Apothecia of ***Monilinia fructicola*** (Wint.) Honey under controlled conditions.

And so, from hour to hour we ripe and ripe,
And then from hour to hour we rot and rot,
And thereby hangs a tale.

As You Like It : Act II, Scene vii

(William Shakespeare)

EPIDEMIOLOGICAL ASPECTS OF MBC RESISTANCE IN *Monilinia fructicola* (Wint.) Honey AND MECHANISMS OF RESISTANCE

A thesis

submitted in fulfilment

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by

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DEDICATION

This thesis is dedicated

to my mother, **CHANTR** and my father, **SAIYOOD SANOAMUANG**

for their love, wish and encouragement.

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Abstract of a thesis submitted in fulfilment of
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EPIDEMIOLOGICAL ASPECTS OF MBC RESISTANCE IN *Monilinia fructicola* (Wint.) Honey AND MECHANISMS OF RESISTANCE

by N. Sanoamuang

Isolates of *Monilinia fructicola* (Wint.) Honey obtained from stone fruit orchards in Hawkes Bay, North Island and from Californian fruit exported to New Zealand, were tested for resistance to methyl benzimidazole carbamate (MBC). Resistant isolates from the North Island had EC_{50} values of >30,000, and most isolates from the imported fruit had of values approximately 1.5 mg a.i./l carbendazim. Sensitive isolates failed to grow on 1 mg a.i./l carbendazim.

A detached peach shoot system was used in controlled conditions for estimation of values for incubation period, latent period and rate of spore production on flowers (*cv* Glohaven). The same variables and the rate of colonisation of host tissue were measured on fruit (*cv* Fantasia) in controlled conditions. An inoculum density of $1x10^4$ spore/flower or fruit greatly increased fitness *in vivo* compared to an inoculum density of $1x10^2$ spore/flower (fruit). Isolates varied considerably, but there was no consistent relationship between the degrees of resistance and fitness. This was in contrast to earlier studies with dicarboximide resistant strains of *M. fructicola.*

The survival in the field of 10 isolates resistant or sensitive to MBC or dicarboximide fungicides on twig cankers and mummified fruit was compared. The ability to produce conidia on twig cankers inoculated in late spring 1989 was maintained by all sensitive and MBC resistant isolates for at least 1 year. The production of conidia on mummified fruit inoculated in February 1990 decreased after 2-3 months in the field but some conidia were still produced on

all fruit in the following spring. Dicarboximide resistant isolates produced less conidia than either the MBC resistant and the sensitive isolates. The pathogenicity and fitness of all isolates were similar to the original values after survival for 1 year.

A technique was developed to produce apothecia reliably from inoculated peach (*cv* Black Boy) and nectarine (*cv* Fantasia) fruit in controlled conditions in the laboratory. The fruit were inoculated with resistant or sensitive isolates, or combinations, and were incubated for 8 weeks at 25 °C (±1 °C) with 12 hours photoperiod of fluorescent light (Sylvania 2x65 W, daylight) to produce mummified fruit. The fruit were then buried in moist autoclaved peat moss for 10 weeks at 25 °C (±1 °C) in the dark to form stromata. These fruit were then hydrated with running tap-water (total hardness ($CaCO_3$) = 47 g/m^3 and conductivity at 20 °C = 12.7 mS/m) for 72 hours. The hydrated mummified fruit were placed in moist peat moss and were incubated for 13-14 weeks at 8 °C (±0.5 °C) in the dark. At the end of this period, stipe initials were visible. Differentiation of stipe initials into mature apothecia occurred within 15-20 days after transfer to 12 °C (±2 °C) with a 12 hour photoperiod of fluorescent and incandescent light. All isolates produced apothecia when treated in this way.

A technique for isolation of ascospore sets in linear arrangement was developed for tetrad analysis of the inheritance of resistance. At least 3 hours of fluorescent and incandescent light at 12 °C (±2 °C) was essential to allow ascospore ejection from individual asci taken from apothecia previously maintained in a 12 hour photoperiod at 12 °C (±1 °C). A water film on the surface of water agar was necessary to hold a set of ejected ascospores in linear sequence. Single ascospores were obtained in sequence with the aid of a micromanipulator.

Genetic analysis of MBC resistant isolates was carried out on ascospores derived from apothecia produced in the laboratory. Analysis of ascospore sets in linear arrangement and ascospore populations indicated that resistance to

>30,000 mg a.i./l carbendazim (high-resistant) is governed by a single major gene and is affected by gene conversion mechanisms. Crossing over was frequent, suggesting that recombination of resistance with other characters, such as pathogenicity and fitness, may occur readily. The segregation ratio (1:1) from most resistant isolates revealed that heterokaryons containing both resistant and sensitive alleles were common in resistant populations and that resistance is dominant. Allozyme analysis of ascospore progeny through electrophoresis revealed a narrow genetic base of *M. fructicola* in New Zealand.

The technique for reliable apothecial production in controlled conditions developed in this study provided an important step for the determination of the biology of *M. fructicola* strains resistant to MBC fungicides, and the complexity of its life cycle. Genetic heterogeneity in field populations can be conserved in one isolate through heterokaryosis, thus providing for adaptability of the pathogen to the changing environmental conditions. Knowledge on genetic variability, overwintering ability, pathogenicity and fitness factors may be useful for future management strategies of stone fruit brown rot. Special emphasis should be made in particular to prevent primary infection on blossoms, which would delay the establishment of recombinant strains of *M. fructicola* and the onset of brown rot epidemics.

KEYWORDS: Methyl benzimidazole carbamate; carbendazim; resistance; nectarine; peach; stone fruit; *Monilinia fructicola*; brown rot; epidemiology; virulence; fitness; competition; survival; twig cankers; mummified fruit; genetic; apothecia; ascospores; mechanisms of resistance; inheritance.

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CHAPTER 1
INTRODUCTION

1.1 GENERAL INTRODUCTION

Pests, including plant pathogens, attack crop plants to complete their life cycle by invading and destroying host tissues. They may cause considerable crop losses, estimated at about 25-45% of production world-wide (Dekker, 1982). Approximately half of these losses are caused by plant pathogenic fungi (Buchenauer, 1990). To ensure adequate world crop production, crop protection measures are taken to prevent these losses. Since the late 1920s, chemical pest control has been one of the measures that has contributed to reduced annual fluctuations of crop production, to increased crop yield and to satisfactory levels of food security in many countries (Koller and Scheinpflug, 1987). However, the pests have defence mechanisms to survive such treatments, which leads to resistance.

Many pest species have become resistant to chemical pesticides, such as the development of resistance by bacteria to antibiotics, weeds to herbicides and insects to insecticides. Resistance of insects to chemical pesticides was recognised nearly 80 years ago in lime sulphur resistant strains of San Jose scale, *Aspidiotus perniciosus* (Melander, 1914). The problem became widespread in the 1940s during extensive use of synthetic organic compounds. There are few problems of resistance to fungicides with multisite inhibitors, even though some of them have been used on a large scale for control of fungal diseases for almost a century (Dekker, 1984). However, a few years after the introduction of synthetic organic fungicides with specific modes of action in the early 1970s, problems with fungicide resistance occurred and there have been many further examples subsequently.

Fungicide resistance is defined as the stable, inheritable adjustment of a fungal population to a fungicide to reduce normal sensitivity to that fungicide (Dekker, 1985). Pathogen populations shift to less sensitivity from repeated

fungicide use in many cases. Resistance to some fungicides develops rapidly and persists permanently, leading to practical problems in the field.

Fungicide resistance in plant pathogenic fungi is a serious problem in crop protection with few solutions currently available (Hollomon, 1986). To understand the problem of fungicide resistance, information on the biochemical mode of action of fungicide, resistance mechanisms in fungi, disease epidemiology and genetics is important.

1.2 FUNGICIDAL MODE OF ACTION AND RESISTANCE

The mode of action of a new fungicide and the likelihood of development of resistant fungal strains is important information for long term resistance management strategies. The probability of emergence of resistance and the behaviour of resistant strains, is determined primarily by the mode of action of the fungicide. Different groups of fungicides interfere with different processes in different groups of fungi. Toxicity of the various biochemically specific fungicides now in use is based on an interference with a variety of cellular activities (Table 1.1). Sisler (1988b) summarised the basic mechanisms of resistance to fungicides as: 1) reduced affinity of target site; 2) reduced uptake or increased efflux of fungicide; 3) detoxification; 4) lack of conversion to active compound; 5) compensation, such as increased production of a target enzyme; and 6) circumvention or block through an alternative pathway. Changes in target affinity are believed to be the most common basis of fungicide resistance with site-specific compounds, but other mechanisms are known. However, if resistance to multisite compounds occurs, it more commonly involves reduced uptake or detoxification mechanisms (Sisler, 1988a; Berg *et al.*, 1990; Hollomon *et al.*, 1990).

Reduction in sensitivity to one fungicide may result in an increased sensitivity to others. The phenomenon is called negatively correlated cross-resistance. This may happen between closely related compounds or between fungicides exhibiting different modes of action (Buchenauer, 1990). The former case

Table 1.1 Modes of action of selective fungicides in target organisms (Buchenauer, 1990).

	Target Sites	Fungicidal Compounds
1	Microtubules	Methyl benzimidazoles carbamate (MBC), N-phenyl carbamate (NPC).
2	DNA-synthesis	Hydroxyisoxazole.
3	RNA-synthesis	2-aminopyrimidine, Phenylamides.
4	Protein synthesis	Cyclohexamide, Blasticidin S, Kasugamycin.
5	Energy production	Nitrophenol derivertives, Fentins, Drazoxolon, Carboxamides, Fenaminosulf, sec-Butylamine.
6	Cytochrome c-reductase	Aromatic hydrocarbons, and related structure, Dicarboximides.
7	Phospholipid synthesis	Epifenphos
8	Sterol synthesis	C-14-demethylation inhibitors (DMI), C(14,15) double bond reduction and ⌂ 8-⌂ 7-isomerization inhibitors, Squalene epioxidase.
9	Cell membranes	Guanidines, Polyene macrolide antibiotics.
10	Cell wall synthesis	Polyoxins, Nikkomycin.
11	Melanin synthesis	Tricyclazole.
12	Primary mode of action of fungicides not completely elucidated	Phosphorothionates, Pyroxychlor, Prothiocarb, Propamocarb, Cymoxanil, Fosetyl-Al, Probenazole, Dichlorocyclopropanes, Mildiomycin, Validamycin A.

happened between MBC and the related N-phenylcarbamate (NPC) fungicides for five diseases (Hollomon, 1986; Kato, 1988; Katan *et al.*, 1989). However, resistant isolates of *Venturia nashicola* (Ishii *et al.*, 1990) and *Botrytis cinerea* (Leroux and Gredt, 1989) to MBC were also resistant to NPC fungicides indicating that the linkage is not absolute. A single change in the ß-tubulin is responsible for the observed phenomenon (Jones *et al.*, 1987; Fujimura *et al.*, 1990). Negative cross resistance also occurred between ethirimol and triazoles in strains of barley powdery mildew (Kramer *et al.*, 1987).

1.3 EPIDEMIOLOGICAL ASPECTS OF FUNGICIDE RESISTANCE.

Epidemics can be divided broadly into two categories. Polycyclic epidemics are those in which many generations of the pathogen occur as it spreads from plant to plant in a single growing season. Outbreaks of powdery mildew resistant to MBC fungicides is an example of polycyclic disease epidemics. Because of their short generation times, massive spore production and reasonably efficient spore dispersal, the pathogen can increase very rapidly. They may increase from below the perceivable threshold to completely destroy the host stands within a single season. In monocyclic epidemics, on the other hand, once the pathogen has gained entry to the host further spread to other hosts does not occur until the following season. Both polycyclic and monocyclic epidemics may occur in a single pathogen. Epidemics of *Monilinia fructicola* (Wint.) Honey, a destructive pathogen of brown rot in stone fruit, in a dry area may be an example of monocyclic disease, but when wet the epidemics may become polycyclic to destroy most of the stone fruit crop.

Plant disease epidemics are dynamic phenomena that may start at different times in different host populations and at different speeds (Burdon, 1987). To understand the population dynamics of pathogens, the components of epidemics must be identified. The biological aspects may begin with the concept of the infection chain. The infection chain consists of a number of infection cycles. Each infection cycle typically comprises germination of a

fungal propagule, infection, growth in the host, and production and release of more propagules. The development of an epidemic integrates all the biological and environmental variables that act and interact on each other.

Epidemics of a particular pathogen strain in the same population are likely to proceed rapidly, if selection pressure favours one strain more than the others. For example, fungicide does not disturb growth of resistant strains but decreases growth of sensitive strains in the pathogen population (Georgopoulos and Skylakakis, 1986; Milgroom *et al.,* 1989). Evidence suggests that in some, but not all pathogen/pathosystems, there is a link between increased resistance and a decrease in fitness and pathogenicity. The slow emergence of MBC resistant strains of *M. laxa,* relative to *M. fructicola,* was associated with reduced fitness of MBC resistant strains (Ogawa *et al.,* 1988; Elmer, 1990). Similarly, the emergence of DMI and dicarboximide resistant strains in several host/pathogen systems has not proceeded as quickly as laboratory studies indicated and was linked with decreased virulence and fitness (Koller and Scheinpflug, 1987; Dekker, 1988). If there are differences between resistant and sensitive strains in a host/pathogen system, the result is the dynamics of the total number of inoculum propagules of pathogenic strains that are produced on infected plant parts.

Inoculum is the variable material that can infect a host (Gilligan, 1987). The inoculum in turn is divided between dispersal and infection units (Zadoks and Schein, 1979). A dispersal unit is any device for spread or survival that can be recognised and counted. An infection unit is the mycelium structure that originates from a dispersal unit after infection of the host. Of the dispersal units that are viable, not all are necessarily capable of infecting a host, even when favourably placed to do so. Garrett (1970) used the term effective inoculum to distinguish the viable and infective fraction from the viable but not-infective fraction. Inoculum potential can be divided into two parts: one dealing with the energy or infective quality of the inoculum, and the other with inoculum density (Lockwood, 1988). Inoculum density is frequently used to describe the number of propagules or dispersal units in inoculum density

experiments.

Primary inoculum that originates from overwintering sources may produce primary infection of the host. During infection, fungal hyphae invade and may destroy host tissues to complete the infection cycle. Large numbers of conidia may be produced from infected tissues for secondary infection. The conidia can be air-borne, splash-borne, insect-borne or dispersed by physical contact depending upon the fungal species. The ability of resistant strains to survive, increase in frequency and dominate the population is dependent upon their ability to function as a pathogen (Gilpatrick, 1983). In the presence of fungicide selection pressure, if resistant strains are pathogenic, virulent and fit, the resistant strains will have a selective advantage over sensitive strains and may quickly dominate the pathogen population.

Definition of the terms pathogenicity, virulence and fitness is necessary because these terms are used interchangeably. Following the definition given in the Commonwealth Mycological Institute (1973): pathogenicity is a qualitative term used to describe the ability to cause disease; virulence is the relative capacity to cause disease and should be used with quantitative adjectives to describe degrees of pathogenicity and fitness is a comparative concept relating the ability of one phenotype to survive and persist under a given set of conditions. Aspects of fitness *in vitro* are the rate of production and proportion of spores which germinate, mycelial growth rate and rate of spore production on agar media (Elmer, 1990). Aspects of fitness *in vivo* are infection efficiency, rate of colonisation, rate of sporulation and length of infectious period (time over which spores are produced) (Lalancette *et al.*, 1987; Kadish and Cohen, 1988b). Other fitness components which may be measured are length of the incubation period and length of the latent period. Analysis of these components of the monocycle have not been reported commonly in fungicide resistance studies. Each of these components may affect the rate of resistance build-up. From a theoretical model of resistance build-up, Skylakakis (1982) concluded that when the rate of growth and sporulation by resistant and sensitive strains were similar, resistance build-up

was faster for the strain with the shorter latent period.

The fitness of a resistant allele in the absence of the fungicide also determines its frequency in the population. There is usually no selection for resistance in a pathogen population unless the fungicide is used. If a resistant strain can not compete with sensitive strains and other organisms under these circumstances, it may not survive to react against the fungicide. In general, accumulation of resistance genes in a pathogen will decrease fitness (Dekker, 1987; Georgopoulos, 1987; Skylakakis, 1987). A mutation to resistance may affect parts of the pathogen chromosome so that some fitness characters may be impaired (Georgopoulos and Skylakakis, 1986). If more point mutations are required for a higher degree of resistance, a greater fitness deficit would be expected. In the field, selection may stabilise the frequency of resistant genes in the resistant population, eliminating those unnecessary for survival. Only those fit resistant strains will survive in the population. Apparently, there is no absolute link between resistance and reduction in fitness for some fungicides, such as resistance to MBC and phenylamide fungicides (Dekker, 1987; Lalancette *et al.*, 1987; Kadish and Cohen, 1988a). Reductions in fitness have been reported in pathogens resistant to dicarboximide and the DMI fungicides (Elmer and Gaunt, 1988; Georgopoulos, 1988; Koller, 1988). Although a short term experiment may show that one strain of a pathogen displaces another over the course of several consecutive generations of asexual spores, it is difficult to prove that this is due to the resistance genes and not to other factors. A pair of isolates of a resistant and a sensitive type may not be representative, especially if it has been in culture for some time (Smilanick and Eckert, 1986).

Many pathogens have a saprophytic phase in addition to a parasitic phase. They must, therefore, survive in the absence of selection pressure on dead host tissues until favourable conditions return and susceptible host tissues reappear. Saprophytic survival, one of the modes, is defined as mycelial survival in infected plant parts invaded during its parasitic phase. Most of the investigations into saprophytic survival in infected tissues have been carried

out with colonised tissues (Garrett, 1970). For such experiments, large numbers of infected plant parts are required. Uniformly colonised materials in large quantities may be obtained by artificial inoculation. Survival of a pathogen in inoculated and naturally infected units seems to produce satisfactory results for experimental purposes (Garrett, 1970). During infection of living tissues, the fungus establishes a mycelial network throughout the tissues. Thereafter, the mycelial network of the pathogen survives saprophytically by a slow but continuous decomposition and exploitation of the tissues. The longevity of the mycelial network may be only a few months or up to a year or more depending upon the infected tissues. For example, the longevity of the mycelium will be shorter for pathogens of annual crops than for pathogens of woody perennials. Primary inoculum may be produced abundantly from those infected tissues when environmental conditions, such as temperatures and moisture are suitable. Primary infection will follow, if susceptible host tissue is available. Many infected plant parts may be present in the tree or on the ground under the crop canopy, however, the quantitative importance of sites of survival and routes of invasion should be known in order to identify the most important sources. In many Ascomycete fungi, primary infection occurs during the winter and early spring from rain-splashed spores or air-borne ascospores produced from overwintering infected tissues, such as in *Botryotinia fuckeliana* on strawberry, *B. squamosa* on onions (Sutton, 1990) and *Typesia yallundae* on wheat (Nicholson *et al.*, 1991).

The use of fungicide must be considered on inherent and management factors (Staub and Sozzi, 1984; Hollomon, 1986). The inherent factors are: high and persistent fungicidal activities and monogenic type of fungicide resistance. The management factors are: long and unfavourable timing of fungicide exposure; use of fungicide alone; overdose of one effective fungicide and extensive use over a large area. The biology of the pathogen is important, such as speed of reproduction, pathogen fitness in the presence and absence of fungicide, degree of resistance to the fungicide, competition for host susceptible sites and movement to new host tissue (Delp, 1980; Hollomon, 1986). The

environment, such as temperature, humidity, precipitation and windspeed etc. must also be considered. Information on how fungicide exposure interacts with the variables on the biology of the disease and cropping practices should provide the basis for practical strategies of fungicide use. Fungicide applications represent an environmental change which leads to a population response.

Generally, fungicide applications exert a selective action upon the pathogen population in favour of resistant strains. In nearly all reports of fungicide resistance there has been a direct relationship between the rate of resistance build-up and the frequency of fungicide applications (Brent, 1987; Dekker, 1987). The selection pressure may be continuous or intermittent, which may also affect the rate of resistance build-up (Dekker, 1982). Interruption of selection pressure during or at the end of the growing season may delay the build-up of resistant strains. However, several reports of persistence of fungicide resistance for several years in the absence of selection pressure there has been linked directly to particular fungicides, such as MBC resistant strains of *M. fructicola* in Australia (Penrose, 1990).

With continuous selection pressure by a fungicide, the sensitive fitter strains that can compete with resistant strains are eliminated, and the resistant strains dominate the pathogen population (Hollomon, 1986). In the case of no fungicide application, stabilising selection may well ensure that a resistant mutant with lowered fitness never survives to bring about reinfection. Information on how fungicide exposure interacts with the variables on the biology of the disease and cropping practices should provide the basis for practical strategies of fungicide use. These variables provide numerous possible interactions going on continuously and changing over time. These data are so complicated that a computer may be necessary to analyze the information.

Most recently, there are two computer simulation models called RESISTAN and SIGATOKA (Arneson *et al.*, 1988; Arneson, 1990). These models were

developed by utilising a number of different mathematical modelling approaches to give quantitative prediction and management options in a specific situation. The models are based ultimately on qualitative response to fungicide selection pressure. The simulation model has a general structure that allows simulation of many fungicides, their effects on different fungi, by pulling in different sets of data rather than having to rewrite the model for each case. The RESISTAN model can be used for pre-season planning of fungicide spray programmes. There are three major parameters. First is the fungicidal effects of the remaining residues that are applied independently to infection, lesion expansion and sporulation. Second is the parameters needed to describe the development of the fungus which has no genetic component to the model. Last is the parameter to enable RESISTAN to be used as a management tool that tallies up the costs and revenue at the end of the season. The SIGATOKA model is more complex than RESISTAN. The model is intended as a research tool and for decision making in day-by-day forecasting for the timing of fungicide applications. Such a model requires detailed knowledge of the growth and development of the host, sporulation of the fungus, spore dissemination, infection, the development of the disease, and the effects of fungicides on the fungus, all in response to appropriate environmental variables. Many of these characteristics are based ultimately on genetic factors.

1.4 GENETICS OF FUNGICIDE RESISTANCE

In a fungal population, a necessary prerequisite for resistance in a normally sensitive population is the existence of less sensitive strains to the fungicide (Koller and Scheinpflug, 1987). Such a decrease in sensitivity is usually associated with genetic changes in the fungal cell. Most of the cases appear to be associated with a spontaneous mutation for resistance of a single major gene, or several minor genes as with the DMI fungicides (Eckert, 1988b). In most pathogen populations, spontaneous mutation rates are sufficient to generate mutants resistant to any highly specific fungicide (Hollomon, 1986).

The frequency of resistance is determined by the mutation rate to resistance, and by the relative fitness of the resistant mutant in competition with sensitive wild types (Skylakakis, 1987). To understand the development of resistant populations, it is important to know whether resistant alleles are likely to arise by mutation, and if so how frequently. Artificially induced mutations probably differ very little qualitatively from spontaneous mutations in the field (Gisi and Staehle-Csech, 1988). Although, mutagen treatment greatly increases the frequency of mutants, there is generally no relation between induced and spontaneous mutation rates (McDonald *et al.,* 1989). Fungicide resistance mutants have been recovered at a rate of $1x10^{-9}$ to $1x10^{-4}$ (Dekker, 1987; Gisi and Staehle-Csech, 1988).

Knowledge on genetic basis of fungicide resistance can be used to describe the relationship between the frequencies of fungicide application and frequencies of resistant genes in the pathogen population. The reproductive system of a pathogen may affect the resistant mutant population (Hollomon, 1986). Where reproduction is asexual, linkage disequilibrium between fungicide resistance and other characters, such as lower pathogen fitness, are likely to persist and possibly delay the build up of resistance in the population. Where reproduction is frequently sexual, linkage disequilibrium between resistance and other characters is likely to be solved, probably by recombination mechanisms during meiosis. Resistance may suddenly be a problem and the effectiveness of fungicides becomes shorter.

Resistance to fungicides can be described either in functional or in genetic terms. The functional terms recognise that resistance may be effective against some fungicides, or gradually developed toward less sensitivity with the others. The terms qualitative and quantitative response were used to describe the differences in response to fungicide selection pressure (Georgopoulos and Skylakakis, 1986; Georgopoulos, 1988). A change in resistance that is required for the organism to acquire the highest resistance is called qualitative response. It is likely that the more widespread fungicide use becomes, the more opportunity for resistance will increase. As the fungicide application

approaches extensive use, only the resistant types will survive. Fungicide selection pressure toward resistance is a destabilising action towards a new equilibrium which might be reached through either a disruptive or directional selection in a pathogen population (Koller and Scheinpflug, 1987; Berg *et al.*, 1990). Disruptive selection occurs when there are at least two distinct resistant and sensitive populations. Under fungicide selection pressure, the sensitive strain will be suppressed, whereas the resistant strain quickly dominates the population (Skylakakis, 1985). In contrast, a directional selection occurs when the initial strains sensitive and less sensitive to a fungicide are overlapping. Fungicide application at a recommended rate initially may affect the entire population. A decrease in effectiveness of fungicide, even very small, may be important in the survival of the less sensitive strains. The effectiveness of fungicide may be slowly decreased by plant mechanisms during translocation: in these instances the strains with less sensitivity become reestablished first. As the frequency of these strains increases, the entire pathogen population will shift towards less sensitivity. An example may be found in spring barley powdery mildew resistance to the DMI fungicide (Brent *et al.*, 1989). However, population shift towards resistance will be gradual and may happen over several seasons, because the less sensitive strains are likely to associate with fitness deficit.

The genetic terms that describe resistance describe the mode of inheritance. There are major-gene and polygenic resistances (Georgopoulos and Skylakakis, 1986). Major-gene resistance is determined by one or a few genes whose individual effects are large and detected readily. It is described as dominant, semi-dominant or recessive (Grindle, 1987). The response is usually qualitative. The degree of resistance is often high enough for the resistant strain to remain unaffected by the recommended rate of fungicide application. Exposure of a fungal population to the fungicide can select resistant mutants and lead to the establishment of a new population totally unaffected by the chemical use at the recommended rate. If such a highly resistant strain is crossed to a sensitive wild type, a Mendelian ratio of distinct phenotypes will be obtained in the progeny. Genes for resistance located at the same point on

a chromosome in the same pathogen could react to different degrees to a fungicide. The different genes were assumed to be multiple alleles at one locus. Various examples for major-gene resistance have been shown in the MBC and phenylamide fungicides (Crute, 1988; Gisi, 1988; Ogawa *et al.*, 1988).

Polygenic resistance is determined by many genes of individually small effect. High resistance to these groups of fungicides requires positive interaction between mutant genes as found in induced mutants of *Aspergillus nidulans* resistant to imazalil (Hollomon *et al.*, 1990) and of *Nectria haematococca* var. *cucurbitae* resistant to fenarimol (Kalamarakis *et al.*, 1991), fenpropimorph and terbinafine (Lasseron-de Falandre *et al.*, 1991). The involvement of several to many resistance genes should maintain a unimodel frequency distribution even after long exposures. The degree of sensitivity of any members of the population is usually inherited by its asexual offspring. The response of resistance is quantitative, expressed through a gradual shift of the distribution toward higher resistance. Such a shift in the sensitivity of *Sphaerotheca fuligenea* to fenarimol has been well documented (Schepers, 1985). As the sensitivity distribution moves gradually towards increased resistance, disease control in the field may also gradually decline. It is unlikely that complete and sudden loss of effectiveness will be observed in practice. The fungus carrying the least sensitive genotype is often the most unfit, so that the strains of intermediate sensitivity become predominant (Georgopoulos and Skylakakis, 1986; Berg *et al.*, 1990).

In fungi with a sexual stage, meiosis promotes genetic recombination through random assortment of chromosomes into haploid sets through crossing over and gene conversion mechanisms (Fincham, 1983a). The former is a strictly reciprocal transfer of genetic information from one chromatid to another whereas the latter seems likely to be nonreciprocal (Fincham, 1983b). If sexual reproduction occurs, even sporadically, genetic recombination of resistance with virulence and high fitness may emerge in a pathogen population.

Many fungi can carry two or more genetically different nuclei in a common cytoplasm. This condition is known as heterokaryosis and often occurs following hyphal anastomosis. Mutation within a homokaryotic mycelium may also cause heterokaryosis. Many Ascomycetes and imperfect fungi have multinucleate cells, and when they are heterokaryotic, the proportions of different nuclei may change in response to the environment, such as in fungicide selection (Grindle, 1987). When a heterokaryon produces conidia, the segregation of dissimilar nuclei in the spores generates variation. Hyphal anastomosis, and heterokaryon formation occurs only between individuals which have alleles at one or more loci which regulate heterokaryon compatibility (Caten, 1987). The restrictions on heterokaryon formation are thought to protect the identity of the individual in much the same way as compatibility mechanisms restrict genetic exchange through fertilisation between the same mating type in heterothallic species (Rayner, 1991). However, heterokaryon incompatibility is not necessarily a barrier to sexual outcrossing in many fungi, such as found in *B. fuckeliana* (Grindle, 1987; Anagnostakis, 1988; Faretra and Pollastro, 1991). Heterokaryosis provides a genetic explanation for secondary homothallism, a self fertile mechanism, in *B. fuckeliana* (Faretra *et al.,* 1988a) and in *Neurospora tetrasperma* (Grindle, 1987). Sexual reproduction in *M. fructicola* might occur after heterokaryosis as it was found that there were two types of nuclei in ascogenous hypha before karyogamy in single field isolate (Heuberger, 1934). However, the *M. fructicola* was concluded to be homothallic (Ezekiel, 1924; Harada, 1977).

