Genetic Studies of Pathogenicity in *Botrytis cinerea* (*Botryotinia fuckeliana*)

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Abstract

Botrytis cinerea is a common pleomorphic fungus causing 'grey mould' disease on a wide range of crops resulting in serious losses both pre- and post-harvest. Traditional control measures rely heavily on frequent fungicide applications. A greater understanding of the infection process and more information on the factors determining successful and unsuccessful host/pathogen interactions is important for the development of new control strategies. Although widely studied, relatively little is known about its genetics and the factors that determine its pathogenic ability.

This study examines the genetics and pathogenicity of *B. cinerea* through mutation and selection. Two new genetic markers were developed based on resistance to the toxic analogues sodium selenate and potassium chlorate. These markers were then utilised in sexual crosses and competition studies *in planta*.

Selenate resistant (SelR) mutants of *Botrytis cinerea* were selected by plating conidia or mycelial plugs onto minimal medium amended with selenate and taurine. Mutants could be divided into three classes based on growth in the presence of selenate or chromate and on improved growth in response to taurine in minimal media. Some mutants grew poorly on minimal media but were responsive to taurine, indicating they were defective in sulphate reduction. Strains showing the SelR phenotype may result from mutations in different genes; the genetic symbol *Sel1* was allocated to one.

Nitrate non-utilising (Nit) mutants, generated as spontaneous sectors on minimal media amended with chlorate, behaved as *nit1* mutants in growth tests (putatively defective in nitrate reductase apoenzyme) and the genetic symbol *nit1* was allocated to one of these mutants. When *nit1* mutants were paired on medium with nitrate as sole nitrogen source, some pairings complemented, behaviour attributed to intragenic complementation.

Selected crosses of SelR and *nit1* mutants with wild type strains gave 1:1 segregation of both phenotypes and no evidence of linkage to either *Mbc1* (benzimidazole resistance) or

Daf1 (dicarboximide resistance) markers; loose linkage was confirmed between *Mbc1* and *Daf1*. Both *Sel1*R and *nit1* mutants were stable following subculture and retained pathogenicity in a French bean leaf assay. Complementation was demonstrated between a taurine responsive SelR mutant and a *nit1* mutant selected from the same parent.

Non-aggressive mutants were isolated from a single-ascospore strain of *B. cinerea* following mutagenic treatment (ultraviolet and 4-nitroquinoline-1-oxide) and screening on French bean leaves. Crosses with reference strains SAS56 or SAS405 revealed one u.v. mutant (Mp97) in which the non-aggressive phenotype segregated 1:1; indicating a single gene of major effect on pathogenicity to which the genotypic symbol *Pat1* was allocated. No evidence of linkage was found between *Pat1* and either *Mbc1*, *Daf1*, *nit1* or *Sel1*.

Further characterisation of this gene in studies involving *Pat1* and wild-type strains revealed various host and temperature responses. *Pat1* strains produced small, restricted lesions on French bean and soybean leaves and slowly spreading lesions on rose flowers. On tomato stems at 20 and 25°C the mutant was essentially non-pathogenic, although a reduced number of invasive infections were produced at 10 and 15°C. *Pat1* strains grow relatively normally, are indistinguishable from wild-type in gross morphology, and grow well on minimal medium indicating no unusual nutrient requirements, and it was concluded from comparison of physiological characteristics that the non-aggressive character is unlikely to be due to gross unfitness.

No difference was found between *Pat1* and wild-type strains in total polygalacturonase activity, and differences in polygalacturonase isozyme profiles were not correlated with the presence of the *Pat1* gene. *Pat1* was found to correlate with low acid production indicating a role for organic acid in pathogenesis of *B. cinerea*. Microscopic examination of 4-day-old lesions showed a distinctly stained ring of mesophyll cells surrounding lesions of Mp97 but not its parent (A4), suggesting a difference in host response. Differences in phytoalexin induction in soybean were not found. It is possible that *Pat1* strains may be deficient in the ability to tolerate or metabolise defence compounds.

Two hypotheses are presented for further investigation. The first that *Pat1* strains may have reduced toxicity due to low production of organic acids, and the second that these strains are non-aggressive due to a reduced ability to metabolise defence compounds.

In competition experiments aggressive and non-aggressive strains were found to co-exist in the same lesion when inoculated at the same time but when challenge inoculations were delayed 6 hours or more the initial inoculation was found to dominate, suggesting non-aggressive strains may be useful as biocontrol agents.

Acknowledgments

This thesis is dedicated to the memory of my brother Ian (Loopy) Weeds whose busy productive life and too early death (●ctober 1987) inspired me to get on with life: this work being one result.

I thank my supervisors Ross Beever and Peter Long for their help and patient encouragement. I am also very grateful to the following people for their important contribution; Keith Sharrock for advice and assistance with the polygalacturonase activity and phytoalexin induction work and his contribution of the PG isozyme studies, Shiva Ganeshanandam for advice and assistance with data analysis, Daryl Cook for help with the monoclonal antibody work, Lorraine Davis & Hugh Nielson for trouble shooting and technical stuff at Massey, Martin Heffer for the photography and for always being there when the fungus was photogenic, Ian Hallett and Paul Sutherland for help with the microscopy, Henry Pak for translating all those German texts, Patrick Connolly & Rod Ball for the logit analysis, Michael Eden for the tomato plants, pots and timely advice, Christoph Janisch & Willie Cornett, for all that computing help, Ken Young for the donation of copious glasshouse roses, Bob Fullerton for supplying pre-conditioned *Sclerotinia sclerotiorum* sclerotia and culturing advice, Molly Dewey for the gift of the BC-KH4 monoclonal antibody, the staff at the Mt Albert library for finding all those urgent references so fast, HortResearch for the use of facilities, Crop and Food for the use of a glass house, AGMARDT for the funds, and Landcare Research Mt Albert for a friendly and supportive working environment, my air conditioned office and the generous use of facilities.

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Last but never least thanks to my friend Heather Samson and my family including; Joy & Arthur, Sandy, Ian, Sally, Janine, Jo and my daughter Lily for your support, understanding and encouragement.

Victory to the truth. Triumph to the invincible sun.

Contents

Tables

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Preface

This thesis consists of an introduction and literature review (Chapter 1), a description of the techniques developed and general methodology (Chapter 2), followed by the main experimental work grouped into three subject areas (Chapters 3 – 5) and written as discrete units to facilitate publication, and ending with a general discussion and outline of possible future work (Chapter 6). To keep the text uncluttered some information has been placed in the appendices; a list of abbreviations (Appendix 1), components, instructions, and references for media and solutions (Appendix 2), source and genotype of B. *cinerea* strains (Appendix 3), a glossary of selected terms (Appendix 4), a summary of genetic nomenclature (Appendix 5), a summary of genetic symbols (Appendix 6), a summary of enzymes related to pathogenicity with relevant references (Appendix 7), and experimental details of the polygalacturonase (PG) cup-plate-assay (Appendix 8)

Chapter 3 has been accepted for publication in Mycological Research co-authored with supervisors Drs Beever (Landcare Research, Mt Albert) and Long (Massey University). Preliminary results and conclusions from the work presented in Chapter 3 were presented at the X1th International Botrytis Symposium held in Wageningen (the Netherlands) in June 1996.

> **Weeds PL, Beever RE, Long PG**. 1996. New genetic markers for *Botrytis cinerea*. In *XIth International Botrytis Symposium Programme and book of abstracts*. Wageningen, the Netherlands. Pp12.
>
> **Weeds PL, Beever RE, Long PG**. 1997. New genetic markers for *Botrytis cinerea* (*Botryotinia fuckeliana*). *Mycological Research*. In press.

Chapter 5 is being prepared for submission to Physiological and Molecular Plant Pathology, co-authored with supervisors Drs Beever and Long, and Dr Sharrock (Hort Research, Ruakura) who advised on PG enzyme and phytoalexin assays and carried out isoelectric focusing experiments on mutant and wild-type strains. Results and conclusions from the PG isoelectric focusing experiments are included within the text (boxed) for

completeness. An extension of these studies, showing segregation patterns of PG isozymes, was presented as a poster at the Australasian Plant Pathology Society 11th Biennial Conference held in Perth (Australia) in September 1997 (Appendix 9).

> **Weeds PL, Beever RE, Sharrock KR, Long PG**. 1997. A major gene controlling pathogenicity in *Botrytis cinerea* (*Botryotinia fuckeliana*). *Physiological and Molecular Plant Pathology*. In preparation.
>
> **Sharrock KR, Weeds PL, Beever RE**. 1997. Segregation of polygalacturonase isozymes in sexual progeny of *B. cinerea* (*Botryotinia fuckeliana*). Proceedings of the 11th Biennial Conference of the Australasian Plant Pathology Society, 29 Sept. - 2 October, Perth, Australia. p. 142.

Clarification of terms

Some terms are used inconsistently through this thesis. Selenate resistant (SelR and Sel1R) mutants are sometimes referred to as selenate mutants. NaSeO4 and SeO4 and selenate are used interchangeably.

The term 'parent' refers to both the wild-type progenitor of mutants and both the sclerotial and fertilising strain in sexual crosses.

Chapter 1 Introduction

Botrytis cinerea

B. cinerea is a common pleomorphic fungus that causes 'grey mould' disease on a wide range of plants in temperate regions [Jarvis, 1977].

Taxonomy

Botryotinia fuckeliana (de Bary) Whetzel (anamorph *Botrytis cinerea* Pers.) is classified in the Ascomycota (Ascomycetes), order Leotiales and family Sclerotiniaceae [Hawksworth *et al.*, 1995].

Micheli established the genus *Botrytis* in 1729. *Botrytis cinerea*, one of Micheli's species, was named by von Haller in 1771 and included by Persoon in 1801 as one of five species of *Botrytis*. In 1822 Persoon increased the number of species to 27, by 1886 this had grown to 128 and was later expanded to 380 species. The genus has since been redefined three times by Whetzel, Buchwald, and Hennebert and 22 species are now recognised [Hennebert, 1973; Jarvis, 1977; Coley-Smith *et al.*, 1980]. For a long time *B. cinerea* was viewed by many as a group of related species, due to the wide variation in vegetative forms and the lack of an established sexual stage, and individuals were commonly referred to 'a *Botrytis* species of the *cinerea* type' [Whetzel, 1945].

De Bary first proposed a connection between the anamorph *B. cinerea* and its teleomorph which he named *Peziza fuckeliana* de Bary [de Bary, 1887]. The teleomorph was later transferred to the genus *Sclerotinia* by Fuckel in 1869 - *Sclerotinia fuckeliana* (de Bary) Fuckel 1869 [Gregory, 1949], and to *Botryotinia* by Whetzel in 1945 - *Botryotinia fuckeliana* (de Bary) Whetzel.

The question of a perfect stage connection, which had been debated for many years, was largely resolved when Groves & Drayton [1939] obtained apothecia from pure cultures of '*Botrytis* of the *cinerea* type' in the laboratory. Unfortunately they were unable to name their fungus because of doubts as to the exact identity of de Bary's *Peziza fuckeliana.*

The connection was not fully accepted until Gregory [1949] located a contemporary description of *Peziza fuckeliana* and *Botrytis cinerea* and re-examined de Bary's original slides of *Peziza fuckeliana*. Gregory also later identified apothecia produced from *B. cinerea* as identical to those of *Peziza fuckeliana* as described by de Bary [Groves & Loveland, 1953]. This has since been confirmed by the work of Faretra and coworkers [Faretra *et al.*, 1988b; Faretra & Pollastro, 1991, and 1993a] who carried out crosses of some 300 field-isolates from various host plants and countries and showed that they could produce viable progeny when crossed with *B. cinerea* reference strains. In a less comprehensive study of New Zealand field-isolates Beever & Parkes [1993] found that some failed to produce apothecia when crossed with reference strains, possibly due to the use of suboptimal growing conditions or alternatively to the inability of some isolates to participate in apothecial production. Theoretically such isolates could represent a distinct species but more information would be needed to demonstrate this. A recent study of field-isolates identified two non-interbreeding sympatric populations (vacuma and transposa), differing slightly in conidial size, co-existing in the Champagne region. The grouping is supported by the presence of two transposable elements, Boty and Flipper, in only one group and by differences in allelic frequency [Giraud *et al.*, 1997]. However, investigations of the two populations are continuing with studies of crosses between vacuma and transposa suggesting the conclusion, of two populations, may have been premature [T Giraud, pers. com.]. Until more information is available the question of speciation within *B. cinerea* will remain unanswered.

According to nomenclatural conventions the scientific name of the sexual stage *Botryotinia fuckeliana* should take precedence over the asexual stage *Botrytis cinerea* [Hawksworth *et al.*, 1995; Yoder *et al.*, 1986]. However, in the case of *B. cinerea* a pragmatic approach has been taken (as with *Aspergillus nidulans*, the asexual stage of *Emericella nidulans*) and the familiar binomial, which is recognised world-wide, has been retained although it is considered appropriate to include both names in publications concerning genetics [Faretra & Grindle, 1992]. Therefore in accordance with common usage the anamorph name *Botrytis cinerea* will be used throughout this thesis.

Morphology

Hyphae of *B. cinerea* are septate with septa perforated by a single pore, hyaline and indistinguishable from the hyphae of many Ascomycotina [Hennebert, 1973; Coley-Smith *et al.*, 1980]. Two asexual spore forms are commonly found; conidia (strictly macroconidia) and microconidia (Figure 1 p6). The main propagules for dispersal are

conidia, which are produced in large numbers on darkly pigmented straight conidiophores comprising a swollen basal cell and alternate branches at the apex. These short dark, septate branches each have a terminal ampulla on which conidia develop synchronously on short fine denticles. Conidia are 9 – 12 x 7 – 10 μm [Harrison, 1988], smooth, hydrophobic, globose, obovate or elliptical shaped, single celled spores with between 3 and 18 nuclei [Coley-Smith *et al.*, 1980]. The genus Botrytis, from the Greek βοτρυς (bunch of grapes), is named after the appearance of the conidia and conidiophores, which resemble a bunch of grapes. Germination is usually by one or two (sometimes up to five) germ-tubes and occurs more readily when supplied with exogenous nutrients (Infection p7). Grey mould, the common name for plant diseases caused by *B. cinerea*, comes from the grey appearance of masses of conidia on plant tissue [Coley-Smith *et al.*, 1980].

Microconidia, (Figure 1 p6) are hyaline, hydrophilic, globose spores 2 – 4 μm in diameter [Urbasch, 1985] produced in chains from short phialides inflated at the base and tapering to the apex [Coley-Smith *et al.*, 1980]. The phialides are produced from germ-tubes, mature hyphae, sclerotia, and also within empty hyphal cells [Brierley, 1918]. Microconidia develop to full maturity after release and when mature contain a single sickle-shaped nucleus and one or two large lipid bodies. Microconidia are believed to function as spermatia in the sexual cycle [Drayton, 1932; Coley-Smith *et al.*, 1980] although a survival function has also been proposed with specific conditions (as yet unknown) necessary to 'break dormancy' [Urbasch, 1985].

Germination and growth of microconidia on artificial medium and successful infection of wounded plant tissue was reported by Brierley [1918]. Grindle [1979] also reported successful germination but other researchers have been unable to repeat this work. Urbasch [1985] found that microconidia occasionally developed a short germ-tube (1 μm) but never observed hyphal formation. Microconidia of *B. fabae* were found to germinate following extended exposure to cold temperatures [Harrison *et al.*, 1977]. However, similar treatment of *B. cinerea* microconidia was unsuccessful in inducing germination [Urbasch, 1985].

In response to adverse conditions the fungus is reported to produce structures made up of aggregations of 1,000 to 3,000 microconidia (30 to 90 μm diam). These are initially held together by mucilage but later a sac forms around them [Urbasch, 1984]. Microconidial aggregates have not been reported by other workers, although structures similar to those pictured by Urbasch were observed in some microconidial suspensions prepared for mating experiments during the course of this study.

Other structures associated with *B. cinerea* include chlamydospores, sclerotia, and apothecia. Chlamydospores of *B. cinerea* have been reported in only a few studies; on dried stems of an Umbellifer [Price, 1911], in nutrient enriched soil solutions [Park, 1954], on malt agar (under stressed growing conditions), on greenhouse tomatoes [Urbasch, 1983], and on greenhouse and outdoor plants of *Fuchsia hybrida* [Urbasch, 1986]. They are described as large (60 – 70 μm), thick walled structures produced as terminal or intercalary cells under adverse conditions and liberated by hyphal disintegration [Price, 1911; Urbasch, 1983]. Mature chlamydospores are resistant to drought, nutrient deficiency, and bacterial invasion and tolerate a wide pH range suggesting a role as survival structures [Park, 1954; Urbasch, 1986]. They appear to be well suited to short term survival of adverse conditions and could have a role in over-summering on crops such as grapes, or as a component in latent infections, but little information is available (Disease development p8). Chlamydospores germinate only when conditions are favourable, to produce either mycelium or microconidia [Urbasch, 1986].

Sclerotia are flat or concave on the attachment surface with a pigmented rind (1 or 2 cells thick), a cortex of loosely packed cells, and a medulla of interwoven cells [Backhouse & Willetts, 1984]. The size varies between strains but is typically between 1 and 6 mm diam. [Harrison, 1988]. Although commonly regarded as important for nutrient storage and survival during adverse conditions there is little quantitative data on the survival of sclerotia in the field. Some reports suggest they can survive long periods on the soil surface but much less if they are buried, especially if the soil is wet [Harrison, 1988]. Sclerotia can germinate in four ways; to produce mycelium (common on agar media at temperatures above 15°C), to produce conidiophores (more common in the field), to produce microconidia, or under special conditions to produce apothecia [Coley-Smith *et al.*, 1980; Lorenz & Eichhorn, 1983; Backhouse & Willetts, 1985].

Apothecia are produced from sclerotia following fertilisation, possibly with microconidia as the spermatia. Stipes grow from the sclerotial surface developing a cap at about 4 – 5 mm which differentiates into a light brown cupulate disk (1 – 6 mm diam.) becoming reflexed and dark brown to black with age (Figure 2 p6). Final height is typically from 3 – 10 mm with a maximum of 25 mm. Long clavate asci with an apical pore are produced on the upper surface each containing eight hyaline, unicellular, ovoid ascospores measuring about 13.5 x 6.7 μm interspersed with sterile paraphyses (Figure 1 p6) [Coley-Smith *et al.*, 1980; Lorenz & Eichhorn, 1983; Jarvis, 1977; Faretra & Antonacci, 1987]. The ascus initial is binucleate; during development the two nuclei fuse leaving one nucleus in

the young ascus, which then divides to produce eight uninucleate ascospores. The nucleus in each ascospore undergoes further division while still in the ascus to produce multinucleate ascospores (typically with 4 nuclei) [Lorenz & Eichhorn, 1983; Coley-Smith *et al.*, 1980; Faretra & Antonacci, 1987]. Apothecia are formed only in light and are positively phototropic [Coley-Smith *et al.*, 1980].

Pathology

B. cinerea is a necrotrophic fungus with an extremely wide host range causing grey mould disease on a large number of economically important vegetable, flower, and fruit crops in temperate regions [Sinclair *et al.*, 1987; Agrios, 1988]. Infection may develop during cultivation in both field-grown and protected crops, and post-harvest in storage or transit. Data on the possible existence of host specificity are conflicting. Field-isolates of *B. cinerea* taken from one host were shown to vary in aggression when inoculated onto another [van den Heuvel, 1976], although no evidence was presented to show that the interactions conform to the 'quadratic check' indicative of a gene-for-gene system [Day, 1974; Elad & Evensen, 1995]. In contrast, a recent analysis of RAPD markers (8 field-isolates from different regions and a variety of hosts) found no evidence for host specialisation and strains did not group according to host or geographical origin [van der Vlugt-Bergmans *et al.*, 1993]. This supports the generally accepted view that host specialisation does not occur in *B. cinerea.*

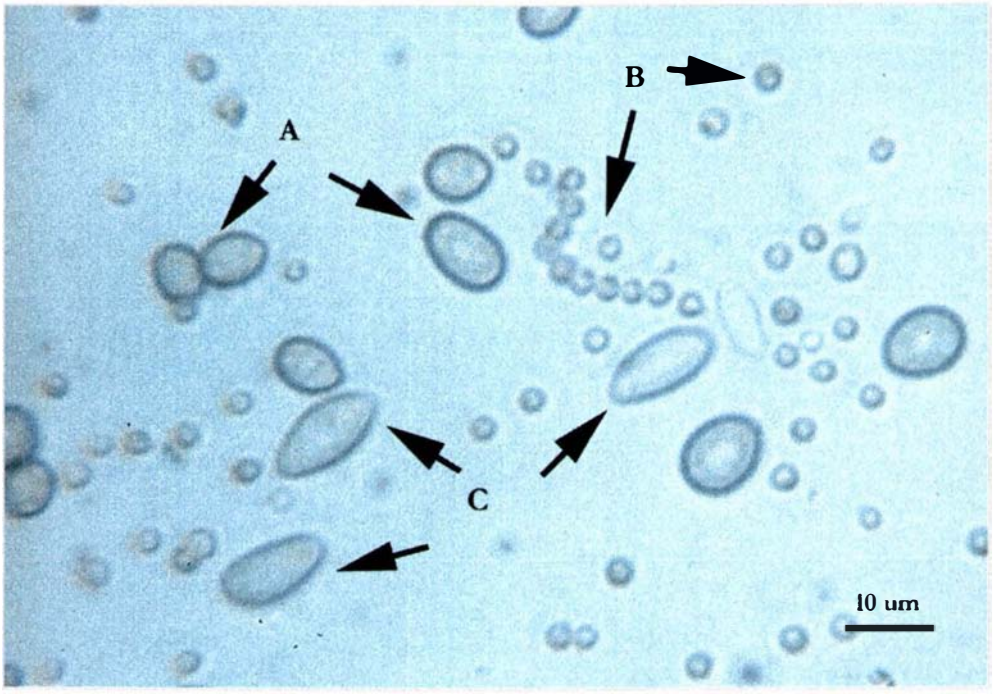


Figure 1 Spore forms of *Botrytis cinerea*. (A) Conidia from 10-day-old cultures grown on MEA at 20°C . (B) Microconidia from 1-month-old cultures grown on MEA in the dark at 15°C. (C) Ascospores isolated from apothecia.

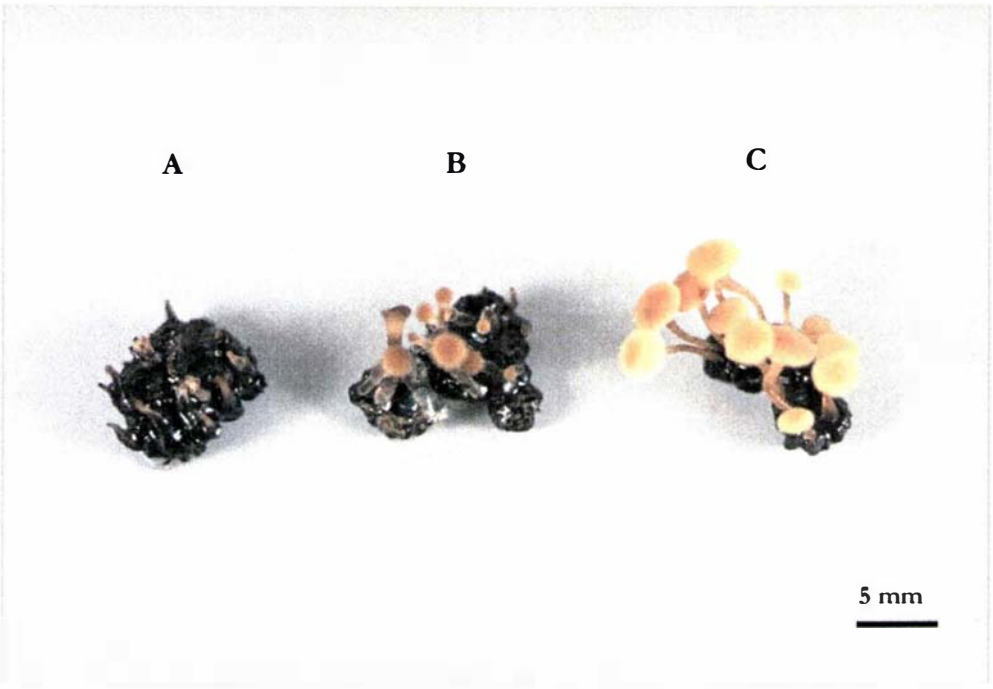


Figure 2 Growth stages of apothecia after 4 months incubation at 10°C following standard mating procedure. (A) Apothecial stipes developing from a sclerotium. (B) Young apothecia. (C) Mature apothecia.

Infection

Grey mould disease is most damaging as a soft rot, blossom blight, or stem canker, but mycelium can also spread internally in stems (e.g. tomatoes, grapes), or grow saprophytically on senescing or dead tissue [Sinclair *et al.*, 1987; Coley-Smith *et al.*, 1980]. In addition *B. cinerea* can cause a pre-emergence damping-off disease under cool wet conditions [Coley-Smith *et al.*, 1980; Agrios, 1988] and is known to be seed transmitted by surface contamination in some crops [Burgess *et al.*, 1997]. Lesions can appear as brown areas under dry conditions, but rapidly become covered in whitish, grey-brown, 'furry mould', consisting of masses of conidia and conidiophores when humidity is high. Infected fruit and soft tissues (e.g. strawberries and lettuce) become soft and watery, while lesions on stems (e.g. tomato) can develop into sunken brown cankers (Figure 3 A-D p9).

B. cinerea requires a nutrient supply for infection of most tissues [Heale, 1992]. Artificial inoculations of conidia usually require a supply of exogenous nutrients to achieve high infection rates [Rossall & Mansfield, 1981; Harper & Strange, 1981] and this requirement may be supplied by leaf or wound exudates under natural infection conditions [Heale, 1992].

Infection occurs, either from germinated conidia through plant cuticles and wounds, or when mycelium invades healthy tissue from an adjacent infection such as saprophytic growth on senescing flower parts or from one grape berry to another in a bunch [Coley-Smith *et al.*, 1980]. Conidia germinate in conditions of high humidity and penetrate the host either directly through the epidermis, as with dry inoculation of rose flowers [Williamson *et al.*, 1995], or via an infection peg in association with single or multi-lobed appressoria [Garcia-Arenal & Sagasta, 1980]. Penetration through stomata has also been observed [Garcia-Arenal & Sagasta, 1980]. The type of penetration (ie. direct or via appressoria) has been shown to be influenced by the concentration of the spore suspension with higher concentrations of conidia giving more direct penetrations [van den Heuvel & Waterreus, 1983]. In addition Garcia-Arenal & Sagasta [1980] found more complex penetration structures with higher nutrient levels in the inoculum. Light and electron microscopy studies of the infection site suggested that both mechanical and enzymatic factors are involved in penetration [van den Heuvel & Waterreus, 1983; Garcia-Arenal & Sagasta, 1980; McKeen, 1974]. In addition conidia inoculated with exogenous nutrients produced a matrix material enclosing the germ-tubes and providing attachment to the leaf surface [McKeen, 1974; Cole *et al.*, 1996]. While no matrix material has been observed with dry inoculated spores, traces were detected at the site of penetration using

immunolabelling techniques and the monoclonal antibody BC-KH4 [Cole *et al.*, 1996]. Infection of damaged plant tissues (e.g. hail damaged grape berries) occurs more readily than through intact plant surfaces [Coley-Smith *et al.*, 1980].

Disease development

Disease development varies between hosts. On grapes the disease first appears in spring on senescing flowers and may become latent in the necrotic flower tissue attached to the developing berry. When the berries ripen the fungus resumes growth and rots the grape, often spreading via mycelium when berries in the bunch are touching [Creasy *et al.*, 1997; McClellan *et al.*, 1973; Coley-Smith *et al.*, 1980] (Figure 3A p9). Conidia produced on the rotting bunch are spread by air currents to healthy berries where infection may occur through wounds or through fine cracks in the cuticle. The fungus over-winters either as mycelium under bud scales and on senescing leaves, or as sclerotia on the plant or among litter on the soil surface. When conditions become suitable in spring these germinate to produce conidia for primary inoculation [Agrios, 1988; Grindle, 1979; Pearson & Goheen, 1988].

B. cinerea infections in greenhouse tomatoes are most troublesome during the winter months, usually beginning in wounds made by the removal of lateral shoots, producing stem cankers that girdle the stem and can kill the plant (Figure 3D p9). Flower and fruit infections are also common, the latter resulting in either ghost-spots caused by latent or abortive infections on young fruit, or post-harvest soft rots [Eden *et al.*, 1996b; Coley-Smith *et al.*, 1980; Jones *et al.*, 1991].

Growth and sporulation are favoured by cool humid conditions (18 – 23°C) but *B. cinerea* is still active at temperatures between 0 and 10°C [Agrios, 1988]. Many crops are stored at these temperatures where plastic lined packaging and poor ventilation result in high humidity around the fruit. Conidia can contaminate wounds or stem-scars in the field and germinate during storage causing a soft rot that can then spread to adjacent fruit (e.g. 'nesting' in kiwifruit Figure 3C p9) [Brook, 1992].

Figure 3 Examples of grey mould disease on various hosts. (A) Bunch rot of grapes (B) Petal fleck on glasshouse grown rose flowers (C) Soft rot of kiwifruit and infection spread during storage resulting in 'nesting'. (D) Stem canker caused by infection of lateral shoot removal wounds in glasshouse tomatoes. (Photos from the collection of the Plant Science Dept. Massey University)

The sexual stage

The importance of the sexual stage in the life cycle of *B. cinerea* and in the epidemiology of grey mould disease has not been established, and reports of apothecia in the field are rare [Verhoeff *et al.*, 1992]. De Bary described apothecia of *Botryotinia fuckeliana* collected from grape in Switzerland in 1864 [de Bary, 1887] and Istvanffi is reported to have collected an apothecium growing from a sclerotium attached to a grapevine in 1930 [Whetzel, 1945]. A more recent report is given by Polach and co-workers [Polach & Abawi, 1974; Polach & Molin, 1975] who found *B. cinerea* apothecia in bean fields (*Phaseolus vulgaris*) and apple orchards at different locations in New York State in two subsequent years. Identification was confirmed morphologically and by pathogenicity tests. In New Zealand the only report of *B. cinerea* apothecia occurring in the field was from a peach mummy in the Hawkes Bay in 1993 [Beever *et al.*, 1997] (Figure 4 p11).

It has been a popular belief among researchers that the sexual stage occurs very infrequently and is generally unimportant in disease epidemiology. The scarcity of reported findings would seem to support this. However, reports from studies that have actively looked for, and failed to find, apothecia are even scarcer [Braun & Sutton, 1987]. The reports available show that environmental conditions suitable for apothecial formation are found in a number of different locations around the world and studies of field-isolates from many different countries have demonstrated the widespread occurrence of both mating-types (*MAT1-1*, *MAT1-2*, Mating-type p21) [Faretra *et al.*, 1988b; Faretra & Pollastro, 1991, and 1993a; Beever & Parkes 1993; Akutsu *et al.*, 1996; Delcan & Melgarejo, 1996].

It may be that apothecia are more common but have been missed due to their small size and ephemeral nature. Or, as apothecia of *B. cinerea* closely resemble apothecia from other fungal species such as *Sclerotinia sclerotiorum* (Figure 5 p11), they may have been wrongly identified. Indirect evidence has been found from a study of 259 field-isolates using a variety of genetic markers, that the sexual stage occurs frequently although a parasexual explanation is also possible [Giraud *et al.*, 1997].

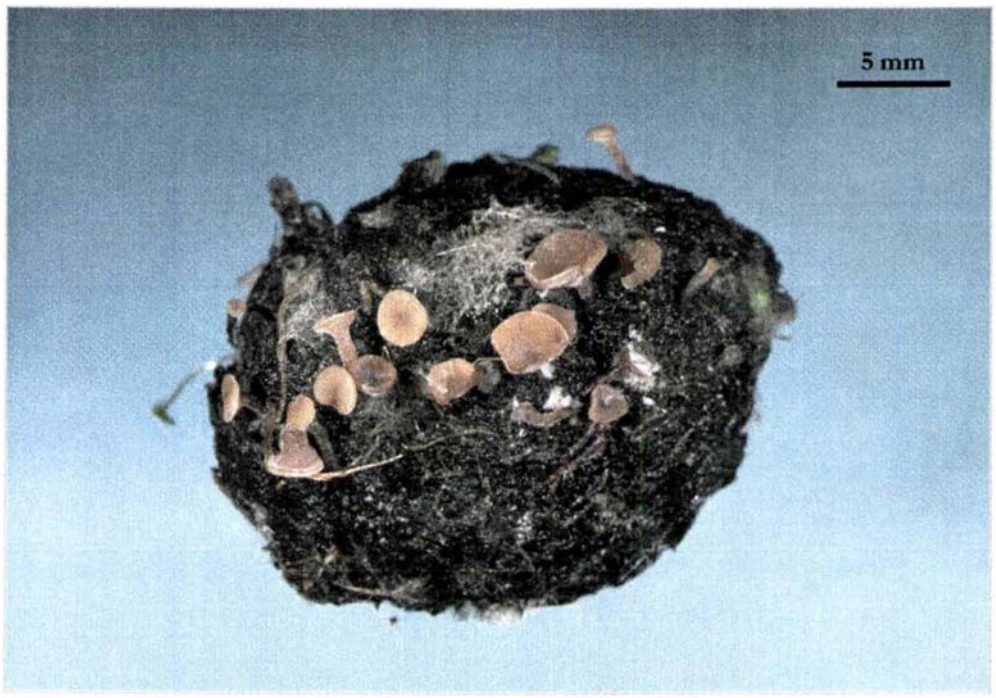


Figure 4 Apothecia growing on a peach 'mummy' found by G. Tate and co-workers in a commercial peach orchard ('Golden Queen'), Hawkes Bay (New Zealand), in September 1993. (Photo R. E. Beever). The specimen has been lodged in the Landcare Fungal Herbarium (Herb. PDD 65768).

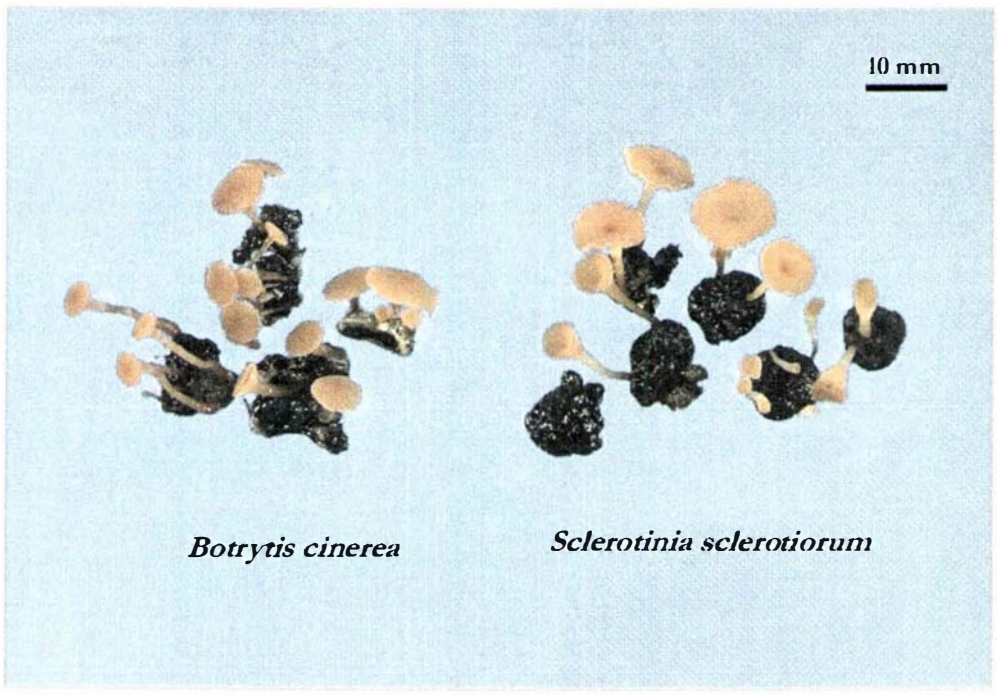


Figure 5 Apothecia of *Botrytis cinerea* (produced in the laboratory as described p33) and *Sclerotinia sclerotiorum* (produced in the laboratory from sclerotia pre-conditioned by burial in soil for 6 months and then incubated on moist paper towels under 12-hours-on/12-hours-off cycled white and long-wave ultraviolet radiation for 10 days).

Host pathogen interactions

Fungal attack compounds

As *B. cinerea* hyphae grow in the host tissue a large number of fungal attack compounds (enzymes and toxins) are secreted which macerate the tissue ahead of the advancing hyphae (expansion zone). Compounds associated with cell death in the expansion zone include; phenol degrading enzymes such as laccase, cell wall degrading enzymes such as polygalacturonase (PG), pectin-lyase (PL), pectin-methyl-esterase (PME), acid-proteinase, cellulase, and phospholipases, and toxins such as organic acids (citric and oxalic acid) and glucans [reviewed Kamoen, 1992]. In addition, multiple isoforms have been identified for many enzymes for example polygalacturonase, pectin-esterase, and pectin-lyase, and the number of isoforms varies both between isolates and in the same isolate under different growing conditions or infection stages [Leone *et al.*, 1990; Leone & van den Heuvel, 1987; Magro *et al.*, 1980; Di Lenna & Fielding, 1983; Marcus & Schejter, 1983]. This suggests either that these enzymes may have more than one function, or that enzyme redundancy is the norm. Redundancy could reflect environmental heterogeneity with specific isozymes conferring an adaptation to a particular niche.

As many attack compounds are secreted early in the infection process (spore germination and penetration) they have been extensively studied as determinants of pathogenicity in *B. cinerea* (summarised in Appendix 7 p137). Most of these works have proposed a pathogenicity-determining role for the enzyme of interest from a correlation of high enzyme activity with aggressiveness on a specific host. Studies using this approach found no evidence of such a role for PG enzymes (4 studies) or PME (4 studies) although one reported a negative correlation for the latter. Examples where a correlation between enzyme activity and aggressiveness was found include; polymethylgalacturonase in 2 of 3 studies, PL (2 studies), protease (3 studies), laccase (2 of 3 studies), and active oxygen species (3 studies). Results obtained by this method are frequently contradictory and definitive evidence of the importance of any one enzyme has been difficult to establish, probably due to differences between isolates and/or hosts and numerous other factors still poorly understood. The situation would be further complicated if enzymes and toxins acted together.

Recent studies seeking to clarify the role of attack compounds have used a gene disruption approach. Van Kan *et al.* [1997] showed that aggressiveness of *B. cinerea* on gerbera and tomato remained unaltered in mutants deficient in the cutinase-A gene. In

contrast disruption of a polygalacturonase gene reduced aggressiveness of *B. cinerea* on tomato leaves [Have *et al.*, 1997].

Host defence compounds

Host plants respond to fungal invasion by forming various defence compounds such as phytoalexins, enzyme inhibitors, and physical barriers, for example lignin. Phytoalexins known to be induced by *B. cinerea* include; resveratrol and pterostilbene in grape [Hoos & Blaich, 1990; Pezet & Pont, 1990], wyerone acid in broad bean [Harrison, 1988], phaseollin, phaseollidin, and phaseollinisoflavan in French bean [van Den Heuvel & Grootveld, 1978, and 1980; Fraile *et al.*, 1980], and 6-methoxymellein, p-hydroxybenzoic acid and polyacetylene in carrot [Defago & Kern, 1983; Mercier *et al.*, 1993]. The effectiveness of these chemicals in plant defence depends on the ability of the plant to respond to infection by rapidly producing high concentrations at the site of infection. The importance of defence chemicals in host/pathogen interactions is supported by their connection with resistance, for example studies on grape showed a strong correlation between resistance to *B. cinerea* infection and the ability to synthesise phytoalexins [Pezet & Pont, 1990]. Also, non-aggressiveness of some field-strains of *B. cinerea* on French bean has been correlated with their inability to break down phytoalexins [van den Heuvel & Grootveld, 1978].

In addition to phytoalexins and fungitoxic substances, compounds inhibitory to fungal attack enzymes are also found. Inhibitors of *B. cinerea* polygalacturonase cell wall degrading enzymes (PGIP's) have been identified in tomato [Stotz *et al.*, 1994], apple, marrow, and grape [Fielding, 1981; Yao *et al.*, 1995], pear [Sharrock & Labavitch, 1994], and raspberry [Johnston *et al.*, 1994]. There is considerable interest in cloning PGIP genes for use in developing crop varieties with increased resistance to infection by *B. cinerea* [Labavitch *et al.*, 1996; Ramanathan *et al.*, 1996].

