Biological control of *Botrytis cinerea* on kiwifruit

A thesis presented in partial fulfilment

of the requirements for the degree

of Master of Horticulture science

at

Massey University

Samir Shamouel Sada

1992

ABSTRACT

Screening for potential antagonists was carried out on plant parts of kiwifruit (*Actinidia deliciosa var. delicisa* [A.Chev]. Lang & Ferguson, cv. Hayward) taken from kiwifruit orchards in four collections.

A range of microorganisms have shown potential activity against *Botrytis cinerea* Persoon. ex Fries, on Potato dextrose agar (PDA) petri dish at various temperatures, including 0°C.
The antagonism was also tested on different media with different pH for antibiosis or mycoparasitism action.
It was found that temperature had a much greater effect on growth and activity of the antagonists than did pH.

Three isolates (FB3, FF9, FO30) which showed good biocontrol activity were tested for ability to inhibit spore germination and germ tube elongation of *B.cinerea* on 2.5% vegetable juices (V.8) medium discs. One of these isolates (FO30: *Fusarium merismoides*) showed such ability.
These isolates were selected for a trial on kiwifruit.

Stem end rot was partially controlled under storage condition when the pathogen (*B.cinerea*) and the antagonist were inoculated simultaneously.
Harvested fruit were inoculated with different inoculum levels and subjected to different curing periods.
The inoculum level of *Fusarium merismoides* isolate FO30 showed a significant affect on the percentage of soft rot caused by *B.cinerea*, and reduced disease incidence on kiwifruit by 17-21% after 13 weeks storage at 0°C.
The curing period did not have any significant effect on the percentage of soft rot except when the fruit was cured for 2 days at ambient temperatures, inoculated, and left 2 further days at ambient temperatures before storage at 0°C.
Further work is required to investigate enhancement of biocontrol of *B.cinerea* on kiwifruit by manipulation of the curing period.

Several microscopic stains including Chlorazol black, Lactophenol cotton blue and phloxine gave good staining of the spores and mycelium of *B.cinerea* and antagonists on 5% V.8 medium and kiwifruit tissue.

ACKNOWLEDGEMENTS

First thanks to God, the creater of humankind.
My sincere thanks go to my supervisor, Dr. P. Long, for his constant support and guidance throughout this study and for his careful assistance during the preparation of this thesis.

I would like also to thank my co-supervisor, Prof. E. W. Hewett, for his instruction during this study.

Thanks are also due to the members of the Department of Horticulture and Plant health who have contributed in this study, among them A. Qadir, B. Dadzie, S. Bautista, and A. Abdulla of Animal Science Department.

I acknowledge also the valuble assistance from the technician of the Plant Health Dept., Hugh Neilson.

Thanks for my loving mother for her faith and her prayer for me.

Special thanks and appreciation to my dear wife Juliette for her love and to my mother in-law Samira Tollo and my four cousins for their support.

TABLE OF CONTENTS

CHAPTER 4 IDENTITY OF ISOLATES

CHAPTER 5 ANTAGONIST ACTIVITY IN DIFFERENT MEDIA

CHAPTER 6 IN VITRO TESTING INHIBITION OF *B.cinerea* SPORE GERMINATION

CHAPTER 7 EFFECT OF BIOCONTROL AGENTS ON INFECTION OF KIWIFRUIT BY *B.cinerea* IN STORAGE

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A.PDA = Potato dextrose agar amended with antibiotic
B = *Botrytis*
CMA = Corn meal agar
CP1 = Curing period one
In = Isolate overgrown by *Botrytis*
In.1 = Inoculum level one
KFJ = Kiwifruit juice
MA = Malt extract agar
MEA = Malt extract agar
µl = Microlitre
µm = Micrometre
NA = Nutrient agar
PDA = Potato dextrose agar
S = *Botrytis* overgrown commencing
SDW = Sterile distiled water
SDWT = Sterile distiled water amended with tween 20
UV = Ultraviolet
V.8 = Juice of eight different vegetables

LIST OF APPENDICES

APPENDIX 1

TABLES

APPENDIX 2

TABLES

CHAPTER ONE

LITERATURE REVIEW

PART 1: BOTRYTIS ROT OF KIWIFRUIT

Botrytis cinerea

1.1 **INTRODUCTION**

Botrytis cinerea Persoon, is one of the most serious pathogens affecting the production and postharvest quality of kiwifruits. It infects most fruits, vegetables and ornamental crops, and first infection could be a result from prior infection of flower. Throughout the world, about a fourth of the fruit harvested is lost due to storage disease (USDA, 1990). Decayed fruit in the overseas market represents a loss of a large financial investment in harvesting, packaging, storage and transportation beside the wastage of fruit and vegetable by microorganisms during movement from harvest to consumption which can be rapid and severe (Wills, *et al.* 1981). Storage rot caused by *Botrytis* is the major cause of postharvest fruit losses in kiwifruit (Hopkirk & Clark, 1990), and costed New Zealand kiwifruit industry $10 million in 1989 (Hopkirk, pers. comm.).

Generally *B.cinerea* lives saprophytically upon the dead leaves and flowers of many plants. It occurs on a wider range of plants than other *Botrytis* species, thus it has received more attention. It grows extensively, or can survive for long periods on the phylloplane prior to penetration (Blackeman, 1980). The primary route of *B.cinerea* into kiwifruit inoculated on the picking scar is via the vasculature of the stem wound (Sharrock & Hallett, 1992). Kiwifruit become infected by *B.cinerea* during harvesting operations, and a picking wound lead to storage rot (Pennycook, 1985). Infections are not always visible at harvest but they develop rapidly in transit under the conditions of high humidity maintained in shipping containers (Redmond, 1987). Products that are actively infected just before or at harvest usually enter the marketing channels because the infections are not visible or have been overlooked (Cappellini & Ceponis, 1985). One of its important features is its adaptation to grow at low temperatures, hence it can grow on fruit in shipping containers at 0•C. Under cool and wet conditions in the field *B.cinerea* can be a limiting factor in the production, marketing and storage of kiwifruit.

Botrytis stem-end rot became a problem in New Zealand in 1978 (Beever, *et al.* 1984). Up to 32% incidence of primary stem-end rots have been recorded in New Zealand kiwifruit (Pennycook, 1985). The problem is that a postharvest wound infection caused by *B.cinerea* can not be directly controlled by preharvest treatments (Pennycook, 1984) because the picking wound is the one point that can not be directly protected by orchard sprays. Freedom from decay is an essential quality because of the potential spread of decay from infected to sound fruits during storage or transport (Harvey, 1978). Avoidance of injuries during harvesting and handling is usually considered an important disease-reduction measure (Lakshminarayana, *et al.* 1987).

Different methods have been used to control this pathogen including biological control.

1.2 CLASSIFICATION, MORPHOLOGY AND GENERAL CHARACTERISTICS

1.2.1 Anatomical form species

Anamorph stage (Imperfect stage or asexual stage-conidial stage):
This stage produces conidia which belongs to the form genus *Botrytis*.

Taxonomy of *Botrytis* :

Division : Eumycota
Subdivision : Deutromycetes
Form-order : Moniliales
Family : Moniliaceae
Form genus : *Botrytis*

1. The conidiophores are more or less straight, slender, septate, branched mostly in the apical region, and become paler near the apex.
The branches are determinate, integrated and the apical cells of the terminal branches enlarged and inflate into ampullae bearing clusters of single celled conidia.

2. The conidia are smooth, attached to the ampullae by sterigmata. The conidia are simple, hyaline, aseptate, and in mass the conidia are grey - brown and appear like grey bunches of grapes and are easily detached by wind.
The diseased areas at first produce abundant conidiophores of the *Botrytis* type and sclerotia are formed later (Brooks, 1908). The sclerotia, upon germination produce either conidiophores of the *Botrytis* type or apothecia of *Botryotinia fuckeliana*.

Telemorph stage (Perfect stage or asexual stage-ascigerous stage):
This stage produces apothecia which belong to the genus *Botryotinia*.

Taxonomy of *Botryotinia* :

Division : Eumycota
Class : Ascomycetes
Sub class : Eu-Ascomycetes
Sub series : Discomycetes
Order : Helotiales
Family : Sclerotiniaceae
Genus : *Botryotinia*
Species : *fuckeliana*

Classification and taxonomy of asexual and sexual stage of *Botrytis* were referred to Groves & Drayton (1939), Groves & Loveland (1953), Webster, J. (1970), Onions, Allsopp, and Eggins (1981), and Moore-Landecker, E. (1982).

1.2.2 Botrytis characteristics

A. Weak pathogen: It has a limited ability to attack healthy plant tissues. Strong pathogens (eg. *Phytophthora infestans*) can attack intact, healthy fruit or other plant parts but *B.cinerea* can only invade damaged or senescing tissue.

B. Necrotrophic pathogen: It produces enzymes (e.g pectinase) which kill host cells in advance of the physical invasion of its mycelium. The plant tissues die immediately and the fungus is living and obtaining nutrients from these recently killed tissues. Biotrophic pathogens (eg. *Podosphaera levcotricha*) can only survive on living host tissue.

C. Facultative pathogen: It can live as a saprophyte on decaying plant material and also as a parasite on living plant material. This contrasts with obligate pathogens (eg. *Podosphaera levcotricha*) which can only live on living tissue and with obligate saprophytes (eg. many species of *Penicillium*) which can only live on dead organic matter.

1.3 SYMPTOMS AND SIGNS ON FRUIT

1.3.1 External symptoms

Stem-end rot originates from contamination and infection of the picking scars at or soon after harvest. No rot appear during the first four weeks, and symptoms appear normally after 6 to 8 weeks storage at 0°C, and after about 12 weeks the remaining healthy fruit are unlikely to develop *Botrytis* rot during continued cold - storage (Pennycook, 1986).
Softening and darkening of the fruit, begins at the stem end but no symptoms appear for 4-5 weeks. After this, the lesion extends along the fruit to 10-22mm at 7 weeks and spread through the entire fruit by 13 weeks in the coolstore (Pennycook, 1986). The affected area retains the normal fruit shape and feels soft but resilient.
After appearance of the first symptoms, there is a rapid increase in the number of additional fruits showing symptoms.
White layer of fungal threads (mycelium) may develop on the skin and spread to adjacent fruits within the tray (nesting), (Pennycook, 1984).
After prolonged cold storage, the mycelium become a grey, fuzzy appearance because of the growth of dark conidiophores bearing numerous powdery grey conidia.

1.3.2 Internal symptoms

The rot advances evenly through the internal tissues, producing a faint pinkish - discoloration of the flesh (Pennycook, 1985). The fruit flesh in the fungus - invaded area is very soft because of the breakdown of tissues by pectinase and cellulase enzymes produced by *B.cinerea.*

1.4 EPIDEMIOLOGY OF INFECTION

1.4.1 **Mechanism of Botrytis on flowers and stored fruits**

a. At blossom, during summer the dying petals of kiwifruit blossoms are frequently covered with a clear grey growth of *Botrytis*, producing millions of spores.
In ripe marketable strawberries, the frequent isolation of *B.cinerea* from the stem end of the fruit and the high percentage of rotten fruit under moist conditions indicate considerable latent infection and these inactive infections may become established in the area where the petals are attached at the stem end of the developing fruit. Five to six months later, the latent infection is reactivated and rot symptoms begins to develop (Pennycook, 1984).

b. At harvest, during autumn the spores are seldom conspicuous in kiwifruit orchards but the fungus (*Botrytis*) is still present on sepals and other flower parts attached to the stem end of the fruits. The fresh picking wound (where each fruit is snapped from its stem), constitutes an ideal infection site for its exposure to the microorganisms which used to occur in the field and cool store environments beside the easier uptake of nutrients. If dry spores are applied to the picking wound of freshly picked fruits, 85-100% of the fruits become rotten during cold storage (Pennycook, 1984).

The results were in favour of the second mechanisms; *Botrytis* infections occur at harvest (Pennycook, 1984).

1.4.2 **Infection condition**

Wounds, senescent flowers and deleafing scars are important saprophytic bases for infection. If the cuticle and the epidermis are broken, *Botrytis* spores can find nutrients and humidity in the fresh wounds ideal for spore germination and colonization. The substrate of flower heads or fruit are more fleshy than leaves, therefore *B.cinerea* may become predominant saprophyte in the early stages of decay of the tissue (Blakeman, 1980).

Any environmental factor that enhances leakage from plant surfaces may often lead to increase infection by facultative parasites such as *Botrytis* species. The conidia may remain in an ungerminated state for long periods, held in check by lack of moisture or by microbial antagonism (Blakeman, 1980).

1.4.3 **Overwintering**

Botrytis overwinter in cold climate as sclerotia. Sclerotia formation is influenced by temperature, light, pH, and nutrient, but itself play no important role in the kiwifruit storage rots. Conidia are usually regarded as short lived propagative spores and are not involved in overwintering.

1.5 **EFFECT OF PHYSICAL AND CHEMICAL ENVIRONMENT**

1.5.1 Physical environment

The environmental factors which affect the survival of the conidia are high temperature, humidity and exposure to direct sunlight (Blakeman, 1980).