The genetic basis of fungicide resistance is considered as an important starting point for the determination of resistance management strategies. In *Bremia lactucae*, for example, phenylamide resistance is associated with the same sexual compatibility type, and incompatibility on lettuce cultivars with the *R-11* resistance gene. The phenylamides have been recommended for use on *R-11* cultivars where it is less likely that a resistant population will be selected. However, recombination may occur, if sexual reproduction is present. Investigation in this area in some fungi, such as *M. fructicola* has not previously been possible because of the difficulty of inducing sexual

reproduction in the laboratory. Genetic basis is an essential step in understanding the epidemiology and the biology of the fungicide resistant strains of *M. fructicola* in the field.

1.5 BIOLOGY OF *M. fructicola*

There are two important phases of brown rot: blossom blight and fruit brown rot. Blossom blight is important because: 1) blossom is the most important target site for infection by ascospores which carry the main source of genetic variation; 2) the number of blighted blossoms may be sufficient to reduce yield; 3) twigs with infected blossoms attached are usually blighted, thus reducing the amount of leaves and fruit bearing surface; and 4) conidia produced on the blighted blossoms and twigs may serve as inoculum for later infections. Fruit decay causes the most severe economic loss in many stone fruit growing areas (Zehr, 1983). Immature fruit at the pit hardening stage are resistant to decay (Biggs and Northover, 1988a), but latent or quiescent infections of green fruit have been reported to be important in Central Otago, of New Zealand (Tate and Corbin, 1978), and South Carolina, United States (Ogawa *et al.,* 1983; Rosenberger, 1983). Green fruit also may be infected after fruit injuries. As fruits ripen, they become increasingly susceptible to infection (Biggs and Northover, 1988a). Because the fungus produces numerous conidia within a few days of infection, explosive outbreaks of brown rot are often observed, especially in very humid climates.

The brown rot fungus *M. fructicola* overwinters as mycelium in mummified fruit and in infected plant parts such as twigs and peduncles (Byrde and Willetts, 1977; Landgraf and Zehr, 1982; Biggs and Northover, 1985). There are two important cycles of reproduction, asexual and sexual. The asexual cycle of the fungus is very common. In the spring, when temperatures and relative humidities are favourable for sporulation, sporodochia bearing chains of conidia develop on the surface of mummified fruit and infected twigs. Mummified fruit on the ground are capable of producing both asexual and sexual spores.

Ascospores are produced erratically by the fungus in stone fruit growing areas in New Zealand (Tate, 1979) and North America (Ogawa *et al.,* 1983; Biggs and Northover, 1985). Apothecia are produced in spring on mummified fruit which have overwintered on the ground in shade (Byrde and Willetts, 1977). Prolonged moisture is considered an important factor for apothecial development in the field. Mummified fruit which remain on the tree do not produce apothecia because desiccation may interrupt the process of production (Atkinson, 1971). The ascospores may be of only minor importance as a source of inoculum in dry areas, such as in Canterbury and Central Otago districts of New Zealand. However, they are very important in the North Island of New Zealand where approximately 60% of blossom blight is initiated by ascospores (Tate, 1979). The liberation of ascospores normally coincides with the emergence of blossoms and young shoots of stone fruit (Byrde and Willetts, 1977).

Ascospores are violently discharged from apothecia on the ground. Ascospore dissemination follows a diurnal rhythm; ascospores are generally absent in significant numbers during the night and until late morning, then peak between noon and late afternoon (Tate, 1979). Bright sunshine, an air temperature above 11 °C and greatest windspeed during the afternoon coincide closely with ascospore peaks. In addition, when relative humidity drops below 85% ascospore numbers increase rapidly. It was found that ascospores of *M. vaccinum-corymbosi* travelled for at least 300 metres from the source under normal prevailing wind (Batra, 1983; Lambert, 1990). The ascospores as well as conidia are important as primary inoculum for flower infection.

Mycelium of the fungus spreads via infected blossoms to fruit peduncles, young fruit and woody tissues. Conidia produced from the mycelium serve as secondary inoculum for fruit infection. Fruit may be infected before pit hardening stage and at the fruit ripening stage (Biggs and Northover, 1988a) and then fruit rot develops during the post-harvest period. The importance of previous year's cankers as primary inoculum sources for blossom blight have not been evaluated.

The conidia are also important as secondary inoculum. They are not violently discharged but are dispersed by air current, wind and water splash. The dissemination pattern of conidia is similar to that of ascospores. Dissemination of the conidia by wind occurs when the temperature is high, relative humidity low and often at high ultraviolet light intensities. The maximum air dispersal of conidia and possibly ascospores occurs at the hottest time of the day which coincides with the lowest relative humidity period (Corbin and Ogawa, 1974; Tate, 1979). Results in the laboratory suggested that light stimulates the development of conidiophores but inhibits conidial formation (Van Den Ende and Cornelis, 1970). Fresh conidia will develop at night coinciding with a minimum air turbulence and high humidity and are probably fully mature and ready to disperse during the day. Transport by air was considered the most important way that brown rot fungi reach their hosts (Roberts and Dunegan, 1932). A distance of 530 metres from the source was reported, if conidia were released from a height of six feet with a wind velocity at eight kilometres/ hour (Roberts and Dunegan, 1932). However, Jenkins (1965) concluded that, in Australia, splash dispersal of conidia of the fungus was more important than aerial dispersal. Water splash dispersal brings about only short-range dispersal; mainly to different parts of the same tree, or between adjacent trees. Spatial autocorrelation analysis of dicarboximide resistant strains of *M. fructicola* also indicated that splash dispersal in several orchards may be important (Elmer, 1990).

Fruit injured mechanically by insects, split pits, limb bruises, etc., are inoculated by insects carrying spores acquired while feeding on fruit with sporulating lesions (Ogawa *et al.,* 1983). A typical example is the fruit injury caused by oriental fruit moth larvae (*Cydia molesta*) and the plum curculio (*Conetrachelus nenuphar*) followed by the visitation of the *M. fructicola* contaminated nitidulid beetles- *Carpophilus* spp. (Tate, 1973). More brown rot caused by *M. fructigena* developed on injured apples which had been visited by *Drosophila subobscura* than on unvisited ones (Lack, 1989). Thrips, principally *Thrips obscuratus* are abundant in New Zealand on diseased fruit which may carry numerous *M. fructicola* conidia to the

healthy fruit (Ellis *et al.*, 1988). These insects may play an important role in the aetiology of the disease and resistance, especially under conditions unsuitable for abiotic spread.

Variation of *M. fructicola* is probably brought about by three mechanisms. Mutation is the basic cause of heritable variation in fungi. Spontaneous resistant mutant strains of *M. fructicola* to MBC and dicarboximide fungicides were detected at an average frequency of $1x10^{-6}$ to $8x10^{-4}$ from both resistant and sensitive strains (Jones, 1983). A mutation rate for resistance to DMI of approximately $1x10^{-7}$ was obtained after treatment with the mutagen nitrosoguanidine (Nuninger-ney *et al.*, 1989). The other two fundamental mechanisms by which such variation may occur in the fungus are: normal sexual recombination and segregation of genes from two different nuclei (Thind and Keitt, 1949); and heterokaryosis resulting from the occurrence of more than one nucleus in the same cell or thallus of the fungus (Byrde and Willetts, 1977).

Heterokaryosis is recognised as a major factor in natural variability, mutant gene conservation, sexuality and parasexuality in fungi (Lorbeer, 1980). However, the sexuality and parasexuality initiated by heterokaryosis apparently have not yet been known for *M. fructicola*.

Normally the hyphal cells in *M. fructicola* are multinucleate (Heuberger, 1934), and they are frequently heterokaryotic in *M. fructigena* (Hoffmann, 1972) and *M. laxa* (Hoffmann, 1974). It is likely to be the same in *M. fructicola* because hyphal anastomoses are frequent (Byrde and Willetts, 1977; Sonoda *et al.*, 1991). Any changes in environment would allow for different patterns of intermingling of the nuclei and hence altered phenotypic expression. Despite these studies the underlying mechanisms for such variation has not been determined, but heterokaryosis is likely to influence phenotypic expression.

Microconidia are produced abundantly, if moist, by the brown rot fungus.

Unlike the conidium and the ascospore, the microconidium does not have the functions of multiplication and dispersal. Whetzel (1929) suggested that the microconidia could act as male gametes in the same way as the pycniospores or spermatia of rust fungi. Microconidia of *Sclerotinia gladioli* (Drayton, 1934a), *S. trifoliorum*, *S. sclerotiorum* (Fujii and Uhm, 1988) and *B. fuckeliana* (Faretra and Antonacci, 1987) are functional spermatia. A similar role has been attributed to microconidia of several fungi belonging to the family Sclerotiniaceae (Drayton, 1937). Possibly microconidia of *M. fructicola* also function as spermatia, although experimental proof for this fungus is lacking. Microconidia of the fungus are produced in abundance in cultures and on the surfaces of mummified fruit (Byrde and Willetts, 1977). Investigation of the role of spermatia is needed as well as a reliable method of sexual induction because there are still important gaps in the knowledge of the development cycle.

Sexual reproduction of the brown rot fungi included in the family Sclerotiniaceae was unknown until the early 1930s. Drayton (1934b) obtained apothecia of *S. gladioli* by placing microconidia from one isolate on the receptive structures that developed from the stromata of another. He demonstrated that there were two mating types of *S. gladioli* with sexual reproduction occurring only when pairs of strains from two different groups of isolates were intercrossed. The inter-group fertility, intra-group sterility and self sterility of Drayton's isolates are typical of a heterothallic fungus.

In the family Sclerotiniaceae self-fertility has been found in various species of the genera *Sclerotinia, Lambertella, Ciboria, Ciborina, Rutstroemia, Septotinia, Verpatinia* and *Botryotinia*. Self fertility has also been found in *M. fructicola* (Ezekiel, 1924; Harada, 1977) and they concluded that the fungus is homothallic. However, not all self fertility means homothallism, it can be by secondary homothallism or by mating type mutation mechanism (Uhm and Fujii, 1983b). Since the microconidia of *S. gladioli* has been shown as a functional spermatia, it has been shown that ascocarp development by many member of the Ascomycotina is preceded by the

copulation of spermatia with hyphae that are physiologically and often morphologically specialised.

The observations in *B. fuckeliana* by Faretra and Pollastro (1991) suggest that homothallic field isolates were actually heterokaryons containing nuclei carrying different alleles of the mating type (*Mat1*) gene. Heterokaryotic co-existence of sexually compatible nuclei can explain why sclerotia of homothallic isolates are able to produce sexual progeny following cross-fertilisation by reference strains or by self-fertilisation. And also it can explain why sclerotia of both *MAT1-1* and *MAT1-2* strains could be fertilised by homothallic isolates to produce sexual progenies with expected genotypes. The same mechanism may exist in *M. fructicola* field isolates but no experimental evidence has been reported.

Ascospore dimorphism was found in many species of *Monilinia,* such as *M. baccarum, M. oxycocci, M. urnula* and *M. type I* (Batra, 1983). Ascospores of *M. vaccinum-corymbosi* are trimorphic (Batra, 1983) and dimorphic and trimorphic ascospores have been reported in *M. gaylussaciae* (Batra, 1988). These provide a means by which heterothallism with a self-fertile mechanism may occur in the genus *Monilinia* as it occurs in large spore strains of *S. trifoliorum* (Uhm and Fujii, 1983b).

1.6 OBJECTIVES

Resistant strains have to compete with sensitive strains to colonise unoccupied tissues in the absence of fungicide selection pressure. Many factors, such as the biology of the strains and management strategies, become involved in the development and epidemiology of the resistant sub-population. Factors delaying the resistance build-up are important, and may be useful as the basis for devising resistance management strategies. The objectives of these studies (Figure 1.1) were to understand any factors relevant to the epidemiological basis of MBC fungicide resistance in *M. fructicola,* to identify the sources of primary inoculum of resistant strains and to develop techniques of

apothecial induction in the laboratory leading to genetical analysis of the mechanisms of inheritance of resistance to the fungicides.

In CHAPTER 2, the incidence of MBC resistance, the degrees of resistance to MBC fungicides in some New Zealand stone fruit orchards and in the Californian-imported nectarine fruit are reported. Components of fitness, including incubation period, latent period, rate of colonisation, rate of spore production and pathogenicity of resistant and sensitive isolates on blossom and fruit are described. The competitive ability of resistant and sensitive isolates on blossom and fruit is also reported.

The frequency of resistant and sensitive sub-populations of the population in the season indicates the fitness of the pathogen. The amount of inoculum of individuals indicates the survival ability and perhaps provides an important indicator of resistant disease epidemiology. In CHAPTER 3, inoculated mummified fruit and twig cankers were exposed to field conditions. Conidia produced under conducive conditions were analyzed quantitatively and survival ability of resistant and sensitive strains is discussed.

Sexual reproduction plays a key role in understanding the genetic basis of fungicide resistance and evolution of resistance. The sexual reproduction of *M. fructicola* is probably rare in nature and is very difficult to induce. Therefore, techniques to induce apothecia under controlled conditions are described in CHAPTER 4. A technique is described to isolate single ascospores in linear sequences from asci.

In CHAPTER 5, the techniques developed from CHAPTER 4 were utilised to analyze genetically the inheritance of resistance. This was used to describe the mechanism of MBC resistance in *M. fructicola*.

CHAPTER 6 is a general discussion on the findings of the thesis and the implications for the successful management of this important pathogen.

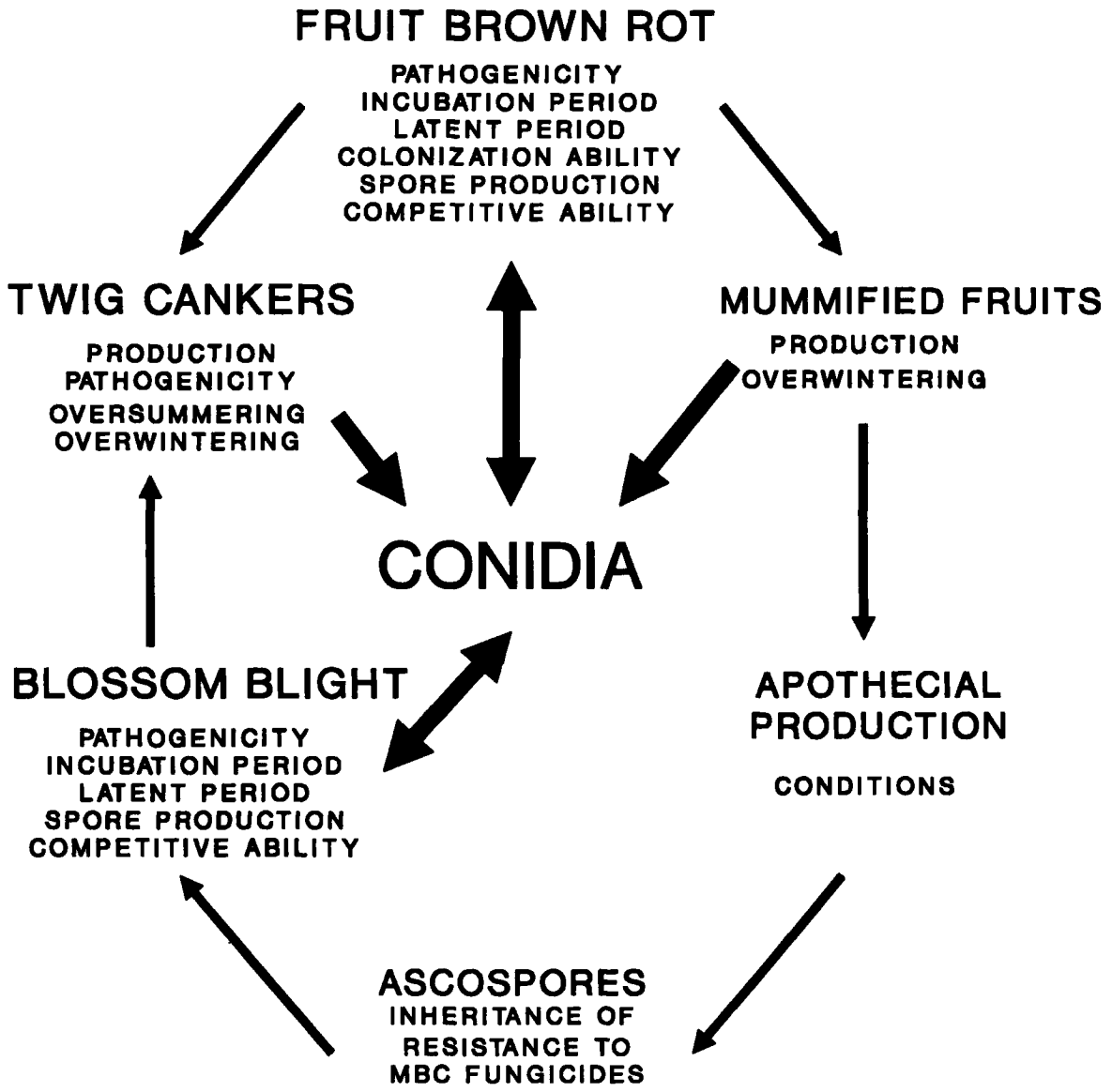

Figure 1.1 Epidemiological aspects of MBC resistance in *Monilinia fructicola* (Wint.) Honey (brown rot of stone fruit) and mechanisms of resistance.

CHAPTER 2
PARASITIC FITNESS AND COMPETITIVE ABILITY OF MBC RESISTANT STRAINS

2.1 INTRODUCTION

Disease control failures associated with fungicide resistance will happen only if resistant strains have a sufficient degree of resistance relative to recommended rates and they become dominant in pathogen populations (Dekker, 1987). In the presence of fungicide selection pressure, build-up of resistant strains may occur very rapidly, slowly, or not at all depending on many factors. The factors which influence the build-up of a resistant population are disease control management, type of pathogen and the biological characteristics of resistant and sensitive strains (Dekker, 1984; Koller and Scheinpflug, 1987). Disease control management, such as good cultural practices, good knowledge of the actions of fungicides and the type of application are essential for the success of any strategies designed to delay resistance (Scheinpflug, 1988; Staub, 1991). Pathogen biological characteristics most frequently measured are the degree of resistance, pathogenicity, virulence and fitness of the resistant strains.

In broad terms, the fitness of an isolate may be considered as being a measure of its capacity to produce viable offspring. This will be influenced by genetic and environmental factors, and the interaction of these two. Genetic factors of importance include the effect of mutation towards resistance on fitness of the resistant strains. Fitness deficits were shown in the case of MBC resistant strains of *Penicillium* spp. on citrus (Eckert, 1988a), *M. laxa* on apricot (Ogawa *et al.*, 1988), *M. fructicola* strains resistant to dicarboximides (Elmer and Gaunt, 1988) and probably in all reported cases of resistance to the DMI fungicides to date. Selection pressure may stabilise the frequency of resistant genes in the resistant population by eliminating the relatively unfit isolates. However, there are pathogens that do not show a decrease in fitness e.g. *V. inaequalis* resistant to MBC (Shabi *et al.*, 1986), *P. infestans*

(Kadish and Cohen, 1988b) and *B. lactucae* (Crute and Harrison, 1988) resistant to phenylamide and *P. digitatum* resistant to sec-Butylamine fungicide (Smilanick and Eckert, 1986). Synergistic or antagonistic interactions between sensitive and resistant strains may occur during growth in the field. In the presence of fungicide selection pressure, sensitive isolates of *P. italicum* predisposed fruit for resistant isolates on citrus (Eckert, 1988a), but reduction in fitness of the resistant strains through competition for nutrients and space was observed in the absence of fungicide application.

In the field, resistant strains have to compete with sensitive strains especially when the fungicide is not present or is at low concentration not effective against the sensitive strains. The fitness of resistant strains is a significant factor responsible for the persistence of resistance. Many parameters have been used to measure fitness of a pathogen, such as infection efficiency, duration of incubation and latent periods, the speed of tissue colonisation and the amount and rate of sporulation (Dekker, 1987; Kadish and Cohen, 1988a; Elmer, 1990). Strains with greater fitness will usually be more competitive than those with less fitness, depending on the relevance of the fitness attribute to the epidemic progress and survival. Competition tests between two or more strains may be the most appropriate indicator of disease epidemics (Beever *et al.*, 1989; Rewal *et al.*, 1991).

Competitiveness is one of the most important factors which may affect the dynamics of resistant and sensitive individuals in a pathogen population (Lynch and Hobbie, 1988). Reversion of a resistant to a sensitive population may be expected if resistant strains are less competitive (Shabi and Katan, 1979). Competition for limited resources may be intra- or inter-specific. An epidemic of strains resistant to fungicide may be delayed compared to sensitive strains because of the differences between competitive ability of resistant individuals.

There are several factors involved in competitive ability. Inoculum density affected the competitiveness of a MBC resistant strain of *V. inaequalis* (Martin

and Oliver, 1981). Differences in the outcome of competitive ability may result from the method of inoculation (Pommer and Lorenz, 1987). Host tissues and pathogen fitnesses influenced the competitive ability of three metalaxyl resistant strains of *P. infestans* (Kadish and Cohen, 1988b). Therefore, investigation of the relationship between method of inoculation, inoculum density and competitive ability on different phases of infection on the host tissues are required and the results may be directly correlated to the field situation.

MBC resistant strains of *M. fructicola* were first reported in Australia (Whan, 1976) and later found in Michigan and New York (Jones and Ehret, 1976) associated with a disease control failure. Strains were resistant to very high degrees with no visual reduction in growth on MBC (100-1,000 mg/l) and there were no obvious differences between resistant and sensitive strains in colony characteristics on agar media and virulence or sporulation on fruit. In addition, strains resistant at low levels (1-4 mg/l) were detected in California in 1977 (Ogawa *et al.*, 1988). Resistance at both high and low levels remained stable after 8-12 years in the absence of MBC use, indicating their equality of fitness and ability to compete with sensitive isolates (Adaskaveg *et al.*, 1987; Penrose, 1990). In contrast, resistant strains were less fit than sensitive strains in South Carolina (Zehr *et al.*, 1991). Such variability may be derived from the fact that only few isolates were tested, and the method of inoculation did not follow the natural pattern of infection. The behaviour of individual isolates may not be representative of the population. Such differences in biological characteristics between resistant populations may reflect the effect of management strategies on the individual population.

Little is known about the degree of resistance, fitness and competitiveness of New Zealand *M. fructicola* strains resistant to the MBC fungicides. It is known that resistant strains have persisted in orchards for at least 12 years after the withdrawal of the MBC fungicides (Elmer and Gaunt, 1986; Braithwaite *et al.*, 1991). The MBC spray programme has been replaced in most areas in New Zealand by one based on DMI and dicarboximide fungicides

since the detection of MBC resistance in 1980 (Elmer and Gaunt, 1986). On the other hand, the MBC fungicides are still included, with caution, in the spray programmes in the South Island because the fungicides are highly efficacious against the sensitive populations and cost effective. Recently, the first occurrence of MBC resistance in South Island properties was reported (Braithwaite *et al.*, 1991). Research is needed to explore management strategies to reduce resistance build-up, and to prevent resistance establishment in the South Island. The purposes of the studies reported here were to clarify the incidence of resistance, degrees of resistance and fitness of resistant strains on blossom and fruit and their competitive ability in relation to the sensitive strains.

2.2 MATERIALS AND METHODS

2.2.1 ISOLATION AND SENSITIVITY TEST

Five hundred and ninety seven diseased samples were collected from eight orchards in New Zealand and from diseased Californian imported nectarines at Bush-Inn Centre, Christchurch. The collection sites for diseased fruit included three properties in the North Island with a history of MBC resistance and five properties in the Canterbury Province of the South Island which had not reported resistance. *M. fructicola* was isolated from these diseased fruit tissue by tissue transplanting. Diseased tissues were surface sterilised in 0.5% sodium hypochlorite (v/v) solution and small pieces were plated onto petri dishes containing 1.5% water agar (Davis). The petri dishes were incubated for 3-5 days at 25 °C (±0.5 °C) with 12 hours photoperiod of NUV and daylight-fluorescent light. Pure cultures were maintained on PDA (Gibco potato dextrose agar) slants at either 1 or 4 °C and were multiplied by transferring mycelial cultures to PDA petri dishes and incubated as above.

All isolates were tested with 0, 1 and 10 mg a.i./l carbendazim (Bavistin 500) in PDA prior to classification as high-resistant, low-resistant and sensitive by a mycelial growth sensitivity test (Dekker, 1988, Smith *et al.*, 1991). Isolates

with no growth on 1 mg a.i./l carbendazim were classified as MBC sensitive strains. Isolates that grew on 1 mg a.i./l MBC but not on 10 mg a.i./l carbendazim were grouped as low-resistant isolates (LR). Isolates with no obvious reduction in growth on 10 mg a.i./l carbendazim were regarded as high-resistant (HR) isolates.

Eight randomly selected MBC sensitive isolates, 15 high-resistant MBC isolates, 21 low-resistant MBC isolates and one dual dicarboximide/ high-resistant MBC isolate were tested for the degree of resistance to the MBC fungicide.

Test media were prepared from a suspension of carbendazim in sterile distilled water and autoclaved PDA. After thorough mixing of the carbendazim suspension in warm (60 °C ± 2 °C), autoclaved PDA, 20 ml was dispensed into 82 mm diameter disposable plastic petri dishes (Gibco). The concentrations of carbendazim-amended medium for sensitive strains were 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1 mg a.i./l and for resistant strains were 0, 0.3, 1, 3, 10, 30, 100, 300, 1,000, 3,000, 10,000 and 30,000 mg a.i./l carbendazim.

Mycelial plugs (6 mm diameter) were cut from the edge of five to seven day old cultures incubated under continuous light at 25 °C (±0.5 °C) on PDA. The plugs were placed with the mycelial surface in contact with the amended and unamended agar prepared as above. Two colony diameters were measured daily at right angles to each other with three replicates per treatment. In all sensitivity tests incubation conditions were standardised to a maximum of five days at 25 °C (±0.5 °C) with 14 hour photoperiod of fluorescent and near ultra-violet (NUV) light.

Growth rates (mm/hr) were derived from linear regression equations of colony diameter (minus the mycelial plug) and time over the linear phase of growth. EC_{50} value were calculated from linear regression equations of growth rate and $\log_{10}$ concentration of carbendazim.

2.2.2 PARASITIC FITNESS INVESTIGATION

Two MBC sensitive strains (S1 and S2) were used as standards in all tests, in comparison with four high-resistant MBC isolates (HR1, HR2, HR3 and HR4), two low-resistant MBC isolates (LR1 and LR2) and one dual dicarboximide/ high-resistant MBC isolate (D/HR). They were selected on the basis of degree of resistance and mycelial growth rate on PDA (Table 2.1).

All isolates were grown on V-8 juice agar at 25 °C (±0.5 °C) with a 14 hour photoperiod of fluorescent and ultra-violet (NUV) light to produce large number of conidia. Conidial suspensions were prepared by flooding five to seven day old cultures with 5 ml sterile distilled water plus 0.05% Tween 20 (v/v). Spore suspension concentrations were adjusted using a haemocytometer. Suspensions were stored until used in these experiments at 1 °C for no longer than 3 hours to prevent germination.

A detached peach shoot system (Elmer, 1990) was used to provide blossoms suitable for measurement of fitness components. Peach (*cv.* Glohaven) shoots with at least 10-20 complete flower buds were collected at the stage of late calyx green to pink tip. The cut ends of these shoots were stood in water (approximately 30-50 mm depth) and stored at >95% relative humidity without light for up to six weeks at 1 °C (±0.5 °C). The shoots were surface sterilised for 2.5 minutes in 0.5% sodium hypochlorite (v/v) plus 0.05% Tween 20 (v/v), and then recut under water to the appropriate length (150 mm). The culture solution contained 300 mg/l sucrose, 20 ml/l ethanol (absolute) and 75 mg/l benzylaminopurine. Three shoots were placed in 150 ml milk bottles with 50 ml of the solution and plugged with sterile cotton wool. They were then allowed to flower in controlled environments at 20 °C (±0.5 °C) and at least 85% relative humidity with a 12 hour photoperiod of fluorescent and halogen light (160 W). At the end of this period, unopened flowers and those at petal-fall were rejected before conducting the experiments.

Inoculum suspensions were adjusted to $1x10^3$ and $1x10^5$ conidia/ml. Flowers

Table 2.1 Isolates and phenotype to fungicides of *M. fructicola* used for determination of fitness attributed.

Isolate code[a]	Phenotype[b]	Culture code	Growth rate[c] (mm/hr)	EC_{50} MBC(mg/l)	Host	Date
S1	MBCS/DS	PDDCC #7640[d]	0.57	0.06	Peach	1982
S2	MBCS/DS	DSW15	0.47	0.17	Peach	1986
HR1	MBCHR/DS	PDDCC #7642	0.50	>30,000	Peach	1982
HR2	MBCHR/DS	JC9[e]	0.38	>30,000	Nectarine	1988
HR3	MBCHR/DS	JC12	0.59	>30,000	Nectarine	1988
HR4	MBCHR/DS	W221	0.59	>30,000	Peach	1989
LR1	MBCLR/DS	CA5	0.43	1.25	Nectarine	1989
LR2	MBCLR/DS	CA4	0.68	1.64	Nectarine	1989
D/HR	MBCHR/DR	H7E	0.50	>30,000	Laboratory induction	1986
DR[f]	MBCS/DR	HJP14C	0.52[f]	159[f] (mg/l iprodione)	Nectarine	1986

[a] S=sensitive isolate, HR=high-resistant MBC isolate, LR=low-resistant MBC isolate, D/HR=dual dicarboximide/high-resistant MBC isolate, DR=dicarboximide resistant isolate.