Physical barriers to fungal invasion involving phenolic substances and lignification have been shown to be overcome by laccase enzymes produced by *B. cinerea* [Viterbo *et al.*, 1992]. Substances that inhibit laccase were found to protect the host plant from infection. Cucumber plants treated with cucurbitacins, which repress the formation of laccase in *B. cinerea*, developed a lignified layer at the infection site preventing fungal infection [Viterbo *et al.*, 1993].

A greater understanding of plant defence mechanisms will assist in developing new strategies for increased plant resistance.

Noble rot

B. cinerea is a serious disease of grapes often causing severe losses and requiring expensive control measures. However, if infection occurs when the grapes are mature and environmental conditions are suitable, the grapes decay slowly and can be used to make sweet dessert wines (e.g. Sauternes and Trockenbeerenauslese) [Jarvis, 1977]. For this beneficial *B. cinerea* infection, known as noble rot (pourriture noble in France), to develop the grapes must be at full maturity and healthy with intact skins when infected. Humid (or foggy) nights ensure growth of the fungus and dry sunny days evaporate water from the berries resulting in high sugar concentrations [Jarvis, 1977; Coley-Smith *et al.*, 1980]. Producing wine by this method is risky as a wet season may result in total loss and even when conditions are favourable the yield is greatly reduced, but the final product is highly prized and can give excellent financial returns. Attempts to artificially induce infection on harvested grapes have only been moderately successful [Coley-Smith *et al.*, 1980].

Control

Control of *B. cinerea* is hampered by its wide host range and ability to infect plants both during growth and in storage [Coley-Smith *et al.*, 1980]. The specific environmental conditions of temperature and humidity required for germination provide a focus for cultural control measures. Cultural practices that reduce humidity and the presence of free water on fruit, leaves, and flowers, such as increased ventilation and leaf removal, have been shown to reduce infection [Hammer & Marois, 1989; English *et al.*, 1993]. In addition, disease pressure from conidia and overwintering sclerotia can be reduced by sanitation practices, and natural host resistance can be enhanced by curing resulting in a significant reduction in storage rots caused by *B. cinerea,* for example in kiwifruit [Pennycook & Manning, 1992; Ippolito *et al.*, 1994].

Despite advances in crop management and storage practices, control of *B. cinerea* diseases still relies heavily on the use of synthetic chemicals. The main systemic chemicals used against *B. cinerea* are the benzimidazole and dicarboximide fungicides, although the rapid development of resistance in fungal populations has limited their efficacy (Fungicide Resistance p21). Fungicide-use strategies recommend the use of mixtures or rotation of fungicides (including protectants such as Euparen which are less prone to resistance problems) to reduce the build up of resistant populations [Walton *et al.*, 1995], and monitoring disease levels to ensure better timing of fungicide applications [Nair *et al.*, 1997]. New approaches to resistance monitoring are also being developed to assist in the implementation of fungicide-use strategies. For example the use of polymerase chain

reaction (PCR) to give rapid identification of benomyl resistant strains to assist in monitoring field populations [Luck & Gillings, 1995].

Increasing public and market concerns about the safety of control chemicals has led to residue limits for many export crops [Sas, 1997] and a growing interest in developing alternative control methods for *B. cinerea* diseases. Numerous studies have shown effective reduction of *B. cinerea* infection using biological control agents such as yeasts, filamentous fungi, or bacteria, although this work is still largely in the developmental stages [reviewed Elad *et al.*, 1996]. A preparation of *Trichoderma harzianum* (brand name Trichodex) is registered for use in greenhouses in Israel, Australia and some other countries, but is not yet available in New Zealand. Biological control organisms have been selected and trialed for use on greenhouse crops in New Zealand [Eden *et al.*, 1996a], but registration is hampered by the high cost of toxicology testing.

Genetic studies of *B. cinerea*

Phenotypic variation

Cultural conditions have been shown to modify some morphological features of *B. cinerea* making them of uncertain value in taxonomy and possibly explaining some of the confusion in this genus (Taxonomy p1) [Paul, 1929; Jarvis, 1977]. Studies of different field-isolates have also shown high levels of variability in morphology, pathogenicity, and quantitative and qualitative production of pathogenicity related enzymes [Hansen & Smith, 1932; Di Lenna *et al.*, 1981; Leone, 1990; Grindle, 1979].

The most widely accepted explanation for variation in field-isolates of *B. cinerea* (and many other fungi) is heterokaryosis. Heterokaryosis exists when two or more genetically different nuclei inhabit the same cell or mycelium, and was first proposed by Hansen & Smith [1932] to explain the variation found in isolates of *B. cinerea* following repeated single-spore isolations. Hansen [1938] studied the phenomenon in 900 isolates of 30 genera of imperfect fungi including *B. cinerea* and concluded that various characteristics of these fungi such as culturing variation, sectoring, reversions and changes in virulence, could occur through heterokaryosis maintained in multinucleate conidia. The work of Summers *et al.*, [1984] which showed that 0.5% of single-conidial progeny from a single-spored dicarboximide sensitive strain were dicarboximide resistant and 0.2% were dicarboximide sensitive from a dicarboximide resistant strain strongly supports an explanation of heterokaryosis. Heterokaryosis may arise in two ways, via somatic mutation (which in multinucleate vegetative fungi could result in significant differences between

nuclei residing in the same mycelium), and via the transfer of nuclei between strains through anastomosis (hyphal fusion).

Since Hansen's study [1938] considerable progress has been made in understanding vegetative incompatibility systems and consequently the factors limiting anastomosis [reviewed by Leslie, 1993]. Little is known about the process in *B. cinerea* although evidence for a vegetative incompatibility system has been demonstrated [Beever & Parkes, 1993]. Beever and Parkes found pigmented interaction zones between isolates paired on NaCl amended malt extract agar indicating mycelial incompatibility, one of several events associated with vegetative incompatibility [Kohn *et al.*, 1990]. These zones appear to be similar in appearance to the interaction zones in the barrage phenomenon as described by Leslie [1993] and also to the reaction lines described by Kohn *et al.* [1990] for *Sclerotinia sclerotiorum*, a fungus in the same family as *Botrytis*. Interpretation of these interactions by analogy with other ascomycete systems suggested at least 5 or 6 vegetative compatibility (VC) loci differences between the strains tested [Beever & Parkes, 1993]. In order for a compatible reaction to occur two strains must be identical at all VC loci suggesting that heterokaryon formation between two strains by anastomosis is probably rare in nature. Studies of vegetative compatibility systems in *B. cinerea* have been hampered by difficulties in obtaining stable Nit mutants [Alfonso *et al.*, 1996].

Other processes that might generate high levels of variability include heteroploidy (variation in chromosome number) or cytoplasmic factors, such as viruses or transposable elements (Cytology p17). Jinks [1959] points out that the evidence for heterokaryosis presented by Hansen & Smith [1932] and Hansen [1938] does not rule out a cytoplasmic origin of variation. DsRNA viruses can be transmitted via hyphal fusion [Ghabrial, 1994]. However, although Howitt *et al.* [1995] found dsRNA in 143 of 200 field-isolates of *B. cinerea* there was considerable variation in profiles both within and between populations indicating that transmission between isolates in the field is probably not common: further evidence that anastomosis between field-isolates is most likely rare. Transposable elements have been identified in *B. cinerea* including 'Boty' a long-terminal repeat retroelement [Diolez *et al.*, 1995] and 'Flipper' resembling a bacterial transposon [Giraud *et al.*, 1997], but as yet little is known of their occurrence and spread or how much they might contribute to genetic variation. Although current research (as discussed above) suggests that anastomosis between strains of *B. cinerea* is unlikely to be common, there is evidence in *Neurospora* of cytoplasmic genetic elements being transmitted without stable cellular

fusion [Kistler, 1992] suggesting that the inability for two strains to form a heterokaryon may not exclude the possibility of some genetic exchange.

Cytology

Light microscopic studies of *B. cinerea* nuclei at metaphase found 16 chromosomes ranging in size from 0.6 to 1.3 μm, with an additional thread-like structure on one chromosome [Shirane *et al.*, 1988]. Further studies showed that *B. cinerea* (5 isolates), *B. byssoidea* (3 isolates), *B. squamosa* (1 isolate), and *B. tulipae* (1 isolate) all had 16 chromosomes, while from a total of 7 *B. allii* isolates tested, 4 had 16 chromosomes and 3 had 32 chromosomes [Shirane *et al.*, 1989]. However, Tolmsoff [1983] suggests that chromosome counts by this method should be regarded as the minimum chromosome number due to the extremely small size of fungal chromosomes and the difficulties inherent in the method.

Although *B. cinerea* usually behaves as a haploid fungus with some genetic markers segregating in a 1:1 Mendelian fashion in sexual crosses, there are a number of reports of aberrant segregation patterns suggesting some form of heteroploidy. Self-fertility of single-ascospore strains has been found in a number of studies [Lorenz & Eichhorn, 1983; Faretra *et al.*, 1988b; Faretra & Pollastro, 1996]. In addition, segregation analysis of DNA polymorphisms in the progeny of one cross showed that while most segregated in a normal Mendelian ratio (1:1), several segregated 1:0, three markers from one parent were not found in any of the progeny, and two new markers (not in either parent) segregated 1:1 in the progeny [van der Vlugt-Bergmans *et al.*, 1993].

Heteroploidy (e.g. aneuploidy, polyploidy) has been found in numerous ascomycete fungi including *Neurospora crassa*, *Aspergillus nidulans*, *Saccharomyces cerevisiae*, *Verticillium dahliae* and *Verticillium albo-atrum* [Tolmsoff, 1983]. In a study of *B. cinerea* nuclei by cytofluorometry using the fluorescence dye DAPI, both field-isolates and single-ascospore strains were found to vary greatly in the DNA content indicating that aneuploidy or polyploidy may be widespread; an hypothesis supported by the increased sensitivity to mutagens of benomyl derived putative haploid strains [Buttner *et al.*, 1994]. In addition, electrophoretic karyotype analysis (showing size and number of chromosomes) using the clamped homogeneous electric field (CHEF) showed a different pattern for each strain indicating aberrant length polymorphisms [van Kan *et al.*, 1993; Vallejo *et al.*, 1996]. It has been suggested that extensive polymorphism may be inversely correlated to the frequency of meiosis as ploidy is defined and enforced at meiosis [Kistler, 1992]. However, the relative ease with which field-isolates of *B. cinerea* from many countries can be crossed

with reference strains in the laboratory demonstrates the potential for meiosis present in these populations [Faretra *et al.*, 1988b; Faretra & Pollastro, 1991, and 1993a; Beever & Parkes [1993]. These findings show that the genetic structure of *B. cinerea* is still poorly understood and many fundamental issues remain unresolved.

Tools for genetic analysis

Sexual crossing

Because the sexual stage of *B. cinerea* was uncommon early genetic studies used field-isolates. The highly variable nature of these field-isolates (Phenotypic variation p15) made it difficult to compare results obtained from one study with those from another. Groves & Drayton [1939] were the first to produce apothecia under laboratory conditions using a complex method involving 5 different temperature and light regimes, growth of sclerotia on sterilised wheat grains and sclerotia incubated under a covering of soil. The original protocol produce apothecia in approximately 7 months, but this was later improved and shortened to 5 – 6 months [Groves & Loveland, 1953]. From their studies Groves & Loveland [1953] demonstrated that *B. cinerea* was heterothallic by showing that apothecia were produced only when pairs of strains from two different groups were crossed. However, Lorenz & Eichhorn [1983] using the same method showed that some isolates of *B. cinerea* are homothallic (22 from 56 field-isolates tested and 5 of 6 single-ascospore strains). Faretra and co-workers made considerable improvements to the technique used to produce carpogenically active sclerotia [Faretra & Pollastro, 1988] and to the conditions necessary for production of apothecia [Faretra *et al.*, 1988a] resulting in more consistent and higher yield of apothecia under controlled environmental conditions (Crossing procedure p33). The improved technique, although still requiring 4 to 5 months for apothecial production, gives relatively reliable, reproducible results and has opened the way for formal genetic analysis of *B. cinerea.*

In a cross of field-isolates Faretra & Pollastro [1992] reported evidence for a major gene causing short apothecia with a rapid synchronous ripening pattern designated *Sma* (small apothecia). Further crosses revealed no evidence of linkage to the mating-type gene (*MAT1*), but indicated that nuclei carrying the *Sma+* (wild-type) allele are dominant over nuclei carrying *Sma-*. Near-isogenic lines carrying *Sma-* may be useful in improved crossing procedures.

Molecular genetics

The molecular genetics of *B. cinerea* is still in the developmental stages in comparison with many other fungi. Attempts to transform *B. cinerea* using protocols from other fungal systems have been successful but generally give a low transformation frequency. Hilber *et al.* [1994] used a biolistic method to transform conidia with the pOHT plasmid carrying hygromycin phosphotransferase gene (*hph,* conferring hygromycin B resistance), giving a maximum transformation frequency of 1.7 transformants per μg of plasmid DNA. This was improved by a protoplast method of Chabani [1991] and Hamada, [1994] using the plasmid pAN7-1 carrying *hph*, which gave 9 and 5 transformants per μg respectively.

Mutagenesis

Genetic studies of *B. cinerea* have been hampered by a lack of suitable genetic markers. Those traditionally used for classical genetics, including morphological, auxotrophic, and fungicide resistant markers, have often proved difficult to obtain. Mutants derived from field-isolates by spontaneous mutagenesis, u.v. irradiation or chemical mutagens such as methanesulphonate and acridine mustard (ICR-170) tended to be unstable and frequently reverted to wild-type after repeated sub-culturing [Grindle, 1979; Geeson, 1978]. Difficulties in obtaining stable mutants of *B. cinerea* have sometimes been attributed to the multinucleate nature of conidia [Grindle, 1979]. The uninucleate microconidia could prove to be a better source of mutants but recent attempts to induce them to germinate have proved unsuccessful (Morphology p2).

Recently, stable auxotrophic and fungicide resistant mutants have been obtained from single-ascospore strains, either as spontaneous sectors or following treatment with u.v. radiation or ethylmethane sulphonic acid (EMS) [Hilber, 1992; Faretra & Pollastro, 1993b; Chabani, 1991; Buttner *et al.*, 1994]. To date only fungicide resistant mutants have been studied in detail.

Mutagenesis of *B. cinerea* using molecular biological techniques has been reported in four very recent studies. Van Kan and co-workers used gene disruption techniques to generate mutants for pathogenesis studies that were deficient in cutinase A (*cutA*), using a transformation vector with the target gene promoter from *B. cinerea* and the GUS reporter gene, and a polygalacturonase enzyme (*Bcpga1*) expressed early in pathogenesis [Van Kan *et al.*, 1997; Have *et al.*, 1997]. Also with the aim of studying pathogenesis, Tudzynski and co-workers have generated non-aggressive mutants using restriction-enzyme mediated integration (REMI) transformation, and are continuing with the process of isolating and analysing the tagged genes [Beermann *et al.*, 1996]. This method has an important

advantage over other methods of mutagenesis as the individual mutation is tagged by the integration event and is thus more easily isolated and identified. The fourth study, another work in progress, involved selection of five insertion (plasmid pAN7-1) non-aggressive mutants screened on apples from 500 transformants [Kunz *et al.*, 1996]. It's likely that improvements in protocols for these and similar techniques will encourage more such studies in the future.

Genetic markers

As yet there are relatively few genetic markers available for *B. cinerea* and most studies have used fungicide resistance (*Mbc1* and *Daf1*) or mating-type (*MAT1*) markers. The absence of readily available, stable auxotrophic or physiological markers has stimulated interest in DNA polymorphic markers. A study of eight field isolates and two single-ascospore strains by RAPD analysis scored 139 markers (using 50 different primers), 65 of which showed polymorphisms [van der Vlugt-Bergmans et al., 1993]. When combined with improved crossing techniques [Faretra et al., 1988a] RAPD analysis can be a powerful tool in genetic analysis.

Terminology for describing pathogenesis

There is ongoing debate on the appropriate terminology for reference to different levels of pathogenicity [Shaner *et al.*, 1992; Andrivon, 1993; Hunt, 1994; Andrivon, 1995], with pathogenicity, virulence and aggressiveness being the most frequently used terms. Pathogenicity has been used qualitatively to describe the capacity of a pathogen to cause disease with reference to an individual strain, to a genus, a species or other commonly accepted grouping [Shaner *et al.*, 1992]. It has also been used quantitatively to describe the amount of infection caused by a disease organism, for example high or low pathogenicity [McDonnell, 1962; Bos & Parlevliet, 1995]. In this study the use of pathogenicity will follow MacHardy [1996]: to express the ability of a population of the pathogen to cause disease under defined conditions. Virulence has most often been used quantitatively as a measure of pathogenicity, usually in reference to gene-for-gene interactions. To avoid confusion, virulence will be restricted to discussions of gene-for-gene interactions and will distinguish individuals of a population that differ in their ability to cause disease. Aggressiveness has been used synonymously with both pathogenicity and virulence. Vanderplank [Federation of British Plant Pathologists, 1973] advocated that aggressiveness be used where races of a pathogen differed in the severity of their pathological effects, but do not interact differentially with host varieties and virulence where there is differential interaction with varieties, a distinction that will be used here.

Therefore, since there is no evidence for gene-for-gene resistance in *B. cinerea* (Pathology p5), strains reduced in pathogenic ability will be referred to as non-aggressive.

The terms isolate and strain are sometimes viewed as synonyms. The Federation of British Plant Pathologists [1973] defines isolate as 'single pure culture made by direct isolation from fresh material and any subcultures made from it' and will be used here to refer to cultures originating from single-spore isolations from diseased material. When the diseased material has been collected in the field the resulting single-spore isolation will be referred to as a field-isolate. The same reference provides five definitions to the term strain including 'a group of similar isolates' and 'the descendants of a single isolation in pure culture' but none were considered suitable for use here. In this study, strain will be used for cultures originating either from a single-ascospore arising from a sexual cross or from mutagenic treatment of a single spore demonstrating defined characteristics.

Mating-type

Faretra *et al.* [1988b] confirmed that *B. cinerea* has a bipolar mating system and designated the two alleles as *MAT1-1* and *MAT1-2*. Although most field-isolates tested were heterothallic (self-sterile, either *MAT1-1* or *MAT1-2* and only able to produce apothecia when fertilised with a strain of the other mating-type), a few were found to be homothallic (self-fertile and also able to produce apothecia when fertilised with strains of either mating-type) and were designated *MAT1-1/2*. This secondary homothallism (self-fertility of normally heterothallic isolates) was attributed to the heterokaryotic nature of many field-isolates (Phenotypic variation p15). However, some strains derived from single-ascospores by Faretra were also found to be homothallic, consistent with the earlier findings of Lorenz & Eichhorn [1983]. Heterokaryosis does not readily explain this behaviour. Faretra & Pollastro [1996] also reported homothallic progeny from a cross of homothallic single-ascospore strains. Possible explanations for homothallism in strains derived from single-ascospores include; (1) heterokaryon formation by the inclusion of two different nuclei in one ascospore during formation in the ascus [Dodge, 1927], (2) heteroploidy [Buttner *et al.*, 1994; Tolmsoff, 1983], and (3) mating-type switching as described for *Schizosaccharomyces pombe* [Beach *et al.*, 1982].

Fungicide Resistance

Studies of reduced efficacy of some fungicides used for *B. cinerea* control revealed widespread development of fungicide resistant field-isolates (Control p14) [reviewed Dekker & Georgopoulos, 1982; Beever *et al.*, 1989]. Faretra and Pollastro [1991] showed

that in *B. cinerea* resistance to benzimidazole (e.g. benomyl, carbendazim) and dicarboximide (e.g. vinclozolin, iprodione) fungicides were each encoded by mutations of a single gene. The gene associated with benzimidazole resistance was designated *Mbc1* (methyl benzimidazole-2-ylcarbamate = carbendazim) with three classes of alleles recognised, *Mbc1S* (sensitive), *Mbc1LR* (low resistance), and *Mbc1HR* (high resistance); and dicarboximide resistance as *Daf1* (dicarboximide and aromatic hydrocarbon fungicides) with the alleles likewise *Daf1S*, *Daf1LR*, and *Daf1HR*. These dicarboximide resistant strains show a pleiotropic effect of high osmotic sensitivity [Faretra & Pollastro, 1991; Beever & Parkes, 1993; Faretra & Pollastro, 1993a]. However, an allele conferring ultra-low level dicarboximide resistance (*Daf1UR*) has been recognised that is wild-type for osmotic sensitivity [Beever & Parkes, 1993]. Most laboratory induced phenylpyrrole CGA 173506 (Ciba-Geigy) mutants were found to be highly osmotically sensitive, cross resistant to dicarboximide fungicides, and cosegregated with *Daf1* in sexual crosses [Hilber, 1992; Faretra & Pollastro, 1993b] suggesting the *Daf1* locus controls resistance to both fungicides. The segregation pattern of one mutant strain suggested the presence of a second *Daf* locus designated *Daf2* [Faretra & Pollastro, 1993b].

Daf1 and *Mbc1* were found to be unlinked to the *MAT1* mating-type gene (Mating-type p21) or to the *Sma* (small apothecia, Sexual crossing p18) gene, but showing loose linkage to each other in some crosses [Faretra & Pollastro, 1991; Faretra & Pollastro, 1992; Faretra & Pollastro, 1993a; Beever & Parkes, 1993].

It has been shown in many fungal systems that the fungicidal activity of benzimidazole compounds results from binding to the β-tubulin protein and inhibition of microtubule function [Davidse, 1986; Koenraadt *et al.*, 1992]. The cloned sequence of the β-tubulin gene from *B. cinerea* showed a high degree of homology with β-tubulin from other filamentous fungi. Carbendazim (Mbc) and diethofencarb (NPC) resistant strains of *B. cinerea* were shown to have single base pair mutations that correlated with the three different phenotypic classes (MbcHR/NPCS, MbcHR/NPCR, and MbcS/NPCR) indicating that the active site in *B. cinerea* is also the β-tubulin gene [Yarden & Katan, 1993].

The mechanism for dicarboximide resistance has not yet been determined [Beever & Byrde, 1982; Ellner, 1994]. However, in a recent paper the *os-1* protein of *Neurospora crassa* was shown to code for an osmo-sensing histidine kinase believed to play an important role in regulation of cell-wall assembly and osmotic responses [Schumacher *et al.*, 1997]. As *os-1*⁻ mutants are osmotically sensitive and resistant to dicarboximide fungicides (similar

to *Daf1* mutants in *B. cinerea*) it is possible that the *Daf1* locus likewise encodes for a histidine kinase protein.

Objectives of this study

The paucity of genetic information and lack of understanding of the factors controlling pathogenicity in *B. cinerea* hampers initiatives aimed at developing alternative, non-chemical based disease control methods. The aim of this work was to use a mutational approach to the study of pathogenesis in *B. cinerea* by:

- Developing new genetic markers.
- Generating non-aggressive mutants.
- Studying the inheritance of pathogenicity through sexual crossing of non-aggressive mutants.
- Investigating morphological and biochemical difference between aggressive and non-aggressive strains.
- Studying the interaction of aggressive and non-aggressive strains on a plant host.

Chapter 2 General Methodology

Storage

Four different methods were used to store *B. cinerea* cultures. Field-isolates, mutants and selected progeny from sexual crosses were routinely stored as dried conidia on silica gel [Perkins, 1977]. Ascospore suspensions from individual apothecia were preserved in 15% glycerol at -80 °C, and large numbers of single-ascospore cultures were stored on malt extract agar (MEA) slopes in sealed plastic tubes at 6°C for up to 18 months. Conidial suspensions in 0.01% Tween 20 or Tween 80 were stored for short periods (1 - 2 d) at 4°C and for longer periods (to 8 months) at -20°C.

Media and growth conditions

Composition and preparation of growth media is given in Appendix 2 (p120). Unless otherwise stated cultures were incubated in plastic petri dishes (85 mm diam.) at 20 – 25°C under a 12-h-on/12-h-off cycle of white fluorescent (Crompton 40 w) and long-wave ultraviolet radiation (Essellese blacklight). Mycelial plugs (5-mm diam.) for inoculation were taken from the margin of colonies actively growing on MEA. Conidial suspensions were prepared by scraping spores from 7 - 10-d-old cultures grown on MEA and flooded with 0.01% Tween 20 or Tween 80. The suspension was filtered through glass wool or 70 µm plastic mesh cell strainers (Falcon) to remove mycelial fragments, and adjusted to the required concentration using a Spencer bright-line haemocytometer. Where appropriate to avoid carry-over of nutrients, spore suspensions were washed by centrifuging twice for 4 min at 130G and resuspended in 0.01% Tween 80.

Botrytis strains

Details of strains are given in Appendix 3 (p127). F. Faretra (Bari, Italy) supplied strains SAS405 and SAS56. The *nit1* mutant Mn012 was supplied by S. L. Parkes (Landcare Research Mt Albert Research Centre). Genetic nomenclature is summarised in Appendix

5 (p134) and the source of genetic symbols used in *B. cinerea* is given in Appendix 6 (p136).

Growth restricting medium

Genetic studies of fast growing filamentous fungi such as *B. cinerea* frequently use a media additive to restrict colony growth in solid media allowing isolation of single spore colonies from plates inoculated with dilute spore suspensions. Sorbose, a commonly used additive, gave inadequate restriction with *B. cinerea* in preliminary tests. This is consistent with Netzer [1973] who found sorbose did not restrict *B. cinerea* and used a sorbose amended medium to differentiate between *B. cinerea* and *B. allii*. However, Madelin [1987] found Triton N-101 was able to restrict growth of *B. cinerea* and other fast growing fungi in air sampling experiments. Triton X100 was tested with two other surfactants, (SBDS and Nonidet) in complete medium at four concentrations between 0.0005% and 5.0% for their restrictive effects.

Triton X100 at 0.05% gave the most useful colony restriction, single spores produced small dense colonies (2 – 3 mm) with compact tufts of conidia after 3 days at 20 – 24°C, and gave good germination (90%). On SBDS and Nonidet the colony size was either too small or the germination was variable.

Mutagenesis

Conidia or mycelial plugs from single-ascospore strains were used in all mutagenesis work to ensure genetic uniformity. Although mature ascospores typically contain around 4 nuclei these nuclei have been shown to originate from a single nucleus (Morphology p2) and are assumed to be genetically identical (homokaryotic). Single-conidial isolates were not used as conidia may be heterokaryotic and single sporing gives no guarantee of homokaryosis [Summers *et al.*, 1984; Hansen & Smith, 1932].

Mutants were selected without mutagenesis (spontaneous), following short wave ultraviolet radiation (u.v.), or chemical mutagenesis using the mutagen 4-nitroquinoline-1-oxide (NQO).

Spontaneous mutations

Mutations occur spontaneously in somatic cells at a low frequency [Fincham & Day, 1963]. Mutants with specific spontaneous mutations can be selected by incubating fungi on selective media on which only the desired mutant is able to grow.

Three-day-old mycelial plugs were inverted on selective medium (3 or 4 per plate). Plates were sealed with laboratory film (Parafilm M, American National Can$_{TM}$) and checked weekly for up to 4 weeks for sectors arising from the plug margins. Sectors were purified by one transfer to fresh selective medium and plugs from the growing edge of the sub-cultures were transferred to MEA and allowed to grow and sporulate before storing on silica gel.

Ultraviolet radiation

Mutagens change DNA by promoting errors in replication or repair. U.v. radiation produces intra-strand cyclobutane-type dimers of adjacent pyrimidines (among other products) which are believed to be the major cause of the mutagenic effect of short wave u.v. light. In *Escherichia coli* three mechanisms have been recognised for repair of pyrimidine dimers; photoreactivation, excision repair, and postreplication repair. When unrepaired damaged DNA replicates it produces discontinuous daughter strands with gaps about 1,000 nucleotides long corresponding to the dimer in the parent strand. These gaps represent secondary damage caused by the replication of DNA-containing dimers and are repaired by a post replication repair system [Witkin, 1976]. If spores are kept in the dark following irradiation the photoreactivation system is unable to function and mutations are induced in the DNA by the inaccurate repair of gaps.

The dose of u.v. radiation required to produce a 2 – 5% survival was determined. Spore suspensions (5 ml, 1x10^6 conidia ml^{-1}) were irradiated in an open petri dish (5-cm diam.) on a magnetic stirrer under a short wave length u.v. lamp (Sylvania germicidal 8w) and aliquots of 100 µl were removed at 0.5, 1 and 1.5 min and then at one-min intervals to 10 min, plated onto complete medium (CM) and incubated at 20°C. Irradiation and plating procedures were carried out under red photograph safe light to avoid photo reactivation repair (Kodak filter no.1).

The percentage of germinated spores was determined from a count of 400 after 8-h incubation and gave a typical survival curve with a sharp decline in percent germination after 1 to 3 min and by 4 min all spores were dead. Subsequent experiments with an exposure time of 2.9 min gave 5% survival (Figure 6 p28). When the u.v. lamp was replaced the procedure was repeated and a time of 2 min was found to give 5% survival.

4-nitroquinoline-1-oxide (NQO)

NQO was chosen for mutagenic work because it can be inactivated with a solution of sodium thiosulphate, making handling and disposal easier and safer than with other

chemicals [Bal *et al.*, 1977]. NQO is known to mimic the biological effects of u.v. light and it has been shown in a number of organisms that DNA damaged by NQO is repaired by the same systems as u.v. damaged DNA [Ikenaga *et al.*, 1975; Prakash *et al.*, 1974]. Mutation by NQO results from covalent binding of NQO to DNA producing NQO-guanine and NQO-adenine adducts [Ikenaga *et al.*, 1975]. Prakash *et al.* [1974] showed that NQO acts specifically on G·C base pairs inducing G·C → A·T transitions and G·C → T·A transversions at a high frequency.

The method used for NQO mutagenesis was as described by Bal *et al.* [1977]. A spore suspension ($1x10^7$ conidia ml^{-1} in a 0.1 M potassium buffer) was incubated in a shaking water bath at 25°C for 30 min before adding NQO to give final concentrations of 4, 5, or 6 μg ml^{-1} (preliminary experiments gave approximately 50% survival for a concentration of 1 μg ml^{-1}). A control treatment without NQO was included. Spore suspensions were incubated with NQO for a further 1 h and an equal volume of 5% sodium thiosulphate was added to inactivate the mutagen. Spore suspensions were then centrifuged (5 min at 130 G) and resuspended in 0.1M phosphate buffer to give a concentration of $1x10^4$ (control) and $1x10^5$ ml^{-1} (NQO treatments). Aliquots of 100 μl were spread onto CM+Triton and incubated for 2 d and colony numbers counted under a dissecting microscope (5 plates per treatment). Colony counts were compared to the unmutated control and the percentage survival calculated.

A concentration of 4 μg ml^{-1} gave a mean survival of 3.5% (Figure 7 p28). Survival percentages were found to vary slightly between experiments so in subsequent experiments NQO treated conidia were incubated in these three concentrations (4, 5, and 6 μg ml^{-1}) and individual colonies were selected from the concentration that gave a survival of between 1 and 5%.

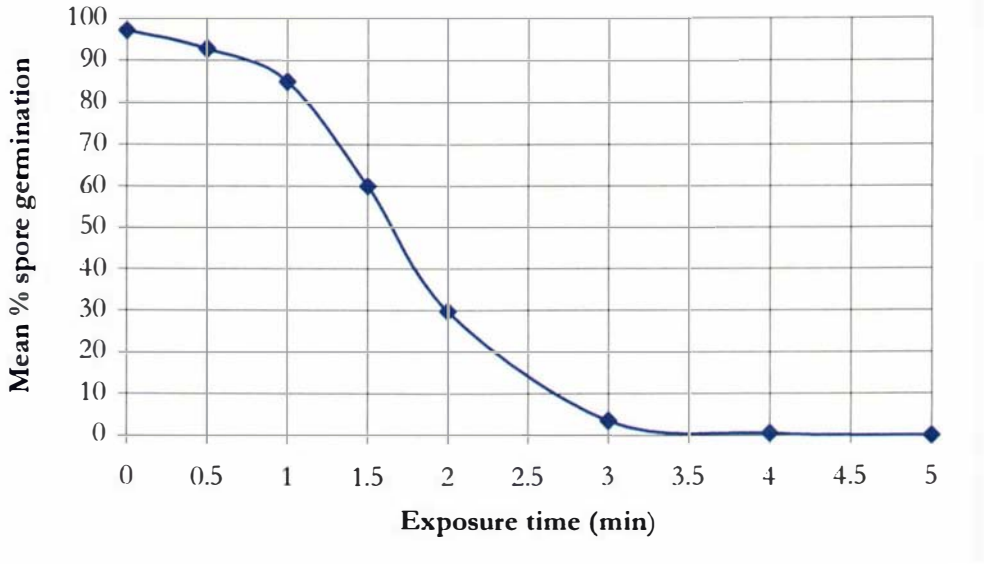


Figure 6 Percentage of conidia surviving after exposure to ultraviolet radiation. Assessed as percent spore germination on CM after 8 hours incubation at 20°C.

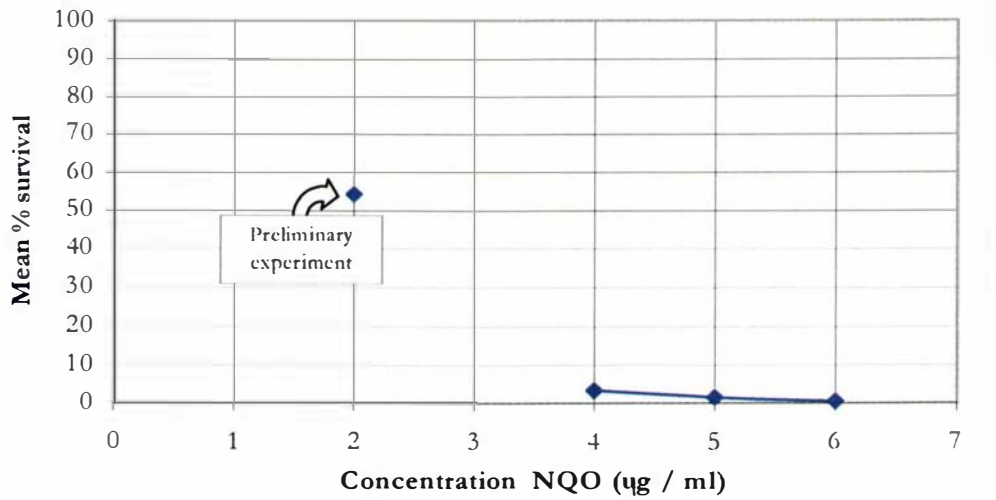


Figure 7 Percentage of conidia surviving after incubation in 4-nitroquinoline-1-oxide for 1 hour. Assessed as colonies counted using a dissecting microscope following 2-days incubation on CM+Triton.

Pathogenicity assays

Assays for pathogenicity were used to screen putative non-aggressive mutants and progeny from crosses, for analysis of aggression, and to investigate competition at the infection site by different strains of *B. cinerea*. The host material chosen needed to be readily available, uniform in cultivation and disease susceptibility, uncontaminated by systemic fungicides, compact (to enable experiments to be carried out in a small space) and give a fast turn around enabling results to be obtained in 3 – 4 d. French bean (*Phaseolus vulgaris*) met the above criteria, its use in pathogenicity tests with *B. cinerea* is well documented and so it was chosen as the main host plant.

French bean

The method followed van den Heuvel [1976]. French bean seedlings (*Phaseolus vulgaris* cv. Top crop) were grown to the two-primary-leaf stage in either washed potting mix grade pumice, or vermiculite, in an unheated glasshouse without added nutrients. The roots were excised and the stem and leaves suspended on a plastic rack over a tray of water with the cut ends immersed. Mycelial plugs of *B. cinerea* were inverted onto the upper leaf surface. A light misting of RO water was applied to the leaves prior to inoculation and although preliminary experiments showed this did not significantly affect lesion development it was helpful in preventing the plugs from falling off the leaves. Inoculated plants were incubated in one of two ways. First, plastic racks of bean seedlings were placed in large metal trays (50 x 810 x 430 mm, 3 racks per tray) which were sealed in plastic bags to maintain a high humidity. This method was suitable for screening large numbers and was used for bulk screening of putative mutants (Figure 21, p85). For all other purposes racks were placed in individual plastic trays (50 x 30 x 15 cm) and enclosed in a plastic bag. Trays were incubated for 3 d at 20 – 25°C on the laboratory bench under a combination of daylight and white fluorescent light during the day. Disease lesions were recorded as radial spread from the plug to the lesion margin. Three aspects of the assay protocol were investigated; the effect of wounding the leaf at the site of inoculation, the importance of the age of the inoculum, and the variability of the assay.

Effect of wounding

The effect of wounding was tested by stabbing beneath the inoculum plug with a needle. Leaves were inoculated with plugs cut from colonies of field-isolate REB658-1 grown on MEA. Wounded and unwounded leaves were arranged in 4 blocks (different trays) of 12 replicate plugs each (one plug per leaf, 2 leaves per plant). The average lesion

radius was 8.29 mm (SE 0.30) and 8.58 mm (SE 0.28) in wounded and unwounded treatments respectively. There was no significant difference between treatments (P = 0.46) so wounding was not used in subsequent experiments.

Age of inoculum

Two protocols were used for experiments involving plug inoculations on French bean. When relatively small numbers of strains were tested or large numbers of replicate plugs were needed, the inoculum was grown on full sized petri dishes of MEA (8.5 cm) where colony growth typically reached the plate margin after 3 – 4 d. When large numbers of strains were tested, cultures were grown in 1.5 cm wells in 24 well tissue culture plates (TC plates, Geiner No. 662160: each well contained 0.5 ml of MEA amended with 4 g l^{-1} additional agar, MEA+A). As colony growth covered the 1.5-cm wells in 1.5 to 2 d at 20°C these plates were grown at 10°C to allow the maximum time window for fast and slow growing strains to produce sufficient mycelium for inoculation onto the bean leaves. At this temperature the well was covered with mycelium in 3 – 4 d when plates were inoculated at the margin and two plugs were taken from the opposite edge of the well for inoculum. The effect of colony age on lesion size was tested with both protocols using strain A4.

On full sized plates at 20°C lesion size was greatest with 2-d-old colonies (8.80 mm SE 0.23), medium for 4-d-old colonies (7.36 mm SE 0.18), but poor for 6-d-old colonies (3.14 mm SE 0.42) (Figure 8 p31). To ensure sufficient inoculation material, plugs were taken after 3 – 4 d as a compromise between optimum lesion size and colony growth. The mean lesion size of 9.1 mm (SE 0.28) caused by 4-d-old plugs from TC plates incubated at 10°C was adequate for measurements, and plugs from this age of colony were used subsequently (Figure 9 p31).

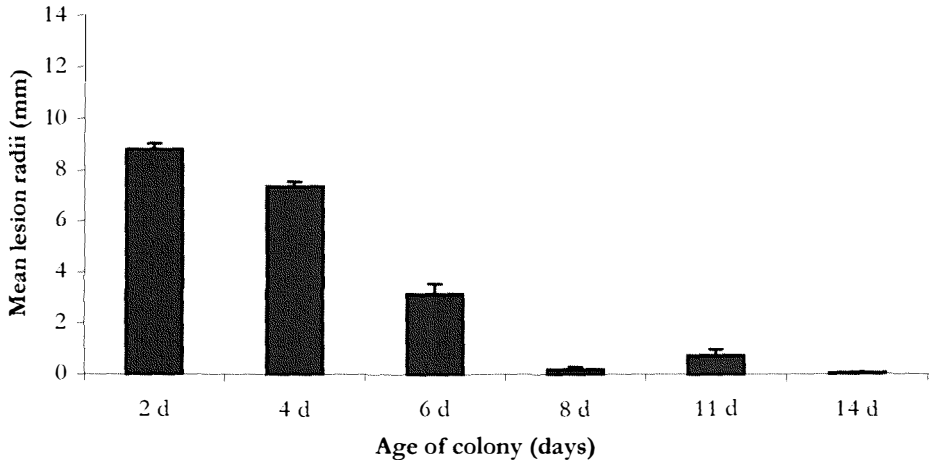


Figure 8 Average lesion radii on French bean leaves at 3d following inoculation with mycelial plugs from the margin of different aged colonies of A4 grown on MEA at 20°C. Bars indicate standard error of means from 32 replications.