Relative humidity (R.H) and availability of free water on the surface of plant organs are the most important factors influencing infection by species of *Botrytis* (Blakeman, 1980). Conidia of *B.cinerea* germinate at relative humidity between 93-100% (Snow, 1949), and the survival percentage of ungerminated conidia is independent of humidity and it decrease with increasing the temperature. The length of the period for which free water present on tokay grapes surfaces was not important if relative humidity was over 94% (Nelson, 1951). *Botrytis* mycelium remains viable for at least 6 months at high relative humidity (94% and above), but dies off rapidly at relative humidity of 85% or below. The greatest infection of *B.cinerea* developed at the high R.H values following the wet period (Nelson, 1951). Germination of *Botrytis* conidia often occurs almost equally well in light or darkness provided adequate water is present (Blackeman, 1980).
Botrytis species possesses an optimum temperature for growth between 20 and 25•C on most horticultural product and it grows slowly at 0 - 2°C. Temperature and humidity have no consistent effect upon the survival of sclerotia.

The adhesion of the young germ tubes to the leaf of *Vicia fabae* was found to be due to the mucilaginous nature of the outer layers of the germ tube wall, and the cuticle ruptured mechanically by the pressure of the tip of the germ tube and after the penetration of the cuticle, the first obvious change on the epidermal cells was usually the swelling of the walls, while disorganization of the protoplast follows later (Blakeman & Welsford, 1916). Germ tubes of *B.cinerea* have been occasionally observed to enter via open stomata of broad bean but there was no evidence of attraction of germ tubes towards stomata (Louis, 1963).

1.5.2 Chemical environment

Substances such as oxalic acid secreted by *Botrytis* are responsible for the primary destruction of lily tissues, and the disorganisation of the lily tissue was due to such substances, and also to a cellulose-dissolving enzyme which is secreted by *B.cinerea* (Brooks, 1908).

The presence of phenols in tomato leaf leachate together with lower levels of sugars and amino acids compared with fruit leachate may partly explain the resistance of leaves to infection. The presence of antifungal phenolic substrates in grape berry tissue is thought to play a part in resistance of fruit to the infection by *B.cinerea*. Antifungal substances can be induced by dipping leaves of chrysanthemum, black currant, birch, broad bean, lettuce, sugar beet, or tomato in chloroform for 5 seconds (Blakeman, 1980).

1.5.3 Nutrient requirements

Endogenous nutrient reserves have an effect on viability and germination of conidia on different *Botrytis* species (Coley-Smith, *et al.* 1980). Nutrients are required not only to stimulate germination of *Botrytis* but also for the development of superficial mycelium over the plant surface and for the formation of appressoria. Abscisic acid detected in strawberry flowers or naturally extracted from strawberry fruit stimulate the conidial germination of *B.cinerea* but a pure sample synthetic abscisic acid depressed the conidial germination of all isolates of *B.cinerea*. Of the hormones tested, only indole-acetic acid caused a small stimulation, but gibberellic acid, pyridoxine, pantothenic acid, nicotine acid, or inositol had little effect. Of the sugars tested, the fructose was found to promote *Botrytis* germination more effectively than glucose or sucrose (Blakeman, 1975).

B.cinerea is largely confined to those plant organs such as flowers and fruits where large amounts of nutrients are present in leachate which stimulate infection (Blakeman, 1980).

CHAPTER TWO

LITERATURE REVIEW

PART 2: BIOLOGICAL CONTROL

2.1 INTRODUCTION

The high cost of developing chemicals, the increasing problem of developing resistant strains by using the fungicide, time-consuming breeding programmes, the expense of conventional methods of disease control and public concern for food free of pesticide residues has attract the attention of many scientists to find another alternative.
Wilson (1989) suggested, that even by breeding and selecting desirable horticultural characteristics, it may develop varieties of fruits and vegetables susceptible to postharvest disease, and he promote the use of nature's own organism against fruit disease (USDA, 1989).
Abiotic factors (temperature, humidity, presence of free water), (Blakeman, 1980), and biotic factors such as host resistance (Mansfield, 1980), and antagonistic microorganism (Redmond, *et al.* 1987), have been used successfully to control *B.cinerea.* These factors still need to be understood for their influence in impeding the development of biological control practises for commercial benefit.

Biological control in its broadest sense has existed as a natural phenomenon since the beginning of life and it exists in natural ecosystems where the pathogens were exist in balance with their higher plant host and other microorganisms in their environment (Campbell, 1989).
The science of biological control began a long time ago. In 1874, W. Roberts, introduced the word antagonism into microbiology after demonstrating antagonistic action of micro-organisms in liquid cultures (Baker, 1987). M.C Potter in 1908, was the first to report an inhibition of plant pathogens by their own metabolites (Baker, 1987). The first international symposium on biological control was in 1963. Since then, five international symposia on biocontrol of plant pathogens in London, Munich and Melbourne, and other fifteen international meetings, five books and three courses have been devoted to biological control (Baker, 1987).

The theory of biological control is to use a living organism to suppress harmful pathogens (Smilanick, 1990). It is necessary to know the strategy of the pathogen before considering whether biological control is possible or not and to understand something about the nature of its specialized microhabitat (e.g phylloplane) in order to enhance the antagonistic effect of foliar microorganisms against pathogens (Blakeman, 1981). Before starting a large-scale application of a biological agent, knowledge of the epidemiology of the pathogen and information on the conditions of the biocontrol agent with mode of action by which the antagonist strain confer control of test pathogen is required (McLaughlin, *et al.* 1990).

It is important to know the morphological and physiological characteristics of an organism which will make it successful in a particular environment. Therefore the first step in the development of a biological control system is to isolate and identify organisms with potential activities for disease suppression (Nelson & Powelson, 1988). Despite the fact that successes have been few and often demonstrated only under experimental conditions, the control of plant pathogens with fungi holds considerable promise (Freeman, 1981). Among potential antagonists, yeasts deserve special attention, because they can survive and colonize effective site for long periods of time under dry conditions (Janisiewicz, 1988).

Stem end rots are important forms of postharvest wastage of many fruits and vegetables (Wills, et al. 1981), *Botrytis* canker is an example. Skin damage is often microscopic but is sufficient for pathogens present on the crop and in the packing shed to gain access to the produce (Wills, *et al.* 1981). After harvest, susceptibility to rotting increase with ripening which enable many of the fungi present on the surface of the fruit to invade the underlying tissue and cause rots (Eckert, 1978). The physiological condition of the produce and the storage temperature significantly affect the development of the infection (Wills, *et al.* 1981).

Wilson & Pusey (1985) reported, most applications of antagonists to control postharvest diseases have been made before harvest.

2.2 OBJECTIVES

The purposes of this study are to :

1. Review the relevant literature for biocontrol of *B.cinerea* on kiwifruit.

2. Isolate, select and identify potential biocontrol agents against *B.cinerea* at various temperatures *in vitro.*

3. Test the biocontrol agent against *B.cinerea* on kiwifruit in coolstore at 0°C *in vivo.*

2.3 WHY IS BIOCONTROL IMPORTANT

Orchard fungicide sprays cannot directly control the disease, but they can influence it indirectly by reducing the level of *Botrytis* inoculum present in the orchard (Pennycook, 1984). The application of a fungicide may actually increase the severity of *Botrytis*-caused diseases because of the presence of fungicide-resistant strains of *Botrytis* and the suppression of natural antagonists by the fungicides (Nelson & Powelson, 1988). The fungicides are not always effective, they could affect the quality of produce in storage, and be hazardous to human's health and detrimental to the environment (USDA, 1990). Fungicides could pose potential oncogenic risk when applied to processed foods (Wilson & Wisniewski, 1989). A number of key fungicides are being withdrawn from the market. Postharvest treatment of fruit with pesticides is not allowed on fruit destined for most of major markets and the regulations are likely to become stricter. Billions of dollars have gone into making fungicides and still many of the fungicides commonly used to control postharvest problems are under review by the environmental protection agency .

Refrigeration is a major way of manipulating the storage environment to control postharvest rot diseases (Droby, *et al.* 1991). *Botrytis* is capable of growth at 0°C which is the recommended storage temperature for kiwifruits. Low temperatures can slow the activity of *Botrytis*, retard its growth and influence the survival of hyphae and propagules, but does not control it.

By using hot water dip there was a trade-off between adequate control of decay and fruit injury. Kiwifruit treated with water at 46°C were not injured, but higher temperatures (50-52°C) although very effective to control *B.cinerea* by 92%, have visibly damaged the fruit and affect the firmness, respiration rate and ethylene production seriously (Cheah, *et al.* 1992).

Acetaldehyde vapour have been used to control *B.cinerea* and *Rhizopus stolonifer* rots of strawberries and raspberries (Prasad & Stadelbacher, 1973), and further researches could be useful on kiwifruit.

Biological control offers an alternative to fungicides use for the control of postharvest diseases of fruits and vegetables (Droby, *et al.* 1991). The antagonist becomes progressively more effective in preventing infection, while the fungicides loses its effectiveness (Tronsmo & Raa, 1977). The strategy of biocontrol needs more knowledge on the epidemiology of the fungus and the host/parasite relationship. Expenditure and investment in biological control is needed to develop effective control methods for postharvest diseases.

Inducing resistance in plant host and natural plant products could be used to control postharvest pathogens (Wilson & Wisniewski, 1989).

All these methods could have the ability to control *B.cinerea* which is responsible for high postharvest losses.

2.4 **WHAT IS BIOCONTROL**

Biological control is the decrease of inoculum or the disease-producing activity of a pathogen accomplished through one or more organisms, including the host plants but excluding man (Baker, 1987).

2.4.1 **Biocontrol agent (Antagonist)**

Is the organism which involves in the exploitation of organisms in the environment to decrease the capacity of the pathogen to cause disease (Parry, 1990).

2.5 MECHANISM OF BIOCONTROL

The following forms of action provided the basis for selection of the most appropriate strain. Effective antagonists may inhibit postharvest pathogens by producing antibiotics, by successfully competing with the pathogens for nutrients and space, by inducing host resistance or by interacting directly with the pathogen (Droby, *et al.* 1991).
Biological control by its definition can cover a broad spectrum of approaches ranging from the use of obligate, facultative parasites to competitors, toxins-producing pathogens and non toxic-producing behaviour-modifying chemicals (Jutsum, 1988).

Antagonism

Antagonism commonly means a relation between organisms in which one organism (the antagonist) creates adverse circumstances for the other (specific organism), (Fokkema, 1976).

Biocontrol mechanism

2.5.1 Antibiosis

Antibiosis is defined as an interaction between organisms whereby a metabolic agent produced by one organism has a harmful effect on other (Schroth & Hancock, 1981). Although antibiosis plays an active role in the biocontrol of plant disease, it is generally not an exclusive role and even antibiosis and competition some times are treated as separate entities, they are considered together on the basis that they function together (Schroth & Hancock, 1981). Antibiosis in culture media does not necessarily indicate antibiosis in fruit. Fravel (1988) reported, coincidental production of antibiosis *in vitro* and biocontrol ability *in vivo* are not necessary causally related. Pusey & Wilson (1984) reported, *Pseudomonas* strains were highly antagonistic to *M.fructicola* on agar media, and the same strains showed minimal or no antagonism on the fruit surface .
Antibiosis or lysis should result in a decrease in inoculum density (Baker, 1968). Antibiosis is particularly suited to the molecular genetics in unravelling the biocontrol mechanisms and there are now sufficient evidence, mostly obtained by genetic methods to indicate that antibiotics do function in biocontrol in nature (Fravel, 1988).

Antibiotics may control plant diseases by acting on the parasite or on the host directly or after undergoing transformation within the plant (Dekker, 1963). Action at a distance apparently caused by secretion of antibiotic substances (Johnson & Curl, 1972).Antibiotics are generally considered to be organic compounds of low molecular weight produced by microbes and vary greatly in their physical and chemical properties and its toxicity (Waksman, 1947). A number of genera of phylloplane microorganisms, including representatives of filamentous fungi, yeast and bacteria have been reported to produce antibiotic *in vitro* (Blakeman & Fokkema, 1982). Usually antibiotic substances prevent growth and reproduction of a specific organism but doesn't kill it immediately. Antibiotic production appears to be a major mode of action of many of the antagonists identified so far (Wilson & Wisniewski, 1989). The action of an antibiotic against microorganisms is selective in nature, some organisms being affected and others not at all or only to a limited degree (Waksman, 1947). If a biocontrol strain produces a single antibiotic, then certainly a resistant strain may occur (Coffey, 1990). Antibiotic substances if produced, could be insufficient to inhibit the growth of *B.cinerea* (Wood, 1951). The production of antibiotics as the mode of action of some bacteria like *Bacillus subtilis* and *Pseudomonas cepacia*, means that licences for their use on fresh commodities may readily be granted (Droby, *et al.* 1991).

2.5.2 **Competition**

Competition for nutrients is a widespread phenomenon in the interaction between microorganisms on the phylloplane (Droby, *et al.* 1991). Competition occurs when two (or more) organisms require the same thing (e.g nutrient, space) and the use of this thing by one reduce the amount available to the other. Smilanick (1990) suggested, a reliable natural microbe that will compete with harmful pathogens for nutrient in the fruit. Related species or species that prefer the same ecological niche usually avoid competition and occupy different geographical areas or different habitats in the same area.

