Pectic enzymes. Advances in Carbohydrate Chemistry **33,** 323-381.

Reynolds, M., Weinhold, A.R. and Morris, T.J. (1983). Comparison of anastomosis groups of Rhizoctonia solani by polyacrylamide gel electrophoresis of soluble proteins. Phytopathology **73,** 903-906.

Ribéreau-Gayon, J., Ribéreau-Gayon, P. and Seguin, G. (1980). Botrytis cinerea in enology. In The Biology of Botrytis. (ed. J.R. Coley-Smith, K. Verhoeff and W.R. Jarvis.) pp. 251-274. Academic Press, London.

Richards, E.G. (1965). Disc electrophoresis of ribonucleic acid in polyacrylamide gel. Analytical Biochemistry **12,** 452-471.

Richmond, D.V. and Pring, R.J. (1980). A comparison between isolates of Botrytis cinerea sensitive and insensitive to benzimidazole fungicides. Annals of Applied Biology **94,** 306-308.

Richter, H. and Schneider, R. (1953). [Studies on the morphological and biological differentiation of Rhizoctonia solani K.] Phytopathologische Zeitschrift **20,** 167-226. Cited in Review of Applied Mycology **33,** 109 (1954).

Ried, J.L. and Collmer, A. (1985). Activity stain for rapid characterization of pectic enzymes in isoelectric focussing and sodium dodecyl sulfate-polyacrylamide gels. Applied and Environmental Microbiology **50,** 615-622.

Roberts, F.A. and Sivasithamparam, K. (1986). Identity and pathogenicity of Rhizoctonia spp. associated with bare patch disease of cereals at a field site in Western Australia. Netherlands Journal of Plant Pathology **92,** 185-195.

Roeb, L. (1974). [A new technique for the in situ determination of enzymes in fungal cultures, demonstrated on polygalacturonases.] Phytopathologische Zeitschrift **79,** 359-363.

Roeb, L. and Stegemann, H. (1975). Pectinmethylesterases: isozymes, properties and detection in polyacrylamide gels. Biochemie und Physiologie der Pflanzen **168,** 607-615.

Ryker, T.C. and Gooch, F.S. (1938). Rhizoctonia sheath spot of rice. Phytopathology **28,** 233-246. Cited in Review of Applied Mycology **17,** 622 (1938).

Sampson, K. and Western, J.H. (1954). Diseases of British Grasses and Herbage Legumes. University Press, London, U.K.

Samson, R.A., Stolk, A.C. and Hadlock, R. (1976). Revision of the subsection Fasciculata of Penicillium and some allied species. Studies in Mycology, Baarn **11,** 1-47.

Scala, F., Cristinzio, G., Marziano, F. and Noviello, C. (1981). Endopolygalacturonase zymograms of Fusarium species. Transactions of the British Mycological Society **77**, 587-591.

Schecter, Y., La ndau, J.W. and Dabrowa, N. (1972). Comparative electrophoresis and numerical taxonomy of some Candida species. Mycologia **64**, 841-853.

Schuster, A. and Jung, J.A. (1981). [Identification of mycelium of wood destroying fungi.] Phytopathologische Zeitschrift **100**, 19-28.

Selvaraj, J.C. and Meyer, J.A. (1974). Electrophoretic protein and enzyme patterns and antigenic structure in Verticillium dahliae and V. alboatrum. Mycopathologia et Mycologia Applicata **54**, 549-558.

Seviour, R.J., Pethica, L.M., Soddell, J.A. and Pitt, D.E. (1985). Electrophoretic patterns of sporangiospore proteins of Rhizopus isolates as taxonomic characters. Transactions of the British Mycological Society **84**, 701-708.

Shidla, L.A. (1972). [Fungi of genus Botrytis Micheli in Lithuania. III. Comparative morphological study.] Lietuvos Tsr Mokslu Akademijos Darbai Serija B. **3**, 31-40. Cited in Biological Abstracts **57**, 35769 (1974).

Shields, C.R., Orton, T.J. and Stuber, C.W. (1983). An outline of general resource needs and procedures for the electrophoretic separation of active enzymes from plant tissues. In Isozymes in Plant Genetics and Breeding, Part A. (ed. S.D. Tanksley and T.J. Orton). pp. 443-516. Elsevier Scientific Publishers, Amsterdam, The Netherlands.

Shipton, W.A. and Fleischmann, G. (1969). Taxonomic significance of protein patterns of rust species and formae speciales, obtained by disc electrophoresis. Canadian Journal of Botany **47,** 1351-1358.

Siemaszko, W. (1929). [Grey mould of onion - Botrytis allii Munn.] Roczniki Nauk Rolniczych i Leśnych **21,** 12. Cited in Review of Applied Mycology **9,** 82 (1930).

Silow, R.A. (1934). A systemic disease of red clover caused by Botrytis anthophila Bond. Transactions of the British Mycrological Society **18,** 239-248.

Smith, A.K. and Circle, S.J. (1938). Peptization of soybean proteins - Extraction of nitrogenous constituents from oil-free meal by acids and bases with and without added salts. Industrial and Engineering Chemistry **30,** 1414-1418.

Snider, R.D. and Kramer, C.L. (1974). An electrophoretic protein analysis and numerical taxonomic study of the genus Taphrina. Mycologia **66,** 754-772.

Söderström, B. and Frisvad, J.C. (1984). Separation of closely related asymmetric Penicillia by pyrolysis gas chromatography and mycotoxin production. Mycologia **76,** 408-419.

Sorenson, W.G., Larsh, H.W. and Hamp, S. (1971). Acrylamide gel electrophoresis of proteins from Aspergillus species. American Journal of Botany **58,** 588-593.

Spek, J. (1965). [Botrytis cinerea, a parasite of flax.] Verslagen van landbowkundige onderzoekingen van rijkslandbowroefstations **651,** 1-146. Cited in Review of Applied Mycology **44,** 3370 (1965).

Stegemann, H. (1967). [Enzyme electrophoresis in inclusive-polymerized acrylamide. B. Polygalacturonases (Pectinases).] Hoppe-Seyler's Zeitschrift füer Physiologische Chemie **348,** 951-952.

Steinberg, R.A. (1950). Growth on synthetic nutrient solutions of some fungi pathogenic to tobacco. American Journal of Botany **37,** 711-714.

Sterling, C. (1970). Crystal-structure of ruthenium red and stereochemistry of its pectin stain. American Journal of Botany **57,** 172-175.

Stipes, R.J. (1970). Comparative mycelial protein and enzyme patterns in four species of Ceratocystis. Mycologia **62,** 987-995.

Stipes, R.J., Emert, G.H. and Brown, R.D. Jr. (1982). Differentiation of Endothia gyrosa and Endothia parasitica by disc electrophoresis of intramycelial enzymes and proteins. Mycologia **74,** 138-141.

Stolk, A.C. and Samson, R.A. (1985). A new taxonomic scheme for Penicillium anamorphs. In Advances in Penicillium and Aspergillus Systematics. (ed. R.A. Samson and J.I. Pitt). pp. 163-192. Plenum Press, New York, London.

Stout, D.L. and Shaw, C.R. (1973). Comparative enzyme patterns in Thamnidium elegans and T. anomalum. Mycologia **65,** 803-808.

Stout, D.L. and Shaw, C.R. (1974). Genetic distance among certain species of Mucor. Mycologia **66,** 969-977.

Sweetingham, M.W., Cruickshank, R.H. and Wong, D.H. (1986). Pectic zymograms and taxonomy and pathogenicity of the Ceratobasidiaceae. Transactions of the British Mycological Society **86,** 305-311.

Tu, C.C. and Kimbrough, J.W. (1978). Systematics and phylogeny of fungi in the Rhizoctonia complex. Botanical Gazette **139,** 454-466.

Voorhees, R.K. (1934). Sclerotial rot of corn caused by Rhizoctonia zeae n. sp. Phytopathology **24,** 1290-1303.

Wade, G.C. and Cruickshank, R.H. (1978). Initiation of latent infections of apricots with Monilinia fructicola and factors influencing their subsequent development. 3rd International Congress of Plant Pathology (Munich), 1978. Abstract 044.

Walker, J.C. and Minter, D.W. (1981). Taxonomy of Nematogonium, Gonatobotrys, Gonatobotryum and Gonatorrhodiella. Transactions of the British Mycological Society **77,** 299-319.

Warcup, J.H. and Talbot, P.H.B. (1962). Ecology and identity of mycelia isolated from soil. Transactions of the British Mycological Society **45,** 495-518.

Webster, A.M. (1945). Webster's New International Dictionary of the English Language. (ed. W.A. Neilson). G. and C. Merriam Co., Springfield, Mass., U.S.A.

Whetzel, H.H. (1945). A synopsis of the genera and species of the Sclerotiniaceae, a family of stromatric inoperculate Discomycetes. Mycologia **37,** 648-714.

Windham, A.S. and Lucas, L.T. (1984). Formation of the perfect state of Rhizoctonia zeae in culture. Phytopathology **74,** 634, Abstract.

Wong, A.L. and Willetts, H.J. (1975). Electrophoretic studies of Australasian, North American and European isolates of Sclerotinia sclerotiorum and related species. Journal of general Microbiology **90,** 355-359.

Zamir, D. and Chet, I. (1985). Application of enzyme electrophoresis for the identification of isolates in Trichoderma harzianum. Canadian Journal of Microbiology **31,** 578-580.

Zuber, M. and Manibhushanrao, K. (1982). Studies on comparative gel electrophoretic patterns of proteins and enzymes from isolates of Rhizoctonia solani causing sheath blight disease of rice. Canadian Journal of Microbiology **28,** 762-771.

XI PUBLICATIONS ON OTHER TOPICS

Cruickshank, R.H. (1953). Chromosome numbers in the genus Pimelia. Papers and Proceedings of the Royal Society of Tasmania **87**, 13-16.

Line, M.A. and Cruickshank, R.H. (1979). Soft-rot fungi from copper-chrome-arsenic treated hardwood transmission poles in Tasmania. International Biodeterioration Bulletin **15**, 113-118.

Marshall, K.C. and Cruickshank, R.H. (1973). Cell surface hydrophobicity and the orientation of certain bacteria at interfaces. Archiv für Mikrobiologie **91**, 29-40.

Marshall, K.C., Cruickshank, R.H. and Bushby, H.V.A. (1975). The orientation of certain root-nodule bacteria at interfaces, including legume root-hair surfaces. Journal of General Microbiology **91**, 198-200.

Wade, G.C. and Cruickshank, R.H. (1978). Initiation of latent infections of apricots with Monilinia fructicola and factors influencing their subsequent development. International Congress of Plant Pathology (Munich) Abstract 044.

XII APPENDICES

Appendix

Appendix

23 Identity of Rhizoctonia cultures from J. Walker, N.S.W.

24 Identify of Rhizoctonia cultures from M.J. Christensen, New Zealand

25 Publications appended.

APPENDIX 1

Culture media for the production of pectic enzymes from *Botrytis* spp.

"P,$(NH_4)_2SO_4$" - Pectin, ammonium sulphate medium:

$(NH_4)_2SO_4$	2.0 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.1 g
Oxoid yeast extract	0.5 g
Citrus pectin	10.0 g
Distilled water	1 litre
pH to 4.0 by 1 N NaOH	

"SP" - Sucrose, pectin medium:

NH_4NO_3	2.0 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.1 g
Oxoid yeast extract	0.5 g
Sucrose	5.0 g
Citrus pectin	5.0 g
Distilled water	1 litre
pH to 4.0 by 1 N NaOH	

APPENDIX 1

continued

"GT" - Glucose, ammonium tartrate medium:

Ammonium tartrate	2.0 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.1 g
Oxoid yeast extract	0.5 g
Glucose	5.0 g
Distilled water	1 litre
pH 5.5	

"PT" - Pectin, ammonium tartrate medium:

As for "GT" medium, substituting pectin (10 g/l) in place of glucose.

"S6" - Malate buffered sucrose medium, pH 6:

Distilled water	1 litre
NaOH	1.0 g
DL-malic acid	1.51 g
NH_4NO_3	2.0 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.1 g
Oxoid yeast extract	0.5 g
Sucrose	10.0 g
pH to 6.0 by malic acid or NaOH	

APPENDIX 1

continued

"P6" - Malate buffered pectin medium, pH 6:

As for "S6" medium, substituting pectin in place of sucrose.

"P4" - Malate buffered pectin medium, pH 4:

Distilled water	1 litre
NaOH	1.0 g
DL-malic acid	3.0 g
NH_4NO_3	2.0 g
KH_2PO_4	1.0 g
$MgSO_v4.7H_2O$	0.1 g
Oxoid yeast extract	0.5 g

pH to 4.0 by malic acid or NaOH

"Maltose" medium:

Distilled water	1 litre
Ammonium tartrate	2.0 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.1 g
Oxoid yeast extract	0.5 g
Citric acid monohydrate	2.0 g
Maltose	10.0 g

pH to 5.5 by KOH

APPENDIX 2

Czapek medium

Sucrose	30.0 g
$NaNO_3$	3.0 g
K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4$	0.01 g
Distilled water	1 litre

APPENDIX 3

Culture medium for the production of pectic enzymes from *Penicillium* spp.

$NH_4H_2PO_4$	0.9 g
$(NH_4)_2HPO_4$	2.0 g
$MgSO_4.7H_2O$	0.1 g
KCl	0.5 g
Citrus pectin	10.0 g
Distilled water	1 litre
pH 6.0	

[Cruickshank and Pitt (1987a)]

APPENDIX 4

Culture medium for the production of pectin enzymes from *Rhizoctonia* isolates

$(NH_4)_2SO_4$	2.64 g
K_2HPO_4	0.34 g
$MgSO_4.7H_2O$	0.14 g
Citrus pectin	10.0 g
Distilled water	1 litre
pH to 5.5 by 1 N NaOH	

[Sweetingham, Cruickshank and Wong (1986)]

APPENDIX 5

Potato decoction

Culture medium for general use in the production of amylase and ribonuclease:

100 g peeled potato tuber (*Solanum tuberosum*) cut into slices about 1 cm thick, is boiled for 30 min in 250 ml water, then strained through cheesecloth. The liquor is diluted to 1 litre.

APPENDIX 6

Buffers used in electrophoresis

System at pH 8.7:

Gel buffer:

tris	4.598 g
citric and monohydrate	0.525 g
deionized water	1 litre

Electrophoresis tank buffer:

boric acid	7.22 g
sodium tetraborate.$10H_2O$	15.75 g
deionized water	1 litre

System at pH 6.5

Gel buffer:

tris	2.5 g
citric acid monohydrate	2.3 g
deionized water	1 litre

Electrophoresis tank buffer:

Na_2HPO_4	4.2 g
KH_2PO_4	13.0 g
deionized water	1 litre

APPENDIX 7

Acrylamide gel composition

(for one 26-well gel)

10.25% acrylamide gel

Gel buffer	30 ml
acrylamide	3.0 g
bis-acrylamide	0.075 g
TEMED	30 µl
ammonium persulphate	0.03 g

15% acrylamide gel

Gel buffer	25 ml
acrylamide	3.656 g
bis-acrylamide	0.094 g
TEMED	25 µl
ammonium persulphate	0.025 g

Components:

Acrylamide:

BDH Electran, specially purified for electrophoresis. Product 44299.

Bis-acrylamide:

N,N'-methylene-bis-acrylamide, Bio-Rad electrophoresis purity reagent, Catalogue 161-0201.

TEMED:

N,N,N',N',-tetramethylethylenediamine Bio-Rad electrophoresis purity reagent, Catalogue 161-0801.

Ammonium persulphate:

Bio-Rad Electrophoresis purity reagent, Catalogue 161-0700.

WARNING Acrylamide is a potent neurotoxin, cumulative and absorbable through unbroken skin.

APPENDIX 8

Electrophoresis and detection of pectic enzymes

Polyacrylamide gel:

10.25% incorporating 0.1% citrus pectin

Electrophoresis buffers:

pH 8.7 system

Incubation:

1 h in 0.1 M DL-malic acid at room temperature. 150 ml per gel.

Enzyme detection:

Stain in 0.01% ruthenium red, overnight at 4°C.

Destain in deionized weater for 1 h.

Oxidize ruthenium red to the yellow brown form by soaking gels 30 min in 0.1% ammonium persulphate.

Polygalacturonase activity revealed by unstained zones.

Pectin esterase activity revealed by zones of intensified staining.

To extend the range of pectic enzymes detected to include lyases active at high pH, separate gels were incubated in 0.05 M tris-HCl buffer, pH 7.5, containing 4 mM $CaCl_2$ for 1 h prior to staining with ruthenium red.

APPENDIX 9

Electrophoresis and detection of amylase

Polyacrylamide gel:

10.25% incorporating 0.1% soluble starch brought into solution by bringing to the boil in gel buffer.

Electrophoresis buffers:

pH 8.7 system.

Incubation:

2 h in 0.1 M acetate, pH 5.5, at room temperature.

Enzyme detection:

Stain in 1.5% potassium iodide with 0.005% iodine.

Amylase activity revealed by unstained zones in a blue-black background.

After Boucher (1975).

APPENDIX 10

Electrophoresis and detection of ribonuclease

Polyacrylamide gel:

15% incorporating 0.03% Na salt of high molecular weight ribosomal RNA from wheat germ (Calbiochem product).

Electrophoresis buffers:

pH 8.7 system.

Incubation:

1 h in 0.1 M acetate, pH 4.0, at room temperature.

Enzyme detection:

Stain overnight at 4°C in 0.05% acridine orange. Destain with changes of distilled water. Ribonuclease activity revealed by unstained zones in the yellow background.

After Boucher (1975).

Use of acridine orange in place of toluidine blue, after Richards (1965).

APPENDIX 11

Electrophoresis and detection of protease

Polyacrylamide gel:

10.25% incorporating 0.05% glycinin*

Electrophoresis buffers:

pH 8.7 system

Incubation:

1 h in 0.1 M acetate buffer, pH 5.0, at room temperature.

Enzyme detection:

Stain 2 h in 0.1% crocein scarlet.

Destain with changes of distilled water.

Protease activity is revealed by unstained zones in the red background.

Method developed by the author.

Crocein scarlet staining, after Crowle and Cline (1977).

*Glycinin was extracted from de-fatted soya bean flour using a method based on that of Smith and Circle (1938).

APPENDIX 11

continued

Soya bean flour was extracted with chloroform then air dried. The de-fatted flour was suspended in saline buffer containing NaCl (1 M), Na_2HPO_4 (0.3 M), and NaH_2PO_4 (0.02 M) and extracted overnight at $4^{o}C$. Insoluble materials were removed by centrifugation. Glycinin was precipitated from the supernatant by 70% saturation with $(NH_4)_2SO_4$ and collected by centrifugation. It was then dissolved in 0.2 M NaCl at pH 7.5 by the phosphate components above at 0.03 M and 0.002 M, respectively, and filtered. The glycinin solution was dialysed against citrate-phosphate buffer at pH 6.0 (0.057 M citric acid, 0.114 M Na_2HPO_4) for 1 h, then against running tap water for 24 h. The precipitated glycinin was collected by centrifugation and was then freeze dried.

APPENDIX 12

Electrophoresis and detection of phosphatidase

Acrylamide gel:

10.25%

Electrophoresis buffers:

pH 8.7 system

Incubation and detection:

After electrophoresis the enzymes were transferred electrophoretically to a 10.25% acrylamide gel containing 0.1% soybean lecithin. The transfer was made using 25 mM Na phosphate buffer at pH 6.5 in apparatus scaled down from that described by Bittner, Kupferer and Morris (1980).

Phosphatidase activity was revealed by the appearance of milky zones of liberated fatty acids.

APPENDIX 13

Electrophoresis and detection of cellulase

Acrylamide gel:

10.25%

Electrophoresis buffer:

pH 8.7 system

Incubation and detection:

Gels were equilibrated to pH 4 in 0.1 M citrate buffer then diffusively printed to agar gels containing 0.5% carboxymethyl cellulose.

Cellulose activity was revealed by precipitation of unreacted substrate by 1% cetrimonium bromide or by staining with congo red using the method of Béguin (1983).

Enzymes that were able to degrade cellulose that had been dissolved in phosphoric acid then precipitated by dilution were detected by diffusive printing to this substrate in agar. Incubation was continued for 3-7 days when enzyme activity was evident as cleared zones in the slightly opaque gel. For the production of photogram records of these results, the residual swollen cellulose was stained with congo red.

APPENDIX 14

Electrophoresis and detection of laccase

Acrylamide gel:

10.25%

Electrophoresis buffers:

pH 6.5 system

Incubation and detection:

By steeping gels in a saturated solution of O-dianisidine in 0.1 M acetate buffer, pH 5.2, laccase activity was revealed by brown zones of oxidized O-dianisidine.

APPENDIX 15

Electrophoresis and detection of esterase

Acrylamide gel:

10.25%

Electrophoresis buffers:

pH 8.7 system

Incubation and detection:

Gels were steeped in 0.1 M acetate buffer, pH 5.0, containing 10 mg alpha-naphthyl acetate (pre-dissolved in a small volume of acetone) per 100 ml of buffer. After 30 min, enzyme activity was revealed by staining with 0.1% naphthanyl diazo blue B in distilled water. Activity was shown by the appearance of red to purple zones.

APPENDIX 16

Electrophoresis and detection of acid phosphatase

Acrylamide gel:

10.25%

Electrophoresis buffers:

pH 8.7 system

Incubation and detection:

Gels were steeped in 0.1 M acetate buffer pH 5.2 containing 20 mg Na salt of alpha-naphthyl acid phosphate, 20 mg $MgCl_2$ and 50 mg naphthanyl diazo blue B in each 100 ml. Zones of acid phosphatase activity appeared red-purple during 60 min incubation at room temperature.

APPENDIX 17

Cultures examined in the study of *Botrytis*

Culture collection codes, by whom determined, and host plants:

Botrytis aclada Fresen., 30 cultures including:

DAR 28780	J. Walker	*Allium cepa*
IMI 15276	N.A. Brown	

B. anthophila Bondarzew.

CBS 122.26	F.H. van Beyma	*Trifolium pratense*
CBS 131.35	R.A. Silow	*Trifolium pratense*

B. bifurcata Miller, Giddens & Foster

IMI 100717	A.A. Foster	Soil, Georgia, U.S.A.

B. byssoidea Walker, 3 cultures including:

CBS 104.23 (T)	J.C. Walker	*Allium cepa*
PDDCC 5601		*Allium cepa*

B. calthae Hennebert

CBS 175.63	G.L. Hennebert	*Caltha palustris*

B. cinerea Pers., 231 cultures

(see separate lists)

B. convallariae (Kleb.) Ondrej

CBS 179.63	G.L. Hennebert	*Convallaria majalis*

B. convoluta Whetzel & Drayton

CBS 285.38	F.L. Drayton	*Iris* sp.
CBS 286.38	F.L. Drayton	
CBS 426.68	G.L. Hennebert	
CBS 428.68	G.L. Hennebert	

APPENDIX 17

continued

B. cryptomeriae Kitajima		
CBS 126.51	K. Kominami	
B. elliptica (Berk.) Cooke		
IMI 145552	H.H. Glasscock	*Lilium* sp.
CBS 128.34	A.D. Cotton	*Lilium regale*
CBS 204.64		*Lilium* sp.
B. fabae Sárdiña, 13 cultures including:		
IMI 225851	J.W. Mansfield	*Vicia faba*
IMI 225852	J.W. Mansfield	*Vicia faba*
B. ficariarum Hennebert		
CBS 176.63 (T)	G.L. Hennebert	*Ficaria verna*
CBS 177.63	G.L. Hennebert	*Ficaria verna*
B. galanthina (Berk. & Br.) Sacc.		
CBS 127.37		*Galanthus nivalis*
CBS 327.78		*Leucojum vernum*
IMI 157565	J.W. Hawkins	*Galanthus nivalis*
B. gladiolorum Timm., 6 cultures including:		
CBS 144.41	A.S. Timmermans	*Gladiolus* sp.
CBS 613.72		*Freesia* sp.
B. globosa Raabe		
CBS 333.52	N.F. Buchwald	
CBS 388.52	J. Webster	
CBS 375.63	G.L. Hennebert	*Allium ursinum* (soil)

APPENDIX 17

continued

B. hyacinthi Westerd. & Beyma		
CBS 128.37		
CBS 549.79		
CBS 632.77 supplied as *Sclerotinia narcissicola* Gregory.		*Polianthes tuberosa*
B. narcissicola Keb. ex Westerd. & Beyma, 7 cultures including:		
IMI 193610	Dr. Humphreys-Jones	*Narcissus*
CBS 376.63	G.L. Hennebert	*Narcissus poeticus*
B. paeoniae Oud.		
CBS 112.47	J.H. Craigie	*Paeonia officinalis*
B. pelargonii Roeed		
CBS 497.50 (T)	H. Roeed	*Pelargonium* sp.
B. polyblastis Dowson		
CBS 287.38 (T)	P.H. Gregory	*Narcissus tazetta*
CBS 377.63	G.L. Hennebert	*Narcissus* sp.
CBS 370.79		
B. porri Buchw.		
CBS 190.26 (T)	F.H. van Beyma	*Allium porrum*
CBS 379.63	G.L. Hennebert	*Allium sativum*
CBS 346.80 supplied as *B. byssoidea*		
B. ranunculi Hennebert		
CBS 178.63 (T)	G.L. Hennebert	*Ranunculus arbortivus*
DAOM/MEE 202		

APPENDIX 17

continued

B. septospora El Helaly *et al.*		
IMI 79154 (T)	A Kilani	*Allium cepa*
B. spermophila Noble		
CBS 219.46	M. Noble	*Trifolium repens*
IMI 63227	M. Noble	*Trifolium repens*
B. sphaerosperma Buchw.		
CBS 381.63	G.L. Hennebert	*Allium triquetrum*
B. squamosa Walker		
CBS 105.23 (T)	J.C. Walker	*Allium cepa*
CBS 113.47		*Allium cepa*
CBS 383.63	G.L. Hennebert	*Allium cepa*
CBS 384.63	G.L. Hennebert	*Allium cepa*
CBS 145.54		*Allium cepa*
B. tulipae Lind., 12 cultures including:		
IMI 143945		
IMI 147187	E. Lester	*Tulipa* sp.
B. viciae Greene		
DAR 31932	B. Quirk	*Vicia atropurpurea*
DAR 32507		
Streptobotrys arisaemae Hennebert		
CBS 111.47	J.H. Craigie	*Arisaema triphyllum*
Amphobotrys ricini (Buchw.) Hennebert		
CBS 352.36	H.N. Hansen	*Euphorbia hirtula*

APPENDIX 17

continued

Verrucobotrys geranii (Seaver) Hennebert		
CBS 168.24	H.H. Whetzel	
CBS 374.63	G.L. Hennebert	*Geranium maculatum*

APPENDIX 18

Isolates representative of the pectic enzyme genotype groups in the *B. cinerea* complex

Genotype	Isolates*
1A1	CBS 179.63 *B. convallariae*
1A3	CBS 261.71 *Gonatobotryum sclerotigenum*; DAR 41980
f coffeae	IMI 100942
1A4	DAR 25722; IMI 169559; PDDCC 7659
1A5	CBS 131.28 f. sp. *lini*; DAR 41982; PDDCC 7663
1B4	PDDCC 7597
1B5	CBS 144.55; DAR 26339 (a); PDDCC 7674
2B2	CBS 810.69; DAR 33857; PDDCC 7669
2B3	CBS 497.50 *B. pelargonii*; PDDCC 7534
2B4	PDDCC 7567
2B5	DAR 27005; PDDCC 7554

*From:

CBS: Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

DAR: Biological and Chemical Research Institute, Department of Agriculture, Rydalmere, N.S.W., Australia.

IMI: Commonwealth Mycological Institute, Surrey, England, United Kingdom.

PDDCC: Plant Diseases Division of D.S.I.R., Auckland, New Zealand.

APPENDIX 19

Cultures examined in the study of *Botrytis cinerea* : frequency of isolation of pectic enzyme genotypes from hosts

Host	1A3	1A4	1A5	1B4	1B5	2B2	2B3	2B4	2B5	Total
Actinidia chinensis			4	2	1	6	5	3		21
Asphodelus fistulosus			1							1
Allium cepa	3	1		1	2		1	1		9
Allium porrum								1		1
Allium sativum			1							1
Allium vineale						1				1
Azalea sp.	1		1							2
Begonia sp.						2	1	1		4
Bergenia cordifolia			1							1
Calendula officinalis			1							1
Capsicum annuum							1	1		2
Chaenomeles sp.				1						1
Cicer arietinum		1								1
Citrus limon								1		1
Coffea sp.			1							1
Cucumis sativus			1	2		2	3	1		9
Cucurbita pepo			1							1
Cyclamen persicum	1					1			1	3
Daucus carota					1					1
Dianthus caryophyllus					1					1

APPENDIX 19

continued

Host	1B3	1A4	1A5	1B4	1B5	2B2	2B3	2B4	2B5	Total
Eucalyptus sp.			2		1	3	1			7
Fragaria cultivars		1	5			3	3	2	1	15
Fragaria vesca					1					1
Geranium sp.					1					1
Hordeum sp.	1									1
Humulus lupulus					1					1
Lactuca sativa			2		1					3
Leucojum sp.			1							1
Lilium candidum	1							1		2
Linum usitatissimum			1							1
Lupinus sp.			2							2
Lycopersicon lycopersicum				1	1	3	6	1	2	14
Malus pumila	1	1								2
Mentha piperita			2							2
Narcissus sp.			2							2
Paeonia mouta̲n	1		1							2
Pelargonium sp.							1			1
Petunia sp.		1								1
Phaseolus vulgaris		1	1				1			3
Primula malacoides			1							1
Prunus armeniaca		1								1
Prunus persica		1								1
Ranunculus sp.			1							1

APPENDIX 19

continued

Host	1A3	1A4	1A5	1B4	1B5	2B2	2B3	2B4	2B5	Total
Rubus idaeus		1								1
Rubus ursinus						1				1
Sekinia sp.			1							1
Trifolium pratense			1							1
Tulipa sp.			2	1						3
Vicia faba	1		2		3					6
Vitis sp.	6	1	6		5	46	6	7	8	85
Pieris larva			1							1
Unspecified					2			1		3
Total	16	10	47	7	22	67	29	20	13	231

APPENDIX 20

Pectic enzyme genotypes of *B. cinerea* in relation to resistance to dicarboxyimide and benzimidazole fungicides, iprodione and carbendazim, respectively

Response to fungicides*	Pectic enzyme genotypes									Total
	1A3	1A4	1A5	1B4	1B5	2B2	2B3	2B4	2B5	
D^S B^S	0	1	2	3	4	6	8	1	1	26
D^S B^R	0	1	1	2	0	12	7	3	0	26
D^L B^S	0	0	0	0	0	2	0	0	0	2
D^L B^R	0	0	0	0	0	4	10	1	0	15

*Isolates indexed for fungicide sensitivity (Beever and Brien, 1983) were provided by Dr. R.E. Beever, D.S.I.R., New Zealand.