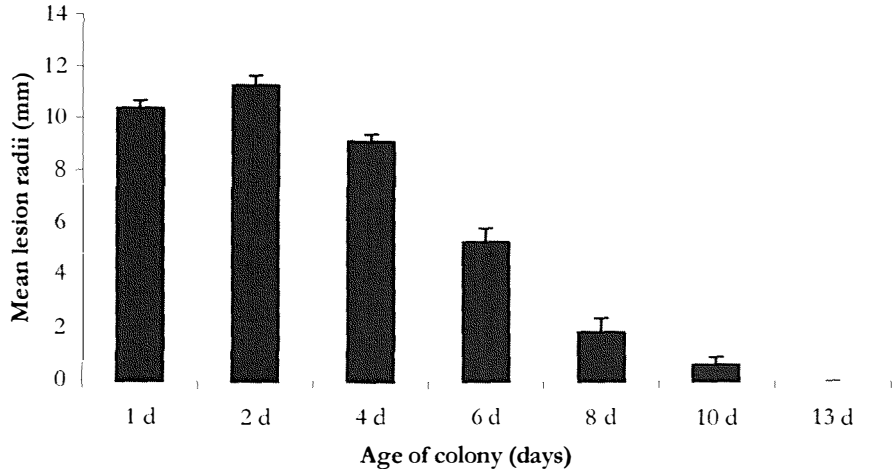


Figure 9 Average lesion radii on French bean leaves at 3d following inoculation with mycelial plugs from the margin of different aged colonies of A4 grown on MEA in tissue culture (TC) plates at 10°C. Bars indicate standard error of means from 24 replications.

Variability of the assay

While the assay method as described consistently gave 100% infection, a moderate amount of variation in lesion size was observed between replicates of the same strain in some experiments. To assess this variation the field-isolate REB658-1 and strain A4 were tested in a fully nested design using 3 blocks (treatments randomised on different racks in one tray) of eight plants, two plugs per leaf to give a total of 32 plugs per block.

No significant difference was found between strains or plants (P = 0.76 and 0.13 respectively) however, there was a significant difference between blocks (racks) and between leaves (P = 0.004 and 0.014 respectively) showing both the position in the tray and differences between leaves affected the lesion size. This variation and the difference in the maximum lesion size observed between the two age experiments described above (Figure 8 p31, Figure 9 p31), highlighted the need to thoroughly test putative mutants in repeated, well replicated assays.

Modifications to French bean assay for spore inoculations

In preliminary experiments inoculation of *B. cinerea* conidia suspended in water or in 0.01% Tween 80 gave a low rate of infection even on wounded leaves. It has been shown that the addition of exogenous nutrients to spore suspensions can markedly improve infection rates [Harper & Strange, 1981; van den Heuvel & Waterreus, 1983]. Therefore, two nutrient solutions were tested for their ability to assist infection by strain A4: phosphate+glucose solution containing 9.12 g l^{-1} of potassium dihydrogen phosphate (KH_2PO_4) with 19.8 g l^{-1} glucose, and Vogel's+sucrose containing 20 ml l^{-1} Vogel's N medium and 15 g l^{-1} sucrose. French bean leaves (not misted) were inoculated with washed spores resuspended in either nutrient solution to give concentrations of $1x10^3$, $1x10^4$, $1x10^5$, and $1x10^6$ spores ml^{-1} (5 µl in the centre of each leaf, 16 leaves per treatment). Inoculations of the nutrient solutions alone were included as controls. The tray was sealed in plastic bags and incubated for 4 d as above. Infection was recorded as percent infection and as mean radial lesion size of successful infections.

In an overall comparison of the two nutrient solutions (lesion radii data) the average lesion size for the Vogel's+sucrose solution was significantly larger than for the phosphate+glucose solution (12.18 mm, SE 3.72 and 8.75, SE 2.18 respectively; P = 0.0315) and the overall rate of infection was also higher (75% and 62% respectively). At lower spore concentrations ($1x10^3$ and $1x10^4$ spores ml^{-1}) there was no significant difference between the two solutions

* Lights were arranged as a bank of six 340 mm long 30 W cool-white fluorescent tubes (Philips) with two 340 mm long black light tubes (Esellese F40T12) and one 60 W tungsten double-life-plus bulb (Sylvania) suspended approximately 800 mm above the vials.

but at $1x10^5$ and $1x10^6$ spores ml^{-1} the Vogel's+sucrose solution significantly increased infection (Figure 10 p34). Vogel's+sucrose at $1x10^6$ spores ml^{-1} was used in all subsequent applications of the assay.

Problems were experienced in these trials with the inoculum droplet rolling off the leaf and in subsequent experiments 4-mm diam. plastic rings (cut from the barrel of 1 ml Beral disposable plastic transfer pipettes) were used to position the inoculum droplet.

Crossing procedure

The procedure for crossing and ascospore isolation was based on Faretra *et al.* [1988a] and Beever & Parkes [1993]. Cultures were grown on MEA in petri dishes at 15°C in the dark for 4 weeks then transferred to 0°C (in the dark) for a further 4 weeks. Crosses were made by transferring 8 – 15 sclerotia to narrow-necked glass McCartney vials (30 ml), each containing 2 ml sterile water, and adding 1 ml of a suspension of microconidia, conidia and hyphal fragments in sterile water prepared from the dishes in which the sclerotia were grown. Sclerotia fertilised with suspensions prepared from their own petri dishes were included as controls. The vials were incubated on their sides with loosely fastened caps under a mixture of white fluorescent, long-wave ultraviolet, and tungsten radiation (12-h-on/12-h-off) at 10°C for 3 – 5 months until apothecia formed (Figure 11 p35). Mating-types (*MAT1-1* or *MAT1-2*) were determined by successful production of apothecia with another strain of known mating-type. Single-ascospore strains that produced apothecia with strains of both mating-types were labelled *MAT1-1/2* (Mating-type p21). Parents for crosses were chosen to include fungicide resistance markers and segregation of these markers in equal ratios were interpreted as evidence of crossing.

Ascospore isolation

Single-ascospore strains were obtained by washing individual apothecia three times in sterile Tween 20 or Tween 80 (0.01 %), and dispersing the spores in 200-µl sterile water by grinding with a glass rod. The resulting ascospore suspension was adjusted to a $1x10^4$ ml^{-1} concentration and 20 µl was spread onto ½ strength PDA (PDA-½). After incubation for 16 h, germlings were transferred individually to MEA in screw capped tubes.

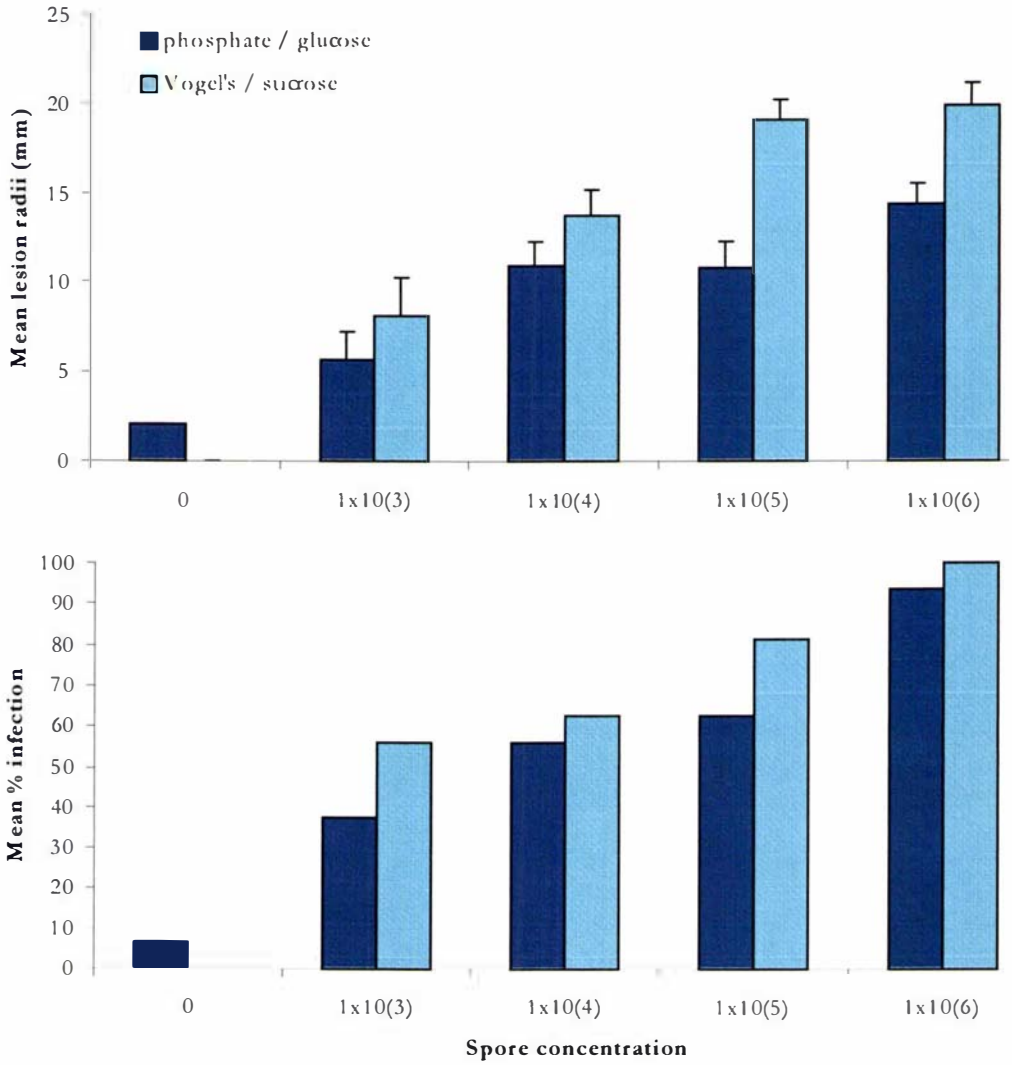


Figure 10 Lesion size and percent infection of *Botrytis cinerea* on French bean leaves following inoculation of conidial suspensions (strain A4) amended with phosphate glucose solution or Vogel's+sucrose. Bars indicate standard error of means from 16 replications.

Figure 11 Agar plate with sclerotia ready for harvesting and mating (left), McCartney vial containing sclerotia and apothecia (right rear), and sclerotia with apothecia (right front).

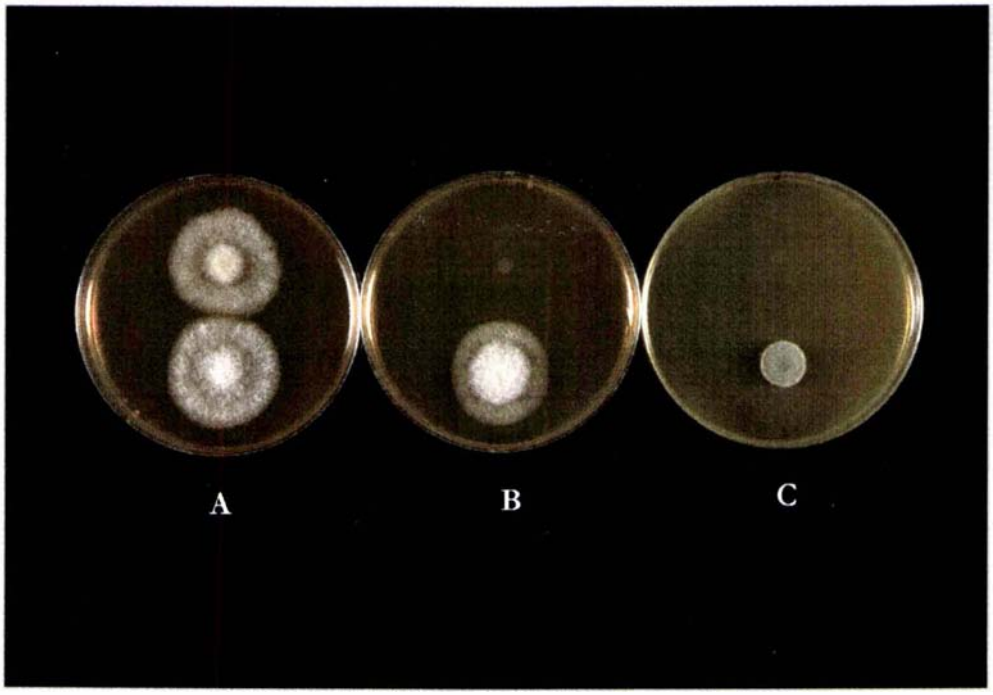


Figure 12 Test for fungicide resistance: SAS56 (*Mbc1S Daf1S*) upper and SAS405 (*Mbc1HR Daf1LR*) lower. (A) MEA. (B) MEA+carbendazim. (C) MEA+vinclozolin.

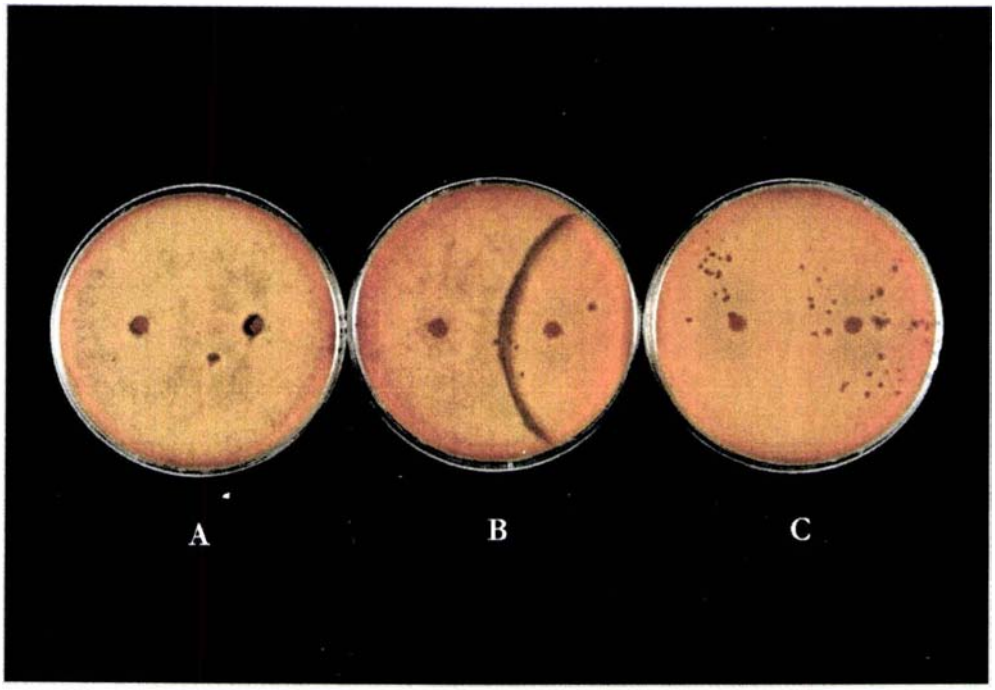


Figure 13 Test for mycelial incompatibility. (A) SAS56 paired with SAS56. (B) SAS56 paired with SAS405. (C) SAS405 paired with SAS405.

Testing for fungicide resistance

Strains were tested for fungicide resistance by adding 10-µl drops of spore suspension to MEA+carbendazim (100-mg ai. l^{-1}) and MEA+vinclozolin (100-mg ai. l^{-1}), [Beever *et al.*, 1989]. Carbendazim resistant strains produced spreading colonies after 2 d and vinclozolin resistant strains a compact pad after 3 d (Figure 12 p36).

Testing for mycelial incompatibility

Mycelial incompatibility (Phenotypic variation, p15) was tested as described by Beever & Parkes [1993]. Mycelial plugs were inverted 35 mm apart on MEA+NaCl medium and incubated in the dark for 14 d. The presence of a distinct dark interaction zone where the two colonies met was interpreted as mycelial incompatibility (Figure 13 p36).

Genetic terminology

Faretra and co-workers adopted the system of genetic nomenclature proposed by Yoder *et al.*, [1986] when naming the mating type (*MAT1-1* and *MAT1-2*) [Faretra & Pollastro, 1991] and fungicide resistance genes (*Mbc1* and *Daf1*) [Faretra & Pollastro, 1993a] of *B. cinerea*. Naming of new genes identified in this study also follows these guidelines (summarised Appendix 5 p134). In brief, a three letter symbol (first letter capitalised) is assigned to each gene/character, in italics for a genotype and in Roman type for a phenotype. A gene is assigned a locus identifying number in order of discovery (for example *Daf1, Daf2*). The letter R and S following indicates resistance or sensitivity and where the dominance relationships are known or plausibly supposed a dominant allele is indicated by three uppercase letters and a recessive by three lower case letters (e.g. *Mbc1HR, MAT1-1*, and *nit1* respectively). Three different nomenclatural systems are currently in use for chlorate resistant mutants (nitrate non-utilising), of filamentous fungi i.e. *Neurospora crassa* [described in Garrett & Amy, 1978], *Aspergillus nidulans* [Pateman & Cove, 1967], and *Fusarium spp.* [Correll *et al.*, 1987; Klittich & Leslie, 1989]. Of these only the latter follows Yoder *et al.* [1986] and is thus adopted here for *B. cinerea*. A summary of genetic symbols for *B. cinerea* is given in Appendix 6 (p136).

Data analysis

Unless otherwise stated all data was analysed using the SAS for Windows statistical package. Where the ANOVA was significant for treatment differences further analysis was carried out using general linear model procedures (GLM), multiple comparison tests

and contrasts. Where necessary data were transformed prior to analysis to stabilise variances and a P value of < 0.05 was interpreted as significant. The χ^2 calculations were performed using Microsoft Excel 97, values greater than 2.71 indicate differences significant at $P = 0.1$.

Chapter 3 New genetic markers for *B. cinerea*

Introduction

There are few markers available for use in genetic analysis of *B. cinerea* and mating-type and fungicide resistance genes have been used in most studies to date [Beever & Parkes, 1993; Faretra & Pollastro, 1993a] (Mating-type p21). The additional markers needed for this work were obtained by mutagenesis and selection using toxic analogues of common nutrients.

Selenium exists in nature as elemental selenium, metal selenides and selenite complexes [Doran, 1982]. Some microorganisms are able to oxidise these to soluble forms such as selenate and selenite, which can be taken up by plants, animals and other microorganisms [Doran, 1982]. Although a small amount is known to be essential to higher animals [Scott, 1973], selenium is toxic or inhibitory in many biological systems where it acts as a sulphate analogue. Selenate resistant mutants, altered in sulphate transport and assimilation, have been characterised in both *Aspergillus nidulans* [Arst, 1968; Gravel et al., 1970] and *Neurospora crassa* [Marzluf, 1970].

Chlorate, as an analogue of nitrate, is likewise inhibitory in various biological systems. Two groups of mutants have been generated in *A. nidulans* [Cove, 1976a] by selection on chlorate amended medium; Nit (nitrate non-utilising) mutants, which are resistant to chlorate and deficient in nitrogen assimilation, and Crn (chlorate resistant but nitrogen normal) mutants which are wild-type in nitrogen assimilation. Chlorate resistant mutants have also been characterised in other fungi including species of *Fusarium* [Puhalla, 1985; Correll *et al.*, 1987; Klittich & Leslie, 1989], and *Colletotrichum* [Brooker *et al.*, 1991; Vaillancourt & Hanau, 1994; Beever *et al.*, 1995].

The objectives of this section of the work were to develop new genetic markers for *B. cinerea* based on resistance to sodium selenate and to potassium chlorate and to cross mutant strains to produce single-ascospore strains carrying these markers.

Materials and Methods

Details of *B. cinerea* strains are given in Appendix 3 (p127). Preparation of spore suspensions and plugs, crossing procedure, ascospore isolation, and fungicide resistance testing are described in Chapter 2.

Selection of selenate resistant mutants

Two procedures were used. First, spontaneous sectors from mycelial plugs were incubated on sodium selenate medium (MM+SeO_4) as described previously (Spontaneous mutations p25). Second, resistant colonies were isolated on selective medium following u.v. mutagenesis of conidia. A spore suspension (1 x 10^7 spore ml^{-1}) was irradiated for 2.9 min (Ultraviolet radiation p26) to give approximately 5% survival. Aliquots of 100 µl were spread on MM+SeO_4, incubated in the dark for 5 d at 20°C, and resulting colonies were purified by transfer to fresh selective medium and then to MEA.

Selection of nitrate non-utilising mutants

Two procedures were used. First, spontaneous sectors were isolated from mycelial plugs as described previously (Spontaneous mutations p25) using chlorate selective medium (MM+ClO_3). Second, NQO mutagenesis was used as described previously (4-nitroquinoline-1-oxide (NQO) p26) and the resulting colonies were purified as above.

Selenate response test

Spore suspension (15 µl of 1 x 10^4 spore ml^{-1}) was spotted onto MM+taurine (0.1 g l^{-1}) amended with various concentrations of $NaSeO_4$ and incubated for 8 h. at 20°C. Percentage germination was determined from a count of 100 spores. EC50 values for selenate medium were determined by Drs P Connolly and R Ball using the statistical program Splus. A binomial generalised linear model was fitted with logit link to the proportion (p) of germinated spores. The model was, logit (p) = a + b*log (concentration) where a and b are intercept and slope parameters respectively [Chambers & Hastie, 1992].

Complementation tests

Two procedures were used. In method A, spore suspensions (1 x 10^6 spores ml^{-1}) of selected pairs of strains to be tested were mixed in a 1:1 ratio then 20 µl was pipetted onto the centre of a plate of MM+NO_3 and incubated for 10 d. In method B, plugs (5-mm diam.) from the selected pair were plated 20 mm apart on MM+NO_3 and incubated for 8 d.

Pathogenicity assay

Mutants were tested for aggressiveness on French bean seedlings (*Phaseolus vulgaris* cv. Topcrop), Racks of inoculated leaves (20 plants per rack) were incubated in individual plastic trays (50 x 30 x 15 cm), enclosed in plastic bags and incubated at 20 – 25°C under a combination of daylight and white fluorescent light (Pathogenicity assays p29)

Results

Sodium selenate resistant mutants

Spontaneous sectors arose readily as dense outgrowths at the plug margin from strains A1 and A5 and a total of 32 resistant sectors were isolated from 70 plugs. A background mutation rate of 1 in 2.8 x 10^7 viable spores was determined by plating conidia of strain A1 onto MM+SeO_4. This rate was increased approximately 20-fold following u.v. mutagenesis, and a further 18 selenate resistant mutants were recovered from this parent.

Selenate resistance was scored routinely by the presence of visible mycelial growth from plugs (5-mm diam.) after 5 d on MM+SeO_4. Three classes of mutants were distinguished by germination and growth on MM+SeO_4 and growth on chromate medium (MM+CrO_4) (Table 1 p42). Mutant classes were not correlated with selection method. Class A mutants had a high germination rate, produced fast growing spreading colonies on MM+SeO_4 (Figure 14 p43), and were resistant to chromate. Class B mutants had a low germination rate and produced slow growing restricted colonies on MM+SeO_4 (Figure 14 p43), but matched group A in resistance to chromate. Class C mutants were resistant to selenate (one mutant showed high resistance similar to group A, and two low resistance similar to group B), and were sensitive to chromate. Some mutants showed a distinctive response to taurine (putative sulphate non-utilisers) with dense growth on MM+taurine compared to thin sparse growth on MM, while others showed relatively thick growth on both MM and MM+taurine. The response of mutants to taurine did not correlate with selenate response (Table 1 p42). All SelR mutants remained vegetatively stable after storage on silica gel (Storage p24).

Table 1 Growth of selenate resistant mutants on minimal medium amended with sodium selenate, potassium chromate or taurine. Control strains (*Sel1S*) were A1, A5, REB678-1, SAS56, and SAS405.

		MM+SeO_4		MM+CrO_4		MM+taurine *
	Number of strains	Germination % (10 h incubation)	Radial growth (mm, 6 days incubation)	Germination % (10 h incubation)	Radial growth (mm, 6 days incubation)	Number of strains
Sel1S	5	0	< 0.5	0	0	-
Class A	8	high (> 80)	17 – 26	high (> 80)	8 – 24	6
Class B	39	low (< 35)	1 – 4	high (> 80)	8 – 24	7
Class C	3	mixed	mixed	0	0	3

* Taurine responsive i.e. thin sparse growth on MM and dense growth on MM+taurine

The response to various levels of selenate was determined for the mutants Ms010 (class A), Ms015 (class B), and a selection of wild-type strains (Figure 16 p44). Logit analysis gave EC 50 values greater than 3.00 mM for strain Ms010 and 0.93 mM (SE 0.087) for strain Ms015. Values for the wild-type strains were as follows: A1, 0.08 mM (SE 0.001); A5, 0.09 mM (SE 0.005); REB678-1, 0.10 mM (SE 0.005); and SAS405, 0.10 mM (SE 0.001).

Crosses of representative SelR mutants from class A and class B with wild-type strains (Table 2 p45, crosses 1 and 2), and one cross with class B recombinant progeny (Table 2 p45, cross 3), gave segregation ratios close to 1:1 with resistant progeny retaining the mutant parental phenotype. It was concluded that selenate resistance in these instances is conferred by a single gene. The genotype symbol *Sel1R* was allocated to class B selenate resistant mutants represented by allele Ms015. The mutant Ms015 was crossed with class A mutants to test for allelism and although a number of combinations produced apothecia the progeny were rejected as authentic crosses because fungicide resistance markers did not segregate 1:1 (i.e. could not be verified as a true cross, Crossing procedure p33). Therefore to test whether class A SelR mutants map to the same locus as *Sel1R* will require further genetic studies. The different phenotypes shown by the classes of SelR mutants suggest that more than one locus may be involved. There was no evidence of linkage between SelR resistance and either *Mbc1* or *Daf1* fungicide markers in any cross (Table 2 p45).

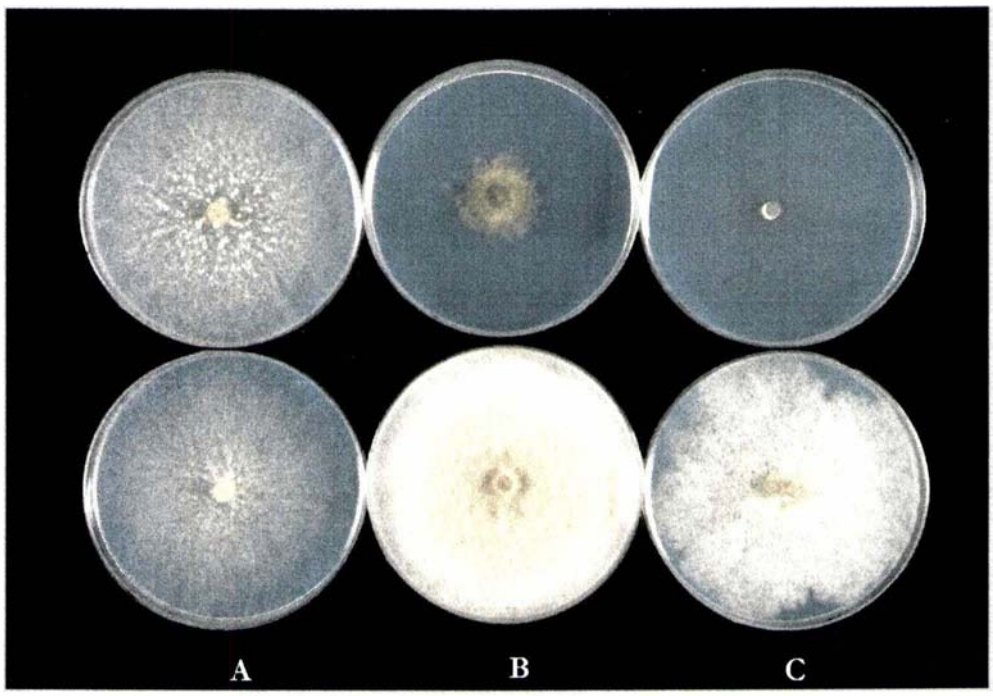


Figure 14 Growth responses of selected selenate resistant mutants of *Botrytis cinerea* on minimal medium amended with selenate plus taurine (MM+SeO_4, upper row) and on minimal medium amended with taurine (MM+taurine, lower row) after 6 days. Strains are; (A) P2-011 (SelR class A), (B) P5-026 (*Sel1R* class B), and (C) A1 (wild-type).

Figure 15 Growth responses of a *nit1* mutant of *Botrytis cinerea* on medium with nitrate as the nitrogen source (MM+NO_3). Strains are; P6-005 (*nit1* mutant) – upper, thin sparse mycelial growth spreading across the plate and abutting the thick dense growth of A5 (wild-type) - lower.

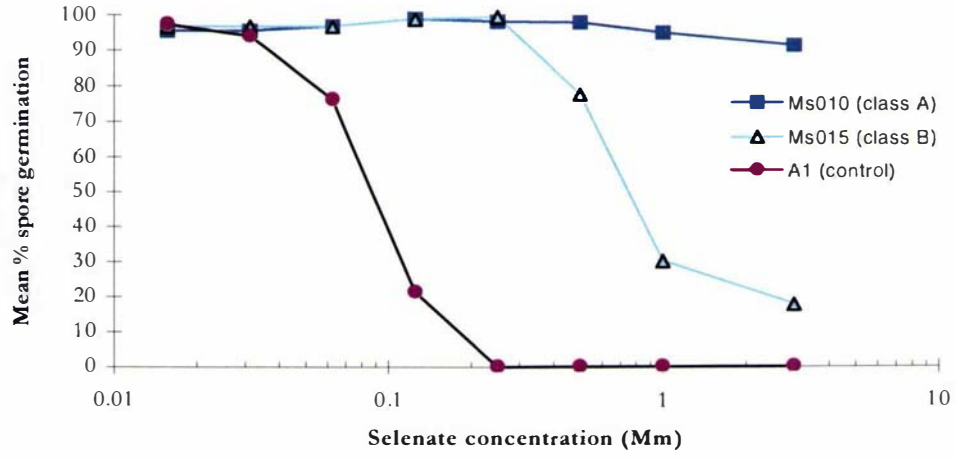


Figure 16 Germination response of selected selenate-resistant mutants and strain A1 of *Botrytis cinerea* on media amended with a range of selenate concentrations.

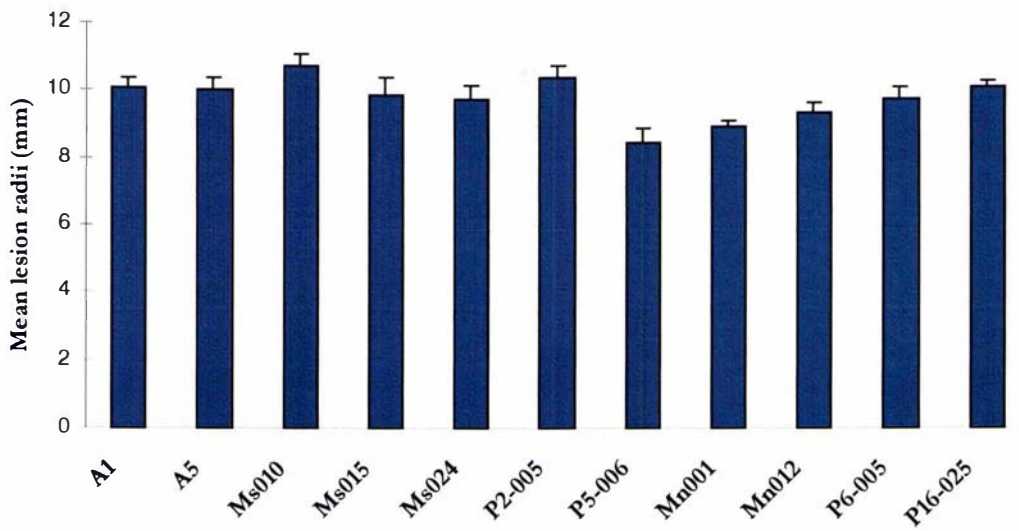


Figure 17 Mean lesion sizes caused by selected strains of *Botrytis cinerea* on French bean leaves after 3-days incubation at 20 – 25°C. Strains are; wild-type strains (A1 and A5), selenate mutants from class A, class B, and class C (Ms010, Ms015, and Ms024 respectively), selenate resistant progeny from crosses of Ms010 (P2-005) and Ms015 (P5-006), *nit1* mutants (Mn001 and Mn012), one *nit1* progeny from cross of Mn001 (P6-005), one progeny carrying both *nit1* and *Sel1*R markers (P16-025). Bars indicate standard error of means from 9 replications.

Table 2 Segregation of selenate (SelR) or nitrate non-utilising (*nit1*) mutants in *Botrytis cinerea* crosses involving known fungicide resistance markers.

Cross No.	Parents		Progeny				Recombination (%) between pairs of genes (χ^2 for independent segregation*)		
	Sclerotial Strain	Fertilising Strain	No. ascospores	Wild-type	Mutant	χ^2 for 1:1 segregation	mutant:*Mbc1*	mutant:*Daf1*	*Mbc1*:*Daf1*
SelR cross									
1	Ms015 (*Mbc1HR-Sel1*R)	REB678-1 (*Mbc1LR-Sel1S*)	105	46	59	1.62	44.8 (1.17)		
2	Ms010 (*Daf1S*-SelR)	SAS405 (*Daf1LR*-SelS)	103	59	44	2.23		44.6 (1.19)	
3	P1-083 (*Mbc1LR-Sel1*R)	P1-105 (*Mbc1HR-Sel1S*)	101	44	57	1.70	53.5 (0.49)		
Nit1 cross									
4	Mn012 (*Mbc1HR-Daf1LR-nit1*)	REB658-1 (*Mbc1S-Daf1S*)	102	47	55	0.63	54.9 (0.92)	58.8 (3.19)	35.3 (9.66)
5	SAS405 (*Mbc1HR-Daf1LR*)	Mn001 (*Mbc1S-Daf1S-nit1*)	102	48	54	0.35	48.0 (0.16)	45.1 (0.99)	32.4 (14.51)
6	P3-018 (*Mbc1HR-Daf1LR*)	P3-009 (*Mbc1S-Daf1S-nit1*)	108	59	48	0.93	50.0 (0.00)	50.0 (0.00)	29.6 (21.50)

* χ^2 values greater than 2.71 (3.84) indicate differences significant at P = 0.1 (0.05).

Nitrate non-utilising mutants

Spontaneous sectors arose as dense outgrowths at the plug margin on MM+ClO_3 from strain A5. A total of 8 chlorate resistant sectors that grew as thin sparse colonies on MM+NO_3 were classified as nitrate-non-utilising mutants and retained for further study. All grew normally on hypoxanthine (MM+Hx) and nitrite medium (MM+NO_2) and it was concluded, by comparison with mutants in other fungi [Correll *et al.,* 1987], that they were defective in the nitrate reductase apoenzyme and the genotypic symbol *nit1* was allocated, represented by the allele Mn001. These mutants were routinely scored by growth on MM+NO_3 (Figure 15 p43). They remained vegetatively stable after storage on silica gel. Eight chlorate resistant mutants that were recovered from NQO treated spores all showed wild-type growth on MM+NO_3, and were classified as Crn (chlorate resistant, nitrogen normal) mutants.

Eight nit1 mutants, derived from strain A5, were tested for complementation in all combinations (method B) and all grew as thin colonies except in 5 combinations (Figure 18A p47) where relatively thick dense mycelial growth was restored along the line of contact. All complementing pairs produced medium to weak growth compared to wild-type strains on the same medium. This dense growth (shown in a mixed spore test, Figure 19 p49) is interpreted as reflecting intragenic complementation between the strains and can be represented as a complementation map with 4 groups (Figure 18B p47) [Fincham, 1966]. Mn012, a *nit1* mutant derived from REB689-1 (Appendix 3 p127) did not complement any of the A5 derived mutants.

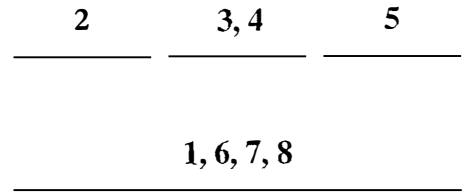
When the *nit1* mutants were crossed, the *nit1* phenotype segregated as a single gene with no evidence of linkage to either *Mbc1* or *Daf1* (Table 2 p45, crosses 4, 5 and 6). The fungicide resistance markers *Mbc1* and *Daf1* showed loose linkage (29.6 – 35.3%) in the same crosses.

Figure 18 Complementation pattern of selected *nit1* mutants derived from strain A5. (A) Score matrix for mutants paired on MM+NO_3 medium. (B) Complementation map derived from data in A. Mutants in non-overlapping groups complement, those within one group or in overlapping groups do not.

A

nit1 mutants Mn001 to Mn008								
	8	7	6	5	4	3	2	1
1	-	-	-	-	-	-	-	-
2	-	-	-	+	+	+	-	
3	-	-	-	+	-	-		
4	-	-	-	+	-			
5	-	-	-	-				
6	-	-	-					
7	-	-						
8	-							

B



Pathogenicity

Selected wild-type strains, primary mutants, and single-ascospore progeny (non-auxotrophs SelR strains) were tested for pathogenicity in a randomised design with nine replications. All were highly aggressive (Figure 17).

Linkage relationships

Crosses of three different *Sel1* (class B) mutants to *nit1* mutants gave 1:1 ratios for segregation of *nit1*, *Sel1*, *Mbc1* and *Daf1* with no evidence of linkage between the two new markers, *nit1* and *Sel1* (Table 3 p48). Crosses 8 and 9 (Table 3 p48) provided evidence for loose linkage between *Mbc1* and *Daf1* similar to that found in crosses 4, 5 and 6 (Table 1 p42). However cross 7 (Table 3 p48) gave a recombination percentage between these genes of 75.2% for which no simple explanation is apparent.

Marker complementation

As some SelR mutants behave as sulphate non-utilisers (demonstrated by increased growth in response to taurine), and *nit1* mutants behave as nitrate non-utilisers, it is feasible to test for "forced" complementation between these mutants on MM+NO_3. In a complementation test (method A) the mutants Ms033 and Mn001 (both from the parent A5) produced relatively dense growth compared to the thin sparse growth observed with spore mixtures of mutants generated from different parents (A5 with mutants from A1 or REB689-1) (Figure 20 p49). The dense growth was interpreted as a result of complementation between the two mutant types.

Table 3 Segregation of class B selenate resistant (*Sel1*), nitrate non-utilising (*nit1*), benzimidazole resistant (*Mbc1*), and dicarboximide resistant (*Daf1*) markers in four-point crosses of *Botrytis cinerea*

	Parents			Progeny					
				Recombination (%) between pairs of genes (χ^2 for independent segregation*)					
Cross No.	Sclerotial strain	Fertilising Strain	No. ascospores	*nit1:Sel1*	*nit1:Mbc1*	*nit1:Daf1*	*Sel1:Mbc1*	*Sel1:Daf1*	*Mbc1:Daf1*
7	P6-022 (*Mbc1HR-Daf1LR- Sel1S-nit1*)	P5-006 (*Mbc1LR-Daf1S-Sel1R*)	101	51.5 (0.10)	53.5 (0.51)	55.4 (1.25)	43.6 (1.75)	49.5 (0.02)	75.2 (35.26)
8	P5-029 (*Mbc1LR-Daf1S-Sel1R*)	P6-037 (*Mbc1HR- Daf1LR-Sel1S-nit1*)	100	56.9 (1.46)	54.0 (0.64)	48.0 (0.16)	46.0 (0.64)	54.0 (0.64)	36.0 (8.51)
9	P6-005 (*Mbc1HR-Daf1LR-Sel1S-nit1*)	P5-026 (*Mbc1LR-Daf1S-Sel1R*)	105	52.4 (0.09)	47.6 (0.24)	53.3 (0.47)	57.1 (2.19)	55.2 (1.17)	30.5 (18.89)

* χ^2 values greater than 2.71 (3.84) indicate differences significant at P = 0.1 (0.05).