2.5.3 Mycoparasitism

Mycoparasitism is the destruction or deterioration of the parasite by predation or enzymic lysis. The practical significance of direct predation and parasitism among microorganisms is not well understood but the phenomenon apparently is widespread in nature (Johnson & Curl, 1972).
Mycoparasites and their possible use in biological control have been recognized since 1932 (Weindling, 1932), and the use of mycoparasites for biological control was first demonstrated by Tribe (1957). Mycoparasites range from the obligate or biotrophic parasite which causes little harm to the host, to the destructive type which first kills and destroys host cells and then penetrate them (Barnett & Lilly, 1962). The parasitic types can be either mycoparasites or hyperparasite (Aronson, 1981). Although many mycoparasites have been isolated and known as biological agents in the laboratory, only a few of them have been successfully exploited for biological of diseases in the field. Researches *in vitro* have shown that the population of an organism has been reduced by parasitism (Papavizas & Lumsden, 1980).

In vitro, antibiosis and mycoparasitism could be observed and lead to the determination of antagonist's potential.
Perhaps the best known mycoparasite is the *Trichoderma* which have been suggested as biocontrol agent against many soil pathogens (Chet & Henis, 1985). An example of *Trichoderma* as mycoparasite is *Trichoderma lignorum* in which its aerial hyphae coil around the host fungus or make close contact causing disintegration of the cellular structure and a release of cell content (Johnson & Curl, 1972).

Hyperparasites are relatively host-specific and may infect biotrophic pathogens such as powdery mildew and rusts. Using hyperparasites to control specific pathogens can be an effective mean of reducing pathogen inoculum (Andrew, 1985). If hyperparasites used for biocontrol process, it must be applied after infection occurred rather than before as in the case of saprotrophs (Rishbeth, 1988).

2.5.4 Lysis

Lysis is the complete or partial destruction of a cell enzymes and observations indicate that lysis of fungi is a common phenomenon in soil (Campbell, 1989). There are two types of lysis, endolysis and exolysis. In exolysis the death is caused by the lysis, but in endolysis the death is the cause of the cell's own lysis. Both forms of lysis may occur at the same time, e.g when a bacterium colonizing a hypha produces an antibiotic that causes endolysis and at the same time produces a chitinase that destroys the fungal wall.
Relatively unspecialized pathogens such as *B.cinerea* can be inhibited by organisms producing lytic enzymes or antibiotic (e.g *T.viride*), but saprophytic organisms possessing these capabilities are relatively rare in the phylloplane (Leben, 1964).

2.5.5 Resistance inducement

The host plant has many methods of defence against the invasion of potential pathogens. Living cell near the injury are stimulated to become very active metabolically and there is convincing evidence of the importance of metabolic activity of adjacent tissue in resistance reactions (Matta, 1971). The failure to obtain protective effects with saprophytic fungi may be associated with their inability to enter the plant and establish physiological contacts with all plants (Matta, 1971). The aim of future research in the field will be to discover microorganisms able to grow inside the xylem vessels and give protection without impairing the plant vigour at a significant economic level (Matta, 1971). Bacteria are well known to induce protective responses in host tissues.

The formation of inhibitory substances by the host, however remains a possible mechanism of antagonism when interactions on wound are concerned (Fokkema, 1976). Some of the compounds produced as a result of wounding are highly toxic to fungi, and normally fruits in high vitality exhibit considerable resistance to fungal attack, while stressed or senescent fruits are often disease prone (Sommer, 1985). It might be that the fruit could have the capability of responding to pathogens with genetically programmed defense mechanisms at least for a period of time (Chalutz & Wisniewski, 1989). When the fruit weaken, it could produce less antifungal chemicals, therefore the infection develops with enlarged lesions covered by characteristic grey mould. Some resistance might be explained by a drying of the wound area (Sommer, 1985).

2.5.6 Siderophores

There is evidence that siderophores can play an active role in the inhibition of one microorganism by another (Leong, 1986). Siderophores are extracelluar, low molecular weight compounds with a very high affinity for ferric iron (Fravel, 1988). Siderophores involved in the biocontrol of pathogen by microorganisms and the prime activity of siderophores is the competition via chelation of iron. Under the condition (which the medium have low available iron), siderophore producers should effectively compete for the limited iron and cause growth inhibition of the pathogen (Campbell, 1989).
In the phyllosphere, siderophores may originate from the plant or from colonizing microorganisms (Fokkema & Schippers, 1986). The siderophores may produce by a saprotroph which may trigger a response that affects the pathogen which arrives later.

2.5.7 Acidity (pH)

The rise of pH was considered to be a possible mechanism of antagonism (Skidmore, 1976). Most bacterial and fungal activity raised the pH of media to 7.8-8.4, a level which is inhibitory for the growth of *B.cinerea* and the activity of any pectinase produced, and the use of buffered media indicated that most bacterial and fungal species were antagonistic because of the production of antibiotic substances independently of the high pH (Newhook, 1951).
Obvious inhibition of *B.cinerea*, required ascorbic acid in the culture medium which lowered the pH to a level which permits the antagonist *T.viride* to antagonize *B.cinerea* (Ale-Agha, *et al.* 1974).
Fravel (1988) reported, understanding the mechanisms of biocontrol of plant disease is important to develop a rational models for the exploitation of antagonists in agro-ecosystems, and biocontrol mechanism is necessary for the manipulation of parameters affecting biocontrol agents and for the genetic improvement of biocontrol agents (Fravel, 1988). Elucidation of modes of action is hampered by lack of knowledge of host/parasite/antagonist interactions in wounds and on the surfaces of fruits and vegetables.

Understanding the mode of action of the antagonists is important for two reasons :
1. It develops more reliable procedures for the effective application of known antagonists.
2. It provides a rationale selecting of more effecting antagonists.

2.6 PRACTICAL APPLICATION

2.6.1 Antagonist strategies

Andrew (1985) reported, the first strategy problem was to select a natural antagonist against fungal pathogens on plant materials, and second to test the antagonists *in vitro* for the inhibition of the target pathogenic fungi and also *in vivo* (green houses or storage).

2.6.2 Antagonists are resident or introduced

Resident antagonists are natural inhabitants of the plant part, soil, rhizosphere, phylloplane, or other sites occupied by pathogens. Biocontrol utilizing these organisms usually involves adjusting cultural practices, such as terminating a niche, encouraging competitors of the pathogen in the niche, or providing the crop with better means to resist to the pathogen.

Introduced antagonists are those cultured under special conditions and applied to sites where needed (e.g soil, seed, sprayed on leaves or any other plant organs). Enormous work has been done in this field during the last 10 years (Wilson & Wisniewski, 1989).

2.6.3 Desirable characteristics of an antagonist

The desirable characteristics of a biocontrol agent for postharvest diseases (Wilson & Wisniewski, 1989) should be:

1. Genetically stabile.
2. Effective at low concentrations.
3. Able to colonize the fruit surface or wound for long periods and survive well under adverse environmental conditions (including storage environments).
4. Amenable to grow on an inexpensive medium in fermenters.
5. Used in a form that can effectively stored and dispersed.
6. Non pathogenic against the plant host and humans.
7. Compatible with commonly used pesticides.
8. Have a high reproductive capacity and be easy to multiply and handle.
9. Effective against a wide range of postharvest diseases.

2.6.4 **Biocontrol manipulation**

Biocontrol agents can be manipulated and controlled by:

2.6.4.1 Manipulation of the environment

Wilson & Pusey (1985) reported, once the antagonist behaviour in different environments have been addressed, the manipulation of these environments to favour the activities of the antagonists can be accomplished, and indirectly will affect disease control. The strategy is to search for the antagonist that functions best at temperatures and relative humidities commonly used in the storage environment (Droby, *et al.* 1991). A better understanding of antagonist behaviour in different storage temperatures can be useful in the manipulation of these environments to favour the activities of the antagonists (Wilson & Pursey, 1985). Tronsmo (1978) indicated, through selection, an isolate of *Trichoderma* that grew at lower temperatures was more effective than other isolates of *Trichoderma* in controlling *Botrytis* rot of strawberries in storage. Success in the future will probably result from the use of a combination of antagonists that could effectively inhibit the target organism during different phases of its life cycle and changing environmental conditions.

2.6.4.2 Manipulation of the antagonist

The wild isolate must be selected, for the stability, adaptability and survivability under field conditions, and the greater the complexity of the biological community, the greater is its stability (Baker, 1987). The pathogenic capability of antagonists against fruit or vegetable products should be taken into account in the selection of potential biocontrol agents, because pathogenic antagonist could affect the products and need to be substitute by another antagonist, and may encounter problems in the registration process for commercialization (Wilson & Wisniewski, 1989).
The number of antagonist propagules (yeast US-7) required to obtain almost complete control of the pathogen *P.digitatum* on grapefruit was three orders of magnitude higher than the number of propagules of the pathogen present (Droby, *et al.* 1991).

Manipulation of antagonists has been attempted by a number of methods :

2.6.4.2.1 Manipulation by salt solutions

Enhancing the activity of biocontrol agents by the addition of salts could be the most important factor in using them successfully for controlling diseases. For example, calcium salts improved the efficacy of the biocontrol strain of the yeast *Candida sp.* strain 87 and 101 against *Botrytis* (McLaughlin, *et al.* 1990).

2.6.4.2.2 Manipulation by integrated biological control

Baker (1987) reported, integrated use of chemical and biological control may metabolically weaken the pathogen and make it vulnerable to an antagonist. An integrated method involving the use of very low doses of selected fungicides in combination with long-term effect of the biocontrol agent beside the manipulation of storage conditions should improve the success of biocontrol procedures in postharvest applications (Droby, *et al.* 1991), and (Tronsmo, 1986).

2.6.4.2.3 Manipulation by genetic engineering

Exploitation of genetic engineering in the service of biocontrol is being done by various workers. Genetic engineering and molecular biology techniques could be used to improve the antagonistic properties of biocontrol agents (Droby, *et al.* 1991). The organism can be altered in some ways including genetic engineering but then there would be severe problems at the present time with getting permission to release it into the environment (Campbell, 1989). Therefore it is better to seek a naturally occurring microorganism that has the desired characteristics than to try to create one by mutation or gene manipulation (Baker, 1987). However, it is possible to combine the most desirable characteristics of several organisms in one agent that will have several mechanisms of attacking a pathogen as well as good survival and colonization characteristics (Campbell, 1989). Ultimately, it may be possible to incorporate the gene or genes for antibiotic biosynthesis into the plant or into other resident organisms which would be a break through of tremendous importance in controlling plant disease (Fravel, 1988).

Enhancement through the use of genetic engineering has to be considered in some instances together with the addition of low level of fungicide (Harvey, 1988). Irradiation with UV light produced mutant spores which develops into mutant colonies and create a new strain.

2.6.4 **Effective antagonist preparation and application methods**

The importance in selecting a microorganism for use as a biocontrol agent comes from considering whether introducing it into a fruit crop will present any hazard to human.
Antagonists have to be applied at relatively high rates to be effective against postharvest diseases (Wilson & Wisniewski, 1989). Therefore it is preferable to find naturally occurring biological agents with no antibiotic-producing or enzyme-producing activity and that are not allergenic (Cork, 1978). However most of the biocontrol agents are typically nontoxic to humans, which is a big advantage during production and application (Scher & Castagno, 1986).

Once effective antagonists are identified for postharvest diseases, methods must be found to prepare, store and apply them commercially (Wilson & Wisniewski, 1989). However, a considerable investment of time and money is required to establish whether an agent has commercial potential (Wilson & Wisniewski, 1989). Biological agents must be cheap, safe and as easy to use as pesticides (Campbell, 1989). The product should be technically and economically feasible and the product should be competitively attractive and should offer some advantage in price or performance than other products in the market place (Scher & Castagno, 1986). Then there must be a probable market before companies will invest in development (USDA, 1990). Therefore, the isolation, screening and selection for potential antagonists deserves careful deliberation (Wilson & Wisniewski, 1989). Detailed studies of commercialization of a biological agent, cost of production, formulation, environmental adaptability and level of persistence are required for the successful of the biocontrol process.

The inoculum may be supplied as a powder or granule in some sort of carrier that is often fine, ground peat, bran, and selected nutrients may be added to encourage growth of the antagonist but not the pathogen (Campbell, 1989).
Release from the formulation must be fast, together with its movement to the site of action (Harvey, 1988). The formulation must be stable and be stored over a long period. Different countries have different rules about the release of organisms into the environment, and the patent protection varies from country to country. There are international conventions and some of the european countries have established a common system (Campbell, 1989).

Once biocontrol procedures have been developed for postharvest disease, they will still need to gain public acceptance (Wilson & Pusey, 1985). Purity and effectiveness of the agent should be confirmed throughout all stages of experimentation and production (Scher & Castagno, 1986). The organism must be identified and its genetical structures should be markers before it can be patented (Campbell, 1989). To obtain a patent it has to be shown that the product has novelty, not previously used, not talked about or published in any way which would allow anyone to gain a knowledge of it (Campbell, 1989). Once having obtained a patent, then a monopoly for 20 years is permitted, and there is possibility to license others to produce or market the invention

(Campbell, 1989). The cost of obtaining registration must be considered, both at a local and even internationally (Harvey, 1988). Collaboration between industries and universities can result in industrial development of university-discovered agents (Scher & Castagno, 1986).