[b] MBCS=MBC sensitive; MBCHR=high-MBC resistance; MBCLR=low-MBC resistance; DS=Dicarboximide sensitive; DR=Dicarboximide resistance.

[c] Slope (b) in regression equation: growth = a + b (hours).

[d] PDDCC isolates obtain from DSIR-New Zealand culture collection.

[e] New isolates held in Department of Biochemistry and Microbiology culture collection.

[f] Data obtained from Elmer (1990) and comparative studies will be appeared in Chapter 3.

were sprayed for three seconds (33 µl/ second) using a handheld airbrush (Badger, Basic spray gun set Model No. 250-1) at 172 kPa to give a final concentration of $1x10^2$ and $1x10^4$ conidia per flower. Inoculated shoots were placed in plastic pots. Transparent plastic chambers were attached to plastic pots using 2 litre beverage bottles. The bottles were cut to (provide a hole) 80 mm diameter at the neck and with 8 small holes, punctured by using a flamed metal needle, at the opposite end. They were prepared to provide very high relative humidity for inoculated shoots. The sleeves containing inoculated shoots (Figure 2.1) were incubated at 20-22 °C at 70% RH in controlled environment chambers, with a 14 hour photoperiod of fluorescent and incandescent light (200 µmol photons / m^2 / second measured at 900 mm above floor level). Relative humidity within the sleeve chambers was adjusted regularly to near saturation by misting the sleeve chambers at 48 hour intervals to encourage sporulation (Elmer, 1990).

Several fitness variables were measured on flowers. The length of the incubation period (IP) was measured as hours from inoculation to the first sign of lesions. Latent period (LP) was also recorded until the first visible sign of conidia. Sub-samples of sporulated flowers on shoots were removed and put into 50% ethanol (v/v) plus 0.05% Tween 20 (v/v) at 18 hour intervals from the end of the latent period. The flowers were shaken mechanically on a Griffin flask shaker (Griffin & George LTD) for 2.5 minute to break conidial chains. The number of conidia produced per flower was quantified with the aid of a haemocytometer. Rate of spore production (number of conidia/ flower/ hour) for each isolate was derived from linear regression equations of the number of conidia produced per flower over the linear phase of spore production. All experiments were conducted by factorial completely randomized block design with three replications per treatment and at least 10 flowers per replication.

Nectarine fruit (*cv.* Fantasia) were harvested and stored at 1 °C (±0.5 °C) and >90% humidity for up to 6 weeks. Deterioration of fruit firmness and moisture content occurred when fruit were stored beyond six weeks. Fruit of uniform maturity were washed thoroughly and surface sterilised as for the blossoms.

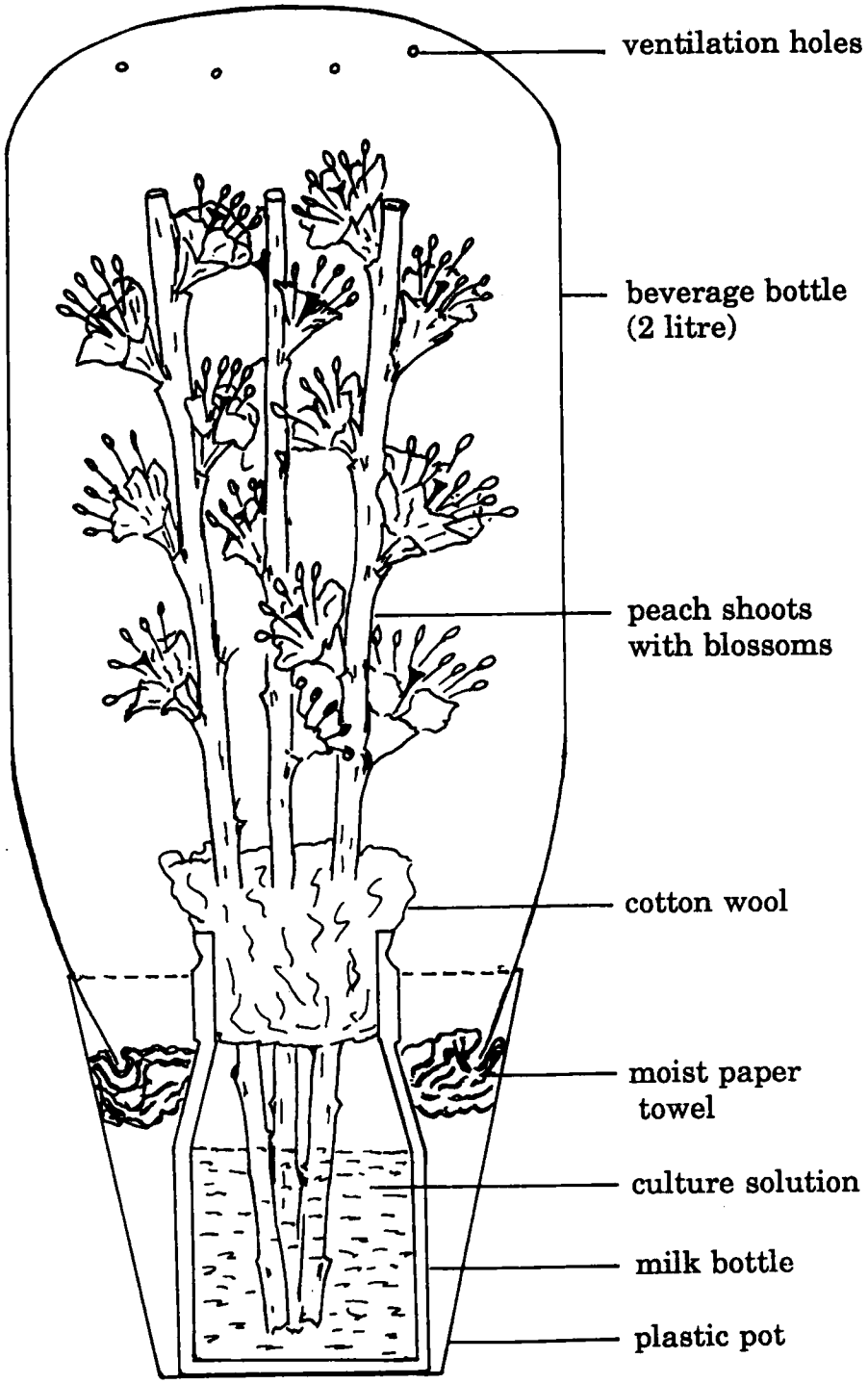

Figure 2.1 A detached peach shoot system for parasitic fitness investigations and competitive ability test.

Aseptic conditions were maintained during all stages of these experiments. Three fruit were placed in sterile semi-transparent 2 litre plastic ice-cream containers containing a moist paper towel to maintain a high relative humidity. Fruit were separated from each other and from the moist paper towel with plastic cups cut from Plix fruit trays. The containers were closed with plastic tops with five holes (10 mm diameter) plugged with sterile cotton wool.

Fruit were wounded with a flame-sterilised cork borer (4 mm diameter) to a depth of 1-2 mm. They were inoculated with 10 µl conidial suspension ($1x10^4$ and $1x10^6$ conidia/ml) with a micropipette to give initial inoculum of $1x10^2$ and $1x10^4$ conidia per wound site. The inoculated fruit were incubated at 25 °C (±0.5 °C) with a 12 hour photoperiod of fluorescent and incandescent light. Fitness was determined as on blossoms. In addition, the rate of fruit tissue colonisation was derived from linear regressions of lesion diameter and time over the linear phase of fruit colonisation.

2.2.3 COMPETITIVE ABILITY ON HOST TISSUES

Two sensitive isolates (S1 and S2), three high-resistant MBC isolates (HR1, HR2, and HR3) and one dual dicarboximide/high-MBC resistant isolate (D/HR) were matched in 8 combinations of resistant and sensitive isolate pairs. Tests were conducted both on blossoms and fruit to evaluate the competitive behaviour of each isolate in the absence of fungicide. A conidial suspension of each isolate was adjusted to $1x10^3$ and $1x10^5$ conidia/ml. Adjusted conidial suspensions of isolate pairs were mixed to give a proportion of resistant strains ranging from 0% to 100% at 10-15% intervals. Each proportion of conidial mixture was applied to flowers for three second using a handheld air brush (Badger, basic spray gun set, Model No 250-1) at 172 kPa to give a final concentration of at $1x10^2$ and $1x10^4$ conidia per flower. Inoculated flowers were incubated in the same manner for fitness experiments. The experiments were conducted with factorial completely randomized block design with three replications per treatment.

A bioassay technique used to determine the competitive ability of strains of *M. fructicola* resistant to dicarboximide fungicide (Elmer, 1990) was modified to determine the competitive ability of the pathogen resistance to MBC fungicide. A monilinia inhibiting medium (MIM) was prepared by adding a suspension of flusilazole (Nustar DF, 200 g a.i./kg) in sterile distilled water to warm (60 °C ± 2 °C), autoclaved PDA to give a final concentration of 15 µg a.i./l (Elmer, 1990). A suspension of carbendazim was added to some of the MIM to give a final concentration of 1 mg a.i./l. Streptomycin sulphate (250 mg/l) was added to all MIM to suppress bacterial growth.

All flowers with spores were harvested, six days after inoculation, and put into sterile distilled water plus 0.05% Tween 20 (v/v). They were shaken mechanically with the aid of Griffin flask shaker for 2.5 minutes to break conidial chains. The suspensions were adjusted to $5x10^2$ conidia/ml. A 500 µl aliquot of suspension was pipetted onto each of two plates of MIM amended with carbendazim and MIM unamended with carbendazim. The suspension was evenly distributed over the surface of each plate with a flamed glass hockey stick. Colony counting was carried out with the aid of a Quebec Colony Counter after three days incubation at 25 °C (±0.5 °C) with a 14 hour photoperiod of fluorescent and NUV light. The ratio of colonies growing on the amended and unamended selective media indicated the proportion of conidia expressing the resistant character.

The proportion of resistance (%) before and after competition on flowers was plotted over the linear phase of competition regression. The slope (b) derived from the regressions over the linear phase of final and initial proportions of resistance, described the competitiveness of resistant and sensitive strains. A *T*-test was used to test the slope parameter (b) of the outcome of competition between two strains and two concentrations against the null hypothesis of equal competitive ability (H_0: $b_1 = 1.0$, $b_2 = 1.0$). The *T*-test was also used to test the slope parameter (b) of the out come of competition between two concentrations against the null hypothesis of equal competitive ability (H_0: $b_1 = b_2$).

Before pathogen inoculation, surface sterilised fruit as described previously were pre-incubated for 48 hours at 20 °C (± 3 °C) and fruit with brown rot symptoms were discarded to avoid confusion caused by latent field infections. Each proportion of conidial mixture was sprayed directly onto the unwounded surface of these fruit for three second as described above for flower studies. Inoculated fruit were incubated in sterile 2 litre plastic ice-cream containers as described in Section 2.2.2. for six days. There were three fruit per replicate, three replicates per treatment with treatments randomized in each of three blocks.

Conidia were removed from the fruit surface using a flame-sterilised scalpel and put into sterile distilled water plus 0.05% Tween 20 (v/v). The proportions of resistant and sensitive conidia was determined as for flowers. The initial and final proportion of resistance (%) on fruit was regressed and competitive ability determined as described previously for flower studies.

2.3 RESULTS

2.3.1 THE INCIDENCE OF RESISTANCE

Thirty three of the 532 isolates obtained from New Zealand fruit grew on the discriminating dose of 1 mg/l carbendazim (Table 2.2). All New Zealand resistant isolates were obtained from Hawkes Bay properties, with 0, 17 and 41 % of the samples being resistant and all of them were resistant at high level (MBCHR). The MBCHR resistance in brown rot of stone fruit has persisted in the area since about 1979. MBC resistance was not found in five commercial orchards sampled in Canterbury.

Forty two of the 65 diseased fruit on the Californian-imported fruit, collected at two separate occasions, grew on 1 mg/l PDA amended carbendazim. Resistant strains were 52 and 75 percent which were later defined as low resistant (MBCLR). One isolate was as highly resistant as the isolates detected in the North Island of New Zealand.

Table 2.2 Fungicide resistance in isolates of *M. fructicola* from commercial orchards and imported fruit in a supermarket.

	Number of isolates assessed	Total number of resistant isolates	Frequency of resistance to MBC for each location (%)	
			HR[a]	LR[b]
COMMERCIAL ORCHARDS				
HAWKES BAY				
J. Clark	56	23	41	0
R. Duncan	60	10	17	0
K. Fulford	56	0	0	0
CANTERBURY				
B. Turner (Lyttelton)	30	0	0	0
B. Dartnals (Rangiora)	100	0	0	0
D. Bell (Rolleston)	40	0	0	0
M. Clements (Springston)	60	0	0	0
N. Thomas (Kaituna)	130	0	0	0
THE CALIFORNIAN-IMPORTED NECTARINE[c]				
Batch I	32	25	3	75
Batch II	33	17	0	52
Total	597	75		

[a] High-resistant MBC isolates that grew on 10 mg a.i./l carbendazim.

[b] Low-resistant MBC isolates that grew on 1 but not on 10 mg a.i./l carbendazim.

[c] Imported nectarines collected from Woolworths supermarket, Bush-Inn Centre, Christchurch.

The sensitivity to MBC fungicides of 37 tested isolates selected randomly was classified into three classes (Table 2.2; Appendix 1). No growth was observed in the most sensitive isolates at 0.1 mg a.i./l carbendazim. The mean EC_{50} of the Californian imported nectarine isolates was 1.5 ± 0.03 mg a.i./l MBC, and the majority of these isolates could not grow at 3 mg a.i./l MBC. One isolate from the imported fruit grew on 30,000 mg a.i./l carbendazim, which was as highly resistant as the MBCHR isolates obtained from properties in the North Island. At a concentration of 30,000 mg a.i./l, the HR isolates sporulated profusely without any obvious reduction in growth. Thus, it was not possible to calculate a EC_{50} value of these isolates.

2.3.2 PARASITIC FITNESS *IN VIVO*

Two main factors, inoculum density and isolate affected fitness components on blossom (Table 2.3). There was no significant difference ($P>0.05$) in interaction between inoculum density and isolate components. Inoculum density at the high level, $1x10^4$ conidia/ flower compared to $1x10^2$ conidia/ flower, significantly ($P<0.01$) reduced the incubation period (IP), the latent period (LP) and significantly increased the rate of spore production per flower. Although there were significant differences ($P<0.01$) between isolates tested in the LP and the rate of spore production parameters, there was no evidence to suggest that the differences were associated with resistant characteristics.

For most variables on fruit, there were significant differences ($P\approx0.01$) in interactions between two main effects (inoculum density and isolate-Table 2.4), but not ($P>0.05$) for the rate of colonisation. However, the main effects ($P<0.001$) were much larger than that of interactions ($P\approx0.01$). Snedecor and Cochran (1982) suggested that there was a minor variation in the effect of factor A (inoculum density) according to factor B (isolate), so in this event, only main effects will be considered. The two main factors significantly affected ($P<0.01$ and $P<0.05$) the four fitness variables. The high inoculum density reduced the duration of IP, LP, and increased the rate of colonisation and the rate of spore production on fruit. A sensitive isolate (S1) predominated

Table 2.3 The fitness components, incubation period, latent period and rate of spore production on blossom for *M. fructicola* field isolates resistant and sensitive to MBC fungicide.

	Incubation period (hours)	Latent period (hours)	Rate of spore production (conidia ($x10^{-4}$ /flower/hour)
INOCULUM DENSITY			
$1x10^2$ spores/flower	22.9	59.4	0.22
$1x10^4$ spores/flower	16.8	43.0	0.27
LSD (*P*=0.05)	0.42	0.94	0.03
ISOLATES[c]			
S1	20.0	50.5	0.31
S2	19.3	54.5	0.18
HR1	20.5	52.2	0.19
HR2	20.5	50.8	0.02
HR3	19.3	48.3	0.32
HR4	19.8	50.0	0.28
LR1	19.8	52.8	0.25
LR2	19.7	50.8	0.43
D/HR	19.5	50.8	0.29
LSD (*P*=0.05)	0.91	1.98	0.07
inoculum density	*P*<0.001	*P*<0.001	*P*=0.001
isolate	*P*=0.087	*P*<0.001	*P*<0.001
interaction	*P*=0.052	*P*=0.242	*P*=0.215
CV %	3.92	3.30	25.09

[a] Each mean of inoculum density is an average of 27 observations, each mean of isolate is an average of 6 observations.

[b] *P*-values for the *F*-test from analysis of variance.

[c] S=sensitive isolates, HR=high-resistant MBC isolates, LR=low-resistant MBC isolates, D/HR=dual dicarboximide/high-resistant MBC isolate.

Table 2.4 The fitness components, incubation period, latent period, rate of colonisation and rate of spore production on fruit of *M. fructicola* field isolates resistant and sensitive to MBC fungicide.

Isolate[c]	Incubation period (hours)		Latent period (hours)		Rate of colonisation (mm-diameter /hour)		Rate of spore production (conidia x10^{-6} / fruit/ hour)	
	inoculum density (conidia/ml)							
	$1x10^2$	$1x10^4$	$1x10^2$	$1x10^4$	$1x10^2$	$1x10^4$	$1x10^2$	$1x10^4$
S1	16.0	14.0	56.0	28.7	1.05	1.04	1.4	2.2
S2	14.7	14.0	57.3	32.0	0.97	1.02	0.2	0.5
HR1	16.0	14.0	53.3	27.7	1.07	1.02	1.8	1.7
HR2	16.0	16.0	63.3	35.0	0.91	1.03	0.1	0.2
HR3	16.0	14.0	42.7	24.3	0.84	0.99	1.1	1.4
HR4	16.0	15.3	45.7	28.7	0.98	0.93	1.2	1.7
LR1	16.0	14.0	45.0	34.0	1.18	1.18	0.7	1.4
LR2	15.3	13.3	50.3	36.7	0.99	1.14	1.3	1.2
D/HR	15.3	14.0	46.7	32.7	0.69	0.88	1.0	1.1
inoculum density	$P<0.001$		$P<0.001$		$P=0.025$		$P<0.001$	
isolate	$P<0.001$		$P<0.001$		$P<0.001$		$P<0.001$	
interaction	$P=0.014$		$P=0.004$		$P=0.230$		$P=0.009$	
LSD ($P=0.05$)	0.92		7.16		0.16		0.38	
CV %	3.7		10.5		0.096		20.6	

[a] Each mean is an average of 3 observations.

[b] *P*-values for the *F* test from analysis of variance.

[c] S=sensitive isolates, HR=high-resistant MBC isolates, LR=low-resistant MBC isolates, D/HR=dual dicarboximide/high-resistant MBC isolate.

($P<0.05$) significantly in the rate of spore production compared to the other isolates.

2.3.3 COMPETITIVE ABILITY *IN VIVO*

The outcome of competition between strains of *M. fructicola* on flowers and fruit is illustrated in the form of linear regression lines as examples (Figure 2.2) and full details were presented in the form of regression equations (Table 2.5) over the linear phase of competition. The slope parameter (b) derived from the regressions of initial and final proportions of resistance described the competitiveness of resistant and sensitive strains.

Inoculum densities, at $1x10^2$ and $1x10^4$ conidia / flower, generally did not significantly ($P>0.05$) affect the outcome of competition in nearly all resistant and sensitive isolate pairs (HR2:S1, D/HR:S1, HR1:S2, HR2:S2, HR3:S2 and D/HR:S2). However, two pairs, HR1:S1 and HR3:S1 showed significant differences at $P<0.05$ and $P<0.001$, respectively. The HR1xS1 pair competed significantly ($P<0.05$) better at the low inoculum density ($1x10^2$) than at the high inoculum density ($1x10^4$), whereas of the S1xHR3 which competed better ($P<0.001$) at the high than the low inoculum densities. Similarly, on fruit there were three isolate pairs (HR3:S1, HR3:S2 and D/HR:S1) that showed significantly differences ($P<0.001$) in their ability to compete at different inoculum concentrations. In contrast to the competition on blossom, all these isolate pairs competed better at the high inoculum concentration ($1x10^4$) than that of at low inoculum concentration ($1x10^2$). The other resistant and sensitive isolate pairs showed no significant ($P>0.05$) differences between inoculum concentrations.

On blossom (Table 2.5), an isolate (HR3) competed significantly ($P<0.001$) better than both sensitive isolates (S1 and S2). A resistant isolate (HR1) was equally ($P>0.05$) competitive with S1, but it was significantly ($P<0.05$) more competitive than S2. The other two resistant isolates, HR2 and D/HR, were significantly ($P<0.05$) less competitive with S1. However, HR2 at low

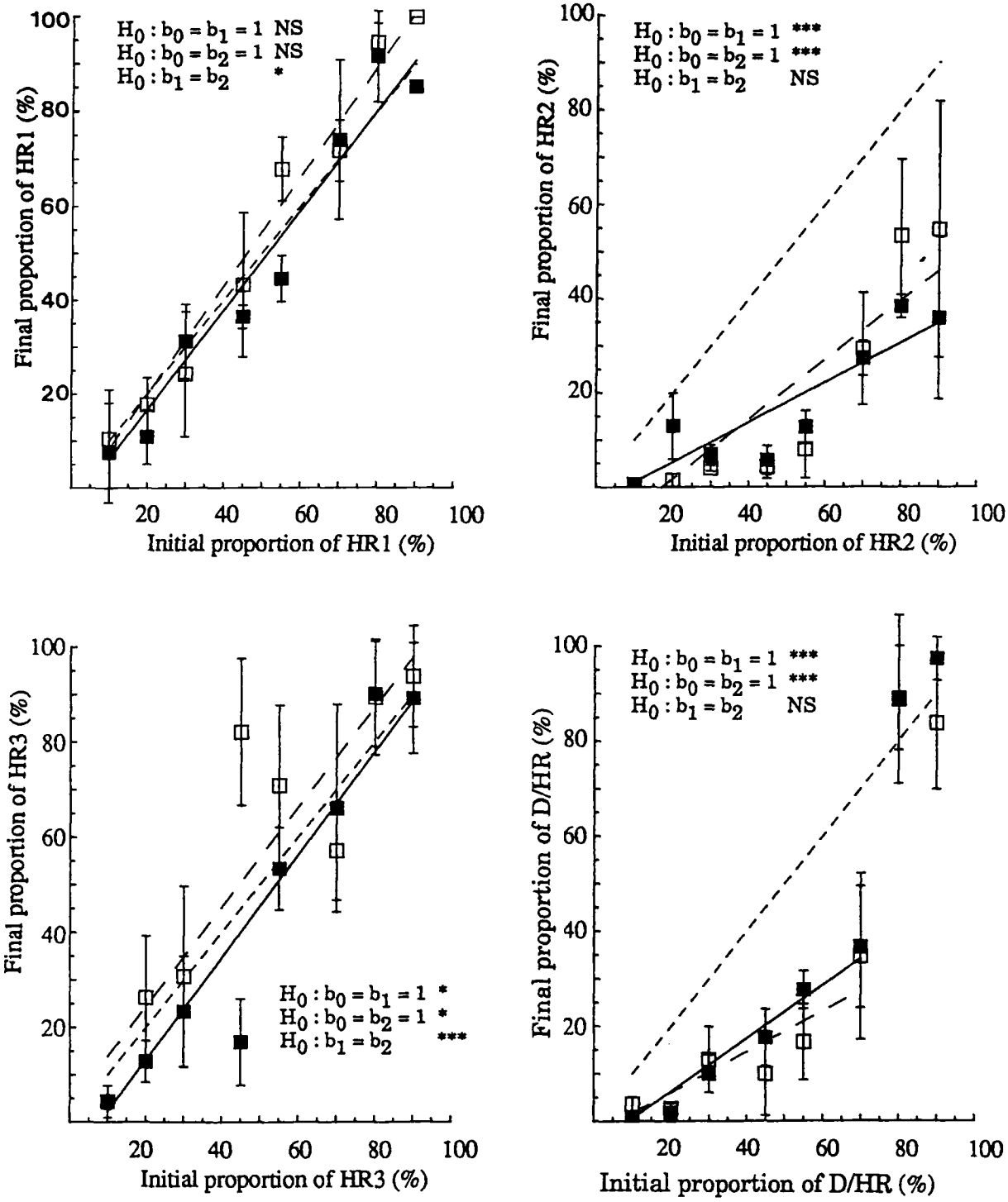

Figure 2.2 Representative patterns of the competitive ability of four MBC resistant *M. fructicola* field isolates with a sensitive (S1) on flower of peach-*cv* Glohaven at $1x10^2$ (b_1)(□——□) and $1x10^4$ (b_2)(■——■) conidia/flower. Error bars are ± the standard error of the sample. Dotted line (-------) is a line of equal competition with slope (b_0)=1. ^{NS}, *, **, and *** stand for *T*-test of H_0: $b_0=b_1=1$, $b_0=b_2=1$ and $b_1=b_2$ at $P>0.05$, $P<0.05$, $P<0.01$ and $P<0.001$, respectively. S= MBC sensitive isolate, HR=high-resistant MBC isolate, D/HR=dual dicarboximide/high-resistant MBC isolate.

Table 2.5 The competitive ability of four MBC resistant *M. fructicola* field isolates with sensitive isolates on peach blossom (*cv* Glohaven) and on nectarine fruit (*cv* Fantasia) at $1x10^2$ and $1x10^4$ inoculum concentrations.

Isolate pairs [a]	Outcome of competition at $1x10^2$ conidia ($H_0:b_1=1$)			Outcome of competition at $1x10^4$ conidia ($H_0:b_2=1$)			
	a [b]	b_1 [c]	r^2 [d]	a [b]	b_2 [c]	r^2 [d]	$H_0:b_1=b_2$
FLOWER							
HR1:S1	-3.4	1.16^{NS}	0.94	-4.6	1.06^{NS}	0.92	*
HR2:S1	-10.8	0.63^{***}	0.66	-3.06	0.42^{***}	0.72	NS
HR3:S1	3.9	1.05^{*}	0.77	-8.5	1.08^{*}	0.87	***
D/HR:S1	-2.6	0.43^{***}	0.83	-4.9	0.56^{***}	0.95	NS
HR1:S2	4.7	1.06^{***}	0.92	-1.7	1.13^{***}	0.96	NS
HR2:S2	2.4	0.90^{NS}	0.92	-1.9	0.94^{*}	0.91	NS
HR3:S2	12.3	1.06^{***}	0.94	18.0	1.15^{***}	0.77	NS
D/HR:S2	-2.8	0.89^{***}	0.86	-6.2	1.01^{***}	0.93	NS
FRUIT							
HR1:S1	7.7	1.07^{***}	0.85	0.1	1.14^{***}	0.89	NS
HR2:S1	-1.0	0.09^{***}	0.83	-0.6	0.05^{***}	0.69	NS
HR3:S1	-9.1	1.08^{***}	0.87	-0.5	1.23^{***}	0.87	***
D/HR:S1	-1.1	0.06^{***}	0.52	-5.7	0.34^{***}	0.66	***
HR1:S2	7.5	0.98^{***}	0.84	2.2	1.09^{*}	0.89	NS
HR2:S2	-1.5	0.12^{***}	0.69	-0.3	0.04^{***}	0.71	NS
HR3:S2	-4.2	0.87^{*}	0.75	-1.0	1.14^{***}	0.92	***
D/HR:S2	-1.5	0.14^{***}	0.66	-3.4	0.26^{***}	0.43	NS

[a] S=sensitive isolates, HR=high-resistant MBC isolates, LR=low-resistant MBC isolates, D/HR=dual dicarboximide/high-resistant MBC isolate.

[b] ordinate (a) from the regression equation, % resistance after competition = a + b(% resistance before competition)

[c] slope (b) from the regression equation, % resistance after competition = a + b(% resistance before competition)

[d] r^2 of the slope, % resistance after competition = a + b(% resistance before competition)

NS ($P>0.05$), * ($P<0.05$), ** ($P<0.01$) and *** ($P<0.001$) for statistic (t-test).

inoculum density was able to compete equally ($P>0.05$) with S2, where the isolate was significantly ($P<0.05$) less competitive than S2 at the high level of inoculum. D/HR was also significantly ($P<0.05$) less competitive with S2. In the D/HR:S1 isolate pair, regression of the final and initial proportions was linear until the initial proportion of 70% resistant mixture was reached (Figure 2.2). Beyond that point, the D/HR regained it's competitive ability, so the plot represented for the competitive outcome may be better by fitting a quadratic curve instead of the linear regression used. The latter regression was used so direct comparison of other isolate pairs could be made.

On fruit, HR1 was significantly ($P\leq0.01$) more competitive than either S1 and S2 at both inoculum concentrations. The competitive ability of HR3 was significantly ($P<0.05$) higher than S1 or S2 at the high inoculum density, but it was significantly ($P<0.05$) less competitive at the low inoculum density than the two sensitive isolates. Both HR2 and D/HR isolates were very poor ($P<0.05$) competitors on fruit compared to S1 and S2 isolates. The competitive patterns of two pairs, HR2:S1 and HR2:S2, performed similarly to the D/HR:S1 as described previously on blossom. The resistant isolate (HR2) at higher proportions in the mixtures, beyond the point of 70% resistant mixture, had gained a chance to compete with those sensitive strains compared to the lower proportions.

