SELECTED STUDIES ON STRAINS OF

BOTRYTIS CINEREA

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ABSTRACT

Some characteristics of benzimidazole and dicarboximide resistant and susceptible strains of Botrytis cinerea and the chemicals that control them were studied.

Difference in sporulation or sclerotial production could be not be used to differentiate between fungicide resistant or susceptible strains. Generally, mycelial growth of dicarboximide low-level resistant strains was slower than that of susceptible strains on unamended malt extract agar and was considerably so on media amended with 0.68 M NaCl. No benzimidazole low-level resistance was detected in the benzimidazole susceptible strains tested.

Fourteen strains of B. cinerea were screened for the ability to sporulate in the dark to assess the feasibility of using material which filtered Ultra-violet light as a glasshouse covering. Eleven of these strains sporulated in complete darkness.

Chlozolinate showed a high degree of protectant and systemic activity against dicarboximide susceptible strains but was poor on low-level resistant strains. PP192 showed high protectant but no systemic activity on both susceptible and low-level resistant strains.

Sub-lethal doses of vinclozolin and iprodione on plant surfaces were shown to stimulate the sporulation of B. cinerea from an inoculum source such as an agar plug.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

CHAPTER 1

INTRODUCTION

1.1 AN OVERVIEW OF BOTRYTIS CINEREA.

Botrytis cinerea Pers. is a ubiquitous plant pathogen found throughout the temperate regions of the world. Since Micheli first erected the genus Botrytis in 1729, this species has been the subject of numerous studies and not without some controversy. It's importance as a plant disease causing agent cannot be understated with many hosts recorded worldwide. In New Zealand, the pathogen has been recorded on 69 different hosts (Dingley, 1969). It is particularly important as a pathogen of vegetables, stored and transported fruits, nursery stocks and ornamental flowers (Jarvis, 1977).

Symptoms (Plates 1.1 - 1.2) can vary depending on the host plant, part of host attacked and environmental conditions. In most cases B.cinerea infections manifest themselves as a soft, wet rotting of the tissue although on some flower crops, such as orchids, a spotting of the petals may occur. These symptoms are often accompanied by masses of grey/brown conidia which cover the infected tissue and so give rise to the common name 'grey mould'. Sclerotia are also commonly produced.

For many years confusion existed over the taxonomic relationships between the hyphomycete genus Botrytis, and the ascomycete genera Botryotinia and Sclerotinia (Jarvis, 1980a). By 1953, studies by a number of authors had clarified this aspect and had established a genetic connection between Botryotinia and Botrytis (Groves and Drayton, 1937; Gregory, 1949; Groves and Loveland, 1953).

Taxonomists now recognize the perfect stage of B. cinerea to be Botryotinia fuckeliana, placed in the Class Discomycete, order Helotiales, family Sclerotiniaceae. This sexual stage is seldom found in nature. (Poach and Abaw, 1975).

The form-genus Botrytis has itself been the subject of controversy (Jarvis, 1980a). The accepted classification is that of Hennebert (1973) which places it in the Hyphomycete family Botrytidaceae.

Structures produced by the thallus of B. cinerea include conidia and conidiophores, microconidia, chlamydospores, appressoria, sclerotia and apothecia.

The conidiophores can be described as being tall (approx. 0.8-2.0mm), dark-coloured and irregularly or dichotomously branched, having a globose basal cell (Jarvis, 1977). Conidia develop synchronously on short, fine, denticles which arise from dark, septate sporogenous branches produced near the apex of each conidiophore. Some conidiophores contain short side branches on which clusters of conidia can arise. The conidia are usually one celled, grey to brown and

globose to ovoid measuring 6-15 x 1-12um (Jarvis, 1977). When mature they are easily detached from the conidiophore by air currents and carried to a new food source.

Microconidia are hyaline, unicellular, 2-3um in diameter and usually borne on phialides in chains from any part of the thallus (Jarvis, 1977). They are not common and although some authors have claimed success in germinating microconidia to form mycelium (Brierley, 1918), their sole function is believed to be in spermatization to produce the sexual stage.

Like many of the discomycete fungi, sclerotia are one feature of this organism. These usually germinate to form conidia, apothecial production being uncommon (Coley-Smith, 1980).

B. cinerea has a high saprophytic ability, and can grow on dead tissue, although it is less competitive than many other saprophytes. Entry to the plant can be gained in three ways, from conidial germ tubes, by mycelium growing on dead parts of the plant or from mycelium established in extraneous organic matter adhering to the plant. The last two pathways are quite common, as B. cinerea seldom attacks fresh green tissue unless conditions are extremely favourable. More often it is moribund tissue adhering to the plant which is infected first, the pathogen then using this tissue as a food base from which to colonize the healthy tissue.

The infection of healthy tissue from conidia depends on many factors

such as the host plant, temperature and humidity, free water and whether or not the tissue attacked is senescing, a wound, or unbroken cuticle. The presence of pollen on the tissues is also known to aid infection by providing carbohydrates and amino acids which stimulate spore germination and growth of germ tubes (Blakeman, 1980). Pollen may also suppress phytoalexins (Verhoeff, 1980). In general, epidemics caused by B. cinerea occur in cool, wet and humid weather. These conditions favour sporulation and infection and may also predispose the host (Jarvis 1980b).

Toxins which kill cells forward of the advancing hyphae, are released during the growth of the pathogen through tissue. How this is achieved and the exact nature of the toxins are not yet known. Organic acids are known to be produced by the fungus and it is thought these acids may change the PH of infected tissues to one more favourable for cell-wall degrading enzymes. Alternatively, it is possible these acids may lead to defence reactions in the plant which involve peroxidase activity, inhibition of the cytochrome oxidase system and effects on nuclei (Verhoeff, 1980).

The pathogen also has the ability to colonize some tissues early and then remain latent in those tissues, reactivating to cause disease at a later stage. In grapes and strawberries for example, the fungus can grow from senescent flower tissue into young fruitlets and remain there as inactive mycelium. When the fruit ripens, a pathogenic relationship is established and disease occurs. Experimental work shows a combination of three factors are likely to be responsible for

this phenomenon. In brief these are; the existence of fungitoxic compounds in unripe berries, nutritional differences between unripe and ripe fruits and an inability of fungal enzymes to hydrolyze pectic material in the cell wall of unripe fruit (Verhoeff, 1980).

Control of B. cinerea has for many years relied on cultural practices such as sanitation, temperature control and fungicides. Fungicide resistance is a new and continuing problem with this pathogen.

1.2 LIGHT REQUIREMENTS FOR THE SPORULATION OF B. CINEREA

Most strains of B. cinerea appear to have specific and complex light requirements for sporulation. Much of the early work was carried out by researchers using a series of filters to determine the relationships (Klein, 1885; Reidemeister, 1909; Moreau, 1913). From this early research it was determined that violet and blue light promote conidiation while green, orange, yellow and red light have no effect.

Other studies which showed that light promoted sporulation of B. cinerea came from Paul (1929), Rabinovitz-Sereni (1932) and Peiris (1947a).

In all these studies visible light only was used with no consideration given to the effect of infa-red or ultra-violet until the work of Leach (1962). Detailed studies on the role of light in the sporulation of B. cinerea have been done by Tan and Epton (1973), Tan (1974), and Suzuki (1977). The conclusions drawn from these studies are listed below.

1. Near-ultra violet radiation (320 - 400nm) promotes sporulation.

2. Blue light inhibits sporulation that has first been promoted by near ultraviolet or far-red.

3. Far-red radiation repromotes sporulation after inhibition by blue light.

4. Red light inhibits sporulation that has been repromoted by far-red, but has no effect on sporulation promoted by near ultraviolet alone, or when near ultra-violet is used followed by far-red.

On the basis of these observations, several models based on the existence of mycochromes, were postulated as to the mechanism of sporulation. Details of these models can be found in Tan and Epton (1973).

Researchers also found that the effect of light on sporulation varied considerably when other factors were changed. Physiological age, (Leach, 1961; Tan and Epton, 1973), spore density and temperature all had an effect. Some isolates were found which sporulated in the dark (Hyre, 1972; Hite, 1973; Honda et al, 1977).

The effect of light quality on sporulation could have important implications in disease control. Honda et al (1977) investigated the use of near-Ultraviolet absorbing vinyl as a glasshouse covering, to reduce sporulation and hence control grey mould disease. These trials were quite successful, with disease incidence in tomatoes and cucumbers being 93 and 83% lower respectivly in greenhouses with near-UV absorbent material, compared to ones with standard coverings.

1.3 VARIATION IN CULTURAL CHARACTERISTICS OF B. CINEREA

Early researchers were quick to observe morphological variation between isolates of B. cinerea when grown in culture (Paul, 1929; Hansen and Smith, 1932). As with most fungi, different environmental and nutritional factors affect the morphology of single isolates quite markedly. B. cinerea can also exhibit gross differences between one isolate and another under the same cultural conditions, a feature shared by other deuteromycete fungi (Hansen, 1938).

Paul (1929) described three main phenotypes of B. cinerea in culture. Sporulating, where under most conditions the colony formed masses of conidia; sclerotial, where few conidia were formed but many sclerotia; and mycelial, where aerial mycelium was formed but few conidia or sclerotia. Paul found that he could manipulate these characteristics to a certain extent by changing the environment. Sporulation could be encouraged on all isolates by illuminating cultures at 27^{o} C or at low relative humidities while sclerotia were more readily formed at 12^{o} C in the dark or at high relative humidities.

Other workers have also observed the three "types" of Paul, but in addition have found isolates which seemed to be combinations of two or all three types (Hansen and Smith, 1932; Hansen, 1938). Indeed one researcher summarized the situation at the time by writing "The species Botrytis cinerea may be visualised as, at any one moment, a cluster of numerous races or strains morphologically congruent on

the host plant but *in vitro* showing marked and constant cultural differences" (Brierley, 1931).

The seemingly consistent phenotypic variation between different isolates led some researchers to assign morphological races to the fungus. (Berkely, 1924; Jorgensen and Weber, 1929; Abdel-Salem, 1934). Only one case is known of a clearly distinct morphological race however: an isolate of *B. cinerea* from *Crassula perforata*, which apparently lacked a phenolic oxidase system and had white sclerotia but was otherwise of typical form and equally as pathogenic as normal black-sclerotial isolates. (Brierley, 1920).

Early researchers, puzzled by the seemingly infinite number of phenotypic isolates which could be produced in culture, devised experiments to determine possible genetic reasons for such a phenomenon. Hansen and Smith (1932) found the hyphal cells and conidia of *B. cinerea* contained large numbers of nuclei ranging from 3 to 18 per cell. They also found anastomosis was a common occurrence. Whilst carrying out a large number of single spore isolations from 47 samples of infected vegetation, they found that some of the strains remained stable, but many continued to give rise to further variations. The stable strains were termed homotypes and the unstable strains were termed heterotypes and were considered to be heterokaryons. By observing the results of repeated single spore isolations and crosses between different stable phenotypes, Hansen (1938) was able to demonstrate that phenotypic differences were most likely conferred by the number and arrangement of two different

elements carried by different nuclei. Isolates whose cells contained entirely one type (homotype a) gave rise to mycelial cultures (M) while isolates, whose cells contained the other type (homotype b) gave rise to cultures which sporulated abundantly (C). These cultures when subcultured using a single spore isolation remained constant. Intermediate types, containing both elements were a combination of the mycelial and sporulating phenotype (MC) and did not remain constant if subcultured. Furthermore, when homotype a was crossed by hyphal anastomosis with homotype b, the result was an unstable intermediate form. The author postulated that many deuteromycete fungi were heterokaryons and that anastomosis may have a role to play in segregation of the nuclei. Phenomenon such as sectoring, reversion or loss of ability to sporulate resulted from a change to the homotype condition.

Since this study, other researchers have found the nuclear number in one cell of an isolate of B. cinerea to be as high as 120 and that nuclei can be transferred across hyphal anastomoses and also from cell to cell through the septal pore (Menzinger, 1965).

A recent study on variation of B. cinerea was carried out by Grindle (1979). He applied modern techniques to try and quantify further the genetic and cytoplasmic features of this organism. Considerable phenotypic instability was found in most morphological variants and no success was achieved in isolating nutritional mutants or in producing the sexual stage of the organism. Grindle concluded that either heterokaryosis or diploidy was responsible for strain

variations and genetic instability. He also postulated that cytoplasmic entities may play a role in variation.

1.4 FUNGICIDE RESISTANCE

First records of fungicide resistant strains of B. cinerea were published by Roy (1947) and Reavill (1950, 1954). These authors recorded laboratory isolates which could tolerate the chlorinated nitrobenzene group of fungicides in the vapour phase and could produce resistant strains in culture in their presence. Other laboratory-induced resistant strains were documented for dicloran (Lankow, 1971), mercury and copper compounds (Parry and Wood, 1958) and the dithiocarbamate group of fungicides (Parry and Wood, 1959a 1959b; Golyshin and Abelentsev, 1973). Strains of B. cinerea with stable inherited resistance to dithiocarbamate fungicides have recently been isolated from crops (Pepin and MacPherson, 1982; Barak and Edgington, 1984).

Even though strains of B. cinerea resistant to the above compounds could be induced in the laboratory, field resistance did not pose a problem. Most fungicides released prior to the introduction of the benzimidazole compounds in the early 1970's were mediocre against B. cinerea anyway, with good control of disease being possible only through a combination of these chemicals and good cultural practices.

The introduction of the fungicide benomyl was a major breakthrough in the control of B. cinerea. The fungicide was extremely toxic to B. cinerea, had high systemic activity and was also very effective on a wide range of other fungi. Unfortunately resistance

to this compound was soon observed in Holland (Bollen and Scholten, 1971) and within four years was widespread within British glasshouses (Miller and Fletcher, 1974; Fletcher and Scholefield, 1976; Geeson, 1976) and indeed in most environments where the fungicide was used regularly. Outbreaks of resistance were accompanied by a complete breakdown of disease control leading to serious crop losses (Schwinn, 1982).

Resistant strains could be readily identified in the laboratory, with both spores and mycelium tolerating benomyl concentrations of >100 mg/l in potato dextrose agar (PDA). Sensitive strains were killed at <1 mg/l (Miller and Jeves, 1979).

The resistance was persistent, and benomyl tolerant strains were found on properties where the fungicide had not been used for up to three years (Miller and Jeves, 1979).

Cross resistance was another feature of these benomyl resistant isolates. Those resistant to benomyl were also resistant to carbendazim, thiabendazole and thiophanate-methyl (Grindle, 1981).

The mode of action of the benzimidazole fungicides is well documented (Davidse, 1982). In the plant, The breakdown products of the fungicides (which with most benzimidazoles is carbendazim) binds strongly to tubulin of certain fungi. This leads to the inhibition of microtubule assembly thus interfering with a great number of processes in which the microtubules are involved, such as nuclear and cellular

division, cell migration and organelle movement. Benzimidazole compounds have a low affinity for plant tubulin.

The interaction between tubulin and the benzimidazole compounds is highly specific and thus very vulnerable to any structural alteration of the binding site. Resistant strains arise when a mutation lowers the binding affinity of tubulin to a benzimidazole compound without any effect on normal tubulin functioning.

During the late 1970's a new group of fungicides were slowly replacing the benzimidazoles for control of B. cinerea. This group (the dicarboximides) is represented by iprodione, vinclozolin, procymidone and chlozolinate, fungicides which show high activity against the Sclerotiniaceae, Moniliaceae and some unrelated fungi. Although released by the chemical industry as protectant compounds, a few workers have found considerable systemic activity in some members of this group. Iprodione has been reported to move from potato roots to the foliage (Cayley and Hide, 1980) while systemic activity of procymidone has been observed in cucumber (Hisada et al, 1977).

It was soon found that dicarboximide resistant strains could be readily formed in the laboratory, by either plating conidial suspensions on sub-lethal dicarboximide supplemented agar plates and continually subculturing onto increasing dose rates or using lethal concentrations from the outset (Pommer and Lorenz, 1982). The first record of field resistance was in 1978 when resistant strains were isolated from treated grapes after only three years dicarboximide

usage (Pommer and Lorenz, 1982). Numerous authors have since reported resistant strains isolated from a number of crops throughout the world.

At first, although dicarboximide resistant strains continued to be isolated from the field, no major control problem occurred. However, failures of chemical control were soon reported where there had been intensive usage of dicarboximides in protected crops. (Katan, 1982; Takeuchi and Nagai, 1982; Beever and Brien, 1983; Hartill et al, 1983; Panagiotaku and Malathrakis, 1983).

While some work has been carried out classifying the characteristics of these resistant strains, the overall picture is confused due to a lack of standard test fungicides and methods.

One isolate, insensitive to vinclozolin, procymidone and iprodione was isolated by Pappas et al (1979) from strawberries which had been inoculated with a benomyl insensitive isolate of the pathogen and sprayed with iprodione. Insensitivity in this case was determined by growing the fungus on fungicide amended malt extract agar (MEA) at a fungicide concentration of 100 mg/l and measuring the final colony size. Sensitive isolates did not grow at this concentration. Tests using agar plugs on unwounded and wounded strawberries showed the resistant strain to be virulent but less pathogenic i.e. while 100% infection occurred from both the dicarboximide sensitive and dicarboximide resistant isolate on the wounded fruit, only 80% infection occurred with the resistant isolate on the unwounded fruit

as compared with 100% from the sensitive isolate.