D^S, dicarboximide-sensitive;

D^L, low-level dicarboximide resistant;

B^S, benzimidazole-sensitive;

B^R, benzimidazole-resistant.

APPENDIX 21

Cultures examined in the study of *Penicillium* subgenus *Penicillium*

		Total
P. arenicola Chalabuda	FRR 3392 T[a]	1
P. aromaticum f. *microsporum*	FRR 1362 T	1
P. atramentosum Thom	FRR 795 T; FRR 1446	2
P. aurantiocandidum Dierckx	FRR 884 T	1
P. aurantiogriseum Dierckx	FRR 971 NT	23
P. aurantiogriseum var. *neoechinulatum* Wicklow	FRR 3589 T	1
P. aurantiogriseum var. *poznoniense* Zaleski	FRR 972 T	1
P. aurantiovirens Biourge	FRR 2138 T	1
P. australicum Sopp ex van Beyma	FRR 935 T	1
P. biforme Thom	FRR 885 T	1
P. brevicompactum Dierckx	FRR 862 NT	7
P. brunneostoloniferum Abe	FRR 1363 T	1
P. brunneoviolaceum Biourge	FRR 2137 T	1
P. camembertii Thom	FRR 877 T; FRR 2160	3
P. camerunense Heim, *apud* Heim *et al.*	FRR 3401 T	1
P. canadense G. Smith	FRR 2553 T	1
P. candidum Roger *apud* Biourge	FRR 876 T	1
P. carneolutescens G. Smith	FRR 2035 T	1
P. casei var. *compactum* Abe	FRR 732a T	1
P. chlorophaeum Biourge	FRR 817 T	1
P. chrysogenum Thom	FRR 807 T	15

APPENDIX 21

continued

P. commune Thom	NRRL 890a T	35
P. conservandi Novobranova	FRR 1480 T	1
P. concentricum Samson et al.	FRR 1715 T; FRR 3217	5
P. crustosum Thom	FRR 1669 T	4
P. crustosum var. spinulosporum Sasaki	FRR 1621 T	1
P. cyclopium Westling	FRR 1888 T	1
P. digitatum (Pers. : Fr.) Sacc.	FRR 288; FRR 1313	4
P. echinulatum Raper & Thom ex Fassatiová	FRR 1151 IT; FRR 1143	4
P. expansum Link	FRR 976 NT	10
P. farinosum Novobranova	FRR 1478 T	1
P. fennelliae Stolk	FRR 521 T	1
P. flavidomarginatum Biourge	FRR 3569 T	1
P. flavoglaucum Biourge	FRR 948 T	1
P. flexuosum Dale apud Biourge	FRR 992 T	1
P. gorgonzolae Weidemann apud Biourge	FRR 857 T	1
P. granulatum Bain.	FRR 2036 NT; FRR 1386	2
P. griseofulvum Dierckx	FRR 3571 T; FRR 1414	8
P. griseofulvum var. dipodomyicola Wicklow	FRR 3580 T	1
P. griseoroseum Dierckx	FRR 820 T	2
P. hagemii Zaleski	FRR 866 T	1
P. harmonense Baghdadi	FRR 512 T	1
P. hirsutum Dierckx	FRR 2032 NT	1

APPENDIX 21

continued

P. hordei Stolk	FRR 815 T; FRR 1343	2
P. italicum Wehmer	FRR 983 NT	3
P. japonicum G. Smith	FRR 3431 T	1
P. lanoso-coeruleum Thom	FRR 888 T	1
P. lanosogriseum Thom	FRR 894 T	1
P. lanosoviride Thom	FRR 879 T	1
P. lanosum Westling	FRR 2009 T	1
P. mali Novobranova	FRR 3121	1
P. martensii Biourge	FRR 2029	1
P. meleagrinum Biourge	FRR 836; FRR 2136	2
P. notatum Westling	FRR 821 T	1
P. ochraceum Bain. var. *macrosporum* Thom	FRR 873 T	1
P. olivicolor Pitt	FRR 3572 T; FRR 870	3
P. olivinoviride Biourge	FRR 2028 T	1
P. olsonii Bain. & Sartory	FRR 432 NT; FRR 3165	2
P. palitans Westling	FRR 2033 T	1
P. palitans var. *echinoconidium* Abe	FRR 637 T	1
P. patris-mei Zaleski	FRR 3575 T	1
P. patulum Bain.	FRR 994 T	1
P. porraceum Biourge	FRR 970 T	1
P. pseudocasei Abe	FRR 3457a T	1
P. psittacinum Thom	FRR 932 T	1
P. puberulum Bain.	FRR 2040 NT	1
P. resticulosum Birkinshaw *et al.*	FRR 2021 T	1

APPENDIX 21

continued

P. roquefortii Thom	FRR 849 T	7
P. roquefortii var. punctatum Abe	FRR 1364a T	1
P. roquefortii var. viride Dattilo-Rubbo	FRR 1775 T	1
P. rubens Biourge	FRR 792 T	1
P. solitum Westling	FRR 937 T	7
P. stoloniferum Thom	FRR 859 T; FRR 3573 T	2
P. terrestre, sensu Raper & Thom	FRR 933; FRR 934	2
P. urticae Bain.	FRR 989 T	1
P. verrucosum Dierckx	FRR 965 NT; FRR 1639	6
P. verrucosum var. melanochlorum Samson et al.	FR 2152 T	1
P. viridicatum Westling	FRR 963 NT; FRR 1636	15
P. viridicyclopium Abe	FRR 1364a T	1
P. volgaense Beljakova & Mil'ko	FRR 3576 T	1
P. sp.	FRR 942; FRR 1668; FRR 3184	3
		233

[a]T, NT, IT indicate cultures ex type, neotype or isotype, respectively.

APPENDIX 22

Cultures examined in the study of the Ceratobasidiaceae

Cultures from the Western Australian wheat belt: see Sweetingham, Cruickshank and Wong (1986).

Cultures from agricultural soils in South Australia: see Neate, Cruickshank and Rovira (1988) and Neate and Cruickshank (1988).

Aquathanatephorus pendulus Tu & Kimbrough

CBS 700.82 ex type. Eichornia azurea, Panama

Ceratobasidium and binucleate Rhizoctonia spp.:

C. anceps Jackson CBS 152.32

C. angustisporum Warcup & Talbot CBS 568.83

Pterostylis mutica, S. Aust.

C. cornigerum (Bourd.) Rogers CBS 148.54

C. globisporum Warcup & Talbot CBS 569.83

Trichoglottis australiensis Qld.

C. papillatum Warcup & Talbot CBS 570.83

Sarcochilus dilatatum Qld.

APPENDIX 22

continued

Ceratobasidium and binucleate *Rhizoctonia* spp., continued:

C. stevensii (Burt.) Talbot CBS 477.82

Malus domestica, U.S.A.

R. cerealis van der Hoeven CBS 236.77 ex type

Triticum aestivum, Japan

CBS 558.77 *Secale cereale*, Germany

CBS 559.77 *Triticum* sp., Germany

R. floccosa Burgeff CBS 336.36 ex type

Myrmechis glabra

R. fraxini Castell. CBS 311.35 ex type, *Fraxinus excelsior*

R. muneratii Castell. CBS 298.37 ex type, *Erigeron canadensis*

R. pini-insignis Castell. CBS 312.35 ex type, *Pinus insignis*

R. ramicola Webster & Roberts CBS 400.51 ex type

Pittosporum tobira

AG-A	SH-6	Rice, Fukuoka
AG-Ba	C-484	Rice, Fukuoka
AG-Bb	C-348	
AG-C	54D25	
AG-D	C-57	Mat rush
AG-E	F-18	Flax, Hokkaido
AG-F	SIR-1	Sweet potato, Tokushima
AG-G	4D9	
AG-I	AV-2	*Artemisia vulgaris*, Tokyo
AG-K	AC-1	Onion, Hokkaido

APPENDIX 22

continued

CAG 1	BN 97	*Agrostis* leaf, Pennsylvania
	NBN 132	
CAG 2		
CAG 3		
CAG 4		
CAG 5	BN 37	*Cucumis* root, Georgia
CAG 6	BN 74	*Erigeron* sp.
CAG 7		
AG-E (Schneider)	CBS 340.84	*Malus* sp.
	CBS 341.84	*Fragaria* sp.
	CBS 342.84	*Rubus* sp.
	CBS 347.84	Original tester strain

Thanatephorus and multinucleate *Rhizoctonia* spp.:

R. mucoroides Bernard CBS 126.08, *Vanda tricolor* CBS 338.36, *Albugo candida*

R. versicolor Mueller & Nueesch. CBS 701.82 ex type *Lorrogloseum hirsini*

AG-1	CBS 323.84	*Lactuca sativa*
	CBS 324.84	Grass
	CBS 325.84	*Daucus carota*
	CBS 360.82	*Hydrangea hortensis*, Berlin
AG-1 (1A)	CS-2	Rice sheath
AG-1 (1B)	B-39	Sugar beet seedling

APPENDIX 22

continued

Thanatephorus and multinucleate Rhizoctonia spp., continued:

AG-1 (1C)	F-2	Sugar beet seedling
AG-2?	CBS 330.84	Calluna sp.
	CBS 331.84	Vriesia sp.
AG-2	CBS 361.82	Brassica oleracea var. saboudia, Berlin
	CBS 362.82	Brassica oleracea var. capitata, Berlin
	CBS 172.83	Calluna vulgaris
	CBS 174.83	Beta vulgaris
	CBS 326.84	Fragaria sp.
	CBS 327.84	Lilium sp.
	CBS 328.84	Brassica campestris
AG-2-1	TG 1	Tulip leaf, Japan
	PS 4	
AG-2-2	C 127	Mat rush, Japan
AG-2-2 (IIIB)	C-330	Rice sheath
AG-2-2 (IV)	R1-64	Sugar beet leaf
AG-2-3	CBS 343.84	Tulipa sp.
AG-3	RS-30	
	CBS 363.82	Solanum tuberosum, Germany
	CBS 163.83	Solanum tuberosum
	CBS 168.83	Solanum tuberosum

APPENDIX 22

continued

Thanatephorus and multinucleate *Rhizoctonia* sp., continued:

AG-4	SN 1	Soil, Nagaro
	CBS 164.83	*Kalonchoë*
	CBS 171.83	*Areca* sp.
	CBS 333.84	*Capsicum frutescens*
	CBS 334.84	*Spinacia oleracea*
	CBS 335.84	*Chrysanthemum* sp.
	CBS 336.84	*Cucumis sativa*
AG-5	R 470	Potato, Japan
	GM 1	Soybean, Japan
	CM 10	
	CBS 159.83	*Lilium* sp.
	CBS 160.83	*Crocus* sp.
	CBS 161.83	*Gladiolus* sp.
	CBS 165.83	*Iris hollandica*
	CBS 337.84	*Lilium* sp.
	CBS 338.84	*Fragaria* sp.
	CBS 339.84	*Zea mays*
AG-6	A01-6	
AG-7	1535	Soil, Kagawa, Japan
	1556	Soil, Kagawa, Japan
AG-B1	CA2-1	

AG-C (Schneider) = AG-4, CBS 364.82, *Annona*, Israel

APPENDIX 22

continued

Not reacting with strains of known AG:

CBS 329.84 *Cactus* sp.

CBS 344.84 *Phaseolus vulgaris*

CBS 346.84 *Spinacia oleracea*

Waitea and *Rhizoctonia* spp. assigned to *Waitea*:

W. circinata Warcup & Talbot, CBS 180.82, Soil, New Zealand

R. oryzae Ryker & Gooch, CBS 273.38 ex type, *Oryza sativa*

CBS 472.38 *Oryza sativa*, U.S.A.

R. zeae Voorhees CBS 384.34 ex type, *Zea mays*

Of uncertain affinity:

[a]*Pseudopapulaspora kendrickii* Sharma

CBS 702.82 ex type, Soil from giner field, India

CBS 566.83

[b]*Rhizoctonia carotae* Rader, CBS 464.48 ex type *Daucus carota*

---

[a]Has characteristics of *Rhizoctonia*. Dr. J.H. Stalpers, personal communication (1985).

[b]Has clamp connections, multinucleate. Burton and Coley-Smith (1985).

APPENDIX 23

Rhizoctonia cultures from Mr. J. Walker, Department of Agriculture, Biological and Chemical Research Institute, Rydalmere, N.S.W. (BCRI).

Isolate	Host	Locality	Gave zymograms of:
DAR 33648	*Davallia* sp.	Springwood	AG-1
DAR 40062	*Solanum tuberosum* L.	Crookwell	AG-2-1
DAR 27909	*Triticum aestivum* L.	Kangiara	AG-3
DAR 40045	*Solanum tuberosum* L.	Deniliquin	AG-3
DAR 40046	*Solanum tuberosum* L.	Four Corners	AG-3
DAR 40047	*Solanum tuberosum* L.	Four Corners	AG-3
DAR 40048	*Solanum tuberosum* L.	Coleambally	AG-3
DAR 40050	*Solanum tuberosum* L.	Coleambally	AG-3
DAR 40051	*Solanum tuberosum* L.	Four Corners	AG-3
DAR 40053	*Solanum tuberosum* L.	Coleambally	AG-3
DAR 40054	*Solanum tuberosum* L.	Spring Hill	AG-3
DAR 40055	*Solanum tuberosum* L.	Spring Hill	AG-3
DAR 40056	*Solanum tuberosum* L.	Orange	AG-3
DAR 40057	*Solanum tuberosum* L.	Guyra	AG-3
DAR 40060	*Solanum tuberosum* L.	Glen Innes	AG-3
DAR 40063	*Solanum tuberosum* L.	Crookwell	AG-3
DAR 40064	*Solanum tuberosum* L.	Crookwell	AG-3
DAR 40065	*Solanum tuberosum* L.	Crookwell	AG-3
DAR 40066	*Solanum tuberosum* L.	Crookwell	AG-3
DAR 41441	*Solanum tuberosum* L.	Coleambally	AG-3

APPENDIX 23

continued

Isolate	Host	Locality	Gave zymograms of:
DAR 41443	Solanum tuberosum L.	Guyra	AG-3
DAR 41444	Solanum tuberosum L.	Guyra	AG-3
DAR 24984	Daucus carota L. var. sativa D.C.	Yanco	AG-4
DAR 34183	Gossipium hirsutum L.	Canberra	AG-4
DAR 34184	Gossipium hirsutum L.	Canberra	AG-4
DAR 37034 (a)	Melaleuca alternifolia Cheel.	Port Macquarie	AG-5
DAR 44676 (b)	Chrysanthemoides monilifera (L.) Norlindh.	Port Macquarie	AG-5
DAR 43049	Triticum aestivum L.	Wagga Wagga	CAG 1
DAR 33240	Comus nuttalliae Aud.	Rydalmere	AG-G
DAR 34185	Gossipium hirsutum L.	Canberra	ZG 1
DAR 35758 (a)	Phaseolus vulgaris L.	Bodalla	ZG 1
DAR 32172	Lupinus angustifolius L.	Binnaway	ZG 4
DAR 55102	Agrostis seed	U.S.A.	W. circinata[a]
DAR 30648 (a)	Papaver bracteata Lind.	Devonport, Tasmania	W. circinata

[a]As from W1-W3, Appendix 24.

APPENDIX 24

Rhizoctonia cultures from Dr. M.J. Christensen, D.S.I.R., Plant Diseases Division, Palmerston North, New Zealand.

Isolate	Host	Locality	Zymogram of
C3, C4 "N.Z.A."	*Lolium perenne*	Palmerston North	CAG 1
C. cornigerum			
C5	Red clover	Palmerston North	CZG 4
C6	Lucerne crown	Manawatu	CZG 5
C7	*Poa annua*	Wellington	CZG 5
C8	White clover root	Warkworth	CZG 5
C9, C17, C18	Red clover tap roots	Palmerston North	CZG 5
C10	White clover root	Palmerston North	CZG 5
C11-C14, C19, C23	*Brassica oleracea* seedlings	Canterbury	CZG 5
C15, C16	*B. oleracea* seedlings	Palmerston North	CZG 5
C20	Red clover tap root	Blenheim	CZG 4
C21	Red clover tap root	Manawatu	CZG 5
C22	Ryegrass root	Palmerston North	CZG 5

APPENDIX 24

continued

Isolate	Host	Locality	Zymogram of
T. cucumeris			
T1-T4, T9-T11	*B. oleracea* seedlings	Canterbury	AG-2-1
T5	*B. oleracea* seedling	Gore	AG-2-1
T6, T7	*B. oleracea* seedlings	Palmerston North	AG-2-3
T8	*B. oleracea* seedling	Palmerston North	AG-2-1
T12	*B. oleracea* seedling	Canterbury	AG-2-3
W. circinata			
W1	*Agrostis palustris*	Palmerston North	'*R. oryzae*', CBS 472.82
W2	*A. tenuis*	Dannevicke	'*R. oryzae*', CBS 472.82
W3	*B. oleracea*	Gore	'*R. oryzae*', CBS 472.82

APPENDIX 25

Publications appended

CRUICKSHANK, R.H. (1983). Distinction between Sclerotinia species by their pectic zymograms. Transactions of the British Mycological Society **80**: 117-119.

CRUICKSHANK, R.H. and PITT, J.I. (1987a). Identification of species in Penicillium subgenus Penicillium by enzyme electrophoresis. Mycologia **79**: 614-620.

CRUICKSHANK, R.H. and PITT, J.I. (1987b). The zymogram technique: isoenzyme patterns as an aid in Penicillium classification. Microbiological Sciences **4**: 14-17.

CRUICKSHANK, R.H. and WADE, G.C. (1980). Detection of pectic enzymes in pectin-acrylamide gels. Analytical Biochemistry **107**: 177-181.

NEATE, S.M. and CRUICKSHANK, R.H. (1988). Pectic enzyme patterns of Ceratobasidium spp. and Rhizoctonias associated with sharp eye spot-like lesions on cereals in South Australia. Transactions of the British Mycological Society: **91**: 267-272.

NEATE, S.M., CRUICKSHANK, R.H. and ROVIRA, A.D. (1988). Pectic enzyme patterns of Rhizoctonia solani isolates from agricultural soils in South Australia. Transactions of the British Mycological Society **90**: 37-42.

APPENDIX 25

continued

PITT, J.I., CRUICKSHANK, R.H. and LEISTNER, L. (1986). Penicillium commune, P. camembertii, the origin of white cheese moulds, and the production of cyclopiazonic acid. Food Microbiology 3: 363-371.

SWEETINGHAM, M.W., CRUICKSHANK, R.H. and WONG, D.H. (1986). Pectic zymograms and taxonomy and pathogenicity of the Ceratobasidiaceae. Transactions of the British Mycological Society **86**: 305-311.

ANALYTICAL BIOCHEMISTRY 107, 177–181 (1980)

Detection of Pectic Enzymes in Pectin–Acrylamide Gels

RALPH H. CRUICKSHANK AND GEORGE C. WADE

Agricultural Science Department, University of Tasmania, G.P.O. Box 252C, Hobart, Tasmania, 7001, Australia

Received January 22, 1980

A rapid, sensitive slab gel electrophoretic method for the study of pectic enzymes is described. By incorporating pectin in acrylamide gels and subsequent staining with ruthenium red, simultaneous detection of esterases, glycosidases, and lyases was possible due to their differing staining reactions. Application of the method to the study of the thermal stability of enzymes is described. Approximate isoelectric points of the enzymes could be determined from their positions in the gel.

Previously described techniques used in studies of pectic enzymes have tended to be time consuming or of low sensitivity. For example, viscometric or chemical analysis of the products of crude enzyme activity may suffer from substrate depletion by the dominant enzymes or from substrate alteration by pectin esterases (PE).[1] Conversely, PE activity may be swamped by the action of depolymerizing enzymes converting the substrate to units too small for PE action (1). Column chromatographic methods have often resulted in only partial resolution of enzyme mixtures and entailed large numbers of determinations of enzyme activity. Column isoelectric focusing has provided greatly improved resolution and concentration of enzymes but again is slow and dependent on assays of many fractions for each enzyme of interest.

Gel electrophoretic methods applicable to pectic enzyme study are available. For example, Rexová-Benková and Marcovič (2) have used electrophoresis in starch gels and Drawert and Krefft (3) have used isoelectric focusing in Bio-gel followed by application of buffered substrates and subsequent staining. Direct detection of polygalacturonase (PG) in pectic acid acrylamide gels by staining with ruthenium red or methylene blue following incubation in a cycled pH gradient was reported by Stegemann (4). In this laboratory a rapid, sensitive, and versatile acrylamide gel electrophoretic method has been developed which allows the simultaneous detection of the isozymes of PE, PG, and pectin lyase (PL), each acting independently on the same substrate. It has been successfully used to follow enzyme production in cultures under varying conditions, to determine the thermal tolerance of pectin enzymes, and for the study of fungal enzymes in infected plant tissues. The technique may be usefully applied to taxonomic studies of fungi.

[1] Abbreviations used: PE, pectin esterase; PG, polygalacturonase; PL, pectin lyase.

MATERIALS AND METHODS

Gel molds. Gel molds were made of glass and Perspex plates 82 mm square, separated by glass strips 3.5 mm thick by 4 mm wide, cemented to the glass plate at each side. Well molds each of 15-μl volume were provided by cementing 10 Perspex blocks 1.5 mm wide, 4.0 mm long, and 2.5 mm deep along a line 15 mm from the bottom edge of the Perspex plate. The plates were sealed together on three sides with lightweight vinyl-backed adhesive tape, with the lower

0003-2697/80/130177-05$02.00/0

corners being further sealed with molten dental wax.

Buffer system. The discontinuous system after Poulik (5) was used. The gels were buffered at pH 8.7, by adding tris(hydroxymethyl)aminomethane, 4.598 g, and citric acid monohydrate, 0.525 g per liter. Electrode tank buffer at pH 8.7 was composed of boric acid, 7.22 g, and sodium tetraborate decahydrate, 15.75 g per liter.

Acrylamide gel mixture. (Caution, acrylamide is highly toxic and can be absorbed through unbroken skin, Stecher (6).) Citrus pectin (0.1 g) was dissolved in 100 ml gel buffer with magnetic stirring, followed by addition of 10 g acrylamide, 0.25 *g* *N,N'*-methylenebisacrylamide and 0.1 ml *N,N,N',N'* - tetramethylethylenediamine. Just prior to pouring, 0.1 g ammonium persulphate crystals were dissolved in the mixture. Gel molds were held obliquely during initial filling to prevent air bubbles from being trapped on the well molds. The mixture was sufficient for six gels which polymerised in about 10 min. Each gel was cooled to 2°C and for use, the tape and Perspex plate were removed leaving the gel supported on the glass plate throughout subsequent treatments.

Electrophoresis. Electrophoresis was carried out at 2–4°C. One or two gels were supported on a 165 × 120-mm glass plate marked with two lines 50 mm apart to correspond with sample wells and the proposed limit of buffer front movement. This plate was supported in a 240 × 240 × 70-mm covered Perspex box by two Perspex bars 30 mm deep cemented across the tank, 7 mm above the tank floor and 25 mm from a central sealed partition having the same height as the bars. This partition formed two electrode tanks each containing 500 ml of tank buffer. A platinum wire electrode was immersed across each tank adjacent to the central partition. Electrical connection was made to the gel ends with eight-layer cheesecloth wicks soaked in the tank buffer. Power was provided by the output resistance constant power method mentioned by Schaffer and Johnson (7), using constant voltage applied to the gels and resistors in series. Resistors of 6980 Ω for one gel or 4200 Ω for two gels allowed electrophoresis at 1 W per gel when 185 V was applied. Alternatively, electrophoresis with a constant current of 12 mA per gel could be used.

Wells were filled with enzyme solution and a 1-μl spot of 0.05% bromophenol blue in gel buffer was applied to the cathodic (sample well) end of the gel. The cheesecloth wicks were applied to the gel ends and power was supplied until the dye, indicating the buffer front, migrated 50 mm beyond the sample wells. This migration took about 1 h.

Development and staining. A cut was made at the buffer front and excess gel discarded. Each gel was incubated at 25°C in 100 ml 0.1 M malic acid which caused a gradual pH change in the gel to pH 3.0 in 90 min, allowing each enzyme to act on the pectin while passing through a suitable pH range. Incubation was for 1–2 h, or extended to 16 h at a suitable pH, depending on the potency of enzymes under examination. Following a brief rinse in distilled water, the gels were stained 1/2–2 h in 0.02% ruthenium red, the former for quick visual examination and the latter to increase contrast for photography. Where lyases active at high pH were likely to occur, a companion gel was treated in the same way but with the addition of 0.002 M calcium chloride to the malic acid. After staining with ruthenium red the gels were washed with several changes of distilled water for 1 h to overnight and examined for evidence of enzyme action. PE produced zones with darker staining than in the background of unaltered pectin. PG action was evident as colorless or pale zones in the stained gel. PL produced yellow zones or cleaned zones with yellow margins when acting at low pH. This reaction was not evident with PL only active at high pH, but activity from these was shown by pale or colorless zones produced only in the presence of Ca^{2+}.

A permanent record of results was made by direct photographic printing onto high-

contrast photographic paper. The illustrations presented were made in this way and hence show negative contrast.

Enzyme preparation. Culture filtrates could be examined directly in most cases although better results were sometimes obtained, depending on enzyme activity, after either dilution with gel buffer or concentration by acetone or alcohol precipitation. Residual pectin in culture filtrates tended to spoil the resolution obtained in the gels and this effect could be reduced by using 0.1% pectin in place of the customary 1.0% in culture media.

Infected plant tissues were blended at −20°C in 2 ml acetone for 1 g of tissue, centrifuged, then enzymes were extracted from the precipitate by soaking 2–16 h at 4°C in saline acetate buffer (NaCl, 4.68 g, Na-acetate trihydrate, 2.64 g, Na-azide, 0.1 g/liter, adjusted to pH 5.0 with acetic acid). This buffer gave good results in a study of *Monilinia fructicola* enzymes in infected apricot fruit but is not necessarily optimal in all cases. Following centrifugation at 10,000*g*, 10 min, 4°C, enzymes were precipitated from the supernatant with 2 vol of chilled acetone, collected by centrifugation under the conditions above, then dissolved in a suitable small volume of gel buffer for electrophoresis.

Variations in the technique were used for special purposes. For example, substrate preference of enzymes could be demonstrated by comparing the results obtained using pectin or Na-polypectate in the gels. The effect of pH on the activity of each enzyme could be demonstrated by comparison of gel strips, each containing the same resolved enzyme mixture, after equilibrating for 11/2 h at 0°C in test tubes containing 20 ml of buffers ranging in pH from 1–10 followed by incubation at 25°C for a suitable time, then staining with ruthenium red. The action of enzyme activators and inhibitors could be demonstrated in a similar fashion.

Using the heat treatment method after Archer and Fielding (8) the thermal tolerance

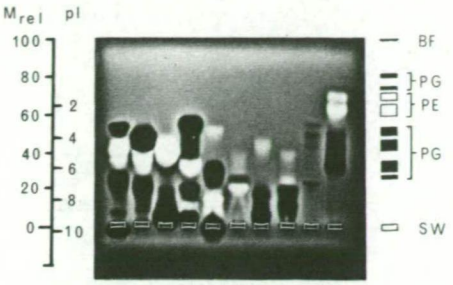
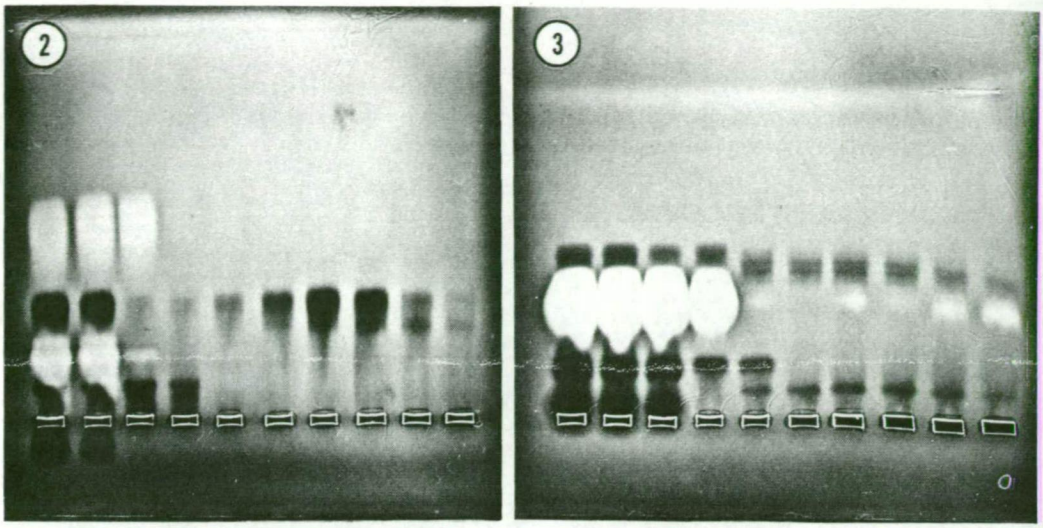


FIG. 1. Contact photographic print (in negative contrast) showing pectic enzyme activities of culture filtrates from 10 fungi. (From the left) *Monilinia fructicola, M. laxa, Sclerotinia sclerotiorum, S. minor, Botrytis cinerea, B. gladiolorum, B. fabae, B. allii, Penicillium atrovenetum, Aspergillus niger*. An interpretative drawing of *A. niger* activity is given on the right. PG, polygalacturonase; PE, pectin esterase; SW, sample well; BF, buffer front. Scale: M_{rel}, mobility relative to that of bromophenol blue as 100; p*I*, approximate isoelectric point. Culture filtrates were tested directly from 7-day stationary cultures at 25°C in: ammonium tartrate, 10 g, KH_2PO_4, 1.0 g, $MgSO_4 \cdot 7H_2O$, 0.5 g, yeast extract, 0.5 g/liter, plus 0.1% apricot powder (alcohol-insoluble residue from mature green fruit). Gel incubated 1 h in 0.1 M malic acid at 25°C, stained 2 h, washed overnight.

of enzymes was examined by heating 0.5-ml aliquots of culture filtrate each to a specified temperature in thin-walled glass tubes for 10 min, then chilling to 0°C prior to electrophoresis. Treatment temperatures ranged from 20°C in 10°C steps to 100°C and extended to include autoclaving at 115°C.