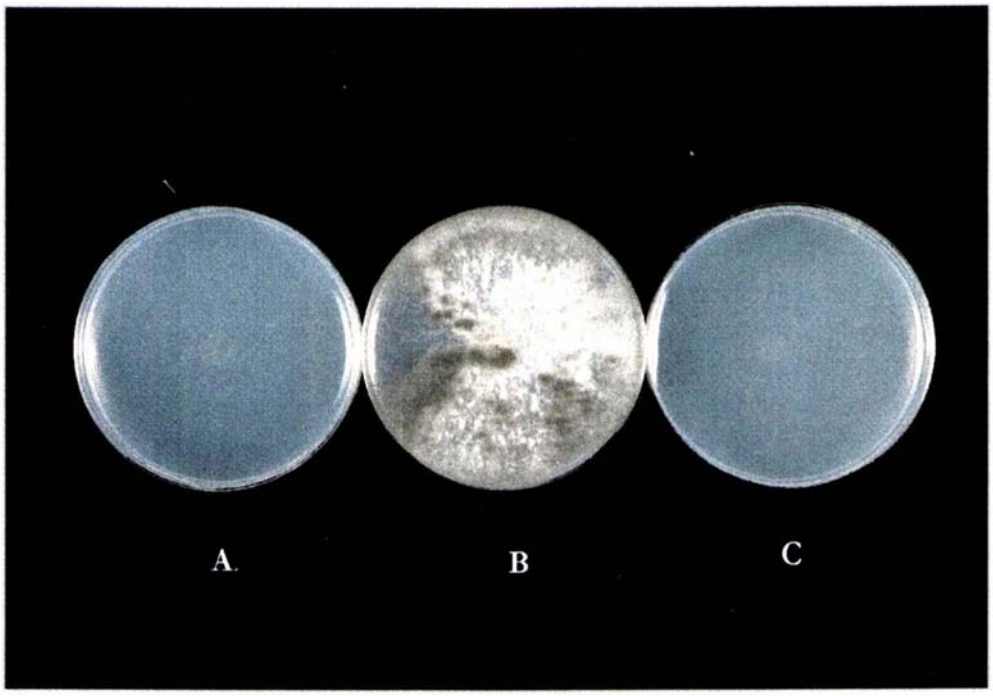


Figure 19 Complementation between *nit1* mutants derived as spontaneous sectors from the parent strain A5 (Mn001, Mn004 and Mn005) or REB689-1 (Mn012). Tested using method A on MM+NO_3. A 1:1 mix of spore suspensions was placed in the centre of each plate. (A) Mn005 paired with Mn001. (B) Mn005 paired with Mn004. (C) Mn004 paired with Mn012.

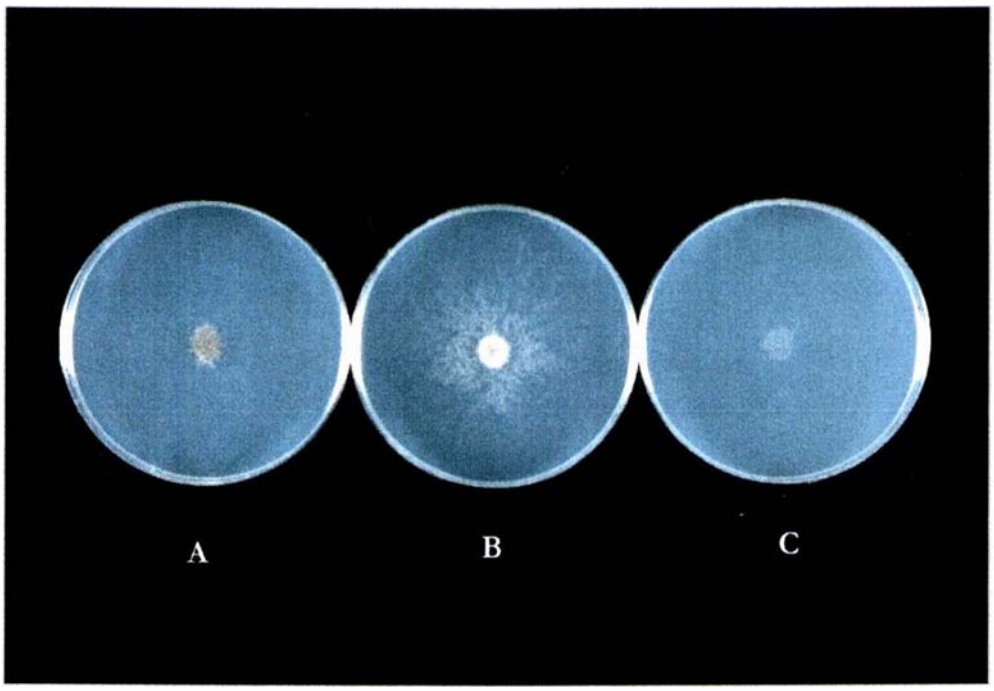


Figure 20 Complementation between SelR (class A) and *nit1* mutants on MM+NO_3 (method A). (A) Ms033 (parent A5) paired with Mn012 (parent REB689-1). (B) Ms033 (parent A5) paired with Mn001 (parent A5). (C) Ms010 (parent A1) paired with Mn001 (parent A5).

Discussion

Selenate has been shown to enter the mycelia of species of *Aspergillus* and *Penicillium* via a permease system that also transports sulphate [Tweedie & Segel, 1970; Shrift, 1961]. This sulphate permease is under metabolic control and is regulated by sulphur status [Renosto *et al.*, 1990]. Once inside the cell selenate can be incorporated in place of sulphate in various metabolites and it is suggested that the major mechanism of selenium toxicity is the instability of the selenium analogues in, for example, the disulphide bridges in proteins [Wilson & Bandurski, 1958; Shrift, 1972].

Although chromate is also transported into the fungal cell by the sulphate permease, it is apparently unable to form stable products when acted on by ATP sulphurylase (the first enzyme in the sulphate assimilation pathway) and therefore is not incorporated into metabolites in place of sulphate. Chromate toxicity is attributed to the intracellular accumulation of chromate causing death, possibly through its strong oxidising effects [Roberts & Marzluf, 1971].

Mutants resistant to chromate and selenate, similar to the class A and B mutants found in this study, have been shown in other fungi to be deficient in sulphate permeases. In *Aspergillus nidulans* the gene *S*-3 encodes a sulphate permease [Arst, 1968] and in *Neurospora crassa* the *cys-13* gene encodes a low affinity sulphate permease [Marzluf, 1970]. Selenate resistant mutants sensitive to chromate, similar to the class C mutants described here, have been found in both *A. nidulans* (*s*-12) and *N. crassa* (*cys-5* and *cys-11*) and identified as deficient in ATP sulphurylase [Arst, 1968; Marzluf, 1970].

Chlorate acts as a nitrate analogue and is apparently reduced by nitrate reductase to chlorite, which is toxic to the cell. It is suggested that chlorate resistant strains are unable to reduce chlorate to chlorite and so are able to grow on chlorate amended media [Cove, 1976b]. Some chlorate resistant mutants recovered in this study were normal in nitrate assimilation (Crn mutants) while others were deficient (Nit mutants) resembling those reported from fungi such as *Fusarium moniliforme* [Klittich & Leslie, 1989] and *F. oxysporum* [Correll *et al.*, 1987].

Nitrogen utilisation tests of chlorate resistant nitrogen non-utilising (Nit) mutants have been widely used to identify three distinct phenotypic classes; *nit1* (putatively defective at the nitrate reductase apoenzyme), *nit3* (putatively defective in a nitrogen pathway-specific regulatory locus), and NitM (putatively defective in loci affecting the assembly of a molybdenum containing cofactor necessary for both nitrate reductase and

purine dehydrogenase activity) [Pateman *et al.*, 1964; Correll *et al.*, 1987; Jacobson & Gordon, 1988]. Here only *nit1* nitrate non-utilising mutants were recovered although examination of further mutants may reveal mutants of other classes. The relative frequency of the various classes of nitrate non-utilising mutants has been found to vary depending on the fungus and the nitrogen source. Brooker *et al.* [1991] found that, while no single phenotypic class predominated in the seven strains of *Colletotrichum* tested, the frequency of the classes varied between strains and on different media, whereas Correll *et al.* [1987] and Klittich & Leslie [1989] found that *nit1* mutants usually predominated in studies done with *Fusarium* species. In this study NQO mutagenesis yielded exclusively Crn mutants and the sector method produced all *nit1* mutants. A preferential effect of mutagen on the selection of chlorate resistant type has been noted previously by Cove [1976a] with *A. nidulans*.

Complementation tests among *nit1* mutants (Figure 18 p47) indicate the presence of a number of groups suggesting intragenic complementation. This is consistent with the findings of Martinelli *et al.* [1984], who found intragenic complementation of *niaD* gene mutants (analogous to *nit1*) of *A. nidulans* and Correll *et al.* [1987] who found intragenic complementation between some *nit1* mutants of *F. oxysporum*. Nitrate reductase was found to be a dimeric enzyme composed of two identical polypeptides in *N. crassa* [Marzluf & Ying-Hui Fu, 1989] and in *A. nidulans* [Kinghorn, 1989], and multimeric in three nitrogen assimilating yeasts [Hipkin, 1989]. It is thus probable that nitrate reductase is at least dimeric in *B. cinerea* and intragenic complementation in this species may be due to the production of hybrid enzyme composed of sub-units derived from each parent, although other explanations are possible [Fincham, 1985].

Further crosses are required to test whether class A and C SelR mutants map to the same locus as *Sel1*. Recovery of selenate sensitive strains from crosses of two SelR mutants from different classes would indicate that the mutations were not allelic.

Subsequent to this work being accepted for publication two studies have reported the use of the *nit1* (nitrate reductase) gene as a genetic marker. Levis *et al.*, [1997] isolated the *nit1* gene from *B. cinerea* using a probe representing conserved domains from the nitrate reductase genes of *N. crassa*, *A. nidulans* and *F. oxysporum* . Sequencing of the *nit1* gene revealed a predicted protein of 907 amino acids with no introns and similarity to many other ascomycete nitrate reductases, the greatest homology (65%) being to the nitrate reductase of *Leptosphaeria maculans*. Chlorate resistant *nit1 B. cinerea* mutants were successfully transformed with the *nit1* genes of both *F. oxysporum* and *B. cinerea* confirming

the identity of this gene. Publication of an abstract of the work described in this thesis in the book of abstracts from the International *Botrytis* Symposium in Wageningen in June of 1996 [Weeds *et al.*, 1996] predates the Levis *et al.* paper and establishes priority for the symbol *nit1*, as used here, over *niaD*. In the second work [Giraud *et al.*, 1997] RFLP sites in the *nit1* gene were successfully used (with 15 other markers) to assess the level of genetic variation in field-isolates from the Champagne region of France.

These studies have shown that both sodium selenate and chlorate resistant mutants can be generated in *B. cinerea*, that the resistance markers segregate as single gene characters in crosses, and are unlinked to each other or to *Mbc1* and *Daf1*. Both markers are easily scored by growth on differential media, and thus have potential for use in genetic analysis of *B. cinerea*.

Chapter 4 Isolation and characterisation of non-aggressive mutants

Introduction

There have been many biochemical studies of the pathogenesis mechanisms of *B. cinerea* [van Kan *et al.*, 1997; Verhoeff & Warren, 1972]. However, knowledge of the genetic factors controlling pathogenicity is limited, in part due to a lack of basic genetic information. Progress in this area has been slowed by the well-documented variability of *B. cinerea* and difficulty in obtaining stable mutants (Phenotypic variation p15, Sexual crossing p18). Early studies used field-isolates for mutagenic work. Recently however, crossing techniques have been refined enabling sexual crossing to be carried out reliably in the laboratory [Faretra *et al.*, 1988a] making single-ascospore strains more readily available and stable auxotrophic and fungicide resistant mutants have been obtained from mutagen treated strains derived from single-ascospore cultures (Mutagenesis p19) [Hilber, 1992; Faretra & Pollastro, 1993b; Chabani, 1991; Buttner *et al.*, 1994]. The value of using single-ascospore isolates is supported by the work described in Chapter 3 where stable mutants resistant to both sodium selenate and potassium chlorate were readily obtained.

It was anticipated that non-aggressive mutants of *B. cinerea* could be obtained in a similar manner and would be valuable genetic tools in the study of the pathogenicity of this fungus. This chapter describes the generation and selection of stable non-aggressive mutants using this approach and examines the inheritance of pathogenicity in five of these mutants.

Materials and Methods

Details of *B. cinerea* strains are given in Appendix 3 (p127). Preparation of spore suspensions and plugs, crossing procedure, ascospore isolation and fungicide resistance testing are described in Chapter 2.

Mutagenesis

Two procedures were used to generate mutants. For u.v. mutagenesis a conidial suspension (5 ml, $1x10^4$ spore ml^{-1}) was irradiated for 2 min to give a spore survival of approximately 5% (Ultraviolet radiation p26). In the second procedure, 4-nitroquinoline-1-oxide (NQO) was used as described on p26. Following mutagenesis single conidial colonies were selected. Aliquots of 100 µl were spread onto plates of triton-amended medium (CM+Triton) and incubated in the dark for 5 d at 20°C. Then individual colonies were transferred to 24 well TC plates (MEA+A) and incubated at 10°C for 4 d in the dark.

Screening of putative non-aggressive strains

Preliminary trials of the French bean assay (Variability of the assay p32) showed variability in lesion sizes, and to ensure a reliable assessment of the aggressiveness of surviving strains, a large number of replications were built into the assay protocol. Non-aggressive mutants were selected in a five-step protocol consisting of three consecutive screenings, storage on silica gel, and re-testing (Figure 21 p55). Screenings involved 2, 4, 10 and 8 plugs respectively, using the French bean leaf assay (two plugs per leaf) as described (Pathogenicity assays p29). The selection criterion at each stage was for strains where all replicates produced lesion radii of less than 5 mm. Wild-type strains typically gave lesions of 7 – 12 mm. Between each screening putative non-aggressive strains were sub-cultured onto fresh MEA in standard petri dishes and grown for 3 d at 20°C.

Growth on minimal medium

Mutants were assessed for auxotrophic growth by inoculating 10 µl of twice washed conidia suspended in 0.01% Tween 80 ($1x10^6$) onto plates of MM with 4 replicate plates per strain. Growth was recorded as colony radii after 4 days incubation at 20 – 25°C.

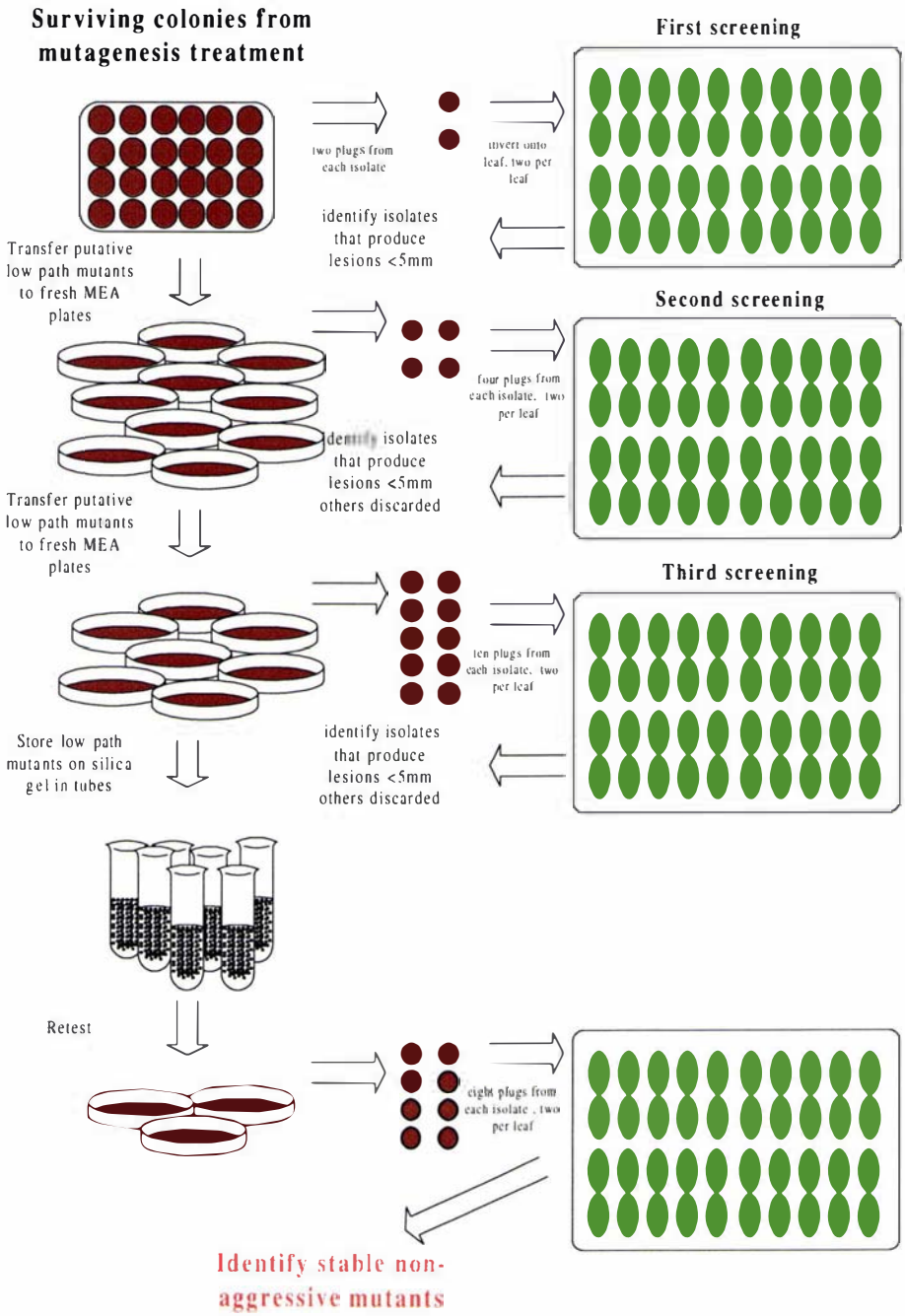


Figure 21 Schematic outline of screening method for non-aggressive mutants on French bean leaves.

Recognition by monoclonal antibodies

B. cinerea mutants were tested for recognition by the monoclonal antibody (MAb) BC-KH4 (gifted by Dr F. M. Dewey, University of Oxford) using indirect immuno-fluorescence [Bossi & Dewey, 1992]. Aliquots of 50 µl of conidia ($5x10^4$ spores ml^{-1} in Vogel's+sucrose medium) were incubated overnight at 10°C (in the dark) on multiwell slides (4 wells per slide, 1 slide per mutant strain) in sealed plastic containers lined with damp paper towels. A slide (4 aliquots) of Vogel's+sucrose medium and 1 slide each (4 replicates) of *Sclerotinia sclerotiorum* and *Colletotrichum musae* were included as controls. After incubation excess liquid was blotted off and the slides were air-dried under sterile conditions in a laminar flow cabinet. Germinated conidia were fixed by adding 50 µl of 3% paraformaldehyde to each well and incubated at room temperature for one hour. The slides were then washed in a gentle stream of PBST for 1 min and air-dried. BC-KH4 antibody (50 µl per well) was added to 3 of the 4 wells on each slide. A second MAb specific for *Penicillium islandicum*, PI-O1, was added to the fourth well as a control. The slides were incubated for 1 h at room temperature, washed in PBST as above and incubated for a further 1 h in 50 µl (per well) of anti-mouse MAb conjugate FITC (Sigma F9006) diluted 1:40 in PBS. Slides were washed again in PBST (as above), mounted in 'Citiflor' and viewed by fluorescence microscopy with a Zeiss fluorescence microscope at 800 times magnification using a mercury vapour lamp with filters to give exciter wavelengths of 350 – 390 nm.

Results

Generation of non-aggressive mutants

Putative mutants were generated from conidia of strain A4. A total of 4,964 survivors were screened for pathogenicity, 2,452 from u.v. mutagenesis and 2,512 from NQO mutagenesis. The yield of non-aggressive mutants was 1.8% from NQO treated spores and 5.8% from u.v. treated spores. In total 133 mutants were stored on silica gel and retested on French bean leaves. Of these 62 mutants (18 from NQO treated spores and 44 from u.v.) maintained a non-aggressive phenotype after recovery from storage. The remaining 71 reverted to an aggressive phenotype and were not investigated further. The high number of putative mutants that reverted to wild-type following storage (>50%) may have been due to heterokaryosis following mutagenesis whereby wild-type nuclei co-exist in low numbers with the mutant nuclei [Summers *et al.*, 1984]. When conidia from putative mutant strains were grown for storage on silica gel the relative proportions of

nuclei may have changed in favour of the wild-type, resulting in an apparent reversion to the wild-type phenotype.

Growth properties of the mutants

Preliminary growth tests on MEA revealed that 34 of the 62 mutants had growth rates of less than 25% of that of the wild-type parent. All but five mutants grew well on MM. Mutants Mp1, Mp17, Mp18, Mp93 and Mp110 produced significant growth on MEA but none on MM, and were tentatively designated as putative auxotrophs and were not examined further.

Recognition by monoclonal antibodies

A total of 25 non-aggressive mutant strains and the parental strain A4 were tested for the presence of the BC-KH4 antigen. Indirect immunofluorescence detected binding of the BC-KH4 MAb in all mutant and wild-type strains. *Sclerotinia sclerotiorum* also gave a positive response suggesting that the BC-KH4 antigen is present on genera other than *Botrytis*. (Figure 22 p58). No binding was observed to Colletotrichum musae

Segregation of pathogenicity

Of the 62 non-aggressive mutants 40 produced apothecia in crosses with single-ascospore reference strains (SAS405 or SAS56), and seven of these were analysed. Approximately 100 single-ascospore progeny were isolated from each cross and tested for segregation of fungicide resistance markers and pathogenicity using the French bean leaf assay (Pathogenicity assays p29). Progeny from a control cross of A4 (the mutant parent) and SAS56 were also analysed.

In the control cross aggressiveness of the progeny was essentially unimodal but with means slightly lower than either parent (Figure 24A p59). In four of the mutant crosses where the fungicide resistance marker segregated 1:1 confirming crossing had occurred, the pattern for the distribution of aggressiveness was also unimodal with the mean lesion sizes for the progeny grouped between the mean lesion size of the parents (Figure 24B p59). It was concluded that non-aggression in these mutants is most likely due to an alteration in more than one gene.

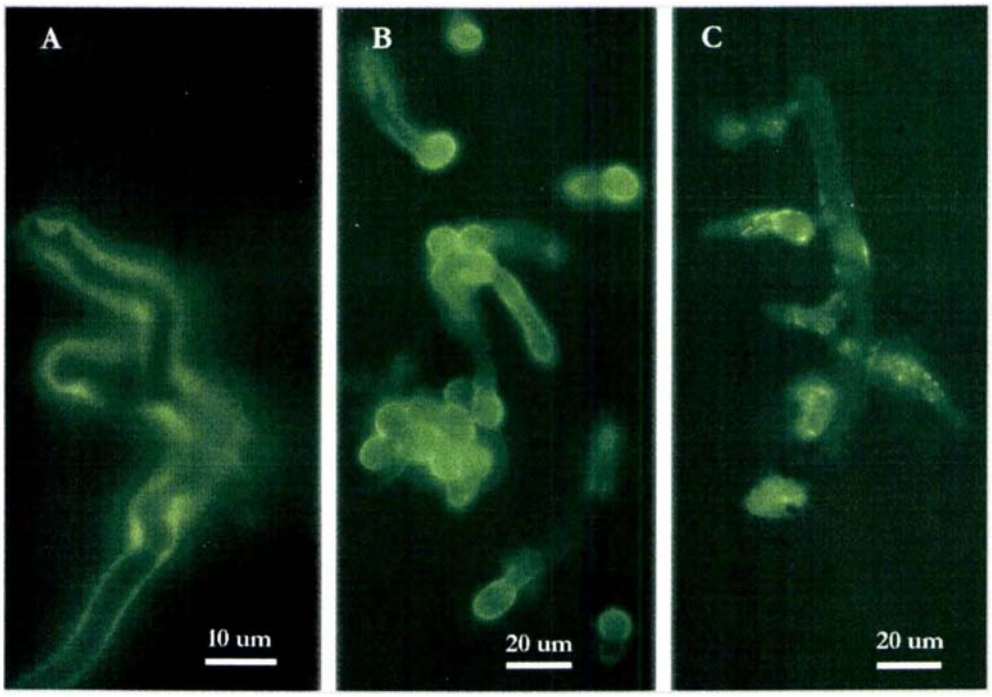


Figure 22 Germinated conidia immuno-stained with BC-KH4 and anti-mouse MAb conjugate FITC viewed under ultraviolet. light (350 – 390 nm). (A) A4 parental wild-type strain. (B) Mp123 (non-aggressive 4-nitroquinoline-1-oxide mutant from A4). (C) *Sclerotinia sclerotiorum.*

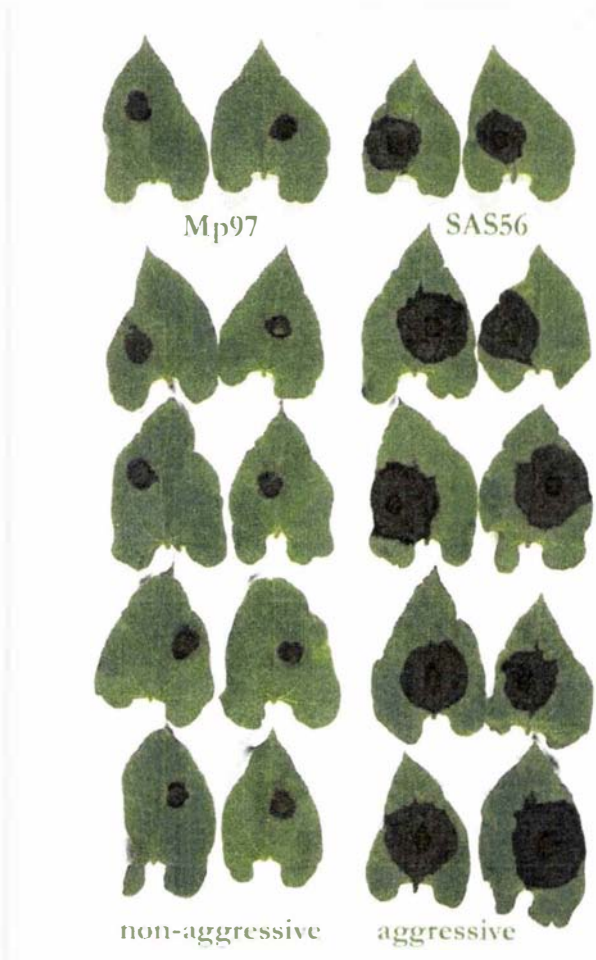


Figure 23 Lesions on French bean leaves inoculated with 5-mm plugs of *Botrytis cinerea.* Strains include Mp97 (non-aggressive), SAS56 (aggressive) and 8 progeny of the cross (Mp97/ SAS56). Four progeny show a non-aggressive phenotype and four show an aggressive phenotype.

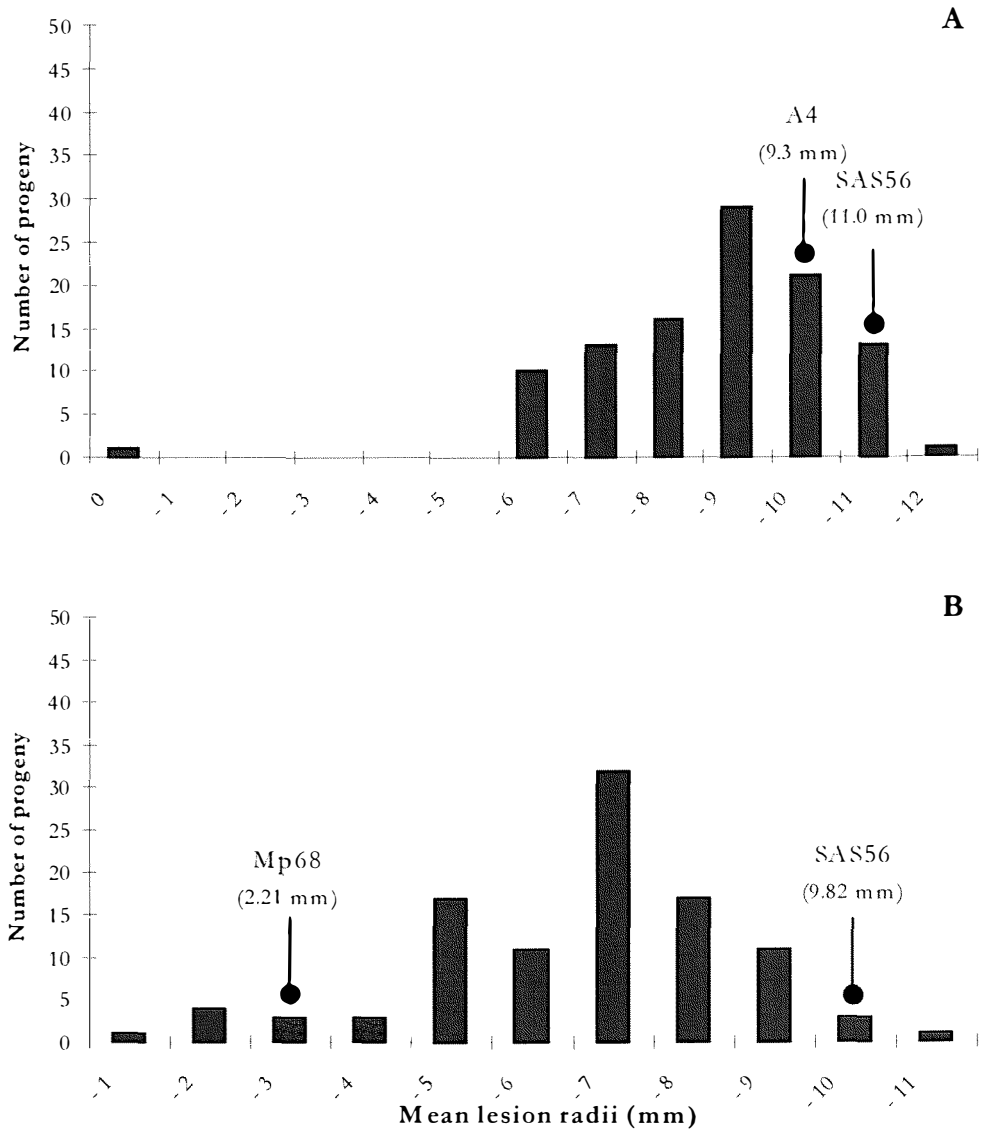


Figure 24 Distribution of lesion sizes in progeny of non-aggressive mutants crossed with aggressive single-ascospore reference strains. (A) Control cross A4 (sclerotial parent) with SAS56 (microconidial parent). (B) Cross SAS56 (sclerotial parent) with Mp68 (microconidial parent). Mean lesion size of parental strains in parentheses.

In one cross (cross 10, Table 4 p61) the fungicide resistance marker *Mbc1* segregated 1:1 and the mean lesion sizes showed an apparent bimodal segregation of aggressiveness (Figure 23 p58, Figure 25A p62) suggesting segregation of a single gene of major effect (χ^2 0.49). Progeny in the lesion classes up to 4mm radius were classified as non-aggressive, those forming larger lesions as aggressive. Progeny analysis of this cross was repeated two more times giving similar results (χ^2 3.37, 0.009). Two repeat analyses of the reciprocal cross (cross 11, Table 4 p61) also gave 1:1 segregation of aggressiveness (χ^2 2.68, 2.68) (Figure 25B p62). On further incubation to a total of 6 d, lesions produced by the non-aggressive strains remained restricted while those of the aggressive strains continued to expand eventually colonising the entire leaf. It was concluded that the non-aggressive character expressed under these conditions is conferred by a single gene and the genotype symbol *Pat1* was allocated, represented by the allele Mp97.

In the remaining two crosses involving other mutants, evidence for sexual recombination was not established as the fungicide resistance markers did not segregate 1:1 in the progeny, and the crosses were not analysed further.

Linkage relationships

In the initial cross involving the mutant Mp97 (cross 10, Table 4 p61), *Pat1* gave 32% recombination with *Mbc1* suggesting possible linkage with this marker, but no linkage was indicated in the reciprocal cross (cross 11, Table 4 p61). Linkage relationships were investigated in a five point cross using markers *Mbc1*, *Daf1*, *Sel1*R and *nit1*. Strain P21-040 (*Pat1-Mbc1S-Daf1S-Sel1S*) was crossed with strain P16-067 (*Mbc1HR-Daf1LR-Sel1R-nit1*). Recombination percentages of *Pat1* were close to 50 indicating no linkage to any of these markers (χ^2 values between 0.15 and 2.25, Table 5 p63). As in previous crosses (Table 2 p45, Table 3 p48) the fungicide resistance markers *Mbc1* and *Daf1* showed loose linkage (33.1%).

Table 4 Progeny analysis of sexual crosses, 10 and 11, reciprocal crosses of mutant (Mp97, *Pat1*) with the reference strain (SAS56).

	Parents		Progeny *		Recombination % between pairs of genes *
Cross No.	Sclerotial Strain	Fertilising Strain	No. Ascospores	*Pat1* as % †	*Pat1:Mbc1* as % ‡
10	Mp97 (*Mbc1HR-Pat1*)	SAS56 (*Mbc1S*)	101	53.5 (0.49)	31.7 (15.98)
11	SAS56 (*Mbc1S*)	Mp97 (*Mbc1HR- Pat1*)	98	58.2 (2.68)	43.9 (1.76)

* χ^2 values greater than 2.71 (3.84) indicate differences significant at P=0.1 (0.05)

† figures in brackets are χ^2 for 1:1 segregation

‡ figures in brackets are χ^2 for independent segregation

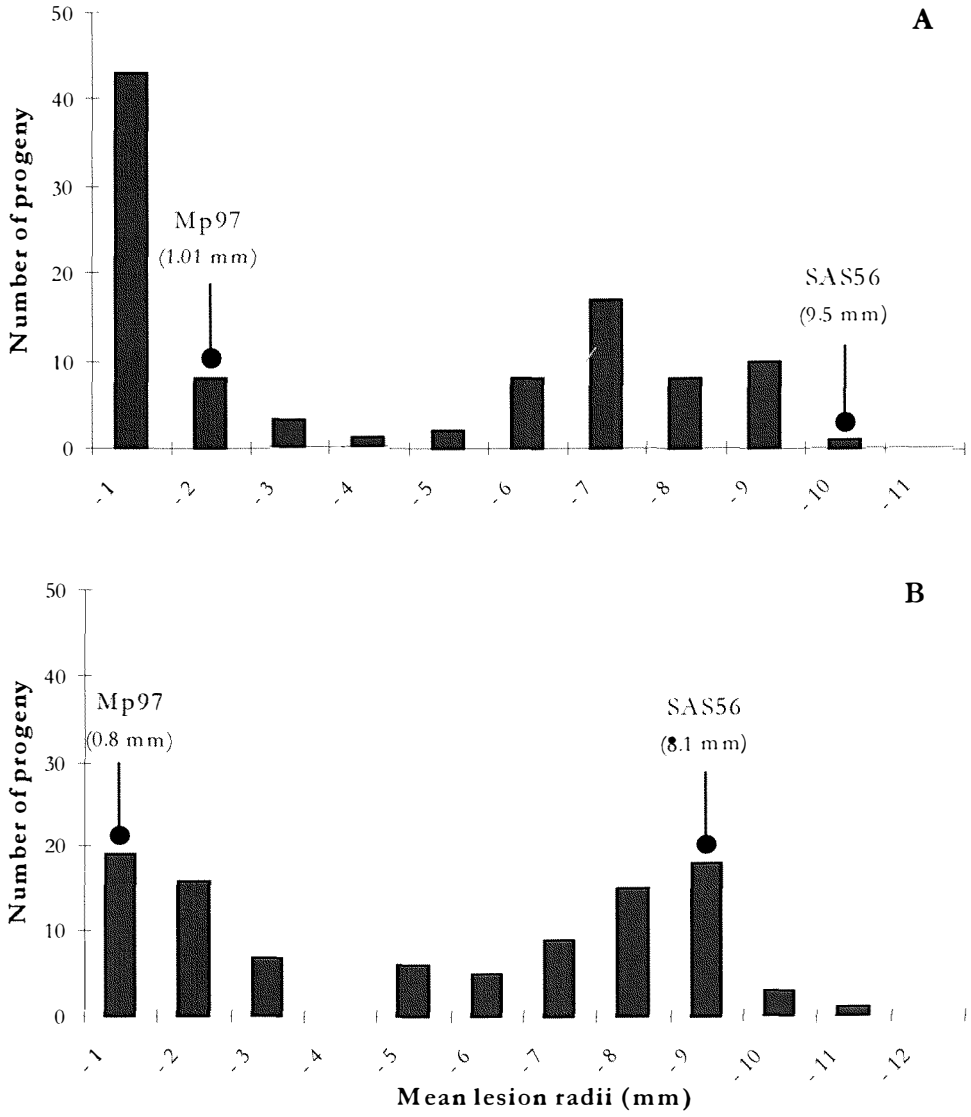


Figure 25 Distribution of lesion sizes in progeny of Mp97 crossed with aggressive single-ascospore reference strain. (A) Cross Mp97 (sclerotial parent) with SAS56 (microconidial parent). (B) Reciprocal cross SAS56 (sclerotial parent) with Mp97 (microconidial parent). Mean lesion size in parentheses.

Table 5 Progeny analysis of sexual cross involving five genetic markers, selenate (*Sel1*), vinclozolin (*Daf1*), and carbendazim resistance (*Mbc1*), *nit1* (nitrate non-utilising), and *Pat1* (pathogenesis).

	Parents		Progeny		Recombination % between pairs of genes (χ^2 for independent segregation *)				
Cross No.	Sclerotial Strain	Fertilising Strain	No. Ascospores	*Pat1* as %[t]	*Pat1:nit1*	*Pat1:Sel1*	*Pat1:Mbc1*	*Pat1:Daf1*	*Mbc1:Daf1*
12	P16-67 (*Mbc1HR-Daf1LR-nit1-Sel1R*)	P21-40 (*Mbc1S-Daf1S-Pat1*)	163	46.0 (1.04)	49.1 (0.06)	48.5 (0.15)	44.2 (2.25)	46.6 (0.75)	29.4 (33.14)
					nit1:Mbc1	*nit1:Daf1*	*Sel1:Mbc1*	*Sel1:Daf1*	*nit1:Sel1*
					45.4 (1.39)	46.6 (0.75)	50.9 (0.55)	49.7 (0.01)	53.4 (0.75)

* χ^2 values greater than 2.71 (3.84) indicate differences significant at P=0.1 (0.05)

[t] figure in brackets shows χ^2 for 1:1 segregation

SAS405 - a homothallic single-ascospore strain

In this study the single-ascospore reference strain SAS405 (*Mbc1HR-Daf1LR*) sometimes produced apothecia in control crosses (i.e. strains 'fertilised' with their own microconidia or with water, Crossing procedure p33). Analysis of fungicide resistance in the progeny of two such crosses revealed the presence of both benzimidazole and dicarboximide sensitive strains in proportions closer to 3:1 than 1:1 segregation (crosses 13 and 14, Table 6 p65). Furthermore, analysis of Mbc and Daf phenotypes in the progeny of cross 14 (SAS405 fertilised with water) gave results consistent with segregation of two genes in a diploid with resistant alleles dominant over sensitive alleles giving a ratio of 9:3:3:1 (Table 7 p66). Recombination of the fungicide markers was 32% similar to that found in haploid analysis (crosses 4, 5, and 6, Table 2 p45 and crosses 8 and 9 Table 3 p48). In cross 13 (SAS405 fertilised with SAS405 microconidia), although all four classes were represented the χ^2 values were much higher due to the low score for one class (Table 7 p66). Further crosses are needed to confirm the validity of these patterns.

Discussion

B. cinerea was more resistant to mutagenesis by NQO than *Aspergillus nidulans* [Bal *et al.*, 1977] and required higher concentrations of the mutagen to achieve similar effects. In addition, approximately half the number of stable mutants were generated from NQO mutagenesis compared to u.v. mutagenesis. In general, mutant strains were fertile as both male and female parents and most mutants crossed readily with reference strains.

Of the 62 mutants obtained in this study five were unable to grow on minimal medium and were identified as putative auxotrophs. Auxotrophic mutants altered in pathogenicity have been demonstrated in other plant pathogenic fungi. Biochemical mutants (induced by both u.v. and chemical mutagens) of *Venturia inaequalis* requiring choline, riboflavin, purines, pyrimidines, arginine, histidine, methionine or proline were found to be avirulent on apple leaves: those requiring biotin, nicotinic acid, pantothenic acid, insitol or reduced sulphur were virulent [Boone *et al.*, 1956; Kline *et al.*, 1957]. A u.v. induced pyrimidine auxotroph of *Sclerotinia sclerotiorum* was avirulent on four of seven susceptible hosts [Miller et al., 1989b]. Further work is needed to determine the nutritional requirements of the putative auxotrophs *B. cinerea* mutants generated in this study.

Table 6 Pattern of fungicide resistance in progeny from SAS405 'fertilised' with SAS405 microconidia or with water.

	Parents		Progeny	% with parental phenotype (χ^2 for independent segregation 1:1*)(χ^2 for independent segregation 3:1*)	
Cross No.	Sclerotial Strain	Fertilising Strain	No. Ascospores	Mbc as %	Daf as %
13	SAS405 (*Mbc1*HR-*Daf*LR)	SAS405 (*Mbc1*HR-*Daf*LR)	101	86.1 (110.47) (10.49)	68.3 (36.96) (2.08)
14	SAS405 (*Mbc1*HR-*Daf*LR)	Water	102	76.5 (39.72) (0.12)	78.4 (48.74)(0.71)

* χ^2 values greater than 2.71 (3.84) indicate differences significant at P=0.1 (0.05)

Table 7 Assessment of progeny from SAS405 'fertilised' with SAS405 microconidia or with water for consistency with segregation of fungicide resistance markers as a diploid with the resistance allele dominant over the sensitive allele.