2.7 VARIOUS RESEARCHES

A number of researchers have accomplished biological control of *B.cinerea* on many horticultural products (using the organs: leaves, flowers, fruits) in pre and post-harvest conditions (Chalutz, *et al.* 1988). Biocontrol agent have been applied to blossoms in the field to protect the fruits from rots during storage. Very limited work has been done for the control of *B.cinerea* on kiwifruits. Biocontrol agents used for this purpose were shown in Table 1.

Table 1. Some examples regards biocontrol of grey mold (*B.cinerea*) on horticultural products.

Antagonists	Crop organs	Source
Antibiotic production (A), Antifungal compounds (F)		
Pseudomonas cepacia	G.D.Apples(fruit)F	Janisiewicz & Roitman 1988
Pseudomonas cepacia	Bosc pear(fruit)F	Janisiewicz & Roitman 1988
Cladosporium herbarum	Tomato(petals)A	Newhook, F.S 1957
Penicillium spp	Tomato(petals)A	Newhook, F.S 1957
Alternaria spp	Tomato(petals)A	Newhook, F.S 1957
Penicillium clavariaeforma	Lettuce(leaves)A	Wood, R.K.S 1951
Fusarium spp	Lettuce(leaves)A	Wood, R.K.S 1951
Volatile compounds (V),Non volatile compounds (T),Hyphal interaction (I) Hyperparasiitic ability (B),Mycoparasite (M)		
Trichoderma hamatum	Bean(blossom)V	Nelson & Powelson 1988
Trichoderma spp	Strawberry(flower)T/I	Tronsmo & Dannis 1983
Trichoderma harzianum	Grapes(flower)B	Dubos, B 1984
Trichoderma spp	Grape(flower)I	Bisiach, *et al.* 1985
Trichoderma pseudokoningii	Apples(flower)T/I	Tronsmo & Raa 1977
Acremonium breve	Apples(fruit)B	Janisiewicz, W. J 1988
Coniothyrium minitans	Field tests M	Baker, K 1987
Nutritional competition (N) and/or induction of host resistance (HR)		
Acremonium breve	Apple(fruit)HR	Janisiewicz, W.J 1988
Debaryomyces hansenii	Apple(fruit)N/HR	Wisniewski, *et al.* 1988
Debaryomyces hansenii	Grape(fruit)N	Chalutz, *et al.* 1988
Debaryomyces hansenii	Tomato(fruit)N	Chalutz, *et al.* 1988
Exophiala jeanselmei	Cut roses(flower)N	Redmond, *et al.* 1987
Candida spp	Apples(fruit)N	Mclaughlin, *et al.* 1990
Aureobasidium pullulans	Strawberry(flower)N	Bhatt & Vaughan 1962
Aureobasidium spp	Grape(flower)N	Bisiach, *et al.* 1985
Cryptococcus laurentii	G.D.Apples(fruit)N	Roberts, R.G 1990

2.8 REASON FOR FAILURE

Failures could be in the field and/or postharvest situations, but some of failures can be solved in postharvest situations.
The main reasons for failure of biocontrol procedures have been :

1. Inability to control environmental conditions in field situation. However in postharvest situation, during shipping and storage for harvested food, exact environmental conditions can be established and maintained.

2. Difficulties of target biocontrol agent to the effective site in the whole plant in the field. However in postharvest situation, application of biocontrol agent can be directed precisely to the target site *in vivo*.

3. Biocontrol procedures may not be economically feasible under field conditions. However in postharvest situation, the high value of harvested crops may make the development and application of biocontrol procedures economically feasible (Droby, *et al.* 1991).

4. In the field and/or postharvest situations, there are problems associated with the limitation of the shelf life of the organism and its sensitive to the changes in temperature or osmotic pressure (Campbell, 1989).
Although low doses of UV irradiation could be useful in postharvest situation, i.e by inducing resistance in sweet potatoes and onions (Stevens, *et al.* 1988). Leben, *et al* (1965) indicated, that destruction of antagonists by UV rays and desiccation are the major reasons for biocontrol failures.

5. In postharvest situation, there are problems regarding the production and storage of antagonists as living organisms in sufficient quantities for large-scale field use (Parry, 1990).

All these failures weaken the ability to have complete control of pathogenic microorganisms responsible for horticultural losses.
Although fungi have been used as food for a long time and have been used in the commercial production of wines, cheese, enzymes and antibiotics, toxin-producing species have caused severe mycotoxicosis in humans when infected plant tissue has subsequently been used as food (Cork, 1978).

Cook & Baker (1983) reported, one potential hazard of antagonists is allergy of workmen in laboratories during mass production of inoculum and when applying it in the field. Major problem of biocontrol is the use of high levels of suppressive microbes which must be added to the system to overcome the fungal pathogens, some times up to 10.000 times as many microbes as would occur naturally (Smilanick, 1990). Considering that most fruits have a number of important pathogens, controlling only one may merely favour another. Neglecting this problem under commercial conditions can make biocontrol ineffective or short-lived, and in the future may rapidly increase the incidence of previously less important disease (Janisiewicz, 1988). Despite numerous reports of successful biocontrol agents for plant pests, few have reached the market place because most agents have developed from a scientific viewpoint and overlooked the industrial perspective (Scher & Castagno, 1986).

In the field, It is just not known how might the biocontrol agents survive, where they go or what they might do?
In general, organism isolated from natural environments and simply returned there are permitted (Campbell, 1989). For example, one of the competitor antagonists used commercially in the UK is *Trichoderma viride* sold as 'Binab T'.
Bio-Care technology company manufactured the product NoGall (a commercial pesticide based on genetically engineered living *Agrobacterium tumefaciens* which protects stone fruits, nuts and roses from crown gall disease) was confident of winning a licence to retail this product through out Australia, and hoped to be approved by US Environmental protection agency to sell it in the USA. But the American ecologist criticized such genetical engineering process for not taking ecological principles into account in predicting risks from releasing genetically modified organisms (GMOs), (Wright, 1989).

2.9 MICROECOSYSTEM AT PLANT SURFACE AND WOUND SITE

The wound site is a special ecological niche for microbial growth, therefore it would be worthwhile to explore naturally occurring micro-organisms at wound sites (Harvey, 1988). A new wound generally forms an ideal environment for antagonists because it provides a moist nutrient-rich substrate (Blakeman & Fokkema, 1982). The microclimate at the plant surface and seasonal variation normally changes the microhabitat of the surface, and colonisation of leaves by microorganisms is affected by varying environmental conditions, therefore the action of the climate on wound response, on antagonist and on the parasite should be taken into account. Plant tissues generally respond to wounding by laying down protective barriers.

Cook & Baker (1983) recommended, that where disease does not occur or is less severe, a natural biocontrol system should be suspected. Biocontrol is based on organismic interactions, therefore it involves ecological studies of factors that regulate or affect these interactions. The abundance of one organism is partly controlled by other organisms and by the environmental factors (Campbell, 1989). To enhance the antagonistic effect of foliar microorganisms against pathogens, it is necessary to understand the nature of their specialized microhabitat, usually referred to phyllosphere (region surrounding the leaf), (Ruinen, 1961), or phylloplane (leaf surfaces), (Blakeman, 1981).

The taxonomic and ecological observations suggest that dew, humidity and leaf surface wetness are more important than temperature in determining the composition of epiphytic populations (Dickinson, 1976). High temperatures and high humidities favour the development of postharvest decay (Wills, *et al.* 1981). Blakeman (1985) reported, relative humidity at the plant surface is likely the most important factor influencing the growth and survival of micro-organisms in this habitat. To support the growth of most micro-organisms, the plant surface must be wet or over 95% R.H. Alteration of dry and wet conditions, reduces the efficiency of biological antagonism. The horticultural necessity for maintaining high humidity in commodity environments is primarily to minimize loss of moisture, which results in shrivelling and loss of turgidity in tissues (Sommer, 1985).

Microbes may compete for space in which water levels are suitable or optimum (Campbell, 1989). Rain and dew on leaves allow the germination, growth, influence the exudation or leaching, and affects spore take-off and dispersal (Dickinson, 1976). The establishment of the antagonist could be more difficult in the field during heavy rain and lower temperatures which would reduce the growth of the antagonist (Blakeman, 1980).

Energy is required for a pathogen to infect a plant, and pathogens satisfy their energy and nutrient requirement from metabolites present in the host (Swinburne, 1978). There are indication that nutrients plays a key role in inhibition of *Botrytis* growth (Waksman, 1947). The principle nutrient limiting

factors are nitrogen, carbon and vitamins (Baker, 1968). Carbohydrate levels on onion leaves were not significantly reduced by the antagonists (*Bacillus*, *Penicillium* or *Trichoderma*), but amino acid levels was declined to a greater extent (Blakeman, 1980).
Baker & Cook (1982) reported, facultative pathogens may obtain nutrients from colonized host tissues (e.g latent infections or dead host remains) or from their own storage organs (e.g sclerotia), and nutrient from these food bases are transferred to the infection site through mycelia, which must remain intact. The presence of antagonists which compete strongly for nutrients may prevent the establishment of the temporary saprophytic phase of the pathogen and thereby prevent or reduce infection (Andrew, 1985).

As the fruit ripens, their constituents change qualitatively in favour of the pathogen (Swinburne, 1978). Unstressed fruits do not become susceptible to *Botrytis* rot until they are nearly ripe, as indicated by fruit softening (Sommer, *et al.* 1983). Pathogens may be directly affected by the stress which weaken them and makes them more vulnerable to antagonists, thus decreased the disease (Baker, 1987).

2.10 CONCLUSIONS

Fungi grew at temperatures lower than the minimum temperature required by bacterial growth (Pusey & Wilson, 1984), and because fungi that cause storage rots grow more slowly than yeast and bacteria, they usually were less desirable biocontrol agents (USDA, 1990).
Wound pathogens causing important postharvest disease of fruits and vegetables can be controlled by naturally occurring microbial antagonists (Janisiewicz & Roitman, 1988), and the wound sites become the battle ground for pathogens and antagonists. To control wound invading pathogens, effective antagonists must be able to colonize wounds and inhibit the pathogen. Most of the important wound parasite have air-dispersed spores and such spores may be present in the store atmosphere, and in consequence the fruit may become infected during the storage period (Eckert, 1978), but storage conditions for food commodities do not present the same hazards for antagonists. This should greatly expand the range of antagonistic organisms that might be useful in postharvest biocontrol. Therefore, possibly, micro-organisms antagonistic to pathogens were of greater potential for disease control after harvest (Pusey & Wilson, 1984).

Disparity between *in vitro* and *in vivo* performance may represent a different survival in the two environments and usually there is no significant correlation between antagonism demonstrable in culture and the effectiveness *in vivo* (Andrew, 1985). It is important to know the pattern of the infection process in order that suitable treatment strategies may be developed to control or eliminate the infection (Wills, *et al.* 1981).
Economical biocontrol will require extensive research on the epidemiology of the disease, ecological studies on both the target pathogen and biocontrol agent, the physiology of the fruit and a realistic approach to the study of biocontrol process itself (Adams, 1990). The behaviour of a particular biocontrol agent is influenced and regulated by any number of associative microorganisms and abiotic factors (Schroth & Hancock, 1981). Pusey & Wilson (1984) reported, many uncontrollable variables exist under the field situation, but the postharvest environment can be more easily manipulated to favour the antagonist. Success of biocontrol process depends upon providing favourable conditions for growth of the antagonist and the ability of its colonization (Bhatt & Vaughan, 1962). However, if infection by *B.cinerea* is prevented at the petal fall stage, fruit rot at harvest could be greatly reduced. For example, application of *Cladosporium* to green strawberries was not effective indicating that the antagonist should be well established before the pathogen arrives (Bhatt & Vaughan, 1962).

Good handling practices, modification of handling techniques and careful supervision of all postharvest operations could be integrated to biocontrol process. The aim of commercial process is to use one organism to replace or antagonize another organism and protect the commodity from damage (Smilanick, 1990). It is probably better to have the desired characteristics in several different isolates than to try by gene management to get them in a single organism (Baker, 1987). Possibly, resistance is less apt to develop towards antagonists that have more than one mode of action such as antibiotic production (Chalutz & Wisniewski, 1989).

The delivery systems need to be tested on the effects of inoculum density of the antagonist, possible at different inoculum potentials of the pathogen to determine the amount needed for control, and relatively high rates of biocontrol agent should be applied to be effective against postharvest diseases (Wilson & Wisniewski, 1989). Inoculum should be in a form that is effective and market-competitive. The agent should prove safe for human consumption at high levels, and if the antagonist is an antibiotic-producing agent, then investigation needed to be looked at the percentage of antibiotic produces in a dynamic system like fruit (Smilanick, 1990).

CHAPTER THREE

ISOLATION OF BIOCONTROL AGENTS

3.1 INTRODUCTION

Selection of antagonistic microorganisms is a principle part of all biological control programs. Screening strategies are essentially the same whether applied to aerial, subterranean or aquatic habitats. They are based on evidence that the candidate organism either interferes with the pathogen or reduces disease (Andrew, 1985).