2.4 DISCUSSION

There were at least three sensitivity groups to the MBC fungicides in the fungus *M. fructicola* found in these diseased samples. They were sensitive (EC_{50} <1 mg a.i./l), low-resistant (EC_{50} <3 mg a.i./l) and high-resistant EC_{50} > 30,000 mg a.i./l). This is in agreement with the reports of resistance in Australia (Penrose *et al.*, 1979) and the United States (Jones and Ehret, 1976; Ogawa *et al.*, 1988). It was found recently (1991) that low resistant MBC isolates were established in home gardens as well as in commercial orchards in Central Otago, New Zealand (Braithwaite, M. pers. comm.). Most resistant isolates from imported nectarine were resistant only at low degree, similar to

similar to the degree reported in Californian orchards, USA. However, one diseased fruit carried a resistance degree as high as the local isolates from the North Island. Various degrees of resistance to the same fungicide may appear in the same populations which suggest that the low resistance may be masked in the high resistant population. The degrees of resistance to the MBC fungicides in *M. fructicola* may be similar to MBC resistance in *V. inaequalis*, *V. nashicola*, *V. pyrina* (Shabi *et al.*, 1983; Shabi *et al.*, 1986) and in *B. fuckeliana* (Faretra and Pollastro, 1991).

Selection pressure may affect resistance at different degrees. In New Zealand, high degrees of resistance were reported in the North Island, whereas, in the South Island resistance at both degrees have only just been found (Braithwaite, M. pers. comm.). Three possible reasons for these regional variations in resistance may be considered. Firstly, the recent emergence of the MBC resistance in the South Island is likely to occur because of an increased frequency of MBC fungicide application (Braithwaite *et al.*, 1991). Secondly, disease pressure in the South Island is much lower than in the North Island because of the difference in weather conditions (Atkinson, 1971). In the North Island, wet weather probably stimulates the fungus to produce numerous conidia and infection cycles and extensive applications of fungicide are needed. It is generally accepted that the more conidia, the higher the chance of mutation. Mutation of different alleles at the same locus may confer different degrees of resistance (Shabi *et al.*, 1986; Faretra and Pollastro, 1991). Intensive applications of the MBC fungicide in the North Island may result in selection of the higher degree of resistance than in the South Island. Lastly, the movement of summer fruit from the North Island and the low-MBC resistant *M. fructicola* isolates detected from the Californian imported fruit may have provided a source of such strains in the South Island.

Movement of resistance from imported fruit may have contributed to the existence and variation of resistance. A total of 52-75% of diseased fruit sampled from California imported in 1989 and sold in the Christchurch district were carrying resistance at a low degree. Although, only two samples were

collected and can not be representative of Californian imported fruit, the figure may indicate a migration possibility for resistance. The possibility of contaminating resistant strains establishing themselves in this new environment is still in question as the imported fruit arrive in New Zealand during winter (July), before any development of blossom. If the diseased fruit were accidentally left on the ground nearby stone fruit trees, the fungus would have a chance to survive and reestablish. Winter is often associated with high humidity because of heavy dew or showers and the *M. fructicola* can survive well under these conditions (Byrde and Willetts, 1977; Penrose *et al.*, 1979). However, if there is very high humidity sporulation will be suppressed so that no freshly produced spores will be available to provide initial inoculum for blossom in the following season. But, if the diseased tissues are positioned in a moist, favourable place, sexual reproduction may develop. Ascospores carrying new resistant genotypes may be produced the following spring. The development will be the same as the larger size diseased fruit or the late infected fruit that could not produce apothecia in the following spring (Byrde and Willetts, 1977; Chapter 4).

Resistance to MBC fungicide was found in Hawkes Bay properties at 0-41% of samples and does not differ (10-35%) from the previous reports (Elmer and Gaunt, 1986; Adaskaveg *et al.*, 1987; Penrose, 1990; Braithwaite *et al.*, 1991). The same percentage found in this report, will probably persist in the absence of fungicide selection pressure. Applications of benomyl greatly increased the frequency of the resistant strains to the point where infections by these strains predominated in the population only after one or two sprays (Kable, 1983; Zehr *et al.*, 1991). A similar situation to that on the properties in Australia and United States involved benomyl resistance which increased explosively from 20% to almost 90% (Ogawa *et al.*, 1988) after a single benomyl application. This pessimistic outcome would be expected if there is any reintroduction of the MBC fungicides in the resistant infested area, especially in the North Island where the frequency of MBC resistance is extremely high.

Most of the resistant strains tested in this study were equally fit and pathogenic as the sensitive strains, although this feature was reported to vary considerably (Jones and Ehret, 1976; Zehr *et al.*, 1991). Individual isolates of both resistant and sensitive strains of *M. fructicola* varied in growth rate on PDA (Appendix 1). Many of these isolates also showed some differences in duration of IP, LP, rate of colonisation and rate of spore production on blossom and fruit. Generally, these results *in vivo* agreed with the work reported by Ogawa *et al.* (1981) that blossom infections were influenced by specific isolates regardless of their resistance or sensitivity to MBC fungicide. Similar results indicated that rate of spore production on fruit is likely to indicate which strains will dominate the pathogen population. Therefore, it can be concluded that a fitness deficit is not associated with any high and low degrees of resistance in the fungus to the MBC fungicide. The difference in fitness factors is probably associated with individual isolate.

Under the conditions of this study, the resistant isolates seemed to be as competitive as the sensitive isolates, although the results were varied. Stabilising selection pressure will probably make sure that the less competitive strains do not survive in the population. Only the equally fit and competitive strains will survive in the population, in the absence of fungicide selection pressure.

Results from this study demonstrate the central role that inoculum density contributes to the development of blossom blight and fruit brown rot in stone fruit. It supports the epidemiological concept that amount of inoculum is an important component in the development of plant disease epidemics (Zadoks and Schein, 1979: Campbell and Madden, 1990). The quantitative frequencies of overwintering and oversummering are important for development of blossom blight in the spring and fruit brown rot in summer. It is also important to investigate the ability of resistant and sensitive strains to overwinter and oversummer and to identify which ones are the major sources of inoculum of the disease. The work will be reported in the following Chapter.

CHAPTER 3
SURVIVAL OF *M. fructicola* ISOLATES RESISTANT TO MBC AND DICARBOXIMIDE FUNGICIDES

3.1 INTRODUCTION

Pathogen inoculum density is a driving component in the development of plant disease epidemics (Campbell and Madden, 1990). The survival ability, viability and dispersal ability of infective propagules are important factors that contribute to the rate of development of the epidemic. Many interacting effects such as types of fungicide used and methods of application, environment, and pathogen fitnesses influence the density and epidemiological competence of pathogen propagules. The result of these interactions is the presence of an amount of primary and secondary inoculum of the pathogen able to infect a potential host plant (Campbell and Madden, 1990; Rayner, 1991). In describing and quantifying epidemic development, it is necessary to quantify inoculum as propagule density at a specified time or monitor frequency of occurrence of propagules continuously or discontinuously throughout an epidemic. Knowledge of the pathosystem, the type of propagules that may be present, viability and infective ability of propagules is required and the method chosen to quantify propagule numbers should be epidemiologically meaningful.

The frequency of resistance to different fungicides in a population is dependent on the relative ability of the resistant strains to persist. In *B. cinerea* on grapes, the incidence of MBC resistance in the United Kingdom has not decreased since 1984 in spite of a considerable reduction in the use of the MBC fungicides (Locke and Fletcher, 1988). The strains resistant to MBC fungicides in plant pathogenic fungi in most cases are very fit, very stable and persist for many years in the absence of fungicide selection pressure (Staub, 1991). On the other hand, resistance to dichlofluanid and to vinclozolin declined steadily after the application of fungicides (Yunis and Elad, 1989; Rewal *et al.*, 1991, Zehr *et al.*, 1991). Therefore, long term investigation may

may be necessary. In *V. inaequalis* strains resistant to the sterol demethylation inhibitor flusilazole, phenotypic expression reversed from resistant to sensitive phenotypes after exposure to cold storage at 2 °C for 7 months (Koller *et al.*, 1991). The reversion of resistant strains is related to a high degree of plasticity in the genetic control of sensitivities to the DMI fungicide. Fitness deficit and instability under certain conditions may be responsible for the inability of resistant strains of a fungus to persist in the field. Similarly, *M. fructicola* laboratory strains resistant to the DMI fungicides, which is associated with lower fitness and instability at high resistant levels (Nuninger-ney *et al.*, 1989), may not persist effectively in the field.

The brown rot fungus overseasons as mycelium in infected plant parts (Byrde and Willetts, 1977; Landgraf and Zehr, 1982; Biggs and Northover, 1985). Primary inoculum produced in spring are in two forms: conidia and ascospores. Conidia are differentiated under favourable conditions from mycelium in the mummified fruit, fruit peduncles, cankers on twigs and branches, leaf scars, and buds (Figure 3.1a, 3.1b). Ascospores are produced only from stromata in mummified fruit on the ground. Secondary inoculum as conidia can arise from any infected tissue in which the moisture content is sufficient for sporulation. Sources of inoculum should be identified not only to control the brown rot disease in stone fruit orchards, but also to prevent significant losses during postharvest storage and marketing. A significant reduction in the number of fungicide applications made possible by good cultural practices will be of great importance to delay the development of fungicide resistance. Good cultural practices may provide effective control strategies against resistant problems, because a decrease in inoculum density is likely to decrease disease pressure.

The relative importance of primary and secondary sources of inoculum is uncertain. There is only limited quantitative information on the importance of the different inoculum sources (Landgraf and Zehr, 1982) and relatively few in association with strains resistant to MBC and dicarboximide fungicides in infected tissues. Kable (1965) considered infected peduncles to be very

important in New South Wales, Australia. He reported that mummified fruit were potential sources but, their importance can be overemphasized. The fungus produced many conidia on infected plant parts if there was adequate moisture content in the diseased tissues (Corbin and Cruickshank, 1962). In contrast, Landgraf and Zehr (1982) failed to induce conidial production from these diseased tissues, except on one fruit peduncle, and as a consequence, sporulation on fruit peduncles, twig cankers and mummified fruit may not be epidemiologically important in South Carolina. Measurement of the amount of sporulation on host tissue might be an appropriate indicator to identify the main sources of inoculum. Comparative experiments can be conducted to distinguish the relative ability of resistant and sensitive isolates to survive under adverse conditions in the field. This chapter describes the potential for inoculum production from inoculated twig cankers and mummified fruit after a period of survival in the field by different isolates resistant and sensitive to MBC and dicarboximide fungicide.

3.2 MATERIALS AND METHODS

The same isolates used in Chapter 2 including DR isolate (Table 2.1) were grown on PDA or V-8 juice agar at 25 °C (±0.5 °C) with 14 hour photoperiod of fluorescent and NUV light for 4-5 days. They were used for twig cankers and mummified fruit inoculation.

Four year old nectarine trees (*cv* Fantasia) were potted in polyurethane planter bags (PB40) with a long term release potting mix. One cubic metre of the potting mix were consisted of 800 litre composted bark, 200 litre fine sand, 7.5 kg fertiliser (12-14 months Osmocote 18-2.1-9.1), 1 kg superphosphate, 6 kg dolomite lime and 300 g Micromax trace elements. The trees were potted before the spring (July) 1989. One year old twigs were wounded to the cambium with a very sharp cork borer, 4 mm diameter. The cortex tissues were peeled off to exposure the cambium tissue. The wounds were inoculated in late spring (October) 1989 by placing a mycelial plug (4 mm diameter) from PDA cultures on to the wounded site and sealed with 18 mm width

transparent adhesive tape (Scotch 600 cellulose tape) for 7-10 days to prevent desiccation (Ogawa and English, 1960; Feliciano *et al.*, 1987). There were twenty wounded sites on each tree which there were only one isolate / tree. The experiment was conducted with a 2 x 2 factorial design (Table 3.1) of 10 isolates and the cankers were collected on 7 occasions after inoculation. The trees were arranged in a randomized complete block design with three replications and 4 trees / isolate / replication. The inoculated trees were placed outdoors with standard orchard row spacing and watered twice a week.

Pathogenicity was assessed by measuring the length of the cankers 7 and 12 days after inoculation. Sporulation was induced in the laboratory on twigs with cankers sampled on 7 occasions at 20, 40, 60, 80, 100, 300 and 370 days after inoculation, using a method described by Corbin and Cruickshank (1962). The twigs with whole cankers were detached, measured and washed under running tap water to remove old conidia and then surface sterilised with 0.5% (v/v) sodium hypochlorite plus 0.05% (v/v) Tween 20 for three minutes. They were then rinsed, soaked with tap water for 6 hours at room temperature and the excess water was removed. The cankers were placed upright individually in a piece of polystyrene in semi-transparent plastic pottles with a lid, containing 10 ml water to provide a moist environment (Figure 3.1c). The twigs were incubated at 25 °C (±0.5 °C) for 48 hours with a 12 hour photoperiod of fluorescent and near ultra-violet (NUV) light until maximum spore production (Corbin and Cruickshank, 1962). Conidia were removed by shaking the whole twigs in 50% aqueous ethanol for 1 minute and the number quantified with the aid of haemocytometer. The number of conidia produced per 10 mm of twig canker length was calculated and results analyzed using two way ANOVA.

All isolates were re-isolated from twig cankers at the fruit ripening period (10 February 1990) and flowering (28 August 1990) in the following season and their pathogenicity and fitness components after survival were investigated using the methods described in Chapter 2. The only inoculum concentration used was $1x10^4$ spore/ fruit or flower; the summer re-isolation was tested on

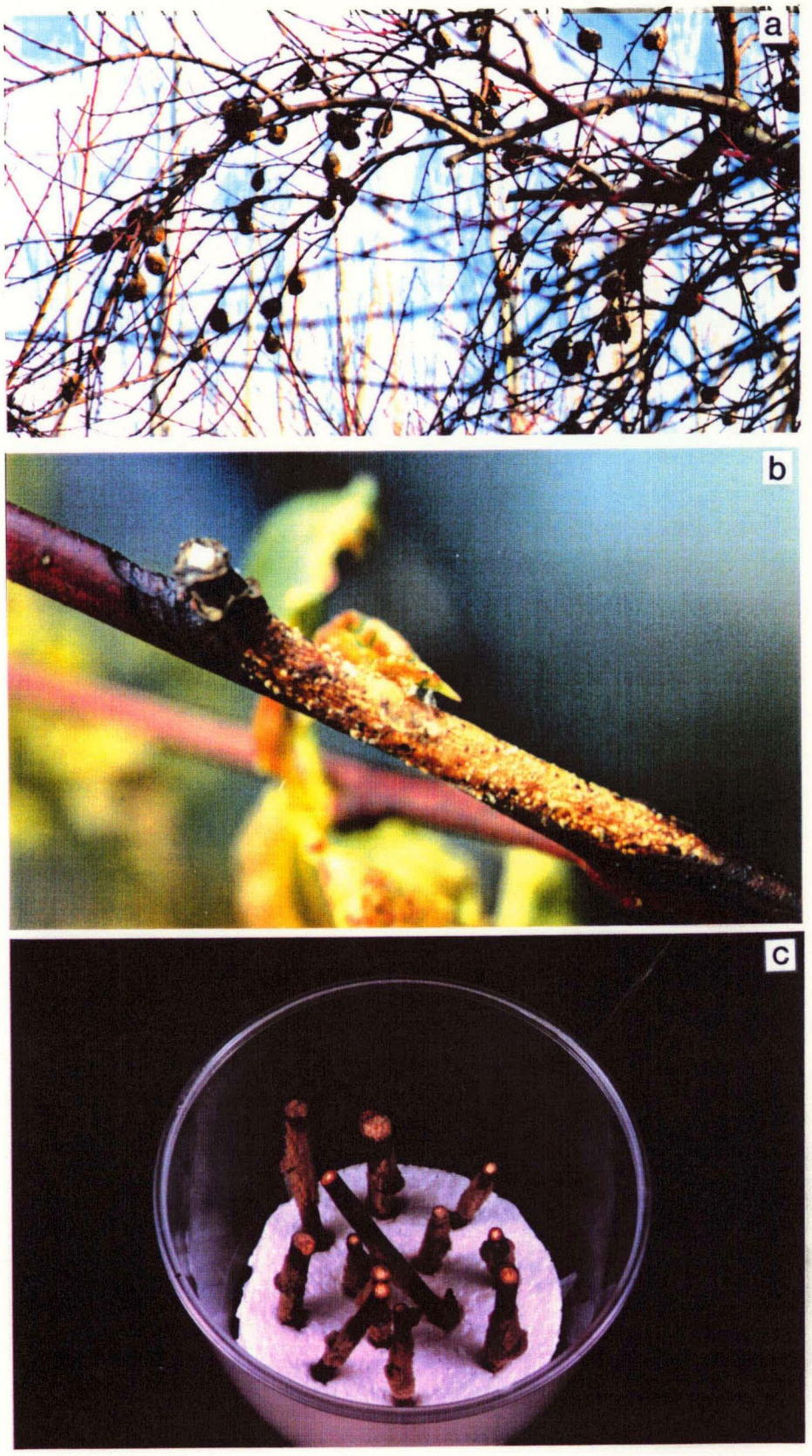

Figure 3.1 Inoculum sources in infected stone fruit trees. a) mummified fruit b) inoculated twig canker and c) sporulation on inoculated twig cankers under conducive conditions.

fruit and the spring re-isolation was tested on blossoms to determine their pathogenicity.

Nectarine fruit (*cv* Fantasia) of uniform maturity and size were washed thoroughly to remove protectant fungicide residues. The fruit were surface sterilised in 0.5% (v/v) sodium hypochlorite plus 0.05% (v/v) Tween 20 for 2.5 minutes. They were allowed to dry at room temperature and were put in open net bags, 5 fruit/bag and the enclosed fruit were placed carefully in the compartment of plastic 'Plix' trays. One hundred trays, 2500 fruit, were prepared and all of the fruit were wounded by pricking 5 times on the fruit surface with a flamed-sterilised set of 10 metal pins to allow infection to occur evenly. The fruit were sprayed with a conidial suspension ($1x10^6$ conidia/ml) of test isolate individuals until run off and were covered with transparent plastic bags to maintain a high relative humidity. These fruit were incubated at room temperature for 5 days until the fruit were fully colonised. The diseased fruit were then hung in the bags in a tree shade (walnut trees) more than 1,000 metre from stone fruit trees to mummify naturally. Six weeks later, when the fruit were fully mummified, the bags were transferred to hang in tree canopies in a peach block isolated from commercial orchards. The mummified fruit were hung in the field in a randomized complete block design with three replications. There were 10 fruit / isolate / harvest / replication with the 2x2 factorial consisting of 10 isolates and 6 harvesting occasions after exposure to the field (Table 3.1). Two bags (ten fruit)/ isolate/ replication were collected monthly from May to November 1990. The collected mummified fruit were washed, surface sterilised, rinsed and soaked by the same method as for twig cankers. The mummified fruit were placed on a wire screen mesh (20x20 mm^2) in plastic trays with moist paper towels in the bottom of the trays and enclosing them in sealed plastic bags. They were incubated to sporulate at 25 °C (±0.5 °C) for 48 hours with 12 hour photoperiod of fluorescent and NUV light until maximum spore production (Corbin and Cruickshank, 1962). Conidia were removed by shaking the mummified fruit mechanically in 50% aqueous ethanol and the conidia were counted by using haemocytometer. The number of conidia produced per mummified fruit was calculated and results

Table 3.1 Experimental designs conducted for survival investigations and subsequent pathogenicity on host tissues.

	Experiments	Designs	Replication
1	Pathogenicity on twig cankers	Randomized completely block design	3 replications with at least 10 cankers / replication
2	Survival on twig cankers	2x2 Factorial design of 10 isolates and 7 harvested times	3 replications with 10 cankers / replication
3	Survival on mummified fruit	2x2 Factorial design of 10 isolates and 6 harvested times	3 replications with 10 mummified fruit / replication
4	Pathogenicity of the survivor on blossom after 1 year on twig	2x2 Factorial design of 10 survivors and their original at $1x10^4$ conidia / flower	3 replications with at least 10 flowers / replication
5	Pathogenicity of the survivor on fruit after 3 months on twig	2x2 Factorial design of 10 survivors and their original at $1x10^4$ conidia / fruit	3 replications with at least 1 fruit / replication

analyzed using two way ANOVA.

3.3 RESULTS

At 7 and 12 days after inoculation, there were significant ($P<0.001$) differences between the 10 isolates in the length of cankers (Table 3.2). The average canker lengths (2100 cankers) observed over the whole year were also significantly ($P<0.001$) different between the ten isolates. One high-resistant MBC isolate (HR2) produced consistently shorter ($P<0.05$) cankers than the others at all stages. One low-resistant MBC isolate (LR1) also produced very short ($P<0.05$) cankers at 7 days after inoculation, however, the development of canker by the isolate increased sharply and the length became no different ($P>0.05$) to the other isolates when measured at 12 days after inoculation. Isolate LR2 was the most pathogenic and produced the largest canker. In addition, significant ($P<0.01$) replicate effects were observed which may have occurred as a result of 2 days between the time of inoculation between individual replications. The mean canker length was 31.4 mm and there was no evidence to suggest that reduced canker length was associated with fungicide resistance (MBC or dicarboximide).

There were highly significant differences ($P<0.001$) in the ability to survive and reproduce conidia on twig cankers between isolates, the length of time after exposed to the field and the interaction of the two components (Table 3.3). The low resistant MBC isolate (LR2) produced significantly ($P<0.05$) larger amounts of conidia compared to all other isolates. One high-resistant MBC (HR2), one dual dicarboximide/high-resistant MBC (D/HR) and a dicarboximide resistant (DR) isolate produced significantly ($P<0.05$) fewer conidia, compared to the sensitive standards (S1 and S2) and the other high and low resistant MBC isolates. There were significant ($P<0.05$) differences within the high resistant MBC isolate group suggesting that the ability to survive and reproduce conidia is associated with each individual isolate. No obvious significant differences between high-resistant MBC and sensitive isolates were detected.

Table 3.2 Mean canker length (mm) on nectarine twigs (*cv* Fantasia) by isolates of *M. fructicola* resistant and sensitive to MBC and dicarboximide fungicides at different intervals after inoculation.

Isolate[a]	Mean twig canker length (mm)		
	7 days[b]	12 days[b]	all year[c]
S1	29.2	38.2	35.1
S2	20.5	30.4	26.1
HR1	23.6	39.1	35.6
HR2	15.5	15.3	20.7
HR3	22.6	31.1	31.2
HR4	32.7	31.2	38.1
LR1	11.1	34.3	28.5
LR2	33.3	45.5	36.5
D/HR	22.4	30.9	28.6
DR	32.4	34.6	33.4
Isolates	$P<0.001$	$P<0.001$	$P<0.001$
Replication	$P=0.005$	$P<0.001$	$P<0.001$
LSD ($P=0.05$)	4.27	4.47	8.17
CV(%)	5.86	4.54	0.18

[a] S=sensitive isolate, HR=high-resistant MBC isolate, LR=low-resistant MBC isolate, D/HR=dual dicarboximide/high-resistant MBC isolate and DR=dicarboximide resistant isolate.

[b] mean of 30 cankers.

[c] mean of 210 cankers measured at 7 occasions for the whole year.

Table 3.3 Number of spores produced[a] (conidia x 10^{-4} / 10 mm canker length) on inoculated twig cankers[b] under conducive conditions in the laboratory of *M. fructicola* isolates resistant and sensitive to MBC and dicarboximide fungicides after exposure to field conditions in 1989/90.

Isolate[c]	Number of conidia[a] ($1x10^{-4}$)							
	1989		1990					**MEAN**
	20 NOV	10 DEC	1 JAN	20 JAN	10 FEB	28 AUG	24 NOV	
S1	67	74	81	130	143	41	32	**81**
S2	62	107	115	98	151	49	14	**85**
HR1	46	189	69	131	79	88	102	**101**
HR2	61	36	27	31	32	43	26	**37**
HR3	70	85	103	116	139	61	96	**96**
HR4	70	90	86	98	95	84	59	**83**
LR1	98	81	52	113	113	61	73	**84**
LR2	176	129	150	276	265	124	103	**175**
D/HR	33	27	37	65	41	41	9	**36**
DR	16	2	19	21	42	25	10	**19**
MEAN	**70**	**82**	**74**	**108**	**110**	**62**	**52**	**80**

SOURCE	df	MS	*F*-ratios	*P*-values	LSD (*P*=0.05)
Isolate	9	40033	35.37	<0.001	24.93
Harvesting	6	14535	12.84	<0.001	17.03
interaction	54	2706	2.39	<0.001	
Replication	2	1668	1.47	0.233	
Error	138	1132			
CV=0.42%,	LSD (*P*=0.05) = 54.39				

[a] mean number of conidia taken from 30 twig cankers.

[b] all twig cankers were inoculated on 24-31 October 1989

[c] S=sensitive isolate, HR=high-resistant MBC isolate, LR=low-resistant MBC isolate, D/HR=dual dicarboximide/high-resistant MBC isolate and DR=dicarboximide resistant isolate.

Table 3.4 Number of spores produced[a] (conidia x 10^{-6} / mummified fruit[b]) on inoculated-mummified fruit under conducive conditions in the laboratory of *M. fructicola* isolates resistant and sensitive to MBC and dicarboximide fungicides after exposure to fields conditions in 1990.

Isolate[c]	Date of collection (1990)						
	10 MAY	4 JUN	28 JUN	22 JUL	20 AUG	6 NOV	**MEAN**
S1	83	85	54	74	15	5	**53**
S2	63	53	76	69	16	4	**47**
HR1	83	87	76	46	31	24	**58**
HR2	53	59	27	27	21	8	**33**
HR3	81	124	85	64	51	8	**69**
HR4	83	117	47	75	72	20	**69**
LR1	97	173	120	94	81	51	**103**
LR2	110	77	79	47	61	51	**71**
D/HR	70	84	49	51	18	7	**47**
DR	52	55	44	42	21	9	**37**
MEAN	**78**	**91**	**66**	**59**	**39**	**19**	**58**

SOURCE	df	MS	*F*-ratios	*P*-values	LSD (*P*=0.05)
Isolate	9	7489.9	16.10	<0.001	14.23
Harvesting	5	20959.3	45.05	<0.001	11.03
interaction	45	774.3	1.66	0.016	
Replication	2	41.5	0.09	0.915	
Error	118	465.2			

CV= 0.36%, LSD (*P*=0.05) = 34.87

[a] mean number of conidia taken from 30 mummified fruit

[b] all mummified fruit were inoculated on 10 February 1990

[c] S=sensitive, HR=high-resistant MBC, LR=low-resistant MBC, D/HR=dual dicarboximide/high-resistant MBC and DR=dicarboximide resistant

The length of time after twig inoculations significantly ($P<0.001$) influenced sporulation ability in all isolates. The quantitative amount of spore production significantly ($P<0.05$) increased during summer and significantly ($P<0.05$) decreased during winter. However, there was no significant ($P>0.05$) differences in the amount of spore production between November 1989 and August and November 1990 one year after exposure under natural conditions. The fluctuating pattern of spore production was similar for all isolates tested.

On fruit, two main factors, isolates and the length of time of exposure of mummified fruit in the field, significantly ($P<0.001$) affected the ability to produce conidia (Table 3.4). There was significant ($P<0.05$) differences in interaction between isolates and the time of harvest. The differences may result from the sporulation ability during exposure to the field. One low resistant MBC isolate (LR1) significantly ($P<0.05$) produced more conidia than the others, whereas one high resistant MBC isolate (HR2) and the dicarboximide resistant isolate (DR) produced significantly ($P<0.05$) fewer conidia. The spore production of all isolates decreased significantly ($P<0.001$) during winter.

The pathogenicity of the re-isolated fungus was maintained ($P>0.05$) for at least one year after overseasoning on twig cankers but significant ($P<0.001$) to differences between isolate individuals on the length of latent period and rate of spore production (Table 3.5). There were no significant effects ($P>0.05$) in all three fitness components on blossom, except for the latent period (LP) between the re-isolated and the original isolates. The re-isolated fungus significantly ($P<0.001$) produced conidia faster than the original isolates. Similarly, there were no significant ($P>0.05$) differences on fruit for all fitness factors after oversummering for 100 days on twig cankers, except for the rate of colonisation (Table 3.6).

Table 3.5 A comparison of fitness components on blossoms of *M. fructicola* isolates resistant and sensitive to MBC and dicarboximide fungicides after oversummering and overwintering on twig cankers in the field.

	Incubation period (hours)	Latent period (hours)	Rate of spore production (conidia $x10^{-4}$/flower)
SURVIVORSHIP			
ORIGINAL[a]	17.0	43.0	0.30
RE-ISOLATED[b]	16.7	41.5	0.32
LSD (*P*=0.05)	0.53	0.87	0.02
ISOLATES[c]			
S1	16.3	40.5	0.36
S2	16.7	44.2	0.32
HR1	16.7	41.8	0.29
HR2	18.0	43.0	0.15
HR3	16.3	39.0	0.37
HR4	17.0	41.3	0.35
LR1	16.2	44.5	0.49
LR2	17.0	42.2	0.24
D/HR	16.5	41.5	0.32
DR	17.7	44.5	0.16
LSD (*P*=0.05)	1.20	1.95	0.06
Isolate	*P*=0.064	*P*<0.001	*P*<0.001
Survivorship	*P*=0.218	*P*=0.001	*P*=0.145
interaction	*P*=0.647	*P*=0.843	*P*=0.253
CV%	0.06	0.04	0.18

[a] original isolates indicated in [c] stored at 4 °C in refrigerator.

[b] *M. fructicola* re-isolated from twig cankers on 28 August 1990.

[c] S=sensitive isolate, HR=high-resistant MBC isolate, LR=low-resistant MBC isolate, D/HR=dual dicarboximide/high-resistant MBC isolate and DR=dicarboximide resistant isolate.