RESULTS

Zymograms from a number of fungi were prepared, an example of which is shown in Fig. 1. The scale shows mobility relative to that of bromophenol blue as 100 (M_{rel}), and adjacent to this, the approximate isoelectric point (p*I*) of the enzymes. This correlation was obtained from comparison of M_{rel} values from this technique, with p*I* values reported for *Monilinia fructicola* and *Monilinia laxa* enzymes by Willetts *et al.* (9) and similarly for p*I* values for PE of *Botrytis cinerea* reported by Drawert and Krefft (3). A linear relationship was found where $pI = 9.8 - 0.12\ M_{rel}$. The illustration shows that all isolates produced



FIGS. 2 and 3. Heat stability of pectic enzymes in culture filtrates of *Botrytis cinerea* (Fig. 2) and *Sclerotinia sclerotiorum* (Fig. 3) heat-treated prior to electrophoresis as described under Methods. Treatment temperatures (from left) 20°C, then 10°C steps to 100°C, with 115°C on extreme right. Negative contrast contact prints. (These illustrations were prepared prior to standardizing on a 50-mm bromophenol blue migration, Fig. 2 having 57 mm, and Fig. 3, 47 mm, the position of the bromophenol blue being the top edge of Fig. 2 and marked by a cut in the upper right region in Fig. 3.)

PGs (black zones in the negative print) and that all except *Penicillium atrovenetum* produced PE isozymes (white zones on the negative print). By suitable dilution, the resolution in zones showing strong activity may be improved. Each species yields a characteristic zymogram and it is evident that *M. fructicola* can be distinguished from *M. laxa* primarily by the difference in mobilities of the PG in the region of p*I* 3–4. These enzymes are most active at pH 2–3. The zymogram of *Sclerotinia sclerotiorum* is considerably different from that of *Sclerotinia minor*. Similarly, each of the *Botrytis* spp. tested produced its own characteristic zymogram which, on testing a range of isolates of each species, was found to be bascially constant. The *P. atrovenetum* zymogram shows four PG zones each of low activity. The *Aspergillus niger* zymogram shows the presence of PE with very low p*I* and two PG bands of low activity and extremely low p*I* as well as three major zones of PG.

Figures 2 and 3 are presented to illustrate the usefulness of the technique in the study of thermal stability of pectic enzymes. The activities of *B. cinerea* enzymes after heat treatments is shown in Fig. 2. It is evident that PEs of this fungus were heat sensitive, their activities being virtually eliminated by treatment at 50°C or above. A PG which migrated toward the cathode was extremely heat labile, its activity being reduced by 30°C and eliminated by 40°C treatments. The PG which remained active after high-temperature treatment illustrates the bimodal stability behavior which has been reported by Archer and Fielding (8), in that intermediate temperatures (40–50°C) were more damaging to the enzyme than were higher temperatures (80–90°C). Figure 3 illustrates the result of the same heat treatment method on the activities of *S. sclerotiorum* enzymes. From this fungus PE was more heat tolerant, the bulk of its activity surviving 50°C and traces of activity even survived autoclaving for 10 min. The three groups of PG enzymes showed variation in stability, the slowest migrating showing bimodal behavior, being inactivated at 50°C but partially surviving higher temperatures.

DISCUSSION

The technique has proved to be very sensitive. For example, proteins were below detectable limits by Coumassie brilliant blue or amido black staining in a gel containing enzymes sufficient to exhibit strong activity as illustrated in Fig. 1. In an experiment using *M. fructicola* enzyme of the activity shown in Fig. 1, it was found that, when the incubation time was increased to 16 h in 0.1 M malic acid, PG was detectable after a dilution of 1:50 with gel buffer and PE was detectable even after a dilution of 1:500. The staining responses used to characterize enzyme types have been investigated. The increased staining produced by PE action is explainable with reference to the work of Sterling (10) who has shown that the action of this enzyme on pectin increases the number of stainable sites available to ruthenium red. PG action either hydrolyzes the pectin chains to units not receptive to staining or else to oligouronides which are more readily leached from the gel than is the undegraded pectin. The change in color of the stain from red to yellow at sites of PL activity at low pH was probably due to intermediate reaction products oxidizing ruthenium red, since continuation of enzyme activity during staining was necessary to produce the effect. This was not produced when enzyme action was inhibited by chilling to 0°C during staining. At high pH, the Ca^{2+} requirement of PL under this condition was used in their detection.

It was thought that enzymes active at high pH might be retarded in their electrophoresis at high pH by coupling to pectin. However, in one test using PE (active at high pH) from *Rhizoctonia solani*, electrophoresis was carried out both in the presence and absence of pectin, and the effect was not found to be significant.

The relationship between relative mobility and isoelectric point has not been rigorously tested and spurious results may be obtained if unusually large enzymes are encountered, when gel pore size might restrict their migration. However, the information is useful in comparing results with those in published work of others, and in designing enzyme purification methods, e.g., in choice of ampholite pH range for isoelectric focusing.

The ease of determining heat tolerance of enzymes using the technique described may be useful in enzyme purification work, e.g., treatment of *B. cinerea* enzymes at 80°C for 10 min was shown to give a high degree of purification of one group of PGs in a single step. If the PEs of *B. cinerea* were of prime interest, then it is evident from Fig. 2 that treatment at 40°C would yield a product enriched for the fast-migrating group of PE isozymes relative to the slow-migrating group, free of PG which migrates toward the cathode and with the otherwise heat stable PG largely depleted. The method may also be useful to those in the food industry interested in designing heat treatment schedules for preserved products, for example to counter fungal enzyme softening of fruit after canning, investigated by Harper *et al.* (11).

ACKNOWLEDGMENT

We wish to thank Dr. W. D. Boucher for the construction of electrophoretic equipment and practical introduction to electrophoresis.

REFERENCES

1. McCready, R. M., and Seegmiller, C. G. (1954) *Arch. Biochem. Biophys.* **50,** 440–450.
2. Rexová-Benková, L., and Markovič, O. (1974) *Advan. Carbohydrate Chem.* **33,** 323–381.
3. Drawert, F., and Krefft, M. (1978) *Phytochemistry* **17,** 887–890.
4. Stegemann, H. (1967) *Hoppe-Seyler's Z. Physiol. Chem.* **348,** 951–952.
5. Poulik, M. D. (1957) *Nature (London)* **180,** 1477–1479.
6. Stecher, P. D. (Ed.) (1968) The Merck Index, 8th ed., Merck and Co., Rahway, N. J.
7. Schaffer, H. E., and Johnson, F. M. (1973) *Anal. Biochem.* **51,** 577–583.
8. Archer, S. A., and Fielding, A. H. (1975) *J. Food Sci.* **40,** 423–424.
9. Willetts, H. J., Byrde, R. J. W., Fielding, A. H., and Wong, A. -L. (1977) *J. Gen. Microbiol.* **103,** 77–83.
10. Sterling, C. (1970) *Amer. J. Bot.* **57,** 172–175.
11. Harper, K. A., Beattie, B. B., Pitt, J. I., and Best, D. J. (1972) *J. Sci. Food Agr.* **23,** 311–320.

Trans. Br. mycol. Soc. 80 (1) 117–119 (1983) Printed in Great Britain

DISTINCTION BETWEEN *SCLEROTINIA* SPECIES BY THEIR PECTIC ZYMOGRAMS

By R. H. CRUICKSHANK

Department of Agricultural Science, University of Tasmania, G.P.O. Box 252C, Hobart, Tasmania, Australia

Zymograms of the extracellular pectic enzymes of *Sclerotina sclerotiorum*, *S. minor* and *S. trifoliorum* were prepared by electrophoresis of untreated fluid from liquid cultures. Each species gave a distinctive zymogram which allowed a rapid and non-subjective identification.

Taxonomic studies on fungi and other forms of life are often complicated by the relatively few stable characters available for comparison. Multigenic involvement and responses to environment may lead to variability within each character and result in overlap between species. A valuable aid in overcoming this difficulty is to use the greatly increased information obtainable by studies on gene complements by electrophoretic examination of the proteins and enzymes for which they code. An example of this approach is the work of Wong & Willetts (1973, 1975) which has shown that *Sclerotinia sclerotiorum* (Lib.) de Bary, *S. minor* Jagger and *S. trifoliorum* Erikss. may be distinguished by electrophoretic examination of proteins and enzymes extracted from sclerotia.

Using similar methods, Scott (1981*a*) found considerable variation between runs and that a degree of subjectivity was required in the interpretation of results from proteins and the enzymes studied with the exception of esterases. The technique used by these authors involved the production of sclerotia, extraction of proteins, protein determination and concentration adjustment prior to electrophoresis. Methods for use in diagnosis are desirably quick and simple as well as being reliable. With this in mind, the electrophoretic method of Cruickshank & Wade (1980) was applied to the extracellular pectic enzymes produced by these fungi.

MATERIALS AND METHODS

Fungi

Cultures identified by the methods of Wong & Willetts (1975) were kindly supplied by Dr J. A.-L. Wong. Apart from three isolates of *S. sclerotiorum* originating from Dr V. P. Singh, Banaras Hindu University, Varanasi, India, the isolates were from various crops and localities in Tasmania. Nine isolates of *S. sclerotiorum*, four of *S. minor*, four of *S. trifoliorum* type *a* (Willetts & Wong, 1980, p. 155) and three of *S. trifoliorum* type *b* were examined.

Enzyme production

A pectin medium buffered to pH 4 was used. In 1 l de-ionized water 1·0 g NaOH and 3·0 g DL-malic acid were dissolved, followed by 2·0 g NH_4NO_3, 1·0 g KH_2PO_4, 0·1 g $MgSO_4 . 7H_2O$ and 0·5 g Oxoid yeast extract. With vigorous mechanical stirring, 10 g citrus pectin was added slowly and dissolved. After autoclaving at 120 °C for 15 min using 100 ml containers, the medium was dispensed to sterile 86 mm diam Petri dishes, 20 ml per dish. Inoculum for each dish was a 3 mm square of mycelium cut from a potato dextrose agar (PDA) plate. After 7 days incubation at 25°, culture fluids were sampled, omitting filtration as unnecessary and subjected to electrophoresis directly or stored at −20 °C.

Electrophoresis and enzyme detection

The method of Cruickshank & Wade (1980) was used. Improved resolution was obtained when enzyme samples were added to the pectin acrylamide gels as a moist pipettable slurry with Sephadex G-150 superfine. Efficient cooling of the gel support plate was essential for linearity of the buffer front. Uniform thermal contact between the gel plate and the cooled support plate was ensured by interposing a thin layer of kerosene. The apparatus, together with the cooling water and circulation pump, was housed in a top-loading deep-freeze cabinet run at 5°.

After electrophoresis, gels were incubated in 0·1 M-DL-malic acid for 1·5 h at 25°, followed by staining overnight in 0·03% aqueous ruthenium red at 5°. Excess ruthenium red was removed by several changes of distilled water over 3–5 h at 5°. Records were made directly from the gels by contact printing under water on to high-contrast photographic paper.

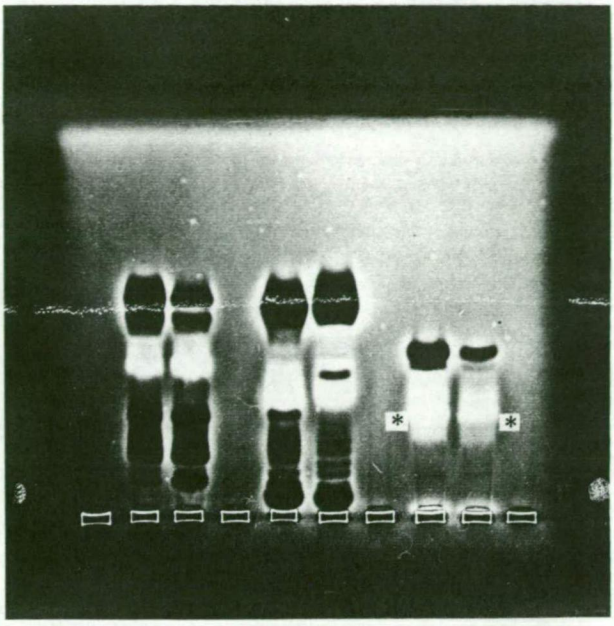


Fig. 1. Pectic zymograms of *Sclerotinia sclerotiorum* (left), *S. minor* (centre) and *S. trifoliorum* (right), from two isolates of each species, using fluids from 7-day liquid cultures. Pectin esterases, white; polygalacturonases, black; pectin lyases,*.

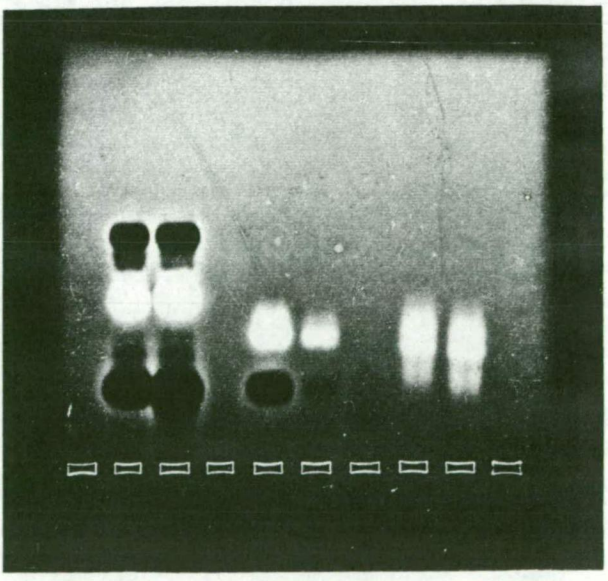


Fig. 2. As in Fig. 1, but using enzymes extracted by diffusion from 3-day PDA cultures of the species. Incubation was extended to 16 h to accentuate the result.

RESULTS AND DISCUSSION

Within each species, all isolates gave the same distinctive and easily recognizable pectic zymogram. Any variations in band intensity were attributable to physiological age. Zymograms from two isolates of each species are illustrated (Fig. 1). Pectin esterase (PE) activity is evident as white bands in the negative contrast print and polygalacturonase (PG) activity as dark bands. Note the differences between species in mobility of the PE bands and the distribution and activity of the PG bands. As a primary distinction between *S. sclerotiorum* and *S. minor*, note the strongly active PG band immediately above the origin wells from the latter. When the stained gels were stood in de-ionized water at room temperature (21°) for 2 days, pectin lyase (PL) activity was shown by all the *S. trifoliorum* isolates. This was evident as orange zones in the red-stained gels in the region marked by asterisks (Fig. 1). The other species showed no PL activity when cultured under the conditions used.

Variation with physiological age is demonstrated in the *S. minor* zymograms (centre group, Fig. 1). The left-hand zymogram was from an isolate of typical growth rate and was representative of most of the isolates of this species. The right-hand zymogram in this group was from an exceptionally fast-growing isolate, of greater physiological age at the time of sampling. When isolates of normal growth rate were sampled 3 days later (at 10 days), they gave zymograms identical to that shown from the fast-growing isolate, with fine banding of PG, marked decline in activity in the PG band at the lower edge of the PE zone and development of the PG band superimposed on the PE zone. With increasing culture age, the activity of the fast-migrating PG zone declined rapidly (absent from the fast-growing isolate by day 10). A particularly slow-growing isolate of *S. trifoliorum* yielded typical PE bands by day 7, but no PG until day 10.

Willetts & Wong (1980), using sclerotial protein evidence, distinguished two types of *S. trifoliorum*, supplied to me as types *a* and *b*. They found no differences between these types in morphology or in the enzymes they examined. The two isolates used for illustration were of type *a* and *b*, left and right respectively, showing only variation in band intensity. These differences were not consistent and the types were indistinguishable.

To shorten the time needed to obtain a result, initial trials were made using enzymes extracted by diffusion to a minimal volume of distilled water from the agar of young PDA cultures. These gave traces of activity which were accentuated when incubation was extended to 16 h prior to staining, and gave sufficient information to distinguish the species (Fig. 2).

Few isolates were examined here, but Wong & Willetts (1975) found each species to be closely homogeneous by their methods when they examined material from Australasia, Europe and North America. With this consideration and since no significant variation within species was detected here, it is reasonable to assume the results are typical for the species.

The results reported here, together with those of Scott (1981*a*, *b*) add further support to the findings of Willetts & Wong (1980) that the species are distinct. The degree of relationship between them remains indefinite. Willetts & Wong (1980) held that *S. trifoliorum* was more closely related to *S. sclerotiorum* and to *S. minor* than the latter were to each other. Scott (1981*b*), using serological methods, found a closer relationship between *S. sclerotiorum* and *S. minor* than between these two species and *S. trifoliorum*. The present work, while showing gross similarities between *S. sclerotiorium* and *S. minor*, was inconclusive in this regard. While both gave numerous PG bands, the presence of isozymes common to the species was dubious, as judged by electrophoretic mobility.

The simplicity and speed of the described methods should be useful in diagnosis in this group and in other genera. Work is in progress on *Botrytis* species, and the zymograms of the sclerotinias are clearly distinct from the characteristic zymograms of each of the 11 species of *Botrytis* so far examined.

REFERENCES

CRUICKSHANK, R. H. & WADE, G. C. (1980). Detection of pectic enzymes in pectin–acrylamide gels. *Analytical Biochemistry* **107**, 177–181.

SCOTT, S. W. (1981*a*). Separation of *Sclerotinia* isolates [illegible]ed from three herbage legume hosts. *Transactions of the British Mycological Society* **76**, 321–323.

SCOTT, S. W. (1981*b*). Serological relationships of three *Sclerotinia* species. *Transactions of the British Mycological Society* **77**, 674–676.

WILLETTS, H. J. & WONG, J. A.-L. (1980). The biology of *Sclerotinia sclerotiorum*, *S. trifoliorum* and *S. minor* with emphasis on specific nomenclature. *Botanical Review* **46**, 101–165.

WONG, A.-L. & WILLETTS, H. J. (1973). Electrophoretic studies of soluble proteins and enzymes of *Sclerotinia* species. *Transactions of the British Mycological Society* **61**, 167–178.

WONG, A.-L. & WILLETTS, H. J. (1975). Electrophoretic studies of Australasian, North American and European isolates of *Sclerotinia sclerotiorum* and related species. *Journal of General Microbiology* **90**, 355–359.

(Received for publication 9 December 1981)

Trans. Br. mycol. Soc. **86** (2), 305–311 (1986) *Printed in Great Britain*

PECTIC ZYMOGRAMS AND TAXONOMY AND PATHOGENICITY OF THE CERATOBASIDIACEAE

BY M. W. SWEETINGHAM

Department of Agriculture, Jarrah Road, South Perth, Western Australia, 6151, Australia

R. H. CRUICKSHANK

Department of Agricultural Science, University of Tasmania, G.P.O. Box 252C, Hobart, Tasmania, 7001, Australia

AND D. H. WONG

School of Agriculture, University of Western Australia, Nedlands, Western Australia, 6009, Australia

The pectic enzymes of 140 isolates of *Rhizoctonia*-like fungi from the Western Australian grainbelt were examined by electrophoresis and found to fall into 11 distinct zymogram groups (ZG). Isolates within a ZG had a similar cultural and morphological appearance and were either all multinucleate or all binucleate. Some isolates from most ZGs sporulated when transferred from potato-dextrose-marmite agar to water agar. Isolates from within a ZG had the same teleomorph. Pathogenicity of the isolates was tested against wheat and lupins. All isolates from within a ZG were consistent in the type of lesions they produced and in their virulence towards these hosts. Rhizoctonia patch disease of cereals and lupins appears to be caused by isolates from ZG 1 and ZG 2. Severe reddish-brown and brown hypocotyl rots of lupins were caused by ZG 3 and ZG 4 isolates respectively. Five *Ceratobasidium* groups (CZG), one *Waitea* group (WZG) and ZG 5 had weak to nil pathogenicity towards wheat and lupins.

The form genus *Rhizoctonia* DC. is a difficult group taxonomically because few reliable morphological characters are available to determine species. *Rhizoctonia solani* Kuhn has been distinguished on the basis of a prominent septal pore apparatus and multinucleate hyphal cells (Parmeter & Whitney, 1970; Tu & Kimbrough, 1978). The teleomorph of *R. solani* has been accepted as *Thanatephorus cucumeris* (Frank) Donk (Talbot, 1970); however, it has become clear that this is a 'collective species' (Anderson, 1982). More recently the anastomosis group concept has been used to subdivide *R. solani* into a number of distinct types which vary in pathogenicity and host specialization (Anderson, 1982). Binucleate *Rhizoctonia* spp. can also be divided into distinctive anastomosis groups (Burpee *et al.*, 1980; Ogoshi *et al.*, 1983).

Unfortunately, the techniques involved in producing teleomorphs and testing for anastomosis group are difficult and time-consuming and do not necessarily work with all field isolates.

The potential to distinguish strains of *Rhizoctonia* by the electrophoresis of extractable proteins in starch gels was demonstrated by de Beer (1965) and Clare, Flentje & Atkinson (1968). Matsuyama *et al.* (1978) found some agreement between anastomosis groups and non-specific esterase zymogram groupings. More recently, Reynolds, Weinhold & Morris (1983) found that soluble protein patterns in polyacrylamide gels could be used to distinguish five anastomosis groups.

Pectic isoenzymes have been used to quickly and reliably distinguish species within the genera *Sclerotinia* Fückel and *Botrytis* Micheli ex Pers. (Cruickshank, 1983*a*, *b*). With this in mind the pectic enzymes of *Rhizoctonia* isolates from diseased roots of wheat and lupins from the Western Australian grainbelt were examined in conjunction with their classical taxonomy and pathogenicity.

MATERIALS AND METHODS

Isolation and examination of fungi

Fungi were isolated from diseased roots of wheat (*Triticum aestivum* L.) and lupins (*Lupinus angustifolius* L.) sampled from a wide range of locations throughout the Western Australian grainbelt. Roots were washed free of soil under running tap water and pieces (approximately 5 mm) were then sequentially washed in three changes of sterile water, blotted dry and plated on to either 2% water agar containing 25 p.p.m. aureomycin HCl or the selective medium of Ko & Hora (1971). After

incubation at 25 °C for 4 days, hyphal tips of *Rhizoctonia*-like fungi were transferred to potato-dextrose agar (PDA). Cultures were maintained for 6–18 months at 5° on PDA plates sealed with thin plastic film and subcultured at three-monthly intervals.

To count the number of nuclei per cell, 1 cm^2 blocks of agar-bearing mycelium were cut from the edge of young PDA cultures and stained with either 0·5 % safranin O in 3 % KOH (Bandoni, 1979) or 0·5 % trypan blue in lactophenol (Burpee *et al.*, 1978) and examined at 400 × magnification. Isolates in which nuclei were difficult to see were examined by fluorescence microscopy using the method of Kangathoralingam & Ferguson (1984).

The diameter of the penultimate cells of the main runner hyphae was measured from 24 h PDA cultures stained with 0·5 % safranin O in 3 % KOH. Cultural pigmentation and sclerotial development were observed after 3 weeks at 25° on Czapek-Dox agar plates amended with 0·5 % Oxoid yeast extract (CDA).

The induction of basidia on 2 % water agar or Difco Cornmeal agar was attempted using the methods described by Murray (1982, 1984). Cultures on sporulation media were incubated on the laboratory bench in diffuse daylight at 15–20° and inspected regularly for the presence of fructifications for up to 30 days. Agar blocks bearing fructifications were mounted on glass slides and stained with lactophenol trypan blue and examined at 400 × magnification using interference light microscopy. The dimensions of basidiospores, sterigmata, metabasidia and the hyphae subtending the basidium were measured.

Pathogenicity tests

Preliminary pathogenicity tests were conducted on four separate occasions with three replicate pots because of the large number of isolates to be examined. In these tests a qualitative description of host reaction was recorded.

All isolates were tested for pathogenicity against wheat (cv. Gamenya) and lupins (cv. Illyarrie) using the method of McDonald & Rovira (1985). Inoculum was prepared by growing the test fungus on sterile moist white millet seed (*Panicum miliaceum* L.) for 2 weeks at 22–25°. Inoculum was mixed with pasteurized (60° for 30 min) sand at two rates: 8 and 32 colonized millet seeds per kg moist sand (2·5 %. w/w, H_2O). Control treatments received sterile millet seed. Free-draining plastic pots (7 cm diam) containing 500 g of inoculated sand were incubated in a phytotron for 14 days prior to sowing. Five seeds were sown per pot at a depth of 3 cm. The phytotron was maintained at 13–17° with a 12 h photoperiod (quantum flux density 450 $\mu E\ s^{-1}\ m^{-2}$). Pots were watered daily to free drainage. Twenty days after sowing the surviving plants were counted and examined for disease.

For a quantitative comparison of pathogenicity 45 representative isolates were tested simultaneously with four replicate pots at the higher rate of inoculum.

Root rot was assessed on the tap for lupins and on each seminal root, excluding lateral branches, for wheat. Both were assessed by two methods.

(1) *Lesion score*. Individual moist roots were placed lengthwise on a white plastic surface at right angles to markings engraved at intervals of 1 cm. The number of intervals containing a whole or portion of a lesion were counted for the top 6 cm of root. Where a root was completely severed by a lesion the missing portion was regarded as totally lesioned.

(2) *Lesion severity*. The most severe lesion on the top 6 cm of the root was recorded on a scale of 0–4. (0 = no lesion; 1 = shallow cortical necrosis; 2 = cortical necrosis as deep as the stele; 3 = cortex completely rotted and some stelar necrosis; 4 = lesion severing the root.)

Similarly, two measures of lupin hypocotyl and wheat coleoptile rot were recorded. Lesion score was assessed as for roots except for the use of 0·5 cm intervals for the first 3 cm of hypocotyl. Lesion severity was assessed by measuring the longest individual lesion lengthwise to the nearest mm.

All measurements were converted to a mean percentage disease index as described by McKinney (1923). Analysis of variance was used to test for treatment effects. Treatments yielding all zero data have no variation and were excluded from the analysis for each measurement.

Pectic enzyme production and electrophoresis

Cultures were grown for 9–10 days at 22° in loosely capped Bijoux bottles each containing 2 ml of culture medium (2·64 g $(NH_4)_2SO_4$, 0·34 g K_2HPO_4, 0·14 g $MgSO_4 . 7H_2O$, 10 g Citrus pectin, 1 l distilled water; pH adjusted to 5·5 with NaOH). Electrophoresis and enzyme detection were conducted using the method of Cruickshank & Wade (1980) with the additional details noted by Cruickshank (1983*a*).

RESULTS

The 140 isolates examined fell into 11 distinct zymogram groups coded ZG for multinucleate isolates, CZG for binucleate isolates and WZG for multinucleate isolates possessing cultural character-

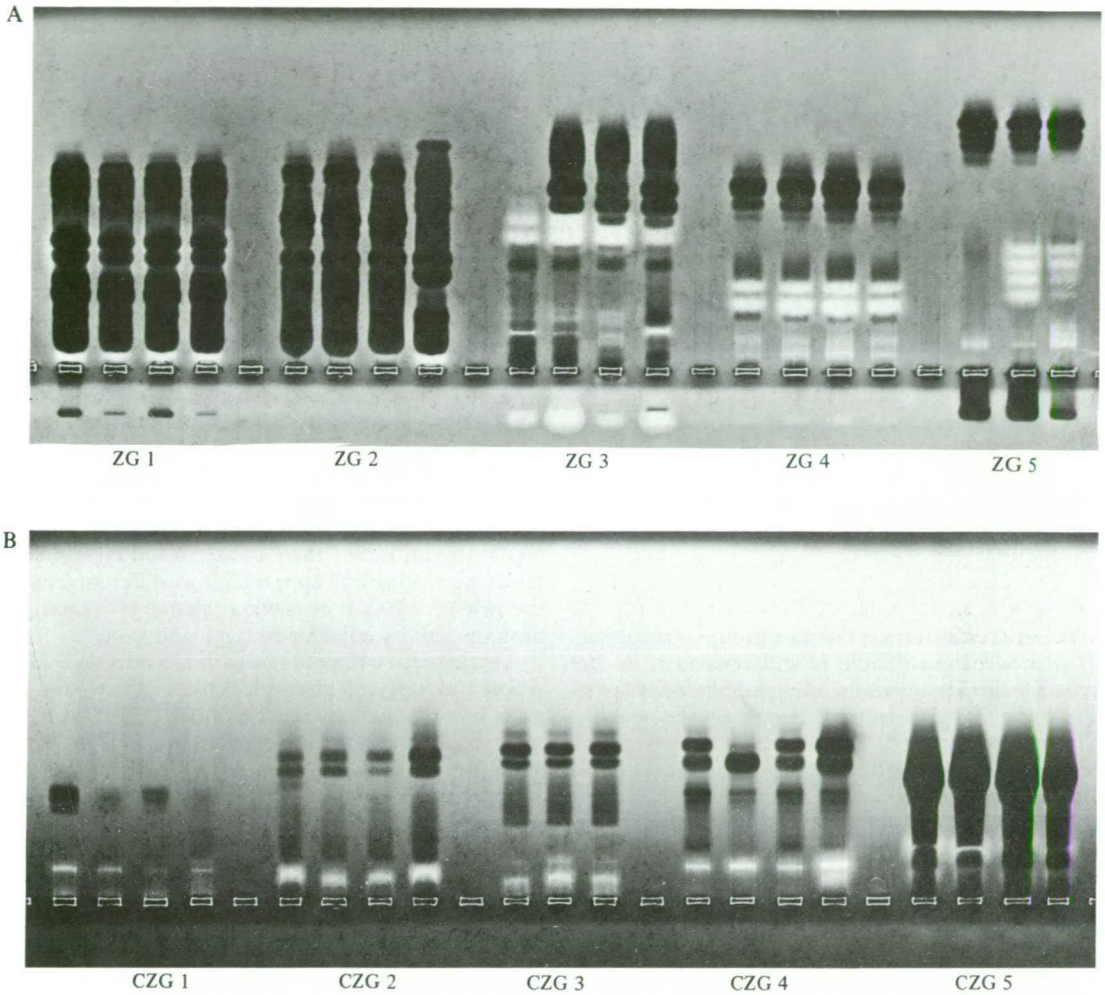
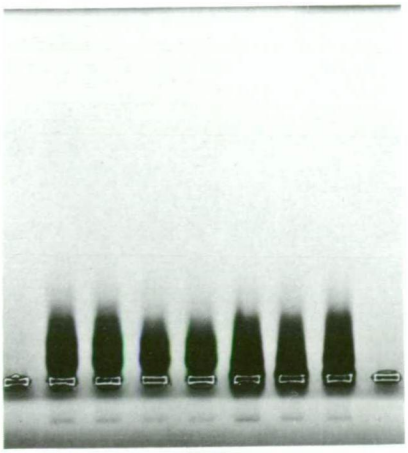


Fig. 1. Pectic zymograms of representative isolates of *Rhizoctonia* from Western Australia illustrating (A) zymogram groups of multinucleate isolates (ZG) and (B) zymogram groups of binucleate isolates (CZG). Actual size.

istics of *Waitea* Warcup & Talbot (Figs 1–2). Isolates within a particular zymogram group shared a similar appearance on CDA plates and were either all multinucleate or all binucleate (Table 1). Twenty-five isolates were induced to form basidia. Isolates from the same zymogram group produced the same teleomorph.