Various researchers have devised techniques for selecting useful isolates in the laboratory, and the majority of their work was screening on PDA petri dishes for antagonistic activities. The A.PDA and NA media were selective because they favoured the growth of certain microorganisms over others. The screen technique on agar petri dishes is aimed at detecting inhibition zones between colonies of pathogen and antagonist because inhibition zones are usually associated with antibiosis (Stessel, *et al.* 1953), or nutrient deprivation (Hsu & Lockwood, 1969). The type of interactions including inhibition or stimulation of pathogen or antagonist can be directly observed in cultures. Agar plate test is a convenient way to compare isolates (Andrew, 1985), and may give some indication of the mechanism of interaction between antagonist and pathogen (e.g antibiotic production, hyperparasitism). *Fusarium sp.* and *Phoma sp.* have stopped growth of *B.cinerea* in dual cultures on PDA either at the point of contact or at a distance (Bhatt & Vaughan, 1962). Wood (1951), designed his screening technique by placing the antagonist on a medium at 3 equidistant around the periphery of the petri dish and the test organism was placed in the centre.

The evidence in soil microbiology and in phylloplane studies have shown no significant correlation between antagonism demonstrated in culture and effectiveness in the field (Andrew, 1985).

In this study, isolates were selected from plant organs, stored fruits and diseased stored fruit. A number of *in vitro* screening techniques [6 or 4 or 3 antagonists plugs (from different colonies) placed around *B.cinerea* on PDA petri dishes] have been used for selection of potential plant pathogen antagonists at various temperatures.

3.2 OBJECTIVES

To isolate potential antagonists that have the ability to reduce activity and growth of *B.cinerea in vitro* at various temperatures.

3.3 MATERIALS AND METHODS

3.3.1 Isolation from kiwifruit orchard

Four collections (mid of March, April, May, and June) of kiwifruit plant organs (old leaves, young leaves, young buds, young stems, fruits, dropped leaves) and soil were taken as samples of Hayward variety from DSIR kiwifruit orchards-Massey University and a Wanganui orchard.

In each collection, suspensions of micro-organisms were made from these samples. Each sample was suspended in 150ml sterilized dist.water (SDW), amended with 0.02% tween 20 (SDWT) in 250ml bottle.

The agar medium used in this test were A.PDA (15.6g/400ml of Potato dextrose agar amended with 1ml of 0.04g oxytetracycline/10ml), PDA (15.6g/400ml of Potato dextrose agar), NA (3.2g nutrient broth + 6g agar/400ml dist.water), and CMA (6.8g/400ml of Corn meal agar). PDA was chosen for its high nutrient content which supports the growth of a wide range of fungal and bacterial species. The antibiotic (oxytetracycline) was used to exclude bacterial growth. NA was used to isolate bacteria. CMA medium was used for *B.cinerea* culture.

Seven bottles of SDWT, one for each type of sample were used in each collection. After shaking for 2 minutes, 0.5ml aliquot of each suspension was transferred aseptically onto the surface of A.PDA and NA petri dishes. The seeded petri dishes of A.PDA and NA were incubated at 20 and 5°C.

At 20°C, after 4-5 days of incubation, distinctive colonies of micro-organisms in A.PDA and NA petri dishes were selected, and fungal isolates were transferred individually and aseptically to fresh PDA petri dishes using a needle and the bacterial isolates were transferred to fresh NA petri dishes using a loop. The isolates were given a code name, for example the second fungal isolate from a young bud sample was coded FB2, and the first bacterial isolate from soil was coded BS1.

After the individual isolates reached an appropriate growth stage, a mycelial disc (5mm diam) was taken from the margin using cork borer and was placed on the surface of the medium to test for antagonistic activity towards *B.cinerea*. *B.cinerea* was isolated from naturally infected kiwifruit in coolstore, its culture was maintained on CMA at 20°C and a 5mm disc of 10 days old culture was used in the screening tests.

In this experiment, six different fungal or bacterial isolates were inoculated at equidistant points at the edge of the agar petri dishes (PDA), and *B.cinerea* was inoculated in the plate centres. The fast growing fungi were tested apart from the slower growing fungi in the screening test to avoid that fast growing isolate overgrowing slower growing isolates.

The PDA and NA Petri dishes were incubated at 0, 5, 10, 15°C (second incubation), and the zones of inhibition were measured at these temperatures. At the incubation of 10 and 15°C, the spacing from the micro-organism isolates to *B.cinerea* was 25-30mm. At 0 and 5°C, the spacing from the micro-organism to *B.cinerea* was 10-15mm because of the slower growth at these temperatures. In preliminary test, six isolates were used on each petri dish. In subsequent tests the numbers were reduced to four to minimize interference between antagonists.

The selection of antagonists was based on their good performance of screening on PDA and NA medium. Every four days the growth of the antagonist colonies and of the *B.cinerea* were recorded by measuring the radial growth of *B.cinerea* towards the antagonist and the radius of any inhibition zones (Table 2 and 3).

3.3.2 Isolation from stored fruit

Suspension was made from microorganisms on the surface of stored fruit taken from wooden boxes and single pack trays held at 0°C for 2 months followed by ambient temperatures (20°C ± 1°C) for 3 days. 0.5ml aliquot was transferred to A.PDA and NA petri dishes, and after 4-5 days, the distinctive colonies of fungal isolates were transferred to fresh PDA, and of bacterial isolates to NA petri dishes. Mycelium plugs and bacterial streaks taken from these colonies were tested against *B.cinerea* at 0, 10 and 20°C (Table 4 and 5).

3.3.3 Isolation from diseased stored fruit

Isolates taken from diseased fruits stored over 2 months at 0°C, were tested against *B.cinerea* on CMA at 0°C (Table 6).

3.4 RESULTS

The kiwifruit organs were dominated by a range of microorganisms, particularly of genera such as *Epicoccum*, *Alternaria*, *Phoma*, *Trichoderma*, *Fusarium*, and of Actinomycete, Yeast and Bacteria.
A total of 115 fungi, 45 bacteria and one actinomycete were isolated and tested for antagonistic activity to *B.cinerea.*
The antagonistic activity of the micro-organisms isolated from kiwifruit plant organs from four collections are given in appendix 1; collection one (table 1, 2, 3), collection two (table 4, 5, 6, 7), collection three (table 8, 9), collection four (table 10, 11).
Collection one, from the orchards of DSIR-Massey University in March, consisted of 21 fungal isolates, 15 bacterial isolates and 2 yeast isolates. Only 9 fungal isolates and a bacterial isolate were successful in their antagonism to *B.cinerea* at various temperatures.
Collection two, from the orchards of DSIR-Massey University in April, consisted of 49 fungal isolates, 8 bacterial isolates and a yeast isolate. Only 19 fungal isolates were successful in their antagonism to *B.cinerea* at various temperatures .
Collection three, from the orchard of Wanganui in May, consisted of 26 fungal isolates and 14 bacterial isolates. Only 18 fungal isolates were successful in their antagonism to *B.cinerea* at various temperatures.
Collection Four, from the orchards of DSIR-Massey University in June, consisted of 16 fungal isolates, 8 bacterial isolates and an actinomycete isolate. Only 3 fungal and a bacterial isolate were successful in their antagonism to *B.cinerea* at various temperatures.

Sporulation of *B.cinerea* began after 6 days of incubation at ambient temperatures (20°C ± 1°C) on PDA petri dish.
The growth of *Botrytis* was rapid and clear at the temperatures (0, 5, 10, 15, 20•C) in comparison to that of other microorganisms. In some petri dishes, the growth of *B.cinerea* stopped some distance away from the specific antagonist to give a distinct zone free of mycelium. The hyphae of *B.cinerea* bordering this zone were often discoloured and malformed. Antagonism by the antibiotic production as indicated by inhibition zones was pronounced in a number of antagonist isolates at 0, 5, 10 and 15°C (Table 2).
The fungi grew at temperature lower than the minimum temperature required for bacterial growth.
Microorganisms producing the largest zone of inhibition during screening *in vitro* were *Fusarium sp*, *Phoma sp*, *Epicoccum sp*, *Alternaria sp* and a bacterial isolate BF21 (Table 3). Antagonist isolates producing zones of inhibition like FF9, FB3 and FO30 have transferred for further study.

In the screen test of isolates from stored kiwifruit, fungal and bacterial isolates were tested at 0, 10 and 20°C. At 0°C, some antagonistic activity to *B.cinerea* was pronounced in the fungal isolates FF22, FF25, FF26, FF31, and in bacterial isolates BF22, BF23, BF24, BF26, BF27 and BF29. At 10°C, some antagonistic activity was pronounced only in the bacterial isolate BF23.

At 20°C, none of the isolates have shown any antagonistic activity towards *B.cinerea*. Thereby, from a total of 11 fungal and 11 bacterial isolates, only FF26 and BF23 at 0°C showed some antagonistic reaction. But these isolates were not identified (Table 4 and 5).

In the screen test of isolates from diseased stored fruit, a total of 8 fungal isolates, none of them showed any antagonistic activity to *B.cinerea* and they were identified as species of ***Penicillium*** (Table 6).

Table 2. Antagonist isolates from four collections tested for antagonism to *B.cinerea* on PDA petri dishes at 0, 5, 10 and 15°C.

Temp	Collection 1	Collection 2	Collection 3	Collection 4
0°C	*Phoma* FF7,FF9,F02-1. *Epicoccum* FF4,FT7. Yeast FB3 Others FT3,F1T-1,F1T-2, B1B-2,F1Y1.	*Phoma* FF-2,FD-2,F1F-10. *Epicoccum* FY20,FY60,FT20, FT50,FF-3. *Alternaria* FB20,FB50,FY50. *Fusarium* FO30,FF-1. Others F1S-10,F1S-20.	*Phoma* FF21,FO21,FO31,FB21. *Epicoccum* FF11,FF31,FD4,FO11. *Alternaria* FD7. Bacteria BF11,BF21. Others FD6,FO41,FB11.	*Phoma* FF-31. *Epicoccum* FD-11. Actinomycete BB-11
5°C	*Phoma* FF7,FF9,F02-1. *Epicoccum* FF4. Others FT4-2.	*Phoma* FD-2,FO-3,F1F-10. *Epicoccum* FY20,FT20,FT50, FF20. *Alternaria* FB20. *Fusarium* FO30. Others F1F-20,F1S-20.	*Phoma* FF21,FO21,FO31. *Epicoccum* FF11,FF31,FO11. Bacteria BF21. Others FT11,FB11.	*Phoma* FF-31. *Epicoccum* FD-11.
10°C	*Phoma* FF9,FO2-1. Others FT4-2.	*Phoma* FF-2,FO-3,FD-2,F1F-10. *Epicoccum* FF20,FY20,FT20, FT50. *Fusarium* FO30.	*Phoma* FO21,FB31. *Epicoccum* FD4. Bacteria BD2. Others FT11,FY31.	None
15°C	None	*Epicoccum* FF20. *Fusarium* FO30	*Phoma* FB31. Others FY31.	None

Others = Unidentified isolates

Table 3. Antagonistic activity of the better performing antagonists from four collections towards *B.cinerea* on PDA petri dishes at various temperatures.
Data is given as width of the inhibition zone (mm).

Collection		One			Two			Three		Four	
Temp	Days	FB3	FF4	FF9	FO30	FB50	F1F-10	FB21	FO41	FD-11	BB-11
0°C	8	8.0	9.0	18.0							
	16	1.5	2.5	15.0	12.0	9.5	4.0	10.0	5.0		
	24	S	1.0	9.0	9.0	6.5	3.0	8.5	3.0	3.5	1.5
	32		1.0	5.0	6.0	5.0	2.0	5.0	2.0	1.5	In
	40		In	2.5	4.0	3.0	1.5	2.0	1.0	S	
	48			S	1.0	2.0	1.5	In	0.5		
	56				1.0	1.0	1.5				
	64				In	S	1.5				
5°C	8	11.0	11.5	11.5	10.0	In	13.0	10.0	3.0	1.5	In
	16	S	4.0	4.0	8.0		1.5	6.5	S	0.5	
	24		2.5	4.0	5.0		1.0	In			
	32			0.5	5.0		In				
	40			0.5	3.0						
	48				1.0						
	56				0.5						
	64				In						
10°C	8	In	1.0	4.5	5.5	In	In	3.5	In	In	In
	16		0.5	2.5	3.0			In			
	24			1.0	In						
	32			S							
15°C	8	In	In	S	4.0	In	In	In	In	In	In
	16				In						

S = *Botrytis* overgrowth commencing. In = Isolate overgrown by *Botrytis*.
FF9, F1F-10 = *Phoma* sp. FF4, FD-11 = *Epicoccum* sp. FB50 = *Alternaria* sp.
FO30 = *Fusarium* sp. BB-11 = Actinomycete. FB3 = Pink yeast. FO41 = unidentified isolate. BF21 = Bacterial isolate

Table 4. Antagonistic activity to *B.cinerea* on PDA petri dishes at various temperatures of fungi isolated from kiwifruit stored for more than two months in wooden boxes and single packing trays.

		Isolates										
Temp	Days	FF22	FF23	FF24	FF25	FF26	FF27	FF28	FF29	FF30	FF31	FF32
0°C	21	3.0	2.0	2.5	5.0	6.0	2.5	2.0	1.0	5.0	4.0	In
	24	1.0	In	S	2.0	5.0	0.5	S	In	1.0	3.0	In
	32	In	In	In	In	1.0	In	In	In	In	S	In
10°C	7	1.0	2.0	In	In	In	In	S	In	In	S	In
	10	In	In	In	In	In	In	In	In	In	In	In
20°C	7	In	In	In	In	In	In	In	In	In	In	In

Table 5. Antagonistic activity to *B.cinerea* on PDA petri dishes at various temperatures of bacteria isolated from kiwifruit stored for more than two months in wooden boxes and single packing trays.