	Parents		Progeny Phenotype					Recombination % (χ^2 for 10:6 segregation *)
Cross No.	Sclerotial Strain	Fertilising Strain	MbcHR / DafLR	MbcHR / DafS	MbcS / DafLR	MbcS / DafS	χ^2 for diploid (2 genes with dominance 9:3:3:1*)	Mbc:Daf
13	SAS405 (*Mbc1HR-DafLR*)	SAS405 (*Mbc1HR-DafLR*)	65	20	4	12	59.56	23.76 (10.52)
14	SAS405 (*Mbc1HR-DafLR*)	Water	60	13	20	9	3.80	32.35 (1.23)

* χ^2 values greater than 2.71 (3.84) indicate differences significant at P=0.1 (0.05)

The monoclonal antibody BC-KH4 was raised for use as a general-purpose immuno-detection assay for *B. cinerea* infections in fresh fruit and flowers [Bossi & Dewey, 1992]. This antibody recognises a carbohydrate epitope on a glycoprotein and binds to surface antigens on germinated conidia and germ-tubes of *B. cinerea* and *B. fabae* [Bossi & Dewey, 1992]. Likewise, binding occurs in regions of the wall and throughout the fibrillar-like matrix material surrounding ascospores within the asci of ripe apothecia [Cole, 1995]. In infection studies of *Vicia faba* by *B. cinerea* the antibody was found to bind to the sheath of fibrillar-like matrix material produced on the leaf surface that provided attachment for the germinating conidium [Cole *et al.*, 1996]. Due to the importance of attachment to the infection process it was considered possible that disruption of the glycoprotein that contains the BC-KH4 antigen could prevent or reduce infection of aggressive strains. Non-aggressive mutants as recovered in this study might lack the glycoprotein. However, for the 25 non-aggressive mutants tested no difference in binding of BC-KH4 was observed. The binding of the antibody to the related fungus *S. sclerotiorum* has not previously been reported.

The aggressive phenotype segregated 1:1 in one mutant (Mp97) indicating the presence of a single gene with a major effect on pathogenicity. No evidence was found for linkage to any of the five markers tested. The presence of a major pathogenicity gene in one out of the seven non-aggressive strains tested suggests other such genes may be present in the remaining, as yet untested, mutants. Ascospores from successful crosses of non-aggressive strains with wild-type strains have been stored at – 80°C (Storage p24) for possible future analysis.

Secondary homothallism in single-ascospore strains as observed in control crosses of SAS405 has been reported previously in some strains (Mating-type p21) relating to SAS405 [Faretra & Pollastro, 1996]. Although other explanations have been proposed the 3:1 segregation of individual markers found in this study supports some form of aneuploidy or heteroploidy. An explanatory model consistent with Table 6 (p65) would be that SAS405 is aneuploid for the chromosome carrying *Mbc1* and *Daf1* (known to be linked, Fungicide Resistance p21) with the second chromosome carrying the *Mbc1S* and *Daf1S* alleles and that these segregate as in a diploid organism. This is supported by the presence of four phenotypic classes in ratios consistent with 9:3:3:1 as expected for two genes with dominance in a diploid (cross 14, Table 7 p66). Further support for this hypothesis comes from estimates of DNA content which show evidence that SAS405 may be diploid or aneuploid [Buttner et al., 1994].

Practical considerations have limited previous studies of pathogenicity determinants to a relatively small number of field-strains. In these strains it is difficult to draw definite conclusions from correlation of pathogenicity determinants with infection severity due to the high levels of natural variation. In this study the 1:1 segregation of non-aggression (Mp97), combined with the fact that the small lesions produced by the non-aggressive strains, appeared to be permanently restricted, gives strong evidence for the importance of *Pat1* in disease development. This mutant is further characterised in chapter 5.

Chapter 5 A major gene controlling pathogenicity in *Botrytis cinerea*

Introduction

B. cinerea has been widely studied but relatively little is known about the factors that determine its pathogenic ability. Studies of pathogenicity determinants have been hampered by the high variability of field isolates (Phenotypic variation p15) making it difficult to separate differences due to alterations in pathogenicity from unrelated natural variation.

The usefulness of artificially induced mutants in pathogenicity studies has been demonstrated in a number of plant pathogenic fungi. For example, an artificially induced non-sclerotial mutant of *Sclerotinia sclerotiorum* retained its wild-type aggressive phenotype on eight different hosts including beans [Miller *et al.*, 1989] while mutants of the same fungus deficient in oxalic acid production produced no lesions on French bean leaves [Godoy *et al.*, 1990]. Artificially induced mutants showed that pectolytic enzymes were not essential for pathogenesis of *Verticillium albo-atrum* on tomato [Durrands & Cooper, 1988] and *V. dahliae* on cotton [Howell, 1976]. Mutants of *Fusarium solani* insensitive to tomatine (a fungi toxic plant resistance chemical) were able to infect green tomato fruit which is normally resistant [Defago & Kern, 1983], while albino mutants of *Colletotrichum lagenarium* have been used to demonstrate that melanin pigments are important for penetration of host tissues [Kubo *et al.*, 1982; Katoh *et al.*, 1988], and cutinase deficient mutants of *C. gloeosporioides* were found to be non-aggressive on papaya fruit [Dickman & Patil, 1986]. Gene disruption techniques have been recently used to induce mutants of *B. cinerea* deficient in either cutinase-A or a polygalacturonase isozyme. While the cutinase-A deficient mutants retained wild-type aggression on gerbera and tomato the polygalacturonase deficient mutants showed reduced aggression in a tomato leaf assay [Van Kan *et al.*, 1997; Have *et al.*, 1997]. Combined with improved crossing techniques

(Sexual crossing p18) and suitable genetic markers (Chapter 3), artificially induced single site mutants may provide answers to many questions relating to pathogenicity in *B. cinerea.*

The previous chapter (Chapter 4) described the selection of a non-aggressive mutant of *B. cinerea* (Mp97) where the aggressive character segregated 1:1 in repeated sexual crosses. This mutant and its sexual progeny, both non-aggressive and aggressive, offer a particular opportunity to investigate the genetic character of pathogenicity in *B. cinerea.* The aims of this chapter initially are to define the genetic relationships of *Pat1* more closely and to better characterise the nature of pathogenesis on bean leaves. Subsequently, a number of experiments are described that seek to distinguish between two broad categories of hypotheses as to the physiological basis of non-aggression. Are *Pat1* strains altered in speed of induction or intensity of response to host defence compounds, or are they defective in production of attack compounds?

Materials and Methods

Details of strains are given in Appendix 3 (p127). Preparation of spore suspensions and inoculum plugs, crossing procedure, ascospore isolation and fungicide resistance testing are described in Chapter 2.

Pathogenesis assays

Four assays were used. For the French bean leaf assay, seedlings (*Phaseolus vulgaris* cv. Top crop) were prepared as described on p29. Fungal inoculum was applied either as plugs, as described on p29, or as spore suspensions. Spore suspensions were washed in 0.01% Tween 80 (Media and growth conditions p24), adjusted to the required concentration, resuspended in Vogel's+sucrose solution and either 15 or 20 µl was inoculated onto the leaf by carefully pipetting into a small plastic ring (approximately 1.5 mm high by 4 mm diam.) placed on the leaf surface. Incubation and lesion assessment was as described on p29 and p32.

In the soybean leaf assay, seedlings (*Glycine max* cv. Amsoy) were grown in a growth cabinet for 8 d in trays of commercial potting mix at 25°C under a 16-h-on/8-h-off light regime. The seed tray was enclosed in polythene until emergence. Plants were prepared by cutting off the roots, suspended on racks, and the primary leaves were then inoculated with plugs as in the French bean assay. Lesion radii were measured after 5 d.

In the rose assay flowers (*Rosa sp.* cv. Delilah, greenhouse grown) were cut with approximately 100 mm of stem and the 4 outermost petals and all but 1 leaf were

* Infection scale was; 0 = no infection, 1 = speckling on outer petals, 2 speckling and few lesions on outer petals, 3 = speckling and numerous lesions on outer petals, 4 = lesions on outer and inner petals, 5 = numerous lesions on inner petals and some outer petals completely infected, 6 = almost completely infected (some tissue still visible), 7 = flower completely infected, 8 = flower completely infected with extensive sporulation and black stem.

discarded. Conidial suspensions in 0.01% Tween 80 ($1x10^6$ spores ml^{-1}) were applied as a spray to run-off with a De Vilbiss atomizer. Flowers were enclosed in individual clear plastic bags (secured around the stem), placed in flasks of water (3 per flask), and incubated at 20 – 25°C under a 12-h-on/12-h-off cycle of white and long-wave (Crompton 40 w) ultraviolet radiation (Essellese blacklight). The number of days to full flower infection was determined by scoring infection on a scale of 0 (no infection) to 8 (completely covered in sporulating mycelium) at 2-d intervals for up to 15 d.

The tomato stem assay followed the procedure of Eden *et al.* [1996b]. Tomatoes (*Lycopersicon esculentum* hybrid cv. Rondello) were planted in a commercial potting mix in 0.6-litre pots and grown outside during summer to the stage of mid to late anthesis of the first or second truss. Freshly cut stem-pieces (40 mm, leaves removed) were dipped up to 5 mm in a $1x10^6$ ml^{-1} concentration of washed conidia in 0.01% Tween 80 and allowed to air dry for 30 min (22 replicate stem-pieces per treatment). Treated stem-pieces were placed in dry petri dishes (3 – 4 per dish) and incubated in sealed plastic boxes lined with damp paper towels at 10, 15, 20 and 25°C. Infection was recorded as the number of stems showing visible softening after 14 d and expressed as a percentage.

Microscopic studies of lesions

French bean leaves were inoculated with conidial suspensions by applying 20 µl of either $1x10^4$ (8-h lesions only) or $1x10^5$ spore ml^{-1} into 4-mm diam. plastic rings. After 8, 18, 24, 48, 62 and 96-h incubation leaf-portions were cut out (approximately 10 x 10 mm, 5 per treatment) to include either the whole inoculation site and surrounding tissue (small lesions), or a section near the lesion edge (large lesions). The leaf-portions were immersed in aniline blue clearing/staining solution [Bruzzese & Hasan, 1983] for 1 d and de-stained for 7 d in chloral hydrate solution (2.5 g ml^{-1}) on a rocking shaker at room temperature. Germ-tube lengths from 8-h incubations were calculated from the average length of 50 germ-tubes per leaf-section (3 leaf-portions per strain) measured on a light microscope using the x20 objective and the image analysing program Optimas (version 4.2). Leaf-portions, incubated for 18 h, were examined under a light microscope and compared for number and complexity of appressoria, and fungal penetrations (determined as clearly visible fungal hyphae below the leaf surface (Figure 29 p82), while in 48, 62 and 96-h leaf-portions the appearance of hyphal structure and surrounding tissue were examined.

Physiological characterisation

Radial growth rate (mm d^{-1}) was assessed on MEA plates inoculated at the margin with mycelial plugs (3 replicate plates per strain) and calculated from growth over a 3 – 5-d interval at 23 – 25°C. Conidial production was assessed from these plates following a further 5 d incubation (10 d in total). The conidia were harvested by homogenising the contents of each plate with 100 ml of Tween 80 (0.01%) in a laboratory homogeniser and the spores counted.

Sclerotial production was assessed on MEA plates inoculated at the margin with mycelial plugs and incubated in the dark for 21 d at 20°C (3 replicate plates per strain). The number of sclerotia per plate was counted, all sclerotia were then removed and weighed and the average weight per sclerotium calculated.

Auxotrophic growth on MM was tested by spotting 10 µl of a twice washed conidial suspension in 0.01% Tween 80 ($1x10^6$ ml^{-1}) onto MM and MEA (4 replicate plates per strain). Growth was recorded by noting colony radius and assessing hyphal density after 4 d incubation at 20 – 25°C.

Spore size was assessed by staining conidia ($1x10^7$ ml^{-1}) with acid fuchsin and calculating the average area from 100 spores per strain (chosen at random) measured on a light microscope with the image analysing program Optimas (version 4.2) using a x40 objective.

Acid production was assessed using the plate method described by Godoy *et al.* [1990]. Potato dextrose agar amended with bromophenol blue (PDA+bromophenol) plates were inoculated in the centre with 10-µl conidial suspensions, ($1x10^6$ ml^{-1}) in 0.01% Tween 80, 2 plates per strain. Plates were incubated for 4 d in the dark at 20°C, assessed visually, and scored on a scale of 0 (low) to 3 (high) for intensity of yellow colour indicative of acid production.

Temperature response was assessed on MEA plates inoculated at the margin with mycelial plugs (5 replicate plates per treatment) incubated in the dark at 10, 15, 20 and 25°C, marked at daily intervals until the mycelium reached the plate edge, and the daily growth rates calculated.

Polygalacturonase activity and lesion extraction

Two methods were used to assess polygalacturonase (PG) activity. Total PG activity in culture filtrates was investigated using a cup-plate gel diffusion assay. Production of PG

isozymes in culture filtrates and *in planta* was investigated using an activity overlay staining method following isoelectric focusing.

Cup plate assay

The cup-plate gel diffusion assay follows the method of Taylor & Secor [1988]. Culture filtrates were prepared by inoculating flasks (150 ml) containing 50 ml of modified-Richardson's-medium[A] with conidia to give a final concentration of 2 x 10^4 spores ml^{-1}. The flasks were incubated on an orbital shaker (100 rpm) at 20°C for 8 and 24 h and harvested by filtering through muslin and transferring 1 ml of the filtrate into fresh tubes containing 100 µl of 0.2% sodium azide as a preservative. Square perspex plates (280 x 280 x 10 mm) containing 240 ml of polygalacturonase substrate gel (p125) were prepared and 64 wells (5-mm diam., 30 cm apart) were cut in each plate using an aspirated cork borer. Wells were loaded with 30 µl of culture filtrate (1 well per culture per plate and 4 replicate plates) and incubated for 18 h at 37°C. Two control wells containing either water or uninoculated growth medium were included in each plate along with a dilution series (10, 20, 50, 100 and 1000-fold dilution in sterile distilled water) of commercially prepared *Aspergillus niger* PG (1440 units mg^{-1} protein; 0.29 mg protein ml^{-1}, Sigma) (p342 9). Following incubation the gels were developed by flooding each plate with 100 ml of ruthenium red (0.3 g l^{-1}) for 45 min. Enzyme activity showed as cleared zones and zone diameters were recorded to an accuracy of 1 mm. A control plate was loaded with boiled filtrate and a dilution series as above. Polygalacturonase standard curves (zone diameter vs. natural logarithm of the concentration of the standard) were prepared from the *A. niger* PG series in order to convert filtrate zone diameters to enzyme activity units (1 unit = the amount of enzyme that catalyses the production of 1 µmole of reducing sugar per min) as described in Appendix 8 (p139).

Isoelectric focusing and detection of PG isozymes

Assessment of PG isozymes by isoelectric focusing was carried out by Dr Keith Sharrock (HortResearch, Ruakura). Culture filtrates were prepared as above. Lesion material for *in planta* analysis was prepared by excising lesions from French bean leaves 4 d after inoculation. The agar plug was removed and all necrotic tissue was cut from the leaf, weighed and homogenised with a ground glass tissue grinder in 10 volumes (v/w) of 10 mM MES (2[N-Morpholino]ethanesulfonic acid, Sigma M-8250) pH 6 buffer. Leaves that developed very small necrotic zones (e.g. after inoculation with Mp97) were cut around the boundary of the visible fungal surface growth or discolouration. Homogenates were centrifuged (15 min at 9873 G at 4°C) and the supernatant stored at -20°C.

Isoelectric focusing of 10 µl samples of culture filtrate (24-hour treatment) or lesion extract, with subsequent detection of PG isozymes was performed as described by Sharrock & Labavitch [1994] with an overlay gel the same size as the isoelectric focusing (IEF) gel. A mixture of protein standards of known pIs (SERVA Cat. No. 39211) was applied to one lane of each gel. After focusing (c. 3000Vh) the overlay gel was placed in contact with the IEF gel for 15 min and then incubated at 37°C (4.5 h for 1-d culture filtrates, 105 min for lesion extracts). The overlay was stained overnight in 0.03% ruthenium red (Sigma), rinsed in distilled water, covered with transparent acetate film to prevent desiccation and allow handling, and the image digitally recorded (Microtek ScanMaker IISP scanner with a SM II TPO transparency attachment). Following removal of the overlay the IEF gel was soaked overnight in fixative containing 30% methanol, 10% trichloroacetic acid, and 3.5% sulphosalicylic acid before silver staining. Proteins were detected using the Bio-Rad Silver Stain kit according to the manufacturer's protocol.

Addition of cell free lesion extract to inoculum plugs on French bean leaves

The ability of lesion extracts to alter lesion development with either non-aggressive or aggressive strains was tested by applying cell free lesion extract to freshly inoculated French bean leaves. The extract was prepared from 4-d-old lesions of Mp97 and A4 infected leaves and from uninfected bean tissue as described for *in planta* detection of PG isozymes (Isoelectric focusing and detection of PG isozymes above). Supernatants were stored overnight at 4°C and applied to leaves the following day. French bean seedlings were inoculated with plugs of either Mp97 or A4 (as described p29) and either left untreated (control) or treated by adding 20 µl of extract from either Mp97, A4 or uninfected leaves, to the plugs (10 replicate leaves for each strain/treatment combination). Leaves were incubated for 3 d and assessed as described (p29).

Phytoalexin induction

Phytoalexin induction was tested using spore suspensions in a modification of the method described by Hahn *et al.* [1992]. Soybean cotyledons were surface sterilised in 3% sodium hypochlorite, rinsed three times in deionised water, patted dry between paper towels and wounded by removing a thin slice of tissue (2 x 10 mm) from the abaxial surface. The wounded cotyledons were arranged on wet filter paper in petri dishes (9 per dish) and inoculated with 50-µl spore suspensions (approximately $5x10^6$ ml^{-1}) suspended in modified-Richardson's-medium[B] (2 replicate plates per treatment). Medium without spores was used as a control. Plates were sealed in plastic and incubated for 16 h at 25°C.

Three cotyledons at a time (5 replicate sets for each treatment) were mixed with 3 ml of water, a 100-µl sample transferred to a curvette with 1 ml water, and the absorbance read at 286 nm.

Competition

Two competition experiments were carried out. In the first French bean leaves were inoculated with spore suspensions of a non-aggressive strain and an aggressive strain. Strains were applied as a spore suspension (15 µl of $1x10^6$ ml^{-1}, washed twice and resuspended in Vogel's+sucrose medium) by inoculating one into 4-mm diam. rings and adding 15 µl of the other strain at 0, 6, 12 and 24 h after the first inoculation (4 replicate applications in each of 4 blocks). Single strain inoculations (at time 0) were included as controls. Lesion radius was recorded after 4 d incubation at 20 – 25°C.

In the second experiment two aggressive strains, differing by two genetic markers, were paired together. The relative proportions of each strain represented in the harvested lesion were analysed by assessing the percentage germination on selective media. Preliminary tests showed that both dicarboximide resistant and sodium selenate resistant strains (class A, Table 1 p42) could be easily distinguished by this method. Initial and challenge strains were applied and leaves incubated as above. Spores were harvested after 7 d, the lesion, excluding the inoculation site, was excised from the healthy tissue, mixed vigorously with 1 ml of sterile 0.01% Tween 80 amended with 100 $mg\ l^{-1}$ of both streptomycin and penicillin, and filtered to remove mycelial fragments. The spores-plus-antibiotic mixtures were plated onto MM+SeO_4^B, MEA+vinclozolin and MEA that had been amended with 200 $mg\ l^{-1}$ streptomycin and penicillin, and incubated at room temperature for 8 h. Percentage germination was calculated from assessment of 300 spores per lesion (3 replicate lesions per treatment).

Mycelial compatibility was tested by pairing strains on MEA+NaCl [Beever & Parkes, 1993] (Testing for mycelial incompatibility p37).

Results

Comparisons are presented between the primary mutant Mp97 and two wild-type strains: the mutant parent A4 and SAS56, a single-ascospore reference strain of different mating type. While the genetic studies, reported in chapter 4, indicate that Mp97 differed from A4 by a single gene affecting pathogenicity, it is possible that genetic changes affecting other properties had also occurred in Mp97, which could confound the interpretation of comparisons that only involved these two strains. Thus progeny of the cross of Mp97 and SAS56 were also included.

Characterising of Pat1 on French bean

Strains Mp97 and A4 and ten aggressive and ten non-aggressive ascospore progeny were retained from cross 10 (Table 4 p61) and their aggressiveness was confirmed on a French bean leaf assay (1 plug per leaf, 5 replicate plugs per strain) (Table 8 p77). After 3 d incubation lesions produced by non-aggressive strains were typically firm (not water soaked), small (<2 mm radius), with a characteristic dark brown ring around the perimeter (Figure 26 p78), and few conidiophores. After further incubation (up to 10 d total) leaves senesced and became yellow but the lesions remained the same size although occasionally the lesion and plug were found to come away from the remainder of the leaf at the ring leaving a 'shot hole' effect. In contrast, lesions from aggressive strains were water soaked, without a clearly marked edge, much larger (7 – 12 mm radius), and had more conidiophores. These lesions expanded to colonise the whole leaf after about 7 d.

Effect of cell free extracts on lesion development

All Mp97 inoculated leaves showed typical non-aggressive lesions (0 – 3-mm radius) with the characteristic dark brown ring (Figure 26 p78) irrespective of which treatment was applied. Likewise, A4 inoculated leaves showed typical aggressive lesions (7 – 10 mm radius) with no significant difference between treatments (untreated control 8.50 mm SE 0.27, Mp97 extract 8.15 mm SE 0.40, A4 extract 8.30 mm SE 0.23, uninfected leaf extract 8.30 mm SE 0.23). No significant difference was found between those receiving extracts from Mp97, A4 or uninfected leaves (1.30 SE 0.23, 1.85 mm SE 0.21, 1.40 SE 0.21 respectively). However, all treatments receiving extracts had lesions that were slightly, although significantly, larger than those of the untreated control of Mp97 plugs alone (0.60 mm SE 0.22 P = 0.0002), most likely due to the effect of the additional nutrients.

Table 8 Comparison of physiological characteristics for 10 aggressive and 10 non-aggressive progeny of the cross Mp97 / SAS56 and of the parental strains Mp97 and SAS56.

	Comparison of aggressive and non-aggressive progeny			Comparison of parental strains		
	Pat1	*Wild-type*	Pr >F	Mp97	SAS56	Pr >Fϕ
Pathogenicity * (mm)	0.74 (0.22)	9.38 (0.33)	0.0001†	1.30 (0.12)	8.0 (0.92)	0.0001†
Radial growth rate at 23-25°C (mm d^{-1})	10.83 (0.24)	14.80 (0.21)	0.0001	10.75 (0.87)	14.5 (0.63)	0.0001
Conidiation x 10^{-8} (no. / petri dish)	1.78 (0.33)	2.22 (0.15)	0.0001‡	2.73 (0.34)	2.27 (0.64)	0.3528‡
Sclerotial no. (/ petri dish)	87.26 (14.88)	36.26 (5.65)	0.0001‡	95.7 (6.01)	39.7 (5.78)	0.0003‡
Sclerotial weight (g)	0.67 (0.07)	1.08 (0.07)	0.0001‡	0.62 (0.03)	1.25 (0.11)	0.0001‡
Weight per sclerotium (g)	0.009 (0.001)	0.037 (0.006)	0.0001‡	0.006 (0.0003)	0.033 (0.0031)	0.0001‡
Spore area (μm^2)	52.03 (0.36)	50.63 (0.32)	✈	65.79 (1.15)	51.24 (0.81)	✈

* on French bean, radial growth from plug

† power transformed (λ= 0.7) for analysis

‡ log transformed for analysis

ϕ Differences were considered significant when Pr>F < 0.05

✈ Statistical results omitted as only a small proportion of the variation (R^2 = 18%) was explained by the model (area = strain) indicating that additional factors, not accounted for in the model, are influencing spore area

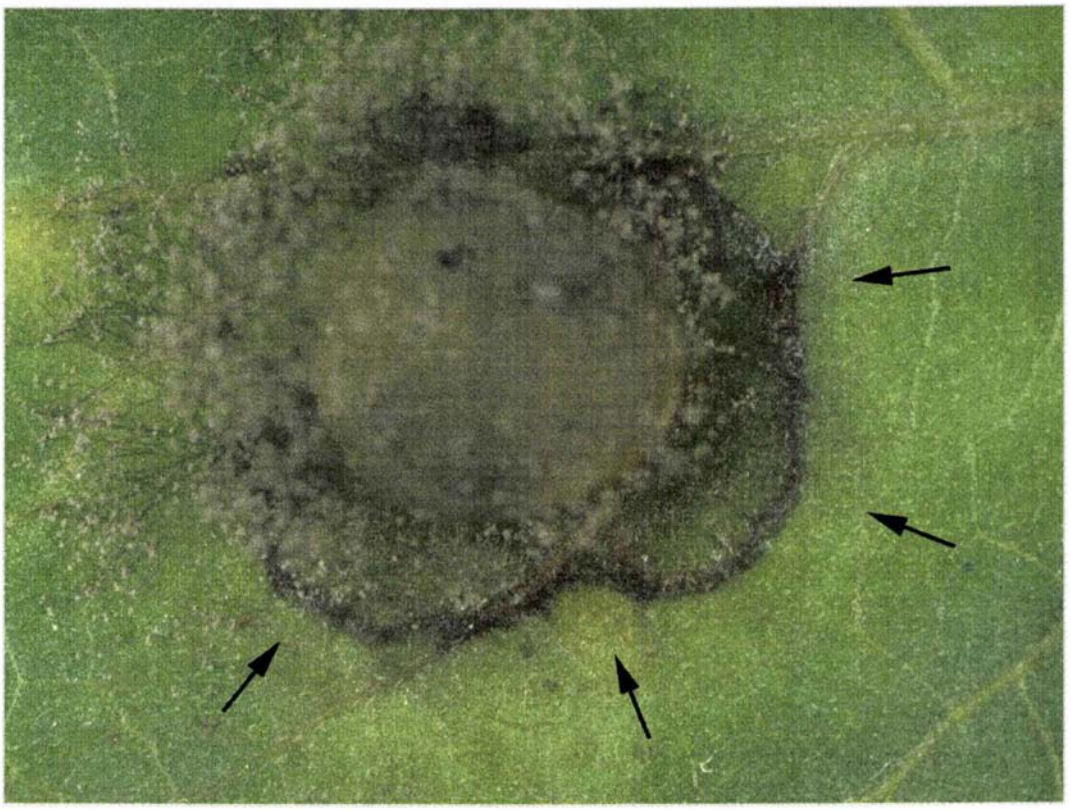


Figure 26 A typical restricted lesion produced on a French bean leaf inoculated with a 5-mm agar plug taken from the actively growing edge of a non-aggressive strain (P21-47) of *Botrytis cinerea* and incubated for 3 days at 20 – 25°C under conditions of high humidity. Arrows indicate the characteristic dark brown ring enclosing the lesion.

Microscopic studies of lesions

Germ-tube length did not differ significantly (P = 0.043) between strains Mp97 and A4 after 8 h incubation on French bean leaves (28.8 μm SE 0.9 and 32.2 μm SE 1.1 respectively). Structures observed on leaf-portions prior to penetration included swollen germ-tube ends, simple oval appressoria, bilobed and multilobed appressoria (Figure 27 p81, Figure 28 p81) which were similar to those described by van den Heuvel & Waterreus [1983] and Garcia-Arenal & Sagasta [1980]. After 16 h incubation numerous single lobed and a few bilobed appressoria were observed in lesions from both Mp97 and A4 and after 18 h incubation bilobed appressoria were common in both. Multilobed appressoria were present after 20 h incubation especially in A4 induced lesions.

Hyphal penetration was evident from both Mp97 and A4 (Figure 29 p82) after 16 h. Few penetration hyphae were observed to originate from bilobed or multilobed appressoria, and although a few hyphae were seen penetrating through stomatal openings most penetrations occurred between epidermal cells. Groups of darkly stained mesophyll cells were frequently associated with mycelium often near the site of penetration of both strains incubated for 18 h. At 2 and 3 days a ring of similarly stained cells was observed surrounding the colony in both Mp97 and A4 infected leaf-portions, although in the latter the mycelium frequently appeared to extend beyond this ring. After 4 d incubation the ring of darkly stained cells was visible surrounding the lesions of Mp97, but not A4, inoculated leaves (Figure 30 p83). No differences were detected in the hyphal structure of Mp97 and A4 in the lesions.

Physiological and morphological characterisation

Following confirmation of their aggressiveness phenotype, 20 strains selected from cross 10 (Table 4 p61, 10 aggressive and 10 non-aggressive, see Characterising of *Pat1* on French bean p76) were compared for a range of physiological characters. Radial growth rate was slower in non-aggressive progeny and the mutant strain (Mp97) compared to wild-type progeny and the wild-type parent (SAS56). Conidial production was also lower in the non-aggressive progeny although there was no significant difference between the mutant strain and the wild-type parent. Sclerotial number was higher and sclerotial weight lower in non-aggressive strains than in wild-type strains in both progeny and parents (Table 8 p77). All strains grew readily as dense spreading colonies on MM and it was concluded the non-aggressive phenotype did not reflect an auxotrophic requirement. Although small differences in spore size were found between strains these did not

correlate with differences in aggressiveness (non-aggressive range of means 45.58 – 59.30 μm^2, aggressive range of means 47.65 – 54.08) (Table 8 p77).

Differences in acid production by Mp97, its parent A4, 20 selected progeny (as above) and wild-type strains (SAS56 and SAS405) were distinctive. All aggressive strains scored 2 or higher for intensity of colour change compared with non-aggressive strains (*Pat1* progeny and Mp97) which scored between 0 and 1.5 indicating a correlation between low acid production and the *Pat1* phenotype.

The mutant Mp97 differed in its response to incubation temperature compared with A4 and SAS56. At 25°C Mp97 showed a significantly slower growth rate (as was shown previously) than both wild-type stains (which also differed from each other), while at 20°C growth rates of Mp97 and A4 were indistinguishable. However, at lower temperatures (10 and 15°C) Mp97 grew significantly faster than both wild-type strains (Figure 31 p84).

Polygalacturonase expression

Cup plate assay

Comparison of total PG activity in filtrates of 8-h cultures showed a slightly larger significant difference between wild-type strains (SAS56 and A4) than between non-aggressive and aggressive progeny (Means 1.17, 1.37 enzyme activity units $P = 0.0004$ and means 1.35, 1.17 enzyme activity units $P = 0.025$ respectively) (Figure 33A p85). This suggests strain variation is the cause of the differences in PG activity rather than the involvement of *Pat1*. In contrast, in filtrates of 24-h cultures, no significant difference was found between wild-type strains ($P = 0.54$) or between the mutant strain (Mp97) and its parent (A4) ($P = 0.408$) but there was a significant difference between non-aggressive and aggressive progeny (mean 11.52 and 15.98 enzyme activity units respectively, $P = 0.0001$) (Figure 33B p85). Variances were stabilised by transforming the data prior to analysis (8-h data was square root transformed, 24-h data was power transformed, $\lambda = -0.0538$).

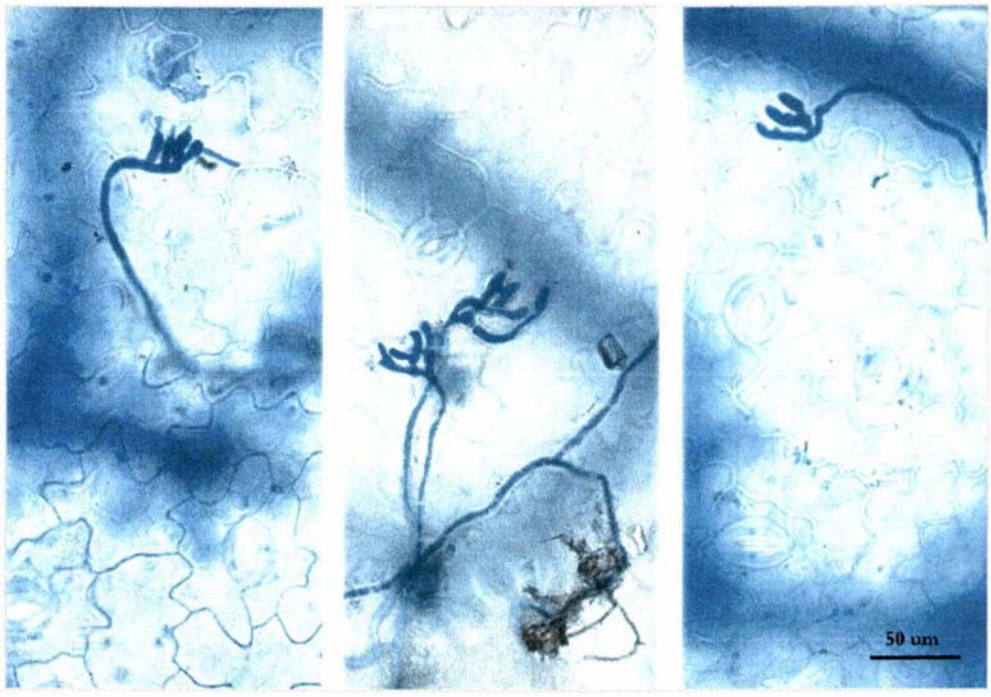


Figure 27 Multilobed appressoria of *Botrytis cinerea* isolate Mp97 on infected French bean leaves incubated for 22 or 24 hours at 20 – 25°C and stained with aniline blue.

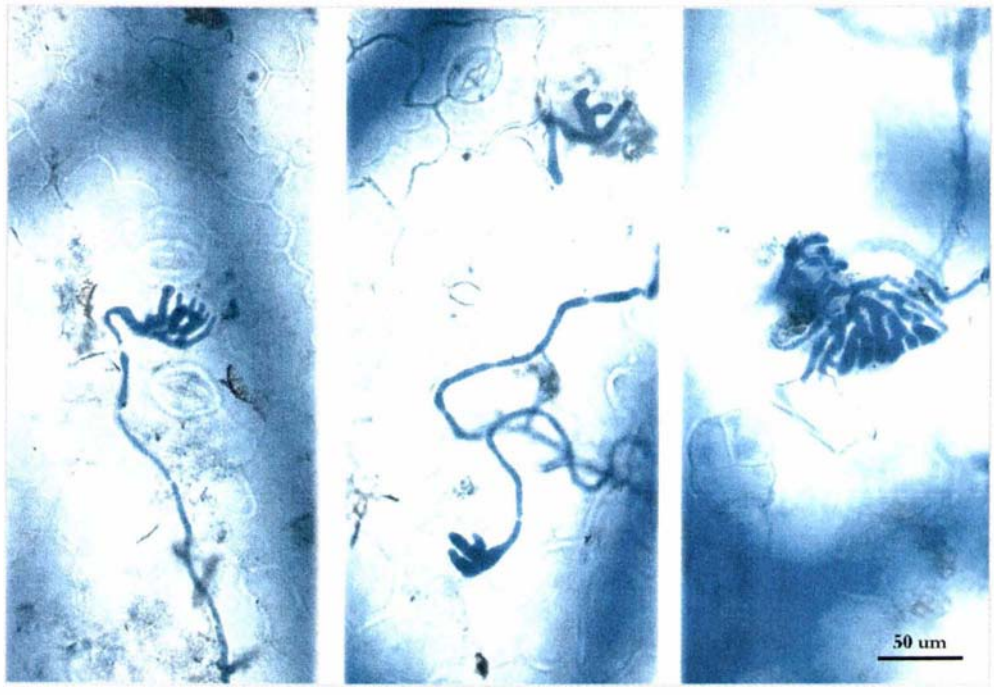


Figure 28 Multilobed appressoria of *Botrytis cinerea* isolate A4 on infected French bean leaves incubated for 22 or 24 hours at 20 – 25°C and stained with aniline blue.

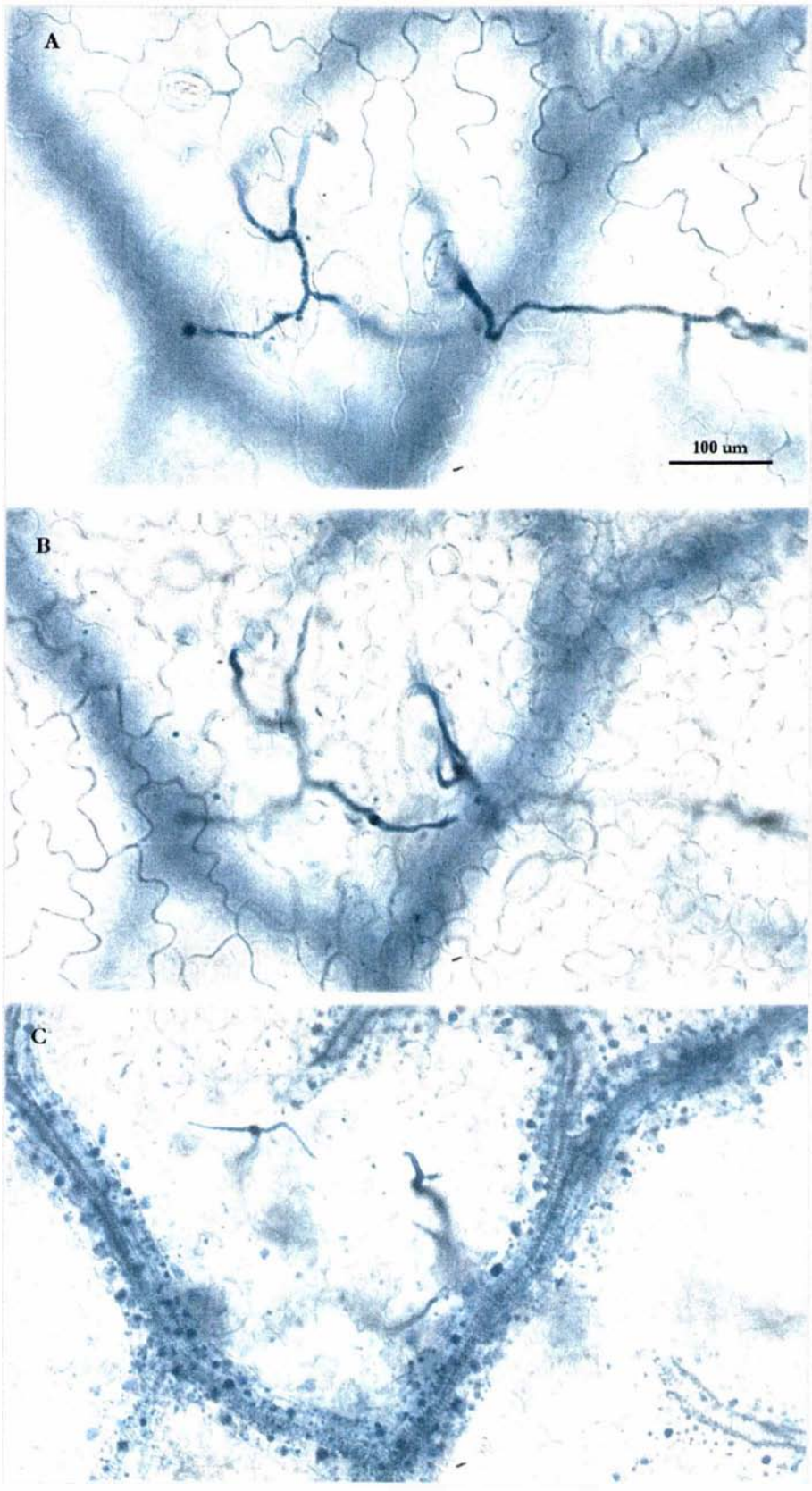


Figure 29 Hyphae of A4 penetrating through successive layers of French bean tissue after 20 hours incubation at 20 – 25°C. (A) Upper epidermis. (B) Palisade parenchyma. (C) Spongy parenchyma.