Davis and Dennis in two studies (1979b, 1981) examined a range of natural and laboratory induced resistant isolates for pathogenicity and other features. Mycelial growth of sensitive isolates was completely inhibited by rates of vinclozolin and iprodione of 2 mg/l. Resistant strains, on the other hand, exhibited EC_{50} values of >100 mg/l. Mycelium of resistant strains were able to infect unwounded strawberry fruits and tomato leaves to a similar extent to that of sensitive strains although infection of carrot roots was markedly less aggressive. Resistant strains were relatively slow growing and showed a marked lack of sporulation compared with most sensitive strains. This is in agreement with Leroux et al (1977) for selected strains in the laboratory. Iprodione reduced the growth of nearly all isolates more than vinclozolin indicating differences of activity between the two chemicals. It is interesting to note that several authors have found or induced resistant strains of fungi with low resistance to iprodione, but high resistance against vinclozolin and procymidone (Rosenberger and Meyer, 1981; Fuchs et al, 1984; Grindle, 1984).

In a separate study, strawberries at different growth stages were sprayed with dicarboximides and then inoculated with agar plugs containing resistant or susceptible strains. All isolates readily infected untreated berries at all stages. Treated berries were attacked by resistant isolates only (Davis and Dennis, 1979a).

Katan (1982), who also carried out an extensive study on dicarboximide

resistant strains, used spore germination on PDA amended with 5 mg/l vinclozolin to determine whether a particular isolate was sensitive or resistant. The EC_{50} values for resistant strains over all dicarboximides were in the range 1.0 - 4.2 mg/l while those for sensitive strains ranged from 0.1 - 0.27 mg/l. On average, resistant strains grew 25-30% slower than sensitive strains. Various plant parts treated with dicarboximide fungicides and then exposed to the fungus by way of agar plugs were protected from sensitive strains but not resistant strains. These findings are in agreement with a study by Panayotakou and Malathrakis (1983).

From these studies by Katan (1982) and Panayotakou and Malathrakis (1983) came the first indication that there was more than one level of resistance. Earlier authors such as Davis and Dennis (1979a) (1979b) (1981) and Leroux (1977) had used high-level resistant strains, mostly laboratory induced and maintained and did not test for lower levels of resistance.

Two classes of resistance were defined by Beever and Brien (1983) in a survey of dicarboximide resistant strains of B. cinerea in New Zealand. A particular strain was classed as having high-level resistance if the EC_{50} value for radial growth in iprodione amended MEA was >5 mg/l while an isolate was classed as having low-level resistance if the EC_{50} value was <5 mg/l under the same conditions. At the same time physiological characteristics of the various strains were also examined. Although dicarboximide resistant strains were noted as having a sharply delimited colony margin on NaCl amended

medium, it was found that as a group they did not differ from sensitive strains in radial growth rate, sporulation or sclerotial production. This agrees with work done by Beever (1983) but not with that of Katan (1982) or Panayotakou and Malathrakis (1983) who found that sporulation and sclerotial production was the same but radial growth rates differed.

Beever and Brien tested 647 isolates from various crops for fungicide resistance. Of these, 150 were classed as having low-level resistance to the dicarboximides. In properties where these strains were prominent, there also appeared to be a loss of disease control. Furthermore, with the exception of one, all low-level resistant strains were abnormally sensitive to high osmotic potential. This relationship has also been found in other studies (Beever, 1983; Grindle, 1983,1984).

Studies in ultrastructure of iprodione resistant isolates of B. cinerea reveal a layer of lipidic material around the circumference of hyphae just inside the plasmalemma. It has been postulated that this may immobilize the fungicide, preventing uptake and subsequent access to target sites (Bishop and Jeves, 1984).

Other recent work has examined dicarboximide resistant mutants of other fungi. Evidence collected by Grindle (1983, 1984) points to the cell wall-plasma membrane complex as being defective in resistant strains, and so susceptible to mechanical stress caused by an isotonic environment. Wild type isolates of B. cinerea can usually

tolerate very high osmotic potential (Jarvis, 1977). Alternatively, some fungi can adjust the osmotic potential of their cytoplasm to suit the surrounding environment by regulating the metabolism of soluble carbohydrates. It may be that dicarboximide resistant fungi cannot regulate the metabolism of soluble carbohydrates effectively and thus are unduly affected by extreme osmotic changes (Grindle, 1984).

High level resistant strains are seldom found outside the laboratory. The main reason for this is probably due to the inability of these strains to compete with wild type sensitive strains, particularly under conditions of high osmotic potential on, say, a ripe strawberry fruit. Low-level resistant strains, which are more competitive but still sensitive to high osmotic potential, will only flourish when wild type populations are depressed by regular applications of dicarboximide fungicide. Furthermore, although low-level resistant strains are not controlled by field rates of dicarboximide fungicide when the inoculum potential is high, say from an infected petal falling on fruit, infection from spores is controlled at standard rates (Hoksbergen and Beever, 1984).

How then do low-level resistant strains relate to disease loss? It seems that where a large percentage of low-level dicarboximide resistant strains exist, crop loss from disease can occur despite the use of a dicarboximide fungicide. This was first observed in protected crops (Katan, 1982; Hartill et al, 1983; Panayotakou and Malathrakis, 1983) and later in outdoor crops such as grapes (Leroux and Clerjeau, 1985; Beever Pers. comm., 1985).

What then is known about the mode of action of the dicarboximide fungicides? Despite extensive study, the literature is confused and conflicting.

It is well known that dicarboximide resistant fungi have cross-resistance to all the dicarboximides and also to other fungicides which contain the N-substituted 3.5-dichlorophenyl structural subunit e.g. dicloran (Leroux et al, 1977; McPhee, 1980; Littley and Rahe, 1984). A common mode of action may therefore exist between all the fungicides with this subunit.

Some researchers have found that nucleic acid synthesis is affected by dicarboximides (Fritz et al, 1977) while others have not (Pappas and Fisher, 1979). Fritz (1977) found procymidone and vinclozolin caused the accumulation of triglycerides and free fatty acids in B. cinerea. Pappas and Fisher (1979), on the other hand reported that the chemicals inhibited triglyceride production and that free fatty acid content was very dependent on the medium used. The exception was iprodione, which only slightly inhibited triglyceride production but inhibited formation of 4-dimethylate steroids. Cook et al (1979) caution that iprodione rearranges into less active isomeric products when dissolved in methanolic or ethanolic solutions, such as were used for these and other experiments, and therefore such biochemical observations should be assessed carefully when using this particular dicarboximide. Other researchers have found no evidence of cross-resistance between ergo-

sterol biosynthesis and dicarboximides (Gullino and de Waard, 1984).

Morphological effects of the dicarboximides on germinating conidia of B. cinerea have been documented by Eichhorn and Lorenz (1978) using vinclozolin; The germ tubes swelled and burst. Albert (1979) showed bursting was prevented by growing the organism in isotonic media where the fungus developed sphaeroplasts and protoplasts but where effective cell wall synthesis still did not take place. Normal growth proceeded after the removal of the vinclozolin.

Pappas and Fisher (1979) found cell membrane permeability to be unaffected by the dicarboximides and so along with Hisada et al (1978) suggested vinclozolin interfered with the cell wall synthesis at a site distinct from that of chitin synthesis.

While the information available is not conclusive, studies on fungicide resistant strains may hold the key to discovering the mode of action of these chemicals.

Plate 1.1: Grape bunches infected by B. cinerea

Plate 1.2: Chrysanthemum flowerheads infected by B. cinerea

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 ISOLATES

All isolates of B. cinerea used in these studies were obtained from the culture collection of D.S.I.R. Plant Diseases Division. The isolates had been obtained from a variety of crops and were a mixture of dicarboximide and benzimidazole sensitive and resistant strains (Table 2.1) as determined by Beever and Brien (1983).

Stock cultures were prepared from freeze dried samples streaked onto Malt agar (MA) and grown in the dark at 20° C +/- 2° C for three days. Mycelial squares 8mm x 8mm were then cut from the actively growing margins with a needle, and transferred aseptically to McCartney bottles containing 12 mls of deionised sterile water. Six to ten squares were placed in each bottle and a total of 6 bottles were prepared for each isolate. They were stored in the dark at room temperature and used when needed. Every 6 months stocks were subcultured by growing the isolates in the dark at 20° C +/- 2° C and re-isolating to new McCartney bottles. When cultures were required, a square was removed from the McCartney bottle and laid mycelial-side down on a petri dish containing MA. This was then incubated for 2-4 days in the dark until the culture had grown.

2.2 GROWTH MEDIA

The basal medium used for all cultures was Malt agar (MA) which contained 15 g/l agar and 30 g/l malt. In most experiments this was supplemented with 5 g/l of peptone and was labelled as Malt extract agar (MEA). The agar was prepared by adding all ingredients to deionised water, bringing to the boil while stirring and then autoclaving at 120° C for 15 minutes.

In some experiments, a commercial preparation was used - Oxoid Malt Extract Agar (OMEA); this contained the same ingredients in the same proportions as MEA.

2.3 INCUBATION CONDITIONS

All cultures and infected plant material were incubated at 20° C +/- 2° C either under near-UV light (Sylvania blacklight blue - F40/BLB, 40W) on a 12 hours on/ 12 hours off cycle or in darkness.

2.4 PLANT MATERIAL

Dwarf beans var. "tender green" were used for all fungicide testing. The plants were grown in peat/pumice medium in 11cm diameter pots in a glasshouse at 20 - 25° C for approximately 3 weeks when the primary leaves were fully expanded.

2.5 FUNGICIDES

Fungicides used were:

(i) iprodione ('Rovral', 50 WP; May and Baker New Zealand Ltd.)

(ii) procymidone ('Sumisclex', 50 WP; ICI New Zealand Ltd.)

(iii) chlozolinate ('Seranil', 40 F; Shell New Zealand Ltd.) which has recently become available commercially in NZ.

(iv) benomyl ('Benlate', 50 WP; Du Pont New Zealand Ltd.)

(v) carbendazim ('Bavistin', 50WP; Du Pont New Zealand Ltd.)

(vi) PP192 (formulated as a 50 % flowable suspension). Formerly known as B1216, it is described by the manufacturer, ICI NZ Ltd, as a broad spectrum, protectant fungicide. Initial trials have shown high activity against many fungi including Botrytis, Sclerotinia, Venturia and Alternaria sp. (Anonymous, 1984).

Fungicides (i) - (iii) are classed as dicarboximides, (iv) - (v) as benzimidazoles and (vi) as a pyridinamine fungicide.

All fungicides were used as the formulated product but the concentrations are expressed as a.i. (active ingredient).

A small hand held garden sprayer was used to spray all plants to runoff with fungicide suspension.

Fungicide suspensions for spray application were prepared by weighing the required amount into a small McCartney bottle, adding 10mls of water and shaking vigorously. This was poured into the spray container and the McCartney bottle then rinsed with tap water, which was also added to the spray container. The container was then filled with tap water to the required amount.

Fungicide amended agar was prepared by adding the powder or liquid fungicide formulation to sterile distilled water and stirring

vigorously until a suspension was formed. Aliquots were then added aseptically to cooled liquid agar and mixed in with a sterile glass stirring rod. The agar was then poured into plastic petri dishes and left to set.

2.6 ASSESSMENT METHODS

(a) Mycelial growth

The margin of growth 24 hours after inoculation was drawn on the plates with a fine felt-tip pen. Mycelial growth in culture was measured with a micrometer on two radii at right angles to each other after a further 24 hours (Figure 2.1).

2.7 STATISTICAL ANALYSIS

Statistical analysis of quantitative data was carried out using GENSTAT or MINITAB.

Figure 2.1: Diagram of petri plate to show the measurement of mycelial growth rate of B. cinerea

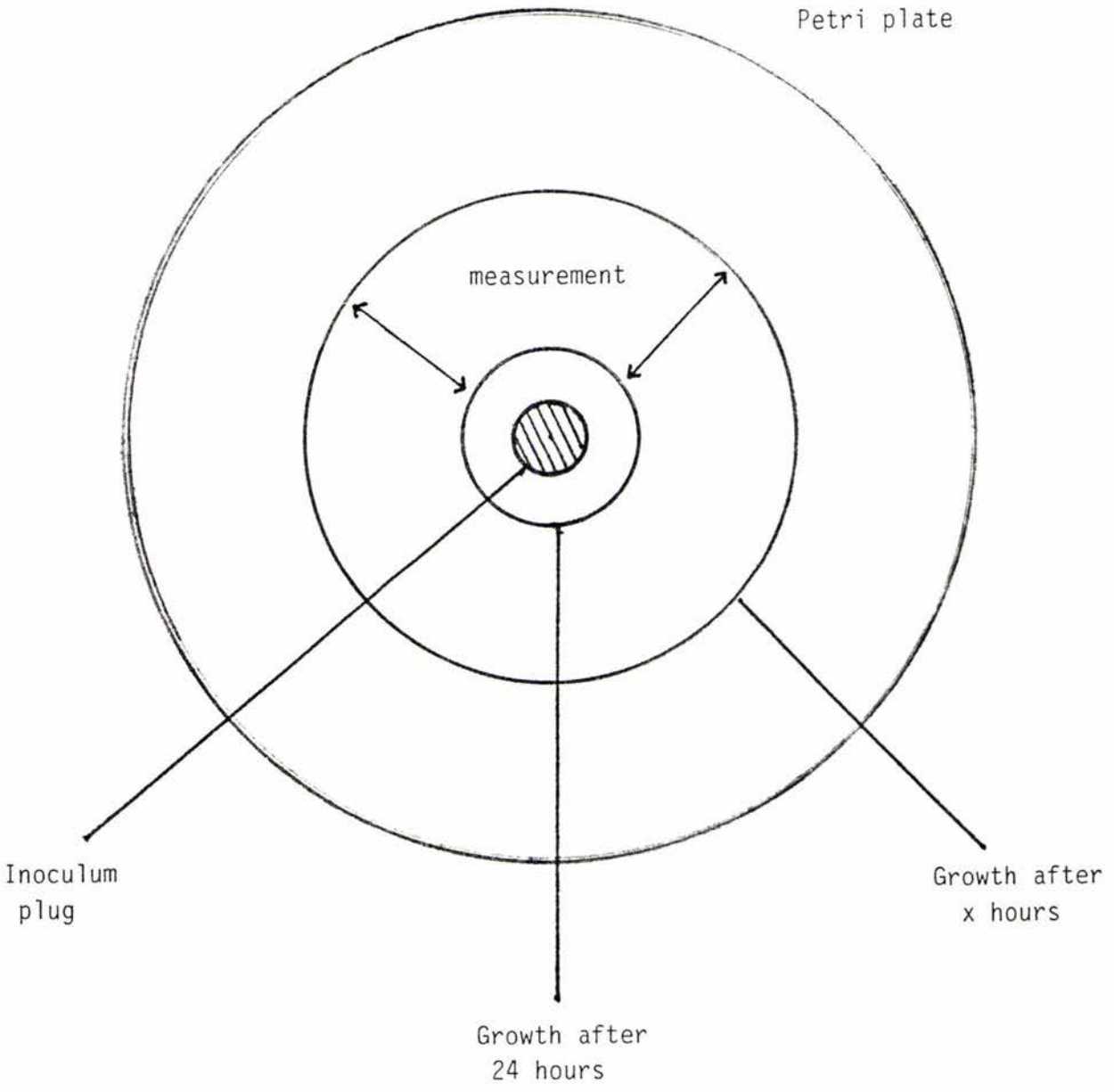


Table 2.1: Isolates of Botrytis cinerea obtained from D.S.I.R. Plant Protection division.

PDD culture code	Host	Phenotype
7516	Grape	D^l B^r
7517	Grape	D^l B^s
7518	Grape	D^s B^s
7667	Grape	D^s B^r
7569	Cucumber	D^l B^r
7668	Cucumber	D^l B^s
7658	Cucumber	D^s B^s
7688	Cucumber	D^s B^r
7550	Tomato	D^l B^r
7551	Tomato	D^s B^r
7554	Tomato	D^s B^s
7664	Phaseolus	D^l B^r
7666	Phaseolus	D^l B^s
7567	Strawberry	D^s B^s
7685	Kiwifruit	D^s B^s
7681	Kiwifruit	D^s B^s
7682	Kiwifruit	D^l B^r
7557	Unknown	D^l B^r
7665	Unknown	D^s B^s
7570	Unknown	D^s B^s
7663	Unknown	D^s B^r
7558	Unknown	

D^l = Dicarboximide resistant (low-level)

D^s = Dicarboximide sensitive

B^s = Benzimidazole resistant

B^r = Benzimidazole susceptible

CHAPTER 3

MORPHOLOGICAL CHARACTERISTICS OF ISOLATES

3.1 INTRODUCTION

Isolates of B. cinerea can exhibit extreme variations in morphology both on the host plant and in culture. Some isolates sporulate heavily, producing abundant conidia but few sclerotia. Those at the other extreme produce abundant sclerotia, or aerial mycelium but few conidia. Many isolates are a mixture of both, producing sclerotia and conidia.

Paul (1929) and Hansen and Smith (1932) studied these phenotypes and their work prepared the ground for recognition of the "Dual phenomenon", now known to be a characteristic of many deuteromycete fungi (Hansen, 1938).