Isolates belonging to ZG 1, ZG 2 and ZG 5 produced medium-to dark-pigmented cultures with multinucleate cells and relatively wide hyphae, however, none produced teleomorphs. Isolates in ZG 3 and ZG 4 had a basidial morphology consistent with descriptions of *Thanatephorus* Donk (Talbot, 1970; Tu & Kimbrough, 1978) and were multinucleate. CZG 1 and CZG 3 isolates produced pale pigmented cultures with binucleate cells and relatively narrow hyphae, but did not produce teleomorphs. Isolates in CZG 2, CZG 4 and CZG 5 had basidial morphology consistent with descriptions of *Ceratobasidium* Rogers (Tu & Kimbrough, 1978) and were binucleate. All WZG 1 isolates had orange-brown sclerotia and multinucleate hyphae characteristic of *Waitea*. One isolate in WZG 1 produced basidia consistent with descriptions of *Waitea* (Warcup & Talbot, 1962; Tu & Kimbrough, 1978).



WZG 1

Fig. 2. As in Fig. 1. Zymogram group of multinucleate isolates with the characteristics of *Waitea* (WZG). Actual size.

All isolates within a zymogram group were similar in their virulence (Table 2) and consistent in the type of lesions they produced in pathogenicity tests. ZG 1 and ZG 2 isolates all caused severe root rot to both wheat and lupins, with lesions often severing the roots at the higher rate of inoculum. ZG 3 isolates produced distinctive reddish-brown lesions on lupin hypocotyls and caused significant pre-emergence damping-off. ZG 3 isolates produced brown coleoptile lesions on wheat and various degrees of leaf distortion and lesioning where leaves were trapped in the damaged coleoptiles. ZG 4 isolates all caused brown lesions on the hypocotyl and occasionally on the cotyledons of lupins, but were avirulent towards wheat. At the higher rate of inoculum ZG 4 isolates also caused pre-emergence damping-off of the lupins. Neither ZG 3 nor ZG 4 isolates produced lesions on the roots of wheat or lupins. Several ZG 4 isolates produced sclerotia on wheat roots without causing any apparent damage to the root tissue. ZG 5 isolates were very weakly pathogenic to both wheat and lupins. CZG 1 isolates were weakly pathogenic towards both wheat and lupin roots. CZG 2 and CZG 3 isolates were non-pathogenic to wheat but caused some taproot rotting on lupins. All isolates from CZG 4 and CZG 5 were considered non-pathogenic towards both wheat and lupins. WZG 1 isolates were the most variable group pathogenically, causing low to moderate levels of root rot. The zymograms of WZG 1 isolates were indistinct in that the polygalacturonase activity around the loading well was smeared, rather than a sharp band or bands. Isolates 47, 69 and 76 appear slightly different from the rest of WZG 1 by having greater polygalacturonase activity adjacent to the loading well.

A quantitative measure of pathogenicity to both wheat and lupins is given in Table 2. There was a good correlation between lesion score and lesion severity (lupin root rot $r = 0{\cdot}94$; lupin hypocotyl rot $r = 0{\cdot}99$; wheat root rot $r = 0{\cdot}95$; wheat coleoptile rot $r = 0{\cdot}99$), thus only lesion score data are presented in the table. Note that the virulence of isolate 13 was markedly lower than the other isolates in ZG 3. In preliminary tests this isolate produced lesions on both wheat coleoptiles and

Table 1. *Zymogram grouping and the morphological and cultural characteristics of 140* Rhizoctonia *isolates from Western Australia*

Zymogram group	*n*	Nuclei*	Teleomorph (no. of isolates)	Pigmentation	Sclerotia	Hyphal diam (μm)†	Basidium/ subtending hypha‡
ZG 1	24	M	Not detected	Medium–dark	–	8·0–(8·8)–12·2	
ZG 2	11	M	Not detected	Medium–dark	–	7·9–(8·2)–8·7	
ZG 3	7	M	*Thanatephorus* (3)	Pale–medium	+	8·2–(8·8)–9·4	1·3
ZG 4	15	M	*Thanatephorus* (3)	Dark	+	7·8–(9·0)–10·2	1·2
ZG 5	3	M	Not detected	Dark	+	7·8–(8·1)–8·6	
CZG 1	16	B	Not detected	Pale	–	4·8–(6·0)–7·4	
CZG 2	21	B	*Ceratobasidium* (9)	Very pale	–	5·0–(6·0)–7·2	2·4
CZG 3	3	B	Not detected	Very pale	–	6·8–(7·0)–7·2	
CZG 4	7	B	*Ceratobasidium* (5)	Pale	–	4·5–(5·9)–7·0	2·4
CZG 5	18	B	*Ceratobasidium* (4)	Pale	–	4·3–(5·5)–7·3	2·1
WZG 1	15	M	*Waitea* (1)	Pink–orange	+	5·8–(6·9)–7·8	1·0

n Number of isolates.
* M = multinucleate cells; B = binucleate cells.
† Values in parentheses are the overall mean of all isolates in the group.
‡ Ratio of the width of the metabasidium to the width of the hypha subtending it.

Table 2. *Zymogram grouping and the pathogenicity of* Rhizoctonia *isolates from Western Australia*

		Lupins			Wheat		
Zymogram group	Isolate no.	Emergence (%)	Root rot disease index (%)	Hypocotyl rot disease index (%)	Emergence (%)	Root rot disease index (%)	Coleoptile rot disease index (%)
ZG 1	37	90	65·4	0·0	85	95·4	0·0
	131	95	62·1	0·0	80	94·2	0·0
	164	95	70·8	0·0	75	88·4	0·0
	179	90	59·2	0·0	80	87·1	0·0
ZG 2	132	95	42·1	0·0	85	78·8	0·0
	134	85	54·2	0·0	90	77·9	0·0
	138	90	47·9	0·0	70	83·8	0·0
	174	90	54·6	0·0	90	84·6	0·0
ZG 3	11	10	0·4	99·8	70	0·0	72·1
	13	85	0·0	32·5	75	0·4	0·0
	16	15	2·6	91·4	90	0·0	95·4
	103	10	2·6	83·0	65	0·0	87·5
ZG 4	52	5	3·1	98·5	60	0·0	0·0
	54	15	0·6	100·0	55	2·9	8·3
	56	15	0·0	100·0	65	1·7	8·3
	87	25	0·0	100·0	65	0·4	6·7
ZG 5	38	95	15·0	5·0	80	1·2	0·0
	98	90	17·5	2·1	80	0·0	1·2
	120	85	11·7	5·4	70	3·3	0·8
CZG 1	23	95	12·9	0·0	85	8·8	0·0
	25	95	17·1	0·0	90	2·9	0·0
	79	90	2·5	0·0	85	0·8	0·0
	130	90	11·2	0·0	75	0·8	0·4
CZG 2	5	95	8·8	0·0	95	0·0	1·7
	78	95	14·6	0·0	70	0·0	0·0
	116	95	12·5	0·0	65	0·0	0·0
	155	95	2·9	0·0	75	0·0	0·0
CZG 3	6	100	20·8	0·0	75	0·0	0·0
	10	90	16·2	0·0	80	0·0	0·0
	12	100	11·7	0·0	75	0·0	9·6
CZG 4	14	90	0·8	0·0	85	0·0	0·0
	55	95	0·0	0·0	95	0·0	1·7
	121	95	7·9	0·0	90	1·7	2·1
	128	100	0·0	0·0	90	0·0	4·2
CZG 5	20	95	0·0	0·0	75	0·0	0·0
	60	90	1·7	0·0	80	0·0	0·8
	71	90	0·0	0·0	75	0·0	5·0
	96	95	0·8	0·0	95	0·0	0·0
WSG 1	65	25	39·6	0·0	60	19·2	0·0
	68	75	48·3	0·0	80	21·2	0·0
	72	100	6·7	0·0	75	1·2	0·0
	137	70	32·1	0·0	90	10·4	0·0
	47	85	33·8	0·0	80	12·5	1·2
	69	100	32·5	0·0	70	37·9	0·0
	76	80	53·3	0·0	80	22·5	0·0
Control		75	0·0	0·0	80	0·0	2·9
5% L.S.D.*		17	19·9	14·8	23	10·8	14·0

The LSD figures are Fisher's Least Significant Difference. They were calculated from analyses which included only treatments with non-zero data and may be used to compare means of these treatments.

lupin hypocotyls, with severity equal to that of all other ZG 3 isolates.

DISCUSSION

Twenty-two of the 24 isolates in ZG 1 originated from rhizoctonia patches in either wheat, lupins or barley crops. All ZG 2 isolates originated from patches in either wheat or barley. Rhizoctonia patch or bare patch disease is a well-known disease of cereals in Australia and is of increasing importance in both cereal and legume crops in Western Australia (MacNish, 1983). The disease has been attributed to a root-attacking strain of *R. solani* (Samuel & Garrett, 1932; de Beer, 1965) which produces distinct patches of stunted plants with markedly reduced root systems with characteristically 'pinched-off' brown pointed tips. The lesions produced by both ZG 1 and ZG 2 isolates in pathogenicity tests were consistent with those observed in the field. Thus it appears that rhizoctonia patch can be caused by these two, perhaps closely related, strains of *R. solani*.

ZG 3 isolates originated from lupin seedling hypocotyl lesions with a distinctive reddish-brown colour identical to those produced in the pathogenicity tests. These lesions frequently girdle the entire hypocotyl, killing the plant and reducing the stand density in lupin crops. The disease is only observed on young plants in the field and does not occur in distinct patches. A 'stem attacking strain' of *R. solani* has been reported from wheat in South Australia (de Beer, 1965). The disease symptoms on wheat in pathogenicity tests described by de Beer were very similar to those caused by ZG 3 isolates in this study. However, de Beer did not report tests of the 'stem strain' against lupins. The loss of virulence of isolate 13 during the course of this study may indicate a lack of stability in virulence of ZG 3 isolates maintained in pure culture.

ZG 4 isolates originated from either brown hypocotyl lesions on seedling lupins or from mature lupins with a collar rot extending up to 2 cm above ground level. One isolate came from a lupin cotyledon lesion similar in appearance to those produced in the pathogenicity tests.

ZG 5, *Ceratobasidium* and *Waitea* isolates originated from wheat and lupin root lesions on plants which did not appear to be suffering severely from root disease. They were also occasionally isolated from lupin hypocotyl lesions in conjunction with ZG 3 types and from bare patches in conjunction with ZG 1 or ZG 2 types. The importance of *Ceratobasidium* and *Waitea* as pathogens in the field is unknown. It would appear from their virulence in pathogenicity tests that they are likely to be of minor importance on wheat but more important on lupins. However, acting with other weak pathogens in a complex, they may be more damaging.

The use of pectic zymograms to characterize *Rhizoctonia* cultures is a relatively quick and simple procedure which gives reproducible results and provides a photographic record which can be used directly by others. It would appear that pectic zymograms are not only a useful tool taxonomically, but can also be used to predict the pathogenicity of *Rhizoctonia* isolates where their zymograms belong to known groups.

This work was supported by the Commonwealth Wheat Industry Research Council and the Reserve Bank of Australia Rural Credits Development Fund. We also wish to thank Miss V. Patroni for her excellent technical assistance and Mr J. Wallace for his assistance with the statistical analyses.

REFERENCES

ANDERSON, N. A. (1982). The genetics and pathology of *Rhizoctonia solani*. *Annual Review of Phytopathology* **20**, 329–347.

BANDONI, R. J. (1979). Safranin O as a rapid nuclear stain for fungi. *Mycologia* **71**, 873–874.

BURPEE, L. L., SANDERS, P. L., COLE, H. & KIM, S. H. (1978). A staining technique for nuclei of *Rhizoctonia solani* and related fungi. *Mycologia* **70**, 1281–1283.

BURPEE, L. L., SANDERS, P. L., COLE, H. & SHERWOOD, R. T. (1980). Anastomosis groups among isolates of *Ceratobasidium cornigerum* and related fungi. *Mycologia* **72**, 689–701.

CLARE, B. G., FLENTJE, N. T. & ATKINSON, M. R. (1968). Electrophoretic patterns of oxidoreductases and other proteins as criteria in fungal taxonomy. *Australian Journal of Biological Sciences* **21**, 275–295.

CRUICKSHANK, R. H. (1983*a*). Distinction between *Sclerotinia* species by their pectic zymograms. *Transactions of the British Mycological Society* **80**, 117–119.

CRUICKSHANK, R. H. (1983*b*). Pectic zymograms and taxonomy of *Botrytis*. *Fourth International Congress of Plant Pathology* (Melbourne), Abstract 708.

CRUICKSHANK, R. H. & WADE, G. C. (1980). Detection of pectic enzymes in pectin-acrylamide gels. *Analytical Biochemistry* **107**, 177–181.

DE BEER, J. F. (1965). Studies on the ecology of *Rhizoctonia solani* Kühn. Ph.D. Thesis, University of Adelaide.

KANGATHORALINGAM, N. & FERGUSON, M. W. (1984). A simple and rapid technique for fluorescence staining of fungal nuclei. *Current Microbiology* **10**, 99–104.

KO, W. & HORA, F. K. (1971). A selective medium for the quantitative determination of *Rhizoctonia solani* in soil. *Phytopathology* **61**, 707–710.

MCDONALD, H. J. & ROVIRA, A. D. (1985). The development of inoculation technique for *Rhizoctonia solani* and its application to screening cereal cultivars for resistance. In *Ecology and Management of Soilborne Plant Pathogens* (ed. C. A. Parker, A. D. Rovira, K. J.

Moore & P. T. W. Wong), pp. 174–176. The American Phytopathological Society. St Paul, Minnesota.

McKINNEY, H. A. (1923). Influence of soil temperature and moisture on infection of wheat seedlings by *Helminthosporium sativum*. *Journal of Agricultural Research* **26**, 195–218.

MACNISH, G. C. (1983). Rhizoctonia patch in the Western Australian grain belt. *Australasian Plant Pathology* **12**, 49–50.

MATSUYAMA, N., MOROMIZATO, Z., OGOSHI, A. & WAKIMOTO, S. (1978). Grouping *Rhizoctonia solani* Kühn with non-specific esterase zymogram. *Annals of the Phytopathological Society of Japan* **44**, 652–658.

MURRAY, D. I. L. (1982). A modified procedure for fruiting *Rhizotonia solani* on agar. *Transactions of the British Mycological Society* **79**, 129–135.

MURRAY, D. I. L. (1984). Cultural conditions influencing basidium formation in the Ceratobasidiaceae. *Australian Journal of Botany* **32**, 101–108.

OGOSHI, A., ONIKI, M., ARAKI, T. & UI, T. (1983). Studies on the anastomosis groups of binucleate *Rhizoctonia* and their perfect state. *Journal of the Faculty of Agriculture, Hokkaido University, Japan* **61**, 244–260.

PARMETER, J. R. JR & WHITNEY, H. S. (1970). Taxonomy and nomenclature of the imperfect state. In *Rhizoctonia solani: Biology and Pathology* (ed. J. R. Parmeter, Jr), pp. 7–19. Berkeley: University of California Press.

REYNOLDS, M., WEINHOLD, A. R. & MORRIS, T. J. (1983). Comparison of anastomosis groups of *Rhizoctonia solani* by polyacrylamide gel electrophoresis of soluble proteins. *Phytopathology* **73**, 903–906.

SAMUEL, G. & GARRETT, S. D. (1932). *Rhizoctonia solani* on cereals in South Australia. *Phytopathology* **22**, 827–836.

TALBOT, P. H. B. (1970). Taxonomy and nomenclature of the perfect state. In *Rhizoctonia solani: Biology and Pathology* (ed. J. R. Parmeter, Jr), pp. 20–31. Berkeley: University of California Press.

TU, C. C. & KIMBROUGH, J. W. (1978). Systematics and phylogeny of fungi in the *Rhizoctonia* complex. *Botanical Gazette* **139**, 454–466.

WARCUP, J. H. & TALBOT, P. H. B. (1962). Ecology and identity of mycelia isolated from soil. *Transactions of the British Mycological Society* **45**, 495–518.

(Received for publication 4 July 1985)

Trans. Br. mycol. Soc. **90** (1), 37–42 (1988) *Printed in Great Britain*

PECTIC ENZYME PATTERNS OF *RHIZOCTONIA SOLANI* ISOLATES FROM AGRICULTURAL SOILS IN SOUTH AUSTRALIA

BY S. M. NEATE,* R. H. CRUICKSHANK† AND A. D. ROVIRA*

* *CSIRO Division of Soils, Glen Osmond, South Australia 5064, and* † *Department of Agricultural Science, University of Tasmania, GPO Box 252C, Hobart, Tasmania*

Pectic enzymes produced by isolates of *Rhizoctonia solani* from many hosts and localities within South Australia were subjected to electrophoresis in pectin acrylamide gels. Seven different zymogram groups (ZG) were detected with the majority of isolates being ZG1-1, ZG1-2, ZG1-3 or ZG2. Representatives of these zymogram groups were found in all localities sampled. Of isolates from cereals and legumes, 92% were ZG1-1, ZG1-2, ZG1-3 or ZG2; in contrast, only 23% of isolates from soil were from these zymogram groups. Isolates in ZG1-1, ZG1-2, ZG1-3 and ZG2 were all anastomosis group AG-8, ZG5 were AG-2-1, ZG7 were AG-3 and ZG8 were AG-4. Pectic zymogram patterns are proposed as a fast and reliable method of determining anastomosis group.

Rhizoctonia solani Kühn is an important pathogen of cereals in southern Australia (Butler, 1961; Meagher, Brown & Rovira, 1978; MacNish, 1983). The 'bare patch' disease caused by *R. solani* results in serious losses to cereals in South Australia (Banyer, 1966) with an estimated yield loss in wheat in southern Australia in the years 1955–60 of 5% (McKnight, 1960). *Rhizoctonia* damage to cereals has become an even greater problem with the introduction in the last 10 years of reduced tillage methods of growing cereals (Neate, 1984; MacNish, 1985; Rovira & Venn, 1985; Weller *et al.*, 1986; Rovira, 1986).

Within wheatfields in South Australia there are at least four species of *Rhizoctonia* DC. (*Thanatephorus cucumeris* (Frank) Donk (anamorph *R. solani*), *Ceratobasidium cornigerum* (Bourd.) Rogers, *Waitea circinata* Warcup & Talbot and *Iodophanus carneus* (Pers.: Fr.) Korf (Neate, 1985)) and three anastomosis groups of *T. cucumeris* (AG-2, AG-4 and AG-8 (Neate & Warcup, 1985)). Rovira, Ogoshi & McDonald (1986), working with a limited number of highly pathogenic isolates from wheat and barley roots, reported that these isolates all belonged to a single anastomosis group, AG-8.

These different species of *Rhizoctonia* and different anastomosis groups of *T. cucumeris* show different pathogenicities (Flentje & Saksena, 1957; Neate, 1985), therefore it is necessary to know the species and anastomosis groups present in a field soil when determining inoculum potential of the soil and for selection of isolates to challenge plants when breeding for resistance. Unfortunately production of teleomorphs of Rhizoctonias is slow and often unsuccessful and determination of anastomosis groups is tedious and also often unsuccessful. Therefore other quicker more reliable means are needed for grouping Rhizoctonias.

Cruickshank (1983) has shown that the production of specific pectic isoenzymes provides a reliable and quick means of identifying species within the genus *Sclerotinia*. Pectic zymograms have also been used for identifying species and pathogenic strains of Rhizoctonias from Western Australia (Sweetingham, Cruickshank & Wong, 1986).

This study examines the pectic zymograms of a range of isolates of *R. solani* sampled from infected roots and agricultural soils in South Australia.

MATERIALS AND METHODS

Fungi

Fungi were isolated from organic matter and hyphae in soil and from plant roots. Roots were washed free of soil in tapwater, blotted dry and placed on weak Czapek-Dox + yeast agar (NDY/6) (Warcup, 1955) containing 100 μg ml^{-1} of streptomycin sulphate and 50 μg ml^{-1} of tetracycline hydrochloride. Incubation was at 10 and 20 °C and plates were inspected for emerging hyphae several times between 18 and 48 h after plating.

Soil was washed through a 710 μm sieve and the debris retained on the sieve was mixed with cooled NDY/6 agar containing antibiotics. Incubation was for 8–36 h at 10° with inspection and removal

of *Rhizoctonia*-like hyphae several times during incubation.

The 184 isolates sampled were from two groups. Twenty-nine isolates made up group (*a*) and were from 14 sites in agricultural soils in South Australia; their anastomosis groups were known (Neate & Warcup, 1985). One hundred and fifty-five isolates making up group (*b*) were sampled from soil and plants in cereal rotations from 50 locations representing most of the cereal growing areas of South Australia. The isolates were grouped according to district of origin: (1) Eyre Peninsula and West Coast, (2) Mid North and Yorke Peninsula, (3) Adelaide and Central, (4) South East and (5) Murray Mallee.

Pectic enzyme production and electrophoresis

Isolates were grown at 22° in Bijoux bottles containing 2 ml of culture medium (2·64 g $(NH_4)_2SO_4$, 0·34 g KH_2PO_4, 0·14 g $MgSO_4.7H_2O$, 10 g citrus pectin, 1 l distilled water, pH adjusted to 5·5 with NaOH) (Sweetingham *et al.*, 1986). After 10–14 d culture fluids were sampled and subjected to electrophoresis.

The electrophoretic method of Cruickshank & Wade (1980) with modifications by Cruickshank (1983) was used. Culture fluids were added to the pectin acrylamide gels as a slurry with Sephadex G-150 superfine. Electrophoresis was carried out at 5° with current kept constant at a level engendered by 8 V cm^{-1} across the gel at the start of electrophoresis. After electrophoresis the gels were incubated in 0·1 M DL-malic acid at 25° for 1·5 h and stained overnight at 5° in 0·01% ruthenium red. The stained gels were washed 1 h with distilled water at 5° and contact printed on photographic paper.

Anastomosis

Anastomosis groups were determined by opposing isolates of known and unknown anastomosis group on cellophane overlying distilled water agar and incubating at 25° for 24–48 h (Sanders, Burpee & Cole, 1978). When advancing hyphae had overlapped, intersecting hyphae were examined at 125 × magnification for anastomoses. An anastomosis was recorded only where the killing reaction and/or a pore could be seen following further investigation at 400 × magnification.

Representative isolates from zymogram groups ZG1, ZG2 and ZG5 were chosen and the frequency of anastomosis between the isolates was recorded.

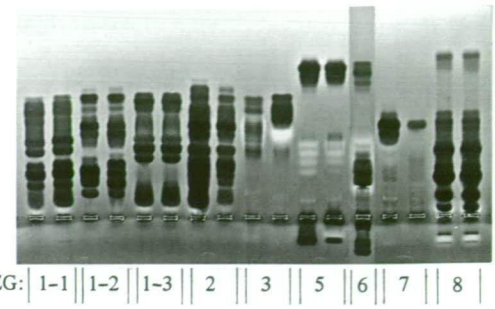


Fig. 1. Pectic zymograms from representatives of the nine zymogram groups detected in *Rhizoctonia solani* from South Australia.

RESULTS

The 184 isolates investigated from group (*a*) and group (*b*) formed nine different zymogram patterns and were placed in four different zymogram groups, ZG1, ZG2, ZG3 and ZG5 of Sweetingham *et al.* (1986). Three subgroups in ZG1 (ZG1-1, ZG1-2 and ZG1-3) and new groups ZG6, ZG7 and ZG8 were recognized (Fig. 1).

The number of isolates of each zymogram group found in different cereal growing areas of South Australia and the total number of isolations from each area are given in Table 1. The majority of isolates were ZG1-1, ZG1-2, ZG1-3, ZG2 and ZG5, with ZG1 making up over 60% of all isolates. Representatives of these groups were

Table 1. *Number of isolates in each zymogram group isolated from different cereal growing areas*

District	ZG1-1	ZG1-2	ZG1-3	ZG2	ZG3	ZG5	ZG6	ZG7	ZG8	Unassigned
Eyre Peninsula and West Coast	26	2	2	5	0	3	0	0	0	0
Mid North and Yorke Peninsula	52	0	8	13	1	9	1	0	4	3
South East	4	1	0	5	0	1	0	0	0	0
Murray Mallee	11	8	1	4	0	6	0	0	2	0
Adelaide and Central	1	2	1	1	1	4	0	2	0	0

Table 2. *Number of isolates in each zymogram group, isolated from each host*

	ZG1-1	ZG1-2	ZG1-3	ZG2	ZG3	ZG5	ZG6	ZG7	ZG8	Unassigned
Arctotheca calendula (L.) Levyas–Cape weed	1	—	—	—	—	—	—	—	—	—
Avena sativa L.–Oat	2	2	—	—	—	—	—	—	—	—
Bromus diandrus Roth–Brome grass	1	—	—	—	—	—	—	—	—	—
Hordeum leporinum Link–Barley grass	2	—	2	—	—	—	—	—	—	—
Hordeum vulgare L.–Barley	26	4	2	4	—	4	—	—	—	—
Lolium rigidum Gaudin–Ryegrass	2	—	—	—	—	1	—	—	—	—
Lupinus angustifolius L.–Lupin	—	—	—	1	—	—	—	—	—	—
Medicago sativa L.–Lucerne	—	—	—	1	—	—	—	—	—	—
Medicago sp.–Medic	3	—	—	—	—	—	—	—	—	—
Pisum sativum L.–Pea	1	—	—	—	—	—	—	—	—	—
Rapistrum sp.–Crucifer	—	—	—	—	—	1	—	—	—	—
Secale cereale L.–Rye	2	—	—	—	—	—	—	—	—	—
Solanum tuberosum L.–Potato	—	—	—	—	—	—	—	2	—	—
Trifolium sp.–Clover	2	—	—	—	—	—	—	—	—	—
Triticosecale sp.–Triticale	1	—	—	—	—	—	1	—	—	—
Triticum aestivum L.–Wheat	45	6	8	21	—	4	—	—	1	2
Vicia sativa L.–Vetch	1	—	—	—	—	2	—	—	—	—
Sclerotium	1	1	—	—	—	—	—	—	—	—
Soil organic matter	4	—	—	1	2	11	—	—	5	1

found in all areas sampled. Isolates of ZG3, ZG6, ZG7 and ZG8 were isolated too infrequently to justify conclusions about their distribution.

The number of isolations of each zymogram group from a host are given in Table 2. Most isolations were made from wheat, barley or organic matter from the soil. The majority of isolates from wheat and barley were in ZG1-1, ZG1-2, ZG1-3 and ZG2. Although there were relatively few isolates from legume roots, all isolates from clover, medic, pea and vetch were in ZG1 and the two isolates from lupin and lucerne in ZG2. In contrast, 19 of the 24 isolates from organic matter in soil were from other zymogram groups.

The zymogram groups and anastomosis groups of group (*a*) isolates are given in Table 3. Isolates in zymogram groups ZG1-1, ZG1-2, ZG1-3 and ZG2 were all AG-8, ZG5 were AG-2-1, ZG7 were AG-3 and ZG8 were AG-4.

The frequency of anastomosis between all combinations of isolates of ZG1-1, ZG1-2, ZG1-3, ZG2 and ZG5 is shown in Table 4. As expected, isolates in ZG5 (AG-2-1) did not or infrequently anastomosed with the other isolates tested (AG-8).