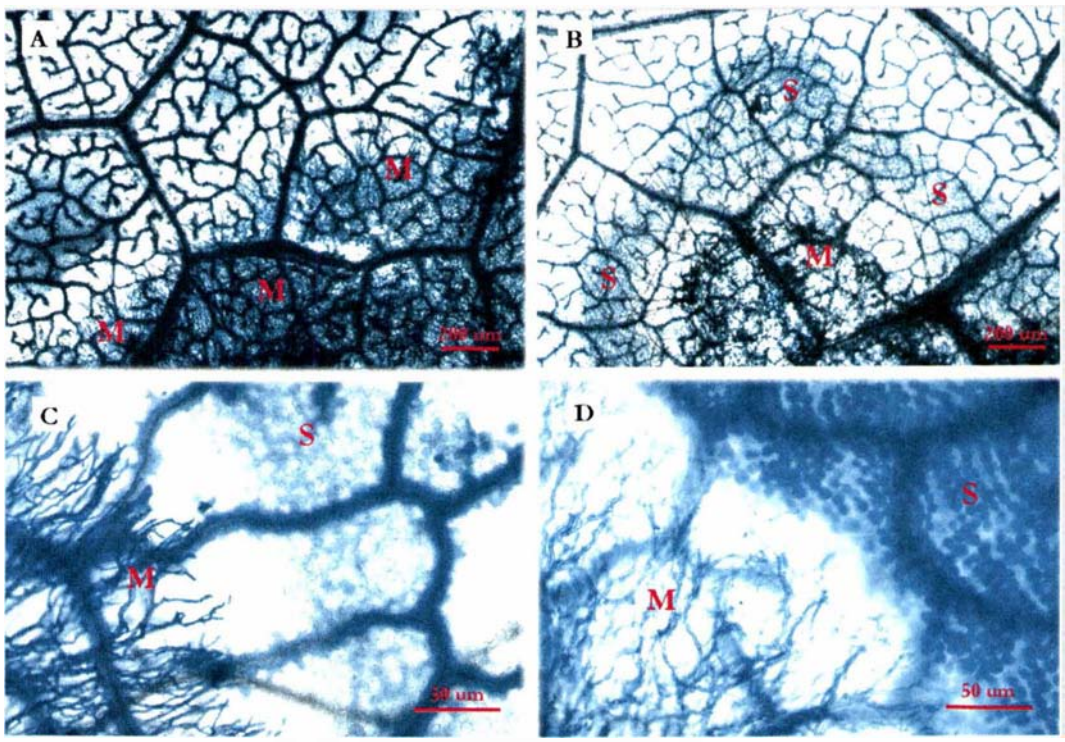


Figure 30 Mycelium of *Botrytis cinerea* in lesions on adaxial surface of French bean leaves incubated for 24 hours and cleared and stained with aniline blue. **M** = mycelium, **S** = darkly stained mesophyll cells. (A and C) A4 aggressive. (B and D) Mp97 non-aggressive.

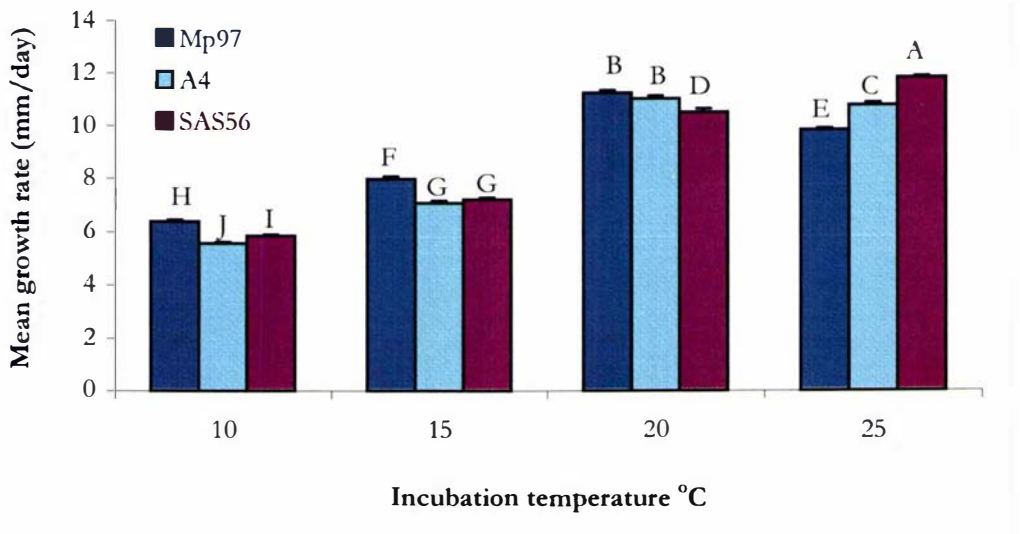


Figure 31 Daily growth rate on MEA of Mp97, A4, and SAS56 averaged over 8 days (10°C), 6 days (15°C), and 4 days (20 and 25°C). Means with the same letter are not significantly different (Duncan's multiple range test, alpha = 0.05). Bars indicate standard error of means from 5 replicate plates.

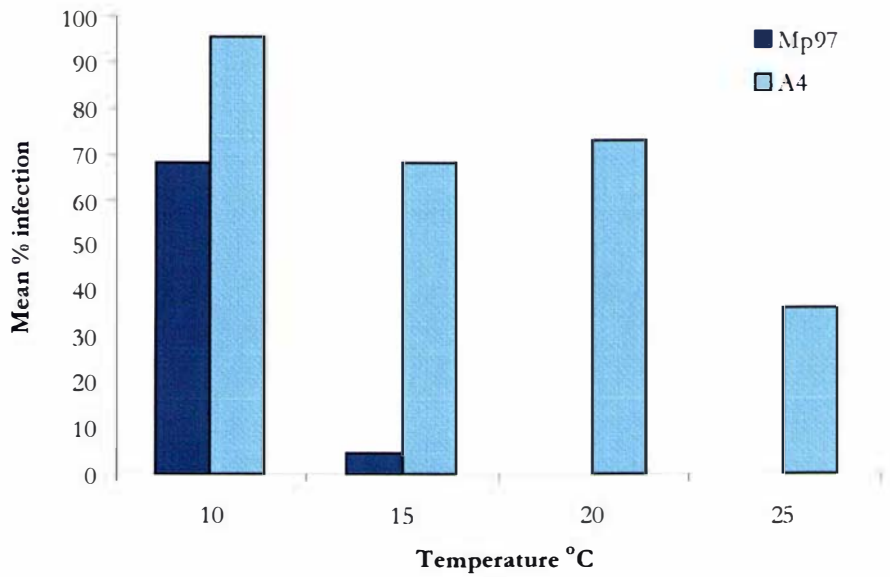


Figure 32 Infection of tomato stem-pieces recorded as a percentage of 22 replications after 14-days incubation.

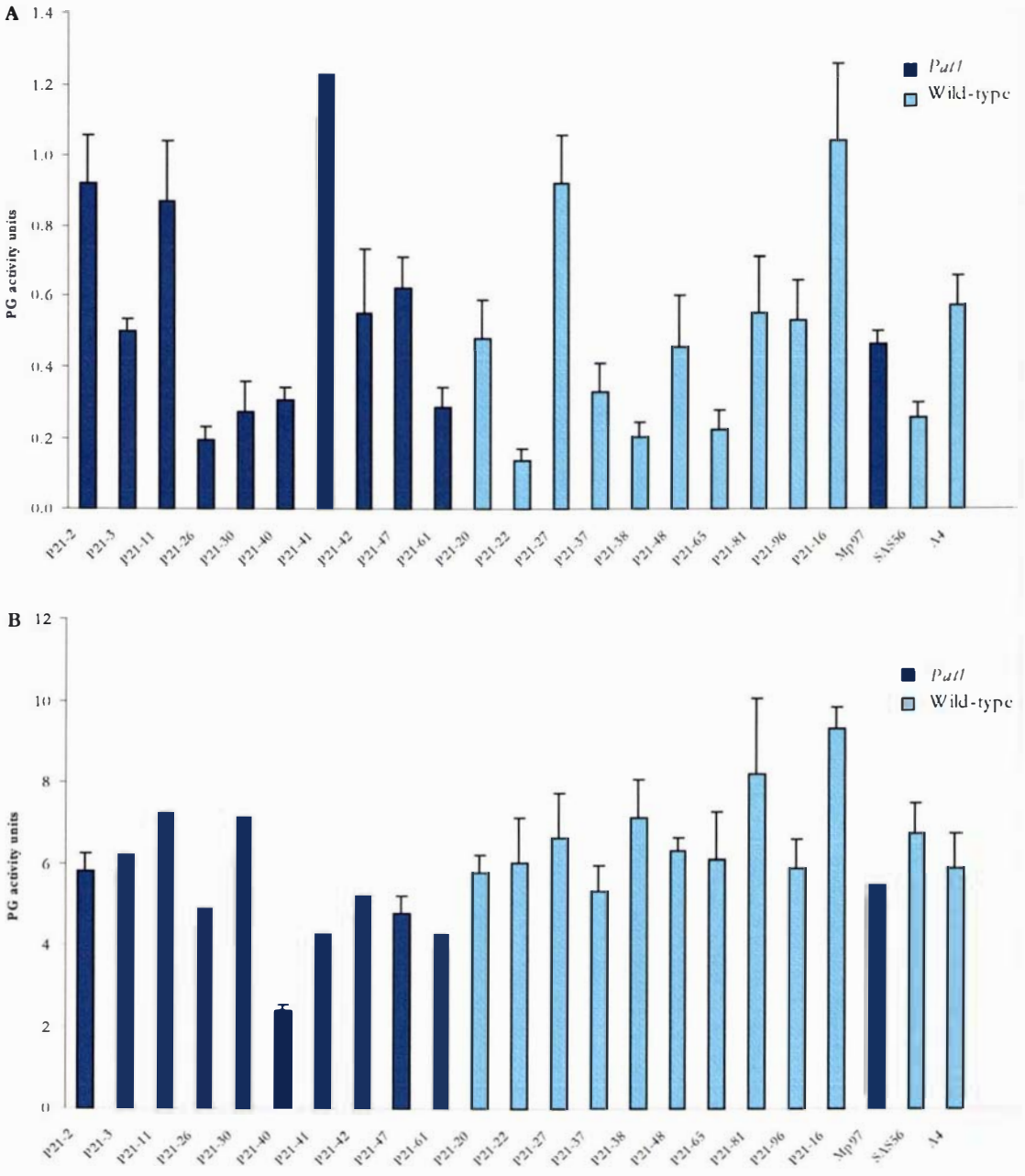


Figure 33 Total polygalacturonase activity of progeny (P-strains), parents (Mp97 and SAS56) from the cross Mp97 x SAS56 and A4 (expressed as activity units of the *Aspergillus* standard). (A) 8-hours incubation. (B) 24-hours incubation. Bars indicate standard error of means from 4 replications.

Isoelectric focusing and detection of PG isozymes

Dr Sharrock found that Mp97 and A4 produced identical isozyme profiles after separation by IEF including a prominent isozyme (pI 8) which was absent from the SAS56 profile (Figure 34 p87). The profiles from extracts of bean leaf-lesions (Figure 35 p87) were very different to those from culture filtrates (Figure 34 p87). Isozymes from SAS56 lesions were predominantly of low pI (<5.3), with relatively little expression of the pI 9 isoenzyme that was prominent in culture filtrate profiles. A4-induced lesions appeared to contain more isozymes, covering a broader pI range (3.5 – 8.3), than were in the culture filtrate. Extracts of Mp97-infected tissue revealed much less PG activity than in the necrotic lesions resulting from the two aggressive strains. The most active isoenzyme (and only one clearly visible) had a pI of about 4.8. This is partly attributable to the relatively short incubation period (105 min) which was more appropriate for the higher levels of PG activity found in extracts from larger lesions.

In order to compare similar PG activities on one gel lesion extracts of SAS56 and A4 were diluted 50 and 100 fold (Figure 35 p87). This revealed that the major isozymes expressed by A4 *in vivo* (pI range 4.2 – 5.0) were also expressed by Mp97 and that differences were restricted to the minor bands. In contrast SAS56 produced a major isozyme band at around pI 5.2 which was not evident in the *in vivo* extracts of the other two strains. Extracts of control leaves (not inoculated) produced no detectable PG bands (data not shown).

The infections caused by strains Mp97 and A4 followed very different courses, which may have resulted in the release of different inducers of PG isoform production. Total protein staining patterns of the IEF separations of Mp97 infected and control leaf extracts were almost identical, confirming that the fungus had caused minimal damage, whereas extracts of A4-infected leaf tissue contained a very different pattern of protein bands from those of the controls

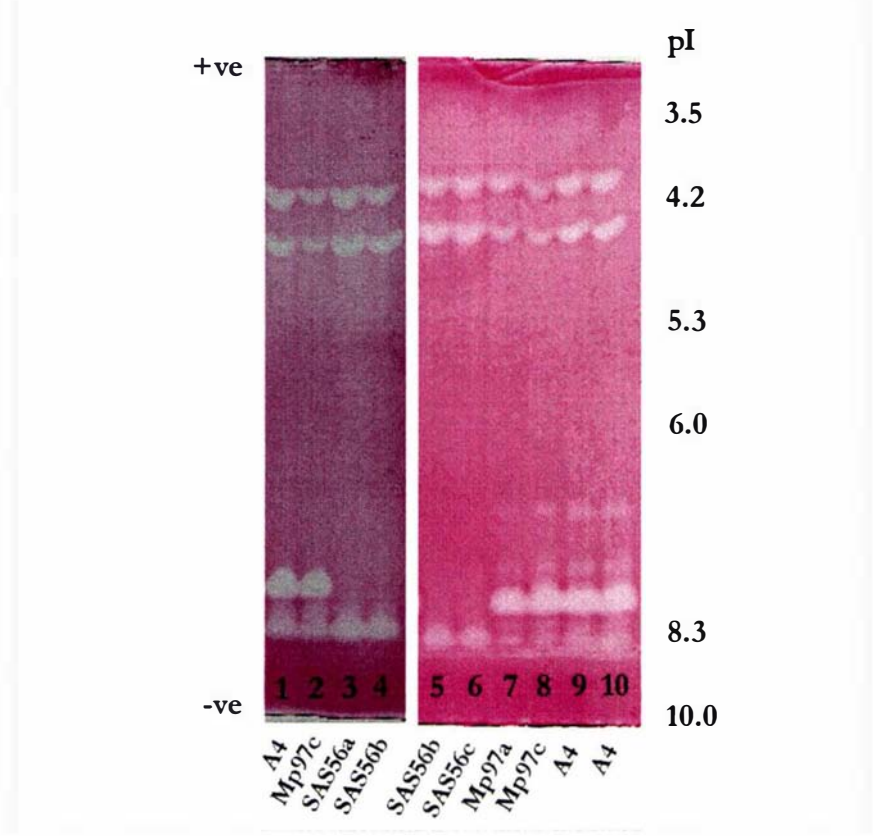


Figure 34 Polygalacturonase (PG) isozymes in 1-day-culture filtrates following isoelectric focusing and incubation for 4.5 hours at 37°C (overlays stained with ruthenium red). A letter after the strain number distinguishes replicated cultures. (Photo Dr Sharrock).

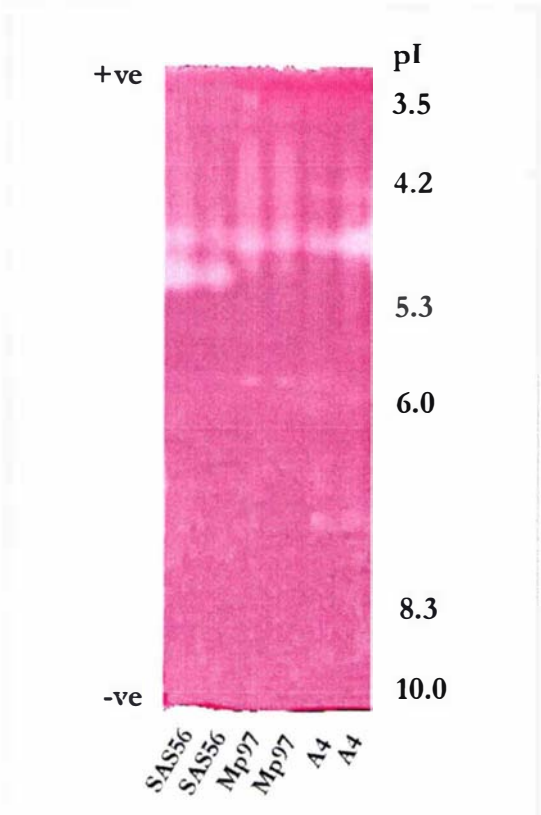


Figure 35 Polygalacturonase isozymes in extracts of 4-day-old lesions of bean leaves inoculated with B. cinerea. Lanes loaded with 10 µl of extract from lesions homogenised in 10 volumes of buffer. SAS56 lesion extract diluted 50 and 100-fold respectively. MP97 lesion extract undiluted. A4 lesion extract diluted 100 and 50-fold respectively (overlay incubated for 15 hours). (Photo Dr Sharrock).

Characterisation of Pat1 on other hosts

Pathogenesis on soybean, rose and tomato

In addition to French bean the aggressiveness of mutant Mp97 and its parent A4 was tested on soybean, rose and tomato. Responses with the soybean leaf assays were similar to that found on French bean leaves (Table 9 below). Typical Mp97 lesions were small and surrounded by a dark ring and remained restricted after 7-d incubation, in comparison with rapidly expanding watery edged lesions found with A4. In the rose assay Mp97 was slower to colonise than strain A4, but all flowers became fully infected by both strains after 15-d (Table 9 below). On tomato the mutant Mp97 did not infect stem-pieces at 20 and 25°C, but did produce infection at lower temperatures, albeit at a lower incidence than that of the parent strain (Figure 32 p84). To confirm that the fungus growing at 15 and 10°C was the strain inoculated, lesions were checked by reisolating (onto MEA) from the lesion margin of all lesions produced on stem-pieces inoculated with Mp97 and one inoculated with A4. These isolates were re-tested on French bean leaves: all lesions produced by Mp97 cultures were consistent with the non-aggressive phenotype, while those produced by the A4 culture were typical of aggressive lesions.

Table 9 Growth of Mp97 and the parental strain A4 on detached leaves of French bean and soybean seedlings, and rose flowers.

Host	Mp97	A4	Pr >F‡
French bean leaves*	1.30 (0.12)	9.60 (0.80)	0.0001
Soybean leaves †	2.85 (0.17)	10.68 (0.31)	0.0001
Rose flowers (days to full infection)	13.86 (0.40)	11.29 (0.29)	0.0002

* Analysis performed on power transformed data (0.8)
† Analysis performed on square root transformed data
‡ Differences were considered significant when Pr>F < 0.05

Phytoalexin induction on Soybean leaves

There was no difference in phytoalexin induction between the mutant Mp97 and its parent A4 (absorbance 0.19, SE 0.019 and 0.18, SE 0.012 respectively), but both differed significantly from the media control (absorbance 0.01, SE 0.001, Duncan's multiple range test, alpha = 0.05).

Competition

The interaction of two strains of *B. cinerea* at the infection site was observed in competition experiments. The effect on infection of an aggressive strain (SAS56) by a

non-aggressive mutant (P33-146) was ascertained by the size of the lesion produced. At 0-h challenge the lesion size in both combinations was part way between that caused by individual (control) inoculation of the two strains suggesting a retarding effect of the non-aggressive strain on the growth of the aggressive strain. Lesion sizes in other challenge combinations were dominated by the strain inoculated first, even if this was the non-aggressive strain. When applied 12 h after the initial inoculation the second strain had no influence on the size of the resulting lesions (Figure 36 p90). The lesion size was larger for the combination treatment involving both strains applied at 0 h when A4 was applied first, compared with the similar treatment when Mp97 was applied first. This was attributed to settling of the conidia from the first inoculation and the lack of mixing when the second strain was added shortly after.

In order to determine whether the dominating effect of the first strain inoculated was a general phenomenon, competition was examined between two genetically marked aggressive strains by estimating the composition of strains in the resulting lesion. The proportion of conidia from the harvested lesion carrying the marker for sodium selenate resistance was interpreted as the proportion of Ms044 biomass present in the lesion and similarly the proportion of conidia carrying vinclozolin resistance was that of SAS405. Where leaves were inoculated with spores from both strains at 0 h the spores harvested had a 50:50 mix of both markers, while lesions from sites where the challenging strain was applied after 6 h contained a small percentage of spores carrying second strain markers, and at 12 h the spores contain no challenge markers. Analysis of lesion radii (8 replicate lesions, using Duncan's multiple range test Alpha = 0.05) revealed no significant difference between Ms044 and SAS405 (Figure 37 p90) indicating the two strains were equally aggressive on French bean. Thus the pattern whereby the first strain inoculated dominates the infection site is apparently the norm for *B. cinerea* infections of French bean leaves.

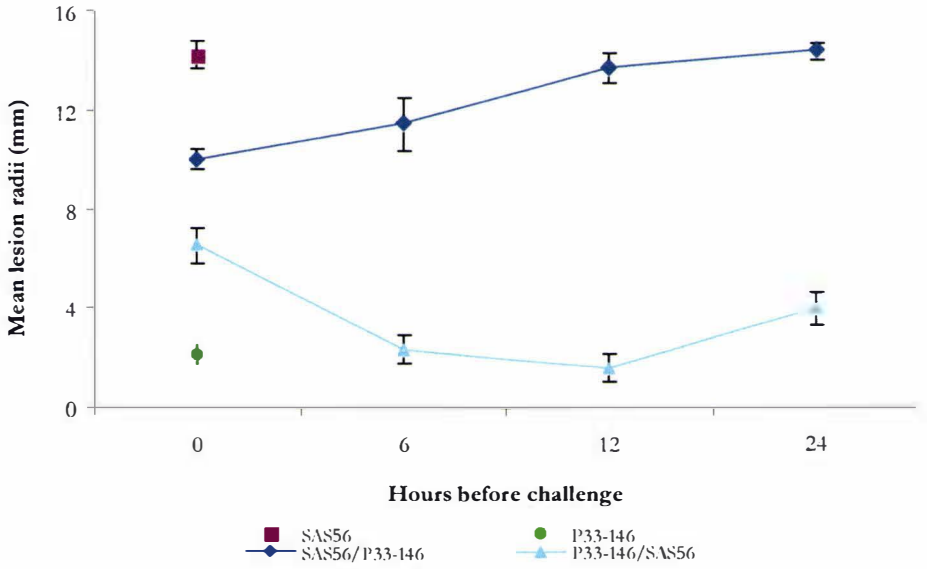


Figure 36 Lesion radii on French bean leaves inoculated with either SAS56 (aggressive) or P33-146 (a non-aggressive single-ascospore strain) and then challenged after 0, 6, 12 or 24-hours incubation with either P33-146 or SAS56 respectively (incubated for 4 days at 20 – 25°C). Bars indicate standard error of means of 4 replications in each of 4 blocks.

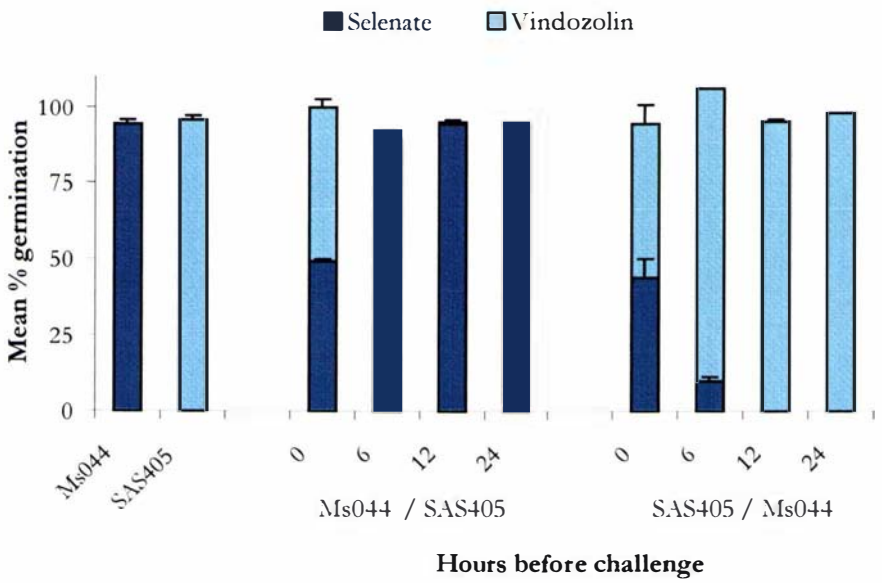


Figure 37 Percent germination on selenate ($MM+SeO_4^{B}$) and vinclozolin (MEA+vinclozolin) differential media, of spore suspensions made from whole leaf-lesions inoculated with either Ms044 (selenate resistant, SelR) or SAS405 (vinclozolin resistant, *Daf1LR*) and then challenged (Ms044 challenged with SAS405, SAS405 challenged with Ms044) after 0, 6, 12 or 24-hours incubation (incubated for 7 days at 20 – 25°C). Bars indicate standard error of means of 3 replications of 300 spores.

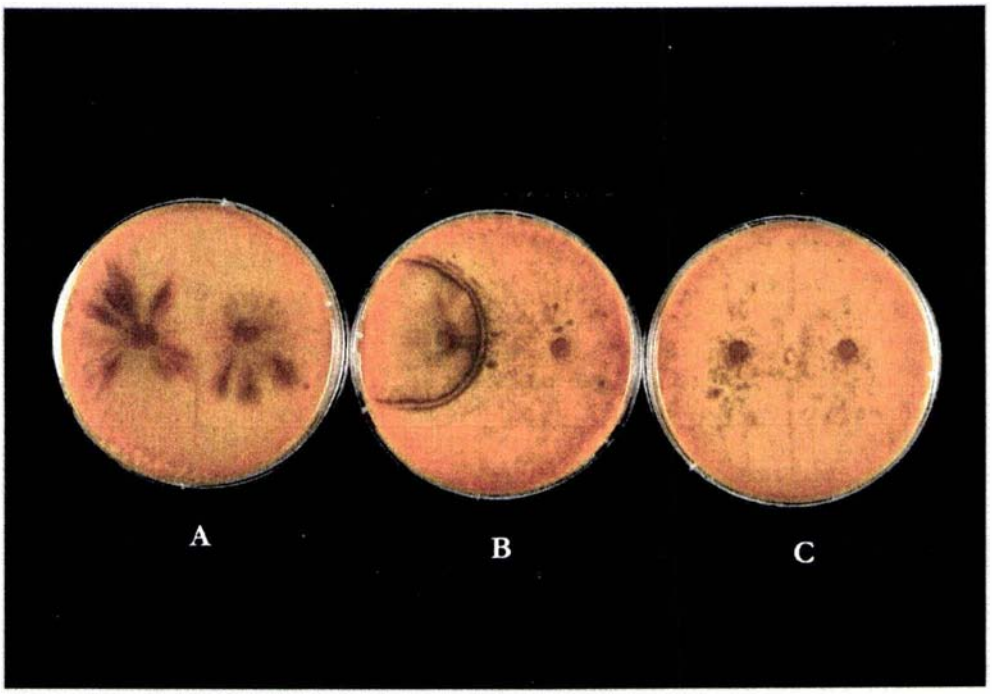


Figure 38 Interaction zone on MEA+NaCl. (A) P33-146 paired with P33-146. (B) P33-146 paired with SAS56. (C) SAS56 paired with SAS56.

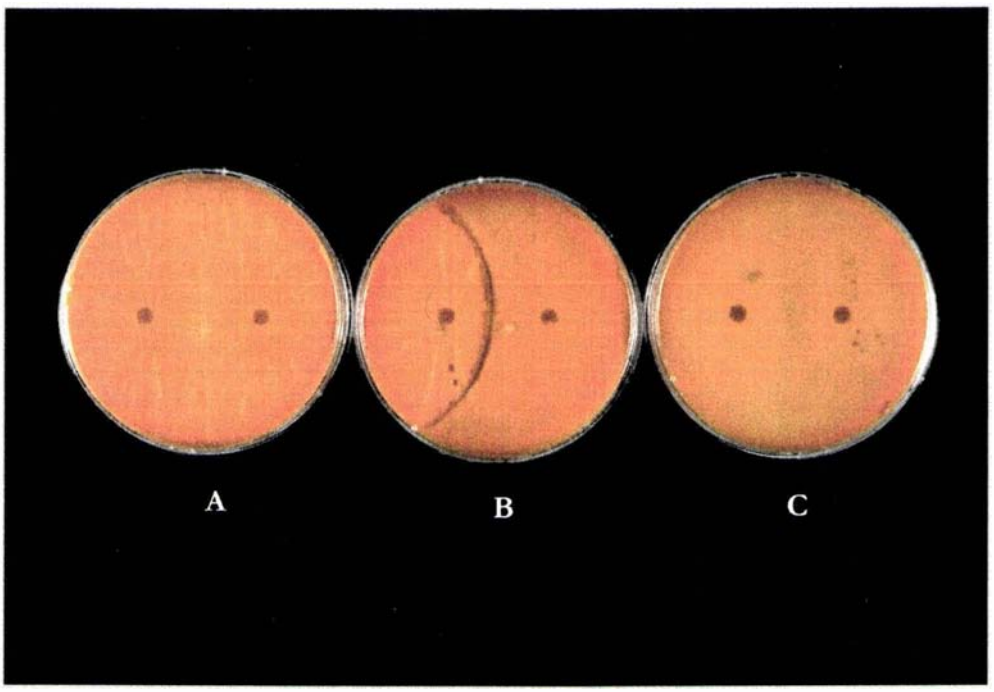


Figure 39 Interaction zone on MEA+NaCl. (A) SAS405 paired with SAS405. (B) SAS405 paired with Ms044. (C) Ms044 paired with Ms044.

Interaction experiments were undertaken to assess the likelihood that heterokaryon formation might confound these results. Both Ms44/SAS405 and P33-146/SAS56 pairings on MEA+NaCl produced distinct dark interaction zones, compared with no interaction zones when two plugs of the same strain were paired (Figure 38 p91, Figure 39 p91). The formation of interaction zones indicates mycelial incompatibility between the strains, and probably that the strains are vegetatively incompatible and thus unable to form heterokaryons [Beever & Parkes, 1993]. This is supported by the results of the second competition experiment where total percent germination of sodium selenate and vinclozolin resistant strains (calculated separately) were close to 100% for all mixed inoculation of Ms44 and SAS405 (Figure 37 p90). If a heterokaryon was formed (assuming resistance is dominant) the total percentage germination (sodium selenate + vinclozolin resistance) would be expected to exceed 100 if appreciable numbers of heterokaryotic conidia were formed.

Discussion

It is apparent that the reduced aggressiveness of *Pat1* isolates is not specific to infections on French beans, indicating that the gene affects a fundamental process of *B. cinerea* pathogenicity. Pathogenesis of *Pat1* strains on soybean closely resembles that on French bean in that limited lesions are formed. On roses spreading lesions were formed, but the rate of lesion spread was slower. At higher temperatures in the tomato test the mutant was essentially non-pathogenic. Its aggressiveness on tomato at 10 and 15°C may reflect reduction in host defence responses as tomato is known to grow poorly at low temperatures [Eden *et al.* [1996b]. It may also reflect the slightly faster growth of the mutant relative to wild-type strains at 10 - 20°C (Figure 31 p84). Although Mp97 consistently produced fewer infections than A4 on tomato stem-pieces over the range of temperatures tested, invasive infections were sometimes established at 10 and \5°C albeit less frequently than with A4. As with rose there was no evidence for the formation of restricted lesions.

While some physiological differences were observed between *Pat1* and aggressive strains (Table 8 p77) none provides an obvious explanation for non-aggressive behaviour. Slower radial growth rate at higher temperatures and more, smaller sclerotia were correlated with non-aggressive lesion formation in parents and progeny suggesting a possible pleiotropic effect between these characteristics and *Pat1*. However, these differences are relatively small and within the range found in wild-type strains [Beever *et al.*, 1989]. Non-aggressive mutants of *Sclerotinia sclerotiorum* also exhibited a reduced growth

rate on agar medium and were defective in sclerotial production [Godoy *et al.*, 1990]. Phillips *et al.*, [1987] reported a correlation between increased spore size and increased aggressiveness in field isolates of *B. cinerea* taken from roses. We found no evidence to suggest a similar difference between Mp97 and A4.

Two broad mechanisms can be identified whereby *Pat1* could affect the infection process. Firstly, through an alteration to the process of host recognition and response either through induction of a faster and/or more intense response or by greater sensitivity to defence compounds. An increased host defence response could lead to acquired resistance in the host resulting in a reduced or restricted lesion size. However, no evidence was found for enhanced induction of phytoalexins in *Pat1* inoculated soybean leaves. It is plausible that *Pat1* strains may have a reduced ability to metabolise phytoalexins in a similar manner to the non-aggressive mutant (BC-5) of van den Heuvel & Grootveld [1978; van den Heuvel, 1976]. Van den Heuvel & Grootveld [1978] studied phytoalexin induction in French bean leaves by aggressive and non-aggressive field-isolates of *B. cinerea*. Phaseollin was produced by the leaf in a zone of apparently healthy cells surrounding the lesion and was metabolised by the fungus to 6a-hydroxyphaseollin (less inhibitory) by aggressive isolates. In contrast to aggressive infections, leaves infected with the non-aggressive strain (BC-5) produced restricted lesions and accumulated twice as much phaseollin by dry weight after 4 d incubation [van den Heuvel & Grootveld, 1978]. Moreover, BC-5 was found to be severely limited in its ability to metabolise phaseollin to 6a-hydroxyphaseollin [van den Heuvel, 1976]. In the current study microscopic examination of leaf-lesions and comparisons of phytoalexin induction suggests that Mp97 is unaffected in its ability to induce or tolerate plant defence responses during the early stages of infection. The presence of a darkly stained ring of cells surrounding Mp97 in 4-d old lesions (Figure 26 p78) is suggestive of a defence response indicating Mp97 may be defective in its ability to either tolerate or metabolise defence compounds at this stage. Increased aggression on tomato stems at lower temperatures could be due to the lower production of defence compounds at temperatures below optimum for plant growth.

The second mechanism by which *Pat1* could affect the infection process is through a quantitative or qualitative deficiency in the production of attack compounds. Quantitative (total activity) and qualitative (isozymes) polygalacturonase activity was investigated for *Pat1* non-aggressive strains and wild-type aggressive strains.

* Total PG activity *in vivo* is relatively low in Mp97 and most non-aggressive progeny (Figure 35, p87; Appendix 9, p143). This is interpreted as an indirect effect of lesion size and development on PG activity, rather than any direct influence of *Pat1*.

** While it is possible that the differences in the minor bands may reflect the direct influence of *Pat1*, further work is needed to clarify the reproducibility of the results and the compounding effect of dilution of the extracts.

Comparison of total PG activity in vitro filtrates of 8 h and 24 h cultures revealed no consistent differences between aggressive and non-aggressive strains. This is in agreement with the findings of some other workers [Leone, 1990; Tiedemann, 1997]. It is possible that measurements of total PG activity could mask important differences in the range of PG isozymes produced by the non-aggressive strains compared with the aggressive strains. An important role in the early stages of infection has been proposed for a constitutively produced PG isozyme of *B. cinerea* [Leone & van den Heuvel, 1987; Leone *et al.*, 1990; van der Cruyssen & Kamoen, 1992]. In addition, Have *et al.* [1997] was able to produce non-aggressive mutants by disrupting a gene coding for a PG isozyme expressed in the early stages of pathogenesis (*Bcpga1*). Thus the *in vitro* and *in vivo* production of PG isozymes by the strains Mp97, A4 and SAS56 was examined in more detail.

The range of PG isozymes produced by different *B. cinerea* strains is known to vary substantially [Magro *et al.*, 1980]. Consistent with this, distinct differences were found between the PG profiles of SAS56 (Italy) and A4 (New Zealand). Mp97, grown *in vitro* and *in vivo* produced identical PG profiles to that of the wild-type A4 parent, with respect to the major bands. This suggests that major PG isoenzymes were not affected by the mutation of A4 to MP97 and that *Pat1* does not encode or modify a PG enzyme. This conclusion is supported by comparison of the polygalacturonase isozyme profiles of ten aggressive and ten non-aggressive progeny of cross 10 (MP97 with SAS56, Table 4 p61), which did not link any particular isoenzyme to pathogenicity (Appendix 9 p141).*

PG isozyme profiles found in extracts of bean leaf lesions in this study differed considerably from those produced by the same strains *in vitro* consistent with the findings of Verhoeff & Warren [1972], Tobias *et al.* [1995], and Sharrock [unpublished]. These differences can be attributed to differences in substrate and to the presence of polygalacturonase inhibitor proteins (PGIPs) *in vivo*. *P. vulgaris* is known to produce a number of forms of PGIP [Pressey, 1996; Cervone *et al.*, 1996] capable of inhibiting PGs from a variety of fungi, including *B. cinerea* (H.U. Stotz, pers. com. - Sharrock). Strains SAS56 and A4 showed substantial differences in the variety of PG isozymes produced but caused similar sized lesions while differences between *in vivo* PG profiles of A4 and Mp97 were restricted to the minor bands, once the lesion extract of A4 had been diluted to a similar total activity.**

Analysis of isozyme patterns from progeny of cross 10 (Mp97 crossed with SAS56) by Dr Sharrock showed that several PG isozymes segregated independently suggesting they are controlled by one gene. This is the first report of segregation of PG isozymes in

progeny of a sexual cross of *B. cinerea* and a poster describing the work was presented at the Australasian Plant Pathology Society 11th Biennial Conference (APPS) held in Perth in September 1997 (Appendix 9).

The low production of organic acids by *Pat1* strains may be the cause of their non-aggressive phenotype. Oxalic acid production is important in the infection process of a number of plant pathogenic fungi including *S. sclerotiorum* [Godoy *et al.* 1990; Noyes & Hancock, 1981], and *Endothia parasitica* [Havir & Anagnostakis, 1983]. *B. cinerea* is known to synthesise oxalic acid [Gentile 1953] which has been correlated with aggressiveness on some hosts but not others [Germeier *et al.*, 1994; Pesis *et al.*, 1991]. These results show a correlation between low acid production and the non-aggressive phenotype suggesting organic acids may play an important role in successful infections. Three types of activity have been proposed for the action of organic acids in fungal infections; acting as a direct toxin on plant tissues, acting in synergy with cell wall degrading enzymes either by lowering the pH and creating conditions favourable to the activity of pectic enzymes (and possibly unfavourable to plant defence chemicals), or assisting in the breakdown of pectic material in cell walls by chelating calcium ions involved in polymer cross-linking [Verhoeff *et al.*, 1988; Havir & Anagnostakis, 1983]. More work is needed to identify and quantify acid production in *Pat1* strains compared to wild-type strains and to identify the nature of its involvement in the infection process.

A number of possible mechanisms for the reduction of aggressiveness of *Pat1* have been studied, although many other compounds putatively important in pathogenesis (Appendix 7 p137) have not yet been investigated. However, addition of cell-free extracts from aggressive lesions to mycelial plug inoculations of the mutant Mp97 onto French bean leaves did not affect the development of non-aggressive lesions. This suggests that if secreted attack compounds are involved, concentration at the site of activity may be important and differences may be difficult to demonstrate.

In summary, *Pat1* was identified as a single gene affecting pathogenicity by 1:1 segregation patterns in repeated sexual crosses. The non-aggressive character is unlikely to be due to gross unfitness as non-aggressive (*Pat1*) strains grow normally, are indistinguishable from wild-type in gross morphology, and grow well on minimal medium indicating no unusual nutrient requirements. The two hypotheses presented, reduced ability to metabolise phytoalexins and reduced toxicity due to low production of organic acids, provide specific primary targets for future investigations into the physiological basis of *Pat1*.

Competition experiments demonstrated that two strains inoculated together can co-exist in the lesion apparently independently. If applied sequentially the strain that becomes established first will continue to dominate, irrespective of the relative aggressiveness of each strain. This could be due to a number of different interactions; the first strain might produce an inhibitor that prevents the germination or growth of the second strain, the presence of mycelium of the first strain may physically exclude the second strain, or a mycelial incompatibility interaction between the two strains leading to cell death might effectively kill the newly germinating strain while the established strain recovers and continues to grow. Further work is needed to fully understand the interaction of different strains of *B. cinerea* at the site of infection.

Domination of the lesion site by the non-aggressive strain suggests a possible use as a biocontrol agent. Infections by *B. cinerea* commonly establish in wounds and then expand into healthy tissue. The inoculation of wounds with a non-aggressive (therefore non-invasive) strain could provide control by preventing subsequent infection by an aggressive strain.

Chapter 6 General Discussion

This study demonstrates that standard mutagenesis methods can generate stable, toxicant-resistant, and non-aggressive mutants of *B. cinerea* from single-ascospore strains and that these mutants can be used in conjunction with sexual crossing procedures to genetically analyse determinants of pathogenicity. Some previous studies [Grindle, 1979; Geeson, 1978] experienced difficulties in obtaining stable mutants. Success in this study is attributed to the use of single-ascospore strains. This is supported by the success of recent workers incorporating mutagenesis of single-ascospore strains [Hilber, 1992; Faretra & Pollastro, 1993b; Chabani, 1991; Buttner *et al.*, 1994].