Table 3.6 A comparison of fitness components on fruit of *M. fructicola* isolates resistant and sensitive to MBC and dicarboximide fungicides after oversummering on twig cankers in the field.

	Incubation Period (hours)	Latent Period (hours)	Rate of colonisation (mm / hour)	Rate of spore production ($x10^{-6}$ / hour)
SURVIVORSHIP				
ORIGINAL[a]	14.3	40.9	0.95	1.12
RE-ISOLATED[b]	14.3	40.0	1.02	1.05
LSD (*P*=0.05)	0.30	1.90	0.03	0.15
ISOLATES[c]				
S1	14.0	27.6	1.07	1.95
S2	14.0	31.0	1.07	0.42
HR1	14.0	27.2	1.04	1.71
HR2	15.7	35.5	0.96	0.18
HR3	13.8	24.5	1.06	1.45
HR4	15.7	29.3	1.04	1.55
LR1	14.3	33.0	1.20	1.32
LR2	13.5	35.0	1.14	1.21
D/HR	14.0	31.6	0.94	1.09
DR	14.3	130.0	0.29	0.0001
LSD (*P*=0.05)	0.67	4.33	0.07	0.34
Isolate	*P*<0.001	*P*<0.001	*P*<0.001	*P*<0.001
Survivorship	*P*=1.00	*P*=0.321	*P*=0.001	*P*=0.386
interaction	*P*=0.455	*P*=0.981	*P*=0.003	*P*=0.699
CV%	0.04	0.09	0.06	0.28

[a] original isolates indicated in [c] stored at 4 °C in refrigerator.

[b] *M. fructicola* re-isolated from twig cankers on 28 August 1990.

[c] S=sensitive isolate, HR=high-resistant MBC isolate, LR=low-resistant MBC isolate, D/HR=dual dicarboximide/high-resistant MBC isolate and DR=dicarboximide resistant isolate.

3.4 DISCUSSION

Environmental factors have a strong influence on the rate of survival and spread of a pathogen. Most stone fruit growing areas have seasonal climates, and conditions favourable for disease development usually last for relatively short periods of time. As a consequence, pathogen populations often display seasonal fluctuations in size. The probability of local survival is considerably higher for facultative parasites compared to obligate ones. For all pathogens, however, the survivors of adverse conditions are the initial sources from which inoculum will be dispersed at the beginning of the next growing season. Then, the number and distribution of these sources is of considerable importance in determining the subsequent distribution of primary infections within newly colonised foci.

The use of twig and fruit inoculation tests for evaluating differences in survival ability of *M. fructicola* resistant and sensitive to MBC and dicarboximide fungicides was demonstrated. The length of twig cankers can be used as an indicator to determine pathogenicity of isolates in association with those fitness characteristics on blossom and fruit. The length of canker has been used to determine differences in reaction to twig inoculation between *Prunus* species by *M. fructicola* (Ogawa and English, 1960) and *M. laxa* on almond and apricot (Crossa-Raynaud, 1969). In contrast, Feliciano *et al.* (1987) failed to demonstrate the differences in resistance or susceptibility to *M. fructicola* among peach genotypes using twig inoculations. The length of canker can be used to determine pathogenicity among resistant and sensitive isolates. The fitness variables on blossoms of MBC resistant isolates re-isolated from twig cankers after one year were as great as the original isolates. Similarly, resistant isolates re-isolated from twig cankers after 4 months showed no reduction in their fitness components on fruit.

All isolates of *M. fructicola*, resistant and sensitive to MBC and dicarboximide fungicides, survived adverse conditions effectively as mycelium in both infected twig cankers and mummified fruit for at least one season.

Since *M. fructicola* overwintered effectively in both twig cankers and mummified fruit in trees, these are likely to be potentially important sources of primary inoculum in the spring and secondary inoculum for fruit brown rot in summer. In addition, the conidia produced from twig canker may infect immature fruit at all stages of fruit development, except at pit hardening, when environmental conditions are suitable (Biggs and Northover, 1988a; 1988b).

The increased sporulation on twig cankers during spring and summer in all isolates may result from the ability of the fungus to penetrate through the epidermal layer. The epidermal layer was gradually rotted after infection, so that the fungus could emerge and sporulate freely when the twig tissues were hydrated.

The reduced sporulation of *M. fructicola* on mummified fruit and twig cankers after winter in relation to previous summer was possibly related to water availability. Very wet weather during winter causes leaching of nutrients from the surface layers of infected tissues (Corbin and Cruickshank, 1962), especially carbohydrate reserves. The lack of carbohydrate reserve could inhibit conidial production (Budge and Whipps, 1991). Willetts and Harada (1984) suggested that under moist conditions, mummified fruit and twig cankers were slowly degraded, thus reducing the potential for conidial production. Saprophytic organisms may affect pathogen overseasoning in the infected tissues, and in some systems the saprophytes may affect survival more than abiotic factors (Rotem, 1988). After prolonged exposure the mummified fruit and twig cankers were contaminated with other organisms. *Fusarium, Cladosporium, Penicillium* and *Bacillus* species were found increasingly on the infected tissues during the period of the experiment.

Isolates resistant to dicarboximide fungicides survived but sporulated poorly on both twig canker and mummified fruit compared with the sensitive and MBC resistant isolates. The dicarboximide resistant isolates were less fit than the sensitive isolates on both flower and on fruit and subsequently declined

steadily in the field in the absence of fungicide selection pressure (Elmer and Gaunt, 1990). A similar result was reported by Zehr *et al.* (1991) for the same fungus in South Carolina. The poor sporulation on infected plant parts may be a further reason why such strains do not persist well in the pathogen population unless dicarboximide fungicides are used frequently (Elmer and Gaunt, 1990). Dicarboximide resistant pathogens are often associated with reduced fitness (Beever *et al.*, 1989; Rewal *et al.*, 1991; Staub 1991). Caution is still required because of the potential for recombination of dicarboximide resistance with enhanced survival and other aspects of fitness.

Grindle and Dolderson (1986) found that a modifier gene in *N. crassa* interacted with the fungicide resistance gene to decrease osmotic sensitivity and increase sporulation without causing a change in resistance to vinclozolin. The theory of genetic homeostasis suggests that many characters of a population in a given environment are held in a complex balance, and consequently an entire population shift will not occur because of fungicide selection alone (Lalancette *et al.*, 1987). There is selection for individuals with resistance regardless of their other traits. The members of this subpopulation may or may not be as fit as the sensitive subpopulation for any given fitness characteristic. The second phase involves a progressive organisation of the genetic background of these individuals for greater overall fitness. Once fit resistant strains occur, the selection proceeds rapidly. However, if stabilising selection pressure is very strong, rate of resistance development may be delayed (Wolfe, 1975).

Resistance may be more likely to dominate the population, if the environmental conditions promote sexual reproduction. During the process of sexual reproduction there is a potential for recombination of characters, such as resistance with fitness and pathogenicity to give progeny which are different from the parents, and in this way new genetic forms are constantly obtained (Byrde and Willetts, 1977).

CHAPTER 4
APOTHECIAL INDUCTION AND SINGLE ASCOSPORE ISOLATION

4.1 INTRODUCTION

M. fructicola strains, both resistant and sensitive to MBC and dicarboximide fungicides, overwintered effectively as mycelium in infected plant parts on trees. The pathogenicity and fitness of the resistant strains are maintained via overwintering. The ability to survive and reproduce conidia (Chapter 3) is essential information to our knowledge on the conidial state (Day and Wolfe, 1987), which may lead to new strategies to control *M. fructicola*. However, the fungus may also overwinter effectively by means of stromata in mummified fruit on the ground and subsequently differentiating into apothecia and ascospores. Airborne ascospores forcibly ejected might provide an alternative inoculum source, as they are likely to be well adapted for long-range dispersal (Hunter, 1989; Lambert, 1990; Nicholson *et al.*, 1991). This together with the capacity of a sexual phase for genetic recombination, would be important in terms of variation in fungicide resistance, pathogenicity and fitness in the brown rot populations. Detailed knowledge on the sexual reproduction of the fungus is lacking, even though the perfect state (apothecia) was first described in the field in 1883 (Winter, 1883) and was subsequently found in many stone fruit growing areas (Tate, 1979; Biggs and Northover, 1985; Zehr *et al.*, 1991).

Apothecia of the fungus were found in Canterbury orchards, New Zealand in August and September 1990 coincident with blossom formation. However, there have been few reports of production of apothecia under controlled conditions (Baxter *et al.*, 1974; Harada, 1977). Apothecial production in the laboratory has not been reliable and the factors involved in apothecial development have not been well understood (Willetts and Harada, 1984).

Knowledge of the sexual reproduction of fungi included in the family *Sclerotiniaceae* has increased gradually since the 1930s. Drayton (1932) obtained apothecia of *S. gladioli* by placing microconidia from one isolate on

the receptive hyphae of another. Sexual reproduction occurred only between strains from different mating types. Drayton's method of apothecial production has been used for subsequent studies with other species. Many of the fungi in the *Sclerotiniaceae* are considered heterothallic, such as *S. trifoliorum* (Uhm and Fujii, 1983a), *S. minor* (Patterson and Grogan, 1984), and *B. fuckeliana* (Faretra *et al.*, 1988b). However, all of these fungi can be self-fertile to various degrees, e.g. *S. trifoliorum* (Uhm and Fujii, 1983b) are 50% self-fertile whereas *B. fuckeliana* is only 6% self-fertile (Faretra *et al.*, 1988b). In *S. sclerotiorum,* which is commonly homothallic, about 40 % of successful crosses can be obtained by single ascosporic strains (Fujii and Uhm, 1988). Mating type mutation (Uhm and Fujii, 1983b) and heterokaryosis (Grindle, 1987; Faretra and Pollastro, 1991) may be involved in the self-fertility mechanisms in these fungi.

Self-fertility has been found in various species of the genus *Monilinia* (Batra, 1983; Faretra *et al.*, 1988). Ezekiel (1924) and Harada (1977) produced apothecia from single ascospore isolates of *M. fructicola* and concluded that the fungus was homothallic. In Japan, the apothecia of species of *Monilinia,* including *M. fructicola,* have been produced *in vitro* (Willetts and Harada, 1984). To date, it has not been possible to duplicate their work (Sanderson and Jeffers, 1992), perhaps due to difficulties in the apothecial induction process. Study of the occurrence of fungicide resistance (Ogawa *et al.*, 1988; Penrose, 1990; Elmer and Gaunt, 1990) and the need to study primary infection (Mylchreest and Wheeler, 1987; Hildebrand and Braun, 1991; Sanderson and Jeffers, 1992) requires a reliable method of apothecial production, thereby producing ascospores which could be used to facilitate the study of pathogen population genetics and resistance evolution (Wade and Delp, 1990).

Resistance to MBC fungicides in *M. fructicola* is an excellent genetic marker to study the inheritance of resistance and recombination with other characters. Detailed genetic analysis of fungicide resistance and other characters may be possible if the sexual stage can be induced. The objectives of this chapter are

to report the induction of apothecia of *M. fructicola* in controlled conditions, the development of a reliable method of apothecial production in the laboratory and the development of a method for isolation of single ascospores in linear order to facilitate genetic analysis.

4.2 MATERIALS AND METHODS

In 1988, surface sterilised ripe fruit (apricot, wild plum, peach *cv* Black Boy, nectarine *cv* Fantasia) and autoclaved green-sour cherry in Erlenmeyer flasks (250 ml) were used as substrate for investigation of apothecial production. Autoclaved potato slices in Erlenmeyer flask (250 ml) acidified with 7.5% malic acid (Byrde and Willetts, 1977) was also used by soaking potato slices with the acid solution for 5 minutes and drained out prior to experimentation. These fruit were placed in cut plastic 'Plix' cups in a moist transparent plastic pottle. Four fruit each for the peach, nectarine and apricot and four cups of wild plum (7-10 fruit / plastic 'Plix' cup) were used. A cross shaped wound was made with a flamed-sterilised scalpel on the upper surface of fruit. The larger fruit were inoculated by placing aliquots (0.5 ml) of the suspension of a mixed inoculum (50:50) of S1 and HR1 isolates prepared as in chapter 2, onto the wounded site. The suspension was dropped directly onto the autoclaved sour cherry and the acidified potato slices. Inoculated fruit and acidified autoclaved potato slices were incubated at 25 °C (±0.5 °C) with 12 hour photoperiod (fluorescent and NUV light). The development of the infected materials was observed approximately every 7 days. At 60 days, all inoculated fruit were placed in moist peat moss and were incubated further at 25 °C (±0.5 °C) for 90 days in the dark before transferring to 8 °C (±0.5 °C) for another 90 days without light (Willetts and Harada, 1984). Peat moss was maintained at less than maximum water holding capacity, thereby allowing good aeration in the incubation chambers.

In 1989, 100 early-ripe nectarine fruit (*cv* Fantasia) and peach fruit (*cv* Black Boy), were surface sterilised and placed in 2 litre plastic ice-cream container as described earlier (Section 2.2.2). Two sets of fruit were inoculated with the

mixed inoculum as described previously and incubated at 24 °C (±2 °C) in a controlled temperature room with 12 hours photoperiod for 20 days. The first set (89a) was transferred to moist peat moss in new surface sterilised plastic ice-cream containers. The peat moss was added until it just covered the fruit. The moist condition in the containers was always less than maximum water holding capacity. According to Willetts and Harada (1984), approximately 30-60 days of diffuse light was required for stromatal development and a further period of 30-60 days incubation without light was needed for stromatal maturation. To find the accurate incubation time these fruit were incubated for 9 possible combinations of 30, 60 and 90 days for stromatal development (25 °C, light) and 30, 60 and 90 days for stromatal maturation (25 °C, dark). Subsequent to this first incubation period, all of these combinations were incubated at 8 °C in the dark for at least 90 days to investigate the development of apothecia.

The second set (89b) of fruit was placed on thoroughly wetted un-autoclaved peat moss in a shaded house in the field in April 1989 (Baxter *et al.*, 1974). The fruit were covered with additional peat moss to a depth of 10 mm and wetted thoroughly. In January 1990, a number of these fruit stromata (89b1) were brought to the laboratory to check their viability by re-isolating onto PDA petri dishes and the rest (89b2) remained in the same condition. The development of the 89b2 that remained in the field was investigated approximately every 7 days until spring (September) 1990.

Because the stromata (89b1) transferred from the field were viable (re-grew on PDA), they were submerged under running tap water, (Lincoln University tap water - 47 g/m^3 hardness of $CaCO_3$ and 12.7 mS/m conductivity tested in 1983 - Dawson, C. unpublished data), for 72 hours to ensure complete hydration (Willetts, H.J. pers. comm.). They were placed in wetted autoclaved peat moss in plastic, 2 litre, ice-cream containers described above, with the upper surface of the fruit just visible. They were incubated at 8 °C in the dark until stipe initials appeared, at approximately 90 days, and then incubated for 20-28 days at 12 °C (±1 °C) under 12 hours dark and 12 hour fluorescent light (Willetts

and Harada, 1984). The fruit remained fully hydrated for the whole period.

A further batch of inoculated fruit (both nectarine cv Fantasia and peach cv Black Boy) were inoculated with individual and mixed isolates in March 1990. There were 24 fruit for each isolate tested and 4 fruit in each plastic container with autoclaved peat moss. The inoculated fruit were maintained, under controlled conditions as follows: 60-90 days at 25 °C under 12 hour photoperiod of fluorescent light : 60-90 days at 25 °C in the dark. They were hydrated completely as for 89b1 and were incubated for 90 days, or until the appearance of stipe initials, at 8 °C in the dark. After stipes appeared, they were transferred into incubation at 12 °C and 12 hours photoperiod (fluorescent and incandescent light) for 15-20 days (Figure 4.1).

Whole fruit stromata, or individual stipe initials, were transplanted to sterilised moist peat moss and kept in transparent moist chambers. The chambers were modified from polystyrene square boxes (300x300x300 mm^3) with one side removed and the top opened. The boxes were sealed with transparent plastic bag to permit maximum light transmission directly through the stipe initials and with ten small holes to permit gas exchange. Wet paper towels were placed inside the boxes to provide moisture.

To monitor the conditions for ascospore ejection from asci, a special moist chamber was made to induce puffing, a phenomenon of spore release in mass, which could be seen as a white cloud. A group of apothecia in a small container was placed in a 14 cm diameter petri dish containing wet paper towels and covered with a glass beaker to provide moisture. The chamber containing mature apothecia was placed under 12 °C with fluorescent and incandescent light for about 3 hours. It was then transferred to room temperature with indirect sunlight for 20 minutes. When the beaker was removed ascospores were released immediately and subsequently. These same conditions were applied for collection of ascospores ejecting from single asci on petri dishes.

To obtain single ascospores in linear sequences from single asci, a small piece of hymenium, taken from an apothecium exposed to fluorescent and incandescent light at 12 °C for at least 3 hours, was placed in a drop of sterilised distilled water on a clean glass slide. The hymenial tissues were teased to separate the individual asci or small rosettes of asci. These asci were picked up with a very sharp bamboo needle, to decrease surface tension of water, under a dissecting microscope. The individual asci or rosettes of asci were placed in a small drop of sterilised distilled water plus 0.05% (v/v) Tween 20 on the surface of 4% water agar. The petri dishes were exposed to indirect sunlight or otherwise were exposed directly to the light under a micromanipulator. Within a few minutes in the drop of water, ascospores ejected in linear order from some asci. If ascospores ejected from a rosette of asci, the rosette was removed immediately to prevent interference by discharge from further asci. Each full set of ascospores was separated by using a micromanipulator and transferred to the opposite side of the petri dishes. They were placed in line separated from each other in their serial order. After 12 hours, the germinated ascospores were transferred to separate PDA plates using a dissecting microscope and a very fine metal pin (Minuten No. 0.02) to avoid interference between the sequence of ascospores during collection. Colonies were transferred to PDA slants and maintained at 4 °C as stock cultures.

4.3 RESULTS

In apricot, within 7 days the whole fruit was completely light brown in colour and covered with a thick mat of mycelium upon which only a few conidia had differentiated. The fruit surface became very soft and juice accumulated on the skin or at the bottom of the containers. At the end of the 14 day the fruit surface was darker and the mycelial mat became darker and thicker which made the fruit surface stronger. Microconidia started to form in approximately 15-20 days and they were numerous on the fruit surface after 30 days. Within 40 days all apricot tissues were colonised completely to the stone. They were black and wrinkled within 50-60 days.

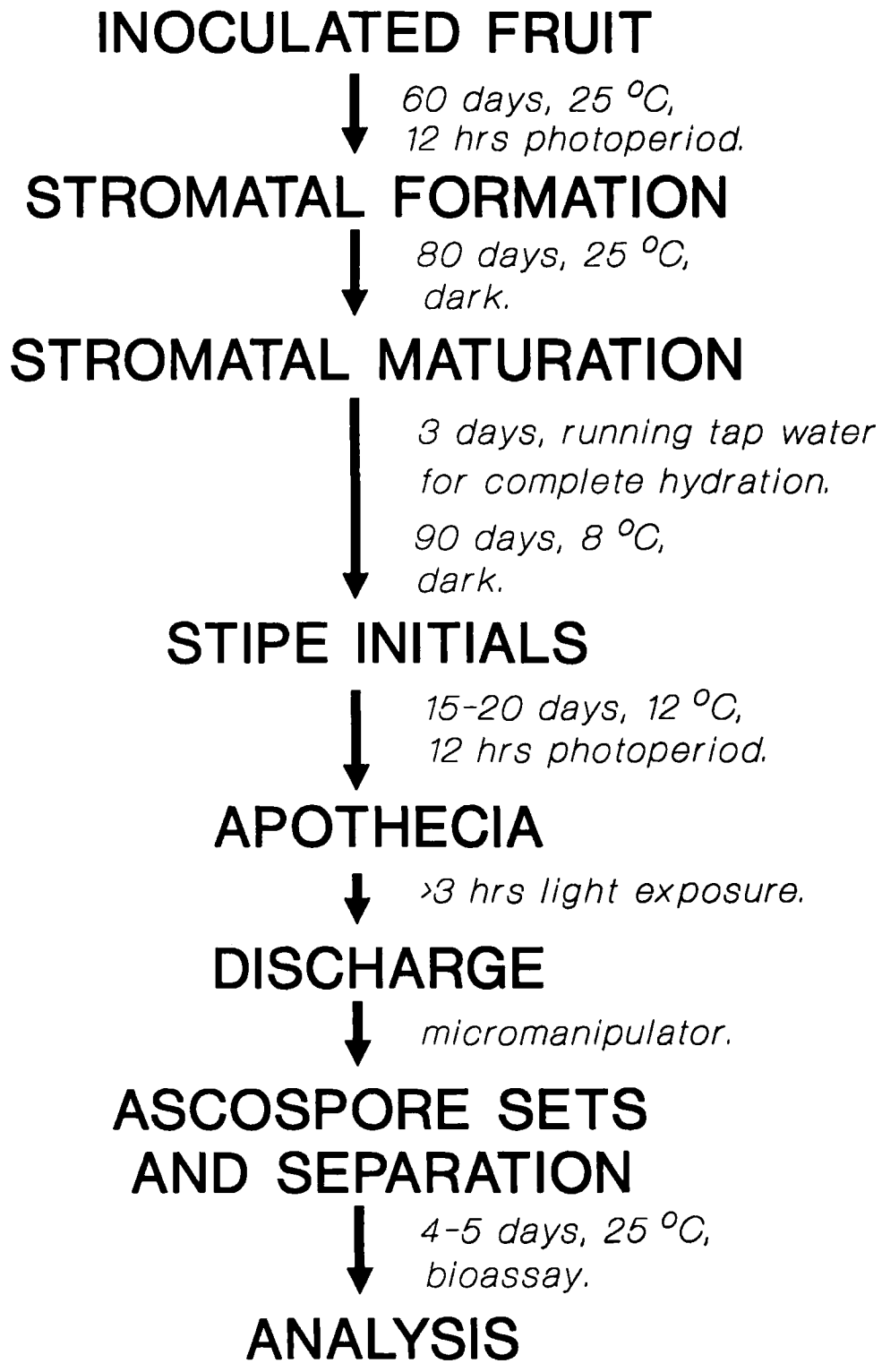

Figure 4.1 Flow diagram of apothecial production from the 1990 experiment and ascospore isolation.

In nectarine (*cv* Fantasia), from 3 to 7 days, light brown soft rot symptoms developed and fluffy mycelium with numerous conidia were observed. Fruit surfaces were as soft as apricots at 6 to 8 days with juice accumulating at the bottom of the containers. Within 20 days, the mycelium and conidia were dried up and a light buff slimy mass of microconidia was found covering the fruit surface. The fruit surface became black and shiny within 50 days. However, the fruit were still very soft compared to the apricot because the fungus colonised nectarines only about 4-5 mm from the fruit surface. The fruit tissue between the stone and colonised tissue was still soft and more or less similar to healthy tissue.

In peach (*cv* Black Boy), the fruit were colonised at the same rate as on apricot and nectarine, however, only small changes in colour from light brown to dark brown was observed during colonisation. No juice drops were observed and sporulation was light. Microconidia were formed on the fruit surface between 20-30 days. They were seen, if the fruit surface was scratched with a scalpel, smeared and observed under microscope. The diseased fruit were nearly as firm as the healthy fruit and became very firm, rubbery-like, when the stromata were mature. All fruit tissues were colonised completely to the stone.

Within 3 days after inoculation of autoclaved sour cherry, white fluffy mycelium was formed which later bore numerous conidia on the outside of the fruits. Twenty days after inoculation the fruit had dried up leaving a light buff slimy mass around the fruit surface. The mass contained numerous microconidia and later became small, dark buff coloured areas over the shiny black surface of the mummified fruit.

In autoclaved potato slices acidified with 7.5% (w/v) malic acid, within 4-5 days the surface of potato slices exposed to the air were covered fully with mycelium approximately 1-2 mm thick but not many conidia had developed. At the end of 14 days, the mat of mycelium turned dark inside but the outside layer was still white. Several light buff-coloured areas of microconidia developed on the

surface within 14 days and were numerous between 20-30 days. Within 30 days the outer mycelium and conidia were almost completely autolysed.

No stipe initials or apothecia were observed on the 1988 experiments, neither from several kind of fruit nor the autoclaved potato slices at the end of the investigation. There were also no stipe initials developed on these fruit, even though, they were re-incubated further under the following conditions: 30 days at 25 °C in the dark and 110 days at 8 °C in the dark. In the 1989 experiments, no stipe initials or apothecial development were noted from the nectarine and peach incubated fully in laboratory conditions (89a) from all combinations.

There was also no apothecial development on the 89b fruit incubated for 300 days (March-December 1989) in the wetted peat moss in a shaded house. However, after the fruit (89b1) were brought back to the laboratory and were hydrated in running tap water for 3 days, stipe initials were visible in early April 1990 from the 89b1 stromata exactly 90 days after they were incubated at 8 °C in the dark. All of these stipe initials (Figure 4.2) developed to fully fertile apothecia (Figure 4.3) after a further 20-28 days under 12 hours photoperiod (fluorescent light).

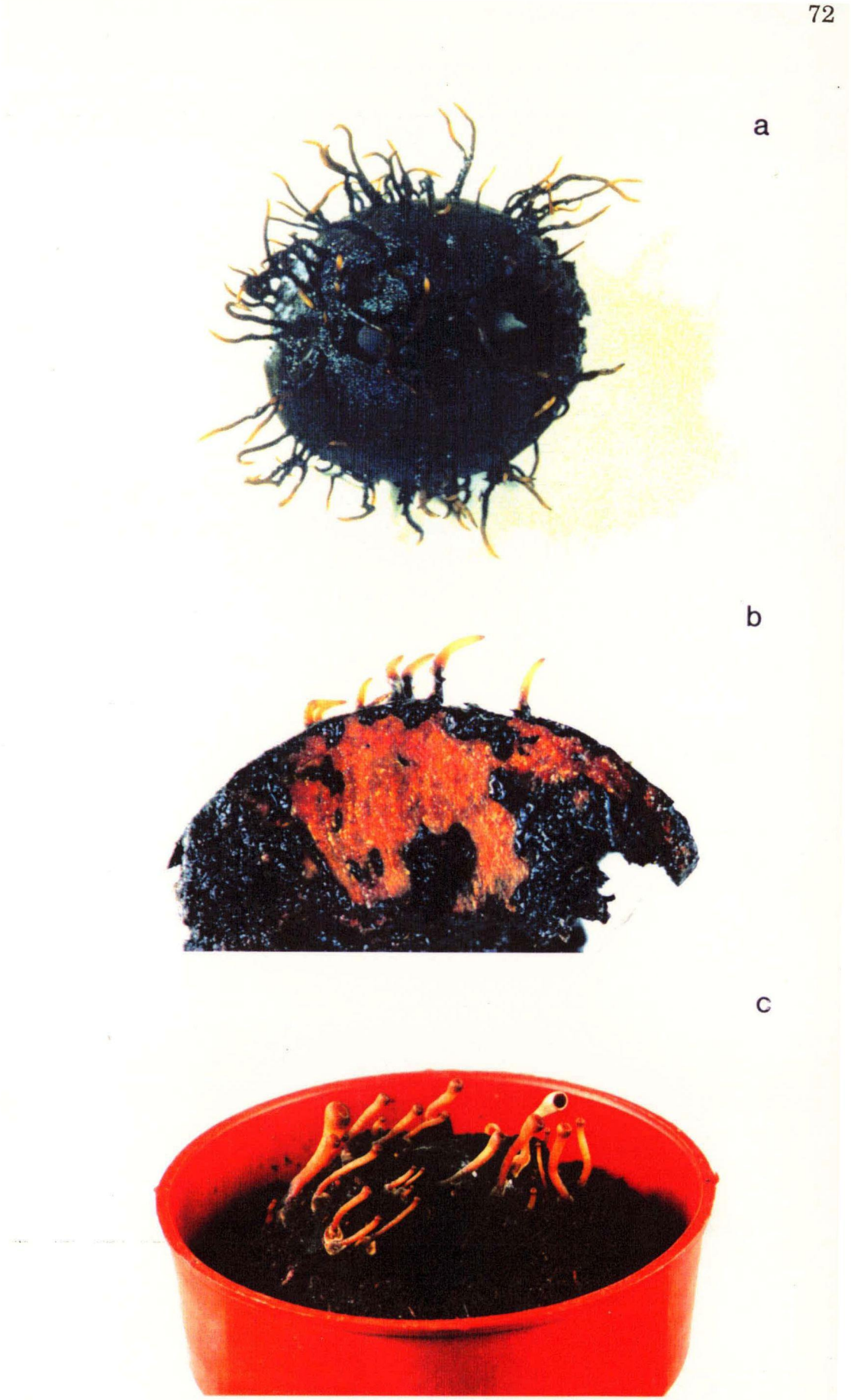

Figure 4.2 Apothecial initials developed from fruit stromata in the laboratory; a) stipe initials, b) cross section view of fruit stroma, and c) young apothecia, 10 days at 12 °C with 12 hr photoperiod.