Table 3. *Zymogram group of group (a) cultures of known anastomosis grouping*

Isolate number	Identity	AG	Host	Location of origin	ZG	Source of isolate*
JW126	*R. solani*	AG-8	Wheat	Eyre Peninsula	ZG1-1	a
JW91	*R. solani*	AG-8	Wheat	Mallee	ZG1-1	a
770	*T. cucumeris*	AG-8	Wheat	Mid North	ZG1-1	a
803	*R. solani*	AG-8	Wheat	Mid North	ZG1-1	a
811	*R. solani*	AG-8	Wheat	Mid North	ZG1-1	a
816	*R. solani*	AG-8	Wheat	Mid North	ZG1-1	a
968	*R. solani*	AG-8	Wheat	Mid North	ZG1-1	a
976	*R. solani*	AG-8	Wheat	Mid North	ZG1-1	a
F95	*R. solani*	AG-8	Wheat	Mid North	ZG1-1	a
F97	*R. solani*	AG-8	Wheat	Mid North	ZG1-1	e
72	*T. cucumeris*	AG-8	Clover	South East	ZG1-1	a
96	*R. solani*	AG-8	Wheat	South East	ZG1-1	a
182	*R. solani*	AG-8	Sclerotium	South East	ZG1-1	a
790	*T. cucumeris*	AG-8	Wheat	Mid North	ZG2	a
F90	*R. solani*	AG-8	Wheat	Mid North	ZG2	d
F92	*R. solani*	AG-8	Wheat	Mid North	ZG2	e
F93	*T. cucumeris*	AG-8	Wheat	Mid North	ZG2	c
F94	*R. solani*	AG-8	Wheat	Mid North	ZG2	a
JW92	*R. solani*	AG-8	Wheat	South East	ZG2	a
F48	*T. cucumeris*	AG-2-1	Soil	Adelaide and Central	ZG5	c
F69	*T. cucumeris*	AG-2-1	Crucifer	Mid North	ZG5	b
JW53	*R. solani*	AG-2-1	Wheat	Mallee	ZG5	a
R63	*T. cucumeris*	AG-2-1	Wheat	Mid North	ZG5	a
F86	*R. solani*	AG-3	Potato	Adelaide and Central	ZG7	d
P12	*R. solani*	AG-3	Potato	Adelaide and Central	ZG7	a
F87	*T. cucumeris*	AG-4	Soil	Mid North	ZG8	d
F88	*T. cucumeris*	AG-4	Soil	Mid North	ZG8	a
L21	*R. solani*	AG-4	Soil	Mid North	ZG8	a
L54	*R. solani*	AG-4	Soil	Mid North	ZG8	f

* a = Neate & Warcup (1985); b = Flentje (1956); c = Flentje & Saksena (1957); d = De Beer (1965); e = Chambers (1966); f = Stretton (1971).

Table 4. *Number of anastomoses in 100 hyphal intersections*

	ZG5			ZG2			ZG1-3			ZG1-2			ZG1-1		
Isolate number...	1481	1493	1519	1491	1337	1335	1440	1461	1512	1454	1504	1509	1506	1513	1536
1536	0	0	0	—	19	14	—	9	9	10	5	12	8	27	
1513	0	1	0	22	19	30	15	19	22	13	16	14	18		
1506	0	0	0	17	24	21	21	4	21	16	24	23			
1509	1	0	0	30	22	10	22	5	21	11	24				
1504	0	0	0	20	24	17	23	21	22	20					
1454	0	0	0	27	3	20	13	26	11						
1512	0	0	0	0	12	16	27	14							
1461	0	0	2	17	26	17	19								
1440	0	0	0	33	11	12									
1335	0	0	1	0	17										
1337	0	0	0	1											
1491	1	0	0												
1519	52	60													
1493	36														
1481															

Anastomosis frequency was as variable within the AG-8 zymogram groups as between them.

DISCUSSION

Zymogram groups ZG1, ZG2, ZG3 and ZG5 have been previously described (Sweetingham *et al.*, 1986) with their ZG1 isolates all ZG1-1, however, ZG1-2, ZG1-3, ZG6 and ZG8 are new groups. In this study ZG7 (AG-3) was only isolated from potatoes and not found associated with cereals.

This work demonstrates that within anastomosis group AG-8 are at least four distinct subgroups based on electrophoresis patterns of pectic enzymes, ZG1-1, ZG1-2, ZG1-3 and ZG2, not distinguishable as subgroups by anastomosis behaviour.

Use of protein and enzyme patterns as an aid in taxonomy of fungi has been slow in acceptance probably because of the variability in patterns obtained from fungi grown under different environmental conditions and at different stages of cultural development (Hall, 1967; Seviour & Codner, 1976). *Rhizoctonia* isolates in the same anastomosis group and even single spore isolates from the same parent isolate have shown protein and isoenzyme patterns which vary markedly between isolates (Matsuyama *et al.*, 1978; Zuber & Manibhushanrao, 1982; Reynolds, Weinhold & Morris, 1983; Clare, Flentje & Atkinson, 1968). This trend is in contrast to the relatively homogeneous pectic zymogram patterns recorded in this study, despite isolates being made from many hosts and locations (Fig. 2). Also, long-term storage and subculturing of many of the 29 isolates of group (*a*) appeared to have little effect on patterns. Group (*a*) isolates which were in culture for between 5 and 32 years gave patterns similar to the freshly isolated group (*b*) isolates (Fig. 3).

Sweetingham *et al.* (1986) showed that ZG3 isolates were capable of causing stem lesions on lupins and suggested that they may be similar to the stem-attacking strain of *R. solani* reported by De Beer (1965). However, Neate & Warcup (1985) showed that the stem-attacking strain was AG-4 and in this study all AG-4 isolates were ZG8, a group not reported in the study of Sweetingham *et al.* (1986).

This study confirms the finding of Neate (1985) and Sweetingham *et al.* (1986) that isolates within AG-8 are pathogenic to cereals and are associated with the bare-patch disease of cereals. It also demonstrates that pectic zymogram patterns in pectin acrylamide gels may be a quick and reliable means of determining the anastomosis groups of isolates of *R. solani*.

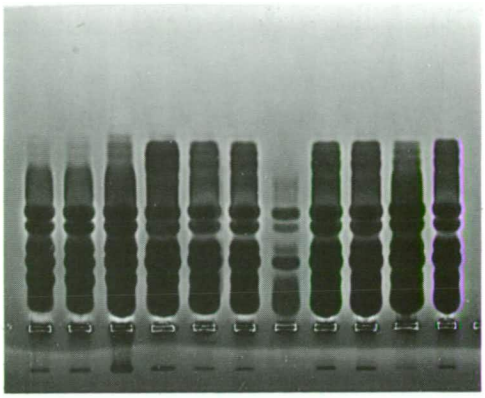
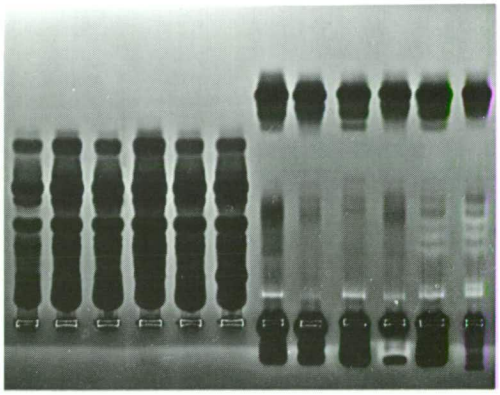


Fig. 2. Pectic zymograms from members of ZG1-1 each isolated from a different host genus and including representatives from the five districts sampled. Note the consistent production of isoenzymes.



1 2 3 4 5 6 7 8 9 10 11 12

Fig. 3. Pectic zymograms from isolates maintained in culture for up to 30 years (lanes 1–4, 7, 8) and from recently isolated cultures in ZG2 and ZG5. Note the minor variations in isoenzymes.

The authors thank Ms J. Bourne for her competent assistance, Ms H. McDonald for isolating and providing cultures from wheat from the Avon Experimental Site, Dr J. Warcup for provision of a number of cultures, and the South Australian Wheat and Barley Industry Research Committees, Wheat Research Council and Reserve Bank of Australia for funding.

REFERENCES

BANYER, R. J. (1966). Cereal diseases and their control. Part 1. *Journal of the Department of Agriculture South Australia* **69**, 310–315.

BUTLER, F. C. (1961). Root and foot rot diseases of wheat. *Scientific Bulletin Department of Agriculture New South Wales* **77**, 98 pp.

CHAMBERS, S. C. (1966). Studies on *Ophiobolus graminis* and a root attacking strain on *Rhizoctonia solani*. Ph.D. Thesis, University of Adelaide.

CLARE, B. G., FLENTJE, N. T. & ATKINSON, M. R. (1968). Electrophoretic patterns of oxidoreductases and other proteins as criteria in fungal taxonomy. *Australian Journal of Biological Sciences* **21**, 275–295.

CRUICKSHANK, R. H. (1983). Distinction between *Sclerotinia* species by their pectic zymograms. *Transactions of the British Mycological Society* **80**, 117–119.

CRUICKSHANK, R. H. & WADE, G. C. (1980). Detection of pectic enzymes in pectin-acrylamide gels. *Analytical Biochemistry* **107**, 177–181.

DE BEER, J. F. (1965). Studies on the ecology of *Rhizoctonia solani* Kühn. Ph.D. Thesis, University of Adelaide.

FLENTJE, N. T. (1956). Studies on the fungus *Pellicularia filamentosa* (Pat.) Rogers. I. Formation of the perfect stage. *Transactions of the British Mycological Society* **39**, 343–356.

FLENTJE, N. T. & SAKSENA, H. K. (1957). Studies on *Pellicularia filamentosa* (Pat.) Rogers. II. Occurrence and distribution of pathogenic strains. *Transactions of the British Mycological Society* **40**, 95–108.

HALL, R. (1967). Protein and catalase isoenzymes from *Fusarium solani* and their taxonomic significance. *Australian Journal of Biological Sciences* **20**, 419–428.

MCKNIGHT, T. (1960). Survey of present incidence, importance and causes of cereal root rots in Australia. *Proceedings of a Conference on Root Rots of Wheat, Wagga Wagga, New South Wales*, pp. 1–12.

MACNISH, G. C. (1983). Rhizoctonia patch in Western Australian grain belt. *Australasian Plant Pathology* **12**, 49–50.

MACNISH, G. C. (1985). Methods of reducing Rhizoctonia patch of cereals in Western Australia. *Plant Pathology* **34**, 175–181.

MATSUYAMA, N., MOROMIZATO, Z., OGOSHI, A. & WAKIMOTO, S. (1978). Grouping *Rhizoctonia solani* Kühn with non-specific esterase zymogram. *Annals of the Phytopathological Society of Japan* **44**, 652–658.

MEAGHER, J. W., BROWN, R. H. & ROVIRA, A. D. (1978). The effects of cereal cyst nematode (*Heterodera avenae*) and *Rhizoctonia solani* on the growth and yield of wheat. *Australian Journal of Agricultural Research* **29**, 1127–1137.

NEATE, S. M. (1984). Minimum cultivation and root diseases of wheat. Ph.D. Thesis, University of Adelaide.

NEATE, S. M. (1985). Rhizoctonias in South Australian wheat fields. In *Ecology and Management of Soil-borne Plant Pathogens* (ed. C. A. Parker, A. D. Rovira, K. J. Moore, P. T. W. Wong & J. F. Kollmorgen). St Paul, U.S.A: American Phytopathological Society.

NEATE, S. M. & WARCUP, J. H. (1985). Anastomosis grouping of some isolates of *Thanatephorus cucumeris* from agricultural soils in South Australia. *Transactions of the British Mycological Society* **85**, 615–620.

REYNOLDS, M., WEINHOLD, A. R. & MORRIS, T. J. (1983). Comparison of anastomosis groups of *Rhizoctonia solani* by polyacrylamide gel electrophoresis of soluble proteins. *Phytopathology* **73**, 903–906.

ROVIRA, A. D. (1986). Influence of crop rotation and tillage on Rhizoctonia bare patch of wheat. *Phytopathology* **76**, 669–673.

ROVIRA, A. D. & VENN, N. R. (1985). Effect of rotation and tillage on take-all and Rhizoctonia root rot of wheat. In *Ecology and Management of Soil-borne Plant Pathogens* (ed. C. A. Parker, A. D. Rovira, K. J. Moore, P. T. W. Wong & J. F. Kollmorgen). St Paul, U.S.A.: American Phytopathological Society.

ROVIRA, A. D., OGOSHI, A. & MCDONALD, H. J. (1986). Characterisation of isolates of *Rhizoctonia solani* from cereal roots in South Australia and New South Wales. *Phytopathology* **76**, 1245–1248.

SANDERS, P. L., BURPEE, L. L. & COLE, H. (1978). Preliminary studies on binucleate turfgrass pathogens that resemble *Rhizoctonia solani*. *Phytopathology* **68**, 145–148.

SEVIOUR, R. J. & CODNER, R. C. (1976). Electrophoresis of soluble proteins from light and dark grown cultures of *Cephalosporium diospyri*. *Microbios Letters* **1**, 103–108.

STRETTON, H. M. (1971). Heterokaryosis in *Thanatephorus cucumeris* (Frank) Donk. Ph.D. Thesis, University of Adelaide.

SWEETINGHAM, M. W., CRUICKSHANK, R. H. & WONG, D. H. (1986). Pectic zymograms and taxonomy and pathogenicity of the Ceratobasidiaceae. *Transactions of the British Mycological Society* **86**, 305–311.

WARCUP, J. H. (1955). Isolation of fungi from hyphae present in soil. *Nature, London* **175**, 953.

WELLER, D. M., COOK, R. J., MACNISH, G. C., BASSETT, E. N., POWELSON, R. L. & PETERSON, R. R. (1986). *Rhizoctonia* bare patch of small grains favored by reduced tillage in the Pacific Northwest. *Plant Disease* **70**, 70–73.

ZUBER, M. & MANIBHUSHANRAO, K. (1982). Studies on comparative gel electrophoretic patterns of proteins and enzymes from isolates of *Rhizoctonia solani* causing sheath blight disease in rice. *Canadian Journal of Microbiology* **28**, 762–771.

(Received for publication 12 February 1987)

Trans. Br. mycol. Soc. **91** (2), 267–272 (1988) *Printed in Great Britain*

PECTIC ENZYME PATTERNS OF *CERATOBASIDIUM* AND *RHIZOCTONIA* SPP. ASSOCIATED WITH SHARP EYESPOT-LIKE LESIONS ON CEREALS IN SOUTH AUSTRALIA

BY S. M. NEATE

CSIRO Division of Soils, Glen Osmond, South Australia 5064

AND R. H. CRUICKSHANK

Department of Agricultural Science, University of Tasmania, Hobart, Tasmania 7000, Australia

As a means of identifying groups within binucleate Rhizoctonias isolated from soil and cereal plants, extracellular pectic enzymes produced by the isolates were subjected to electrophoresis in pectin acrylamide gels. Five binucleate zymogram groups CZG1 to CZG5 were isolated from both soil and cereals. Several binucleate zymogram groups and zymogram groups within *Thanatephorus cucumeris* were isolated from sharp eyespot-like lesions on cereals but *Rhizoctonia cerealis* was not found. Pathogenicity testing showed that isolates of *Ceratobasidium cornigerum* and *T. cucumeris*, particularly AG-4 the stem strain, produced sharp eyespot-like lesions on cereal stems but they were not as pathogenic as authentic isolates of *R. cerealis* from Europe.

Although much is known of the taxonomy, pathogenicity and ecology of *Thanatephorus cucumeris* (Frank) Donk (anamorph: *Rhizoctonia solani* Kuhn) (Parmeter, 1970) there is little information about other species of Rhizoctonias such as *Ceratobasidium cornigerum* (Bourd.) Rogers which is commonly found in wheatfields (Warcup & Talbot, 1965; Neate, 1985). *Ceratobasidium cornigerum* can be a diverse species; many isolates of *C. cornigerum* studied by Warcup & Talbot (1965) showed marked variability in basidiospore shape and type of basal hyphae. This is not surprising, as using anastomosis to group *Ceratobasidium* spp. and other binucleate *Rhizoctonia*-like fungi, Ogoshi, Oniki, Sakai & Ui (1979) described 16 groups, AG-A to AG-O and Burpee, Sanders, Cole & Sherwood (1980) described 7 groups, CAG-1 to CAG-7. Ogoshi, Oniki, Araki & Ui (1983) and Oniki, Ogoshi & Araki (1982) showed that AG-A corresponded to CAG-2, AG-D to CAG-1, AG-E to CAG-3 and CAG-6 while AG-F corresponded to CAG-4; the rest of the groups were unique.

Induction of teleomorphs and determination of anastomosis groups of Rhizoctonias for their identification is slow and not always successful. Production of pectic zymogram patterns produced on pectin acrylamide gels has been used to separate groups within genera and species, of a number of fungi including Rhizoctonias (Cruickshank, 1983; Sweetingham, Cruickshank & Wong 1986; Neate, Cruickshank & Rovira, 1988). Sweetingham *et al.* (1986) showed that pectic isoenzymes quickly and reliably distinguished five groups, CZG1 to CZG5, within the genus *Ceratobasidium* Rogers from 65 binucleate isolates from Western Australia.

Although Blair (1942) in Canada and Glynne & Ritchie (1943) in England both isolated what was thought to be *R. solani* from wheat stems with sharp eyespot symptoms, it is now known that the cause of sharp eyespot disease in Europe and America is usually *Rhizoctonia cerealis* van der Hoeven (teleomorph: *Ceratobasidium cereale* Murray & Burpee; anastomosis group CAG-1 or AG-D).

The identity of the fungus causing sharp eyespot-like lesions on cereals in Australia is confused. McKnight (1960) and Banyer (1966) recorded symptoms such as lesions on the plumule, coleoptile and lower leaf sheaths of cereal plants. A stem-attacking strain of *R. solani* associated with sharp eyespot-like lesions on wheat was identified by Flentje & Saksena (1957) and De Beer (1965). The only reports of *R. cerealis* in Australia are by Wong & Sivasithamparam (1985) and Roberts & Sivasithamparam (1986) who isolated the fungus from plant roots in Western Australia. Other studies on the identity of Rhizoctonias in South Australia (Warcup & Talbot, 1965, 1966; Neate, 1984, 1985) have not found this fungus.

The aims of this study were to use gel electrophoresis of pectic enzymes to determine what

groups of binucleate *Rhizoctonia* are present on plants and in soil in cereal fields in South Australia and to test the ability of *C. cornigerum*, *R. cerealis* and the stem- and root-attacking strains of *T. cucumeris* to produce sharp eyespot-like lesions on cereals.

MATERIALS AND METHODS

Isolation

The fungi were isolated from roots, stems and organic matter in soil onto weak Czapek-Dox+-yeast agar (NDY/6, Warcup, 1955) containing 100 μg ml^{-1} of streptomycin sulphate and 50 μg ml^{-1} of tetracycline hydrochloride. Incubation was at 10 and 20 °C.

Pectic enzyme production and electrophoresis

Isolates were grown in a low-nutrient medium containing pectin and adjusted to pH 5·5, which is within the optimum range for growth of *R. solani*. After 10–14 d, culture fluid was subjected to electrophoresis at 5° with a current constant at the level needed to maintain 8 V cm^{-1} across the gel at the start of electrophoresis. After electrophoresis gels were incubated on 0·1 M DL-malic acid at 25° for 1·5 h and stained overnight at 5° in 0·01 % ruthenium red. Gels were washed 1 h in distilled water at 5° and contact printed on photographic paper (Neate *et al.*, 1988).

Pathogenicity testing

The *Rhizoctonia* isolates tested are listed in Table 1. Isolates were grown for 14 d at 25° in 50 ml of nutrient medium (0·6 g KH_2PO_4, 0·87 g K_2HPO_4, 0·133 g $MgSO_4.H_2O$, 17 g sucrose, 7 g peptone, 1 l distilled water; De Beer, 1965) in stationary 250 ml conical flasks. Hyphal mats formed on the surface of the solution were washed in three changes of distilled water and suction filtered between washes. Hyphal mats were cut into 5 mm square pieces for inoculum.

Wheat seed (*Triticum aestivum* L. cv. Condor) were surface sterilized in 1 % available chlorine solution of NaOCl for 2 min, rinsed in distilled water and germinated on moistened sterile filter paper for 36 h at 20°. Four germinated seeds were placed 2 cm below the sand surface in 250 ml plastic cups filled with sterilized, washed, fine river sand. When the wheat coleoptiles had just emerged from the sand a 5 mm square mat of inoculum was placed in the sand 5 mm from each wheat coleoptile (Fig. 1). The sand was moistened to field capacity and watered to weight every second day. Plants were grown in controlled environment at 15° with a 12 h day and a photosynthetic photon flux density of 250 μE s^{-1} m^{-2} at plant height. Experimental design was a split plot with isolates as main plot treatments and harvest date as subplot treatments with 5 replicate plots per treatment. Data

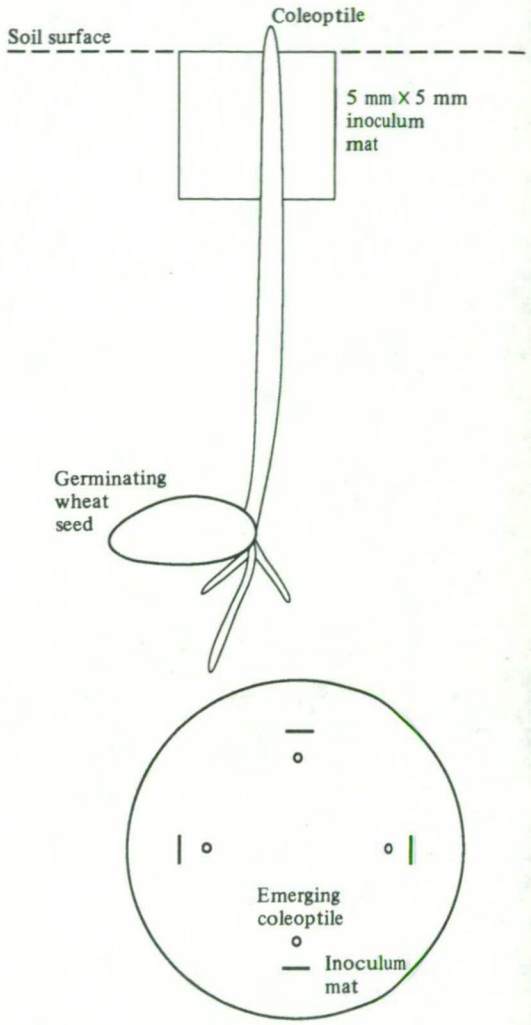


Fig. 1. Placement of inoculum mat in relation to seed and sand in the pot. (A) side view; (B) top view.

were analysed by analysis of variance after square root transformation.

Seedlings were washed free of sand after 26 or 37 d. Seedlings were rated for stem and coleoptile lesions using a modification of the scale of Pitt (1964):

	Scale
No infection	0
1 lesion 0·2–0·5 cm	1
2–3 lesions < 0·5 cm	2
1–2 lesions 0·5–1·0 cm	3
1 lesion 1·0–1·5 cm	4
1 lesion 1·5–2·0 cm	5
Coleoptile sheath rotted, lesion continuous and sometimes spreading to leaves	6

Table 1. *Isolates used in sharp eyespot pathogenicity testing*

Isolate number	Identity	Host	Location of origin*	ZG
805	*T. cucumeris*	Wheat stem	Avon	ZG1-1
806	*T. cucumeris*	Wheat stem	Avon	ZG2
521	*T. cucumeris*	Wheat root	Avon	ZG1-1
F87	*T. cucumeris*	Soil	Moonta	ZG8
F88	*T. cucumeris*	Soil	Moonta	ZG8
748	*C. cornigerum*	Wheat root	Avon	—
550	*C. cornigerum*	Wheat root	Avon	CZG5
531	*C. cornigerum*	Wheat root	Avon	CZG5
1029	*R. cerealis*	Wheat stem	Germany	—
1030	*R. cerealis*	Wheat stem	Germany	—

* Avon and Moonta are in South Australia.

Table 2. *Number of isolates in each binucleate zymogram group from different cereal growing areas*

	CZG1	CZG2	CZG3	CZG4	CZG5	Unassigned
Eyre Peninsula and West Coast	7	5	3	—	—	—
Mid North and Yorke Peninsula	1	1	1	2	16	1
South East	—	—	—	1	6	—
Mallee	1	2	7	3	—	—
Adelaide and Central	2	1	—	—	2	—
Total	11	9	11	6	24	1

Table 3. *Number of isolates of each binucleate zymogram group from different hosts*

	CZG1	CZG2	CZG3	CZG4	CZG5	Unassigned
Avena sativa L. (oat)	—	2	3	—	1	—
Hordeum leporinum Link (barley grass)	—	—	1	1	—	—
Hordeum vulgare L. (barley)	4	1	3	2	—	1
Lolium rigidum Gaudin (ryegrass)	1	—	—	—	—	—
Lupinus angustifolius L. (lupin)	—	—	—	1	2	—
Medicago sativa L. (lucerne)	—	—	—	—	2	—
Medicago sp. (medic)	—	—	1	—	3	—
Rapistrum sp. (crucifer)	—	1	1	—	—	—
Trifolium sp. (clover)	2	—	—	—	—	—
Triticum aestivum L. (wheat)	2	3	1	1	12	—
Vicia sativa L. (vetch)	—	1	—	—	—	—
Soil	2	1	1	1	4	—

The rating of 7, where plants were damped-off, was excluded as Rhizoctonias which can produce no sharp eyespot lesions can cause damping-off.

RESULTS AND DISCUSSION

Sixty-one of the 62 binucleate isolates investigated were placed in the five zymogram groups CZG1 to CZG5 previously described in Western Australia (Sweetingham *et al.*, 1986); the remaining isolate was unassigned. These results are in contrast to the findings with multinucleate zymogram groups from agricultural soils in South Australia where several new groups were found (Neate *et al.*, 1988).

The frequency of isolation of each binucleate zymogram group from the different cereal growing areas in South Australia is presented in Table 2. Isolation of Rhizoctonias from plants harvested from the major cereal growing areas showed no pattern in numbers or incidence of CAG groups within the areas. Similar results were found with multinucleate isolates (Neate *et al.*, 1988).

The number of isolates of each binucleate zymogram group isolated from a particular host are given in Table 3. There appears to be a lack of specificity of CZG group for host; this is in contrast to the results found for multinucleate isolates (Neate *et al.*, 1988). However, total numbers of isolations of binucleate isolates were

Table 4. *Number of isolations of each zymogram group from stem and root lesions of different cereals*

Lesion	Host	Multinucleate								Binucleate					
		ZG1-1	ZG1-2	ZG1-3	ZG2	ZG5	ZG6	ZG8	Un.*	CZG1	CZG2	CZG3	CZG4	CZG5	Un.*
Stem	Oat	—	—	—	—	—	—	—	—	—	—	2	—	—	—
	Barley	2	—	—	1	4	—	—	—	3	—	2	2	—	—
	Wheat	1	—	—	2	—	—	—	—	—	3	—	—	—	—
	Rye	2	—	—	—	—	—	—	—	—	—	1	—	—	—
	Triticale	—	—	—	—	—	1	—	—	—	—	—	—	—	—
Root	Oat	2	2	—	—	—	—	—	—	—	2	1	—	1	—
	Barley	2	4	2	3	—	—	—	—	1	1	1	—	—	1
	Wheat	44	6	8	19	4	—	1	2	2	—	—	1	12	—
	Rye	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Triticale	1	—	—	—	—	—	—	—	—	—	—	—	—	—

* Unassigned isolate.

low, so that further investigations are needed to confirm these observations.

The number of isolates of each zymogram group from stems and roots of cereals is given in Table 4. The 122 root isolates were in six multinucleate zymogram groups and five binucleate zymogram groups and the 26 stem isolates were in four multinucleate zymogram groups and four binucleate zymogram groups as described previously (Sweetingham *et al.*, 1986; Neate *et al.*, 1988). The numbers of binucleate and multinucleate isolates obtained from stems were equal which is in contrast to isolations from roots where there were 100 multinucleate isolates and 22 binucleate isolates.

Confusion exists about the presence of sharp eyespot disease of cereals in Australia, as the fungus which causes the disease overseas (*R. cerealis*) has never been associated with sharp eyespot-like lesions on cereals in Australia. In this study *R. cerealis* was not found, but a wide range of zymogram groups within *R. solani* and CZG groups of binucleate Rhizoctonias were isolated from sharp eyespot-like lesions on cereals (Fig. 2) and many of these fungi are also found regularly on cereal roots (Table 4). In Western Australia, Wong & Sivasithamparam (1985) isolated *R. cerealis* from diseased subterranean clover roots and Roberts & Sivasithamparam (1986) isolated the fungus from cereal roots. We have assayed the zymogram pattern of culture 128 supplied by D. Wong from subterranean clover roots and found it to match the pattern of authentic cultures of *R. cerealis* from Europe. However, Sweetingham *et al.* (1986) failed to isolate *R. cerealis* from cereal roots sampled from the same cropping areas of Western Australia from which Wong & Sivasithamparam (1985) and Roberts & Sivasithamparam (1986) made their isolations. Many attempts to isolate the fungus in South Australia have also been unsuccessful. More work needs to be done on the ecology and pathogenicity of the *R. cerealis* found in Western Australia to determine why the fungus is not associated with the disease under field conditions.

Results are presented in Table 5 of pathogenicity testing of the isolates of *T. cucumeris*, *C. cornigerum* and *R. cerealis* listed in Table 1. There was a significant difference ($P = 0{\cdot}001$) between isolates and between incubation periods, and a significant interaction ($P = 0{\cdot}01$) between incubation period and isolate. It was found that all isolates produced sharp eyespot-like lesions but *R. cerealis* was the most pathogenic. The other isolates capable of producing severe lesions were an isolate of *T. cucumeris* from wheat stems (805) and the isolates F87 and F88 (both ZG8 or AG4) which have been classified in the past as the 'stem' strain (Flentje & Saksena, 1957). It is interesting to note, however,

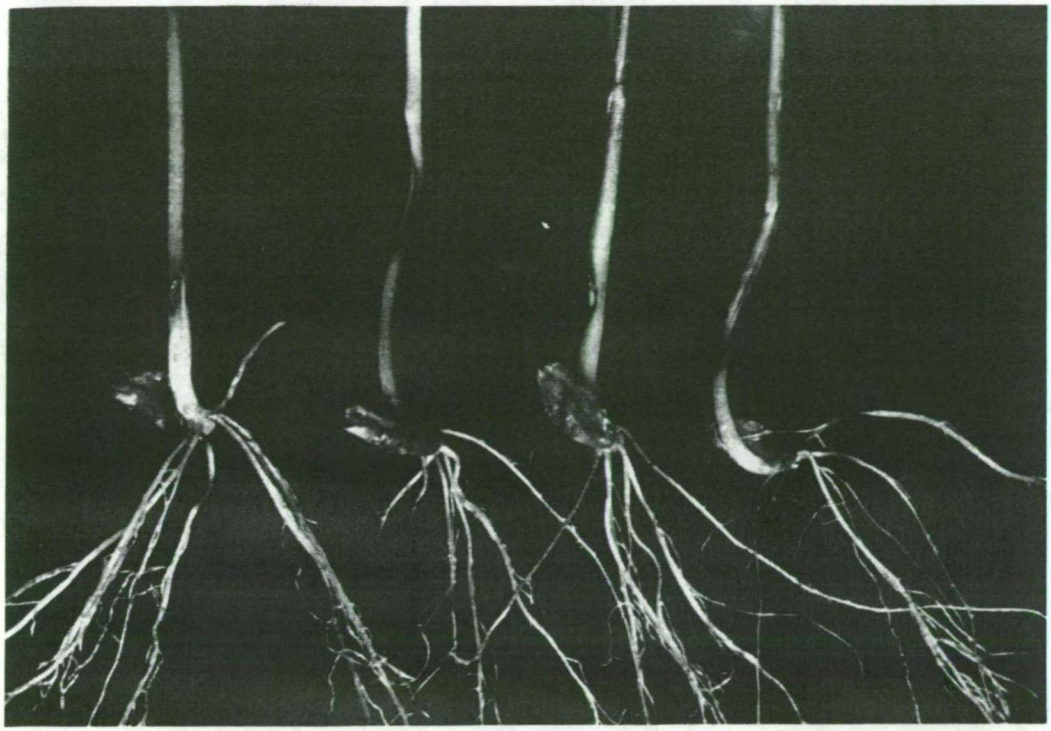


Fig. 2. Typical sharp eyespot-like symptoms on the coleoptile and stem of field-grown triticale seedlings.