While it is clear that the morphology of any one isolate of B. cinerea is determined by the arrangement and number of nuclei containing either of the two "elements", M or C, recognised by Hansen (1938), the expression of these can be modified to a certain degree according to the environment which the fungus finds itself in (Paul 1929). As a general rule, the organism sporulates best (and sclerotia are formed least) when grown in low humidities at high temperatures

while under a diurnal light regime. Conversely, low temperatures, high humidity and darkness favour the formation of sclerotia. Both sclerotial formation and sporulation are also determined by the level of organic nutrients in the substrate (Jarvis, 1977).

Some authors have examined the morphological characteristics of fungicide resistant strains of B. cinerea to determine if there is any relationship between morphological expression and resistance. With benzimidazole resistant strains there is no such relationship. Wild types and resistant strains are indistinguishable in culture and both types are also ecologically "fit" in the field (Maude, 1980).

Is this also the case with dicarboximide resistant strains? Could there be a correlation between phenotype and dicarboximide resistance? Davis and Dennis (1982) found that high level dicarboximide resistant strains produced fewer conidia than their susceptible counterparts, both on plant tissue and on laboratory media. Pappas et al., (1979) found the same characteristic in dicarboximide resistant strains isolated from strawberries, when grown on malt agar.

Katan (1982) and Beever and Brien (1983), on the other hand, found colony morphology could not be used to distinguish between dicarboximide sensitive and low-level resistant strains. Beever (1983), examined dicarboximide resistant strains of Aspergillus nidulans, and came to the same conclusion with this organism.

An attempt was made to resolve some of these conflicting results by setting up a series of experiments with the following objectives:-

(a) To document the phenotypes of the isolates used in this study, for future experiments.

(b) To investigate whether any relationship exists between dicarboximide sensitive or low-level resistant strains as regards the production of sclerotia or conidia.

(c) To determine the effect on sporulation of peptone in malt agar and thereby obtain an optimal medium for sporulation of B. cinerea for use in subsequent experiments.

3.2 MATERIALS AND METHODS

The sixteen isolates of B. cinerea used for this study, represent a wide spectrum of benzimidazole and dicarboximide resistant and susceptible strains.

Each strain of B. cinerea was transferred from stock onto MA and incubated in the dark until a colony had formed. Plugs, 4mm in diameter, were then taken from the edge of the growing colony and placed face down in the centre of MA plates containing 0, 3, 5, or 7 g/l of peptone. Each concentration was replicated 3 times.

After 2 weeks incubation under near-UV light in a 12 hours on/ 12 hours off regime, the plates were removed and total spore and sclerotial production assessed.

Sporulation was measured by homogenising the whole plate in 100mls of a 0.1% Tween solution, diluting by 1:10 with water if necessary, and counting the conidia in two aliquots with a haemocytometer.

Sclerotia were counted in each dish using an electronic colony counter.

3.3 RESULTS

(a) Colony morphology

The isolates represented a varied range of morphological types (Tables 3.1 - 3.6) but they could be grouped into three categories on the basis of their growth on unamended MA: -

Sclerotial (Sc) (Plate 3.1) - Sparse sporulation (<1.0 x 10^8) and high sclerotial numbers (>50) represented by isolates 7658, 7551, 7550 and 7682.

Sclerotial + sporulating (Sp/Sc) - Moderate sporulation (1.0 - 2.0 x 10^8) with some (10 - 100) sclerotia produced represented by isolates 7668, 7664, 7666 and 7567.

Sporulating (Sp) (Plate 3.2) - Heavy sporulation (>1.0 x 10^8) with few (<10) or no sclerotia represented by 7516, 7517, 7518, 7667, 7554, 7688 and 7685.

For most isolates, all areas of the colony were fairly consistent with regards to sporulation and sclerotia production with the exception of 7569. This isolate showed a high degree of saltation, with clear

sporulating and mycelial sectors (Plate 3.3).

It was thought that these sectors could show differences in resistance to dicarboximides and/or benzimidazoles. To test this theory, mass transfers of spores and mycelium were taken from each "type" of sector (Sp or Sc) and plated onto MA containing 50 g/l carbendazim or 5g/l iprodione and growth rates measured. There was no change in fungicide resistance of any sector from the parent colony (Table 3.7).

(b) Morphology and resistance

The production of sclerotia differed considerably between the strains, and bore no relationship to the absence or presence of either benzimidazole or dicarboximide resistance. The large standard errors of the means at each peptone concentration (Table 3.8) show just how variable this feature was!

For sporulation there was little difference between D^l and D^s at low peptone concentrations but sporulation was less in D^l strains at higher concentrations (Fig 3.1). A t-test between D^s and D^l strains at each concentration did not show significance however, even at peptone 7g/l ($P=0.36$). No significant differences in sporulation were observed between B^r and B^s strains (Fig 3.2).

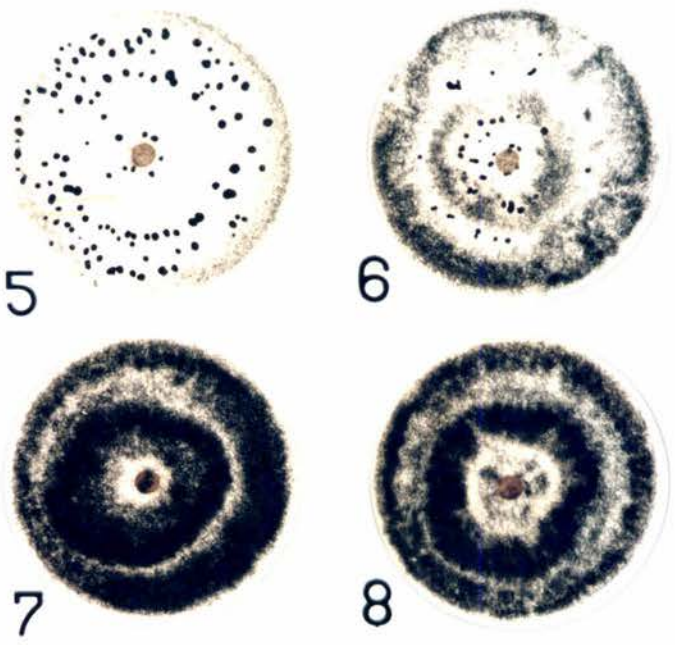
(c) Effect of peptone

The effect of peptone on sporulation and sclerotial production was clear. Sporulation increased significantly between peptone 0 g/l and 3g/l (Figs 3.1 - 3.2) but was much the same between peptone 3 g/l and 5 g/l. Sporulation significantly decreased between 5 g/l and 7 g/l for the D^l isolates ($P = 0.05$) but not for the D^s ones.

Sclerotial production decreased significantly for nearly all isolates from 0 to 3 g/l peptone (Fig 3.3). No statistical analysis was done between different susceptible and resistant strains regarding sclerotial production. As many isolates did not produce sclerotia at all, and extreme variation was present between those that did, any significance test would be invalid.

For the purpose of displaying sclerotial production in graph form, isolate 7682 was omitted. Not only were the numbers of sclerotia very high and mostly immature with this isolate, but they were also contrary to those of all other isolates. Because of the large numbers of sclerotia produced, to include this isolate in the graph would give a misleading picture of the behaviour of the fungus as a whole.

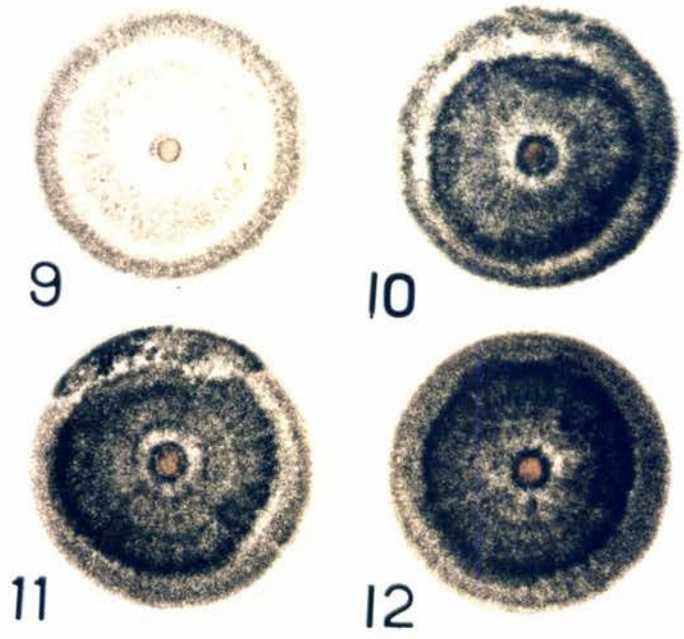
Plate 3.1: Isolate 7551 showing an example of a typical sclerotial (Sc) strain of B. cinerea over 4 peptone levels in MA.



Key

5 = 0 g/l peptone
6 = 3 " "
7 = 5 " "
8 = 6 " "

Plate 3.2: Isolate 7516 showing an example of a typical sporulating (Sp) strain of B. cinerea over 4 peptone levels in MA.



Key

9 = 0 g/l peptone
10 = 3 " "
11 = 5 " "
12 = 6 " "

Plate 3.3: Isolate 7569 on MA showing saltation.

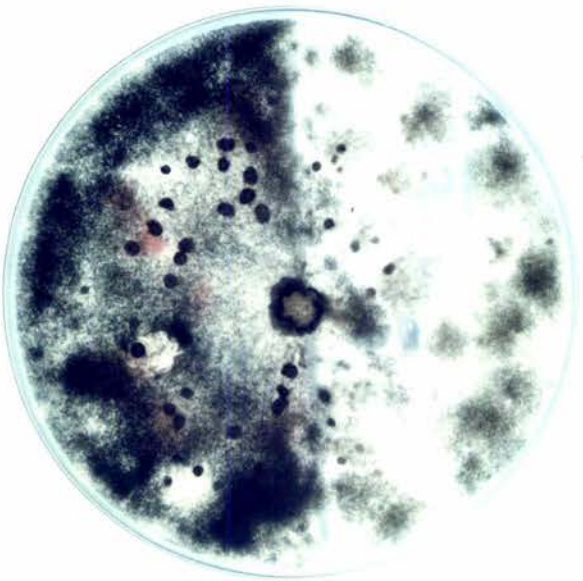


Figure 3.1: Sporulation of Ds and Dl strains of B. cinerea on peptone amended MA

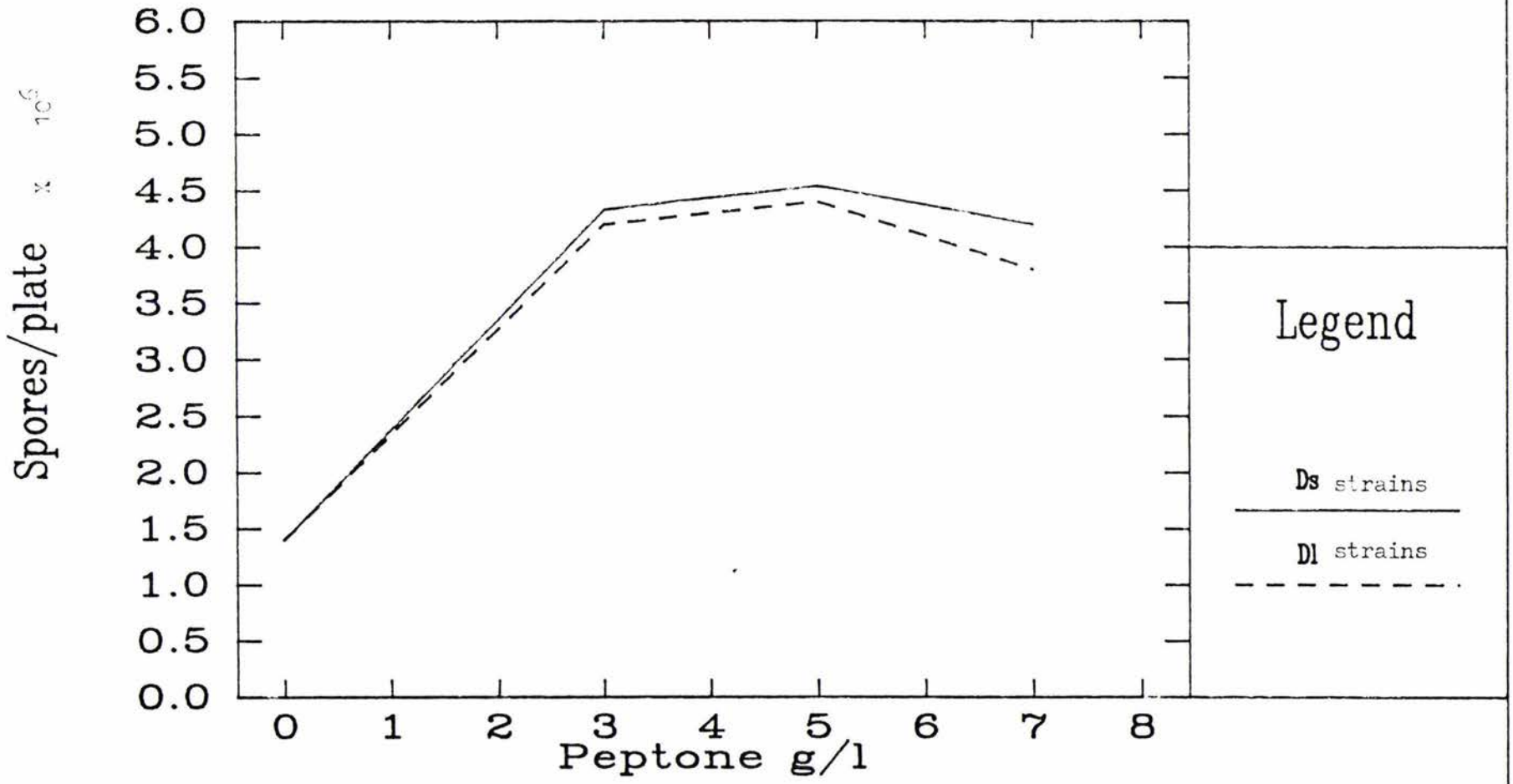


Figure 3.2: Sporulation of Bs and Br strains of <u>B. cinerea</u> on peptone amended MA