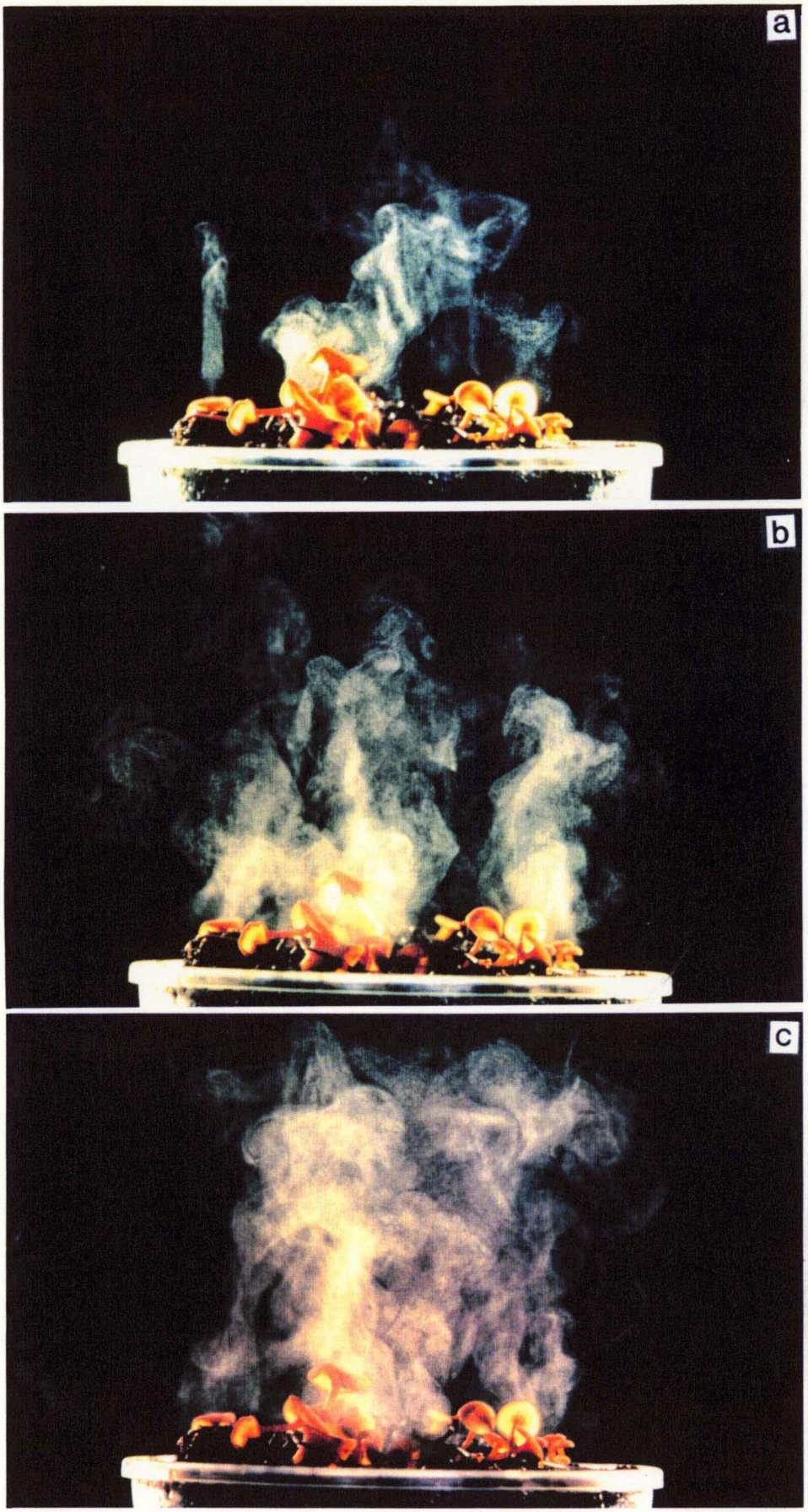

Figure 4.3 Ascospore discharge by puffing. a) 0:67 second, b) 1:00 second, and c) 1:67 second.

Stipe initials first appeared, on 8 May 1990, from the undisturbed-inoculated nectarine (89b2) and peach which were incubated fully in the shaded house. Incubation continued in the shaded house without any change in their environmental condition. All of the stipe initials developed fully into mature apothecia approximately 15 days before blooming period in the spring 1990 (15 August 1990). These apothecia actively discharged ascospores until full bloom, approximately 20-25 days after apothecia were matured fully.

Apothecia were obtained under fully controlled environments in the laboratory in 1990 from single, and combinations of, field isolates (Table 4.1). Apothecial initials formed on 85% of inoculated fruit, and from every combination, after 90 days at 8 °C in the dark after stromata were mature. Apothecia were mature within 20 days of transfer of stipe initials to 12 °C under 12 hour photoperiod (fluorescent and incandescent light). The number of apothecia per fruit varied considerably (Table 4.1) and was related to isolate individuals, such as HR2 and DR isolates produced only 1 apothecium per fruit. However, up to 530 apothecia were counted on a mummified fruit produced from mixed isolates S1xHR1.

Following the requirement of spore puffing conditions, ascospores were forced to eject and 576 single ascospore isolates in their serial order were obtained from 72 asci. Sixty four asci were obtained from apothecia produced from four single parents, HR1, HR3, HR4 and D/HR isolates. The other eight asci were derived from S1xHR1 and S1xLR1 mixed inoculum.

4.4 DISCUSSION

The nature of substrates in which stromata were produced appeared to be of great importance (Willetts and Harada, 1984; Faretra and Pollastro, 1988). Willetts and Harada (1984) suggested that the physical structure of the substrate, such as fruit, should remain firm after autoclaving. In order to maintain fruit structure, the use of 0.5% (v/v) sodium hypochlorite proved a better method of fruit surface sterilisation than autoclaving.

Table 4.1 Number of apothecial initials produced by fungicide resistant and sensitive isolates of *M. fructicola* on mummified fruit (peach *cv* Black Boy), under controlled conditions in the laboratory.

Isolate[a]	Number of stipes/fruit	Isolate combination[b]	Number of stipes/fruit
S1	29	S1xHR1	131
S2	26	S1xHR2	19
S3	24	S1xHR3	42
S4	26	S1xLR1	36
HR1	21	S2xHR4	3
HR2	1	S2xLR4	42
HR3	33	S2xDR	21
HR4	26		
LR1	34		
LR2	38		
LR3	42		
LR4	48		
D/HR	30		
DR	1		

[a] S=sensitive isolate, HR=high-resistant MBC isolate, LR=low- resistant MBC isolate, D/HR=dual dicarboximide/high-resistant MBC isolate and DR=dicarboximide resistant isolate.

[b] mixed inoculum, 1:1, before inoculation onto fruit.

Green sour cherry could be sterilised by autoclaving (Willetts and Harada, 1984). In addition, the autoclaved cherry can be kept for a very long time if they are held at 4 °C. Well developed stromata were found in all fruit used in experiments within 30 days or less. Black Boy peach and apricot were considered the best substrate for stroma production because all fruit tissues were colonised fully to the stone.

No apothecia were obtained in the first experiment in 1988 with different types of substrate and on the second trial (89a) with Black Boy peach and Fantasia nectarine incubated at different combinations in the laboratory. There may not have been enough moisture in the peat moss which was kept moist approximately at less than maximum water holding capacity.

Morphologically fully developed stromata required a further period of 30-60 days incubation at temperature above 20 °C before they were fully mature (Willetts and Harada, 1984). Even though the field-incubated fruit that had been inoculated were physically mature, the temperature during autumn and winter could have delayed or suppressed the maturation of stromata. Subsequently, there was no apothecial development in the following spring. Although field-incubated fruit (89b) were wetted thoroughly at the beginning of the experiment by watering and raining during incubation, no apothecial initials developed in the subsequent spring. The temperatures may have been too cold for the stromata to be matured. However, when stromata were fully mature in January 1990, the temperatures were warmer than 15 °C. The temperatures will be too warm for apothecial initials to develop (Willetts and Harada, 1984). Batra (1983) found that mummies of blueberries (*M. vaccinii-corymbosi*) must be on the ground before the end of summer in order to produce apothecia in the next spring. Byrde and Willetts (1977) concluded that fruit infected by *M. fructicola* late in the season, or from diseased larger fruit, needed more time to reach maturity and did not produce apothecia in the subsequent spring. This finding has shown that apothecia can develop from second year mummified fruit, if the conditions are conducive.

Microconidia were found on all fruit inoculated in experiments, however, they were visually abundant on autoclaved sour-cherry and autoclaved potato-slices. The findings were similar to the review of Willetts and Harada (1984) but in contrast with the report of Willetts (1968) in which no microconidia were noted. The stage of ripening of fruit may be responsible for the differences. On the surface of the other kinds of fruit microconidia will be abundant if there has been very high humidity. Also, on the surface of these mummified fruit were produced numerous microconidia which may function as spermatia (Drayton, 1934a).

Although, microconidia in many species of the *Sclerotiniaceae* are functional spermatia, such as *S. sclerotiorum, S. trifoliorum* (Fujii and Uhm, 1988), *S. minor* (Patterson and Grogan, 1984) and *B. fuckeliana* (Faretra and Pollastro, 1991), there is no convincing evidence to suggest that they are necessarily a precursor to apothecial initiation in *M. fructicola* (Willetts and Harada, 1984). Spermatisation to form dikaryotic phase may arise directly from vegetative hypha (Willetts and Wong, 1980). The availability of microconidia on substrate may be useful for further investigation of whether they are functional spermatia.

Complete hydration may be essential for apothecial production (Abawi and Grogan, 1975; Willetts, H.J. pers. comm.). During autumn and winter 1989, the Lincoln University stone fruit orchard floor was carefully monitored by observing the differences between shaded and non shaded trees. A prolonged wetness was always associated with the shaded areas and it was hypothesized that water saturation may be essential for apothecial development. The condition was similar to the 1989 field incubated fruit in the shaded house, which was always wet. Similar wet condition in the shaded trees was also found in 1990.

The water quality may also be important to the formation of apothecial initials (Willetts, H.J. pers. comm.). Soaking of mature stromata probably stimulated sexual structures to develop. Conidia or ascospores will germinate directly

into microconidia, if there is excess water (Willetts and Calonge, 1969; Fujii and Uhm, 1988). Apothecia were produced on a majority of Black Boy peach and Fantasia nectarine that were incubated fully in the field under continuous hydration.

Temperature is one of the most important physical factors affecting apothecial production in the Sclerotiniaceae (Willetts and Harada, 1984; Faretra and Pollastro, 1988; Huang, 1991). Chilling temperatures ranging from 4-10 °C is required to trigger apothecial initials to develop in many apothecial producing fungi (Willetts and Harada, 1984; Mylchreest and Wheeler, 1987; Nicholson *et al.*, 1991). Temperatures of above 15 °C encourage mycelial growth and stromatal development but inhibit apothecial initiation (Willetts and Harada, 1984).

Apothecia were obtained reliably under controlled conditions in the laboratory on three separate occasions. The minimum time for all the combinations of these conditions requires further investigation. From this findings it may be concluded that at least 60 days at 24 °C (±2 °C) with 12 hour photoperiod (fluorescent light) was required for stromatal development and at least 60 days at 24 °C (±2 °C) in the dark for stromatal maturation. Apothecial initials were developed approximately 90 days following complete hydration of the stromata in tap water and incubation at 8 °C in the dark. Complete hydration appeared to be essential for the development of apothecial initials. Apothecia were fully matured within 20 days after the apothecial initials were transferred to 12 °C with 12 hour photoperiod (fluorescent and incandescent light). In the second and the third occasions, apothecia were obtained from every combination within a total of 220 (on nectarine *cv* Fantasia) to 250 (peach *cv* Black Boy) days.

There were two isolates, HR2 and DR, which produced considerably fewer apothecia than the other isolates (Table 4.1). These isolates were generally less fit for other pathogenicity and fitness attributes (Chapter 3), especially, the rate of colonisation on fruit. If the stromatal development and maturation

proceeds slowly, the stromata will be vulnerable to attack by other organisms (Willetts and Harada, 1984). The stromata of these two isolates were formed poorly (tissue easily disintegrated) and this may affect apothecial production, the firmer and the more rubbery-like the stromata, the more apothecial initials were observed.

Light and moisture were the major factors for ascospore release from asci. When single ascospore sets, in linear sequence, are required, conditions needed to eject ascospores from an ascus must be known (Sharvelle and Chen, 1943). Exposure of mature apothecia in moist environment for at least three hours at 12 °C with fluorescent and incandescent light was found to be essential. It seems that the more hours of light exposure, the faster the rate of ejection. Further investigation on the effect of light is needed to verify this matter. A film of water on the surface of water agar was needed to trap the ejected ascospores. The phenotypes and genotypes of these single ascospore isolates obtained from this experiment will be analyzed in detail in the following chapter.

CHAPTER 5
INHERITANCE OF RESISTANCE AND MECHANISMS OF RESISTANCE TO MBC FUNGICIDE

5.1 INTRODUCTION

All biological phenomena are based ultimately on genetic controls. The function of these controls in plant pathogenic fungi is a key factor in the occurrence of strains resistant to fungicide and their epidemics (Day and Wolfe, 1987). A fungus can acquire fungicide resistance either in one step or in multisteps (Georgopoulos, 1988) as a result of spontaneous mutation in the field. The process can be enhanced for research studies by induced mutation in the laboratory. High degrees of resistance may result from mutation, either of a single major gene (Georgopoulos, 1988), or from the result of an additive (Kalamarakis *et al.,* 1991; Lasseron-de Farandre *et al.,* 1991) or synergistic interaction (Molnar, *et al.,* 1985) between several mutant genes.

Although many fungal plant pathogens are primarily asexual throughout most of the life cycle, there are some examples where sexual reproduction occurs regularly. The importance of sexual reproduction to the dynamics of resistance depends on the genetic basis of resistance and on the opportunities for sexual recombination. A diploid phase is brought about by the fusion of two haploid nuclei. If the two haploid nuclei are provided by two genetically different individuals, new combinations of alleles can arise in the next generation, because of segregation and crossing over mechanisms bringing about recombination at meiosis (Fincham, 1983b). As a consequence, the recombination processes which occurs in natural populations can lead to bringing together resistance and fitness, arising originally in separate strains. The occurrence of resistance may not necessarily reduce fitness (Lalancette *et al.,* 1987). Moreover, two separate mechanisms may contribute to the development of fungicide resistance and fitness. First, it is possible that the ability to repair damaged chromosomes may be present, and this process may assist in decreasing sensitivity to a fungicide. Second, if there are modifier

genes, a decreased hypersensitivity to high osmotic pressure mechanism could occur such as those found in *N. crassa* resistant to dicarboximide fungicides (Grindle and Dolderson, 1986) and *A. nidulans* resistant to imazalil (Van Tuyl, 1977). These mechanisms will eliminate unwanted characters, but an increase in fitness will result in effective survival of the resistant populations in the fields.

The phenotypic characters of fungicide resistant plant pathogenic fungi has been reported in more than 80 papers, but the associated genotypes were seldom analyzed (Hollomon, 1986; Grindle, 1987). Handling difficulties, slow and unreliable procedures or even lack of methods to generate recombinant progeny and isolate instability have limited genetic work on most plant pathogenic fungi. Consequently, much of the genetic information on fungicide resistance has been generated through work with *N. crassa*, *A. nidulans* and *S. cerevisiae*. This information may be directly applicable to plant pathogens, but this requires verification.

The inheritance of fungicide resistance has been investigated for only a small number of plant pathogens (Grindle, 1987; Milgroom *et al.*, 1989). Resistance may be controlled at a single locus or may be polygenic. In *Venturia* spp., MBC resistance is controlled by three or four different resistance alleles at the same locus (Stanis and Jones, 1984; Shabi *et al.*, 1986). Three different resistance alleles at the same locus in *B. fuckeliana* conferred resistance to MBC and two alleles at the same locus controlled resistance to dicarboximide fungicides (Faretra and Pollastro, 1991). The resistance of *F. oxysporum* f.sp. *lycopersici* to MBC fungicide is affected by mutations at other loci which interact synergistically between two major genes conferring high degrees of resistance to the fungicide (Molnar *et al.*, 1985). Single-locus resistance has also been shown for phenylamide resistance in *B. lactucae* (Crute, 1989), and *P. infestans* (Shattock, 1986). Kasugamycin resistance in *P. oryzae* is controlled by three loci where a resistance allele at any one locus confers resistance (Taga *et al.*, 1979). Polygenic resistance were demonstrated in *E. graminis* f.sp. *hordei* to ethirimol (Hollomon, 1981) and triadimenol

(Hollomon *et al.,* 1984). In contrast, Brown *et al.* (1992) found only three different loci control the resistance in *E. graminis* f.sp. *hordei* to ethirimol and triadiminol and the resistance may not be polygenic. Resistance to dodine is polygenic in *N. haematococca* var. *cucurbitae* (Kappas and Georgopoulos, 1970). Ultraviolet-induced mutants of *N. haematococca* var. *cucurbitae* also show polygenic inheritance for resistance to fenarimol (Kalamarakis *et al.,* 1991), fenpropimorph and terbinafine (Lasseron-de Falandre *et al.,* 1991). At least 4-9 independent genes have been identified and two highly resistant mutants carried mutations in two unlinked genes, which showed an important additive effect (Kalamarakis *et al.,* 1991; Lasseron-de Falandre *et al.,* 1991).

Genetic studies have also provided evidence of dominance arising through interaction of a resistance allele with its wild-type allele (Borck and Braymer, 1974). Although many important plant pathogens are haploid for most of the life cycle, dominance can be of practical significance where heterokaryons and dikaryons exist, since dominance may ensure that all cells are resistant even though varying properties of wild type sensitive nuclei may be present (Grindle, 1987). Genetic information in many plant pathogenic fungi including *M. fructicola* are lacking and the information is needed for future management strategies (Wade and Delp, 1990; Grove and Boal, 1991).

There is no information available on genetic controls of resistance to the MBC fungicide for *M. fructicola,* perhaps because it is difficult to produce ascospore progeny for genetic analysis in controlled conditions. The techniques described in Chapter 4 provides an opportunity to investigate the genetic basis of many characteristics including the determination of the mechanisms of inheritance of resistance. In this chapter, the use of sexual progeny of field isolates to investigate the genetics of resistance to MBC and dicarboximide fungicides is reported.

5.2 MATERIALS AND METHODS

Apothecia of *M. fructicola*, derived from single parent isolates and attempted crosses were obtained as described in Chapter 4. They were crushed individually to release ascospores from asci in plastic vials containing 2-3 ml sterile distilled water plus 0.05% Tween 20 (v/v). The suspension was filtered through sterilised lens tissue to remove hymenial tissue and unbroken asci. Ascospore numbers were adjusted to $5x10^2$ ascospores / ml by using a haemocytometer. Aliquots (0.5 ml) of the suspension were plated on a monilinia inhibiting medium (MIM) (chapter 2, section 2.2.3) unamended and amended with 1 mg/l carbendazim to distinguish resistant and sensitive phenotypes (Smith *et al.*, 1991). The ratio of colonies growing on the amended and unamended MIM indicated the proportion of ascospores carrying the resistant character in the original apothecium.

A technique to isolate single ascospores in linear sets (Chapter 4) was used to dissect full sets of eight ascospores. Five hundred and eighty four single ascospore isolates from 73 asci were tested on PDA amended with 1 and 100 mg/l carbendazim for resistance expression. The number and patterns of resistant and sensitive ascospores in each ascus indicated the segregation of genes and the inheritance of resistance.

An isozyme analysis, modified from May and Royse, (1982), was used to detect whether cross- or self-fertilisation had occurred in the formation of apothecia. One ascospore set derived from a single parent HR1 isolate, one ascospore set of an attempted cross (S1xHR1) and parent isolates, S1 and HR1, were selected. Two plugs of mycelium (5 mm diameter) taken from the edge of 4-5 day old cultures of each on PDA were inoculated in 100 ml Oxoid malt extract broth (MEB) in 250 ml Erlenmeyer flask. The inoculated flasks were placed on a shaking machine set to rotate at 200 rpm held at 24 °C (±2 °C) for 7 days. Wet mycelium (0.25 mg) was harvested from submerged cultures via vacuum filtration through Whatman No. 1 filter paper, and was placed in an agate mortar

and frozen with liquid nitrogen. The frozen mycelium was then immersed in 0.05 M Tris-HCl pH 7.1, 500 mg PVP (polyvinyl pyrrolidone), 2 drops 2-mercapto-ethanol and about 0.1 mg of acid washed sand, and macerated until a homogenate was obtained. The homogenate was placed in Eppendorf tubes centrifuged at 10,000 G for 12 minutes, and the supernatant containing soluble enzymes from all parents/progeny were tested electrophoretically. Two methods of electrophoresis were carried out using horizontal starch gel electrophoresis (HSE) and vertical one-dimensional polyacrylamide gel electrophoresis (PAGE).

The HSE technique was used to investigate cross- or self-fertilisation in ascospore progeny. Starch gels (11%) were prepared by mixing 25 g hydrolysed potato starch (Sigma-4501) in 225 ml of S4 gel buffer (8 mM tris, 3 mM citric acid, adjusted to pH 6.7 with NaOH). The starch was mixed with approximately 75 ml of the S4 gel buffer in a 1 litre conical flask. The remainder of the buffer, approximately 150 ml, was heated over a bunsen burner until boiling, and then added to the unheated starch/buffer suspension while constantly swirling the flask. The 1 litre flask containing the full 225 ml gel buffer and 25 g starch was then immediately heated over bunsen burner, with swirling every 5 second, until boiling. This boiling starch suspension was degassed under vacuum created by a water aspirator for approximately 1 minute. The starch gel suspension was then poured into two open horizontal perspex forms, 220x110x3 mm internal volume clamped one above the other to a glass plate 240x130x5 mm. The starch gel was then allowed to cool to room temperature, for approximately 1 hr, before using.

The gel was laid flat onto the surface of the cooling pad of a LKB 2117 Multiphor II horizontal electrophoresis unit. The edge of a perspex ruler was then placed 25 mm from the cathodal side of the gel and used to guide a scalpel cut across the gels length. Filter paper (MFS No. 526) was cut, 5x15 mm, to create sample wicks. The individual wicks were then loaded with 25 µl of each sample. A wick was loaded with 0.1% bromophenol blue as a dye marker to trace the progress of electrophoretic migration. The gel was

separated at the cut and the loaded wicks were placed vertically in the cut surface. A LKB Bromma 2197 electrofocusing constant power supply was used, set at 250 V and 60 mA. Cheese cloths were used to conduct the electric current from the electrode buffer and the gel. After 10-15 min, when the enzymes had moved from the wicks into the starch, electrophoresis was temporarily terminated to remove the wicks and the gel was then pushed firmly back together. Electrophoresis was continued until the dye marker had migrated about 80 mm from the cut ends (origin), after approximately 4 hours.

At the completion of electrophoresis, the gel was sliced horizontally into 2 pieces by removing the upper form, and drawing a piece of thin 20 SWG Nichrome wire through the gel. The slices were placed into individual trays, and after addition of the specific enzyme stain, incubation was carried out at 30 °C in the dark until enzyme activity became visually evident. The enzymes alcohol dehydrogenase (ADH), glucokinase (GK), glucose phosphate isomerase (GPI), phosphogluconate dehydrogenase (PGD), phosphoglucomutase (PGM) and malate dehydrogenase (MDH) were selected to distinguish cross- or self-fertilisation (May and Royse, 1982).

One-dimensional polyacrylamide gel electrophoresis (PAGE) was carried out using a LKB Bromma 2001 vertical electrophoresis unit, and a LKB Bromma 2197 constant power supply unit. The acrylamide stock solution for PAGE contained 30 g acrylamide, 0.8 g bisacrylamide, and 100 ml distilled water. The running gel buffer contained 1.5 M Tris-HCl, pH 8.8 and the stacking gel buffer 0.5 M Tris-HCl, pH 6.8. Gels containing running and stacking gels were prepared. The running gel contained 10% acrylamide (10 ml acrylamide stock, 3.75 ml running gel buffer, 1.5 ml of 1.5% ammonium persulphate, 15 µl Tetramethyl- ethylenediamine (TEMED), and 14.75 ml distilled water). The stacking gel contained 2.5 ml acrylamide stock, 5 ml stacking gel buffer, 1 ml of 1.5% ammonium persulphate, 15 ul TEMED and 9 ml water, added onto the running gel. The upper glycine buffer was 1.5 g Tris and 7.2 g glycine in 500 ml water, pH 8.3. Samples were loaded into gel slots, and electrophoresis carried out at 250 V with 30 mA and 15 °C for 5 hour. Gels were stained

immediately for general protein using 0.125% (w/v) Coomassie brillient blue R-250 (Sigma), 50% (v/v) methanol, 10% (v/v) acetic acid overnight. Gels were destained in 50% methanol, 10% (v/v) acetic acid.

5.3 RESULTS

Analysis of populations of ascospores from self-fertile isolates and attempted-crosses showed several segregation patterns (Table 5.1). All of the ascospores derived from sensitive parent isolates were sensitive to 1 mg/l MBC fungicide. The ascospore progeny generated from high-level resistant MBC (HR) isolates segregated into three types. The segregations of 39 apothecia of HR1, HR3 and HR4 were not significantly ($P>0.05$) different from a 1:1 ratio. In all ascospore populations derived from HR3 and HR4 isolates, resistant and sensitive segregation occurred in a 1:1 ratio. The segregation ratio, 1:1, was also found in most apothecia (14 out of 15) in isolate HR1, indicating a single gene control of the resistant phenotype. Two apothecia of HR2 deviated slightly ($P\leq0.05$) from a 1:1 ratio and the segregation of 3 apothecia of D/HR were significantly ($P\leq0.01$) different from a 1:1 ratio of resistant and sensitive phenotypes. In addition, thirteen apothecia (D/HR^c isolate) produced only the resistant phenotype. Segregation was significantly ($P\leq0.01$) different from either 1:1 or 9:7 ratios for resistant and sensitive phenotypes in ascospore populations obtained from the low-level resistant MBC isolates, LR1 and LR2.

The ascospore progeny derived from 8 attempted crosses between sensitive and high resistant parents exhibited the same manner of segregation as the ascospore progeny of the high-level resistant MBC isolates. Two pairs, S1xHR1 and S2xDR, of the attempted-cross produced only the sensitive phenotype. No difference from the segregation ratio 1:1 was observed in ascospore progeny of S1xLR1, whereas, in S2xLR4 was significantly different ($P\leq0.01$) from the 1:1 ratio. There were two types of segregation in the progeny of S1xHR2 and S1xHR3 isolate pairs: one only showed the sensitive phenotype and the other was not significantly different from the 1:1 segregation ratio.

Table 5.1 Frequency of MBC resistant progeny in ascospore populations derived from resistant and sensitive parental isolates of *M. fructicola*.

Parent isolate[a]	Number of apothecia	Number of colonies resistant	Number of colonies sensitive	Chi-square Test[d] 1:1	Chi-square Test[d] 9:7
1 S1	4	0	759	-	-
2 S2	4	0	830	-	-
3 S3	3	0	538	-	-
4 S4	3	0	753	-	-
5 HR1	14	1939	1796	2.74^{NS}	12.5^{**}
6 HR1[b]	1	0	378	-	-
7 HR2	2	230	209	5.79^{*}	0.11^{NS}
8 HR2[b]	2	0	891	-	-
9 HR3	9	1384	1271	2.40^{NS}	8.02^{**}
10 HR4	16	1897	1838	0.46^{NS}	19.8^{**}
11 LR1	6	707	290	87.2^{**}	38.1^{**}
12 LR2	6	597	362	28.8^{**}	6.14^{*}
13 D/HR	3	773	370	71.1^{**}	26.3^{**}
14 D/HR[b]	1	0	172	-	-
15 D/HR[c]	13	2384	0	-	-
16 DR	-	-	-	-	-
17 S1xHR1	8	0	1827	-	-
18 S1xHR2	2	0	289	-	-
19 S1xHR2[b]	1	50	68	1.37^{NS}	4.04^{*}
20 S1xHR3	4	760	697	1.36^{NS}	4.33^{*}
21 S1xHR3[b]	2	0	295	-	-
22 S1xLR1	3	226	187	1.84^{NS}	0.17^{NS}
23 S2xLR4	2	264	112	30.7^{**}	13.0^{**}
24 S2xDR	8	0	1656	-	-

[a] S=sensitive isolate, HR=high-resistant MBC isolate, LR=low-resistant MBC isolate, D/HR=dual dicarboximide/high-resistant MBC isolate and DR=dicarboximide resistant isolate.

[b] resistant isolate produced only sensitive progeny.

[c] resistant isolate produced only resistant progeny.

[d] Chi-square Test, NS ($P>0.05$), * ($P\leq0.05$), ** ($P\leq0.01$).

Results obtained from many apothecia deviated from expected ratios, which were all sensitive, all resistant and segregated, 1:1, or deviated from 1:1 in ascospore progeny. Thus, to verify the previous results, single ascospore sets were used for tetrad analysis. Bioassays with 1 mg/l MBC fungicide showed that ascospore sets from eight asci derived from two attempted-crosses, S1xHR1 and S1xLR1, only exhibited the sensitive phenotype. In contrast, the segregation ratio of the 512 ascospores derived from 64 asci derived from single parents deviated from 1:1 resistant and sensitive phenotypes. In these asci, the resistant phenotype was always numerous (Table 5.2). However, when tested at 100 mg/l MBC, the segregation ratio became obviously four resistant: four sensitive phenotypes in most cases (Table 5.2). Six of the 64 asci derived from single parents, HR3, HR4 and D/HR segregated in the ratio 5:3, one ascus segregated 6:2 and another was 8:0. Tetrad analysis was used to analyze the patterns of ascospore arrangement in linear sequence. The ascospore arrangements in most of the ascospore sets fell into six patterns of first and second meiotic division (Figure 5.1 and Figure 5.2).