		Isolates										
Temp	Days	BF22	BF23	BF24	BF25	BF26	BF27	BF28	BF29	BF30	BF31	BF32
0°C	21	6.0	5.5	5.0	In	4.0	6.0	S	5.5	2.5	0.5	In
	24	4.0	5.0	1.5	In	2.0	4.0	In	4.0	In	S	In
	32	1.0	1.5	In	In	In	In	In	In	In	In	In
10°C	7	In	7.0	2.0	In	In	In	In	0.5	In	3.0	In
	10	In	2.0	In	In	In	In	In	In	In	In	In
	16	In	In	In	In	In	In	In	In	In	In	In
20°C	7	In	In	In	In	In	In	In	In	In	In	In

Table 6. Antagonistic activity to *B.cinerea* on CMA petri dishes at 0°C of Fungi isolated from diseased, coolestore fruit for more than two months in wooden boxes.

		Isolates							
Temp	days	FF22	FF32	FF42	FF52	FF62	FF72	FF82	FF92
0°C	31	In	In	In	In	In	1.0	In	S
	35	In	In	In	In	In	In	In	In

3.5 DISCUSSION

Pathogens vary in their adaptability to grow and survive at different temperatures. Temperature affects hyphal growth, sporulation, antibiotic release and rate of substrate decay. Factors to be considered include the growth of the antagonist and test organism, type of agar medium used, depth of medium in petri dish and the temperature of incubation, because the lack of essential nutrients and undesirable temperatures may inhibit the antagonist growth and its activity.

Laboratory screening procedure could be useful as a means to eliminate ineffective or slightly effective isolates, therefore it is possible that with a larger screening, better isolates could be found (Weller, *et al.* 1985).

Normally, the medium should support the development of the antagonists and the growth of the test organism (*B.cinerea*).

In general, the isolates of *Fusarium*, *Phoma*, *Epicoccum*, *Alternaria*, and the bacterial isolate BF21 were the most potential antagonists towards *B.cinerea* on PDA petri dishes at 0°C (Table 3). The bacterium isolate BF21 from all bacterial isolation have shown antagonistic activity against *B.cinerea* at 0°C, but an organism showing strong antagonism against another on agar does not necessary behave similar under natural conditions. The enzymes of fungi vary in their activity in response to environmental condition, thus their behaviour in an agar culture might not reflect their action in a natural substrate (Campbell, 1989).

The occurrence of inhibition zones between an antagonist and a pathogen on an agar medium was commonly considered to be the result of the production of antibiotics (Fokkema, 1976). If there is inhibition in plate culture then it is difficult to prove whether it is nutrient competition or antibiotic production except by showing that the inhibition disappears when excess general or particular nutrient are applied (Campbell, 1989). Inhibition of fungi i.e *Glomerella cingulata* by some isolates of *streptomyces* in agar, in some cases appears to be due to nutrient deprivation (Hsu & Lockwood, 1969). Large inhibition zones unlikely to be nutrient deficiency, however further study is needed to prove whether the inhibition zones on medium is the result of antibiotic production or nutrient deprivation and if the nutrients were the reason, then the biocontrol process would become more reliable because there is still some suspect of antibiotic production *in vivo* test.

Failure of candidate antagonists to produce inhibition zones on agar petri dishes is not necessarily an indication of their effectiveness *in vivo* since the agar petri dish test does not discern other aspects of antagonism such as competition for nutrients. Competition for nutrients which not detected *in vitro* can be detected *in vivo* (Fokkema, 1973). Also antibiotic production or mycoparasitism action *in vitro* does not guarantee a successful biocontrol action *in vivo*. *In vitro* tests should be followed by tests on the fruit (or other plant parts) *in vivo* to confirm the effectiveness of the organisms.

Isolates of micro-organisms from stored fruit didn't show any positive antagonism, therefore they were not used in further studies. In my opinion selecting isolates from stored fruit did not succeed in biocontrol because the microorganism's microhabitat would change in the new environment (coolstore), and this would change the activity and behaviour of both pathogens and biocontrol agents.

The isolates FF9 (*Phoma sp*), FO30 (*Fusarium sp*), and FB3 (pink yeast) were selected to evaluate their ability to reduce the percentage of infection caused by *B.cinerea* on kiwifruits.

CHAPTER FOUR

IDENTITY OF ISOLATES

Tests were made to identify the species of ***Fusarium*** isolate FO30, the genus of yeast isolate FB3, the genus of *Phoma* isolate FF9 and the genus of bacteria isolate BF21.

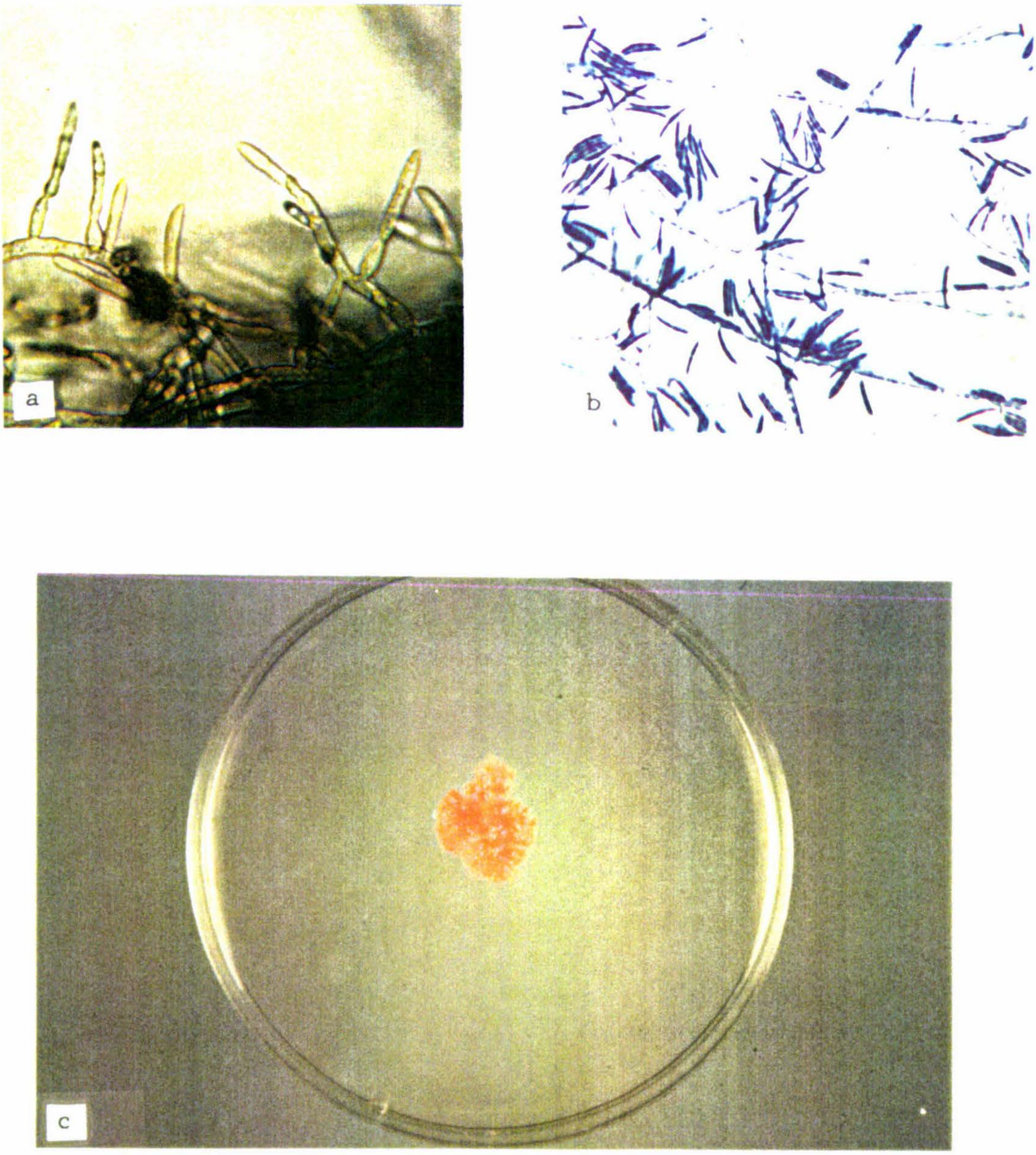
4.1 *Fusarium* **ISOLATE FO30**

Characteristics assessed were :

1. Growth rate; about 1.10-1.20cm on PDA after 4 days incubation at ambient temperature (20 ± 1°C) under near UV light.
2. Colony morphology; the cultures were slimy cream, peach to orange colour without any visible mycelium on PDA agar (Fig 1c).
3. Conidial development; macroconidia were formed on single phialides (Fig 1a).
4. Conidial morphology; macroconidia length and width were 25-38 x 3.7-5.2μm, straight to curved with a rounded apex and a marked food cell. Macroconidia septate 3-4 and some were 1 septate.
5. Conidiophores were simple lateral phialides.
6. Chlamydospores not observed, microconidia were absent.
7. No perfect state has been found.

On the basis of these observations, the fungus was indicated as *Fusarium merismoides*, using the keys and descriptions of Booth (1971).

Fig 1. Isolate of FO30 (*Fusarium merismoides*): (a) Macroconidia emerged from single phialides (x 250). (b) Macroconidia stained with lactophenol cotton blue on PDA medium. (c) Colony from a drop of liquid suspension on PDA, 13d, at 20°C ± 1°C.



4.2 YEAST ISOLATE FB3

Characteristics assessed were:
1. Ability to utilize inositol; filter discs of 0.5cm diam were suspended in inositol solution (1g/5ml) and were left 24hr at ambient temperature (20 ±1°C). The filter discs were transferred to empty petri dish and were incubated at 25°C for one day to dry. The medium used was a nitrogen base medium (KH2Po4 1g, MgSo4.7H2O 0.5g, NH4So4 5g, agar 25g, dist.water 1000ml). The autoclaved medium was transferred to waterbath to stabilise at 39-41°C. A loopful of the yeast growth was suspended in 3ml SDW, the yeast suspension was transferred to a sterilize empty petri dish and the medium was poured on the yeast suspension and gently rotated to have a mixed suspension, and were left for 24hr at ambient temperature, then the dried filter discs were placed at 3 points on the surface of the medium plate and the plate was incubated at 25°C for 2 days.

2. Ability to utilize D-Mannitol; filter discs were suspended in D-Mannitol solution (1g/5ml), the medium used was nitrogen base medium, and the same procedure was proceed as above.

3. Ability to utilize nitrate; filter discs were suspended in KNo3 solution (0.2g/5ml), the medium used was carbon base medium (KH2Po4 1g, MgSo4.7H20 0.5g, NH4So4 5g, agar 25g, dist water 1000ml), and the same procedure was proceed as above.

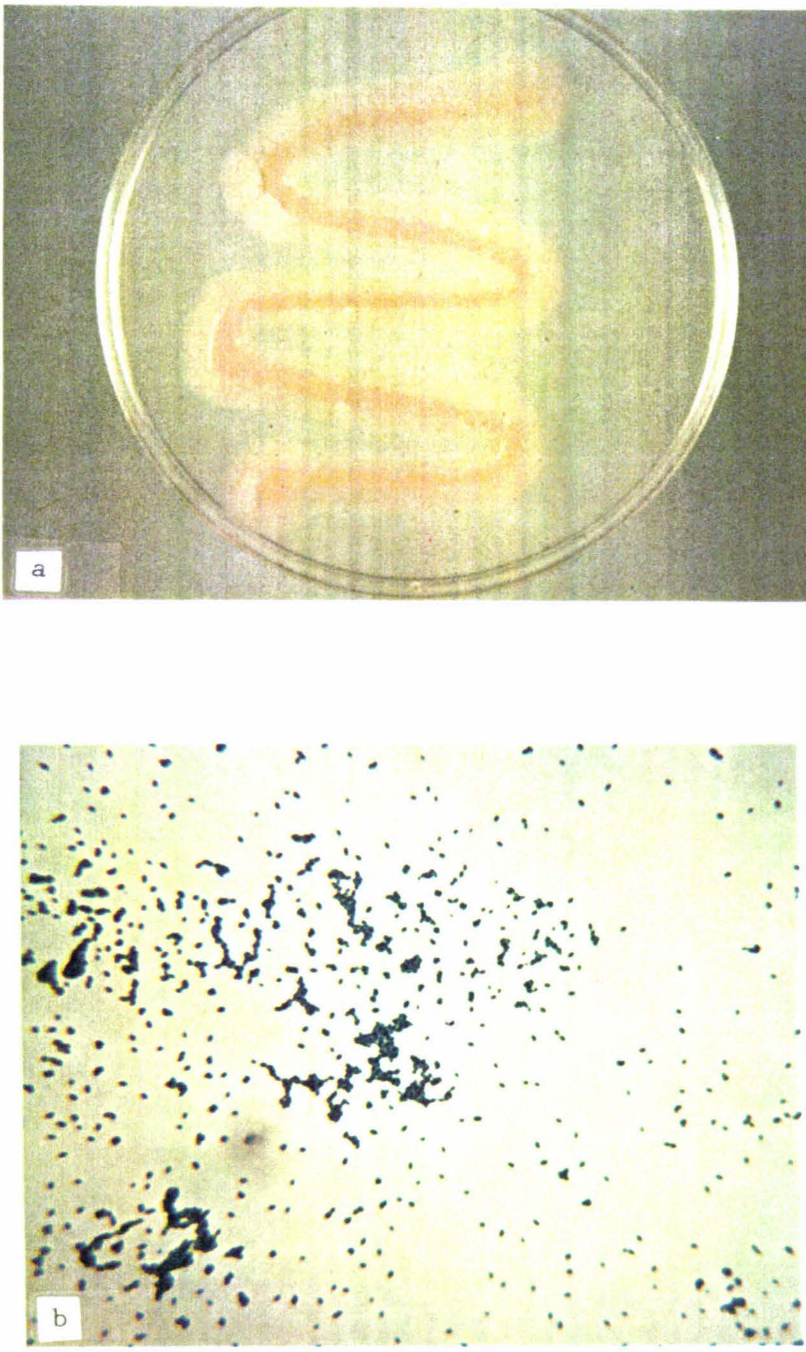
The results of these tests on yeast isolate FB3 were :

1. Inositol assimilation; positive.
2. D-Mannitol assimilation; positive.
3. Nitrate assimilation; positive.
4. Cell development; no ballistospores and no capsules were produced. Obvious mycelium was produced on PDA.
5. Colony morphology; light pink colour on PDA (Fig 2a).
6. Origin; very common in kiwifruit orchard.