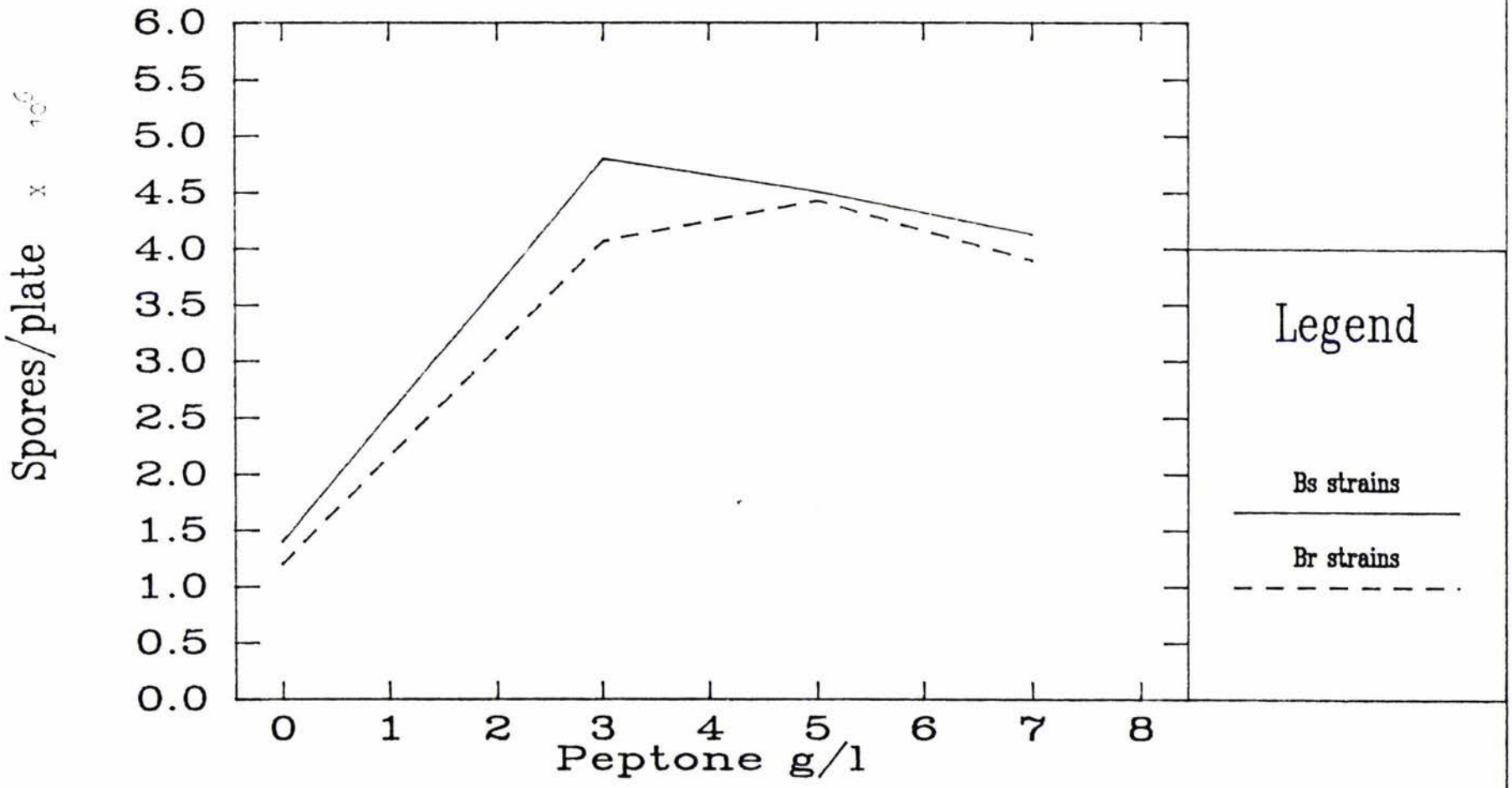


Figure 3.3: Average sclerotial production of B. cinerea on peptone amended MA

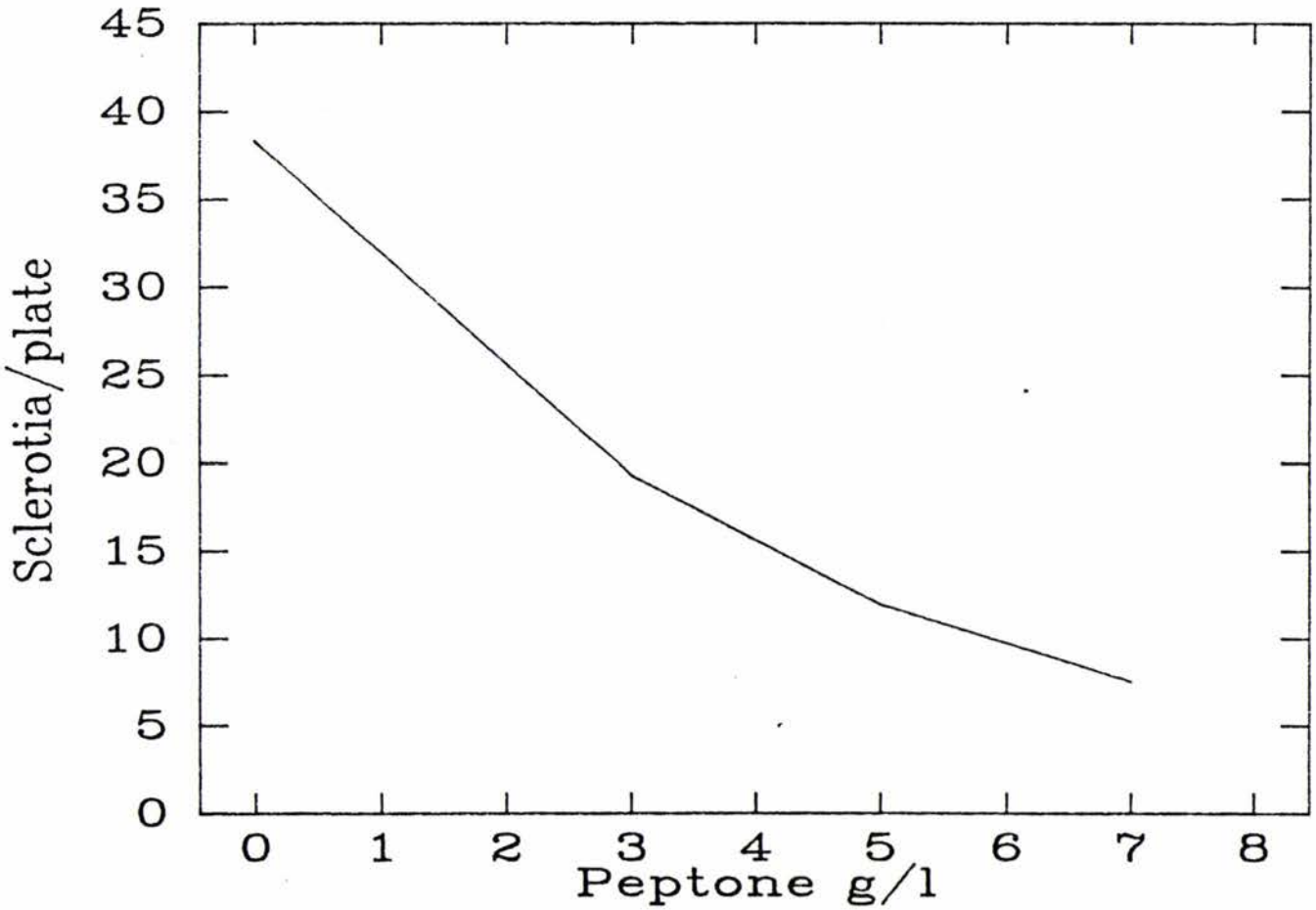


Table 3.1: Sporulation and sclerotial production of strains of *B. cinerea* isolated from grapes, with 4 levels of peptone added to the media.

Isolate	Resistance	peptone added g/l	Conidia x 10^8	Sclerotia produced
7516	D^l B^r	0	1.68 (0.08)	0
"	"	3	5.17 (0.57)	0
"	"	5	4.13 (0.29)	0
"	"	7	4.01 (0.40)	0
7517	D^l B^s	0	2.32 (0.49)	0
"	"	3	5.50 (0.40)	0
"	"	5	6.07 (0.88)	0
"	"	7	6.10 (0.48)	0
7518	D^s B^s	0	2.56 (0.77)	0
"	"	3	6.07 (0.41)	0
"	"	5	7.86 (1.70)	0
"	"	7	7.30 (0.80)	0
7667	D^s B^r	0	1.81 (0.06)	0
"	"	3	6.83 (0.64)	0
"	"	5	5.72 (0.66)	0
"	"	7	5.23 (0.68)	0

Figures in brackets = S.D.

Table 3.2: Sporulation and sclerotial production of strains of *B. cinerea* isolated from cucumber, with 4 levels of peptone added to the media.

Isolate	Resistance	peptone added g/l	Conidia x 10^8	Sclerotia produced
7569	D^l B^r	0	0.31 (0.10)	157 (144)
"	"	3	0.47 (0.49)	112 (8.1)
"	"	5	1.89 (0.66)	52 (23)
"	"	7	1.89 (1.56)	20 (20)
7668	D^l B^s	0	1.60 (0.41)	15 (14)
"	"	3	6.40 (1.95)	18 (25)
"	"	5	5.20 (1.10)	2 (2.1)
"	"	7	3.41 (1.43)	1 (1.1)
7658	D^s B^s	0	0.17 (0.06)	96 (2.1)
"	"	3	1.46 (0.45)	81 (13)
"	"	5	2.13 (0.62)	64 (14)
"	"	7	2.39 (0.06)	77 (20)
7688	D^s B^r	0	2.01 (0.19)	4 (6.4)
"	"	3	6.50 (0.48)	0
"	"	5	6.40 (0.61)	0
"	"	7	4.78 (0.49)	0

Figures in brackets = S.D.

Table 3.3: Sporulation and sclerotial production of strains of *B. cinerea* isolated from tomato, with 4 levels of peptone added to the media.

Isolate	Resistance	peptone added g/l	Conidia x 10^8	Sclerotia produced
7550	D^l B^r	0	0.73 (0.13)	55 (2.5)
"	"	3	4.41 (0.40)	7 (4.5)
"	"	5	5.70 (0.94)	6 (6.6)
"	"	7	3.95 (1.11)	4 (1.7)
7551	D^s B^r	0	0.87 (0.22)	91 (29)
"	"	3	3.31 (0.74)	19 (15)
"	"	5	5.11 (1.12)	4 (3)
"	"	7	4.98 (0.29)	1 (1)
7554	D^s B^s	0	1.62 (0.05)	0
"	"	3	5.15 (0.61)	0
"	"	5	3.58 (0.43)	0
"	"	7	4.33 (0.73)	0

Figures in brackets = S.D.

Table 3.4 Sporulation and sclerotial production of strains of B. cinerea isolated from phaseolus, with 4 levels of peptone added to the media.

Isolate	Resistance	peptone added g/l	Conidia x 10^8	Sclerotia produced
7664	D^1 B^r	0	1.17 (0.49)	45 (47)
"	"	3	1.82 (0.97)	40 (11)
"	"	5	2.24 (1.61)	47 (25)
"	"	7	2.53 (0.15)	8 (2.5)
7666	D^1 B^s	0	1.30 (0.25)	73 (35)
"	"	3	5.80 (0.62)	13 (12)
"	"	5	5.72 (0.42)	4 (2.5)
"	"	7	4.62 (1.15)	2 (2.6)

Figures in brackets = S.D.

Table 3.5: Sporulation and sclerotial production of a D^S B^S strain of B. cinerea isolated from strawberry, with 4 levels of peptone added to the media.

Isolate	peptone added g/l	Conidia x 10^8	Sclerotia produced
7567	0	1.00 (0.23)	37 (34)
"	3	4.47 (0.66)	0
"	5	4.50 (0.94)	0
"	7	4.07 (0.52)	0

Table 3.6: Sporulation and sclerotial production of D^S B^S strains of B. cinerea isolated from kiwifruit, with 4 levels of peptone added to the media.

Isolate	peptone added g/l	Conidia x 10^8	Sclerotia produced
7685	0	1.67 (0.48)	1 (1.7)
"	3	4.69 (0.08)	0
"	5	4.59 (0.13)	0
"	7	4.10 (0.62)	0
7682	0	0.40 (0.15)	138 (33)
"	3	0.96 (0.61)	283 (46)
"	5	1.08 (0.13)	238 (51)
"	7	0.92 (0.25)	301 (136)

Figures in brackets = S.D.

Table 3.7: Growth over 3 days of subcultures of 7569 (D^1B^r) taken from Sc or Sp sectors of two colonies growing on MA with 5 g/l peptone. Growth measured in mm.

	carbendazim		iprodione	
Inoculum	spores	mycelium	spores	mycelium
Origin				
(Sc)				
Plate 1	>45 (0)	>45 (0)	21 (1.9)	17 (2.8)
Plate 2	>45 (0)	>45 (0)	29 (2.8)	24 (4.2)
(Sp)				
Plate 1	>45 (0)	>45 (0)	22 (4.3)	22 (0)
Plate 2	>45 (0)	>45 (0)	24 (6.4)	21 (2.5)

Figures in brackets = S.D.

Table 3.8: Means and standard errors of spore and sclerotial production over differing levels of peptone.

Peptone conc	Isolates	Means	
		Spores	Sclerotia
0	Ds	1.34 (0.15)	28.5 (8.6)
	Dl	1.30 (0.15)	49.3 (14.5)
	Bs	1.40 (0.16)	27.7 (8.0)
	Br	1.23 (0.14)	50.3 (15.0)
3	Ds	4.38 (0.40)	12.5 (5.7)
	Dl	4.22 (0.49)	27.3 (8.5)
	Bs	4.50 (0.39)	14.0 (5.7)
	Br	4.07 (0.50)	25.6 (8.6)
5	Ds	4.55 (0.41)	8.5 (4.5)
	Dl	4.42 (0.42)	16.0 (5.4)
	Bs	4.52 (0.40)	8.8 (4.5)
	Br	4.45 (0.41)	15.6 (5.4)
7	Ds	4.23 (0.34)	9.7 (5.5)
	Dl	3.79 (0.34)	4.9 (2.0)
	Bs	4.14 (0.34)	10.0 (5.4)
	Br	3.91 (0.30)	4.6 (2.1)

Figures in brackets = S.E.

3.4 DISCUSSION

The extreme variation in morphological types noted by other authors was apparent in these experiments. The three categories determined, Sc, Sp, Sc/Sp are broad, but do serve as a useful way of classifying the strains for future work. They correspond roughly to the M, MC, and C types described by Hansen (1938).

Sclerotial production is affected by the C:N ratio, probably due to changes in PH which in turn effect metabolism. Work by a number of authors has shown that, in general, a high C:N ratio in the substrate tends to favour sclerotial production of B. cinerea (Peiris, 1947b; Townsend, 1952; Vanev, 1966). In this study, the numbers of sclerotia produced decreased with increasing levels of peptone. This supports the above findings with the addition of peptone, a nitrogen source, lowering the C:N ratio in the media and decreasing the numbers of sclerotia produced. Isolate 7682 was the exception to the rule. It is possible that some isolates of B. cinerea do not follow the conditions discussed above, just as some isolates can sporulate in the dark, when light is a normal requirement. It is also interesting to note that most of the sclerotia produced remained immature. This is a common phenomenon where large numbers of sclerotia are produced on enriched media (Jarvis, 1977).

There are few reports of factors other than light having any effect on sporulation (Epton and Richmond, 1980). Maas and Powelson (1972) found sporulation of Botrytis convoluta was most profuse on

enriched media containing a carbon or nitrogen source, the same conditions which supported maximum growth. The malt (maltose) in malt agar provides a high carbon source and peptone is used in media as a principle source of organic nitrogen. The fact that sporulation increased when peptone was added suggests the presence of both carbon and nitrogen sources in the ratio's used here (malt 3%/peptone 0.3-0.7%), encouraged maximum production of spores.

It could be argued that the presence of peptone as a nitrogen source in these experiments also supported maximum mycelial growth therefore sporulation was initiated faster and more spores produced. The cultures were grown under near-UV light on a 12 hour on/ 12 hour off cycle however, conditions which are known to be very conducive to spore production (Epton and Richmond, 1980). Furthermore, they were incubated under this regime for 2 weeks before assessment. This should have been long enough for the fungus to reach it's full potential for spore production, as even the slow growing cultures reached the edge of the petri dish after 4 days.

Peptone at 5 g/l appeared to be the optimum concentration for sporulation of B. cinerea and was used in all further experiments where high numbers of conidia were required.

The observation that D^l isolates seem to sporulate less than the D^s isolates with 7 g/l peptone added to the medium is interesting. Sporulation by B. fabae is known to be promoted by high concentrations of organic salts or very high concentrations of

glucose, an observation which Leach and Moore (1966) interpreted in terms of a high osmotic requirement for sporulation (Jarvis, 1977). Bearing in mind that D^1 resistant isolates of B. cinerea are abnormally sensitive to osmotic pressure, high levels of an osmotica such as peptone could reduce sporulation compared with sensitive strains. This phenomena of poorer sporulation of D^1 strains compared to D^S strains on media amended with organic nutrients has also been observed by other authors (Beever, 1983; Grindle, 1984, 1985).

Do these findings have any implications as regards the epidemiology of B. cinerea? If high osmotic potential in the host substrate leads to a reduction in conidiation for D^1 strains (as for mycelial growth) then their competitiveness against the D^S wild type will be reduced. Under a regime of low dicarboximide usage, say 2 or 3 applications a season, D^S strains should predominate. If dicarboximides are used heavily however, a predominance of D^1 strains could result and disease control be lost! Field observations in New Zealand over the past year (Beever pers. comm.) and in other parts of the world (Leroux and Clerjeau, 1985) indicate that this is happening.

When Beever (1983) tested a number of organic and inorganic osmotica, glycerol was the only one which did not reduce growth or sporulation of D^1 strains compared to sensitive ones. He postulated that B. cinerea may act very much like yeasts in their response to high osmotic potential i.e. increasing their intracellular pool of glycerol. High extracellular glycerol therefore, could directly or indirectly increase the intracellular glycerol pool and thus increase

the ability of the fungus to grow at high osmotic potentials.

Glycerol is produced by B. cinerea in infected grapes. There is a possibility that D^1 organisms may sporulate and grow as well as D^s strains on grape berries due to this glycerol production. If this is the case then, these strains would be competitive with the wild type strains in this environment and persist longer. Many of the control problems being experienced with the dicarboximides have occurred in grapes (Beever, pers. comm.; Leroux and Clerjeau, 1985) where there has been heavy dicarboximide usage and it would be worth investigating the role of glycerol production by the pathogen in this situation.

capable of sporulation in the dark. The objective in this experiment was to establish the preponderance of these dark sporulating strains and therefore assess the feasibility of using UVA film as a control practice.

4.2 MATERIALS AND METHODS

(a) Ability of *B. cinerea* isolates to sporulate in the dark

Inoculum was prepared by transferring 14 different isolates from stock culture onto MEA plates. Isolates were selected on the basis of their abundant sporulation and fungicide resistance. After 3 days growth in the dark, 4mm diameter mycelial plugs were taken from the edge of each colony and placed mycelial face down onto the centre of an MEA plate, one plug per plate.

Isolates were divided into batches with three replicates per batch. One batch was exposed to near-UV light on a 12 hours on/ 12 hours off cycle while the other batch was incubated in the dark. Incubation in the dark was achieved by placing the plates in 38 x 22.5cm cardboard filing boxes, which were sealed with masking tape. These filing boxes were then placed in one larger box, which in turn was sealed and placed in a larger box again. As *B. cinerea* is known to have have the capability of responding to light exposures of <1 minute (Tan and Epton 1973) these elaborate procedures were necessary to eliminate any possible stray light.

After 14 days, the plates were assessed for sporulation on the following scale (Plate 4.1).

0 - No sporulation

1 - Sporulation sparse

2 - Moderate sporulation

3 - Heavy sporulation

Three single plates of representative cultures were selected from groups 1,2 and 3. Spore counts were carried out on these cultures to check on the reliability of the visual assessments.

Sporulation was measured in the following way: Whole cultures (including agar) were lifted from petri dishes and gently placed in a wearing blender. Two hundred mls (in the case of groups 2 and 3) or 100mls (in the case of group 1) of a 0.1% solution of Tween 80 was added to the blender and the mixture blended for 1 minute at low speed and 15 seconds at high speed. Two aliquots were taken from this mixture and the spores counted in a haemocytometer.

(b) Effect of type of inoculum on sporulation in the dark.