The protein staining with Coomassie blue had an identical overall appearance in both parents and ascospore progeny in the banding patterns (Figure 5.3a, 5.3b). Staining intensity of the fastest and the slowest moving bands was weak in all isolates when compared to the moderate moving bands. Bands observed at the same distance are presumed to be identical proteins, with respect of the intensity of staining (May and Rose, 1982). The amount of enzyme activity is responsible for the intensity of allozyme expression (Mitchell, A. pers. comm.).

All isolates showed a single, common electromorph or allozyme at GK, GPI, MDH, PGD and PGM loci, although there the common alcohol dehydrogenase (ADH) electromorph was segregated 4:4 for presence and absence in progeny derived from both a resistant parent (HR1-Figure 5.4a) and an attempted cross (S1xHR1-Figure 5.4b). All of these bands seemed to express in the same manner as their parents, ie: intensity and migration.

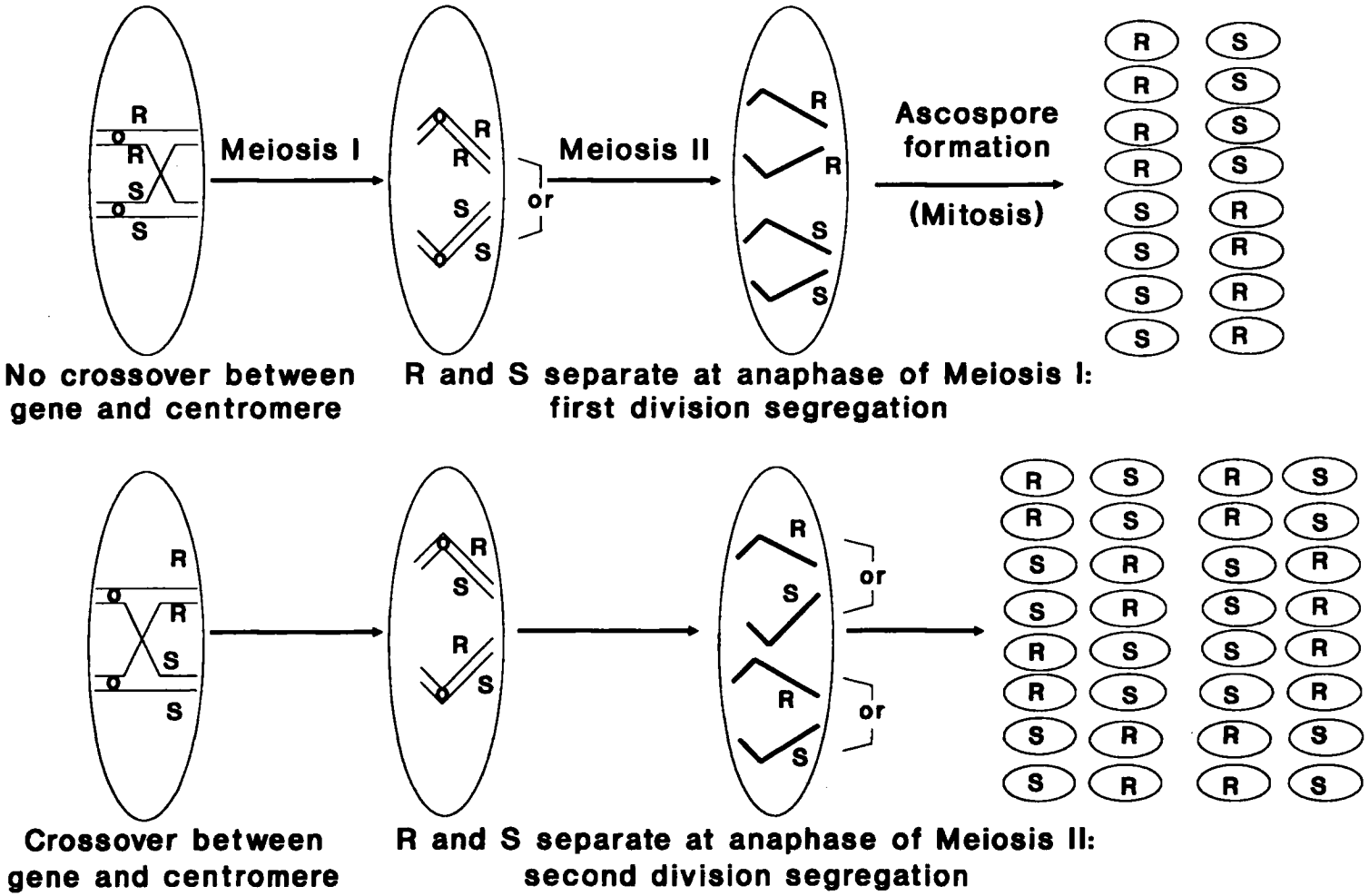

Figure 5.1 First and second division segregation patterns in asci. The symbol R stands for a resistant genotype and S for a sensitive genotype to fungicide.

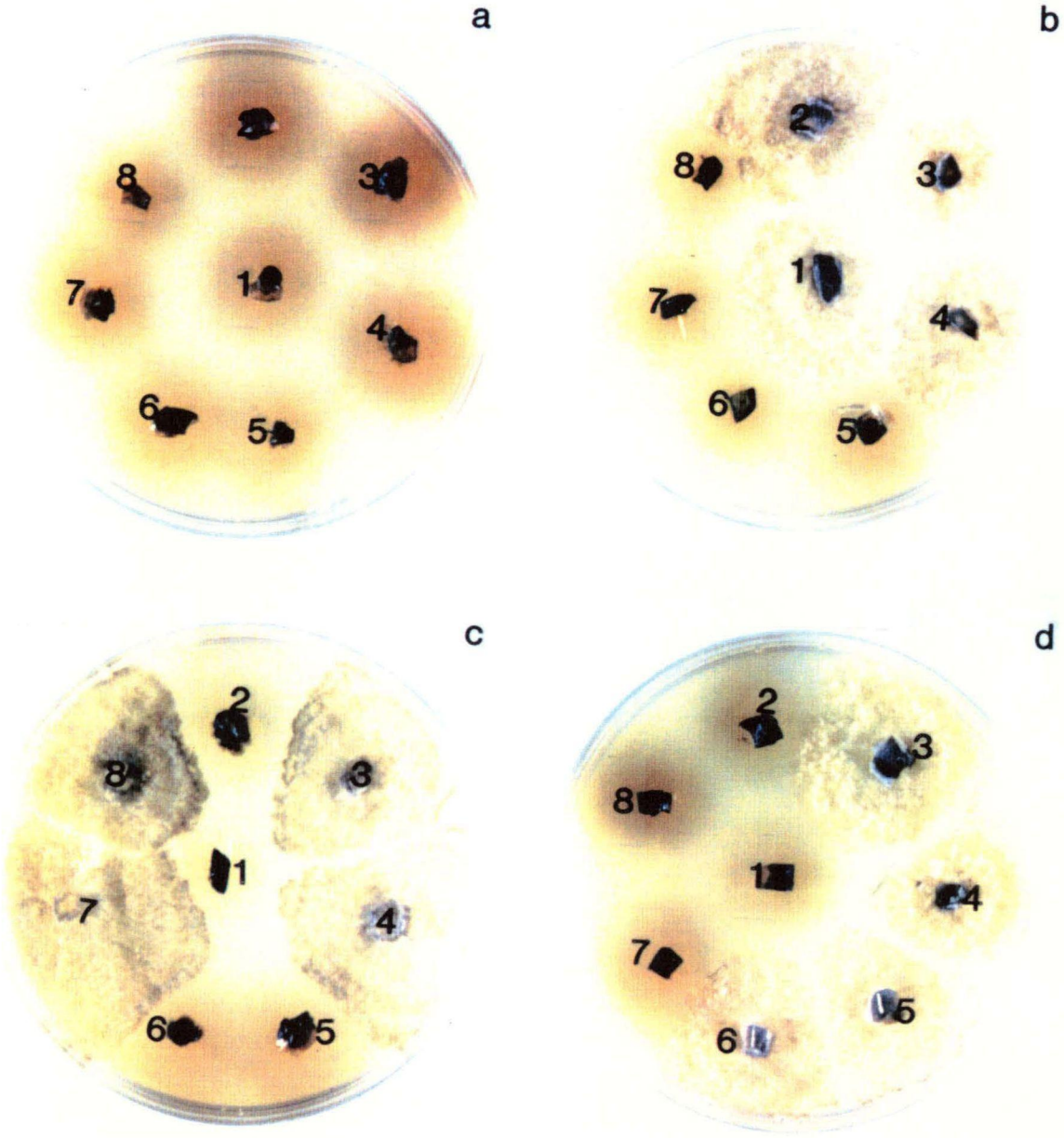

Figure 5.2 Patterns of ascospore arrangements in linear sequence as indicated by numbers, from single asci grew on MBC fungicide amended PDA for 5 days. a) All sensitive, b) first division segregation, c) and d) second division segregation.

Table 5.2 Frequency of phenotypic expression, segregation division and ratio (R:S) of ascospore sets in linear arrangement derived from apothecia derived from single resistant isolates of *M. fructicola*.

Isolates[a]	No. of asci	Phenotype of ascospore		Division of segregation at 100 mg/l carbendazim (asci)		Segregation ratios at 100 mg/l carbendazim (asci)				
		1 (mg/l carbendazim)	100			(R:S)				
		R:S	R:S	First	Second	0:8	4:4	5:3	6:2	8:0
HR1	4	17:15	16:16	0	4	0	4	0	0	0
HR3	14	66:46	57:55	6	8	0	12	2	0	0
HR4	26	114:94	108:100	9	17	0	22	4	0	0
D/HR	20	112:48	86:74	6	12	0	18	0	1	1
S1xHR1	5	0:40	0:40	-	-	5	0	0	0	0
S1xLR1	3	0:24	0:24	-	-	3	0	0	0	0
TOTAL	72	309:267	267:309	21	41	8	56	6	1	1

[a] R=resistant isolate, S=sensitive isolate, HR=high-resistant MBC isolate, LR=low-resistant MBC isolate, D/HR=dual dicarboximide/high-resistant MBC isolate.

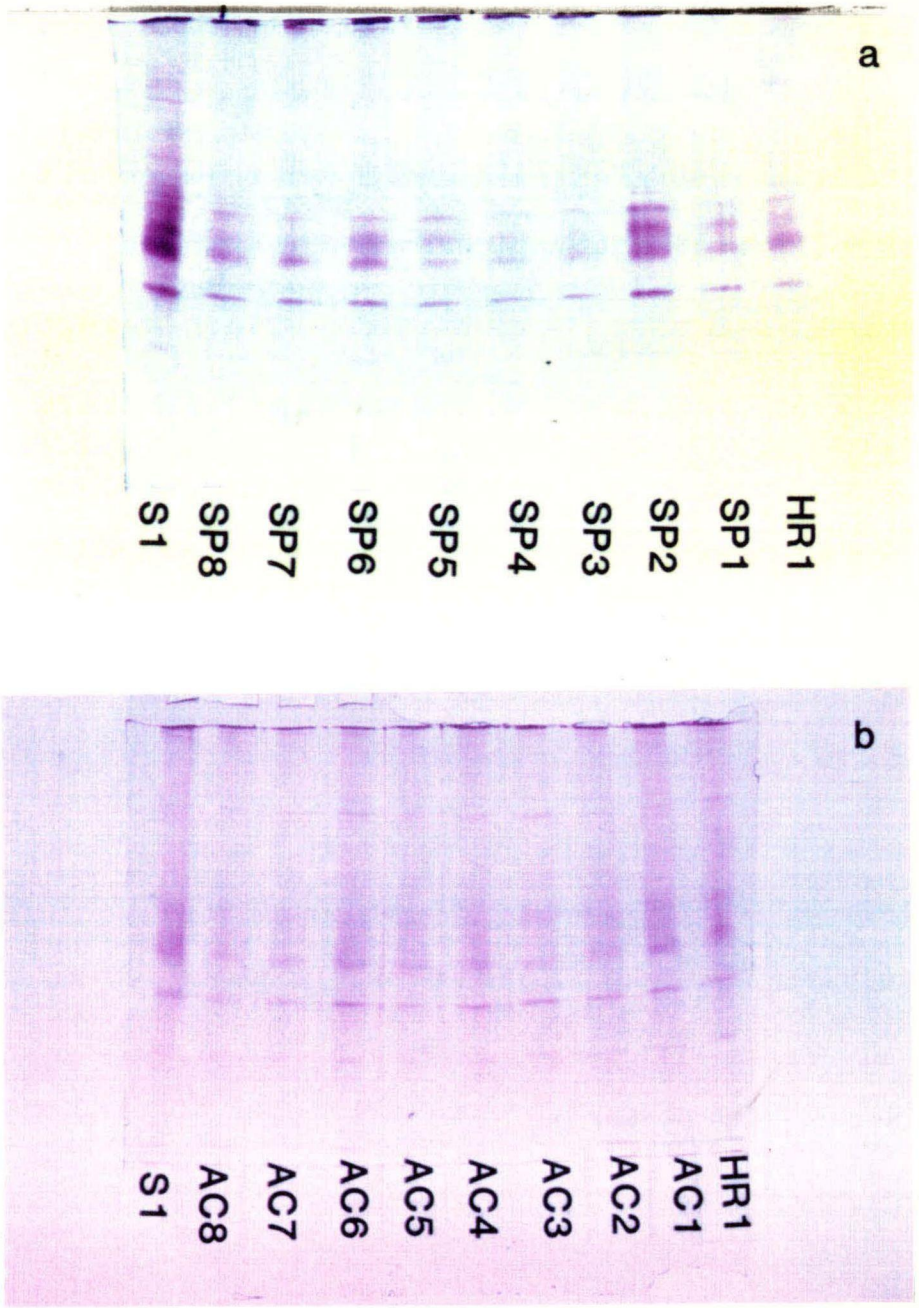

Figure 5.3 One-dimensional polyacrylamide gel electrophoresis of total proteins extracted from mycelium of *M. fructicola* resistant (HR1), sensitive (S1) field isolates and single ascospore isolates in linear sequences derived from; a) single parent of HR1, and b) attempted cross of S1xHR1. S=MBC sensitive isolate, HR=high MBC resistant isolate, SP=single ascospore isolate derived from single parent, and AC=single ascospore isolate derived from attempted-cross.

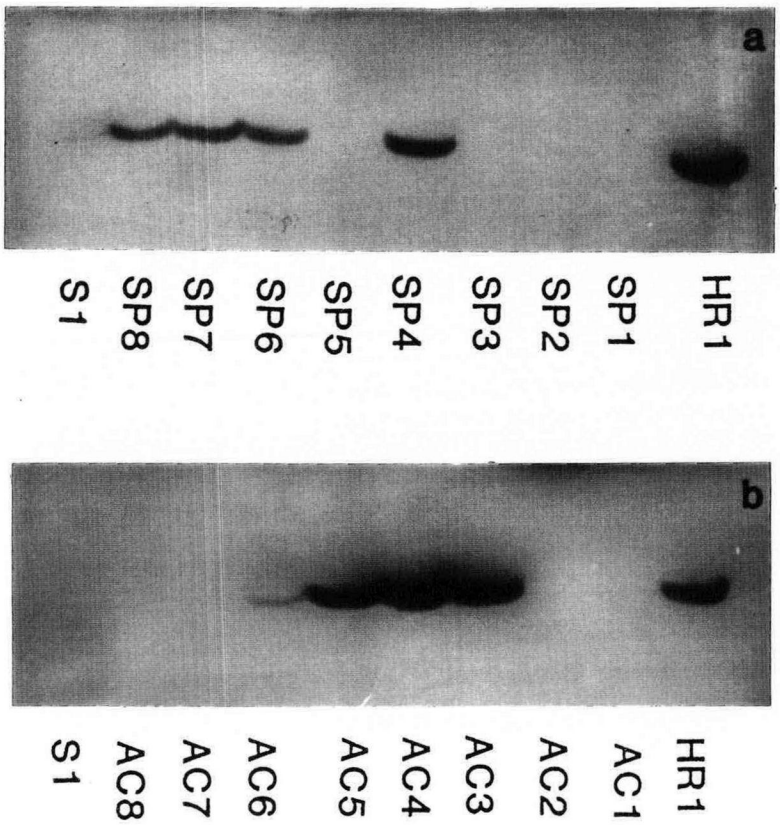

Figure 5.4 Starch gel electrophoresis of alcohol dehydrogenase (ADH) allozyme extracted from mycelium of *M. fructicola* resistant (HR1), sensitive (S1) field isolates and single ascospore isolates in linear sequences derived from; a) single parent of HR1, and b) attempted cross of S1xHR1. S=MBC sensitive isolate, HR=high MBC resistant isolate, SP=single ascospore isolate derived from single parent, and AC=single ascospore isolate derived from attempted-cross.

5.4 DISCUSSION

Resistance to the MBC fungicide appeared to be governed by a single gene which was inherited in Mendelian fashion in meiotic progeny. Evidence of segregation, 1:1, of resistant and sensitive phenotype was shown in most of the ascospore population (70%) derived from single resistant parents and attempted crosses (Table 5.1). The six patterns of segregation in ascospore progeny obtained from typical ordered tetrad analysis also supported the hypothesis that only one gene governs the resistance at high level (Table 5.2). The genetic basis of resistance to MBC fungicide and the phenotypes of *M. fructicola* resistant strains may be similar to those of species of plant pathogenic fungi belonging to the genus *Venturia* spp. (Stanis and Jones, 1984; Shabi *et al.,* 1986) and *B. fuckeliana* (Faretra and Pollastro, 1991). The resistance to MBC fungicide in *M. fructicola* is likely to be controlled by a polymorphic series consisting of at least two allelic mutations in a single Mendelian gene.

Aberrant segregation ratios, 5:3 and 6:2, for resistant and sensitive markers were observed in seven out of 64 asci. These deviations (from 1:1) indicated that a conversion mechanism occurred during meiosis or postmeiotic mitoses and the conversion was only to the resistant phenotype and not *vice versa.* Those asci with 5:3 segregations show segregation in only one of the four postmeiotic mitoses in the ascus. The 5:3 segregation is quite common in *Ascobolus immersus* (Fincham, 1983a) which is an apothecial producing fungus. On the other hand, the 6:2 segregation can be interpreted as a case of conversion of a whole product from one allelic type to other (Fincham, 1983a). The overall frequency of aberrant tetrads (10.9%) was comparable to the higher rates reported in yeast and in *V. inaequalis* (14.6%) (Boone and Keitt, 1956), although it was considerably higher than frequencies of gene conversion found in *N. crassa* or *Sordaria fimicola, S. brevicollis* (0.2-0.63%) (Fincham, 1983a). The occurrence of the conversion mechanism is likely to increase the chance of gene recombination between characters that are necessary for the fungus. An explanation for conversion given in Fincham (1983a) is that some

mechanism exists for correcting mismatches in DNA base-pairs. For example, the base-pair at a wild-type site is A-T, and that it changes by mutation to G-C, then hybrid DNA formed at that site in a wild/mutant heterozygote will contain either an A-C or G-T mismatch. Correction would bring about the removal of a mismatch base followed by the filling of the gap by repair synthesis using the surviving stand as a template.

Recombination frequency of characteristics can be calculated precisely using tetrad analysis (Fincham, 1983b; Bainbridge, 1987). Since only one gene governed the resistance, six segregation patterns (Figure 5.2) should be observed (Fincham, 1983b; Uhm and Fujii, 1983a). Although, there were few aberrant segregation ratios for the resistant marker, those six segregation patterns still could be recognised in 5:3 segregated asci. However, it was not possible to recognise the patterns in the 6:2 or 8:0 segregated asci because there was not enough marker to distinguish them. Therefore, the two asci were omitted from the sample. The ratios of segregation patterns were 21:41 of first and segregation divisions, respectively, suggesting that crossing- over occurred quite frequently in approximately 33% of ascospore progeny. High recombination frequency may eventually be expected between resistance and any characters relevant to the evolution of resistant population, such as pathogenicity and fitness during meiosis. This is an advantage of using tetrad analysis over the use of 1 mg/l carbendazim only in the ascospore populations study.

According to the reaction of ascospore progenies, the majority of ascospores in single ascus and most of the ascospore populations derived from high resistant parents were segregated (Table 5.1, 5.2), although, Ezekiel (1924) and Harada (1977) reported that the fungus *M. fructicola* is homothallic. If the fungus is homothallic and the perfect stages are formed by autogamy, no segregation in their asci would be expected. Therefore, in order to explain segregation in the asci of the fungus, self-fertility must be interpreted by a mechanism other than homothallism. For example, secondarily homothallism found in *N. tetrasperma* (Grindle, 1987) and *B. fuckeliana* (Faretra *et al.*, 1988b) or the mating type

mutation mechanism in *S. sclerotiorum* (Uhm and Fujii, 1983a) may occur. The segregation, 1:1, between resistant and sensitive phenotypes suggested that there are at least two types of nuclei in the same thallus. Therefore, this evidence supports the hypothesis of Byrde and Willetts (1977) that the fungus is heterokaryotic. This finding was quite different from the conclusion of Ezekiel (1924) and Harada (1977) and the fungus might not be a homothallic species.

There was other evidence in support of the heterokaryotic condition existing in resistant isolates and perhaps also in all isolates. Heterokaryosis is probably the mechanism responsible for the incidence of only resistant, only sensitive or segregated apothecia on the same fruit. This was similar to the situation found in *B. fuckeliana,* strains resistant to MBC and dicarboximide fungicides in approximately 6% in the field and in the laboratory (Faretra *et al.,* 1988b; Faretra and Pollastro, 1991). In addition, this finding suggested that the resistant gene is dominant because the only phenotype expressed was resistant.

The relationship between a single resistant gene with dominant character and heterokaryosis is potentially important to the evolution of the pathogen resistance to fungicide. The dominance of MBC resistant genes was reported in *N. crassa* (Borck and Braymer, 1974) and in nematodes (Driscoll *et al.,* 1989). The gene with dominant character encoding for ß-tubulin appears to be non-essential for growth in nematodes (Driscoll *et al.,* 1989) and is consistent with the lack of fitness deficit in many fungi possessing resistance to MBC fungicide (Shabi and Katan, 1979) including this fungus (Chapter 2; Jones and Ehret, 1976; Penrose *et al.,* 1979). Since the heterokaryotic condition occurred regularly in the *M. fructicola* resistant subpopulation it is likely to dominate the pathogen population because the phenotypic expression will be only resistant. If heterosis occurs in some heterokaryotic strains, the strains must then be more superior than the homokaryotic strains (Falconer, 1989). Even though, evidence in other fungi suggested that heterokaryosis may be confined to the same vegetative compatibility group (Caten, 1987; Boone, 1988; Brasier,

1988), heterokaryosis in *Monilinia* spp. occurs regularly in the field (Hoffmann, 1972; 1974). The occurrence of resistance to MBC fungicide provides more opportunity to grow normally in both presence and absence of fungicide selection pressure. The sensitive genotype will be well protected by the resistant one because resistant phenotype is dominant.

No differences between protein patterns of general activity suggested that the genetic basis of the *M. fructicola* being studied may be very narrow. Although, the test was conducted with a small number of isolates aiming at only investigating self- or cross-fertilisation, the result indicated low genetic variability in the pathogen population. Considering when there is a chance introduction of a fungus into a complete new region, the genetic variability of that population is much less than that of the large population of the fungus in the region from where it originated (Byrde and Willetts, 1977). In a host-pathogen relationship, such changes in the genotype of the one will affect the genotype of the other. However, commercially planted stone fruit trees are quite genetically uniform, the lack of change in host plants probably lead to reduced selection pressure.

Preliminary investigation into the potential usefulness of allozyme data as an indicator of outcrossing was investigated. Allozyme analysis revealed the same electromorphs for the parental field isolates and all the progeny tested except at the ADH locus. The segregation 4:4 of the ADH electromorph presence and absence in 8 monoascosporic lines derived from single asci potentially indicated cross-fertilisation. In progeny obtained from the single parent (HR1), a heterokaryotic condition for ADH activity was demonstrated. These nuclei are probably responsible for the production of apothecia. In the attempted cross (S1xHR1), the progeny may be derived from either cross-fertilisation between the two isolates or otherwise from only the HR1 isolate. The resistant parent sometime produced only sensitive progeny (Table 5.1), because two compatible sensitive nuclei in the heterokaryotic resistant parent possibly have equal chances for the production of apothecia. The ADH banding reaction may be useful as a polymorphic marker for *M. fructicola*. If

the ADH and the MBC resistant markers are used together in ascospore progeny, it may be possible to demonstrate recombination between resistance and other characters. Further screening of enzyme staining and buffer systems are needed to establish the usefulness of allozyme data as an indicator of outcrossing, and/or population variation.

Although, the genetic base of *M. fructicola* in New Zealand is possibly narrow, there have been several reports of cultural variations between single ascospore isolates (Sharvelle and Chen, 1943; Thind and Keitt, 1949). In the North Island of New Zealand where ascocarps of the fungus have been found occasionally (Tate, 1979), variation and recombination of characters are likely to occur frequently. The introduction of the low resistant MBC isolates (Chapter 2) through imported fruit, the occurrence of high resistant MBC, dicarboximide resistant and dual dicarboximide/high MBC resistant isolates, are likely to provide additional genetic variably into the gene pool of New Zealand brown rot populations. More recently, the emergence of a DMI triforine resistant subpopulation of *M. fructicola* was detected in Hastings orchards (Elmer *et al.*, 1992), although a disease control failure has not been reported. Recombination between the alleles coding for resistance to a particular fungicide and fitness characters might happen after sexual reproduction. It appears that the significance of apothecia and ascospores should be re-evaluated (Batra and Harada, 1986), as the basis for improvement of control strategies combating resistance in brown rot of stone fruit.

CHAPTER 6
GENERAL DISCUSSION

6.1 CURRENT STATUS OF *M. fructicola* RESISTANCE TO MBC FUNGICIDE IN NEW ZEALAND

The introduction of MBC fungicides in the late 1960's (Prince *et al.,* 1989) and their intensive use led to resistance problems in the North Island of New Zealand in 1979 (Anon., 1980). The presence of resistant strains led to complete control failures similar to those that occurred in Australia (Penrose *et al.,* 1979) and the United States (Ogawa *et al.,* 1988). The circumstances were similar to the emergence of MBC resistance in a number of plant pathogens (Smith, 1988), such as cercospora leaf spot in Greece (Georgopoulos and Dovas, 1973) and in the United states (Delp, 1980), *V. inaequalis* in Germany (Staub, 1991), penicillium fruit rot in citrus (Eckert, 1988a) and botrytis rot in European vineyards (Staub, 1991). Consequently, the MBC fungicides were replaced by dicarboximide fungicides in New Zealand stone fruit spray programmes in 1980 (Beever and Brien, 1983). However, in 1985 *M. fructicola* strains resistant to dicarboximide were detected (Elmer and Gaunt, 1986). Occasionally, dicarboximide resistance developed in strains already resistant to MBC fungicide (Jones, 1983; Elmer, 1990; Braithwaite *et al.,* 1991). The use of a negatively cross-resistant fungicide, such as N phenyl carbarmate (NPC) may lead to the development of triple resistance to all fungicides (Elad *et al.,* 1992). The dual- or triple-resistant isolates may complicate the control measures in brown rot of stone fruit, because fruit growers have less choice to control the pathogen.

Resistance to MBC has been detected recently in the South Island of New Zealand (Braithwaite *et al.,* 1991). Resistance is likely to occur as a result of an increased frequency of MBC fungicide applications in the South Island. The movement of infected fruit from areas where MBC resistance was detected, such as fruit imported from overseas, or the North Island, are possible further sources of such resistant isolates.

The MBC resistant strains still remain in many orchards in the North Island, New Zealand, even though dicarboximide and DMI fungicides have totally replaced MBC products in many New Zealand spray programmes (Elmer and Gaunt, 1986; Braithwaite *et al.,* 1991). The MBC resistance has persisted in orchards for at least 12 years as was reported in Australia (Penrose, 1990) and the United States (Ogawa *et al.,* 1988). The persistence of resistant strains in *M. fructicola* populations not exposed to selection pressure implies that these strains are as fit and pathogenic as the sensitive strains.

Three important aspects of resistance in *M. fructicola* have been investigated: 1) reliable apothecial production in the laboratory and single ascospore isolation in linear sequence from single asci for detailed studies of the sexual reproduction of *M. fructicola,* the genetic structure and the basis of resistance to MBC fungicide; 2) some epidemiological aspects, such as pathogenicity, incubation period (IP), latent period (LP), rate of spore production on blossom and fruit, rate of colonisation on fruit and competitive ability of these sup-populations; and the ability of the resistant and sensitive strains in the absence of selection pressure to survive on twig cankers and mummified fruit; 3) the first study on the inheritance of MBC resistance in *M. fructicola* and the basis of resistant mechanisms in the pathogen populations. These factors provide fundamental information to the long-term understanding of the epidemiology of resistance and the design of resistance management strategies for stone fruit orchards. Moreover, these achievements can be applied to study the resistance to dicarboximide and DMI fungicides, of which the genetic basis of resistance to these fungicides in *M. fructicola* have not yet reported.