B. cinerea appears to be relatively resistant to mutagenesis by NQO requiring a concentration of 4 – 6 μg ml^{-1} to give a 1 – 5% survival relative to *Aspergillus nidulans* which gave a survival of 0.1% when incubated in 1 μg ml^{-1}. [Bal *et al.*, 1977]. In addition, NQO produced non-aggressive mutants at the rate of 1.8% of the survivors screened compared with 5.8% for u.v. mutagenesis. In a similar study the overall rate for isolating non-aggressive mutants of *Fulvia fulva* was lower, u.v. mutagenesis giving a rate of 1.4% and chemical mutagens, N-Methyl-N'-Nitro-N-nitrosoguanidine (NTG) and 1,3-butadiene diepoxide (DEB), giving 0.06% and 0.8% respectively [Lenyon, 1990].

Mating experiments between the new markers (*nit1*, *Sel1R* and *Pat1*) and fungicide resistance genes (*Mbc1* and *Daf1*) revealed no evidence of linkage. However, crosses 4, 5, 6 (Table 2 p45), and 12 (Table 5 p63) showed a loose linkage between the *Mbc1* and *Daf1* genes of 29 – 35% in agreement with results reported by Beever & Parkes [1993]. Faretra & Pollastro [1991; 1992] recorded recombination values between 17 and 48% in 12 crosses and recently confirmed the linkage of these genes by tetrad analysis suggesting a distance of 47 map units [Faretra & Pollastro, 1996].

The absence of NitM mutants amongst the Nit mutants generated in this study (Nitrate non-utilising mutants p46) prevented the investigation of vegetative compatibility

with complementation tests using *nit1* and NitM paired mutants as has been commonly accomplished with other fungi [Brooker *et al.*, 1991]. Problems in obtaining stable Nit mutants in *B. cinerea* have been reported previously [Alfonso *et al.*, 1996].

Selenate resistant sulphur non-utilising mutants were able to complement Nit mutants in vegetative compatibility tests of *Fusarium spp* [Correll & Leslie, 1987; Jacobson & Gordon, 1988]. In this work it was found that some selenate resistant mutants of *B. cinerea*, especially in classes A and C, produce thin expansive growth on minimal medium and it was demonstrated that one such mutant had the ability to complement a *nit1* mutant generated from the same strain, but not a *nit1* mutant generated from another parental strain. This is the first report of the successful use of auxotrophic mutants to demonstrate complementation in *B. cinerea*. These results provide support for the suggestion of Beever & Parkes [1993] that a vegetative incompatibility system may be operating in *B. cinerea*, although further work will be needed to evaluate the practicality of this method to investigate vegetative incompatibility amongst field strains.

During the course of this study new genetic markers were produced for *B. cinerea* based on resistance to sodium selenate and potassium chlorate which were shown to be useful (in addition to fungicide markers) in genetic studies and competition experiments. Selected strains with different marker combinations (fungicide resistant, selenate resistant and nitrate utilisation) have been lodged in the International Collection of Micro-organisms from Plants (ICMP, Appendix 3 p127) maintained by Landcare Research at Mt Albert in Auckland (NZ).

Stable non-aggressive mutants were isolated from the single-ascospore strain A4 following mutagenic treatment and selection on French bean leaves. Mutants crossed readily with reference strains and analysis of segregation patterns in progeny revealed one mutant (Mp97) where the non-aggressive phenotype segregated 1:1 indicating a single gene (*Pat1*) of major effect. Mp97 was similarly non-aggressive on French bean and soybean leaves and produced fewer infections on tomato stems, but caused slow spreading lesions on rose flowers, suggesting a differential host response and implicating the involvement of host resistance in the mechanism of *Pat1* non-aggression. This, combined with microscopic observations showing a distinctly stained ring of mesophyll cells surrounding bean lesions, suggests that *Pat1* strains may be defective in the ability to either tolerate or metabolise defence substances [van den Heuvel & Grootveld, 1978]. In addition, the non-aggressive phenotype was correlated with low production of organic acids on agar medium. In light of the importance of organic acids in the infection process

of other plant pathogenic fungi, especially the closely related organism *Sclerotinia sclerotiorum*, this result warrants further investigation. Selected *Pat1* strains have been lodged in the ICMP (Appendix 3 p127).

French bean leaves pre-inoculated with a *Pat1* (non-aggressive) strain were protected against infection by a wild-type aggressive strain suggesting a possible use for *Pat1* strains as biocontrol agents. Significant reduction of symptoms in a number of pathogen/plant interactions have been reported following pre- or concurrent-inoculation with a weakened or non-aggressive strain of the pathogen. A local inoculation of tomato and potato leaves with *Phytophthora infestans* under unfavourable fungal growth conditions gave 30 to 70% protection [Stromberg & Bishammar, 1991], suppressed sporulation and delayed penetration by formation of papillae [Heller & Gessler, 1986; Kovats *et al.*, 1991]. In mixed inoculations of Tn-5 induced avirulent mutants and wild-type virulent strains of *Pseudomonas solanacearum* on tomato the avirulent mutant was invasive but caused no disease symptoms while the virulent strain was unable to colonise [Trigalet & Trigalet-Demery, 1990]. An endophytic non-pathogenic mutant of *Colletotrichum magna* protected plants from diseases caused by *Colletotrichum* and *Fusarium spp.*[Freeman & Rodriguez, 1993; Prusky *et al.*, 1994]. A similar result was reported for an attenuated strain of *B. cinerea,* produced by culturing under environmental and cultural extremes, which induced systemic acquired resistance in begonia [Beauverie and Ray in 1889 and 1901 reported by Chester 1933]. To determine the potential of *Pat1* strains as biocontrol agents more work is needed to identify the mechanism and to evaluate the effect of host and environment on the interaction.

Epidemiological studies of *B. cinerea* are hampered by a lack of suitable markers. Fungicide resistance markers are usually unacceptable due to the extensive use of such sprays on horticultural properties, the presence of resistant strains in the existing fungal populations, and because the release of resistant strains could result in subsequent loss of disease control. Because the chlorate (*nit1*) and selenate resistant mutants (SelR) developed in this study retain high levels of pathogenicity they have the potential for use as marked strains in these studies. Selenate resistant spores of Ms044 were easily quantified in competition experiments (Competition p88) demonstrating how marked strains might be utilised in epidemiological work. Although class A SelR 'raw' mutants (i.e. from direct mutation, not generated from crosses) such as Ms044 could be used, there is a high probability that these strains are 'multiple-hit' mutants, i.e. have mutations in more than one gene, and may therefore prove less fit in the field. Better characterised strains with a

range of additional markers (e.g. *nit1* and fungicide resistance) could be developed by crossing and then selecting vigorous progeny with a demonstrated ability to compete with field strains.

Future work

The following section outlines possible ways that different aspects of this work might be developed further and extended into new areas.

Although the biochemical basis of non-aggression of *Pat1* strains was not determined in this study the results do offer some intriguing clues suggesting a direction for further work. Correlation between low acid production and the non-aggressive phenotype needs to be further investigated. Culture filtrates could be examined for organic acids to determine the difference in nature and quantity of acids secreted by *Pat1* and wild-type strains *in vivo* and *in vitro* using gas chromatography and high performance liquid chromatography [Godoy *et al.*, 1990; Redgwell, 1980].

Enzymes that detoxify host plant phytoalexins have been identified from many plant pathogenic fungi including stilbene oxidase from *B. cinerea* (detoxifies pterostilbene and resveratrol phytoalexins of grape) and phaseollidin hydratase from *Fusarium solani* f. sp. *phaseoli* (detoxifies phaseollidin from French bean) [VanEtten *et al.*, 1995]. Mutants defective in a bean phytoalexin detoxifying enzyme might produce small non-invasive lesions (contained by phytoalexin build up in plant tissues) and exhibit a localised cross protection effect as found with Mp97. The ability of *Pat1* and aggressive strains to grow in the presence of bean phytoalexins could be tested by a toxicology test where growth responses are evaluated in media containing various concentrations of bean phytoalexins. In addition, measurements of bean phytoalexins (phaseollin, phaseollidin and phaseollinisoflavan) could show whether there is a build up of phytoalexins in beans infected with non-aggressive strains compared with aggressive strains. This has been demonstrated in a non-aggressive field-isolate (BC-5) tested by van den Heuvel & Grootveld [1978]. The workers in van den Heuvel's study were unable to finally conclude that the deficiency in phytoalexin metabolism in BC-5 was the direct cause of the non-aggressive phenotype. However, a study as outlined above, where results could be demonstrated in a number of *Pat1* strains, would be much more robust in demonstrating the importance of such a mechanism.

Activity of pathogenesis related enzymes such as polymethylgalacturonase, pectin-lyase, laccase and protease (Appendix 7) could be assessed in a similar manner to the

polygalacturonase assays already described. In addition, recent work by Tiedemann [1997] demonstrated that aggressiveness of field-isolates of *B. cinerea* was closely correlated with high levels of active oxygen species (H_2O_2 and OH* radicals) in the host tissue during infection and that such strains were more suppressive of plant peroxidase activity than non-aggressive isolates. The importance of peroxidase in the resistance of ripe tomatoes to *B. cinerea* infection is supported by studies of heat treated fruit (38°C for 3 d) where the heat treatment was shown to enhance the level of peroxidase (compared with fruit held at 20°C) and give greater resistance to infection [Lurie, 1997]. In light of these findings it may be productive to examine bean lesions of *Pat1* and wild-type aggressive strains for levels of active oxygen species.

A molecular biological approach to identify markers linked to *Pat1* might include the use of bulked segregant analysis (a gene isolation method devised for use in situations where there is no prior knowledge of the gene product) [Michelmore *et al.*, 1991; Tanksley *et al.*, 1995]. DNA from the progeny of a *Pat1* strain crossed with a genetically distinct wild-type strain would be bulked to give two samples, aggressive and non-aggressive, and screened for differences using molecular markers such as RFLP probes or RAPDs. As the two populations are expected to be heterozygous for all regions except the *Pat1* gene polymorphic differences between the banding patterns for the two populations would identify markers either side of the gene with a sensitivity of 10% recombination frequency [Michelmore *et al.*, 1991]. These markers could then be used to screen a *B. cinerea* DNA library and isolate clones carrying the *Pat1* gene, which could be sequenced and identified from homology with known gene sequences on the data base. Alternatively, they could be used to transform a non-aggressive strain demonstrating restoration of the aggressive phenotype. However, this would be a challenging task.

As a result of the success of complementation tests in this study, SelR (class A) and *nit1* mutants are being selected and used to test vegetative compatibility in a study of the genetic basis of mycelial incompatibility in *B. cinerea* [R.E. Beever, pers. com.]. The effectiveness of SelR/*nit1* complementation in grouping field-isolates into vegetative compatibility groups could be determined by selecting *nit1* and SelR (class A) sectors for each isolate and pairing them in all combinations on a medium with nitrate as the sole nitrogen source. Development of a fast reliable method of establishing VCG groups would facilitate studies of population genetics and epidemiology of *B. cinerea* isolates.

Selenate and chromate resistant mutants have been used extensively in genetic analysis of the early steps of sulphur metabolism in *A. nidulans* and *N. crassa* [Arst, 1968; Roberts

& Marzluf, 1971; Marzluf, 1970]. In this study sodium selenate resistant mutants were grouped into three classes showing characteristics corresponding to defined mutants from both of these fungi. This suggests that the mechanism of sulphur transport and assimilation in *B. cinerea* is similar to that found in *Aspergillus nidulans* and *Neurospora crassa*. Genetic analysis of sulphur metabolism in *B. cinerea* could be carried out using the collection of sodium selenate resistant mutants isolated in this work.

Of the 62 non-aggressive mutants selected (Chapter 4) five were unable to grow on minimal medium suggesting an auxotrophic mutation. Requirements for single growth factors can be tested following the method described by Holliday [1956] where washed spore suspensions are plated onto 12 different agar media based on MM each supplemented with different combinations of six of 36 growth factors. Characterisation of these strains as defined auxotrophic mutants would provide additional markers for use in genetic studies and also clarify the importance of the growth factor in pathogenesis.

In this study 40 non-aggressive mutants were successfully crossed with single-ascospore reference strains (SAS405 or SAS56), but only seven of these crosses have so far been analysed. Individual apothecia from the remaining crosses have been stored in 15% glycerol at –80°C. Analysis of progeny from these crosses may reveal additional single-gene mutations, which could be investigated in their own right or tested for allelism with Mp97.

Evidence in this study suggests that at least some strains of *B. cinerea* may be aneuploid [Buttner *et al.*, 1994]. This evidence could be tested by screening DNA from SAS405 and selected self-fertilised progeny (cross 13 and 14, Table 6 p65) with primers specific to the *Mbc1HR* and *Mbc1S* alleles of the β-tubulin gene. This has the potential to improve our understanding of the genetic structure of this economically important pathogen with possible implications for epidemiology and disease control. Positive identification of both alleles in one strain would strongly support the aneuploid hypothesis.

The use of a *Pat1* strain of *B. cinerea* to control grey mould disease has significant advantages over other control strategies, including the absence of fungicide residues and the likelihood that environmental conditions conducive to the disease would also suit the biocontrol agent. It would be appropriate to investigate such control in the first instance in glasshouses where the fungus could be contained. A particular focus could be the lateral shoot removal wounds of tomatoes as investigated by Eden *et al.* [1996a]. As tomatoes are an economically important host for *B. cinerea* (Disease development p8) findings would be directly transferable to the horticultural industry. Particular attention would need to be

paid to the effect of different glasshouse temperatures on interaction of the *Pat1* and aggressive strains of *B. cinerea* and host plants as results in this study suggests *Pat1* strains may be more aggressive at temperatures below 20°C. Another system offering many of the same advantages would be the pre-inoculation of stem-scars of stored kiwifruit with a *Pat1* strain. Unlike tomatoes cured kiwifruit may have pre-synthesised defence compounds in the region of the stem-scar [Pennycook & Manning, 1992; Ippolito *et al.*, 1994] resulting in the non-aggressive phenotype being maintained at lower temperatures.

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Index

Appendix 1 Abbreviations

ai	active ingredient
cm	centimetre
conc.	concentration
cv.	cultivar
d	day(s)
diam.	diameter
et al.	et alia, and others
G	gravitational force
g	gram(s)
h	hour(s)
ICMP	International Collection of Micro-organisms from Plants
l	litre
m	metre
min.	minute(s)
mm	millimetre
n.d.	not determined
nm	nanometre
no	number
°C	centigrade
p	page
pers. com.	personal communication
PGIP	polygalacturonase inhibitor protein
RAPD	random amplified polymorphic DNA
REMI	restriction-enzyme mediated integration
RFLP	restriction fragment length polymorphism
RO water	water treated by reverse osmosis
SE	standard error
TC plates	tissue culture plates
u.v.	ultraviolet
v/w	volume/weight
wt	weight
y	year(s)
µg	microgram
µl	microlitre
µm	micromolar

Appendix 2 Media and solutions

Quantities to make one litre of medium unless otherwise stated.

Fungal growth media

CM (Complete medium)

Based on Vogel's medium N

Vogel's medium N	1 litre
Sucrose	20 g
Yeast extract	5 g
Peptone	5 g
Agar	15 g

Use: A defined full nutrient medium.

CM+Triton (Triton medium)

Based on CM

CM	1 litre
Triton X100	5 ml

Use: A colony restricting medium
Reference: Madelin [1987], p25, p26, 54.

MEA (Malt extract agar, Oxoid)

Oxoid malt extract (30 g l^{-1}) contained

Mycological peptone	5 g
Agar	15 g

Use: routine culturing

MEA+A (Malt extract agar, Oxoid no. 2)

Based on MEA

MEA	1 litre
Additional agar	4 g

Use: In 24-well tissue culture plates (Geiner No. 662160, 15-mm diam. wells each containing 0.5 ml media).
Reference: p54.

MEA+carbendazim (Benzimidazole)

Based on MEA

MEA	1 litre
Bavistin	100 mg ai.

Fungicide was a wettable powder formulation dispersed in sterile water and added to media after autoclaving.

Use: selective test medium for benzimidazole resistance, resistant strains produce spreading colonies after 2 days.
Reference: Beever & Parkes [1993], p37.

MEA+NaCl (Sodium chloride medium)

Based on MEA

MEA	1 litre	
NaCl	40 g	Sodium chloride

Use: putative indicator of mycelial incompatibility shows as dark line(s) at the interaction zone where two strains meet.
Reference: Beever & Parkes [1993], p37, p75.

MEA+vinclozolin (Dicarboximide)

Based on MEA

MEA	1 litre
Ronilan	100mg ai

Fungicide was a wettable powder formulation dispersed in sterile water and added to media after autoclaving.
Use: Selective test medium for dicarboximide resistance: resistant strains produce a compact pad of mycelium after 3 d.
Reference: Beever & Parkes [1993], p37.

MM (Minimal medium)

Based on Vogel's medium N

Vogels medium N	20 ml
Sucrose	15 g
Agar	15 g

Use: A defined minimal nutrient medium.

MM+ClO_3 (Chlorate medium)

MM	1 litre	
Potassium chlorate	30 g	$KClO_3$

Use: Selective medium for selection of nitrate non-utilising mutants.
Reference: Modified from Puhalla [1985], p40.

MM+CrO_4 (Chromate medium)

MM	1 litre	
Potassium chromate	1 g	$KCrO_4$
Methionine	0.04 g	$C_5H_{11}NO_2S$

Use: Test medium for sodium selenate resistant mutants
Reference: Roberts & Marzluf [1971], p41.

MM+Hx (Hypoxanthine medium)

Based on Vogel's medium N-

Vogel's medium N-	20 ml	
Sucrose	15 g	
Hypoxanthine	0.1 g	$C_5H_4N_4O$

Use: A nitrogen utilisation test to determine the class of chlorate resistant derived mutants.
Reference: Pateman *et al.* [1964], p46, p50.

MM+NH_4 (Ammonium medium)

Based on Vogel's medium N-

Vogel's medium N-	20 ml

Sucrose	15 g	
Ammonium tartrate	0.92 g	$C_4H_{12}N_2O_6$

Use: A nitrogen utilisation test to determine the class of chlorate resistant derived mutants.
Reference: Pateman *et al.* [1964], p46, p50.

MM+NO_2 (Nitrite medium)

Based on Vogel's medium N-

Vogel's medium N-	20 ml	
Sucrose	15 g	
Potassium nitrite	0.85 g	KNO_2

Use: A nitrogen utilisation test to determine the class of chlorate resistant derived mutants.
Reference: Pateman *et al.* [1964], p46, p50.

MM+NO_3 (Nitrate medium)

Based on Vogel's medium N-

Vogel's medium N-	20 ml	
Sucrose	15 g	
Potassium nitrate	1.01 g	KNO_3

Use: A nitrogen utilisation test to determine the class of chlorate resistant derived mutants.
Reference: Pateman *et al.* [1964], p46, p50.

MM+SeO_4 (Selenate medium)

MM	1 litre	
Sodium selenate	1 g	Na_2SeO_4
Taurine	0.1 g	$C_2H_7NO_3S$

Use: A selection medium for selenate resistant strains.
Reference: Modified from Jacobson & Gordon [1988], p40.

MM+SeO_4^B (Selenate medium B)

MM	1 litre	
Sodium selenate	1.5 g	Na_2SeO_4
Taurine	0.1 g	$C_2H_7NO_3S$

Use: Identification of SelR strains in mixed inoculations onto French bean leaves.
Reference: Modified from Jacobson & Gordon [1988], p75.

MM+taurine (Taurine medium)

MM	1 litre	
Taurine	1g	$C_2H_7NO_3S$

Use: A characterisation medium for selenate resistant strains.
Reference: Modified from Jacobson & Gordon [1988], p40.

Modified-Richardson's-medium [A]

Potassium nitrate	10 g	KNO_3
Potassium dihydrogen orthophosphate	5 g	KH_2PO_4
Magnesium sulphate	0.25 g	$MgSO_4 \cdot 7\ H_2O$
Ferric chloride	0.4 mg	$FeCl_3$
Citrus pectin	5 g	

Use: Liquid growth medium used to induce polygalacturonase activity in flask cultures.
Reference: p73

Modified-Richardson's-medium[B]

Potassium nitrate	10 g	KNO_3
Potassium dihydrogen orthophosphate	5 g	KH_2PO_4
Magnesium sulphate	0.25 g	$MgSO_4 \cdot 7\ H_2O$
Ferric chloride	0.4 mg	$FeCl_3$
Sucrose	1 g	

Use: Nutrient solution added to spore suspensions on soybean leaves in a test for phytoalexin induction.
Reference: p74

PDA (Potato dextrose agar)

Difco potato dextrose agar contained:

Potatoes, infusion from	200 g
Bacto dextrose	20 g
Bacto agar	15 g

Use: Base for other medium (see below).

PDA+bromophenol

Based on PDA

PDA	1 litre
Bromophenol blue	
Adjusted to pH7 with KOH_2.	

Use: Indicator medium for rapid evaluation of acid production
Reference: Godoy *et al.* [1990], p71.

PDA-½ (Half-potato dextrose agar)

Based on potato dextrose agar

RO water	1 litre
PDA	19.5 g
Agar	22.5 g

Use: Ascospore isolation
Reference: p33.

Vogel's

Reference: Vogel [1964]

Vogel's salts N (makes 1 litre)

Distilled water	775 ml	
Trisodium citrate	125 g	$C_6H_5Na_3O_7 \cdot 2\ H_2O$
Potassium dihydrogen orthophosphate	250 g	KH_2PO_4 anhydrous
Magnesium sulphate	10 g	$MgSO_4 \cdot 7\ H_2O$
Calcium chloride	5 g	$CaCl_2 \cdot 2\ H_2O$
	added 1g at time on a magnetic stirrer	
Ammonium nitrate	5 g	NH_4NO_3 anhydrous

Stir with gentle heat to dissolve each compound before adding next, can be stored indefinitely as a 50x solution

Vogel's salts N-

Omit NH_4NO_3 for nitrogen free media

Vogel's trace elements (makes 100 ml)

Distilled water	95 ml

Citric acid	5 g	$C_6H_8O_7 \cdot 1\ H_2O$
Zinc sulphate	5 g	$ZnSO_4 \cdot 7\ H_2O$
Ferrous ammonium sulphate	1 g	$Fe\ (NH_4)_2(SO_4)_2 \cdot 6\ H_2O$
Cupric sulphate	0.25 g	$CuSO_4 \cdot 5\ H_2O$
Manganese sulphate	50 g	$MnSO_4 \cdot 1\ H_2O$
Boric acid	50 mg	H_2BO_3 anhydrous
Sodium molybdate	50 mg	$Na_2MoO_4 \cdot 2\ H_2O$

Dissolve in distilled water and adjust volume to 100 ml.

Vogel's biotin (makes 100 ml)

50% ethanol	100ml	
Biotin	5 mg	$C_{10}H_{16}N_2O_3S$

Dissolve biotin in ethanol and store in refrigerator.

Vogel's concentrate N

Salt solution (Vogel's salts N)	1 litre
Trace element solution	5 ml
Vogels biotin	2.5 ml
Chloroform (preservative)	1 ml

If necessary adjust to 1litre.
Note: This is a 50x concentration dilute - 20 ml to 1 litre RO water.

Vogel's concentrate N-

Salt solution (Vogel's salts N-)	1 litre
Trace element solution	5 ml
Vogels biotin	2.5 ml
Chloroform (preservative)	1 ml

If necessary adjust to 1litre.
Note: This is a 50x concentration dilute 20 ml to 1 litre RO water.

Vogel's medium N

Vogel's N	20 ml

Use: Base for other media (see above).

Vogel's medium N-

Vogel's N-	20 ml

Use: Base for other media (see above).

Vogel's+sucrose

Vogel's N-	20 ml
Sucrose	15 g l^{-1}

Use: Nutrient solution added to spore suspensions inoculated onto French bean leaves.
Reference: p32, 34, 56, 70, 75.

Buffers

Potassium phosphate buffer (0.1 M)

Potassium dihydrogen orthophosphate
13.6 g KH_2PO_4
Adjust to pH7 with KOH
Use: Buffer for spore suspensions in NQO mutagenesis
Reference: Bal *et al.* [1977], p26.

Others

Aniline blue clearing/staining solution

For approximately 1 litre

95% ethanol	300 ml
Chloroform	150 ml
90% lactic acid	125 ml
Phenol	150 g
Chloral hydrate	450 g
Aniline blue	0.6 g

Use: As a clearing / staining solution for portions of French bean leaves infected with *B. cinerea.*
Reference: Bruzzese & Hasan [1983], p71.

NQO (4-Nitro-1-quinaline oxide)

Stock solution

NQO 10mg 4-Nitro-1-quinaline oxide
Dissolve in 1ml of acetone
Keeps for 6mths at 4°C

Working solution

Based on phosphate buffer

Phosphate buffer	0.9 ml
Stock solution	100 µl

Gives concentration of 1mg/ml (1 mg = 1000 µg),
1 µl per 1 ml spore suspension to give a concentration of 1 µg NQO/ml
Use: As a mutagen for fungi
Reference: Bal *et al.* [1977], p26.

Polygalacturonase cup plate assay substrate

Based on 0.1 M Sodium acetate buffer (pH5).

0.1M Sodium acetate buffer	1 litre	
Sodium pectate	0.1 g	
Purified agar	15 g	
EDTA sodium salt	3.72 g	
Azide	0.2 g	(to prevent fungal bacterial contamination)

Use: Substrate gel for detection of polygalacturonase activity. Enzyme activity shows as cleared zones in a pink background.
Reference: Taylor & Secor [1988], p73.

Sodium thiosulphate solution

Sodium thiosulfate 50g
Use: to inactivate NQO before disposal making the mutagen much safer and easier to handle.

Reference: Bal *et al.* [1977], p26.

PBS

Makes a x 10 concentration stock solution

NaCl	80g
KCl	2g
Na_2HPO_4	11.5g
KH_2PO_4	2g

Add chemicals individually then add distilled water. Store at room temp

Use: A buffered washing solution for use in the monoclonal antibody procedure.
Reference: Bossi & Dewey [1992], p56.

PBST

Based on PBS

PBS concentrate	1 litre
Tween 20	5 ml

Make up when needed as will not store.

Use: A buffered washing solution for use in the monoclonal antibody procedure.
Reference: Bossi & Dewey [1992], p56.

Appendix 3 Botrytis strains

Table 10 **Sources** and genotypes of *Botrytis cinerea* strains.

	Strain	ICMP No. *	Type	Source / Parent	Mating -type †	Genotype ‡
Wild Type	REB658-1	7577	field isolate	kiwifruit, NZ	*MAT1-2*	*Mbc1S-Daf1S*
	REB666-1	7596	field isolate	kiwifruit, NZ	*MAT1-1*	*Mbc1HR-Daf1S*
	REB678-1	7869	field isolate	tomato, NZ	*MAT1-2*	*Mbc1LR-Daf1S*
	REB689-1	8569	field isolate	begonia, NZ	*MAT1-1*	*Mbc1HR-Daf1LR*
	REB702-1	9430	field isolate	grape, NZ	*MAT1-1*	*Mbc1S-Daf1S*
	REB705-1	9433	field isolate	grape, NZ	*MAT1-2*	*Mbc1HR-Daf1UL*
	SAS405		single-ascospore	lab cross, Italy	*MAT1-2*	*Mbc1HR-Daf1LR*
	SAS56		single-ascospore	lab cross, Italy	*MAT1-1*	*Mbc1S-Daf1S*
	A1		single-ascospore	REB702-1 x REB705-1	*MAT1-1*	*Mbc1HR-Daf1S*
	A4		single-ascospore	REB658-1 x REB666-1	*MAT1-1/2*	*Mbc1HR-Daf1S*
	A5		single-ascospore	REB658-1 x REB666-1	n.d.	*Mbc1S-Daf1S*
	P1-105		single-ascospore	Ms015 x REB678-1	*MAT1-1/2*	*Mbc1HR-Daf1S*
	P3-018		single-ascospore	Mn012 x REB658-1	n.d.	*Mbc1HR-Daf1LR*
SelR strains	Ms010		UV mutant	A1	*MAT1-1*	*Mbc1HR-Daf1S*-SelR
	Ms015		sector mutant	A1	*MAT1-1*	*Mbc1HR-Daf1S-Sel1*R
	Ms024	13063	sector mutant	A1	n.d.	*Mbc1HR-Daf1S*-SelR
	Ms033		sector mutant	A5	n.d.	*Mbc1S-Daf1S*-SelR
	Ms044	13369	sector mutant	A5	n.d.	*Mbc1S-Daf1S*-SelR
	P1-083		single-ascospore	Ms015 x REB678-1	n.d.	*Mbc1LR-Daf1S-Sel1*R
	P2-005	13165	single-ascospore	Ms010 x SAS405	*MAT1-1/2*	*Mbc1HR-Daf1S*-SelR
	P2-011	13166	single-ascospore	Ms010 x SAS405	*MAT1-1/2*	*Mbc1HR-Daf1LR*-SelR
	P5-006	13066	single-ascospore	P1-083 x P1-105	n.d.	*Mbc1LR-Daf1S-Sel1*R
	P5-026		single-ascospore	P1-083 x P1-105	n.d.	*Mbc1LR-Daf1S-Sel1*R
	P5-029	13167	single-ascospore	P1-083 x P1-105	*MAT1-1/2*	*Mbc1HR-Daf1S-Sel1*R
nit1 strains	Mn001		sector mutant	A5	*MAT1-1*	*Mbc1S-Daf1S-nit1*
	Mn002		sector mutant	A5	n.d.	*Mbc1S-Daf1S-nit1*
	Mn003		sector mutant	A5	n.d.	*Mbc1S-Daf1S-nit1*
	Mn004		sector mutant	A5	*MAT1-1*	*Mbc1S-Daf1S-nit1*
	Mn005		sector mutant	A5	n.d.	*Mbc1S-Daf1S-nit1*
	Mn006		sector mutant	A5	n.d.	*Mbc1S-Daf1S-nit1*
	Mn007		sector mutant	A5	*MAT1-1*	*Mbc1S-Daf1S-nit1*
	Mn008		sector mutant	A5	n.d.	*Mbc1S-Daf1S-nit1*
	Mn012		sector mutant	REB689-1	*MAT1-1/2*	*Mbc1HR-Daf1LR-nit1*
	P3-009		single-ascospore	Mn012 x REB658-1	n.d.	*Mbc1S-Daf1S-nit1*
	P6-005	13069	single-ascospore	SAS405 x Mn001	*MAT1-1/2*	*Mbc1HR-Daf1LR-nit1*
	P6-022		single-ascospore	SAS405 x Mn001	*MAT1-1/2*	*Mbc1HR-Daf1LR-nit1*
	P6-037		single-ascospore	SAS405 x Mn001	*MAT1-1/2*	*Mbc1HR-Daf1LR-nit1*
	P6-067	13168	single-ascospore	SAS405 x Mn001	*MAT1-1*	*Mbc1S-Daf1S-nit1*
*Sel1*R - *nit1*	P16-025	13169	single-ascospore	P6-005 x P5-026	n.d.	*Mbc1S-Daf1S-nit1-Sel1*R
	P16-067	13170	single-ascospore	P6-005 x P5-026	n.d.	*Mbc1HR-Daf1LR-nit1-Sel1*R

	Strain	ICMP No.*	Type	Source / Parent	Mating -type †	Genotype ‡
Non-aggressive	Mp1		NQO mutant	A4	*MAT1-2*	*Mbc1*HR-*Daf1S*
	Mp17		NQO mutant	A4	n.d.	*Mbc1*HR-*Daf1S*
	Mp18		NQO mutant	A4	n.d.	*Mbc1*HR-*Daf1S*
	Mp68		u.v. mutant	A4	n.d.	*Mbc1*HR-*Daf1S*
	Mp93		u.v. mutant	A4	n.d.	*Mbc1*HR-*Daf1S*
	Mp97	13370	u.v. mutant	A4	*MAT1-2*	*Mbc1*HR-*Daf1S-Pat1*
	Mp110		u.v. mutant	A4	n.d.	*Mbc1*HR-*Daf1S*
	P21-002	13371	single-ascospore	Mp097 x SAS56	*MAT1-1*	*Mbc1*HR-*Daf1S-Pat1*
	P21-003		single-ascospore	Mp097 x SAS56	n.d.	*Mbc1*HR-*Daf1S-Pat1*
	P21-011		single-ascospore	Mp097 x SAS56	*MAT1-1*	*Mbc1*HR-*Daf1S-Pat1*
	P21-012	13372	single-ascospore	Mp097 x SAS56	*MAT1-2*	*Mbc1S-Daf1S-Pat1*
	P21-024		single-ascospore	Mp097 x SAS56	*MAT1-2*	*Mbc1S-Daf1S-Pat1*
	P21-026		single-ascospore	Mp097 x SAS56	n.d.	*Mbc1S-Daf1S-Pat1*
	P21-030		single-ascospore	Mp097 x SAS56	*MAT1-1*	*Mbc1*HR-*Daf1S-Pat1*
	P21-040		single-ascospore	Mp097 x SAS56	*MAT1-1*	*Mbc1S-Daf1S-Pat1*
	P21-042		single-ascospore	Mp097 x SAS56	n.d.	*Mbc1*HR- *Daf1S-Pat1*
	P21-047		single-ascospore	Mp097 x SAS56	*MAT1-1*	*Mbc1S- Daf1S-Pat1*
	P21-061		single-ascospore	Mp097 x SAS56	*MAT1-1*	*Mbc1S- Daf1S-Pat1*
	P33-146	13373	single-ascospore	P16-067 x P21-040	n.d.	*Mbc1*HR-*Daf1*LR-*Pat1*
	P33-141	13374	single-ascospore	P16-067 x P21-040	n.d.	*Mbc1*HR-*Daf1*LR-*nit1-Sel1*R-*Pat1*
	P33-034	13375	single-ascospore	P16-067 x P21-040	n.d.	*Mbc1*HR-*Daf1S-nit1-Sel1*R-*Pat1*
	P33-124	13376	single-ascospore	P16-067 x P21-040	n.d.	*Mbc1S-Daf1S-nit1-Sel1*R-*Pat1*
Aggressive	P21-016		single-ascospore	Mp097 x SAS56	n.d.	*Mbc1*HR-*Daf1S*
	P21-020		single-ascospore	Mp097 x SAS56	n.d.	*Mbc1*HR-*Daf1S*
	P21-022		single-ascospore	Mp097 x SAS56	n.d.	*Mbc1S-Daf1S*
	P21-027		single-ascospore	Mp097 x SAS56	n.d.	*Mbc1S-Daf1S*
	P21-037		single-ascospore	Mp097 x SAS56	n.d.	*Mbc1*HR-*Daf1S*
	P21-038		single-ascospore	Mp097 x SAS56	n.d.	*Mbc1S-Daf1S*
	P21-048		single-ascospore	Mp097 x SAS56	n.d.	*Mbc1S-Daf1S*
	P21-065		single-ascospore	Mp097 x SAS56	n.d.	*Mbc1S-Daf1S*
	P21-081		single-ascospore	Mp097 x SAS56	n.d.	*Mbc1*HR-*Daf1S*
	P21-096		single-ascospore	Mp097 x SAS56	n.d.	*Mbc1*HR-*Daf1S*

* International Collection of Micro-organisms from Plants (ICMP) maintained by Landcare Research.

† *MAT1-1* = mating-type 1, *MAT1-2* = mating-type 2, *MAT1-1/2* = mating-type 1 and 2.

‡ *Mbc1HR* = high-level benzimidazole resistance, *Mbc1*LR = low-level benzimidazole resistance, *Mbc1S* = benzimidazole sensitive, *Daf1*LR = low-level dicarboximide resistance, *Daf1S* = dicarboximide sensitive, *Pat1* = reduced pathogenicity, SelR = selenate resistance phenotype, *Sel1*R = selenate resistance, *nit1* = nitrate reductase deficient, n.d. = not determined.

Summary of sexual crosses (*Botrytis cinerea*)

Cross No.	Sclerotial Strain	Fertilising Strain	Progeny Series	Page
1	Ms015 (*Mbc1HR-Sel1R*)	REB678-1 (*Mbc1LR-Sel1S*)	P1-	45
2	Ms010 (*Daf1S*-SelR)	SAS405 (*Daf1LR*-SelS)	P2-	45
3	P1-083 (*Mbc1LR-Sel1R*)	P1-105 (*Mbc1HR-Sel1S*)	P5-	45
4	Mn012 (*Mbc1HR-Daf1LR-nit1*)	REB658-1 (*Mbc1S-Daf1S*)	P3-	45
5	SAS405 (*Mbc1HR-Daf1LR*)	Mn001 (*Mbc1S-Daf1S-nit1*)	P6-	45
6	P3-018 (*Mbc1HR-Daf1LR*)	P3-009 (*Mbc1S-Daf1S-nit1*)	P23	45
7	P6-022 (*Mbc1HR-Daf1LR- Sel1S-nit1*)	P5-006 (*Mbc1LR-Daf1S-Sel1R*)	P14	48
8	P5-029 (*Mbc1LR-Daf1S-Sel1R*)	P6-037 (*Mbc1HR- Daf1LR-Sel1S-nit1*)	P15	48
9	P6-005 (*Mbc1HR-Daf1LR-Sel1S-nit1*)	P5-026 (*Mbc1LR-Daf1S-Sel1R*)	P16-	48
10	Mp97 (*Mbc1HR-Pat1*)	SAS56 (*Mbc1S*)	P21-	61, 62
11	SAS56 (*Mbc1S*)	Mp97 (*Mbc1HR- Pat1*)	P28	61, 62
12	P16-67 (*Mbc1HR-Daf1LR-nit1-Sel1R*)	P21-40 (Mbc1S-Daf1S-Pat1)	P33-	63
13	SAS405 (*Mbc1HR-Daf1LR*)	SAS405 (*Mbc1HR-Daf1LR*)	P7-	65
14	SAS405 (*Mbc1HR-Daf1LR*)	Water	P8-	65
	SAS56 (*Mbc1S*)	Mp68	P19	59
	SAS56 (*Mbc1S*)	A4	P26-	59

Summary of selenate resistant mutants

Mutant	*	Class	Taurine†	Mutant	*	Class	Taurine†	Mutant	*	Class	Taurine†
Ms001	s	B	-	Ms019	u	A	+	Ms039	s	C	+
Ms002	u	B	-	Ms020	u	B	-	Ms040	s	C	+
Ms003	u	B	-	Ms021	s	B	-	Ms041	s	B	-
Ms005	u	B	-	Ms022	u	B	-	Ms042	s	A	+
Ms006	u	B	-	Ms023	u	B	-	Ms043	s	B	+
Ms007	u	B	-	Ms024	s	C	+	Ms044	s	A	-
Ms008	u	B	-	Ms025	u	B	-	Ms045	s	B	+
Ms009	s	B	-	Ms026	u	A	-	Ms046	s	B	+
Ms010	u	A	+	Ms027	s	B	-	Ms047	s	B	-
Ms011	u	B	-	Ms029	u	B	-	Ms048	s	B	-
Ms012	u	B	-	Ms031	s	B	-	Ms050	s	B	-
Ms013	s	B	-	Ms032	s	A	+	Ms051	s	B	-
Ms014	s	B	-	Ms033	s	A	+	Ms052	s	B	-
Ms015	s	B	-	Ms035	s	B	+	Ms053	s	B	-
Ms016	s	B	-	Ms036	s	B	+	Ms054	s	B	-
Ms017	u	B	-	Ms037	s	A	+	Ms055	s	B	+
Ms018	u	B	-	Ms038	s	B	+				

* s = sector mutant, u = u.v. mutant

† A score of + indicates increased growth on MM+taurine compared with growth MM

Summary of chlorate resistant mutants

Mutant	*	Class	Mutant	*	Class	Mutant	*	Class
Mn001	s	*nit1*	Mn007	s	*nit1*	Mn016	q	Cm
Mn002	s	*nit1*	Mn008	s	*nit1*	Mn017	q	Cm
Mn003	s	*nit1*	Mn012	SP	*nit1*	Mn018	q	Cm
Mn004	s	*nit1*	Mn013	SP	*nit1*	Mn019	q	Cm
Mn005	s	*nit1*	Mn014	q	Cm	Mn020	q	Cm
Mn006	s	*nit1*	Mn015	q	Cm	Mn021	q	Cm

* s = sector mutant, q = NQO mutant, SP = supplied by S. Parkes

Summary of non-aggressive mutants

Mutant	*	Stable	BC-KH4	Mutant	*	Stable	BC-KH4	Mutant	*	Stable	BC-KH4
Mp1	q	+		Mp47	u	+		Mp92	u	+	✔
Mp2	q	-		Mp48	u	-		Mp93	u	+	
Mp3	q	+		Mp49	u	+		Mp94	u	-	
Mp4	q	+	✔	Mp50	u	+		Mp95	u	-	
Mp5	q	-		Mp51	u	-		Mp96	u	+	✔
Mp6	q	-		Mp52	u	+		Mp97	u	+	✔
Mp7	q	-		Mp53	u	+		Mp98	u	+	✔
Mp8	q	-		Mp54	u	+	✔	Mp99	u	-	
Mp9	q	-		Mp55	u	-		Mp100	u	-	
Mp10	q	+	✔	Mp56	u	+	✔	Mp101	u	+	
Mp11	q	-		Mp57	u	+	✔	Mp102	u	+	
Mp12	q	-		Mp58	u	-		Mp103	u	-	
Mp13	q	-		Mp59	u	+		Mp104	u	-	
Mp14	q	+	✔	Mp60	u	-		Mp105	u	+	
Mp15	q	-		Mp61	u	+		Mp106	u	-	
Mp16	q	-		Mp62	u	-		Mp107	u	+	
Mp17	q	+		Mp63	u	+		Mp108	u	-	
Mp18	q	+	✔	Mp64	u	+	✔	Mp109	u	+	✔
Mp19	q	+		Mp65	u	-		Mp110	u	+	
Mp20	q	-		Mp66	u	+		Mp111	u	-	
Mp21	q	-		Mp67	u	+	✔	Mp112	u	+	
Mp22	q	-		Mp68	u	+	✔	Mp113	u	+	
Mp23	q	-		Mp69	u	+		Mp114	u	+	✔
Mp24	q	-		Mp70	u	-		Mp115	u	+	
Mp25	q	-		Mp71	u	+		Mp116	u	-	
Mp26	q	-		Mp72	u	-		Mp117	u	-	
Mp27	q	+	✔	Mp73	u	-		Mp118	u	+	
Mp29	q	-		Mp74	u	-		Mp119	u	-	
Mp30	q	-		Mp75	u	-		Mp120	u	+	✔
Mp31	q	+		Mp76	u	+		Mp121	u	-	
Mp32	q	-		Mp77	q	+	✔	Mp122	u	-	
Mp33	q	+		Mp78	u	-		Mp123	q	+	✔
Mp34	q	-		Mp79	u	-		Mp124	u	-	
Mp35	q	+		Mp80	u	-		Mp125	u	+	
Mp36	q	-		Mp81	u	-		Mp126	u	-	
Mp37	q	+	✔	Mp82	u	-		Mp127	q	+	
Mp38	q	-		Mp83	u	-		Mp128	q	+	
Mp39	q	-		Mp84	u	-		Mp129	u	-	
Mp40	q	-		Mp85	u	+	✔	Mp130	u	-	
Mp41	q	-		Mp86	u	-		Mp131	q	+	
Mp42	q	-		Mp87	u	-		Mp132	u	-	
Mp43	q	-		Mp88	u	+	✔	Mp133	u	+	
Mp44	q	-		Mp89	u	+		Mp134	u	+	
Mp45	u	+	✔	Mp90	u	+	✔				
Mp46	u	+		Mp91	u	-					

* u = u.v. mutant, q = NQO mutant, Stable + = the mutant retained the non-aggressive phenotype after storage on silica gel, BC-KH4 ✔ = tested with the antibody BC-KH4 (p57).