MEA plates were centre inoculated with four high-sporulating isolates of B. cinerea using either 4mm mycelial plugs containing hyphal tips from the very edge of a 3 day old culture grown in the dark or mass transfer of conidia from 14 day old cultures grown under near-UV

light on a 12 hours on / 12 hours off cycle.

These dishes, replicated 3 times, were incubated for 2 weeks in the dark and then scored for sporulation as in the previous experiment.

4.3 RESULTS

(a) Ability of B. cinerea isolates to sporulate in the dark

Quantitative spore counts showed the visual ratings to be fairly accurate (Table 4.1).

All isolates showed moderate to high sporulation under the light regime but sporulated to a lesser degree in the dark (Table 4.2) (Plate 4.2). The response to darkness however, varied considerably from isolate to isolate ranging from no sporulation at all for 7569, 7550 and 7557 to heavy sporulation which was almost as high as that under light for 7518 and 7667 (Table 4.2). All other isolates fell between these two extremes.

Often sclerotia or aerial mycelium was produced from those isolates which sporulated poorly in the dark although this was not always the case, e.g. 7517 (Table 4.2).

There were some differences observed between the isolates carrying fungicide resistance or susceptibility. Double resistant strains (D^1 B^r) showed only moderate sporulation under near-UV light, and three out of four did not sporulate in the dark. Strains resistant to dicarboximides, but susceptible to benzimidazoles (D^1 B^s) sporulate abundantly under light, but poorly in the dark. Benzimidazole

resistant but dicarboximide sensitive (D^S B^r) strains sporulated abundantly under light but varied considerably in their response to darkness. Finally, wild type strains (D^S B^S) sporulated well in the light, but three of the four isolates also showed considerable sporulation in the dark.

(b) Effect of type of inoculum on sporulation in the dark.

The results showed no difference in amount of sporulation, regardless of whether the initial inoculum was from hyphal tips or from conidia. However, there were some differences in the pattern of development. Colonies which developed from spores showed even sporulation from the point of inoculation outwards but colonies which developed from mycelial tips exhibited a discrete 2mm "ring" of lesser sporulation around the agar plug which could be seen when the plates were held up to a strong light.

Overall sporulation in the dark by isolates 7516, 7517, 7518 and 7667 was the same as in the previous experiment but 7517, 7516 and 7667 formed sclerotia in this experiment.

Plate 4.1: A series of isolates showing scale of sporulation

Key

0 = No sporulation

1 = Sporulation sparse

2 = Moderate sporulation

3 = Heavy sporulation

Plate 4.2: Comparison of two isolates grown under light and dark conditions. (Note heavy sporulation in the dark for 7554).

Table 4.1: Spore counts from representative cultures to ascertain the reliability of visual assessment procedure.

Visual assessment category	Isolate	light regime	Conidia x 10^8
1	7551	dark	0.089
1	7517	dark	0.129
1	7685	dark	0.191
2	7668	dark	1.025
2	7567	dark	0.330
2	7550	light	0.830
3	7685	light	3.416
3	7551	light	3.072
3	7551	light	2.804

Table 4.2: Visual assessment of sporulation of 14 strains of *B. cinerea* on a 0 - 3 scale. Means of three replicates.

Isolate	Fungicide resistance	Light regime	
		Light	Dark
7569	$D_1^l B^r$	2.0	0.0(1)
7550	$D_1^l B^r$	2.0	0.0(1,2)
7557	$D_1^l B^r$	2.0	0.0(1)
7516	$D^l B^r$	2.3	1.3(1,2)
7517	$D_1^l B^s$	3.0	1.3
7668	$D_1^l B^s$	3.0	1.3(1)
7666	$D^l B^s$	3.0	1.3(2)
7551	$D^s B^r$	3.0	0.7(1)
7667	$D^s B^r$	3.0	2.3
7688	$D^s B^r$	2.7	1.3(1)
7567	$D^s B^s$	3.0	2.0(1)
7554	$D^s B^s$	3.0	2.0(2)
7685	$D^s B^s$	3.0	1.0(1,2)
7518	$D^s B^s$	3.0	2.7

(1) = Sclerotia present (2) = Aerial mycelium present

Table 4.3: Visual assessment of sporulation on a 0 - 3 scale of 4 strains of B. cinerea grown from 2 different sources of inoculum. Means of three replicates.

Isolate	Fungicide resistance	Source of inoculum	
		Hyphae	Spores
7516	D^l B^r	1.3(1,2)	1.7(1,2)
7517	D^l B^s	1.0(1)	1.3(1)
7518	D^s B^s	2.7	2.3
7667	D^s B^r	2.0(1)	2.0(1)

(1) = Sclerotia present (2) = Aerial mycelium present

4.4 DISCUSSION

Isolates exposed to near-UV light sporulated more abundantly than those grown in the dark. What was surprising in the first experiment however, was that out of this random sample of 14 isolates collected in the field, 11 were able to sporulate in complete darkness, 4 of them to a high degree!

Most previous research would indicate that strains which sporulate in the dark are fairly rare (Paul, 1929; Hite, 1973; Honda et al 1977). However, Hyre (1972) using a strain of B. cinerea isolated from geranium found sporulation from infected tissue was nearly as profuse in darkness as under cool-white fluorescent lighting. Clearly spore production can occur without photoinduction and the results of experiment (b) show it makes no difference whether the initial inoculum is spores or mycelium.

Are any differences evident between the different resistant strains? At first glance, it seems the double resistant strains (D^1 B^r) are poor sporulators in the dark whereas wild type strains are not. It is difficult to draw conclusions from such a small sample however. Davis and Dennis (1981) observed that resistant strains sporulated less well than susceptible ones although it is clear they were studying high-level resistant strains, not the low-level resistant strains which I have used in this experiment. Most authors agree that although mycelial growth on unamended media is often slower, neither sporulation nor any other morphological feature can be used to

distinguish either low-level resistant dicarboximide strains or benzimidazole resistant strains, when grown on unamended agar (Grindle, 1981; Katan, 1982; Beever and Brien, 1983; Panayotakou and Malathrakis, 1983).

The existence of B. cinerea strains which are capable of sporulation in the dark raises doubts about the feasibility of using special plastic glasshouse coverings as a means of controlling this species. Before growing crops under UVA vinyl, Honda et al (1977) collected a random sample of 200 isolates of B. cinerea from various plant material and found that none of these isolates were able to sporulate in the dark. When sampling from cucumbers which had been grown for a season in the UVA vinyl glasshouses however, they found 1 isolate out of a sample of 15 which did sporulate well in the dark! Was the filtered light selecting for these isolates? The results from these experiments show isolates which sporulate in the dark may be fairly common. Is it not probable therefore, that over a few growing seasons in greenhouses under UVA vinyl, strains which sporulate in the dark will be selected for and thereby reduce the effectiveness of the control procedure? There is no evidence to suggest the ability to sporulate in the dark is connected with any lessening of pathogenicity. Like differences in growth rate and morphology it merely indicates "basic biochemical variation between single spore isolates of B. cinerea" (Epton and Richmond, 1980).

Table 4.2 would suggest D^l isolates may sporulate less in the dark than D^s ones. The sample in this experiment is too small to draw a

definite conclusion but this aspect would be worth investigating.

As a result of the apparent common occurrence of strains able to sporulate in the dark, it was decided not to pursue studies into near-UV light absorbent greenhouse covers any further.

CHAPTER 5

FUNGICIDE RESISTANCE

5.1 INTRODUCTION

The development of resistance to the benzimidazole and dicarboximide compounds in B. cinerea has been covered in some detail in the main introduction to this work. Benzimidazole resistant strains do not differ from susceptible strains in their pathogenicity, sporulation or growth rate and so can compete equally in the field (Maude, 1980).

Dicarboximide resistant strains on the other hand, show different levels of resistance which are correlated with an inability to grow well in conditions of high osmotic potential. High-level resistant strains grow and sporulate poorly compared with susceptible isolates regardless of substrate. Low-level resistant strains as a whole do not differ in sclerotial production and sporulation from the wild type but are still abnormally sensitive to osmotica. Some authors (Katan, 1983; Panayotakou and Malathrakis, 1983) have found they differ in mycelial growth rate on laboratory media, while others have not (Beever, 1983;, Beever and Brien, 1983). In a non-dicarboximide environment therefore, these strains would appear to be less "fit".

From the literature, benzimidazole resistance would appear to be "all or nothing", with strains being either completely resistant or completely susceptible. However, it seems that strains of some fungi, such as Pseudocercosporella herpotrichoides (Long, Pers. comm.) may have low-level resistance to the benzimidazoles. If this is so, it raises the question of whether some other mode of action may be present in this compound along with tubulin affinity, or that certain strains may exist where the binding site for tubulin is controlled by more than one allele.

To investigate the possibility of low-level resistance in B. cinerea, a range of isolates classified as B^s, were grown on agar plates containing low concentrations of carbendazim. At the same time, isolates classified as B^r were grown on plates containing very high concentrations of carbendazim to see if any difference in mycelial growth ensured. These isolates were a mixture of D^1 or D^s to determine whether there was any correlation between dicarboximide and benzimidazole resistance.

Eichhorn and Lorenz (1978) observed that germ tubes from spores of sensitive strains of B. cinerea seeded onto dicarboximide supplemented agar swelled and burst. When Albert (1979) grew sensitive isolates in isotonic media, cell wall synthesis still did not take place, the fungus forming only sphaeroplasts and protoplasts.

Since resistant strains are susceptible to high osmotic pressure they could have a "leaky" cell wall, which would counter the effect of a

dicarboximide fungicide on a substrate with a high osmotic potential. An experiment was carried out to test this possibility.

The aims of these two experiments therefore were to:

(a) Investigate the existence of carbendazim, low-level resistant strains.

(b) Investigate the growth of D^l strains in isotonic media supplemented with dicarboximide, to see if one factor may balance out the other.

(c) To compare and contrast the mycelial growth of D^l and D^s strains.

5.2 MATERIALS AND METHODS

(a) Resistance to carbendazim

Twenty isolates of B. cinerea were used for this experiment, representing a wide spectrum of benzimidazole and dicarboximide resistant and susceptible strains.

Each strain of B. cinerea was isolated from stock onto MA and incubated in the dark until a colony had formed.

Four mm diam. plugs were then taken from the edge of the growing colony and placed face down in the centre of MEA plates containing varying concentrations of carbendazim. Concentrations of 0, 1, 5 and 10 mg/l carbendazim were used for all B^s isolates while 0, 100, 500 and 1000 mg/l were used for B^r ones. All treatments were replicated 3 times per isolate.

The plates were incubated in the dark and mycelial growth measured every 24 hours over 100 hours to determine a growth rate in mm/hr.

(b) Growth on high osmotic media supplemented with iprodione

Four morphologically stable isolates from grape were grown as outlined above on MEA unamended, supplemented with 0.68M NaCl, iprodione at

5 g/l or NaCl and iprodione together at these concentrations. All treatments were replicated 3 times for every isolate.

Colonies were measured every 24 hours over 100 hours to determine a growth rate in mm/hr.

5.3 RESULTS

(a) Resistance to carbendazim

With the exception of the controls (Table 5.1), no growth occurred on any of the carbendazim supplemented plates with the B^s isolates. With the B^r isolates mycelial growth significantly decreased in most strains at concentrations of 500 and 1000 mg/l (Tables 5.2 - 5.3).

Differences were evident between D^s and D^l strains. Overall, D^l strains exhibited significantly slower mycelial growth on unamended agar compared to D^s strains ($P<0.01$). This trend was evident also, at every concentration of carbendazim (Fig 5.1). Also less variation was apparent in the D^s isolates. Regression analysis showed a high linear relationship between carbendazim concentration (mg/l) and mycelial growth (mm/hr) for D^s isolates according to the equation Log(Av. mycelial growth) = -0.295 - 0.000093 x carbendazim conc. Linear regression was not found for the D^l isolates over all the concentrations but a straight line could be fitted from 100 to 1000 mg/l giving the equation Log(Av. mycelial growth) = -0.464 - 0.000122 x carbendazim conc..

As Beever and Brien (1983) found no difference in mycelial growth rate between D^s and D^l strains on unamended agar, a small trial was carried out subsequent to these experiments to examine any difference in growth between four (2 D^s and 2 D^l) isolates grown on the MEA

used in this work and the OMEA used by the authors above. All isolates grew better on the OMEA than on the MEA but the D^1 isolates grew relatively better when compared to the D^S ones on OMEA (Table 5.4).

(b) Growth on high osmotic media supplemented with iprodione

The two D^S isolates did not grow on iprodione amended MEA (Table 5.5). Growth did occur with the D^1 isolates 7517 and 7516 however, being 25% and 20% of that on unamended agar respectively.

Isolate 7518 (D^S) grew significantly faster than the other three on unamended MEA.

On NaCl supplemented plates, the D^1 isolates grew significantly slower than the D^S isolates although the latter only grew 63% - 65% of their growth on unamended MEA (Table 5.5).

No growth occurred on the iprodione/NaCl plates with any of the 4 isolates.

Figure 5.1: Average mycelial growth of Br isolates at different carbendazim concentrations

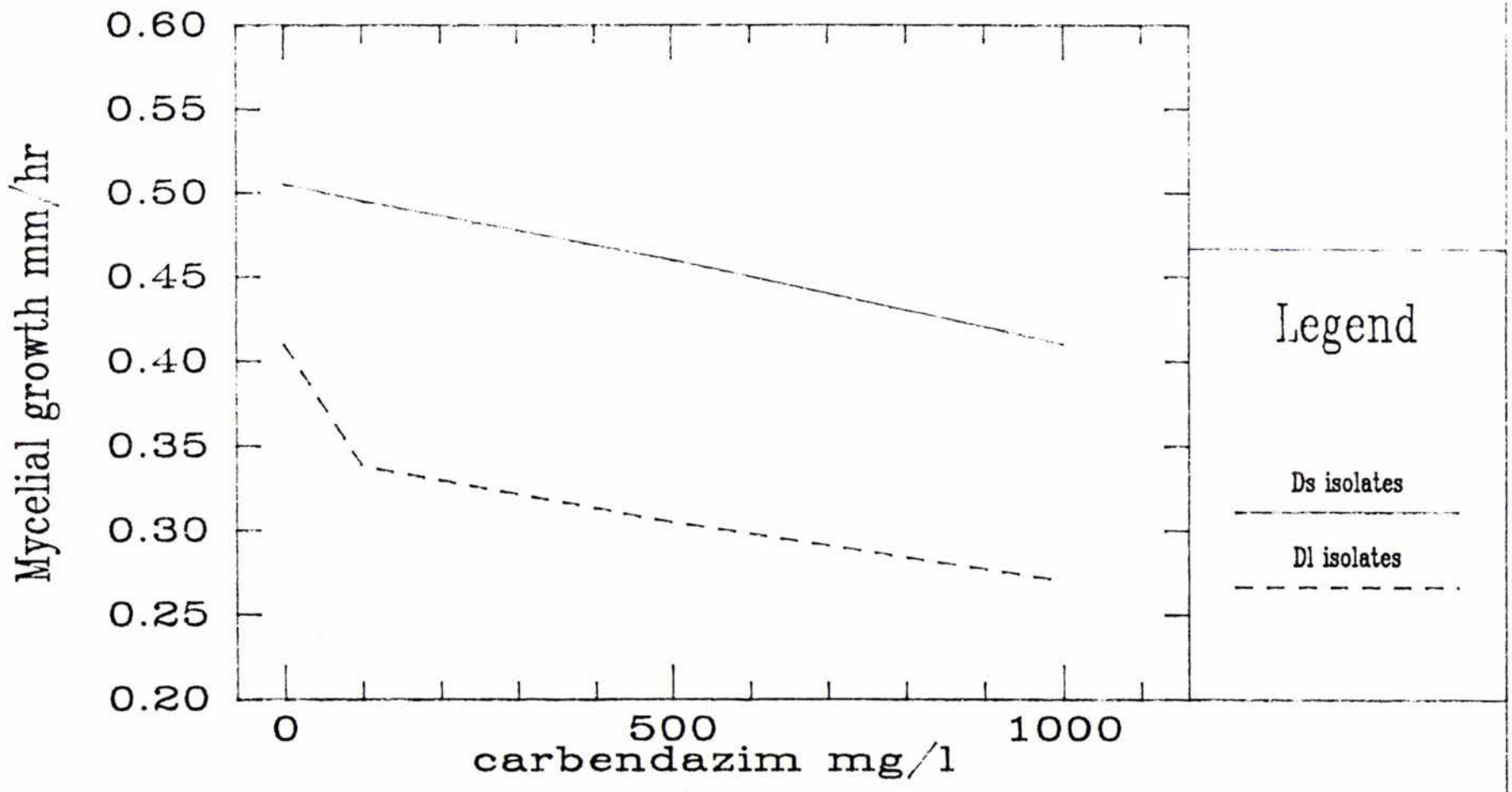


Table 5.1: Mycelial growth in mm/hr of benzimidazole susceptible isolates of B. cinerea on unamended MEA.

Isolate	growth in mm/hr
7517 (B^S D^1)	0.47 (0.01)
7668 "	0.47 (0.04)
7666 "	0.49 (0.0)
7682 (B^S D^S)	0.52 (0.01)
7663 "	0.50 (0.08)
7658 "	0.63 (0.01)
7685 "	0.59 (0.01)
7681 "	0.61 (0.02)
7570 "	0.55 (0.03)
7518 "	0.59 (0.01)
7554 "	0.58 (0.01)
7567 "	0.58 (0.01)

Figures in brackets = S.D

Table 5.2: Mycelial growth in mm/hr of B^r D^1 isolates of B. cinerea on fungicide amended MEA.