Table 5. *Variation in pathogenicity* of several isolates of* Rhizoctonia *on stem and coleoptile of wheat seedlings*

Isolate	Incubation period	
	26 d	37 d
805	0† (0·7)‡	1·2 (1·2)
806	0·3 (0·8)	0·2 (0·8)
521	0 (0·7)	0·1 (0·8)
F87	2·0 (1·5)	2·0 (1·5)
F88	0·7 (1·0)	1·2 (1·2)
550	0 (0·7)	0·3 (0·9)
531	0·2 (0·8)	0 (0·7)
748	0 (0·7)	0·1 (0·8)
1029	2·7 (1·7)	4·8 (2·3)
1030	2·1 (1·4)	6·0 (2·5)

SE of mean difference (transformed data) = 0·2.
* Visual rating of increasing severity from 0 to 6.
† Untransformed data (x).
‡ Transformed data (sqrt ($x+0·5$)).

that the only ZG8 isolated was from wheat roots and none was isolated from lesions on cereal stems. This may indicate that although ZG8 are capable of causing severe stem lesions, most stem lesions are caused by other Rhizoctonias or a complex of Rhizoctonias.

The authors wish to thank Ms J. Bourne for her competent assistance and the South Australian Wheat and Barley Industry Research Committees and The Reserve Bank of Australia for funding.

REFERENCES

BANYER, R. J. (1966). Cereal diseases and their control. Part 1. *Journal of the Department of Agriculture South Australia* **69**, 310–315.

BLAIR, I. D. (1942). Studies on the growth in soil and the parasitic action of certain *Rhizoctonia solani* isolates from wheat. *Canadian Journal of Botany* **20**, 174–185.

BURPEE, L. L., SANDERS, P. L., COLE, H. JR & SHERWOOD, R. T. (1980). Anastomosis groups among isolates of *Ceratobasidium cornigerum* (Bourd.) Rogers and related fungi. *Mycologia* **72**, 689–701.

CRUICKSHANK, R. H. (1983). Distinction between *Sclerotinia* species by their pectic zymograms. *Transactions of the British Mycological Society* **80**, 117–119.

DE BEER, J. F. (1965). Studies on the ecology of *Rhizoctonia solani* Kuhn. Ph.D. Thesis, University of Adelaide.

FLENTJE, N. T. & SAKSENA, H. K. (1957). Studies on *Pellicularia filamentosa* (Pat.) Rogers. II. Occurrence and distribution of pathogenic strains. *Transactions of the British Mycological Society* **40**, 95–108.

GLYNNE, M. D. & RITCHIE, W. M. (1943). Sharp eyespot of wheat caused by *Corticium* (*Rhizoctonia*) *solani*. *Nature, London* **152**, 161.

MCKNIGHT, T. (1960). Survey of present incidence, importance and causes of cereal root rots in Australia. *Proceedings of a Conference on Root Rots of Wheat, Wagga Wagga, New South Wales*, 1–12.

NEATE, S. M. (1984). Minimum cultivation and root diseases of wheat. Ph.D. Thesis, University of Adelaide.

NEATE, S. M. (1985). *Rhizoctonia* in South Australian wheat fields. In *Ecology and Management of Soilborne Plant Pathogens* (ed. C. A. Parker, A. D. Rovira, K. J. Moore, P. T. W. Wong & J. F. Kollmorgen), pp. 54–56. St Paul, U.S.A.: American Phytopathological Society.

NEATE, S. M., CRUICKSHANK, R. H. & ROVIRA, A. D. (1988). Pectic enzyme patterns of *Rhizoctonia solani* isolates from agricultural soils in South Australia. *Transactions of the British Mycological Society* **90**, 37–42.

OGOSHI, A., ONIKI, M., SAKAI, R. & UI, T. (1979). Anastomosis grouping among isolates of binucleate *Rhizoctonia*. *Transactions of the Mycological Society of Japan* **20**, 33–39.

OGOSHI, A., ONIKI, M., ARAKI, T. & UI, T. (1983). Studies on the anastomosis groups of binucleate *Rhizoctonia* and their perfect states. *Journal of the Faculty of Agriculture, Hokkaido University* **61**, 244–260.

ONIKI, M., OGOSHI, A. & ARAKI, T. (1982). Formation of the perfect state of AG-D (causal fungus of foot rot of cereals) of binucleate *Rhizoctonia*. *Annals of the Phytopathological Society of Japan* **48**, 357.

PARMETER, J. R. JR ed. (1970). *Rhizoctonia solani, Biology and Pathology*. Berkeley, U.S.A.: University of California Press.

PITT, D. (1964). Studies on sharp eyespot disease of cereals. I. Disease symptoms and pathogenicity of isolates of *Rhizoctonia solani* Kuhn and the influence of soil factors and temperature on disease development. *Annals of Applied Biology* **54**, 77–89.

ROBERTS, F. A. & SIVASITHAMPARAM, K. (1986). Identity and pathogenicity of *Rhizoctonia* spp. associated with bare patch of cereals at a field site in Western Australia. *Netherlands Journal of Plant Pathology* **92**, 185–195.

SWEETINGHAM, M. W., CRUICKSHANK, R. H. & WONG, D. H. (1986). Pectic zymograms and taxonomy and pathogenicity of the Ceratobasidiaceae. *Transactions of the British Mycological Society* **86**, 305–311.

WARCUP, J. H. (1955). Isolation of fungi from hyphae present in soil. *Nature, London* **175**, 953.

WARCUP, J. H. & TALBOT, P. H. B. (1965). Ecology and identity of mycelia from soil. III. *Transactions of the British Mycological Society* **48**, 249–259.

WARCUP, J. H. & TALBOT, P. H. B. (1966). Perfect states of some Rhizoctonias. *Transactions of the British Mycological Society* **49**, 427–435.

WONG, D. H. & SIVASITHAMPARAM, K. (1985). *Rhizoctonia* spp. associated with root rots of subterranean clover in Western Australia. *Transactions of the British Mycological Society* **85**, 21–27.

(*Received for publication 16 September 1987*)

Food Microbiology, 1986, **3**, 363–371

Penicillium commune, P. camembertii, the origin of white cheese moulds, and the production of cyclopiazonic acid

J. I. Pitt[1], R. H. Cruickshank[2] and L. Leistner[3]

[1] *CSIRO Division of Food Research, P.O. Box 52, North Ryde, N.S.W. 2113, Australia*
[2] *Department of Agricultural Science, University of Tasmania, Hobart 7000, Australia*, and
[3] *Federal Centre for Meat Research, 8650 Kulmbach, Federal Republic of Germany*

Received 21 September 1986

The taxonomy of Penicillia producing the mycotoxin cyclopiazonic acid, including isolates classified as Penicillium aurantiogriseum *and* P. puberulum, *is reviewed on the basis of morphology, physiology, mycotoxin production and isoenzyme profiles. It is concluded that* P. puberulum, *as neotypified by Pitt in his 1979 monograph, is a synonym of* P. aurantiogriseum. *The correct name for saprophytic Penicillia producing cyclopiazonic acid is* P. commune *with* P. palitans *as a synonym. The moulds used in the manufacture of white cheeses, which are all classified in* P. camembertii, *and which also produce cyclopiazonic acid, are domesticated fungi derived from* P. commune.

Introduction

The white moulds which are used in the production of cheeses such as Camembert, Brie, Weißschimmelkäse and Neufchatel have long been regarded as domesticated fungi. Thom (1906), in describing *Penicillium camembertii*, stated 'Persistent search has failed to find a single colony [of *P. camembertii*] in America whose presence can be attributed to anything but Camembert cheese imported from Europe'. These moulds have been known by various names in cheese industries around the world: Thom (1930) and Raper and Thom (1949) accepted two species, *P. caseicola*, which produces white conidia, and *P. camembertii*, in which conidia are pale grey. Samson et al. (1977) combined the two species under the older of the two names, *P. camembertii*. Pitt (1979) agreed, considering that 'the two species are so strikingly similar that it is difficult to escape the conclusion that the strains with white conidia are mutants of the grey green: selected for, perpetuated in, and apparently confined to, cheese manufacture'.

The origin of the moulds used in the manufacture of white cheeses has aroused curiosity, for domesticated fungi are rare. Pitt (1979) speculated that FRR 2160, an isolate from spoiled New Zealand cheddar cheese, was a 'wild' *P. camembertii*. This isolate differed in a number of morphological and microscopic properties from the domesticated species, but similarities suggested a common ancestor.

The origin of *P. camembertii* became of more than academic interest when it was discovered by Still et al. (1978) that white cheese moulds may produce the mycotoxin cyclopiazonic acid. In a subsequent study of 69 *P. camembertii* isolates of both white and grey spored types,

0740–0020/86/040363 + 09 $02.00/0

drawn from a wide variety of sources, Leistner and Eckardt (1979) failed to find a single isolate which was not capable of cyclopiazonic acid production. Apparently all known starter cultures of *P. camembertii* used in cheese manufacture can produce cyclopiazonic acid (Scott 1981). The discovery of a *P. camembertii* ancestor which was not mycotoxigenic could be of great potential value in producing new strains suitable for cheese manufacture.

The taxonomy of Penicillia producing cyclopiazonic acid has proved to be difficult. Leistner and Pitt (1977) reported *Penicillium cyclopium* Westling (= *P. aurantiogriseum* Dierckx) to be the main producer of this toxin, but some isolates identified as *P. puberulum* Bainier, *P. viridicatum* Westling, *P. crustosum* Thom and *P. patulum* Bainier (= *P. griseofulvum* Dierckx) produced it also. Pitt (1979) regarded all of these species as distinct, though with the name changes indicated above in brackets. Of particular interest here is his retention of *P. puberulum* as a separate species from *P. aurantiogriseum* on the basis of relatively minor differences in growth rates, colony texture and conidial colours. Toxin production was not considered.

In their study of secondary metabolite production by species in subgenus *Penicillum*, Frisvad and Filtenborg (1983) 'provisionally included' isolates of *P. puberulum* in *P. aurantiogrisum*, a species they regarded as the major producer of penicillic acid. Isolates producing cyclopiazonic acid were all assigned to a single species, *P. camembertii*, which they divided into two 'groups'. '*P. camembertii* Group I' included all of the cheese moulds placed in this species by modern taxonomists, while '*P. camembertii* Group II', centred on FRR 2160, was introduced for creatine positive isolates (Frisvad, 1981) drawn from several species accepted by Pitt (1979). Frisvad (1986) placed the neotype of *P. puberulum* in his subspecies '*P. aurantiogriseum* Group II'. In his view, the cyclopiazonic acid producers were not related to *P. puberulum*.

Williams and Pitt (1986), working from traditional taxonomic bases, enlarged the concept of *P. aurantiogriseum* so that it effectively included all *P. puberulum* isolates, but they did not resolve the fundamental confusion between isolates which produced penicillic acid, and those which produced cyclopiazonic acid.

Two studies which together resolved this question, and established the origins of *P. camembertii*, are reported in this paper. One study was carried out in the Federal Republic of Germany by two of us, while at the same time the third (R. H. C.) independently worked in Tasmania with cultures supplied from the collection at the first author's institute.

Materials and Methods

Studies in culture

About 250 isolates producing penicillic acid and/or S-toxin (Leistner 1984), or cyclopiazonic acid was studied in pure culture using the morphological and gross physiological methods of Pitt (1979). Cultures were grown on the standard plating regime of Czapek yeast extract agar (CYA) at 5, 25 and 37°C, and on malt extract agar (MEA) and 25% glycerol nitrate agar at 25°C. Cultures were examined macroscopically and microscopically from CYA and MEA at 25°C after 7 days incubation, and colony diameters were measured from all five standard conditions. Colour names in capitals and other colour nomenclature used below are from the 'Methuen Handbook of Colour' (Kornerup and Wanscher 1978). Nearly all of the cultures examined were from the collection at the Federal Centre for Meat Research, Kulmbach.

Mycotoxin assays

The cultures indicated above had previously been examined for mycotoxin production at

Kulmbach, by thin layer chromatography and other methods outlined by Leistner and Pitt (1977) and Leistner and Eckardt (1979).

Enzyme profiles

Production of pectic enzymes, amylases and ribonucleases were studied by growing cultures in the presence of suitable substrates, citrus pectin or wheat grains, followed by electrophoretic separation at low temperature and visualization by staining. Isoenzymes were then photographed and compared. The methods are given in detail by Cruickshank and Pitt (Mycologia, submitted). Cultures examined included type, authentic and other cultures classified in relevant species by the first author, including some regarded as synonyms in Pitt (1979). All cultures came from the FRR collection at CSIRO Division of Food Research, North Ryde, N.S.W., but included some originally obtained from the collection at Kulmbach.

Results

Studies at Kulmbach

At Kulmbach, detailed taxonomic studies on isolates producing either penicillic acid and/or S-toxin showed that the great majority could confidently be placed in *Penicillium aurantiogriseum*. Conidial colours of colonies grown on CYA and MEA at 25°C for 7 days were consistently greyish blue to blue green (24-25D-E3-4). Under the high power microscope, stipes from colonies grown on both media were smooth to finely roughened. This feature was not recognized by Pitt (1979) but was emphasized by Williams and Pitt (1986). Conidial colours of isolates producing cyclopiazonic acid, however, were more greenish in colour: on CYA, colours ranged from grey blue (24D-E3-4) to green (27E3), while on MEA, colours could almost always be considered to be true greens (26-27D-E3-4). Stipes of cyclopiazonic acid producers were usually distinctly, though not prominently, roughened when examined from colonies on both CYA and MEA. It became clear that two species of very similar appearance were being observed, separated readily by clear cut differences in mycotoxin production and, with care, on morphological criteria as well (Table 1).

Examination of a culture ex neotype of *P. puberulum* present in the collection at Kulmbach, Sp 916 (= FRR 2040), indicated that this should be placed in *P. aurantiogriseum* on these revised morphological grounds, as it had been on the

Table 1. Features distinguishing *Penicillium* isolates producing cyclopiazonic acid from those producing penicillic acid.

Mycotoxin	Penicillic acid	Cyclopiazonic acid
Conidial colour, CYA	Bluish green 24-25D–E3-4[a]	Bluish green to green 24-27D–E3-4
Conidial colour, MEA	Bluish green 24-25D–E3-4	Green 26-27D–E3-4
Colony diameters, mm[b]		
absolute range, CYA	20–44	24–40
absolute range, MEA	16–41	18–35
80% of isolates, CYA	30–37	30–37
80% of isolates, MEA	24–37	23–30
Stipe roughening, CYA	Smooth to finely rough (rarely rough)	Finely rough to rough (rarely smooth)
Conidia, length	Not exceeding 4 μm	Often up to 4·5 μm

[a] Colour codes from Kornerup and Wanscher (1978).

[b] Data from 167 isolates producing penicillic acid, and 94 producing cyclopiazonic acid. Cultures examined included many old and deteriorating isolates, with colony diameters outside the ranges to be expected from fresh isolates.

basis of secondary metabolism by Frisvad and Filtenborg (1983). Examination of the FRR strain of the *P. puberulum* neotype (FRR 2040) confirmed this. It was now clear that the name *P. puberulum*, neotypified by Pitt (1979) using an isolate (NRRL 1889) regarded as representative of the species by Raper and Thom (1949), is a synonym of *P. aurantiogriseum*. An equally important corollary is that the name *P. puberulum* is unavailable for the producers of cyclopiazonic acid.

The collection at Kulmbach also contained a culture ex type of *p. palitans* Westling 1911, Sp 915 (= CBS 107.11, IMI 40215, FRR 2033), which has been established to be a cyclopiazonic acid producer. Based principally on the examination of several isolates regarded by Raper and Thom (1949) as authentic for this species, Pitt (1979) had placed *P. palitans* in synonymy with *P. viridicatum*; however, in the opinion of R. A. Samson (pers. comm.) the type of *P. palitans* could not be classified in the latter species. Morphological examination of Sp 915 showed Samson's opinion to be correct: conidia on both CYA and MEA were dull green (27E4 and 26½E4 respectively), with definitely rough stipes. The early original publication date for *P. palitans*, and its morphology, together with the production of cyclopiazonic acid, indicated that it was an appropriate name for the cyclopiazonic acid producers. The possibility that an earlier valid and typified name existed could not be dismissed, but deterioration of old types in culture made accurate assessment of earlier species names difficult.

At the same time as the studies outlined above were in progress, studies on the enzyme patterns of these and other species in subgenus *Penicillium* were being undertaken at the University of Tasmania. These studies will be reported in detail elsewhere (Cruickshank and Pitt, Mycologia, submitted). With regard to the species of interest here, these studies independently produced the same conclusions as those outlined above: that isolates producing cyclopiazonic acid gave zymograms distinct from those isolates of *P. aurantiogriseum* which produced penicillic acid; that the neotype of *P. puberulum* produced zymograms characteristic of *P. aurantiogriseum*; and that the type of *P. palitans* produced zymograms characteristic of the isolates forming cyclopiazonic acid (Fig. 1). Furthermore, the cultures of *P. camembertii* examined all produced zymograms identical with, or very similar to, those of the other cyclopiazonic acid producers. Further study of type isolates of some species placed by Pitt (1979) in synonymy with *P. aurantiogriseum* and *p. puberulum* showed that the type of *P. commune* Thom 1910 produced zymograms characteristic of the cyclopiazonic acid producers also (Fig. 1). This isolate (FRR 890) was examined subsequently in the North Ryde laboratory: its morphology was also characteristic of the cyclopiazonic acid producers. Moreover, it produced a low level of cyclopiazonic acid, despite having been maintained in culture for most of the past 80 years. It can be confidently concluded that the earliest identifiable valid names for *Penicillium* isolates producing cyclopiazonic acid are *P. camembertii* and *P. commune*.

Discussion

It has been shown above that the earliest recognizable species producing cyclopiazonic acid are *P. camembertii* and *P. commune*. It is considered both logical and expedient to reserve the name *P. camembertii* for the domesticated moulds with which cheeses such as Camembert and Brie are produced, and to revive the

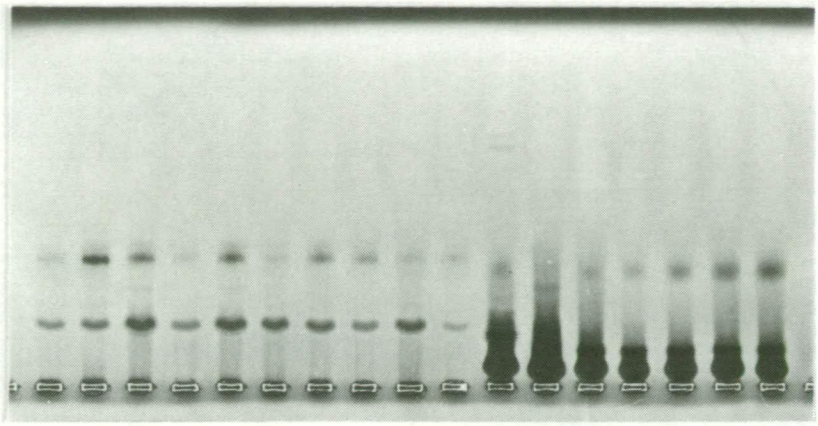


Fig. 1. Pectic (polygalacturonase) zymograms of *Penicillium* isolates. Lanes 1–10, cyclopiazonic acid producers; lanes 11–17, penicillic acid producers. 1, *P. palitans*, FRR 2033, type isolate; 2, *P. commune*, NRRL 890a, type isolate; 3–7, various isolates grouped in *P. commune* by zymogram; 8, *P. camembertii*, FRR 2160, considered to be a 'wild type' by Pitt (1979), now placed in *P. commune*; 9, *P. camembertii*, recently isolated from Camembert cheese; 10, *P. camembertii*, FRR 877, type isolate; 11, *P. aurantiogriseum*, FRR 971, neotype isolate; 12, *P. puberulum*, FRR 2040, neotype isolate; 13–17, isolates grouped in *P. aurantiogriseum* by zymogram.

name *P. commune* for isolates which are not specifically cheese moulds but which occur as ubiquitous saprophytes. The common origin of these two species is evident, both from their production of a single mycotoxin, rarely produced by other *Penicillium* species and, perhaps more convincingly, by unique and virtually identical patterns of zymograms (Fig. 1). If it is accepted that *P. camembertii* is a domesticated species, then *P. commune* must logically be its ancestral wild type. It is interesting and relevant that the type isolate of *P. commune* was isolated from cheese, and that FRR 2160, regarded by Pitt (1979) as a wild *P. camembertii*, was also isolated as a cheese spoilage fungus. Taking into account the extreme age and floccose habit of FRR 890, the type of *P. commune*, the morphological and physiological resemblance of this isolate to FRR 2160 is quite striking.

Previously, the taxonomy of the species referred to here as *P. commune* has been confused. Thom (1910, 1930) regarded *P. commune* as a ubiquitous species. Raper and Thom (1949), however, classified it in their subsection *Lanata*, to which floccose (and therefore deteriorating) species were consigned. It is probable that, in their classification, producers of cyclopiazonic acid were assigned to *P. cyclopium*, *P. puberulum*, *P. palitans* (perhaps only the type), and also other floccose species as well as *P. commune*. Samson et al. (1977) maintained *P. commune* as a rare species characterized by its floccose habit and ellipsoidal conidia. In their taxonomy, most freshly isolated cyclopiazonic acid producers would be identified, with a variety of other mycotoxigenic isolates, as *P. verrucosum* var. *cyclopium*. Pitt (1979) assigned the cyclopiazonic acid producers to either *P. aurantiogriseum* or *P. puberulum*, or in one case, FRR 2160, to *P. camembertii*, while Frisvad and Filtenborg (1983) logically created '*P. camembertii* Group II' for them. Williams and Pitt (1986) as-

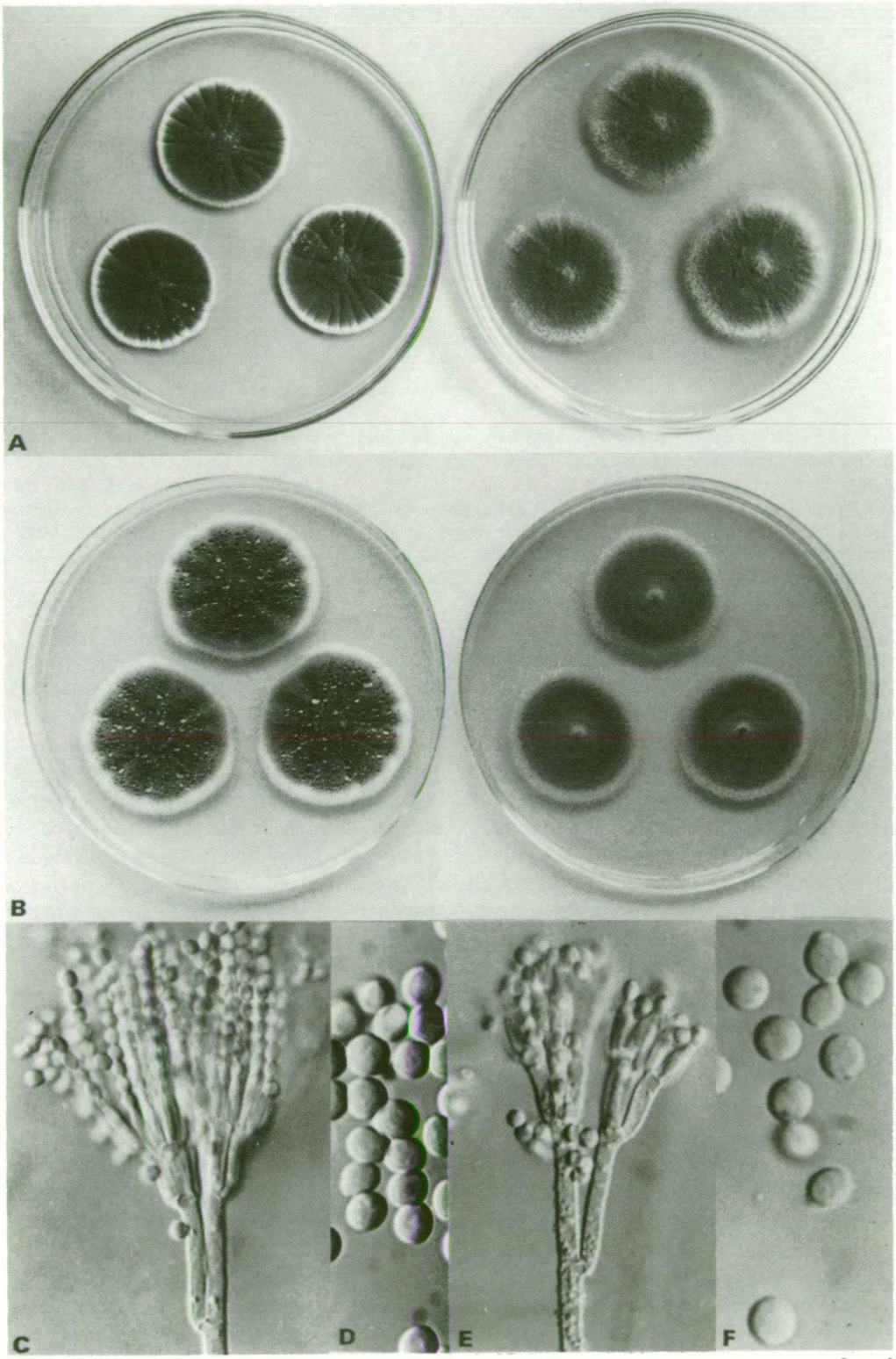


Fig. 2. *Penicillium aurantiogriseum*: (A) colonies on Czapek yeast extract agar and malt extract agar at 25°C, 7 days; (C) penicillus, ×750; (D) conidia, ×1875. *Penicillium commune*: (B) colonies on Czapek yeast extract agar and malt extract agar at 25°C, 7 days; (E) penicillus, ×750; (F) conidia, ×1875.

signed them to an enlarged *P. aurantiogriseum*.

More than 160 isolates known to produce cyclopiazonic acid, but not identifiable as *P. camembertii*, have been examined in this study. A high proportion of them, in excess of 80%, can be identified morphologically as a single species, now recognized as *P. commune*. This species is very similar morphologically to *P. aurantiogriseum*, usually differing from the latter by the features detailed in Table 1, although some degree of morphological overlap occurs. *P. commune* also resembles *P. viridicatum*: *P. viridicatum* produces brighter green conidial colours and more delicate penicilli. A small number of isolates have been examined which morphologically are indistinguishable from *P. viridicatum*, but which produce cyclopiazonic acid. In our present state of knowledge it is considered preferable to maintain the morphological basis to this species, and to accept that a minority of *P. viridicatum* isolates produce cyclopiazonic acid rather than to attempt to 'force' such isolates into *P. commune*.

Descriptions of *P. aurantiogriseum*, as now emended, and *P. commune*, as now revived, follow. Both species are illustrated in Fig. 2.

Penicillium aurantiogriseum Dierckx Annls Soc. Sci. Brux. 25: 88, 1901.

Penicillium puberulum Bainier, Bull. trimest. Soc. mycol. Fr. 23: 16, 1907.

Penicillium cyclopium Westling, *op cit.* 11: 90, 1911.

Penicillium aurantiovirens Biourge, Cellule 33: 119, 1923.

Penicillium martensii Biourge, *op. cit.* 33: 152, 1923.

Penicillium lanoso-coeruleum Thom, Penicillia: 322, 1930.

Penicillium verrucosum var. *cyclopium* (Westling) Samson et al., Stud. Mycol., Baarn 11: 37, 1976.

Penicillium commune Thom Bull. Bur. Anim. Ind. US Dep. Agric. 118: 56, 1910.

Penicillium palitans Westling, Ark. Bot. 11: 83, 1911.

Penicillium lanosum Westling, *op. cit.* 11: 97, 1911.

Penicillium lanosogriseum Thom, Penicillia: 327, 1930.

Colonies on CYA 30–37 mm diam, radially sulcate, moderately deep, texture velutinous to fasciculate; mycelium white, usually inconspicuous; conidiogenesis moderate to heavy, Greyish Turquoise to Dull Green (24-25D–E3-4); exudate usually conspicuous, clear or pale brown; soluble pigment produced by some isolates, brown to reddish brown; reverse pale, light to brilliant orange, or reddish to violet brown. Colonies on MEA 24–37 mm diam, plane or rarely radially sulcate, low and relatively sparse, surface texture velutinous to fasciculate; mycelium usually subsurface, occasionally conspicuous and then bright yellow; conidiogenesis usually moderate to heavy, Greyish Turquoise to Dull Green (24-25D–E4-5); soluble pigment sometimes produced, yellow brown to reddish brown; reverse pale, orange, or reddish brown. Colonies on G25N 18–24 mm diam, usually radially sulcate, moderately deep, dense, velutinous to fasciculate; reverse pale, yellow or brown. At 5°, colonies 2–5 mm diam, of white mycelium. No growth at 37°.