These results were compared with the yeast characteristics given by Vanbreuseghem *et al.*(1978), Davenport R.R (1981), and Barnett, *et al.* (1990).

The tests indicate, the yeast isolate FB3 belong to the genus *Rhodotorula* .

Fig 2. Isolate of FB3 (*Rhodotorula sp.*): (a) Colony streak on PDA, 13d, at 20°C ± 1°C. (b) cells stained with lactophenol cotton blue on PDA medium (x 100).



4.3 *Phoma* **ISOLATE FF9**

The taxonomy of the genus of *Phoma* is uncertain and the identification of species is unreliable (Sutton, 1980), hence this isolate was identified to genus only.

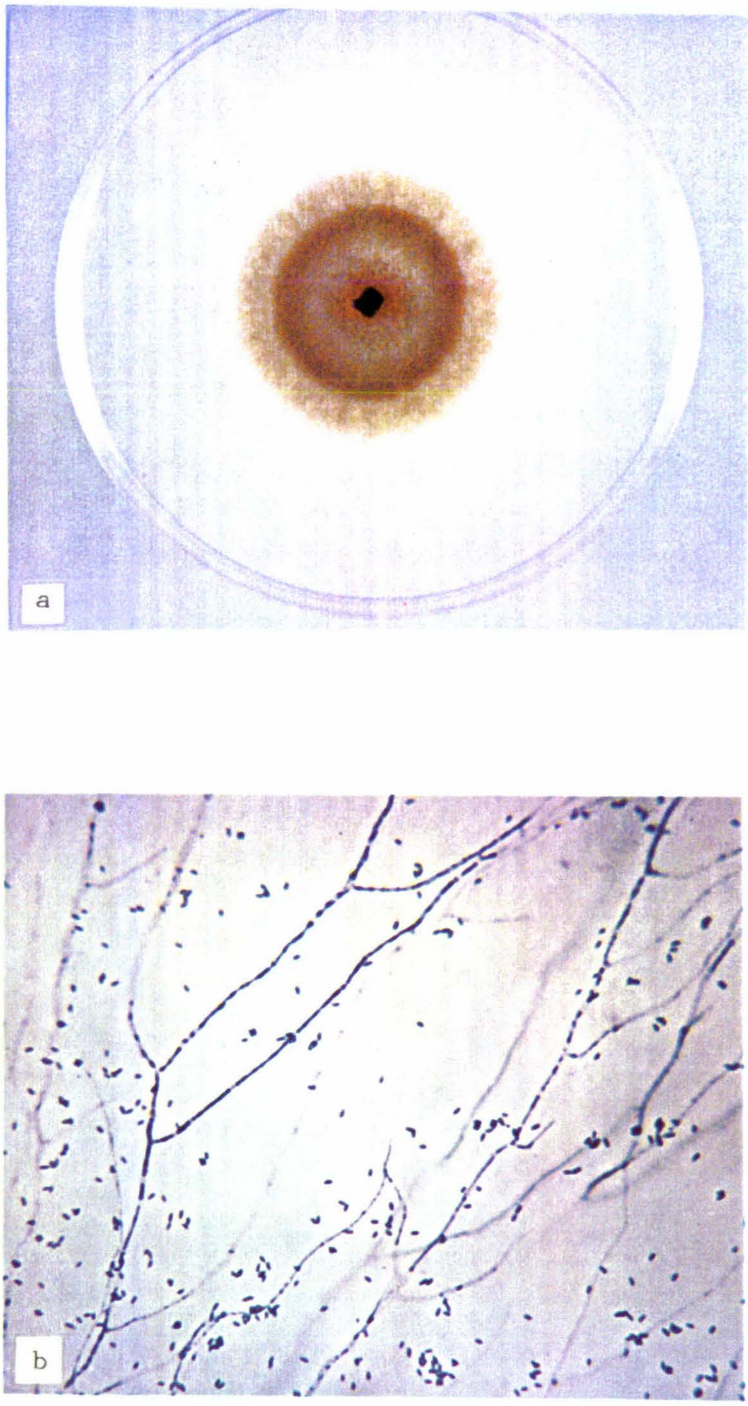
Phoma species are characterised by the production of black subsurface pycnidia which exude small cylindrical conidia in slime.

Pycnidia have one or more orifices (ostioles) and the production of pycnidia were abundantly on malt extract agar (MEA), just beneath the agar surface.

Colonies of *Phoma* on MEA and PDA have grey green or olive mycelium, and the sporulation of *Phoma* species have stimulated by its growth on PDA (Fig 3a).

The conidia are cylindrical, narrow, 4-5 x 2-2.5µm (Pitt & Hocking, 1985), and the conidia are hyaline with thin smooth walls.

Fig 3. Isolate of FF9 (*Phoma sp.*): (a) Colony on PDA, 13d, at 20°C ± 1°C. (b) Mycelium and cells stained with lactophenol cotton blue on PDA medium.



4.4 **BACTERIA ISOLATE BF21**

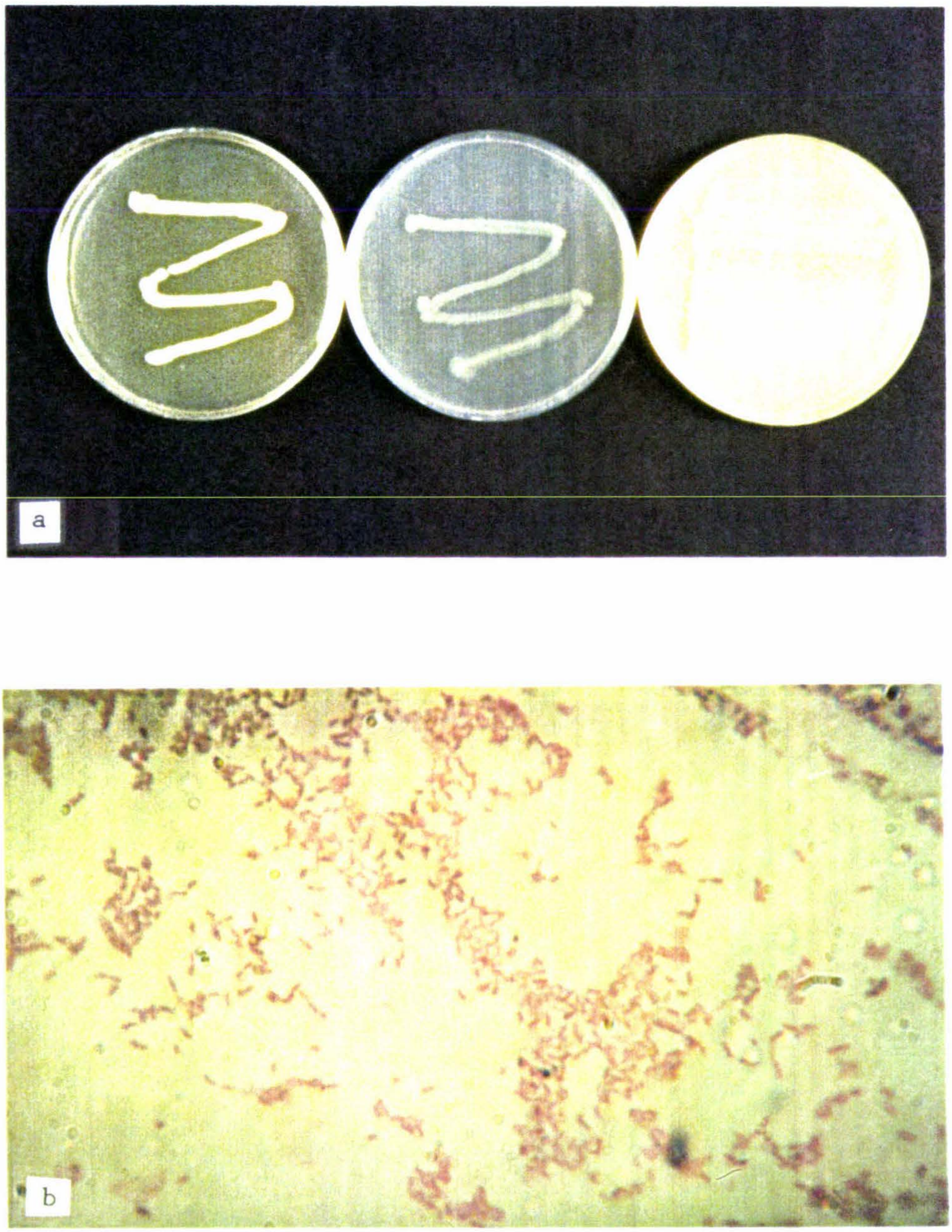
Characteristics assessed were:

1. Gram stain; the isolate stained red (gram-negative).
2. Kovacs oxidase test; a dark bluish violet colour formed within 10sec after smearing small amount of BF21 on a dampened filter paper with 1% aqueous tetramethyl-p-phenylene diamine dihydrochloride; indicated positive.
3. Heat test; a loopful of BF21 (in 3ml dist.water) in a thin sterile tube was heated to 80°C for 15min in a waterbath, streaked before and after heating in NA medium, no spore was formed after heating; indicated negative.
4. Florescent pigment; florescent pigment was not visible on King B medium [agar 1.5%, Difco proteose peptone No.3, 2%, glycerol (A.R. grade) 1%, K2HPo4 anhydrous 0.15%, MgSo4.7H20 0.15%]; indicated negative.
5. Acid production from glucose; the medium [oxoid bacteriological peptone 0.1, NH4H2Po4 0.1, KCl 0.02, MgSo4.7H20 0.02, bromothymol blue 0.003, oxoid agar No.3 0.3 (pH 7-7.2)] was poured in two tubes (one tube was sealed to 1cm thickness of sterile mineral oil), the tubes were incubated at 28°C for 2d, a loopful of BF21 was stabbed down to the tube bases, glucose was oxidised aerobically in the open tube by turning to yellow colour.
6. Nitrate reduction test; a loopful of BF21 was stabbed in the medium (oxoid bacteriological peptone 1%, K2HPo4 0.5, Difco yeast extract 0.1%, KNo3 0.1%, oxoid agar No.3 0.2%) within screw-capped bottle and the bottle was incubated at 28°C for 2d. A drop of Gram's iodine and 0.5ml of 0.6% dimethyl-a-napthylamine in 5N acetic acid was added, no nitrate was detected (no red colour was developed), and with small amount of zinc dust (5ml/ml of culture) added by using a needle, a red coloration was developed after a few minutes indicated the presence of nitrate in the medium (zinc has reduced very little of nitrate to nitrite); indicated negative.
7. Cell morphology; straight rod shape cells, motile (Fig 4b).
8. Colony morphology; yellow growth on Yeast extract-dextrose-CaCo3 agar (YDCA), (Fig 4a, right), and on Nutrient glucose agar (NGA) with opaque colonies.
9. No mycelium present.
10. Origin; fruit surface.

The results were compared with the bacteria characteristics given by Skerman V. B. D (1967), Bradbury J.F (1970), and Shaad N.W (1988).

The tests indicated, the bacteria isolate BF21 belong to genus *Xanthomonas*.

Fig 4. Isolate of BF21 (*Xanthomonas sp.*): (a) Colony streak on (left) PDA, 2d, (middle) NA, 2d, and (right) YDCA, 1d, at 20°C ± 1°C. (b) Rod cell (x 100), coloured with gram stain.



5.3 MATERIALS AND METHODS

5.3.2 Test on various media at different temperatures

Five millimetre diameter mycelial plugs of *B.cinerea* and of the antagonist isolates FB3, FF9 and FO30 were cut from the margins of 2 week old growing colonies on PDA medium using a cork borer. The antagonist plugs (discs) were transferred aseptically and placed in equidistance at the edge of PDA petri dish of various media (3 mycelial discs per petri dish) using a needle. *B.cinerea* plugs were inoculated in the centre of the plate.