Isolate	carbendazim mg/l	growth in mm/hr
7569	0	0.31 a
"	100	0.29 a
"	500	0.22 b
"	1000	0.21 b
7557	0	0.45 a
"	100	0.33 b
"	500	0.31 bc
"	1000	0.30 c
7550	0	0.43 a
"	100	0.32 b
"	500	0.31 b
"	1000	0.23 c
7516	0	0.45 a
"	100	0.42 b
"	500	0.38 c
"	1000	0.35 d

Letters after means = Duncans Multiple range test within each isolate

Table 5.3: Mycelial growth in mm/hr of B^r D^s isolates of B. cinerea on fungicide amended MEA.

Isolate	carbendazim mg/l	growth in mm/hr
7688	0	0.53 a
"	100	0.51 a
"	500	0.50 a
"	1000	0.45 b
7551	0	0.52 a
"	100	0.52 a
"	500	0.47 b
"	1000	0.41 b
7667	0	0.50 a
"	100	0.49 a
"	500	0.45 b
"	1000	0.38 c
7558	0	0.47 a
"	100	0.46 a
"	500	0.43 ab
"	1000	0.39 b

Letters after means = Duncans Multiple Range test within each isolate

Table 5.4: Mycelial growth in mm/hr with 4 isolates on OMEA and MEA

Isolate	Agar	Growth	OMEA as a % of MEA
7517 (D1)	OMEA	0.51 (0.02)	113
"	MEA	0.45 (0.03)	
7516 (D1)	OMEA	0.54 (0)	117
"	MEA	0.46 (0.02)	
7518 (Ds)	OMEA	0.55 (0.02)	110
"	MEA	0.50 (0)	
7667 (Ds)	OMEA	0.33 (0.04)	110
"	MEA	0.30 (0.01)	

Numbers in brackets = S.D.

Table 5.5: Mycelial growth in mm/hr of isolates on fungicide and NaCl amended MEA - Comparison of treatments.

Treatment	Isolate	Resistance	growth in mm/hr	% of untreated
Unamended	7518	D^s B^s	0.55 a	
"	7667	D^s B^r	0.48 b	
"	7517	D^1 B^s	0.47 b	
"	7516	D^1 B^r	0.49 b	
iprodione	7518	D^s B^s	0.0	
"	7667	D^s B^r	0.0	
"	7517	D^1 B^s	0.12 a	25.6
"	7516	D^1 B^r	0.10 a	20.4
NaCl	7518	D^s B^s	0.34 a	61.8
"	7667	D^s B^r	0.31 a	64.5
"	7517	D^1 B^s	0.23 b	48.9
"	7516	D^1 B^r	0.21 b	42.9
ipro/NaCl	7518	D^s B^s	0.0	
	7667	D^s B^r	0.0	
"	7517	D^1 B^s	0.0	
"	7516	D^1 B^r	0.0	

Letters after means = Duncans Multiple Range test within each isolate

5.4 DISCUSSION

(a) Resistance to carbendazim

Of the 12 B^S strains tested here, none grew in agar supplemented with carbendazim. It could be that a rate under 1 mg/l would have shown growth but as the EC_{50} of benomyl is about 0.02 mg/l it is unlikely this would be reflecting low-level resistance but rather very low-level resistance or merely natural variation in growth between sensitive strains.

Whilst none of the isolates tested here were of benzimidazole low-level resistance it is possible that such strains do exist but that large numbers of isolates must be screened to detect them. A similar situation was that where Honda et al (1977) tested 200 isolates of B. cinerea and not one sporulated in the dark yet these type of strains have been found elsewhere.

Mycelial growth of the B^r strains was affected by high concentrations of carbendazim but not even 1000 mg/l stopped growth and all strains were similarly affected. It is not likely that any differences in benzimidazole resistance occurs between these B^r strains although the same comments as above apply. The tubulin of carbendazim resistant fungi do have some affinity to carbendazim (Davidse, 1982) and even though this is very low it is enough to reduce growth at very high concentrations.

The interesting result to come out of this experiment was the difference between D^s and D^l isolates. With all isolates on unamended media and with B^r isolates on carbendazim amended media, mycelial growth of D^l strains was significantly lower than that of D^s strains. This agrees with the findings of Katan (1983) and Panayotakou and Malathrakis (1983) for low-level resistant isolates on potato dextrose agar but is contrary to that found by Beever (1983) and Beever and Brien (1983) on OMEA. Results of the small MEA vrs OMEA trial suggest laboratory media can differ in it's ability to distinguish between resistant and susceptible strains. Although in this trial one D^s isolate grew slower than the D^l ones, the latter grew relatively faster on OMEA than MEA. If this is typical, then the difference in growth rates of D^l and D^s isolates would be less on OMEA than on MEA and this could explain why Beever and Brien (1983) did not find differences in growth rates between the two groups of isolates on unamended media. Clearly, nutrition is a very critical factor in mycelial growth of low-level resistant strains.

(b) Growth on high osmotic media supplemented with iprodione

The results here reflect the slower growth of the D^l isolates compared to D^s isolates on isotonic media as found in other work (Beever, 1983; Beever and Brien, 1983; Grindle, 1983,1984).

That no growth occurred with D^1 isolates on media amended with NaCl and iprodione shows that at these concentrations their effect was additive, not of one nullifying the other.

CHAPTER 6

FUNGICIDES FOR THE CONTROL OF *B. CINEREA*

6.1 INTRODUCTION

Chemical control of *B. cinerea* has been widely practiced for over 30 years. The early fungicides such as the dithiocarbamates were effective as protectants but did not always lead to increased marketable yields (Maude, 1980). A high level of control relied on an integrated approach involving chemical application with a good spraying technique combined with cultural practices such as temperature and humidity control (in the greenhouse) or growth habit and correct siting of plants (outdoors).

With the release of the benzimidazole fungicides, chemical control on it's own became far more effective. Higher yields with higher quality produce, free from grey mould disease came to be expected as the norm.

The development of resistance of *B. cinerea* to the benzimidazole compounds and now also the new dicarboximide compounds which replaced them, are of great concern to horticultural industry. New fungicides are always being sought and an evaluation of two of these new chemicals, chlozolinate and PP192, is the subject of the following experimental work.

Chlozolinate (Serinal 50WP from Shell NZ Ltd.) has recently become available commercially in NZ. It is a dicarboximide fungicide, and in common with others in this group it shows high activity against the Sclerotiniaceae and has been the subject of past studies under the common name dichlozolinate (Hartill, Tompkins and Kleinsman 1983, Katan 1982).

The other fungicide, PP192 is described by the manufacturer as a broad spectrum, protectant fungicide. It belongs to the new pyridinamine group of fungicides and has shown good activity against many fungi including Botrytis, Sclerotinia, Venturia and Alternaria sp..

The objectives of the following experiments were to evaluate chlozolinate and PP192 and compare them with two established dicarboximide fungicides, iprodione and procymidone.

6.2 MATERIALS AND METHODS

(a) EC_{50} determinations

The EC_{50}'s of PP192 and chlozolinate were determined on fungicide amended Oxoid malt extract agar (OMEA) for 11 D^S and 7 D^1 *B. cinerea* strains. Final concentrations of fungicide were : 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, and 100.0 ug/ml.

Plugs 4mm diameter were taken from the margins of actively growing colonies of *B. cinerea* on malt agar, and inverted onto the edge of fungicide-amended plates. The plates were incubated in the dark for 48 hours and mycelial growth measured.

Each isolate was tested using a minimum of 3 replications per treatment and the mean growth over 24 hours was used to determine EC_{50} values by regression analysis.

(b) Protectant Activity

A total of 18 *B. cinerea* isolates (6 D^1 and 12 D^S) were tested. The method used was a modification of that reported by Hoskbergen and Beever (1984).

Dwarf beans at the 4-6 leaf stage were sprayed to runoff with aqueous suspensions (no surfactants added) of PP192 or chlozolinate at concentrations of 0.065 mg/ml or 0.125 mg/ml and left to dry for 24 hours. These rates are equivalent to field concentrations of 0.065 and 0.125 Kg/ha respectivly if the crop is sprayed to runoff.

The primary leaves of each plant were then detached and placed on supports in a small humid chamber (a small clear perspex sandwich box - Plate 6.1) so that the petioles of each leaf were in contact with the wet tissue paper at the bottom. Two half primary leaves on each of 3 plants were inoculated with five 4mm MEA plugs of an isolate to give three replications of 10 sites per plant per isolate. The chambers were sealed, and incubated at 22^{o} C for 3 days under near-UV light on a 12 hours on/off diurnal cycle.

Some tests were also made using spore suspensions as inoculum. As spore suspensions applied to unwounded, untreated leaves have been found to give irregular infections by other researchers (Hoksbergen and Beever, 1984) leaves in this experiment were wounded with a fine needle immediately prior to fungicide application. After 24 hours a 5ul drop of nutrient solution containing 10^{4} conidia was placed on each wound as described by Hoksbergen and Beever (1984).

The experiment was assessed after 3 days by scoring the number of successful infections at each inoculation site.

(c) Systemic activity

Two isolates of B. cinerea were used: one double susceptible (D^S B^S) and one benzimidazole resistant but dicarboximide sensitive (D^S B^r).

Dwarf Beans were grown as described above. An aqueous suspension of 0.5 mg/ml of each fungicide was prepared and added to the soil at the rate of 50ml per pot. A period of three days was allowed for any systemic uptake and translocation to the foliage and then the two primary leaves were detached from each plant. They were placed in humid chambers, inoculated with mycelial plugs, and incubated as for the protectant experiments.

Replication was the same as in the previous experiment but assessment was based on measurement of lesion size since there were small but detectable infections at most inoculation sites. Two measurements of radial growth were made at right angles to each other for each lesion.

(d) Translaminar activity

Dwarf Beans were grown as above. Aqueous suspensions of fungicides at 0.5 mg/ml were then applied to runoff to the lower surface only of the primary leaves using a small artist's paint brush. These were then

left undisturbed in order for the fungicide to dry, and thus prevent runoff or creep to the top surface. After three days the primary leaves were detached, inoculated, incubated and assessed as in the systemic experiment. The same two strains of B. cinerea were used and replication was the same as in the protectant experiment.

(e) Eradicant activity

Dwarf beans were grown as above and the primary leaves inoculated while still attached to the plant. MEA mycelial plugs of a D^S B^S isolate of B. cinerea were placed at eight points on the top surface of each primary leaf. The inoculated plants were placed in a moist chamber for 2 days at 22° C under a 12 hour natural light cycle. The radius of each lesion was then measured (mean of 2 measurements at right angles), and the plants were sprayed to runoff with aqueous suspensions of fungicides at rates of 0.5 mg/ml (2 plants per fungicide). They were returned to the humid chamber for a further 4 days, the lesions were remeasured and the increase in lesion radius was calculated.

Plate 6.1: Detached inoculated bean leaves in humid chamber before incubation.

6.3 RESULTS

(a) EC_{50} determinations

The EC_{50} values for chlozolinate fell into 2 major groups based on susceptibility or low-level resistance to dicarboximides (Figs 6.1a and 6.2). The mean EC_{50} value for D^S isolates was 1.0 ug/ml while that for D^1 isolates was 13.7 ug/ml. These results clearly show the 'family' relationship of chlozolinate to the other dicarboximide fungicides.

The EC_{50} values for PP192 show no correlation with dicarboximide resistance (Fig 6.2). Many of the isolates were also benzimidazole resistant (B^r) and there was no correlation of either fungicide with this resistance.

PP192 exhibited very high activity in malt agar with a mean EC_{50} value of 0.030 ug/ml (Fig 6.1b). Mycelial growth was very irregular at higher concentrations with microscopic examination revealing hyphae which was short, profusely branched and very tightly packed (Plate 6.2 - 6.3). Germ tubes from spores seeded onto OMEA amended with 25 ug/ml PP192 were highly distorted (Plate 6.4).

(b) Protectant Activity

Dicarboximide resistant isolates were unaffected by chlozolinate at any concentration (Fig 6.3). However, D^S isolates caused fewer infections at the higher concentrations of this fungicide.

Protectant activity of PP192 increased at the higher concentrations, with no difference detected between D^S and D^1 isolates.

A follow-up experiment examined the level of control of D^1 isolates gained by using a range of chlozolinate concentrations up to twice the field recommendation. There was no control, at even twice the field rate although the sensitive isolate was completely controlled at concentrations of 0.25 mg/ml and above.

When spores were used as inoculum the results were more variable than those obtained for agar plugs (Table 6.1a). The D^S isolate was controlled at 0.063 mg/ml but complete control of D^1 isolates was not obtained, even at 1.0 mg/ml. There was however, a progressive reduction in infections at fungicide rates greater than 0.125 mg/ml.

In similar tests using PP192 some control of mycelial inoculum was obtained at the lowest concentration used leading to complete control of both isolates at 0.5 mg/ml (Table 6.1b). There was complete control of both isolates, even at the lowest fungicide rate, when spore suspensions were placed on wounded leaves.

(c) Systemic activity

Toxophores (Edgington, 1981) of the dicarboximide fungicides, and chlozolinate in particular, were taken up by the roots and translocated upwards to the leaves in sufficient quantities to substantially reduce development of infections (Table 6.2). PP192 had no effect and benomyl, a known systemic, substantially slowed lesion development of the B^s isolate but not of the B^r one.

(d) Translaminar activity

The translaminar experiment (Table 6.3) gave a similar result to the systemic one. Chlozolinate showed evidence of strong translaminar movement, iprodione and procymidone showed some activity and PP192 exhibited none. Benomyl, as before, arrested the development of the B^r isolate, but not the B^s one.

(e) Eradicant activity

Lesion development was inhibited and lesions dried up on plants treated with benomyl, chlozolinate and procymidone (Table 6.4). Iprodione checked the spread of the lesion but PP192 had no effect.

Plate 6.2: Colony margin of B. cinerea on OMEA plate supplemented with 25 ug/ml PP192 after 7 days growth (mag. x 100). (Bottom photo shows growth on unamended agar).

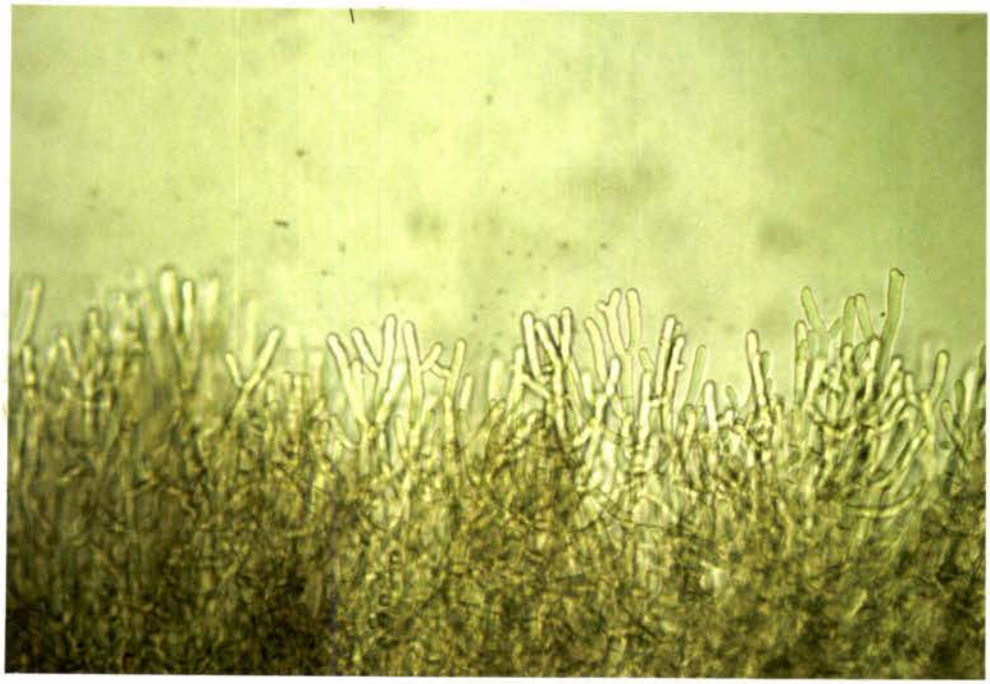


Plate 6.3: Colony margin of B. cinerea on unamended OMEA plates after 7 days growth (mag. x 100).