Conidiophores borne singly or in fascicles, mostly from subsurface hyphae, stipes 200–400 μm long, or of indeterminate length in fascicles, with walls smooth to finely roughened, only rarely rough, bearing terminal terverticillate or less commonly biverticillate penicilli; rami 15–25(–30) μm long; metulae 10–15(–18) μm long; phialides slender, ampulliform, mostly 7–10 μm long; coni-

dia spherical to subspheroidal, less commonly ellipsoidal, usually 3·0–4·0 μm long, with smooth walls, mostly borne in long, well defined columns.

Colonies on CYA 30–37 mm diam, radially sulcate, usually fasciculate, less commonly velutinous; mycelium white, usually inconspicuous; conidiogenesis moderate, of variable colour, Greyish Turquoise to Dull Green (24-27D–F3-5); exudate usually present, clear to pale brown; soluble pigment not produced; reverse usually pale, occasionally yellow, brown or purple. Colonies on MEA 23–30 mm diam, plane or lightly sulcate, low and dense, surface velutinous or lightly fasciculate; mycelium inconspicuous, white; conidiogenesis moderate, Dull Green (26-27D–E3-4); exudate and soluble pigment absent; reverse usually uncoloured. Colonies on G25N 18–22 mm diam, plane, sulcate or wrinkled, low to moderately deep, dense, usually fasciculate; mycelium white to yellowish; reverse pale to orange brown. At 5°, at least microcolony formation; typically colonies of 2–4 mm diam formed. No growth at 37°.

Conidiophores borne singly or in fascicles, mostly from subsurface hyphae, stipes 200–400 μm long or of indeterminate length in fascicles, with walls finely to conspicuously roughened, typically bearing terminal terverticillate penicilli; rami 15–20(–30) μm long; metulae 10–15(–18) μm long; phialides ampulliform, 9–11 μm long; conidia spherical, uncommonly subspheroidal, 3·5–4·0(-5) μm diam, smooth walled, borne in disordered chains.

Acknowledgements

Support from the Adalbert Raps Foundation, Kulmbach, Federal Republic of Germany, is gratefully acknowledged by J. I. P. during studies at the Federal Centre for Meat Research, Kulmbach. Support for R. H. C. came from the Rural Credits Development Fund of the Reserve Bank of Australia, and a research grant from the University of Tasmania. Ailsa D. Hocking supplied cultures from the FRR culture collection for R. H. C.

References

Cruickshank, R. H. and Pitt, J. I. (1986) Identification of terverticillate *Penicillium* species by enzyme electrophoresis. Mycologia (submitted).

Frisvad, J. C. (1981) Physiological criteria and mycotoxin production as aids in identification of common asymmetric Penicillia. *Appl. Environ. Microbiol.* **41**, 568–579.

Frisvad, J. C. (1986) Profiles of primary and secondary metabolites of value in classification of *Penicillium viridicatum* and related species. In *Advances* in Penicillium *and* Aspergillus *systematics*. (Eds Samson, R. A. and Pitt, J. I.) pp. 311–325. New York, Plenum Press.

Frisvad, J. C. and Filtenborg, O. (1983) Classification of terverticillate Penicillia based on profiles of mycotoxins and other secondary metabolites. *Appl. Environ. Microbiol.* **46**, 1301–1310.

Kornerup, A. and Wanscher, J. H. (1978) *Methuen handbook of colour*. 3rd ed. London, Eyre Methuen.

Leistner, L. (1984) Toxigenic Penicillia occurring in foods and feeds: a review. *Food Technol. Aust.* **36**, 404–406, 413.

Leistner, L. and Eckardt, C. (1979) Vorkommen toxigener Penicillien bei Fleischerzeugnissen. *Fleischwirtschaft* **59**, 1892–1896.

Leistner, L. and Pitt, J. I. (1977) Miscellaneous *Penicillium* toxins. In *Mycotoxins in human and animal health*. (Eds Rodricks, J. V., Hesseltine, C. W. and Mehlman, M. A.) pp. 639–653. Park Forest South, Illinois, Pathotox Publishers.

Pitt, J. I. (1979) *The genus* Penicillium *and its teleomorphic states* Eupenicillium *and* Talaromyces. London, Academic Press.

Raper, K. B. and Thom, C. (1949) *A manual of the Penicillia*. Baltimore, Williams and Wilkins Co.

Samson, R. A., Eckardt, C. and Orth, R. (1977) The taxonomy of *Penicillium* species from fermented cheeses. *Antonie van Leeuwenhoek* **43**, 341–350.

Scott, P. M. (1981) Toxins of *Penicillium* species used in cheese manufacture. *J. Food Protect.* **44**, 702–710.

Still, P., Eckardt, C. and Leistner, L. (1978) Bildung von Cyclopiazonsaure durch *Penicillium camembertii*-Isolate von Käse. *Fleischwirtschaft* **58**, 876–877.

Thom, C. (1906) Fungi in cheese ripening: Camembert and Roquefort. *Bull. Bur. Anim. Ind. U.S. Dep. Agric.* **82**, 1–39.

Thom, C. (1910) Cultural studies of species of *Penicillium*. *Bull. Bur. Anim. Ind. U.S. Dep. Agric.* **118**, 1–109.

Thom, C. (1930) *The Penicillia*. Baltimore, Williams and Wilkins Co.

Williams, A. P. and Pitt, J. I. (1986) A revised key to *Penicillium* subgenus *Penicillium*. In *Advances in* Penicillium *and* Aspergillus *systematics*. (Eds Samson, R. A. and Pitt, J. I.) pp. 129–134. New York, Plenum Press.

Mycologia, 79(4), 1987, pp. 614–620.

IDENTIFICATION OF SPECIES IN *PENICILLIUM* SUBGENUS *PENICILLIUM* BY ENZYME ELECTROPHORESIS

RALPH H. CRUICKSHANK

Department of Agricultural Science, University of Tasmania, Hobart, Tasmania 7000, Australia

AND

JOHN I. PITT

Commonwealth Scientific and Industrial Research Organisation, Division of Food Research, North Ryde, N.S.W. 2113, Australia

ABSTRACT

The extracellular enzymes polygalacturonase, pectinesterase, amylase and ribonuclease from cultures of species belonging to *Penicillium* subg. *Penicillium* were examined by polyacrylamide gel electrophoresis. Isolates which gave similar pectic zymograms were grouped; amylase and ribonuclease zymograms produced groupings consistent with these. Groups corresponding to accepted species were: *P. arenicola, P. atramentosum, P. brevicompactum, P. crustosum, P. digitatum, P. echinulatum, P. expansum* (including *P. resticulosum*), *P. fennelliae, P. granulatum, P. griseofulvum, P. griseoroseum* (including *P. chrysogenum*), *P. hirsutum, P. italicum, P. olsonii, P. roquefortii* and *P. verrucosum*. Some closely related species including *P. aurantiogriseum* and *P. viridicatum* were divisible into several enzymatically distinct groups which did not correspond well with accepted morphological species. For example, a group typified by *P. commune* included many isolates regarded as *P. aurantiogriseum*; enzymic patterns indicated that *P. camembertii* is closely related to this group. The clear correlations shown between most currently accepted species and the enzyme patterns reported here reinforce current species concepts, and also show that enzyme patterns are a useful taxonomic tool. The poor correlations with a minority of species, especially *P. aurantiogriseum* and *P. viridicatum*, may indicate an overreliance on conidial colors in the traditional taxonomy of these species.

Key Words: *Penicillium*, enzyme electrophoresis, *Penicillium* taxonomy.

In the taxonomy of the important fungal genus *Penicillium*, morphological methods appear to be inadequate for separating some physiologically distinct taxa. Consequently, readily accessible, broader expressions of the genotype have been sought to supplement the morphological methods. Colony diameters of isolates grown under standardized temperature conditions on three media have been used in *Penicillium* taxonomy (**11**, **12**) and recently extended to other genera (**14**). Use of this standardized system increases the probability that *Penicillium* isolates grouped as a species have a larger proportion of common genes than could be expected using morphology alone.

A variety of physiological and chemical properties of fungi have been studied, with potential value in the taxonomy of *Penicillium* (**3**, **13**). Of particular interest have been studies on the production of mycotoxins and other secondary metabolites, for example by Ciegler *et al.* (**4**) and Frisvad and co-workers (**7**, **8**). This approach shows promise, but has proved to be very complex (**18**).

A more direct approach is to study patterns of electrophoretically separated enzymes or total soluble proteins, which are the initial expressions of the genome in a tangible form. Limited investigations of this nature have been carried out (**1**, **2**, **9**), with encouraging results. This paper reports on the electrophoretic examination of extracellular enzymes from all species included in subgenus *Penicillium* by Pitt (**12**).

MATERIAL AND METHODS

Fungal cultures.—Isolates studied were from the FRR collection at CSIRO Division of Food Research, North Ryde, N.S.W., Australia (TABLE I). They were maintained on Czapek malt mainte-

TABLE I

CULTURES EXAMINED AND THEIR DISPOSITION

Species[a]	Type and authentic cultures	Others
1. *P. arenicola* Chalabuda	FRR 3392 *ex* type	
2. *P. canadense* G. Smith	FRR 2553 *ex* type	
3. *P. atramentosum* Thom	FRR 795 *ex* type; FRR 1446	
4. *P. aurantiogriseum* Dierckx	FRR 971 *ex* neotype	22[b]
5. *P. brunneoviolaceum* Biourge	FRR 2137 *ex* type	
6. *P. cyclopium* Westling	FRR 1888 *ex* type	
7. *P. porraceum* Biourge	FRR 970 *ex* type	
8. *P. puberulum* Bain.[c]	FRR 2040 *ex* neotype	
9. *P. brevicompactum* Dierckx	FRR 862 *ex* neotype	6
10. *P. camembertii* Thom	FRR 877 *ex* type; FRR 2160	1
11. *P. commune* Thom	NRRL 890a *ex* type	34[b]
12. *P. palitans* Westling	FRR 2033 *ex* type	
13. *P. lanoso-coeruleum* Thom	FRR 888 *ex* type	
14. *P. lanosogriseum* Thom	FRR 894 *ex* type	
15. *P. ochraceum* Bain. var. *macrosporum* Thom	FRR 873 *ex* type	
16. *P. crustosum* Thom	FRR 1669 *ex* type	3
17. *P. digitatum* (Pers.: Fr.) Sacc.	FRR 1313; FRR 288	2
18. *P. echinulatum* Fassatiova	FRR 1151 *ex* isotype; FRR 1143	2
19. *P. expansum* Link	FRR 976 *ex* neotype	9
20. *P. resticulosum* Birkinshaw *et al.*[c]	FRR 2021 *ex* type	
21. *P. fennelliae* Stolk	FRR 521 *ex* type	
22. *P. granulatum* Bain.	FRR 2036 *ex* neotype; FRR 1386	
23. *P. griseofulvum* Dierckx	FRR 1414; FRR 2002	5
24. *P. griseoroseum* Dierckx	FRR 820 *ex* type	1
25. *P. chrysogenum* Thom[c]	FRR 807 *ex* type	14
26. *P. hirsutum* Dierckx	FRR 2032 *ex* neotype	
27. *P. hordei* Stolk	FRR 815 *ex* type; FRR 1343	
28. *P. italicum* Wehmer	FRR 983 *ex* neotype	2
29. *P. japonicum* G. Smith	FRR 3431 *ex* type	
30. *P. olsonii* Bain. and Sartory	FRR 432 *ex* neotype; FRR 3165	
31. *P. roquefortii* Thom	FRR 849 *ex* type	6
32. *P. solitum* Westling	FRR 937 *ex* type	6[b]
33. *P. mali* Novobranova		1
34. *P. verrucosum* var. *melanochlorum* Samson *et al.*	FRR 2152 *ex* type	
35. *P. verrucosum* Dierckx	FRR 965 *ex* neotype; FRR 1639	4
36. *P. viridicatum* Westling	FRR 963 *ex* neotype; FRR 1636	13[b]
37. *P. olivicolor* Pitt[c]	FRR 870; FRR 882	
38. *Penicillium* sp.		3[b]

[a] Taxa indented following a species were grouped with that species by zymograms. Numbers refer to zymograms in FIG. 2 and FIG. 3.

[b] Including those otherwise identified but placed in this species by zymograms, see TABLE II.

[c] Species accepted by Pitt (**12**), but not distinct by zymograms.

nance medium (**12**). In all, 181 isolates were studied.

Enzyme production.—For enzyme production, cultures were grown in loosely capped 7 ml Bijoux bottles, each containing 2 ml of culture medium, autoclaved at 121 C for 15 min, inoculated by needle point and incubated at 22 C.

For pectic enzymes the medium contained $NH_4H_2PO_4$, 0.9 g; $(NH_4)_2HPO_4$, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; KCl 0.5 g; and citrus pectin, 10.0 g, per liter of distilled water (pH 6.0). Cultures were incubated for 7 da.

For amylase and ribonuclease production, the medium consisted of five wheat grains in 2 ml distilled water in each bottle. Cultures were incubated for 10 da.

Electrophoresis.—Electrophoresis was performed in horizontal slab gels of polyacrylamide 2 mm thick and 84 mm long. Sample wells were 3 mm × 1 mm, 1.5 mm deep, spaced 3 mm apart, 15 mm from the cathodic edge, across gels 160 mm wide.

A discontinuous pH 8.7 buffer system after Poulik (**15**) was used: gel buffer consisted of tris

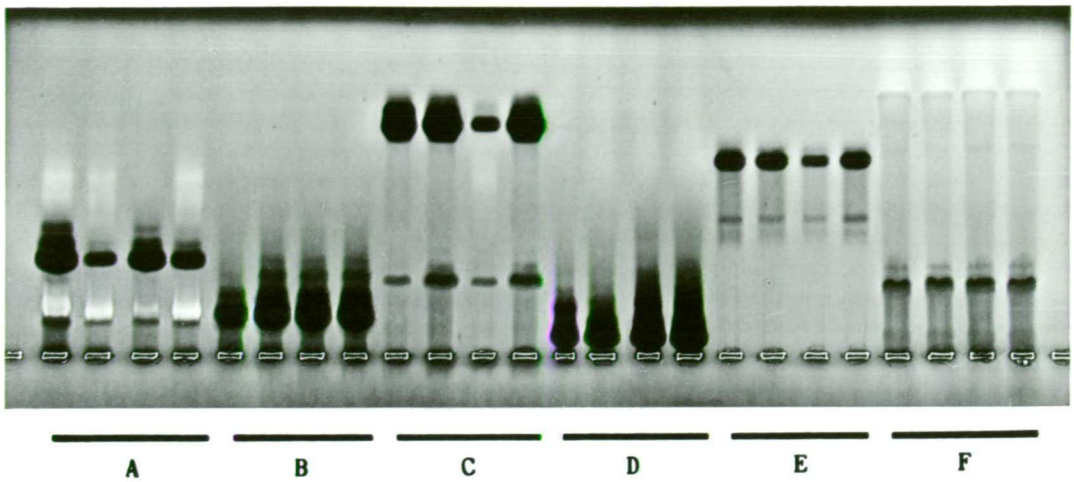


FIG. 1. Pectic zymograms from 24 *Penicillium* isolates from six species, illustrating homogeneity within species and clear differences between species. (A) *P. crustosum*; (B) *P. expansum*; (C) *P. brevicompactum*; (D) *P. aurantiogriseum*; (E) *P. roquefortii*; (F) *P. viridicatum*. The first in each group is from an ex type or ex neotype culture. Black: polygalacturonase; white: pectinesterase, ×1.

base, 4.598 g, and citric acid monohydrate, 0.525 g/L of deionized water; the electrode buffer contained boric acid, 7.22 g, and sodium tetraborate decahydrate, 15.75 g/L of deionized water.

Wells in the gel were filled from mixtures of 50 μl of culture fluid with 2.5 mg Sephadex G-150 superfine (**5**). In all cases, electrophoresis was at 4 C with constant current and initial potential difference of 70 V, continued until bromophenol blue tracker dye spots applied across the gel near the cathodic edge had migrated with the buffer front 50 mm beyond the sample wells.

Pectic enzymes.—Pectic enzymes were examined by the method of Cruickshank and Wade (**6**). Electrophoresis was performed in 10.25% acrylamide (2.5% bisacrylamide) gels containing 0.1% low methoxyl citrus pectin. After electrophoresis, gels were incubated for 1 h at room temperature in 0.1 M malic acid, stained overnight in 0.01% ruthenium red solution at 4 C, then washed for 1 h in distilled water. To increase contrast in photograms, gels were then treated for 30 min in 0.1% ammonium persulphate solution.

Amylase.—Amylase was examined in 10.25% acrylamide (2.5% bisacrylamide) gels containing 0.1% soluble starch in solution. After electrophoresis, gels were incubated for 2 h at room temperature in 0.1 M acetate buffer, pH 5.5, then stained overnight at 4 C in a solution containing 1.5% potassium iodide and 0.005% iodine.

Ribonuclease.—Electrophoresis was performed in 15% acrylamide (2.5% bisacrylamide) gels containing 0.03% Na salt of high molecular weight ribosomal RNA from wheat germ. Following electrophoresis, gels were incubated for 1 h at room temperature in 0.1 M acetate buffer, pH 4.0, then stained overnight at 4 C in 0.05% acridine orange solution. Excess stain was removed by changes of distilled water.

Photographic recording.—Photograms of gels were prepared by direct printing under water onto high contrast (No. 5) Ilfoprint paper.

RESULTS

Response to media.—Different nitrogen sources and buffers in the media influenced the presence or concentrations of the various isozymes of polygalacturonase (PG). Pectinesterase (PE) was also influenced in those species shown to produce this enzyme, notably *P. arenicola, P. canadense, P. crustosum, P. digitatum, P. italicum* and the traces of PE produced by *P. atramentosum, P. brevicompactum* and *P. olsonii.*

The response to medium variation ranged from high in *P. italicum* to very low in *P. expansum.* The latter produced a set of PG isozymes constitutively in the presence of either sugars or pectin in a wide range of media. The inclusion of yeast extract gave no improvement in enzyme yields from the species examined. The most gen-

TABLE II

THE CASES OF DIFFERENCE BETWEEN GROUPING BY CLASSICAL MEANS AND BY ZYMOGRAMS

	No. classically identified as:				
In zymogram group	*P. aurantio-griseum*	*P. crustosum*	*P. puberulum*	*P. verrucosum*	*P. viridicatum*
P. aurantiogriseum	—	0	3	0	4[a]
P. commune	23	4	2	1	4
P. solitum	3	1	0	2[b]	0
P. viridicatum	0	0	2	1	—
Penicillium sp.	1[c]	0	0	0	1[d]

[a] Including FRR 1637; FRR 1641; FRR 1642.
[b] FRR 2574; FRR 2575.
[c] FRR 942.
[d] FRR 1668. This zymogram group included *P.* sp. FRR 3184.

erally useful medium of those tested is given in Materials and Methods.

Yields of amylase were scant from several semi-synthetic media containing starch or maltose, but good yields were obtained on natural substrates such as grains of wheat or maize or potato tuber tissue. Poor yields were obtained from polished rice. The best amylase and ribonuclease results were obtained from most species when wheat was used in the growth medium (see Materials and Methods). The species which produced barely detectable amylase activity, *P. italicum, P. digitatum* and *P. roquefortii,* gave improved yields of ribonuclease when 0.2% glucose was added to the medium.

Initial tests of grouping by zymogram evidence.— An assortment of 56 isolates was separated into evident groups, primarily on the basis of similarities in pectic zymograms, but reinforced by ribonuclease and amylase results. Subsequent comparision showed excellent agreement between these groups and species as recognized by Pitt (**12**). An exception was the recognition of two distinct zymogram types from isolates assigned to *P. aurantiogriseum,* and a lack of separation of *P. aurantiogriseum* and *P. puberulum.*

Further tests.—This approach was continued and extended to the examination of cultures from type or neotype isolates. All isolates examined are listed in TABLE I. From this material 22 zymogram groups were recognized. Within each group pectic zymograms were similar, as illustrated by the examples in FIG. 1. Zymograms of amylase and ribonuclease showed similar uniformity. Correspondence between zymogram groups and species was excellent for most species. However, correlations were less clear in *P. aurantiogriseum, P. viridicatum* and some related species (TABLE II). A distinct group which has yet to be linked with a species is listed in TABLES I and II as *Penicillium* sp.

Zymograms of each of the recognizably different groups are illustrated in FIG. 2, while those from some groups found to include more than one taxon (TABLE I) and isolates differently identified by classical means (TABLE II) are illustrated in FIG. 3.

DISCUSSION

Zymograms, evidence of particular genes, were found to be distinctive for the majority of accepted species. Perhaps all true species should be distinguishable this way. However, like other characters, the enzymes studied here involve only a sample of the genome. Similar zymograms may indicate very close relationships, but may be insufficient evidence, taken alone, to delimit species on the basis of the total genome. However, the results shown here suggest that some accepted taxa are related so closely that they may be synonymous. Conversely, distinctive zymograms obtained here do not support some cases where synonymy has been postulated.

The results generally support the classification of Pitt (**12**) but with some exceptions. The culture from the type of *P. resticulosum* produced zymograms similar to *P. expansum* (FIG. 3), supporting the conclusion (**8**, **17**) that these species are synonymous. The second of the two cultures regarded as *P. resticulosum* (**12**), that from the type of *P. japonicum,* produced zymograms typical of *P. italicum* (FIG. 3).

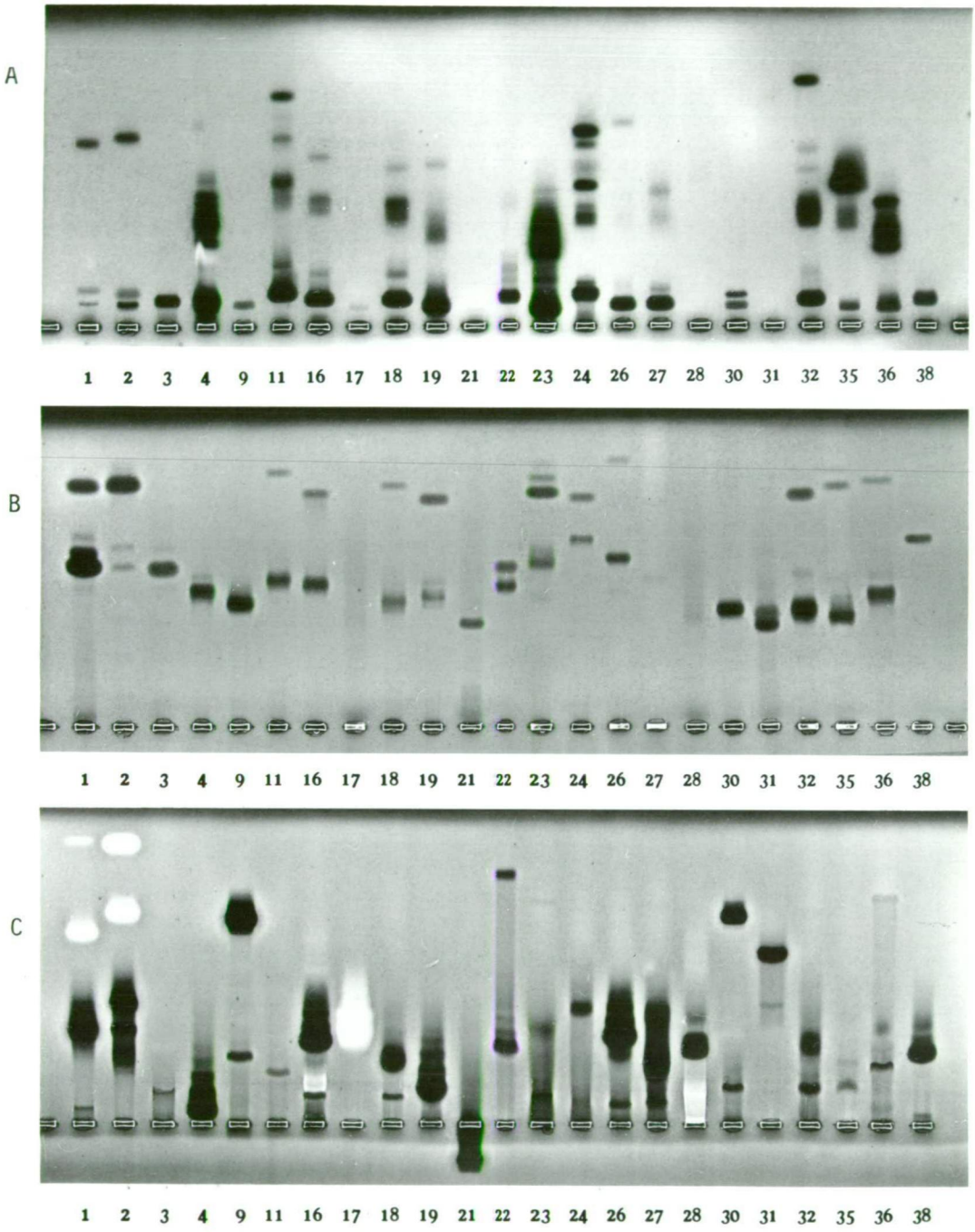


FIG. 2. Zymograms of species producing distinct patterns in this study. Key to species: see TABLE I. (A) amylase; (B) ribonuclease; (C) pectic enzymes, ×1.

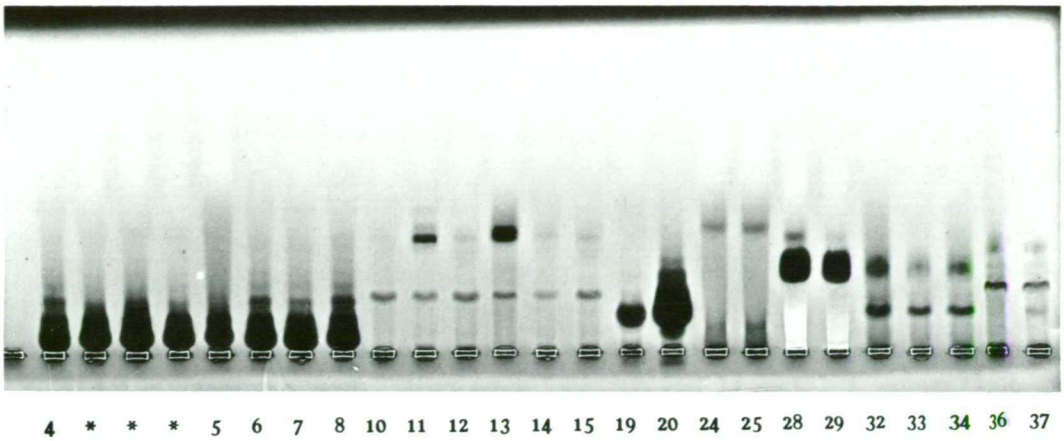


FIG. 3. Pectic zymogram evidence for the grouping of taxa and identification of isolates. As examples from TABLE II, zymograms in lanes marked * were from isolates identified by classical means as *P. viridicatum* but by zymograms as *P. aurantiogriseum*. Key to zymogram numbers: see TABLE I.

Pitt (**12**) regarded *P. hirsutum* and *P. hordei* as synonymous. From their zymograms these species do not appear closely related, in keeping with current thought. A degree of relatedness between *P. olsonii* and *P. brevicompactum* is indicated by their pectic zymograms. Single isolates of *P. arenicola* and *P. canadense* have been examined. These show a degree of relationship in Rf correspondence of two of the three major bands of ribonuclease. Their amylase and pectic zymograms show similarities in their appearance but differences in Rf (FIG. 2). Synonymy of the species (**12**) is tentatively supported. Zymograms of *P. chrysogenum* and *P. griseoroseum* were the same, supporting the known close relationship (**12**). If synonymy were accepted, a strong case could be prepared for conservation of *P. chrysogenum* despite the priority of *P. griseoroseum*.

The results support maintenance of *P. aurantiogriseum, P. crustosum* and *P. viridicatum* as distinct species (**12**). These species were placed together in *P. verrucosum* var. *cyclopium* by Samson *et al.* (**17**) and accepted by Ramirez (**16**). The culture from the neotype of *P. puberulum* produced zymograms suggesting synonymy with *P. aurantiogriseum* (FIG. 3); however, the existence of a distinct species, incorrectly considered to be *P. puberulum* by Pitt (**12**), was clearly supported. This species is considered to be correctly named *P. commune,* a species placed in synonymy with *P. puberulum* by Pitt (**12**). This taxon will be considered further in a separate paper.

The difficulty of separating the species considered in this section by morphology, growth characteristics or secondary metabolites is well known. The results presented here indicate that zymograms may be of great value in resolving these difficulties.

The zymogram technique has one other feature of great potential utility: the positive placement of old or deteriorating cultures whose identification is often conjectural on the basis of morphology alone. For example, in considering some species previously regarded as synonyms of *P. aurantiogriseum* or *P. puberulum* (**12**), zymograms (FIG. 3) indicated synonymy of *P. porraceum, P. cyclopium* and *P. brunneoviolaceum* with *P. aurantiogriseum,* while *P. commune* included *P. palitans, P. lanoso-coeruleum, P. lanosogriseum* and *P. ochraceum* var. *macrosporum.* The species *P. solitum* was found to be distinct from *P. aurantiogriseum.* Zymograms of *P. camembertii* indicated a close relationship with *P. commune,* but the distinctive character and commercial usage of this species militates against considering it as a synonym.

The zymogram technique appears to provide an excellent base for further studies by other means, for example, as proposed by Onions *et al.* (**10**).

ACKNOWLEDGMENTS

This work was supported by the Rural Credits Development Fund of the Reserve Bank of Aus-

tralia, Grant No. UT/2084, and a research grant from the University of Tasmania.

Dr. Ailsa D. Hocking is thanked for her interest and for the provision of cultures.