Appendix 4 Glossary of selected terms

Aggressiveness The differences in the capacity of a parasite to invade and grow in its host plant and to reproduce on it (Gaumann, 1950 [from MacHardy, 1996]).

Allele One of two or more alternate forms of a *gene* occupying the same *locus* on a chromosome [Agrios, 1988].

Ampulla A conidiophore which develops a number of short branches or discrete conidiogenous cells [Hawksworth *et al.*, 1995].

Anamorph The asexual reproductive manifestation of a fungus: usually produces *conidia*, but may also be *sclerotial* [Kendrick, 1992].

Anastomosis Fusion of *somatic* hyphae [Kendrick, 1992]. The union of a hypha or vessel with another resulting in intercommunication of their contents [Agrios, 1988].

Aneuploid Having a chromosome number that is not a multiple of the *haploid* set [Kendrick, 1992].

Antibody A protein induced in the blood of warm-blooded animal by injection of an *antigen* and capable of reacting specifically with that *antigen* [Agrios, 1988].

Antigen A substance, usually a protein or polysaccharide which, when injected into the body of a living warm-blooded animal, induces the formation in the blood of proteins (*antibodies*) with which the antigen reacts specifically [Federation of British Plant Pathologists, 1973].

Apothecium An open cup- or saucer-shaped *ascocarp* of some *ascomycetes* [Agrios, 1988].

Appressorium A swelling on a germ-tube or hypha, which adheres to the surface of a host, and facilitates subsequent penetration [Kendrick, 1992].

Ascocarp The fruiting body of *ascomycetes* bearing or containing *asci* [Agrios, 1988].

Ascomycetes A group of fungi producing their sexual spores within *asci* (a sac-like cell of a hypha in which meiosis occurs and which contains the ascospores) [Agrios, 1988].

Ascus (pl asci) A saclike cell of a hypha in which meiosis occurs and which contains the ascospores (usually eight) [Agrios, 1988].

Attenuated Having reduced *pathogenicity* or *virulence* (non-aggressive) [Kendrick, 1992].

Auxotrophic Having nutritional requirements (e.g. for growth factors) additional to those of the '*wild-type*' [Federation of British Plant Pathologists, 1973]. A biochemical *mutant* deficient for one or more substances; it will grow on minimal medium only if it has been supplemented with these substances [Kendrick, 1992].

Canker A sunken necrotic *lesion* of a main root, stem or branch arising from disintegration of tissues outside the xylem cylinder [Federation of British Plant Pathologists, 1973].

Chlamydospore A thick-walled asexual spore formed by the modification of a fungal hyphal cell [Agrios, 1988].

Conidiophore A specialised hypha, simple or branched, on which *conidia* are formed [Kendrick, 1992].

Conidium (pl conidia) A non-motile fungal mitospore (from mitosis, asexual) [Kendrick, 1992].

Cross protection The phenomenon in which plant tissues infected with one strain of an organism are protected from infection by other strains of the same organism [Agrios, 1988].

Curing Holding fruit at ambient temperature for a time prior to storage. For example kiwifruit held at 15°C for 48 h following picking and prior to storage at 0 °C. [Pennycook & Manning, 1992]

Damping-off Collapse and death of seedling plants resulting from the development of a stem *lesion* at soil level [Federation of British Plant Pathologists, 1973].

Dominant (of an *allele*) Exerting its full *phenotypic* effect despite the presence of another *allele* of the same *gene*, whose *phenotypic* expression it blocks [Kendrick, 1992].

EC 50 Effective concentration for a 50% kill.

Gene A unit of heredity in the chromosome; a sequence of nucleotides in a DNA molecule that codes for a polypeptide [Kendrick, 1992].

Gene-for-gene concept The concept that corresponding *genes* for *resistance* and *virulence* exist in host and pathogen respectively [Federation of British Plant Pathologists, 1973].

Genotype The hereditary potential of an organism [Federation of British Plant Pathologists, 1973]. The genetic constitution of an organism [King, 1974].

Germ-tube The early growth of mycelium produced by a germinating fungal spore [Agrios, 1988].

Haploid A cell or organism whose nuclei have a single complete set of chromosomes [Agrios, 1988].

Heterogeneity Diverse in character [Concise Oxford Dictionary].

Heterokaryosis The occurrence of two nuclei of different *genotypes* in a fungal cell or mycelium [Blackmore & Tootill, 1986].

Heteroploidy Any deviation from the normal chromosome number in a cell, tissue or whole organism. Modified definition for fungi: a frequent shifting of chromosome numbers per nucleus, ranging from nullisomics (n-x) through the euploid series of 1n, 2n, 3n etc. and including various *aneuploids* [Tolmsoff, 1983].

Heterothallic Describes a fungi in which two genetically distinct but compatible mycelia must meet before sexual reproduction can take place [Kendrick, 1992].

Homothallic Describes fungi in which a single strain can undertake sexual reproduction, self-compatible [Kendrick, 1992].

Host range The range of plant species known to serve as hosts for a given pathogen [Federation of British Plant Pathologists, 1973].

Hyaline Colourless, transparent [Agrios, 1988].

Hydrophilic Having an affinity for water [Daintith, 1976].

Hydrophobic Having no affinity for water [Daintith, 1976].

Hypersensitivity The violent reaction of an organism to attack by a pathogenic organism or a virus resulting in prompt death of invaded tissue, thus preventing further spread of infection [Federation of British Plant Pathologists, 1973].

In vitro In culture. Outside the host [Agrios, 1988].

In vivo In the host [Agrios, 1988].

Intercalary Formed along and within the mycelium [Agrios, 1988].

Isolate Cultures originating from single-spore isolations from diseased material (Terminology for describing pathogenesis p20).

Latent infection An apparent infection that is chronic and in which a certain host-parasite relationship is established [Federation of British Plant Pathologists, 1973]. A symptomless *non-aggressive* infection [Coley-Smith *et al.*, 1980].

Lesion A localised area of diseased or disordered tissue [Federation of British Plant Pathologists, 1973].

Locus (pl loci) The position that a *gene* occupies in a chromosome [King, 1974].

Mendelian segregation A normal Mendelian segregation pattern for a genetic marker in a *haploid* organism is 1:1.

Monoclonal antibodies Identical antibodies produced by a single clone of lymphocytes [Agrios, 1988].

Mummy A dried shrivelled fruit [Agrios, 1988].

Mutagen An agent that increases the mutation rate [Kendrick, 1992].

Mutant An organism showing one or more discrete heritable differences from a standard type (*wild-type*) [Federation of British Plant Pathologists, 1973].

Necrotrophic Utilising dead plant or animal tissues as a source of nutrients [Federation of British Plant Pathologists, 1973].

Obovate Ovate with the narrower end at the base [Concise Oxford Dictionary].

Paraphyses Sterile hyphae growing up between the *asci* in the hymenium of many *ascomycetes* [Kendrick, 1992].

Pathogenicity The quality or characteristic of being able to cause disease. Should be regarded as an attribute of a genus, species or some other commonly accepted grouping irrespective of the fact that particular entities within the group may not cause disease under defined conditions [Federation of British Plant Pathologists, 1973].

Permease Transport protein or carrier molecule that assists in the movement of substances across cellular membranes; not permanently altered in the process [Kendrick, 1992].

Phenotype The observable properties of an organism [King, 1974].

Phialides A conidiogenous cell which produces a basipetal succession of blastic *conidia* from an open end without any change in the length of the cell [Kendrick, 1992].

Phytoalexin Low molecular weight anti-microbial compounds [Hahn *et al.*, 1992]. Substances which inhibit the growth of certain microorganisms and which are produced in higher plants in response to a number of chemical, physical and biological stimuli [Federation of British Plant Pathologists, 1973].

Plasmid Self-replicating, extra chromosomal, hereditary circular DNA found in certain bacteria and fungi, generally not required for survival of the organism [Agrios, 1988].

Pleiotropic The control of several apparently unrelated characteristics by a single *gene* [Blackmore & Tootill, 1986].

pleomorphic Having more than one independent form or spore stage in the life cycle [Federation of British Plant Pathologists, 1973].

Polyploidy An individual having more than two sets of chromosomes [King, 1974].

Positive phototropism Growing towards a light source [Blackmore & Tootill, 1986].

Promoter A nucleotide sequence in a gene to which RNA polymerase attaches in order to begin transcription of mRNA [Kendrick, 1992].

Protoplast A cell from which the cell wall has been removed [Agrios, 1988].

Recessive A gene whose *phenotypic* expression is masked by a dominant *allele* [Kendrick, 1992].

Resistance The ability of an organism to withstand or oppose the operation of or to lessen or overcome the effects of an injurious or pathogenic factor. Or the ability of the host to suppress or retard the activity of a pathogenic organism or virus [Federation of British Plant Pathologists, 1973].

Saprophytic Utilising non-living materials as nutrients [Federation of British Plant Pathologists, 1973].

Sclerotium A compact mass of hyphae with or without host tissue, usually with a darkened rind, and capable of surviving under unfavourable environmental conditions [Agrios, 1988].

Secondary homothallism Self-fertility of normally heterothallic isolates (Mating-type p21).

Septa Cell walls or partitions [Hawksworth *et al.*, 1995], septate – having septa.

Somatic mutation Genetic changes occurring in somatic (cells other than the gametic cells) cells [Blackmore & Tootill, 1986].

Spermatia Non-motile male gametes [Kendrick, 1992].

Strain Cultures originating either from a single-ascospore arising from a sexual cross or from *mutagenic* treatment of a single spore demonstrating stable defined characteristics (Terminology for describing pathogenesis p20).

Sympatric Two or more populations that could interbreed but do not do so because of various differences [Blackmore & Tootill, 1986].

Systemic Occurring throughout the plant [Federation of British Plant Pathologists, 1973].

Teleomorph The sexual stage of the life cycle of a fungus [Agrios, 1988].

Toxin An organic substance usually acting at a low concentration, which deleteriously and irreversibly affects the normal processes of a living organism. Usually applied to

such substances produced by living organism [Federation of British Plant Pathologists, 1973].

Vegetative incompatibility The inability of vegetative hyphae to *anastomose* and form a stable *heterokaryon* [Hawksworth *et al.*, 1995].

Virulence The relative capacity to cause disease; degree or measure of pathogenicity. Used in reference to *gene-for-gene* interactions [Federation of British Plant Pathologists, 1973; MacHardy, 1996].

Wild-type The common form of a *gene* or organism in natural (wild) populations (usually designated +) [Blackmore & Tootill, 1986]. Predominating in the wild population [Federation of British Plant Pathologists, 1973].

Appendix 5 Summary of genetic nomenclature

Nomenclature conventions used in this study were based on the recommendations of Yoder *et al.* [1986], summarised below.

Genotype designation

- The **investigator who first identifies the gene** has the responsibility to **name it**. The name should be consistent with designations for other genes in the host / parasite system under study and all investigators working on the system should agree to use the generally accepted symbols.
- Each locus should be defined genetically and then assigned **a unique three-letter symbol** that recalls the genotype associated with the mutant allele.
- Often mutants at different loci lead to similar phenotypes – each locus should be identified by a **number unique to that locus**. Locus-identifying numbers will usually be assigned in the order in which the loci are discovered – the number should immediately follow the three letter symbol without a space or hyphen
- The symbol should be written in ***italics*** with the **first letter capitalised** and the others lowercase (if dominance relationships are unknown).
- When **dominance** relationships are known, the **dominant allele** is indicated by writing the symbol as **three uppercase letters** and a **recessive allele** as **three lowercase letters**
- The **wild-type allele** can be identified by a **plus sign** following the locus number and a **mutant allele by a minus sign**.
- The letters ***R*** and ***S*** can be used following the gene symbol and allele number to **indicate resistance or sensitivity** associated with a particular allele.
- The authors recommend that genes controlling **pathogenicity or virulence** should **not be assigned special symbols** because it would be difficult or

impossible to devise notations suitable for all systems and anticipatory of all circumstances and because there is no reason to suspect fundamental differences between pathogenicity or virulence genes and other genes in the organism. But modifications can be used (to the three-letter symbol) where necessary to accommodate special cases.

- Each new mutant allele can be assigned a unique isolation number immediately upon discovery (could include a letter).

Phenotype designation

- The phenotype of a strain should be described using a three-letter symbol for the gene controlling that phenotype.
- The symbol should be written in Roman type with the first letter uppercase and the other two lowercase.
- A plus sign should be used to indicate the wild-type phenotype and a minus to indicate the mutant phenotype.

Mating-type designation

- Suggest the symbol *MAT* for the locus with the single locus found in many fungi called *MAT1* and the two known alleles at that locus *MAT1-1* and *MAT1-2* (all letters in the locus symbol of both alleles are uppercase since they are co-dominant - both are needed for activity).

Appendix 6 Summary of genetic symbols

Table 11 Genetic symbols used for genes of *B. cinerea.*

Gene	Allele	Genotype/Phenotype	Page	Reference
Mbc1	*Mbc1HR*	Benzimidazole (carbendazim) high resistance	14, 20, 21, 37, Figure 12,Table 2, 47, Table 3, Table 4, Table 6, 60, Table 5	Faretra & Pollastro, 1991
	Mbc1LR	Benzimidazole (carbendazim) low resistance	14, 20, 21, Table 2, 47, Table 3	
	Mbc1S	Benzimidazole (carbendazim) sensitive	14, 20, 21, 37, Figure 12, Table 2, 47, Table 3, Table 4, 60, Table 5	
Daf1	*Daf1HR*	Dicarboximide (vinclozolin) high resistance	14, 20, 21, 37	Faretra & Pollastro, 1991
	Daf1LR	Dicarboximide (vinclozolin) low resistance	14, 20, 21, 37, Figure 12, Table 2, 47, Table 3, Table 6, 60, Table 5	
	Daf1UR	Dicarboximide (vinclozolin) ultra low resistance	14, 20, 21, 37	Beever & Parkes, 1993
	Daf1S	Dicarboximide (vinclozolin) sensitive	14, 20, 21, 37, Table 2, 47, Table 3, Table 4, 60, Table 5	Faretra & Pollastro, 1991
Daf2	-	Dicarboximide (vinclozolin) sensitive	21	Faretra & Pollastro, 193
MAT1	*MAT1-1*	Mating-type 1 (able to mate with *Mat1-2*)	18, 20, 21, 33	Faretra *et al.*, 1988b
	MAT1-2	Mating-type 2 (able to mate with *Mat1-1*)	18, 20, 21, 33`	
	MAT1-1/2	Mating-type 1/2 (able to mate with *Mat1-1* and *Mat1-2*)	18, 20, 21, 33	
Sma	-	Small apothecia gene – wild-type	18, 21	Faretra & Pollastro, 1992
cutA	-	Cutinase A	19	Van Kan *et al.*, 1997
Bcpgal	-	A polygalacturonase expressed early in pathogenesis.	19	Have *et al.*, 1997
Sel1	*Sel1R*	Sodium selenate resistance class B	Chapter 3-39, 60, Table 5, 88, Figure 37	Weeds *et al.*, 1997
	Sel1S	Sodium selenate sensitive	Chapter 3-39, 60, Table 5, 88, Figure 37	
nit1	-	Nitrogen utilisation gene – defective in nitrate reductase	Chapter 3-39, 60, Table 5, 88	Weeds *et al.*, 1997
Pat1	*Pat1*	Pathogenicity related gene – non-aggressive	58, Table 4, Figure 25, Chapter 5-69	Weeds *et al.*, in prep

Appendix 7 Pathogenicity related fungal attack chemicals

Table 12 Summary of references investigating the importance of *B. cinerea* attack chemicals to their aggressiveness on a particular host (correlation 'yes' indicates positive correlation unless otherwise stated).

Attack chemical	Reference	No. isolates	Host	Comment	Aggressiveness correlation
PG	Zalewska-Sobczak *et al.*, 1981	2	Apple	-	No
	Di Lenna *et al.*, 1981	3	Grape Strawberry Bean	-	No
	Di Lenna & Fielding, 1983	3	Apple Carrot	4 isoforms	No
	Kovacs & Tuske, 1988	27	Apple Paprika Bean	-	No
	Have *et al.*, 1997	2		Gene disruption mutant	Yes
PME	Wasfy *et al.*, 1978	3	Strawberry Apricot Bean	-	Yes (neg)
	Di Lenna *et al.*, 1981	3	Grape Strawberry Bean Lettuce	-	No
	Lorenz & Pommer, 1987	10	Grape Pepper	-	No
	Kunz *et al.*, 1996	5	Apple	Insertion mutants	No
PMG	Wasfy *et al.*, 1978	3	Strawberry Apricot Bean	-	Yes
	Zalewska-Sobczak *et al.*, 1981	2	Apple	-	No
	Lorenz & Pommer, 1987	10	Grape Pepper	-	Yes

Attack chemical	Reference	No. isolates	Host	Comment	Aggressiveness correlation
PL	Di Lenna *et al.*, 1981	3	GrapeStrawberry Bean Lettuce	2 isoforms	Yes
	Movahedi & Heale, 1990	8	Carrot Cabbage Strawberry Raspberry Grape Broad bean	-	Yes
Protease	Zalewska-Sobczak *et al.*, 1981	2	Apple	-	Yes
	Lorenz & Pommer, 1987	10	Grape Pepper	-	Yes
	Movahedi & Heale, 1990	8	Carrot Cabbage Strawberry Raspberry Grape Broad bean	-	Yes
Laccase	Kovacs & Tuske, 1988	27	Bean	-	Yes
	Faretra & Mayer, 1992	1	Grape	Cream mutant with low laccase production	No
	Sbaghi *et al.*, 1996	8	Grape	Also ability to degrade stilbene-type phytoalexin	Yes
AOS	Edlich *et al.* 1989	13	*Vicia faba*	-	Yes
	Weigend & Lyr, 1996	1	*Vicia faba*	-	Yes
	Tiedemann, 1997	6	Grape Sunflower Barley Broad bean	-	Yes
Cutinase	Van Kan *et al.*, 1997	2	Gerbera Tomato	Gene disruption	No
Maltase	Wasfy *et al.*, 1978	3	Strawberry Apricot Bean	-	Yes
Phenol oxidase	Wasfy *et al.*, 1978	3	Strawberry Apricot Bean	-	Yes (neg)
Xylase	Zalewska-Sobczak *et al.*, 1981	2	Apple	-	No
Catalase	Wasfy *et al.*, 1978	3	Strawberry Apricot Bean	-	Yes
β-Glucosidase	Sasaki & Nagayama, 1994 and 1996	11	Apples Grapes Lettuce	-	Yes

PG = Polygalacturonase
PL = Pectin-lyase
AOS = Active oxygen species
PMG = Polymethylgalacturonase
PME = Pectin-methyl-esterase

Appendix 8 Polygalacturonase standard curves

Preparation of PG standard curves follows Taylor & Secor [1988]. A dilution series (10, 20, 50, 100 and 1000 fold) of standard PG solution (*Aspergillus nidulans* PG, 1440 units mg^{-1} protein; 0.29 mg protein ml^{-1}, Sigma) was included in each plate of *B. cinerea* filtrates assayed for PG activity (73). Zone diameters for each series (one series from each of four plates) were plotted against the enzyme concentration expressed as a percentage of the PG standard. A best-fit line was plotted using Microsoft Excel 97 (Figure 40 p140) and the regression equation rearranged making enzyme concentration the subject as shown.

Plate 1 [Enzyme] = antilog ((diam-17.092)/2.4639)
Plate 2 [Enzyme] = antilog ((diam-16.411)/2.7102)
Plate 3 [Enzyme] = antilog ((diam-16.442)/2.4822)
Plate 4 [Enzyme] = antilog ((diam-16.264)/2.5827)

The equation was then used to convert zone diameters to a percentage of the standard, using the formula from the appropriate plate, and then to PG activity units using the formula: units = % (mg protein ml^{-1}) (units *) from the PG standard. The converted data was analysed statistically (p37).

* 1 unit = the amount of enzyme required to catalyse the production of 1 μmole of reducing sugar per min.

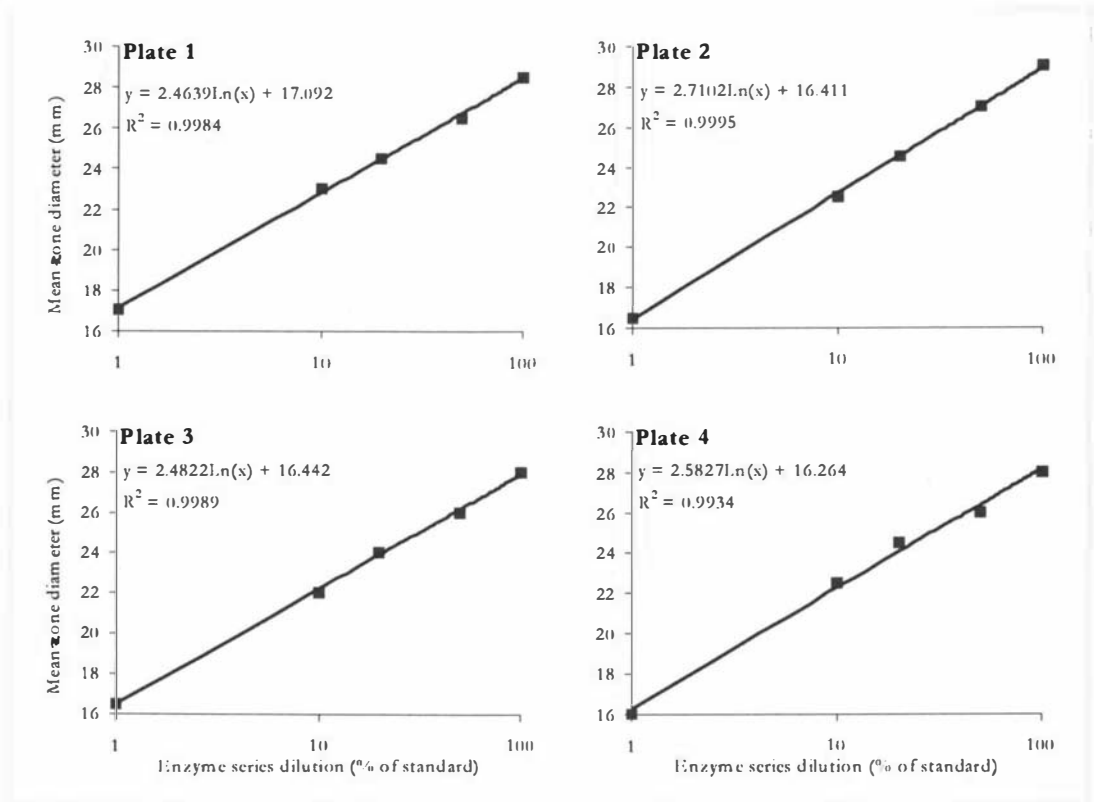

Figure 40 Polygalacturonase standard curves generated from zone diameters produced on substrate gel by a series of dilutions of the *A. niger* PG standard. R^2 values indicate the appropriateness of the fit (the closer to 1 the better the fit).

Appendix 9 Segregation of polygalacturonase isozymes

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Abstract

SEGREGATION OF POLYGALACTURONASE ISOZYMES IN SEXUAL PROGENY OF *BOTRYTIS CINEREA (BOTRYOTINIA FUCKELIANA)*

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[A]The Horticulture and Food Research Institute of New Zealand, Private Bag 3123, Hamilton, New Zealand
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[C]Manaaki Whenua - Landcare Research, Private Bag 92170, Auckland, New Zealand

INTRODUCTION

Some strains of *Botrytis cinerea* can be readily crossed in the laboratory. We have compared polygalacturonase (PG) isozymes of a New Zealand and an Italian strain and found marked differences in certain areas of their PG isozyme profiles. Segregation of individual PG isozymes and linkage relationships with benzimidazole resistance and pathogenesis markers in single-ascospore progeny from the cross of these two strains are discussed.

Little is known of the genetic basis for the wide range of isozymes of PG produced by many plant pathogenic fungi, including *B. cinerea.* It is possible that some of the diversity of PGs could be attributable to post-translational modification, with a single gene thereby giving rise to several PG isozymes. In this case, such isozymes would segregate together in progeny of a sexual cross, whereas those, which are separate gene products, should show greater independence in segregation. To our knowledge, there have been no previous reports of the segregation of PG isozymes between progeny of a sexual cross in *B. cinerea.*

MATERIALS AND METHODS

***B. cinerea* strains** SAS56 was a single-ascospore-derived reference strain supplied by F. Faretra (Bari, Italy). Mp97 was a non-aggressive mutant produced by u.v. mutagenesis from A4 (a single-ascospore strain from a cross of two NZ field isolates). SAS56 and Mp97 were crossed and 20 single spore progeny were selected for further study.

Growth of the fungi *in vitro* and *in vivo* Liquid growth medium used for *in vitro* culture was modified Richard's solution with citrus pectin (0.5% w/v) as the carbon source. Flasks were inoculated with spores (2 x 10^4/ml final concentration) and filtrates were collected after 24 h growth on an orbital shaker at 20°C.

French beans (*Phaseolus vulgaris* cv. Top Crop) were grown to the two true leaf stage. Plastic rings (3 mm diam.) placed on the adaxial leaf surfaces were loaded with 20 µl containing 10,000 spores suspended in half normal strength Vogel's medium + sucrose (0.75% w/v). After 48 h incubation under humid conditions at 23°C, the inoculum droplets were retrieved from above the developing lesions, and assessed for PG content.

Separation and detection of PG isozymes Isoelectric focusing of culture filtrates and leaf infection droplets, with subsequent detection of PG isozymes, was performed as previously described. After focusing, an overlay gel containing buffered sodium polypectate (0.04% w/v) was placed in contact with the IEF gel for 15 min and then incubated at 37°C for 4.5 h (for culture filtrates) or 1 h (leaf infection fluids). The overlay was then stained for at least 2 h in 0.03% ruthenium red.

RESULTS AND DISCUSSION

PG isozyme profiles distinguished parent strains. A distinctive profile of PG isozymes was produced by each of the parent strains both *in vitro* and *in vivo*. *In vitro* the most obvious differences between the two were in the pI 8 vicinity, with Mp97 producing a prominent isozyme of pI 8.0 which was consistently absent from the SAS56 cultures. *In vivo* this distinction was not as clear-cut; the band at pI 8.0 was still prominent in Mp97, but SAS56 did produce a minor band at this pI. SAS56 *in vivo* produced a distinctive band at pI 5, which was not detected in the Mp97 inoculation droplet. Direct comparison of the range of isozymes produced *in vivo* was complicated by the large differences in growth and lesion development between the non-aggressive and aggressive strains, so *in vitro* profiles were used for analysis of the segregation of individual isozymes in the progeny.

Segregation Patterns The pI 8 band which distinguished the parents *in vitro* segregated 1:1 in the progeny, indicating it is controlled by a single gene with a recombination pattern suggesting linkage to *Mbc1* (benzimidazole resistance).

Two other PG isozymes with pIs of ca. 8.4 and 8.8 were expressed in various relative intensities by the progeny. Almost half of the progeny appeared to lack the pI 8.4 isozyme that was expressed by both parents. As *B. cinerea* is haploid, this apparent loss of an isozyme in approximately half of the progeny is difficult to explain.

The pI 8.8 isozyme was produced in both parents but was more intense in SAS56. The progeny matched one or other parental type in roughly equal proportions, except for one strain in which this particular band was extremely faint. More work is needed to determine if this is indicative of multiple isozymes of similar pI.

No link between any PG isozyme and pathogenicity Progeny of the Mp97 x SAS56 cross were clearly distinguishable on bean leaves as either non-aggressive (Mp97 type) or invasively pathogenic (SAS56 type). This difference in pathogenicity could not be linked to any one PG isozyme produced either *in vitro* or *in vivo*.

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Segregation of polygalacturonase isozymes in sexual progeny of *Botrytis cinerea (Botryotinia fuckeliana)*

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Aims

- To determine the nature of inheritance of polygalacturonase (PG) isozymes of *Botrytis cinerea*.
- To determine whether a particular PG isozyme can be linked to pathogenicity differences.

Materials and methods

Single-ascospore *B. cinerea* strains SAS56 (aggressive) and Mp97 (non-aggressive mutant of strain A4 (1)) were crossed and 20 single spore progeny were selected.

In vitro sampling

Culture filtrates were collected after 24 h growth at 20°C in liquid medium with 0.5% citrus pectin as the carbon source.

In vivo sampling

Infection droplets were recovered from leaves of French bean (*Phaseolus vulgaris* cv. Top Crop) 48 h after inoculation.

Separation and detection of PG isozymes

Isozymes were separated by isoelectric focusing and PG activity was revealed by staining a polypectate overlay (2).

Results and discussion

PG isozyme profiles distinguished parent strains

A distinctive profile of PG isozymes was produced by each of the parent strains both *in vitro* and *in vivo* (Figs 1&2). The most obvious differences *in vitro* were in the pI 8 vicinity (Table 1). A greater variety of isozymes was produced by each strain *in vivo* (Fig. 2).

Segregation Patterns

Analysis of isozyme segregation in the progeny (Table 1) was performed using *in vitro* profiles (Fig. 1). Isozymes in the pI 8 vicinity fell into two distinct classes in the progeny. Those at pI 8.0 and pI 8.8 resembled one or other parent suggesting control by a single gene. The pI 8.4 isozyme (present in both parents) was undetectable in some progeny, suggesting multigene control.

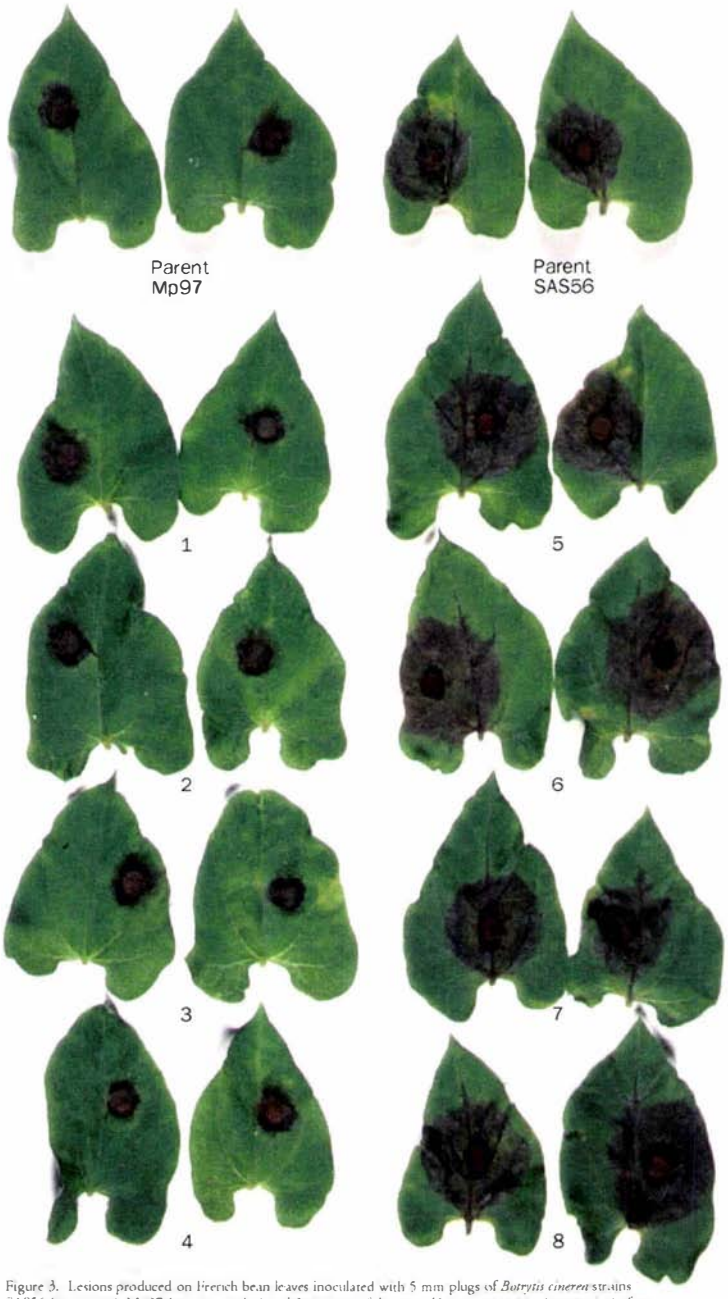

Figure 3. Lesions produced on French bean leaves inoculated with 5 mm plugs of *Botrytis cinerea* strains SAS56 (aggressive), Mp97 (non-aggressive) and 8 progeny of the cross (4 non-aggressive, 4 aggressive) after incubation at 20-24°C for 3 days.

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No link between any PG isozyme and pathogenicity

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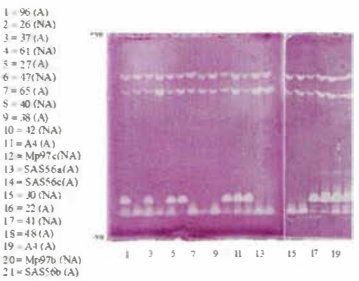

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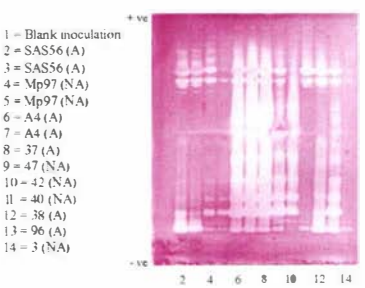

Figure 2. *In vivo* PG activity revealed by a polypectate overlay after isoelectric focusing of 48 h infection droplets from bean leaves inoculated with parent strains and a selection of progeny. A = aggressive; NA = non-aggressive.

Conclusions

- Several PG isozymes segregated independently, suggesting that they are controlled by distinct genes.
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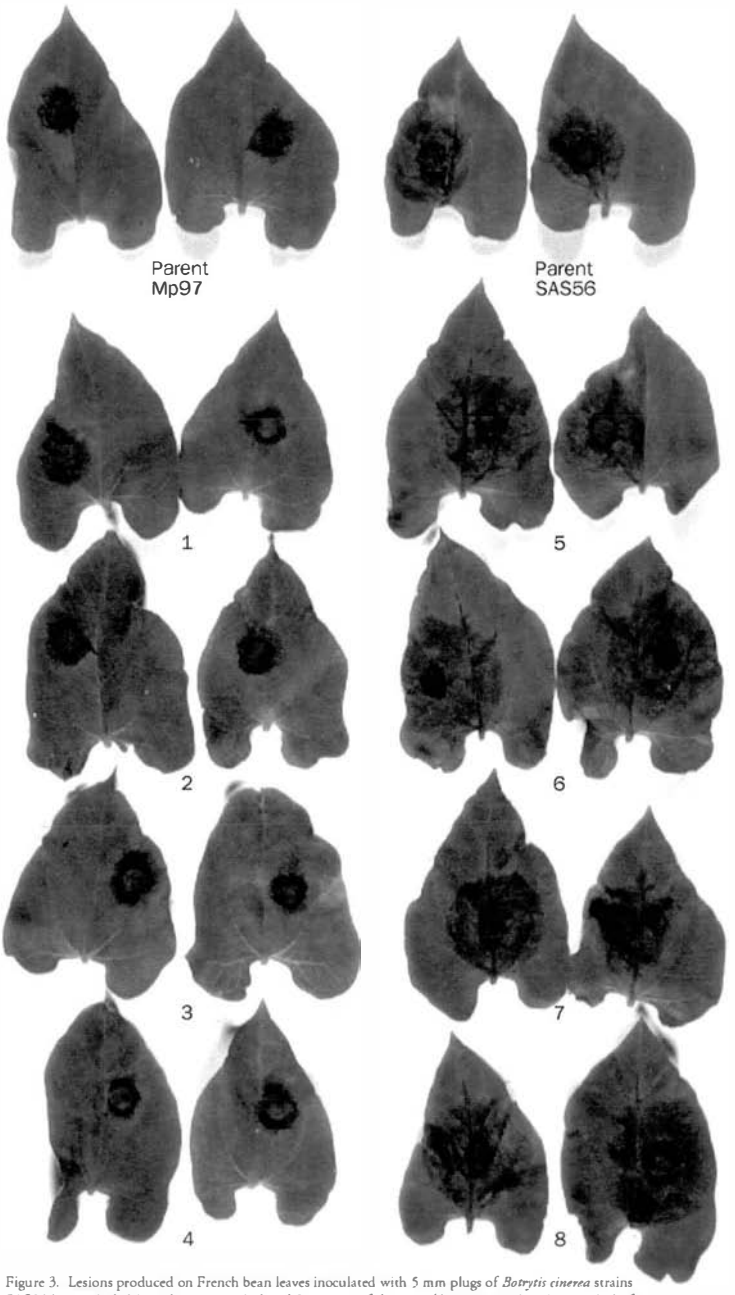

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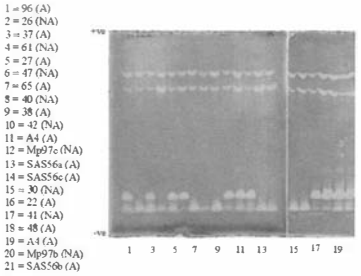

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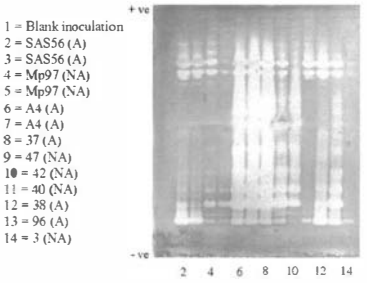

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