6.2 TECHNIQUES FOR DETAILED STUDIES OF THE SEXUAL REPRODUCTION AND THE BASIS OF RESISTANCE TO FUNGICIDE.

A technique was developed so that apothecia can be induced reliably from inoculated fruit under controlled conditions in the laboratory and in the field (Chapter 4). This technique, in the laboratory, permits the further study of many important details on the developmental cycle of *M. fructicola* which still

require further investigation. Methods for experimental crossing of isolates may be established to study the combinations and segregation of genetic factors that occur in the development of the ascigerous stage. In addition, it may be possible to use the technique to duplicate the production of apothecia in related species of the *Monilinia* genus, such as *M. laxa, M. fructigena, M. oxycocci* etc. To study primary infection by ascospores (Mylchreest and Wheeler, 1987; Sanderson and Jeffers, 1992) and genetic recombination of characters, such as fungicide resistance, pathogenicity and fitness (Hunter, 1989; Nicholson *et al.,* 1991), a reliable method of producing apothecia is required. The technique is primarily important for ascospore progeny production, which leads to a study on components of fitness and selection within and between generations (Wade and Kalisz, 1990). The technique was used reliably to study the genetic basis of MBC resistance in *M. fructicola* which is likely to be useful as a preliminary for dicarboximide and DMI resistance studies.

A technique to isolate single ascospores from asci in linear order was developed. The technique provides an opportunity to use tetrad analysis which is a critical step in the genetical analysis of many characters. The occurrence of MBC resistant strains has offered a useful genetic marker, allowing us to recognise segregation patterns after meiosis. In addition, tetrad analysis is a useful approximation of the frequency of first- or second-division segregation for a character being studied. The second-division segregation frequency is significant to determine the crossing over and the recombination frequencies (Fincham, 1983b; Bainbridge, 1987). Recombination is important (Cockburn, 1991) for the following reasons. First, it disrupts linkage, breaking up favourable gene combinations. Second, it allows the transfer of new alleles derived from mutation, such as resistant gene, or gene flow throughout the population. Any mechanisms occurring during meiosis may be detected, such as the direction of gene conversion and its frequency. The technique was utilised to obtain recombination frequencies (34% chapter 5) which is likely to indicate a chance of recombination among characters to occur in ascospore progeny.

Recombination frequencies between characters were very high and allozyme analysis, used to distinguish between parents and progeny, suggested a narrow genetic basis of the brown rot population in New Zealand. It is probable that the disease was introduced from the United States into South America and Australasia more than a hundred year ago (Byrde and Willetts, 1977). From an evolutionary view point, New Zealand is regarded as a relatively new habitat for the pathogen and the genetic variability of a *M. fructicola* population is likely to be much less than that of the pathogen populations in the region of origins. It's most likely that the majority of isolates consisting in natural populations are fit and pathogenic because they co-adapt successfully to the local hosts. If resistant mutants emerge with lower fitness, the fitness deficit is likely to be repaired soon after sexual reproduction. However, in many instances the fungus was unable to survive in the new habitat so not many phenotypes existed. If so, further study by using allozymes, or some other molecular method, such as recombinant DNA analysis should be investigated intensively to ensure there is a narrow genetic base of *M. fructicola* in New Zealand. The information generated from such techniques is likely to be vitally important to the resistance breeding programme or new resistant varieties being introduced from other places.

6.3 EPIDEMIOLOGICAL ASPECTS OF MBC AND DICARBOXIMIDE RESISTANT AND SENSITIVE ISOLATES

Selection pressure is considered the most important driving factor influencing the dynamics of a pathogen population (Milgroom *et al.*, 1989). In the presence of fungicide selection pressure, a resistant sub-population is likely to dominate the population. In the absence of fungicide applications, however, natural selection may continue to operate in the pathogen population, because there are some phenotypic and genotypic variations in individual isolates (Wade and Kalisz, 1990; Stratton, 1992a; 1992b).

Survival ability, pathogenicity, fitness and natural selection may be highly correlated (Primack and Kang, 1989). In this study, the survival ability was indicated by the spore production from infected plant parts (Table 3.3, 3.4). All isolates resistant and sensitive to MBC and dicarboximide fungicides survived effectively for at least one year as mycelium in twig cankers and for at least one season in mummified fruit. Conidia produced from these sources were highly viable and pathogenic, so that mummified fruit, twig cankers and infected fruit peduncles are important as sources of primary inoculum for blossom blight in the spring. Although, their pathogenicity appeared to be maintained for at least a year (Chapter 3), the number of conidia recovered from infected tissues was found to be strain-related. The dicarboximide resistant isolate produced fewer conidia on both twig cankers and mummified fruit than the MBC resistant and sensitive isolates. The sensitive strains produced fewer conidia on both tissue than the MBC resistant strains. Therefore, MBC resistance is likely to dominate the pathogen population or at least the ratio of resistance will be maintained from generation to generation. The amount of spore production per unit area and the area of colonisation may be important indicators for measuring fitness, and perhaps persistence of resistant strains. These findings explained why MBC resistance has persisted for at least 12 years (Adaskaveg *et al.*, 1987; Penrose, 1990; Braithwaite *et al.*, 1991) in the absence of selection pressure, whereas the dicarboximide resistance decreased dramatically after the use of dicarboximide fungicides had ceased (Elmer, 1990).

The fitness, pathogenicity and survival ability of MBC and dicarboximide resistant strains were documented. Range of infection cycle components, such as incubation period (IP), latent period (LP), rate of colonisation and rate of spore production in different phases of disease cycle were investigated individually. These investigations covered blossom blight, fruit brown rot and survival phases. Such studies of multiple characters may eventually identify which specific characters are the targets of selection.

In the short term, the proportion of MBC resistant strains in a population may

remain low compared to the most sensitive strains, if there is no selective advantage. The strong competitive ability of strains would maintain the larger sensitive proportion in the population. These results indicated that resistant isolates were as fit, pathogenic and equally competitive as sensitive isolates on blossom and fruit. However, there were some differences in fitness, pathogenicity, competitive ability and survival ability of individual isolates. In the long run, selection for an isolate to dominate the population should favour the one that has greater virulence and fitness. Evidences for recombination frequency (34% in Chapter 5) and variation in colony characteristics (Thind and Keitt, 1949) were quite high after sexual reproduction. If recombination of resistance with great virulence and fitness has occurred, resistance is likely to dominate the pathogen population.

Considering the host, commercially planted stone fruit trees are relatively uniform genetically, perennially and spatially. The pathogenicity of a given strain may vary depending on host genotype (Augspurger, 1990). Host populations can act as powerful selective forces on pathogen populations (Burdon *et al.,* 1989; McDonald *et al.,* 1989). Hence, selection pressure by the host is likely to select strains of high fitness and virulence. The virulent and fit strains survive adverse winter conditions on the host tissues or on the ground nearby. Infection may occur repeatedly by the same strains year after year. There may be considerable variation in the number of infection cycles on flowers in Spring, or on fruit during the Summer (Elmer, 1990). Despite this variation, the population will be dominated in each case by the fittest strains of the pathogen.

At both blossom blight and fruit brown rot phases, the rate of spore production will be greater by the virulent and fitter genotypes than by the slower reproducing genotypes. If infection occurs at all, the rate should be proportional to the number of inoculum propagules. Hence, the basic reproductive rate, R_0 (Van der Plank, 1963; Zadoks and Schein, 1979), or the rate at which secondary infections are produced, of the virulent pathogen is

expected to be greater than the R_0 of the less reproductive genotype. Although lower fitness could delay the initial advance of virulent strains and subpopulation extinction and replacement may happen (Gale, 1987), no lower fitness associated with the MBC resistant isolates was observed. Four assumptions can be made based on this argument: 1) higher epidemic rates in strains are achieved by higher reproductive rates; 2) the reproductive activities (fitness) of the strain is possibly linked to virulence; 3) competition among genotypes *in vivo* favours the most virulent one; and 4) winter survivorship is the most important factor determining the relative proportion of resistant and sensitive strains in a population, consequently the success of the pathogen population as a whole.

In general, those pathogen individuals that have a rapid spore production rate are more successful at generating progeny for secondary infections than pathogen individuals with lower rates of reproduction. For example, isolates S1, HR1, HR3 and LR2 that had higher rates of spore production are expected to generate more propagules than isolates S2, HR2, LR1, D/HR and DR under conducive conditions (Table, 2.3, 2.4, 3.5, 3.6). The tests, however, were in optimal conditions and several factors influencing reproduction rate may vary considerably in nature. For instance, relative humidities and wetness duration are known to influence the rate of infection and spore production (Biggs and Northover, 1988b; Wilcox, 1989). Natural selection among isolates of the pathogen is likely to favour the isolate that has the greatest number of inoculum, which are highly viable and most pathogenic.

There were several arguments supporting the generality of the second assumption suggested in this study. Inoculum density was the major effect influencing virulence and fitness of the pathogen on both flower and fruit. The greater the spore production rate, the higher the inoculum density on the host tissue. The larger the pathogen burden the greater the severity to the host plant. In this study, the high inoculum concentration ($1x10^4$ spores / infection site) caused significantly shorter incubation, shorter latent periods, higher rates of spore production on both flower and fruit than the low inoculum

concentration ($1x10^2$ spores / infection site). In addition, the greater the rate of spore production and the amount of the inoculum present, the more severe the disease due to secondary infection. The quantitative data (Table 2.3, 2.4) show the pronounced effect of inoculum density in all resistant and sensitive isolates tested.

The third assumption is that competition amongst isolates present together in the same niche favours the most virulent and the fittest strains. Disease manifestation in the host tissue was caused by the most pathogenic isolate. The fittest strain is most likely to be dispersed to a new host. Inoculum propagules affected the competitive outcome and the higher the proportion, the greater the competitive ability. The most virulent isolate will occupy the majority of the host tissue and reproduce more progeny than the less virulent and less pathogenic isolates. There is no opportunity for less fit isolates to colonise host tissue at a later time when the niche for food is limited.

The last assumption is winter survivorship which is considered the most important component of selection. Survival ability is important to a pathogen in order to remain in the population. The overwintering ability is indicated by the amount of initial inoculum density. The initial inoculum density was considered one of the main factors in determining if a blossom blight or brown rot epidemic will occur (Chapter 2). Even though, wet and cold weather during winter drastically reduced initial inoculum (conidia but not ascospores) (Budge and Whipps, 1991), there were some differences in the ability of isolates to produce conidia in the spring. Thus, when conditions favour the pathogen during the critical blossom infection in the spring, only the isolates that survive can cause an epidemic. The main sources of inoculum were identified as conidia produced from infected plant parts (Chapter 3), and as ascospores from fruit stromata on the ground at Lincoln, Canterbury (Chapter 4).

In this study, the MBC isolates survived more effectively than the sensitive and the dicarboximide isolate (Table 3.3, 3.4). Conidia and possibly ascospores

are the initial source of infection for blossom blight in spring (Tate, 1979; Zehr, 1983). The link between blossom blight and fruit brown rot may be by latent infection and quiescent infection on young or immature fruit (Ogawa *et al.*, 1983; Rosenberger, 1983) and cankers on twigs. Conidia produced from twig cankers during late spring and summer may infect immature fruit and fruit at ripening stage. No conidial or apothecial development occurs in the absence of adequate moisture.

Ascospores are very important inoculum for blossom blight in the spring, but not for fruit brown rot in summer (Zehr, 1983). The most important factor is the possibility that a change might occur with the transmission of phenotypes across generations, from parent to offspring, owing to the genetic phenomena of segregation, recombination, chromosomal rearrangement, and perhaps, genetic drift (Batra and Harada, 1986; Wade and Kalisz, 1990). The most virulent and fit isolate whether carrying resistant character or not, may become established and subsequently acted upon by selection pressure, which will proceed to dominate the population.

M. fructicola isolates resistant to MBC fungicide potentially dominate the pathogen population in both the presence and the absence of fungicide selection pressure. It is likely to cause disease control failures in the areas where the MBC fungicide is still in use. The high resistance factor and the high fitness of resistant strains probably result in the domination of MBC resistance. Resistance may quickly build up to high frequencies in the populations, where it remains stable, in the absence of fungicide selection pressure. After the development of resistant foci, the resistant strains overwintered effectively in both infected twig cankers and mummified fruit. Summer and winter conditions did not limit survival to any degree, such as sporulation quantitatively, pathogenicity and fitness on both blossom and fruit. The MBC resistant isolates produced apothecia in similar numbers to the sensitive isolates. Hence, the MBC resistant population is likely to co-exist or dominate in the brown rot population. In contrast, isolates resistant to dicarboximide fungicide survived and sporulated poorly compared with the

sensitive isolates. The dicarboximide resistant population is likely to decline sharply unless dicarboximide fungicides are used frequently (Elmer and Gaunt, 1990). However, the dicarboximide resistant genotype will not disappear from the *M. fructicola* population as found in *B. cinerea* (Grindle, 1987; Akutsu *et al.*, 1988a; 1988b).

6.4 GENETIC BASIS OF MBC RESISTANCE IN *M. fructicola*

The inheritance of resistance and mechanisms of inheritance were identified by using those techniques mentioned previously. Resistance to MBC fungicide was completely dominant and governed by a single major gene. Recombination percentage in ascospore progeny was quite high. A gene conversion mechanism was detected in only one direction from sensitive to resistant character. In terms of resistance dynamics, recombination may affect the fitness of resistant individuals and subsequently alter phenotypic frequencies. Heterokaryosis occurs frequently and is a very important condition in *M. fructicola.*

Heterokaryosis is considered the most important mechanism influencing the life cycle of the brown rot fungus resistant and sensitive to MBC fungicide. Hyphal anastomosis between different strains to form multinucleate heterokaryons is likely to occur regularly in the field and may take place during blossom colonisation by ascospores. Conidia that are already heterokaryotic may continue to combine with the other during colonisation. It was found that approximately 1-4 vegetative incompatibility groups (VCG) of isolates co-infected on a single flower to form blossom blight in the spring (Sonoda *et al.,* 1991). Three important phenomena may have followed: 1) mating success between compatible germ tubes from different ascospores; 2) multinucleate homokaryon become a multinucleate heterokaryon which may bring about heterosis (hybrid vigour) in the heterokaryon; and 3) the sensitive nuclei become well protected and the only phenotypic expression is resistant.

Mating success between compatible germ tubes from different ascospores may

be possible through hyphal anastomosis, which may bring together two different nuclei into the same thallus. Sexual reproduction and genetic variation in ascospore progeny will occur whenever the conditions are suitable. This study revealed that apothecia could be produced readily from single parent field isolates. The situation is similar to the finding of Faretra and Pollastro (1991) that heterokaryosis in *B. fuckeliana* stimulates sexual reproduction through a self-fertile mechanism. Mating in *Pyrenopeziza brassicae* must take place soon after ascospore germination (Courtice *et al.*, 1988).

A multinucleate homokaryon may become a multinucleate heterokaryon, if there is multiple infection on the same niche which may bring about heterosis in the heterokaryon. When two compatible strains of *N. crassa* were inoculated together, fusion of hyphae occurred resulting in the mixing of two kinds of nuclei in a common cytoplasm (Fincham, 1983a). The two strains were mutant in different genes for non function and the heterokaryon grew like the wild type since each component supplied the normal function which was lacking in the other. Infection behaviour of *M. vaccinii-corymbosi* may be evidence supporting heterosis in the heterokaryotic mycelium. Infection by ascospores caused leaf and shoot blight whereas conidia caused mummification of fruit, because only the conidia can cause infection of the ovary (Batra, 1983; Lambert, 1990). The homokaryotic and the heterokaryotic multinucleate isolates may behave differently in host tissues and the differences in characters, such as pathogenicity and fitness are most likely to show on fruit tissue. If the fungus is considered a heterothallic species, a consequence of inbreeding almost universally observed is a reduction of fitness (Falconer, 1989). Heterokaryosis can increase fitness, and is considered equivalent to the formation of heterozygous individuals. A consequence, in contrast, is heterosis which may improve pathogen fitness and subsequently acted upon by selection pressure.

The sensitive nuclei become well protected because; 1) heterokaryosis is quite common in field isolates (Hoffmann, 1972; 1974; Byrde and Willetts, 1977) and

heterokaryosis is quite common in a few *Sclerotiniaceae* (Fujii and Uhm, 1988; Faretra and Pollastro, 1991); 2) resistance is dominant; and 3) a segregation, 1:1, of resistant and sensitive phenotypes reappeared in most ascospore progeny (Chapter 5). An obvious evidence was demonstrated by Borck and Braymer (1974) that fusion of MBC resistant and sensitive hyphae of *N. crassa* resulted in a heterokaryotic strain which was able to grow normally in the presence of fungicide. Similar results were investigated in *B. cinerea* strains sensitive to MBC and dicarboximide fungicides (Grindle, 1987; Akutsu *et al.*, 1988a; 1988b). Hyphal anastomosis was not only changing the degree of resistance but also brought about differences in pathogenicity. The other supporting evidence is derived from competitive experiments conducted by Penrose *et al.* (1979). Fruit were inoculated using spore suspensions mixed in ratios of 1:9, 1:1 and 9:1 of susceptible and resistant isolates. In most cases, the isolate inoculated in the larger proportion predominated at the end of the first successive generation. However, only the resistant phenotypes were observed after 3 consecutive cycles of competition on fruit. Apart from this argument, vegetative compatibility groups and any associated factors should be understood clearly before conducting competitive experiments. Complementation groups which each group corresponds to a different functional gene, ie. a different degrees of resistance are likely to complicate the outcome of competition. Care should be taken to overcome the complexity of *M. fructicola* life cycle and perhaps to the other related species.

6.4 MANAGEMENT OF RESISTANCE IN *M. fructicola*

The overwintering ability of *M. fructicola* populations is likely to be the most important factor determining the severity of a brown rot epidemic in following reason. The more overwintered diseased tissues, the more initial inoculum will be produced. Blossoms are vulnerable to attack and if there are numerous inoculum sources on trees or on the ground, blossom infection will inevitably occur in the spring. High humidity and free water due to rain and heavy dew frequently occur which, in most areas, favour apothecial production (Chapter 4), and conidial production on infected plant parts (Chapter 3; Corbin and

Cruickshank, 1962). Bright sunshine with mild prevailing wind the following day results in the release and dispersal of ascospores and conidia to host tissues during the day (Corbin and Ogawa, 1974; Tate, 1979). Morning frost in spring is likely to follow and cause floral injuries. Injured tissues result from the intracellular infiltration of water and solutes (Palta, 1990). Leakages of solutes from injured tissues are likely to stimulate ascospore or conidial germination, resulting in increased infection incidence (Hildebrand and Braun, 1991). Blossom infection leads to reduced yield directly and to the formation of twig cankers which harbour inoculum until fruit are susceptible to infection. This suggests three control strategies for protecting stone fruit and for anti-resistance strategies: 1) infected plant parts should be removed from trees in orchards and nearby home gardens; 2) good sanitation practice of orchard floor in combination with watering system; and 3) applications of protective fungicide should be implemented before infection occurs.

Good crop production practices are essential for resistance management. Isolates resistant to MBC fungicide survive adverse conditions effectively on twig cankers and mummified fruit (Chapter 3), thus any infected plant parts on the tree could be sources of resistant inoculum. Removal of mummified fruit, infected fruit peduncles, blighted twigs and stem cankers should be practiced for resistant management. A decrease in diseased plant parts not only reduces resistance sources, but also decreases initial inoculum quantitatively which may delay brown rot epidemics. Autumn pruning and knocking mummified fruit onto the ground are very common practice in some blocks for orchardists (Elmer, 1990). Although pruning systems which remove some diseased tissues have been practiced in many New Zealand stone fruit orchards, overwintering twig cankers and mummified fruit hanging in trees were sometimes numerous in the spring. They were often found on trees in home gardens. Sporulation ability of resistant and sensitive isolates from overwintering mummified fruit and one year old twig cankers was relatively high during blossoming. These findings call for careful pruning practice or other management practices designed to eliminate the resistant strains from

stone fruit orchards. Although, careful pruning is likely to increase labour costs at first, it may provide effective control management, proving economical in the long run. The efficacy and commercial life of fungicides will be prolonged.

Apothecia were found naturally in the Lincoln University stone fruit orchard, Canterbury, during this study from 10 August till 15 September 1990. The ability of *M. fructicola* to produce apothecia in the field was recognised by Winter (1883) and subsequently, the occurrence of apothecia in nature have been reported sporadically in many countries (Landgraf and Zehr, 1982; Tate, 1979; Biggs and Northover, 1985). In addition, ascospores produced from mummified fruit on the ground in early spring provided more inoculum and may enhance genetic diversity for the pathogen population. Apothecia were often found in shaded areas in very humid climates. This finding is important because air borne ascospores may permit spread of the pathogen over relatively large distances (Hunter, 1989; Lambert, 1990; Nicholson *et al.*, 1991). Hence, this might explain the presence of fungicide resistant isolates in fields with no history of fungicide applications as found in *Tapesia yallundae* strains resistant to MBC fungicide (Sanderson and King, 1988). The present discovery of apothecia in the field in a dry production region calls for diligent search for their presence and reassessment of the contribution to the pool of primary inoculum in the region where blossom blight is prevalent. To interrupt the process of apothecial production, irrigation systems should be limited around tree canopy and the moist condition should be interrupted for sometime. It is important that the orchard floor should be clear (without grasses), so that no sexual reproduction develop from mummified fruit exposed to dry conditions and that mummified fruit be cleared up.

In the field, production of apothecia in controlled condition was demonstrated (Baxter *et al.*, 1974) and it was modified reliably using known strains in this study. The method can be used to monitor and detect the occurrence of natural apothecia where ascospores are considered the most important source of primary inoculum for blossom blight, such as in the North Island, New

Zealand (Tate, 1979). In the spring 1990, mature apothecia were found approximately two weeks earlier suggesting that primary inoculum in the orchard air may be present before the initial spray programme. Perhaps an earlier application of protective fungicides during the primary infection periods would provide better control of the disease by preventing primary infection and thus possibly delaying the onset of epidemics.

In addition, control strategies may be implemented, such as application of DMI, dicarboximide or other protective fungicides which are registered for the control of stone fruit brown rot may be practiced when the condition is conducive. Current recommendations for blossom blight spray program may be not appropriate because blossoming is not a synchronous event and even a first bloom in some flowers have been opened for some time. Because prolonged wet periods may be frequent and probably be of long duration in stone fruit orchards in spring, spore production or ascospore release are likely to occur. Blossom infection is likely to take place the following day or two after frost injuries (Hildebrand and Braun, 1991). Monitoring of weather forecasting may be necessary in order to reveal a correlation between spore release, frost injuries and infection. By speculation, blossom infection would be likely to occur the night after bright sunshine day which is often associated with early morning frost, so that control strategies should be practiced to prevent ascospore and conidial infection. More research to verify this speculation should be conducted in order to assist the present stone fruit management spray programme.

6.5 FUTURE RESEARCH

Several factors remain for intensive investigation in order to predict which strains are going to dominate the pathogen population in the absence of fungicide selection pressure. Genetic information is considered an important factor to understand the biology of the fungus and the fitness variables being studied. Data are needed on: 1) sexual behaviour in order to determine pair-wise mating isolates, 2) role of microconidia which may be important to

initiate cross-fertility, and 3) the improvement of apothecial production techniques. Details are discussed below.

Mating systems can be used to determine isolate pairs for experimental genetic study from one generation to the next, and the mating system in *M. fructicola* is not known. Even though most field isolates examined produced apothecia by self-fertile mechanism, two different mating types may exist in the population. Two different nuclei carrying different mating types may co-exist in the same thallus via anastomosis between hyphae originating from different single ascospores. The mating behaviour of the fungus should be determined before conducting research on the fungus genetic system.

Although microconidia were found abundantly on fruit surface in the laboratory and they were abundant in the dark line wherever members of the different pairs were adjacent to each other on PDA (Thind and Keitt, 1949), their function in *M. fructicola* have not been investigated. Microconidia of many fungi belonging to the *Sclerotiniaceae* function as spermatia, such as in *S. trifoliorum, S. minor, S. sclerotiorum* (Fujii and Uhm, 1988), *S. gladioli* (Drayton, 1932) and *B. fuckeliana* (Faretra and Pollastro, 1991). Microconidial function and genesis in *M. fructicola* should be investigated to assist any research involved with sexual outcrossing.

The production of apothecia under laboratory conditions is a lengthy and tedious process. The processes may be speeded up if various factors involved in the process are clearly understood. Incubation temperatures during stromata maturation and cooling treatment may influence the processes of sexual induction. Vernalisation, a cooling treatment, of mature stromata which were done in *S. sclerotiorum* at 4 °C for 4 weeks (Mylchreest and Wheeler, 1987; Smith and Boland, 1989; Sanford and Coley-Smith, 1992) or in *B. fuckeliana* at 0 °C for 30 days (Faretra *et al.*, 1988a) may trigger the development of apothecial initials in *M. fructicola*. Combinations of temperature and incubation time may be investigated to speed up the sexual induction process.

The inheritance of many characters of the fungus resistant and sensitive to MBC fungicide are left to be investigated. For examples, the inheritance of pathogenicity of resistant and sensitive isolates and the resistance of stone fruit cultivars to these strains are not known. Many characters may be used as important genetic markers, and if use in combination with the resistant marker, recombination between characters could be determined. The nature of inheritance of any of these characters may indicate the response to selection.

Heritability of many fitness characters may be polygenic, quantitative genetic approaches may be useful to analyze multiple components of selection in natural population. Selection target should be identified. Changes within and between generations are likely to be the intensive study areas in order to understand clearly how and why selection operates in the pathogen populations. An understanding of selection can be applied readily to study of dicarboximide resistant strains in *M. fructicola,* already occurring in New Zealand stone fruit orchards. Moreover, *M. fructicola* strains resistant to the DMI fungicide which developed easily in the laboratory (Nuninger-ney *et al.*, 1989), have the potential to emerge in the field. There is now some evidences that this may already have occurred in New Zealand orchards (Elmer *et al.*, 1992). The results obtained from this study are directly useful and applicable to study and to manage the resistance to the DMI which is expected to be more complicated than the MBC resistant pathogen.

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Appendix 1. General characteristic of tested isolates of *M. fructicola* to Methyl benzimidazole carbamate (MBC) fungicide.

Isolates[a]	Phenotypes[b]	Growth rate at 0 mg a.i/l carbendazim	EC50 mg a.i/l carbendazim	Maximum concentration inhibition mg a.i./l carbendazim	Date of collection
S1	MBCS	0.57±0.01	0.06	0.1	1982
S2	MBCS	0.47±0.02	0.17	0.3	1986
S3	MBCS	0.57±0.01	0.05	0.1	1990
S4	MBCS	0.50±0.01	0.05	0.1	1990
S5	MBCS	0.53±0.07	0.07	0.1	1986
S6	MBCS	0.51±0.01	0.05	0.1	1986
RD10	MBCS	0.45±0.02	0.08	0.1	1988
LR1	MBCLR	0.43±0.01	1.25	3	1989
LR2	MBCLR	0.68±0.01	1.64	3	1989
CA1	MBCLR	0.69±0.01	1.44	3	1989
CA2	MBCLR	0.67±0.01	1.50	3	1989
CA3	MBCLR	0.68±0.01	1.52	3	1989
CA6	MBCHR	0.58±0.01	>30000	>30000	1989
CA7	MBCLR	0.70±0.02	1.44	3	1989
CA8	MBCLR	0.69±0.02	1.49	3	1989
CA9	MBCLR	0.60±0.01	1.55	3	1989
CA10	MBCLR	0.68±0.01	1.48	3	1989
CA11	MBCLR	0.65±0.01	1.47	3	1989
CA12	MBCLR	0.65±0.01	1.44	3	1989
CA13	MBCS	0.58±0.01	0.05	0.1	1989
CA14	MBCLR	0.45±0.05	1.33	3	1989
CA15	MBCLR	0.69±0.01	1.38	3	1989
CA16	MBCLR	0.56±0.01	1.69	3	1989
CA17	MBCLR	0.66±0.01	1.53	3	1989
CA18	MBCLR	0.37±0.02	1.82	3	1989

(continued on following page)

Appendix 1 (*continued*)

Isolates[a]	Phenotypes[b]	Growth rate at 0 mg a.i./l carbendazim	EC50 mg a.i./l carbendazim	Maximum concentration inhibition mg a.i./l carbendazim	Date of collection
CA19	MBCLR	0.50±0.01	1.80	3	1989
CA20	MBCLR	0.55±0.01	1.50	3	1989
CA21	MBCLR	0.63±0.01	1.48	3	1989
CA22	MBCLR	0.63±0.01	1.46	3	1989
HR1	MBCHR	0.05±0.01	>30000	>30000	1988
HR2	MBCHR	0.38±0.02	>30000	>30000	1988
HR3	MBCHR	0.59±0.01	>30000	>30000	1988
HR4	MBCHR	0.59±0.02	>30000	>30000	1988
JC1	MBCHR	0.48±0.01	>30000	>30000	1988
JC4	MBCHR	0.46±0.01	>30000	>30000	1988
JC8	MBCHR	0.48±0.01	>30000	>30000	1988
JC10	MBCHR	0.44±0.01	>30000	>30000	1988
JC13	MBCHR	0.42±0.01	>30000	>30000	1988
RD6	MBCHR	0.52±0.01	>30000	>30000	1988
RD8	MBCHR	0.40±0.01	>30000	>30000	1988
RD12	MBCHR	0.38±0.02	>30000	>30000	1988
W124	MBCHR	0.63±0.01	>30000	>30000	1989
W211	MBCHR	0.59±0.01	>30000	>30000	1989
W225	MBCHR	0.30±0.01	>30000	>30000	1989
D/HR	DMR/ MBCHR	0.50±0.02	>30000	>30000	1986

[a] S=MBC sensitive isolate, LR=low MBC resistant isolate, HR=high MBC resistant isolate, D/HR=dicarboximide/high MBC resistant isolate, RD=R. Duncan isolate, CA=Californian isolate, JC=J. Clarke isolate, W=Waikato isolate

[b] MBCS=MBC sensitive phenotype, MBCLR=low degree MBC resistant phenotype, MBCHR=high degree MBC resistant phenotype, DMR=dicarboximide resistant phenotype