LITERATURE CITED

1. **Anné, J., and J. F. Peberdy.** 1981. Characterisation of inter-specific hybrids between *Penicillium chrysogenum* and *P. roqueforti* by isoenzyme analysis. *Trans. Brit. Mycol. Soc.* **77**: 401–408.
2. **Bent, K. J.** 1967. Electrophoresis of proteins of 3 *Penicillium* species on acrylamide. *J. Gen. Microbiol.* **49**: 195–200.
3. **Bridge, P. D.** 1985. An evaluation of some physiological and biochemical methods as an aid to the characterization of species of *Penicillium* subsection Fasciculata. *J. Gen. Microbiol.* **131**: 1887–1895.
4. **Ciegler, A., L. S. Lee, and J. J. Dunn.** 1981. Production of naphthoquinone mycotoxins and taxonomy of *Penicillium viridicatum*. *Appl. Environ. Microbiol.* **42**: 446–449.
5. **Cruickshank, R. H.** 1983. Distinction between *Sclerotinia* species by their pectic zymograms. *Trans. Brit. Mycol. Soc.* **80**: 117–119.
6. ———, **and G. C. Wade.** 1980. Detection of pectic enzymes in pectin-acrylamide gels. *Anal. Biochem.* **107**: 177–181.
7. **Frisvad, J. C.** 1981. Physiological criteria and mycotoxin production as aids in identification of common asymmetric Penicillia. *Appl. Environ. Microbiol.* **41**: 568–579.
8. ———, **and O. Filtenborg.** 1983. Classification of terverticillate penicillia based on profiles of mycotoxins and other secondary metabolites. *Appl. Environ. Microbiol.* **46**: 1301–1310.
9. **Garber, E. D., and L. Beraha.** 1966. Genetics of pathogenic fungi. Pectolytic enzymes of virulent and avirulent strains of 3 phytopathogenic penicillia. *Canad. J. Bot.* **44**: 1645–1650.
10. **Onions, A. H. S., P. D. Bridge, and R. R. Paterson.** 1984. Problems and prospects for the taxonomy of *Penicillium*. *Microbiol. Sci.* **1**: 185–189.
11. **Pitt, J. I.** 1973. An appraisal of identification methods for *Penicillium* species: novel taxonomic criteria based on temperature and water relations. *Mycologia* **65**: 1135–1157.
12. ———. 1979. *The genus Penicillium and its teleomorphic states Eupenicillium and Talaromyces*. Academic Press, London.
13. ———. 1984. The value of physiological characters in the taxonomy of *Penicillium*. Pp. 107–118. *In: Toxigenic fungi—their toxins and health hazard*. Eds., H. Kurata and Y. Ueno. Kodansha, Tokyo.
14. ———, **and A. D. Hocking.** 1985. *Fungi and food spoilage*. Academic Press, Australia, North Ryde, N.S.W.
15. **Poulik, M. D.** 1957. Starch gel electrophoresis in a discontinuous system of buffers. *Nature* (London) **180**: 1477–1479.
16. **Ramirez, C.** 1982. *Manual and atlas of the penicillia*. Elsevier Biomedical Press, Amsterdam.
17. **Samson, R. A., A. C. Stolk, and R. Hadlok.** 1976. Revision of the subsection Fasciculata of *Penicillium* and some allied species. *Stud. Mycol., Baarn* **11**: 1–47.
18. **Söderström, B., and J. C. Frisvad.** 1984. Separation of closely related asymmetric Penicillia by hydrolysis gas chromatography and mycotoxin production. *Mycologia* **76**: 408–419.

Accepted for publication March 10, 1987

The zymogram technique: isoenzyme patterns as an aid in *Penicillium* classification

RH Cruickshank* & JI Pitt

The zymogram technique, a means of visualizing isoenzymes and comparing differences between related species, is described. The technique has been applied to the very difficult Penicillium *subgenus* Penicillium *with notable success. Results have reinforced some aspects of accepted taxonomies and provided a basis for re-examination of others. The zymogram technique should have broad applicability to other fungal genera.*

Introduction

The taxonomy of terverticillate *Penicillium* species, i.e. those which produce penicilli bearing well defined rami, is notoriously difficult.[1] Raper & Thom[2] classified species fitting this description in four subsections of their very large section *Asymmetrica*. This classification was largely unworkable because the subsections were established on differences in colony texture, a character difficult to assess, of unreliable stability, and of doubtful taxonomic significance at the subsectional level. Nevertheless, their classification was in almost exclusive use for 30 years.

Samson *et al.*[3] approached the problem of speciation of terverticillate Penicillia by reducing a number of well defined species to varietal status, and placing many others in synonymy. Only 65% of terverticillate species accepted by Raper & Thom[2] were treated by them, however. Pitt[4] disagreed with both the Raper & Thom reliance on texture[2] and the varietal approach of Samson *et al.*[3] He combined all of the terverticillate species into a single subgenus, subgenus *Penicillium,* so named because it included the type species of the genus, *P. expansum*.

For distinguishing between species, Pitt's classification relied heavily on colony diameters and morphology of cultures grown for a standard time under standard conditions. However, in subgenus *Penicillium,* responses of most species to three of the five conditions used were very similar: in consequence, differentiation often relied on such characters as colony colours, especially conidial colours, wall texture of stipes and conidia, and other minutiae.

The majority of accepted *Penicillium* species were described more than 50 years ago. Despite having at his disposal the best strains available, many of which were freeze dried by K.B. Raper and his colleagues in the 1940s, Pitt[4] was often forced to rely for identifications and species placements on old type and authentic isolates. In many cases, these were floccose, with morphological properties having undergone varying degrees of deterioration over time. The choice of correct names for species he accepted, and the disposition of the many synonyms, was therefore very difficult and often subjective. When the types on which taxonomy relies are in poor condition, it is also very difficult to make decisions on the placement of interface isolates which do not totally fit recognizable species. Morphological taxonomy, even with the new approaches introduced by Pitt,[4] is stretched to the limit by degree of similarity and lack of distinctive features found in species belonging to subgenus *Penicillium*.

Ciegler *et al.*[5] suggested the use of secondary metabolite production as a taxonomic criterion, and applied it on a limited scale to *Penicillium viridicatum* Westling.[5,6] Frisvad & Filtenborg[7–9] extended this concept to embrace most species in subgenus *Penicillium*. However, such is the variety of metabolites produced by these particular species that Frisvad's schemes have become very complex[10,11] and have failed thus far to provide a basis for a practical taxonomy.

Among other recent developments, a group under the leadership of D.L. Hawksworth at the Commonwealth Mycological Institute (now CAB International Mycological Institute), Kew, Surrey, UK are undertaking a revision of the subgenus using a wide variety of taxonomic criteria.[1,12] They plan to assess their very extensive data by numerical taxonomic methods. Also, Samson[13] has recently accepted several of Pitt's species concepts in place of the earlier varieties,[3] while Pitt (see discussion)[14] has now accepted some species recognized by Samson *et al.*,[3] but which he had placed in synonymy[4] (Table 1).

The zymogram technique

Examination of protein profiles and isoenzyme profiles, or 'zymograms', prepared by electrophoresis provides valuable evidence of taxonomic relationships.[15] This paper describes the use of the zymogram technique to obtain a more objective assessment of speciation in the difficult subgenus *Penicillium*.

Pectic enzymes were examined using methods developed by Cruickshank & Wade[16] and Cruickshank[17] for separation of species of *Sclerotinia, Botrytis* and other genera. Fungal isolates were grown in liquid media formulated to encourage the secretion of pectic

*Department of Agricultural Science, University of Tasmania, Hobart, Tasmania, Australia.
Received 4 September, 1986.

galactosidase when they were treated with mitomycin C, a DNA-alkylating agent. Twenty such operon fusions were identified; none of them were expressed in the absence of DNA damage, and one of them was found to be in the *recE* gene, the functional equivalent of the *E. coli recA* gene, a central control element in the SOS circuit. Yasbin described an elegant experiment to show that *B. subtilis din-lacZ* fusions were expressed in competent cells: cultures were allowed to attain competence, at which time competent cells (20% of the population) were separated from non-competent cells by gradient centrifugation in the presence of a chromogenic β-galactosidase substrate. The band of competent cells was yellow (*din-lacZ* expressed) while the non-competent cells produced a white band (*din-lacZ* not expressed). The relevance of this phenomenon to competence for DNA transformation is not understood; *din* expression in competent cells seems to depend on a cascade reaction initiated by DNA-damage-independent increased expression of *recE*. It is almost as though the cells were preparing themselves for a massive influx of foreign DNA which they might have to degrade, repair or recombine with their chromosome.

Anthony P. Pugsley

*Papers presented at the GIM meeting will be published in a symposium volume in June, 1987.

Gene matching in plant pathology

The most complex problem in microbial pathology is simply stated: why don't all members of a species come down with the same diseases? Howard Flor's classic studies of fungal infections of plants proved that, for these organisms, pathogenicity is a two-way street. Or, rather, *lack* of pathogenicity is. A plant will be infected (in the confusing terminology of the field, have a 'compatible' interaction) with a fungus unless the plant carries a specific resistance (*R*) gene corresponding to the specific avirulence (*A*) gene carried by the particular fungus. These are 'gene-for-gene' interactions: there is, generally speaking, only one *A* gene with which a particular *R* allele will have incompatible interactions, resulting in plant protection.

Flor's studies of fungus-plant interactions were performed in the 1940s and '50s. Dean Gabriel and his colleagues at the University of Florida, Gainesville, have only now extended the gene-for-gene pattern to bacterial pathogens. Their studies of cotton resistance to a *Xanthomonas campestris* pathovar demonstrate that incompatibility is on a gene-for-gene basis, and raise interesting questions about the mechanism and meaning of avirulence.[1]

The studies of Gabriel *et al.* required both a host plant with well-determined resistance genetics, and a bacterial pathogen with defined avirulence genes. For the host, they used a series of ten congenic cotton strains that differ only in their genes for resistance to *Xanthomonas campestris* pv. *malvacearum* (Xcm). The bacterial pathogen was a problem: a single strain of Xcm was incompatible (avirulent) on all the cotton strains. If the gene-for-gene pattern was to hold true, this Xcm isolate must be carrying multiple *A* genes corresponding to each of the different cotton *R* genes.

The Florida researchers cloned Xcm DNA from an avirulent strain and introduced various fragments into a uniformly virulent Xcm isolate. They were able to isolate five *A* genes that specifically interact with five of the nine cotton *R* genes tested. All the *A* genes are chromosomal: three were found linked on the same piece of cloned DNA. When Gabriel *et al.* compared restriction digests of virulent and avirulent Xcm strains they found few differences, suggesting that the virulent strain carries recessive *a* alleles for the *A* incompatibility genes.

What light do these experiments shed on the nature of virulence? One striking fact about plant pathogenicity is that both avirulence and resistance tend to be dominant traits. This is startling if our model of pathogenicity is that of a virus and its cellular receptor, where the lack of corresponding components would produce a recessive incompatibility. The existence of dominant *A* and *R* genes suggests a model more like the vertebrate immune system, where defence (incompatibility) requires both an antigen (*A*) and an appropriate immune response gene (*R*).

Gabriel *et al.* point out that if the immune system model is correct, one might expect that all *A*/*R* reactions would be qualitatively similar, because the specific interaction would trigger a generalized, non-specific defence response—just as all good antigen-antibody reactions can trigger the same kind of complement cascade. But the Florida scientists find that, instead, every *A*/*R* combination has a different phenotype, almost as though each *A*/*R* interaction produces incompatibility by a different mechanism.

This exciting work is the kind that raises many questions and presents a system that can be used to answer them. Useful as any research on diseases of important crops must be, the Florida studies have even more interesting prospects in general phytopathology. It is even possible that their work will, in years to come, be part of a new field: plant immunology.

Mary Ellen Curtin

1 Gabriel DW, Burges A, Lazo GR. Gene-for-gene interactions of five cloned avirulence genes from *Xanthomonas campestris* pv. *malvacearum* with specific resistance genes in cotton. *Proceedings of the National Academy of Sciences USA* 1986; **83**: 6415–19.

FORTHCOMING REVIEWS

Enzymes and genes of microbial autotrophy *GA Codd & D Vakeria (UK)*

Novel antibacterial toxins produced by insects *JA Hoffmann & D Hoffmann (France)*

Diagnostic methods for human antimycosis *K Holmberg (Sweden)*

Problems and potential for *in situ* treatment of environmental pollutants by engineered microorganisms *RK Jain & GS Sayler (USA)*

Hepatitis B virus proteins eliciting protective immunity *AR Neurath, BA Jameson & T Huima (USA)*

Biotechnology in Australia: promise of performance *I Stevenson (Australia)*

Table 1. *Species in subgenus* Penicillium *for which agreement on concept and name has recently been reached.*[13]

Consensus name	Pitt[4]	Samson *et al.*[3]	Raper & Thom[2]
P. aurantiogriseum	*P. aurantiogriseum* *P. puberulum pro parte*	*P. verrucosum* var. *cyclopium*	*P. cyclopium* *P. aurantiovirens* *P. martensii* *P. puberulum* *P. lanoso-coeruleum*
P. commune[a]	*P. puberulum pro parte*	*P. commune*	*P. commune*
P. coprophilum[b]	Syn. of *P. italicum*	*P. concentricum*	—
P. crustosum	*P. crustosum*	*P. verrucosum* var. *cyclopium pro parte*	*P. crustosum*
P. hirsutum	*P. hirsutum*	*P. verrucosum* var. *corymbiferum*	*P. corymbiferum*
P. hordei	Syn. of *P. hirsutum*	*P. hordei*	—
P. olivicolor	*P. olivicolor*	*P. verrucosum* var. *ochraceum*	*P. ochraceum*

[a]R.H. Cruickshank and J.I. Pitt, unpublished results; [b]reference 14.

enzymes. 'Microcultures' using 2 ml of medium were adequate. Small samples of culture fluid were then subjected to electrophoresis at low temperature in polyacrylamide slab gels: the separated enzymes were allowed to act on methoxy pectin incorporated in the gels, and the sites of enzyme action visualized by ruthenium red staining.

To support the differentiation of the many species in subgenus *Penicillium*, the technique was broadened by including the examination of amylase and ribonuclease isoenzymes. These were also examined by electrophoresis in gels incorporating specific substrates: for amylases, soluble starch with visualization by staining with iodine, and for ribonucleases, ribosomal RNA visualized by staining with acridine orange (R.H. Cruickshank and J.I. Pitt, unpublished results). Microcultures containing wheat grains were found to be suitable for the production of amylases and ribonucleases. Permanent records of all the isoenzyme patterns observed were obtained as photograms by contact printing of the gels directly onto photographic paper under water. Representative examples of isoenzyme patterns are shown in Figure 1.

Zymograms of species in subgenus *Penicillium*

A study of zymograms produced by all species accepted in subgenus *Penicillium*[4] has now been completed (R.H. Cruickshank and J.I. Pitt, unpublished results). The study began with the acquisition by the first author of a group of 50 isolates from subgenus *Penicillium*, supplied without identifications from the FRR culture collection (CSIRO Division of Food Research, North Ryde, N.S.W.) by the second author. Suitable enzyme systems and tests were developed, as outlined above. The isolates were then grouped according to similarities in pectic zymograms. Groupings showed promise, i.e. the pectic zymograms showed a discrete number of recognizable patterns. Moreover, the other enzyme systems produced groupings consistent with those from the pectic enzymes. Subsequent examination of known type cultures permitted the assignment of almost all groupings to known species. Further type and reference isolates from all species in subgenus *Penicillium* were then examined, and the reproducibility of zymogram patterns within a single species was confirmed. Finally, a series of isolates with known mycotoxin profiles,

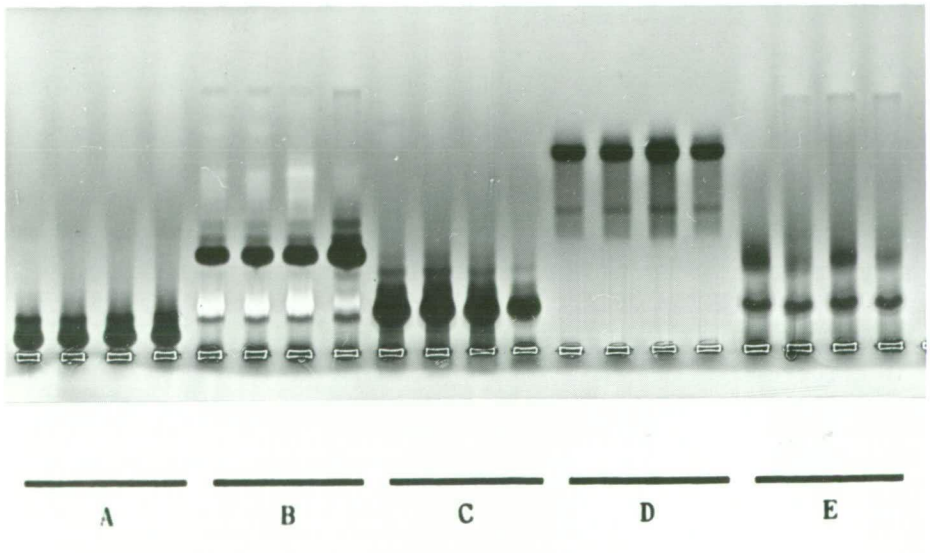


Figure 1. *Pectic zymograms from 20 isolates representing five species in subgenus* Penicillium, *showing similarities within species and distinct differences between species. A:* P. aurantiogriseum; *B:* P. crustosum; *C:* P. expansum; *D:* P. roquefortii; *E:* P. solitum. *Black bands: polygalacturonase; white bands: pectinesterase.*

especially chosen as difficult to assign to recognized morphological species, were examined. The correlations obtained between zymograms and mycotoxins confirmed both the fundamental nature of the production of specific isoenzymes and mycotoxins, and the utility of zymograms and metabolite profiles as aids to classical taxonomy.

This study was remarkably successful. For the first time with this subgenus, it has been possible to correlate a reasonably objective test of specific physiological properties with traditional morphological species concepts. The extent of the correlations observed to date is shown in Table 2.

All isolates examined from most species accepted by Pitt[4] and now agreed on by Samson & Pitt[13] showed a definite 1:1 correlation with distinctive zymograms, greatly reinforcing our confidence in current species concepts in this subgenus. Correspondence in some species is less clear, particularly in *P. aurantiogriseum* Dierckx and *P. viridicatum,* and the accurate morphological delimitation of these species will require further study.

The position of the species called *P. puberulum* Bain. by Pitt[4] but not recognized more recently[9,13] has been clarified (J.I. Pitt, R.H. Cruickshank and L. Leistner, unpublished results). This clarification will mean the revival of *P. commune* Thom, a species accepted by Raper & Thom[2] only as a rare, floccose taxon. Its status as a species was retained by Samson *et al.*,[3] again as a rare species, and without a clear definition. It was placed in synonymy with *P. puberulum* by Pitt.[4] Frisvad[10] suggested the revival of *P. commune* on the basis of its secondary metabolism: its status as an independent species has been confirmed by its distinctive zymogram patterns. Our recent studies (J.I. Pitt, R.H. Cruickshank and L. Leistner, unpublished results) have shown that *P. commune* is a common species and that, with care, most isolates can be identified by morphological techniques as well.

Another species which will be revived as a result of this work is *P. solitum* Westling, which was not regarded as distinct by Samson *et al.*,[3] or Pitt.[4] Again, the zymogram patterns of the long-maintained type isolate are readily distinguished from all others examined, and provide strong evidence that it is a distinct species. A recent morphological reappraisal (J.I. Pitt, unpublished results) has supported this conclusion.

Table 2. *Correlations in subgenus* Penicillium *between distinctive isoenzyme profiles and species.*

No. of species accepted by Pitt.[4]	22
No. of groupings with distinct zymograms.	22
No. of species with agreement in identification by classical criteria and zymograms for all isolates.	18
Species accepted by Pitt,[4] but not distinct by zymograms.	*P. chrysogenum*[a]
	P. olivicolor[b]
	P. puberulum[c]
	P. resticulosum[d]
Species synonymized by Pitt,[4] but distinct by zymograms.	*P. hordei*[e]
	P. solitum[f]
	P. commune[g]

[a]Zymograms not distinct from those of *P. griseoroseum;* [b]zymograms not distinct from those of *P. viridicatum;* [c]zymograms not distinct from those of *P. aurantiogriseum;* [d]zymograms not distinct from those of *P. expansum;* [e]considered synonym of *P. hirsutum;*[4] [f]considered synonym of *P. aurantiogriseum;*[4] [g]see text.

Conclusions

Some very significant results stem from this work. First, the zymogram technique provides strong support for many of the species concepts established by Pitt[4] on morphological and broadly physiological grounds. Second, zymograms will assist in the correction of some errors, misconceptions and grey areas in Pitt's work[4] where morphologically very similar species have been shown recently to have distinct habitats or mycotoxin profiles.[9,13,14] Third, the zymogram technique can very effectively and accurately permit disposition of old and deteriorated type or other isolates into accepted species, or attest to their distinctiveness. Fourth, and perhaps most important, if the zymogram technique can be used successfully in a taxonomic area as difficult as subgenus *Penicillium,* it can be used with confidence for many other genera. It may be possible, indeed, to use the technique with some fungi which cannot be grown in axenic culture but which produce enzymes such as pectic enzymes, amylases, ribonucleases or other enzymes in plant tissue. Its value as a taxonomic aid and criterion with such fungi could be very great.

Acknowledgements

This work was supported by the Rural Credits Development Fund of the Reserve Bank of Australia, Grant No. UT/2084, and a research grant from the University of Tasmania.

References

1 Onions AHS, Bridge PD, Paterson RR. Problems and prospects for the taxonomy of *Penicillium. Microbiological Sciences* 1984; **1**: 185–9.

2 Raper KB, Thom C. *A Manual of the Penicillia.* Baltimore: The Williams and Wilkins Co., 1949.

3 Samson RA, Stolk AC, Hadlok R. Revision of the subsection *Fasciculata* of *Penicillium* and some allied species. *Studies in Mycology,* Baarn 1976; **11**: 1–47.

4 Pitt, JI. *The Genus* Penicillium *and its Teleomorphic States,* Eupenicillium *and* Talaromyces. London: Academic Press, 1979.

5 Ciegler A, Fennell DI, Sansing GA, Detroy RW, Bennett GA. Mycotoxin-producing strains of *Penicillium viridicatum:* classification into subgroups. *Applied Microbiology* 1973; **26**: 271–8.

6 Ciegler A, Lee LS, Dunn JJ. Production of naphthoquinone mycotoxins and taxonomy of *Penicillium viridicatum. Applied and Environmental Microbiology* 1981; **42**: 446–9.

7 Filtenborg O, Frisvad JC. A simple screening method for toxigenic moulds in pure cultures. *Lebensmittel Wissenschaft Technologie* 1980; **13**: 128–30.

8 Frisvad JC. Physiological criteria and mycotoxin production as aids in identification of common asymmetric Penicillia. *Applied and Environmental Microbiology* 1981; **41**: 568–79.

9 Frisvad JC, Filtenborg O. Classification of terverticillate Penicillia based on profiles of mycotoxins and other secondary metabolites. *Applied and Environmental Microbiology* 1983; **46**: 1301–10.

10 Frisvad JC. Profiles of primary and secondary metabolites of value in classification of *Penicillium viridicatum* and related species. In: Samson RA, Pitt JI, eds. *Advances in* Penicillium *and* Aspergillus *Systematics*. New York: Plenum Press, 1986: 311–25.

11 Frisvad JC. Classification of asymmetric Penicillia using expressions of differentiation. In: Samson RA, Pitt JI, eds. *Advances in* Penicillium *and* Aspergillus *Systematics*. New York: Plenum Press, 1986: 327–35.

12 Bridge PD, Hawksworth DL, Kozakiewicz Z, Onions AHS, Paterson RRM, Sackin MJ. An integrated approach to *Penicillium* systematics. In: Samson RA, Pitt JI, eds. *Advances in* Penicillium *and* Aspergillus *Systematics*. New York: Plenum Press, 1986: 281–309.

13 Samson RA, Pitt JI. Check list of common *Penicillium* species. In: Samson RA, Pitt JI, eds. *Advances in* Penicillium *and* Aspergillus *Systematics*. New York: Plenum Press, 1986: 461–3.

14 Seifert KA, Samson RA. The genus *Coremium* and the synnematous Penicillia. In: Samson RA, Pitt JI, eds. *Advances in* Penicillium *and* Aspergillus *Systematics*. New York: Plenum Press, 1986: 143–54.

15 Garber ED. Symposium on the use of electrophoresis in the taxonomy of algae and fungi. VIII. Electrophoresis as a taxonomic and genetic tool for fungi. *Bulletin of the Torrey Botanical Club* 1973; **100**: 308–12.

16 Cruickshank RH, Wade GC. Detection of pectic enzymes in pectin-acrylamide gels. *Analytical Biochemistry* 1980; **107**: 177–81.

17 Cruickshank RH. Distinction between *Sclerotinia* species by their pectic zymograms. *Transactions of the British Mycological Society* 1983; **80**: 117–9.

FORTHCOMING COLOUR CENTRESPREADS

Genetic analysis of *E. coli* surface structures important in the pathogenesis of urinary tract infections *G Boulnois (UK)*

Time-resolved fluroimmunoassay *P Halonen (Finland)*

Antibiotic action in bacteria *WB Hugo (UK)*

The preS region of hepatitis B virus *AR Neurath (USA)*

Microbes and the treatment of toxic wastes *T Peyton (USA)*

Gastrointestinal epithelial surfaces as microbial habitats *DC Savage (USA)*

Geminivirus genomes

JW Davies*

The geminiviruses are unusual plant viruses containing circular single-stranded DNA. The sequences and organization of several of their genomes are described, and recent experiments towards understanding their replication, gene expression and gene functions are discussed.

Introduction

The geminiviruses are plant viruses containing a small (2.5 to 3.0 kb) single-stranded circular DNA genome encapsidated in a small geminate particle (Figure 1). Sixteen (confirmed and potential) geminiviruses have been reported, about half of which are transmitted by the whitefly *Bemisia tabaci*, and infect only dicotyledonous plants. The other members of the group are transmitted by several genera of leafhopper, and infect specifically either dicotyledonous or monocotyledonous hosts. The DNA genomes of several geminiviruses have now been cloned and sequenced. These data provide clues concerning their replication and gene expression strategies, and reveal differences which may relate to the host plant subclass or insect vector specificity. An understanding of such processes should facilitate the construction of gene vectors for plant genetic manipulation.

*John Innes Institute, Colney Lane, Norwich NR4 7UH, UK.
Received 23 September, 1986.

Genome structure

Cassava latent virus (CLV), also known as African cassava mosaic virus, was the first geminivirus to be cloned and sequenced.[1] The sequence revealed two types of DNA circle, DNA 1 and DNA 2, both of which were required for systemic infection of the experimental host, *Nicotiana benthamiana*.[2] This whitefly-transmitted virus which infects only dicotyledonous hosts therefore has a truly bipartite genome. There was no known precedent for this phenomenon among DNA viruses of any kind. Furthermore, the sequence also showed that both the virion-sense (+) and complementary sense (−) DNA carried potential genes (open reading frames, or ORFs) (Figure 2). Twelve ORFs coding potentially for proteins of $\leq$ 10000 kDal were evident from the primary sequence of CLV. However, comparison with the sequences of tomato golden mosaic virus (TGMV) and bean golden mosaic virus (BGMV) which have also been sequenced,[3,4] revealed a conserved arrangement of six ORFs, suggesting common or similar functions and

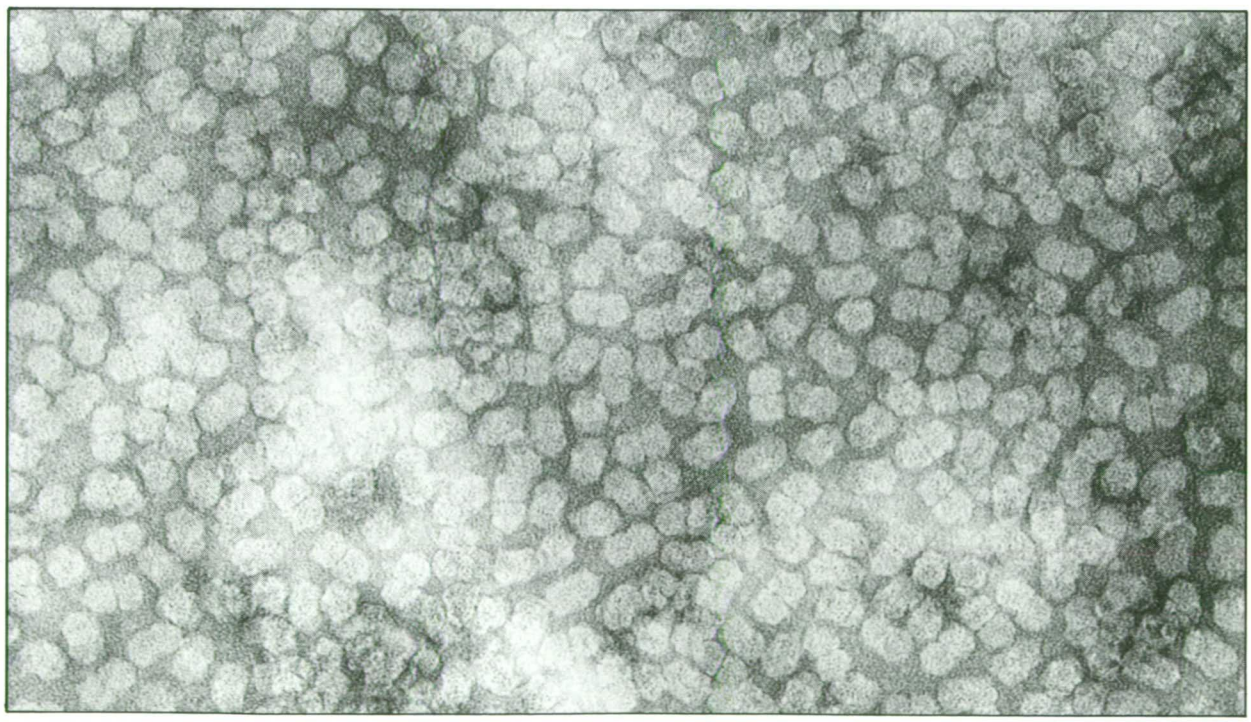


Figure 1. *Electron micrograph of maize streak virus (MSV) type member of the geminiviruses. Magnification ×126000. Each geminate pair of two ~ 20 nm quasi-icosahedral units comprises one virion particle containing small single-stranded DNA. (EM provided by Margaret Boulton.)*