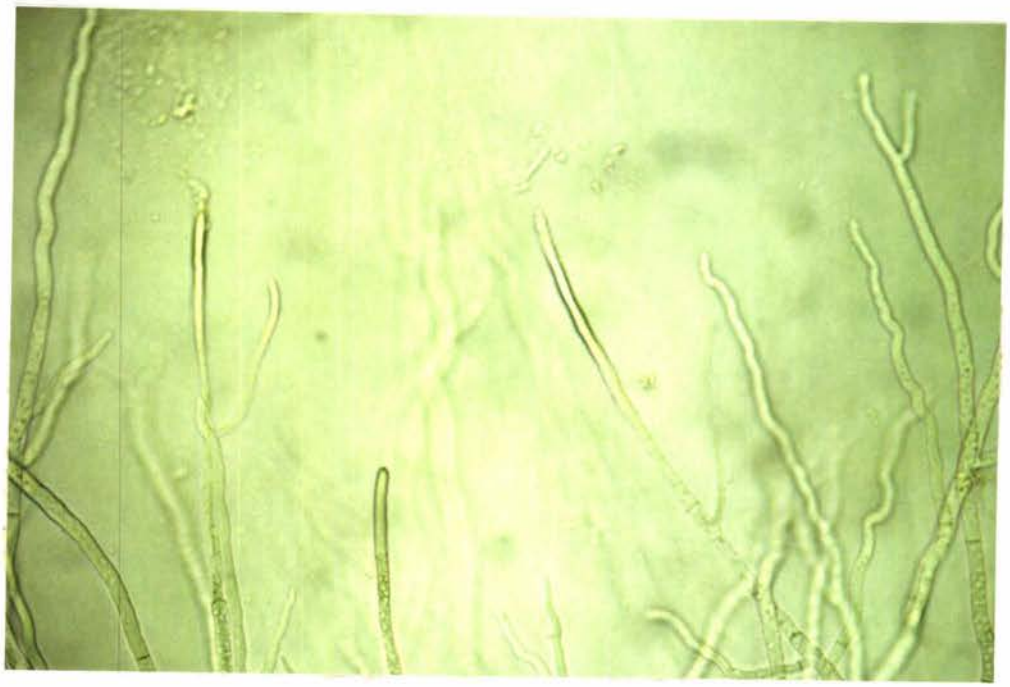


Plate 6.4: Germ tubes from spores after 7 days on OMEA supplemented with 25 ug/ml of PP192 (mag x 400).

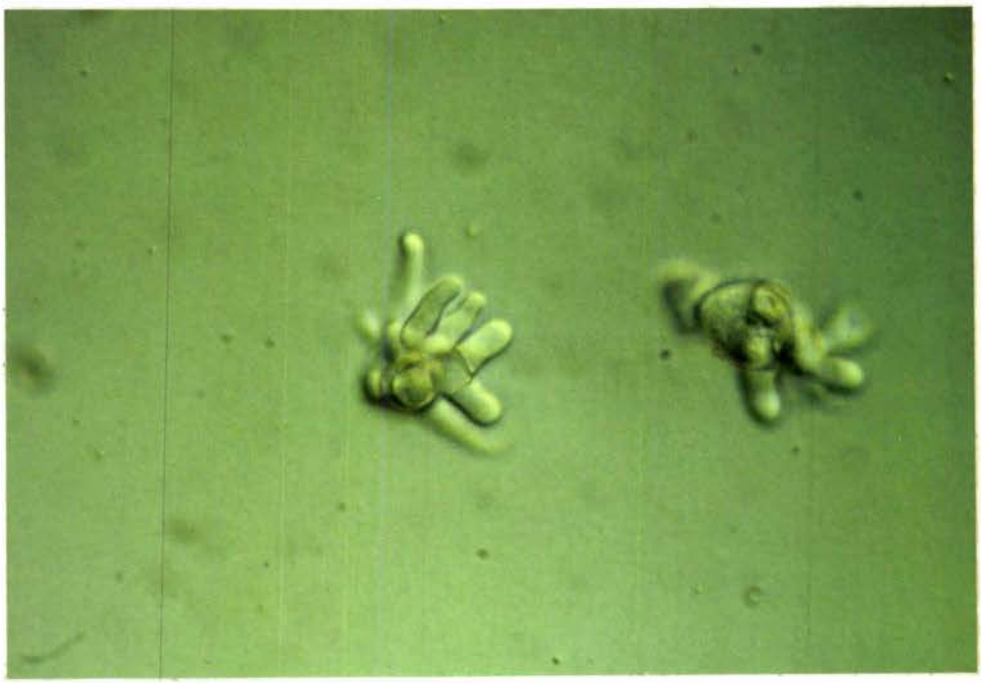


Figure 6.1a: Dose response curve for mycelial growth in chlozolinate amended OMEA

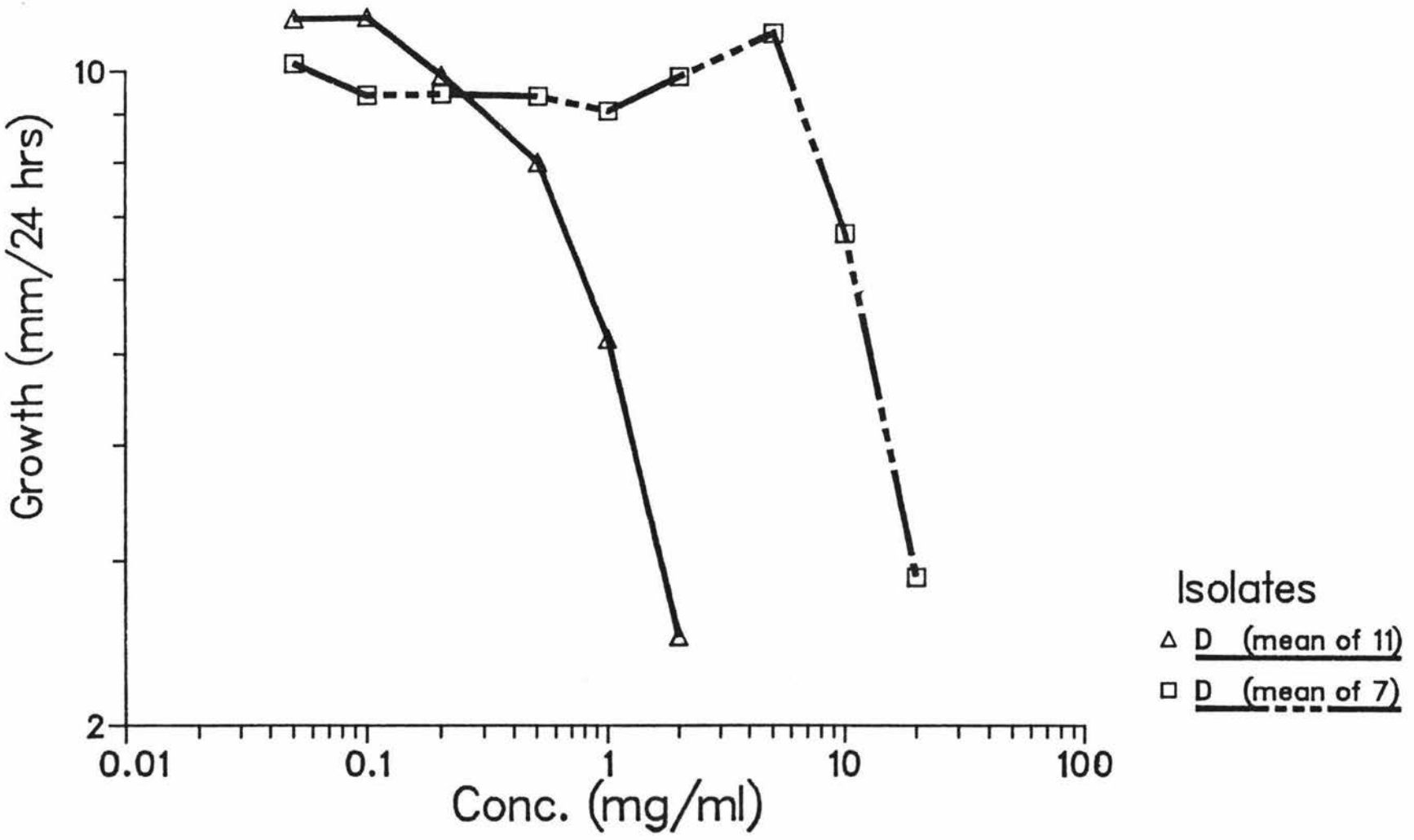
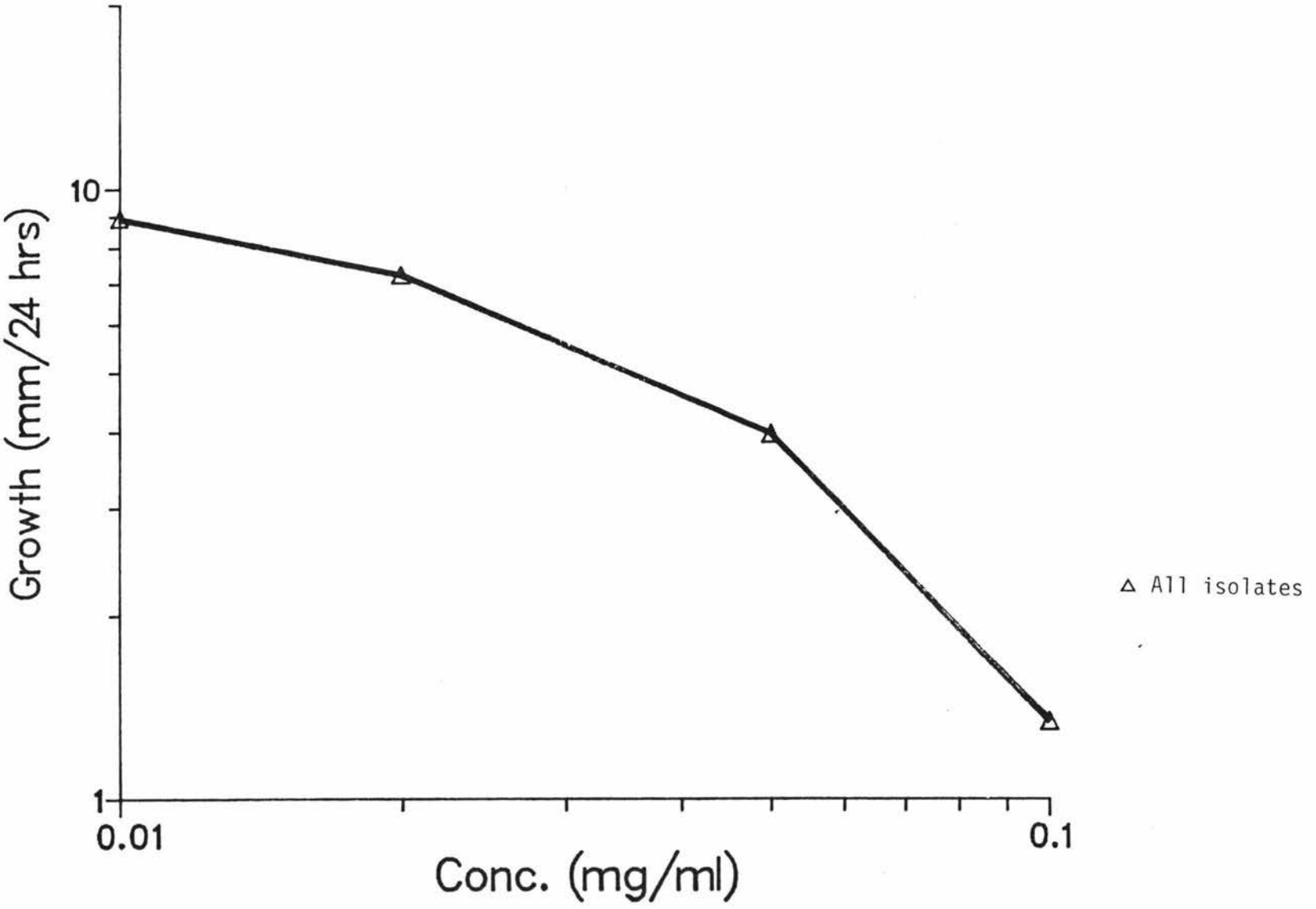


Figure 6.1b: Dose response curve for mycelial growth in PP192 amended OMEA



KEY TO FIGURE 6.2

D^S isolates:	1 = 7554	2 = 7667	3 = 7558
	4 = 7663	5 = 7570	6 = 7551
	7 = 7518	8 = 7681	9 = 7685
	10 = 7682	11 = 7688	
D^1 isolates:	12 = 7550	13 = 7666	14 = 7557
	15 = 7517	16 = 7516	17 = 7665
	18 = 7668		

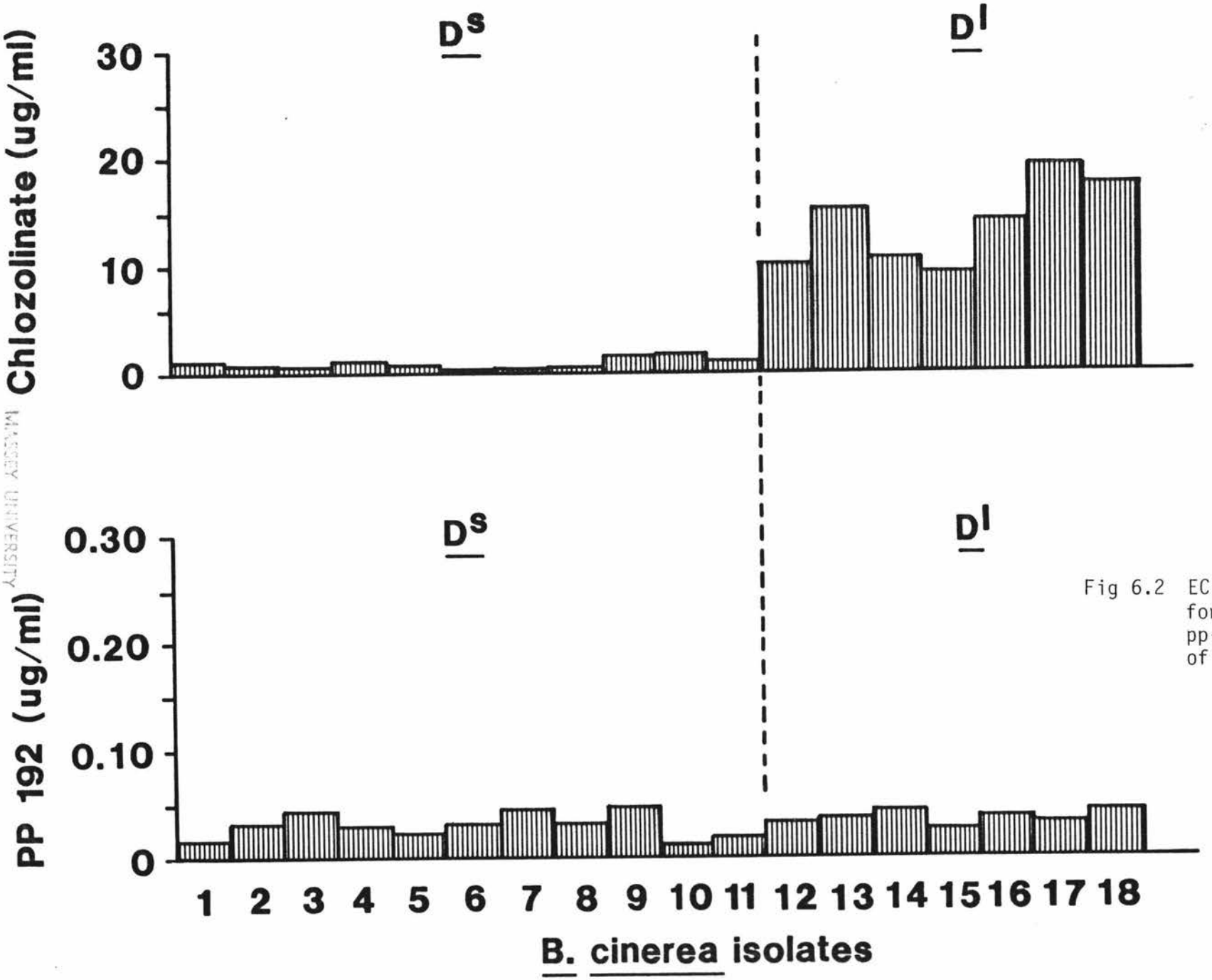
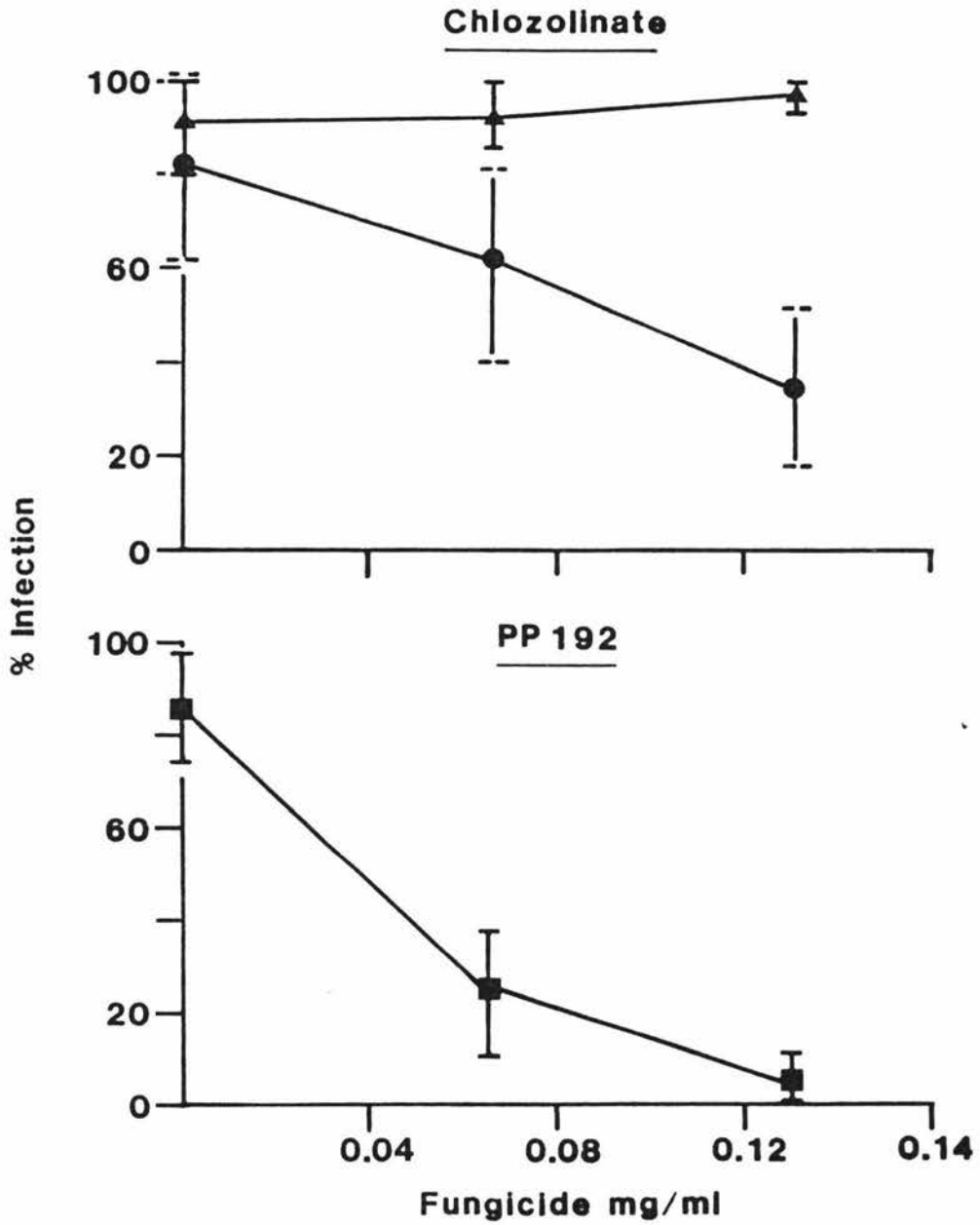


Fig 6.2 EC_{50} values in ug/ml for chlozolinate and pp_{192} over 18 isolates of B. cinerea

Fig. 6.3 Graph showing % infection of dwarf bean leaves 3 days after inoculation with agar plugs of B. cinerea on to fungicide treated surface.



Symbols: ▲- dicarboximide resistant isolates (mean of 6);
● - dicarboximide sensistive isolates (mean of 12);
■ all isolates (mean of 18)
95% confidence intervals determined by two tailed T-test.

Table 6.1a Protectant activity of chlozolinate against dicarboximide resistant isolates.

Concentration (mg/ml)	% Infection spore suspensions on wounded dwarf bean leaves				
	dicarboximide sensitive isolates	dicarboximide resistant isolates			
	7681	7517	7516	7668	7557
0	87(±23)	60(±17)	80(±10)	80(±0)	97(±6)
0.063	0	87(±10)	93(±11)	83(±15)	93(±6)
0.125	0	60(±20)	73(±15)	84(±6)	93(±6)
0.250	0	73(±25)	33(±21)	30(±10)	50(±20)
0.500	0	53(±35)	57(±15)	53(±21)	47(±15)
1.000	0	30(±20)	35(±5)	20(±17)	30(±20)

Values in brackets = SD of 3 replicate means

Table 6.1b: Protectant activity of PP192 on dwarf bean leaves.

Concentration (mg/ml)	% Infection			
	Agar plugs (unwounded)		Spore Suspensions (wounded)	
	Isolate 7517	Isolate 7681	Isolate 7517	Isolate 7681
0	100	86(±11)	93(±12)	80(±17)
0.063	20(±10)	23(±32)	0	0
0.125	7(±11)		0	0
0.25	3(±6)	0	0	0
0.5	0	0	0	0
1.0	0	0	0	0

Table 6.2 Systemic activity of fungicides applied as a soil drench to pots of dwarf beans 3 days before inoculation of leaves with agar plugs.

Fungicide	Lesion radius in mm after 3 days	
	Isolate 7667(D^SB^r)	Isolate 7554(D^SB^S)
untreated	4.6 a*	7.6 a*
benomyl	3.2 b	1.6 d
chlozolinate	0.2 d	1.3 d
iprodione	1.5 c	4.6 bc
PP192	4.7 a	6.8 a
procymidone	1.6 c	3.0 cd

* Duncan's Multiple Range Test at 5%

Values are means of 30 infection points.

Table 6.3 Translaminar activity of fungicides applied to undersides of dwarf bean leaves 2 days before inoculation on the top surface with agar plugs.

Fungicide	Lesion radius in mm after 3 days	
	Isolate 7667(D^SB^r)	Isolate 7554(D^SB^S)
untreated	6.0 a*	7.6 a*
benomyl	5.7 a	0.2 c
chlozolinate	2.6 cd	0.9 c
iprodione	3.6 c	5.0 ab
PP192	5.3 ab	6.0 ab
procymidone	4.1 bc	4.3 b

* Duncan's Multiple Range Test at 5%

Values are means of 30 infection points.

Table 6.4 Eradicant activity of fungicides on established infections of isolate 7663(D^SB^S) on dwarf bean leaves. All fungicides were used at a rate of 0.5 mg/ml.

Fungicide	Lesion radius increase in mm four days after treatment
untreated	15.4 ab*
benomyl	2.9 d
chlozolinate	1.4 d
iprodione	7.0 c
PP192	12.5 b
procymidone	2.2 d

* Duncan's Multiple Range Test at 5%

Values are means of 32 infection points.

6.4 DISCUSSION

The modified technique of Hoksbergen and Beever (1984) appeared to work well for distinguishing protectant, systemic and translaminar activity of the fungicides. One criticism of the method for detection of systemic and translaminar activity is that lesion development is not just a reflection of chemical movement in the plant but of the interaction between movement and effectiveness. However a comparison with EC_{50} values or protectant activity shows whether ineffectiveness is due to lack of fungitoxic action or lack of systemic movement. This is clearly seen in the results with PP192 where the 'in vitro' EC_{50} was very low and it was an extremely effective protectant against spores and mycelium but the fungicide exhibited no systemic, translaminar or eradicant activity. Since it was also effective against dicarboximide and benzimidazole resistant isolates of B. cinerea and is recorded as having anti-sporulant activity on this organism (Anonymous, 1984), PP192 would appear to have considerable potential as a useful commercial fungicide although strict adherence to a protectant type schedule would probably be required.

Chlozolinate had high protectant activity against dicarboximide susceptible isolates. Unfortunately, the control of low-level resistant strains, whether from mycelium and spore inoculum, was poor even at field rates and above. In this respect it differs from iprodione which has been found to provide adequate control from infection by spores, but not agar plugs, at field rates (Hoksbergen

and Beever 1984). The lack of effectiveness against low-level resistant strains may be a great handicap in the field, where such strains are becoming common (Beever Pers. comm.). This may limit chlozolinate's usefulness despite the strong systemic, translaminar and eradicant activity shown against sensitive strains. Like other dicarboximides, it's use should be limited to periods of high disease pressure preferably mixed or rotated with a suitable protectant fungicide of a different group such as dichlofluanid.

Iprodione and procymidone were first marketed by the chemical industry as protectant fungicides only. Some workers, however, have noted some systemic activity of these materials in plant tissue (Hisada et al, 1977; Katan 1982). There was strong evidence of systemic activity in all the dicarboximide fungicides tested in this work.

CHAPTER 7

THE EFFECT OF SUB-LETHAL CONCENTRATIONS OF DICARBOXIMIDE FUNGICIDES ON THE SPORULATION OF *B. CINEREA*

7.1 INTRODUCTION

In previous experiments it was observed that agar plugs containing isolates of *B. cinerea* sporulated profusely when placed for three days on bean leaves treated with a dicarboximide fungicide.

Dennis and Davis (1979) observed a stimulation of mycelial growth when resistant strains were grown in agar plates supplemented with sub-lethal doses of iprodione and vinclozolin. Also, Beever (1983) observed strains of *Aspergillus nidulans* which were almost aconidial on basal medium, but conidiated abundantly on medium amended with iprodione. Were these fungicides stimulating sporulation at sub-lethal concentrations? A series of experiments was devised to investigate this phenomenon.

7.2 MATERIALS AND METHODS

Beans were grown in pots (3 plants per pot) until the primary leaves were fully expanded. The plants were then sprayed with fungicide suspensions and left to dry.

Fungicide concentrations sprayed onto leaves were 0, 0.125, 0.25 and 0.5 g/l using three plants per treatment. Each treatment contained 30 replicate plugs per isolate.

After 24 hours, the leaves were detached and, using a cork borer and cardboard as a base, ten 8mm leaf discs were cut out of each leaf and mounted in a petri dish on water agar. Each plant therefore provided 20 leaf discs. Half of these discs (5 from one leaf and 5 from the other) were inoculated with 5mm MEA plugs containing B. cinerea isolates 7518 (D^s) or 7516 (D^l) and the other half with plugs containing 7667 (D^s) or 7517 (D^l).

After inoculation, the plates were stored at 20° C under a 12 hour on/ 12 hour off near-UV light cycle.

After a period of 4 days the treatments were assessed. Ten inoculation plugs were bulked into a MaCartney bottle with 3mls of an 0.1% Tween solution together with one drop of formalin. Over the next two days, spores from each 10 plug sample were counted by shaking vigorously for 15 seconds in a mechanical shaker and then transferring two aliquots to a haemocytometer.

7.3 RESULTS

The D^S isolates responded in one of two ways to fungicide on the treated leaves. Either sporulation from the plugs was very sparse or absent, or the plugs sporulated profusely. In plugs which showed no sporulation, the fungus was presumed dead. No growth was observed when these plugs were transferred to unamended MEA and incubated. In order to achieve a direct comparison between sporulation from the plugs on untreated leaves versus sporulation from plugs on treated leaves, all spore counts have been expressed as spores per plug, (Table 7.1) to avoid bias from those plugs which did not sporulate because the fungus was killed.

The results show that with D^S isolates, where the fungus was not killed, sporulation increased significantly following exposured to fungicide. The conidia produced at these sub-lethal levels were viable and germinated on MEA. The 0.25 g/l rate of vinclozolin resulted in a reduction of sporulation but this was not evident on iprodione at the equivalent concentration.

Sporulation of the D^1 isolates did not increase significantly when exposed to fungicide, although the mean spore count was higher than the untreated.

Table 7.1: Average number of spores/plug x 10^6 from MEA plugs which showed sporulation on treated bean leaves.

Treatment	Isolates			
	7682 (D^s)	7685 (D^s)	7516 (D^1)	7517(D^1)
Untreated	0.08 c	0.12 b	0.09 a	0.13 a
vinclo 0.062	0.28 a	0.25 ab	0.11 a	0.12 a
vinclo 0.125	0.28 a	0.33 a	0.13 a	0.11 a
vinclo 0.250	0.13 bc	0.27 a	0.12 a	0.12 a
iprodi 0.062	0.25 ab	0.27 a	0.13 a	0.15 a
iprodi 0.125	0.22 ab	0.21 ab	0.15 a	0.11 a
iprodi 0.250	0.28 a	0.30 a	0.16 a	0.12 a

Letters beside means = Duncan's multiple range test at 5% significance

7.4 DISCUSSION

Sub-lethal levels of the dicarboximide fungicides stimulated the sporulation of D^s strains of B. cinerea in these experiments. Preliminary trials using higher fungicide concentrations from 0.5 upwards showed little or no sporulation from the plugs due to the death of these strains. It would appear that the rate which produces the maximum numbers of spores lies between 0.062 and 0.125 for vinclozolin and 0.062 and 0.25 for iprodione. This may reflect the higher activity of vinclozolin since stimulation of sporulation was diminished at 0.25 g/l with this chemical but not with iprodione.

The D^1 strains were unaffected by these low levels of fungicide, although sporulation in 7516 did seem to increase with fungicide concentration.

What are the practical implications of these findings? If the fungus in diseased tissue exhibits the same behaviour as in agar plugs, it is possible disease pressure may increase if the fungicides are applied carelessly or at less than field rates. In the epidemiology of B. cinerea conidia play a major role and any factor which leads to their increase can only assist infection and spread. Correct rates and careful application would assume more than usual importance. Equally, the effect of chemical breakdown with time could lead to lengthy periods when sub-lethal doses stimulate sporulation on surviving disease lesions.

The experiments above are only preliminary, and more work outside the scope of this study waits to be done in this particular aspect of the dicarboximides fungicides. Many questions can be asked. For example, do resistant strains exhibit the same behaviour at higher concentrations as the D^S strains do with the concentrations tested here? While spores produced at sub-lethal fungicide concentrations are viable, do the same percentage germinate successfully compared to conidia produced from untreated tissue? Does the fungus exhibit similar sporulation in tissue as in agar plugs when exposed to sub-lethal doses of fungicide? Do other fungicides give the same effect? Increased sporulation may be simply a response of the fungus to stress. Many simple organisms switch from vegetative to reproductive growth when the environment becomes unfavourable.

Experiments devised to answer these questions may lead us closer to understanding the mode of action of these fungicides, and the nature of dicarboximide resistance.

CHAPTER 8

GENERAL DISCUSSION

The major elements of this study involved an investigation of the various facets of fungicide resistant strains of B. cinerea and the fungicides themselves. The subject is a large and complex one, and therefore exploration in limited areas only was possible within the scope of this work.

The morphological characteristics of fungicide resistant strains used in this study demonstrated the high variability present within this species. This supports the findings of Hansen and Smith (1932) and Paul (1929) with regard to phenotypes and Beever (1983) and Grindle (1984, 1985) regarding the poorer sporulation of D^1 strains on media amended with rich organic nutrients. The stimulatory effect of peptone on spore production and inhibitory effect on sclerotial production supports other work which has examined nutrition of Botrytis cinerea and other Botrytis sp. (Mass and Powelson, 1972; Peiris, 1947b; Towsend, 1952; Vanev, 1966).

That the study found no correlation between low-level dicarboximide resistance or benzimidazole resistance on the basis of sporulation or sclerotial production is in agreement with the work of Katan (1982), Grindle, (1981), Panayotakou and Malathrakis (1983) and

Beever and Brien (1983). Significant differences in average mycelial growth rate between D^S and D^1 strains on MEA were recorded.

A correlation was also found between dicarboximide resistance and slow growth in media with high osmotic potential, a characteristic which has been noted by other researchers (Beever, 1983; Beever and Brien, 1983; Grindle; 1983, 1984).

Studies of fungicide activity showed that dicarboximide compounds have significant systemic activity, as was noted by Cayley and Hide (1980) and Hisada et al (1977). The new dicarboximide, chlozolinate, showed high systemic activity and was very effective against D^S strains but not D^1 ones. PP192 had very high protectant activity on all strains but no systemic activity was found. This compound will be a useful addition to the arsenal of fungicides used for B. cinerea control.

The techniques developed for assessing fungicide activity should also be useful for studies involving other plant pathogens and fungicides.

Some interesting results arising from the fungicide studies showed that iprodione and vinclozolin stimulated sporulation of the fungus at sub-lethal levels. This interaction could be important in situations of high disease pressure where sporulation from surviving fungi in moribund tissue may increase after fungicide application.

UVA film as a glasshouse covering would appear, on the surface, to be

an effective control measure as an alternative to, or in combination with, fungicide application (Hite, 1973). This study shows that the ability of B. cinerea to sporulate in complete darkness is perhaps more common than past literature would indicate (Paul, 1929; Hite, 1973; Honda et al, 1977). Such strains would be unaffected by UVA coverings and, as it is improbable that sporulation in the dark is connected with a lessening of pathogenicity (Epton and Richmond, 1980), loss of control would result if these strains constituted a large proportion of the population. Furthermore, it is likely that the UVA would apply selection pressure thereby encouraging these strains, as the results of one study would suggest (Honda et al, 1977). At present, UVA greenhouse film as a control measure would not appear to be feasible unless the indication that D^1 isolates may be less able to sporulate in the dark is confirmed by more extensive trials. In such a case a management programme integrating the use of UVA film and dicarboximide fungicides may be feasible.

Several interesting facets of B. cinerea have emerged out of this work which could be studied further. For example, what biochemical mechanism triggers sporulation in those strains which sporulate in the dark? Are they as competitive as those which require light as has been suggested?

What of dicarboximide resistance? Although no difference was noted in sporulation and conidiation for dicarboximide resistant strains on laboratory media, is this also true for plant tissue? Nutrition plays a role in both these functions and differences may be evident on

different substrates. Glycerol seems to be an osmotica that D^1 strains can tolerate (Beever, 1983). Are D^1 strains as competitive with D^S strains in an environment where glycerol occurs, such as ripe grapes?

Finally, why do the dicarboximide fungicides cause heavy sporulation from colonies they do not kill? Could the answer to this question give a lead into the mode of action of this group?

The understanding of fungicide resistance in B. cinerea is hindered by the variability and poor genetic understanding of the organism (Grindle, 1981). Future work on the genetics of resistance would be best carried out using fungi with an easily producible sexual stage, thus facilitating genetic analysis. Such techniques may reveal the secrets of dicarboximide resistance, and the mode of action of the fungicides themselves.

CHAPTER 9

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ADDENDA

NOTE

These addenda consist of references cited in the text but not recorded in the main bibliography

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