The media were, Potato dextrose agar (PDA): 15.6g PDA/400ml dist.water, pH 5.6, Malt extract agar (MA): 8g malt extract + 6g agar/400ml dist.water, pH 5.6, Juice of eight different vegetables (V.8): 10ml V.8 + 8g agar/400ml dist.water, pH 6.3, Corn meal agar (CMA): 6.85g CMA/400ml dist.water, pH 6.1, Kiwifruit juice (KFJ 1:20): 20ml pure KFJ + 380ml dist.water + 12g agar, pH 3.8, Kiwifruit juice (KFJ 1:30): 20ml pure KFJ + 580ml dist.water + 8g agar, pH 4.

Five petri dishes of each medium of PDA, MA, V.8, CMA, KFJ (1:20), KFJ (1:30) were used to ensure adequate replication in case of contamination, and were incubated at 0, 5, 10, 15 and 20°C (Table 7a, b, c, d, e).

At 10, 15 and 20°C the isolates of FF9, FO30 and FB3 were placed at a distance of 25-30mm from the centre of the petri dish. At 0 and 5°C, the distance was reduced to 10-15mm because of the slower growth at low temperatures.

5.3.1 Test at various pH levels

In the second test of various pH levels, the antagonists were placed around *B.cinerea* on PDA petri dishes with pH 4.5, 5.0, 5.6 and 6.2 and the antagonistic activity was observed at the temperatures 0, 5, 10, 15 and 20°C (Table 8).

5.4 RESULTS

In some petri dishes, *B.cinerea* grew through the colony of the antagonist with no indication of inhibition, in others its growth stopped some distance away from the antagonist to give a distinct zone free of mycelium.

Table 7a, b, c, d, e, showed the antagonistic activity of FF9, FO30 and FB3 towards *B.cinerea* in different media at various temperatures, after some period of incubation.

The antibiotic production (indicated by the width of inhibition zones) of FF9 and FO30 towards *B.cinerea* was higher in KFJ 1:30 and PDA medium than other nutrient media used at 0°C, but FF9 and FO30 differed in their reactions at other temperatures. Isolate FF9 showed some antagonism on KFJ (1:30) at all temperatures used except 20°C, and also on PDA medium at 0, 5, 10°C but not at 15 and 20°C. Isolate FF9 inhibited *B.cinerea* better than FO30 on V.8

medium at all temperatures except 20°C. Isolate FO30 showed some antagonism on KFJ (1:20) at 0, 5, 10, 20°C, but not at 15°C. The yeast isolate FB3 did not react antagonistically against *B.cinerea* on any agar petri dishes at any temperatures used (Table 7). A typical screening test of *B.cinerea* and antagonist isolates FB3, FF9, FO30 on different media incubated at 0, 10, 20°C is shown in Fig 5.

General Linear Models Procedure showed that temperature and time period did not significantly ($P< 0.05$) affect the antagonistic action on any media used. The isolates FF9, FO30 showed significant antagonism to *B.cinerea* on those media at 5% level (Table 7). For more statistical information see Appendix 2 (Table 1, 2, 3).

The second test of the isolates FB3, FF9, FO30 at various pH levels, has shown that isolates FF9 and FO30 were more antagonistic than FB3 on PDA medium at various pH levels and temperatures. At 0•C, the isolates FF9 and FO30 showed more antagonistic activity to *B.cinerea* at pH 5.6 and 6.2 than at 4.5 and 5.0 (Table 8). At 5, 10, 15 and 20°C, the isolates FB3, FF9, FO30 did not show any antagonistic activity.

Table 7. Antagonistic activity of the antagonists FF9, FO30, FB3 towards *B.cinerea* shown by the width of the inhibition zones (mm) in different media at various temperatures.

Table 7a.

At 0°C

Media	Antagonists											
	FF9				FO30				FB3			
	I.Z(mm) in Days				I.Z(mm) in Days				I.Z(mm) in Days			
	26	36	49	65	26	36	49	65	26	36	49	65
PDA	3.5	2.5	1.0	In	5.0	4.5	2.0	In	In	In	In	In
MA	4.0	3.0	1.0	In	0.2	In	In	In	S	In	In	In
V.8	7.0	4.0	S	In	6.0	3.0	In	In	1.5	S	In	In
CMA	3.0	0.5	In	In	2.5	1.0	1.0	0.5	1.0	In	In	In
KFJ(1:20)	1.5	In	In	In	5.0	1.5	S	In	In	In	In	In
KFJ(1:30)	5.0	4.5	3.5	In	5.0	3.0	2.0	In	In	In	In	In

In = Isolate overgrown by *Botrytis*
S = *Botrytis* overgrowth commencing

Table 7b.

At 5°C

Media	Antagonists								
	FF9			FO30			FB3		
	I.Z(mm) in Days			I.Z(mm) in Days			I.Z(mm) in Days		
	14	26	36	14	26	36	14	26	36
PDA	3.5	1.0	In	2.0	In	In	In	In	In
MA	2.0	In	In	1.5	In	In	In	In	In
V.8	2.0	1.0	0.5	S	In	In	In	In	In
CMA	2.0	In	In	2.0	In	In	In	In	In
KFJ(1:20)	2.0	In	In	3.5	0.2	In	In	In	In
KFJ(1:30)	5.0	3.0	1.0	3.5	In	In	In	In	In

Table 7c.

At 10°C

Media	Antagonists								
	FF9			FO30			FB3		
	I.Z(mm) in Days			I.Z(mm) in Days			I.Z(mm) in Days		
	9	14	26	9	14	26	9	14	26
PDA	4.5	4.0	In	7.0	7.0	In	In	In	In
MA	6.0	S	In	3.0	1.0	In	In	In	In
V.8	9.0	7.0	In	2.0	S	In	In	In	In
CMA	9.0	4.0	In	3.0	0.2	In	In	In	In
KFJ(1:20)	9.0	5.0	In	6.5	0.2	In	In	In	In
KFJ(1:30)	7.0	4.0	In	4.0	S	In	In	In	In

Table 7d.

At 15°C

Media	Antagonists					
	FF9		FO30		FB3	
	I.Z(mm) in Days		I.Z(mm) in Days		I.Z(mm) in Days	
	5	9	5	9	5	9
PDA	4.0	In	3.5	In	In	In
MA	4.5	In	1.5	In	In	In
V.8	4.5	0.5	S	In	In	In
CMA	6.0	In	6.0	In	S	In
KFJ(1:20)	4.0	S	4.0	In	In	In
KFJ(1:30)	6.0	1.0	5.5	S	S	In

Table 7e.

At 20°C

Media	Antagonists					
	FF9		FO30		FB3	
	I.Z(mm) in Days		I.Z(mm) in Days		I.Z(mm) in Days	
	5	9	5	9	5	9
PDA	2.5	In	2.0	In	S	In
MA	In	In	S	In	In	In
V.8	0.5	In	3.5	0.5	In	In
CMA	0.5	In	0.5	In	S	In
KFJ(1:20)	3.0	In	3.0	1.0	S	In
KFJ(1:30)	3.0	In	1.5	1.0	S	In

Fig 5. Antagonistic activity towards *B.cinerea* on different media by using the antagonists, (left) FF9 (*Phoma sp.*), (right) FO30 (*Fusarium merismoides*), (bottom) FB3 (*Rhodotorula sp.*) incubated for (a) 26d at 0°C, (b) 9d at 10°C, (c) 5d at 20°C.

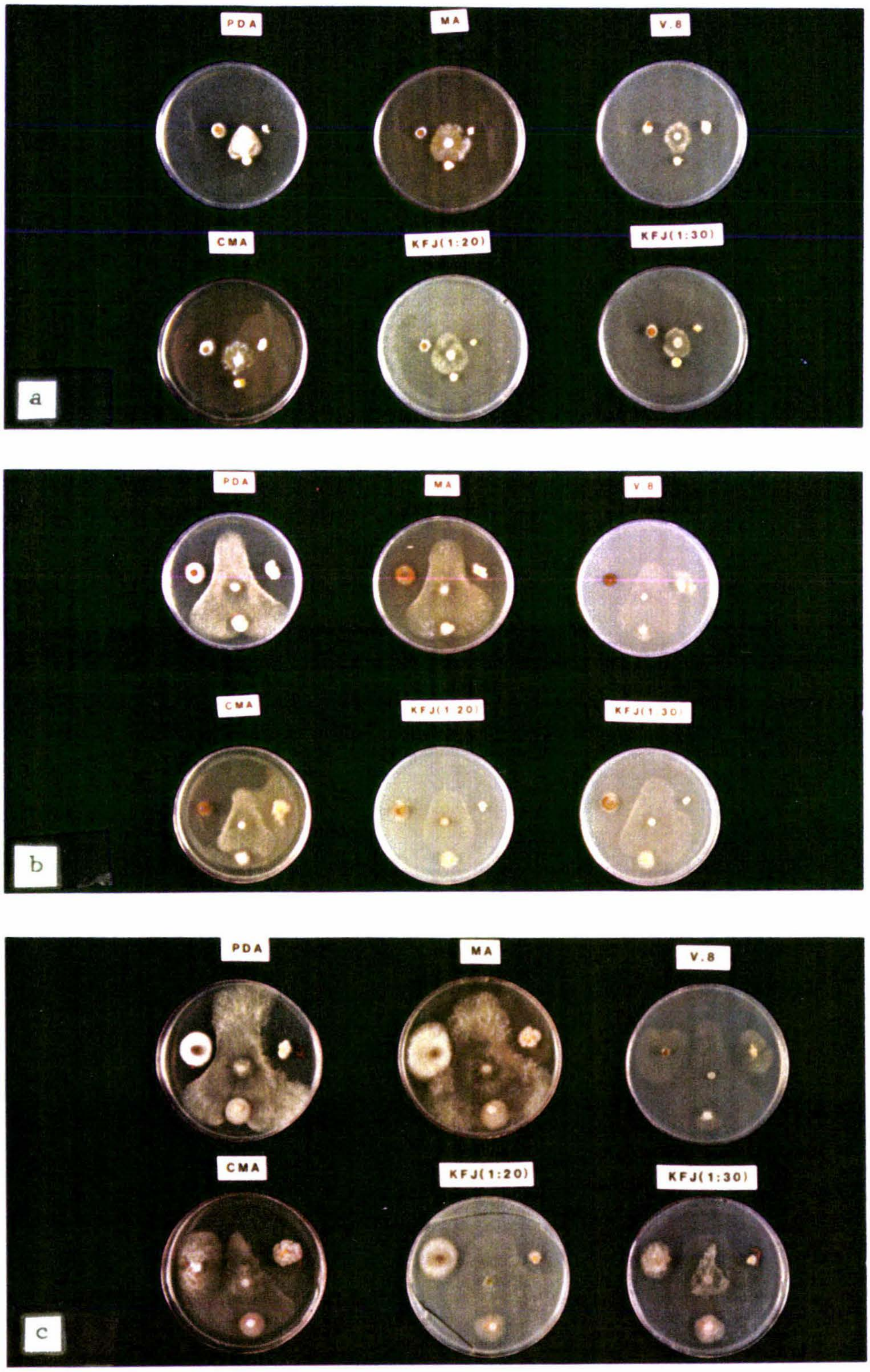


Table 8. Antagonistic activity of FB3, FF9 and FO30 tested against *B.cinerea* on PDA medium in different PH at various temperatures.

		PH 4.5			PH 5.0			PH 5.6			PH 6.2		
Temp	Days	FB3	FF9	FO30	FB3	FF9	FO30	FB3	FF9	FO30	FB3	FF9	F030
0°C	19	2.0	6.0	5.0	4.0	3.0	4.0	4.0	8.0	6.0	3.0	6.0	5.0
	25	In	In	2.5	In	In	0.5	1.5	4.0	4.0	In	1.0	3.5
	30	In	In	S	In	In	In	0.5	0.5	1.5	In	In	0.5
	34	In	In	In	In	In	In	In	In	In	In	In	In
5°C	9	2.0	4.0	7.0	In	3.0	6.0	4.0	7.0	5.0	2.0	6.0	6.0
	16	In	In	In	In	In	S	In	In	In	In	In	S
10°C	5	2.0	9.0	8.0	1.0	6.0	8.0	0.5	8.5	6.0	2.0	8.0	4.0
	9	In	1.0	1.0	In	0.5	1.0	In	In	2.0	In	S	S
15°C	5	In	S	1.0	In	0.5	In	In	In	In	In	S	In
	9	In	In	In	In	In	In	In	In	In	In	In	In
20°C	5	In	In	In	In	In	In	In	In	5.0	In	In	In
	9	In	In	In	In	In	In	In	In	1.0	In	In	In
	16	In	In	In	In	In	In	In	In	In	In	In	In

5.5 DISCUSSION

Of the media tested, the nutrient rich media PDA, V.8 and KFJ were the most favourable for the antagonists to act against *B.cinerea*. It has been postulated that sugars and/or amino acids provided to *B.cinerea* spores, could enhance the activity of *B.cinerea* enzymes and removed the inhibitions attributed to antibiotics produced by the antagonists (Fokkema, 1973), but that was not the case in the present work.

Isolates of *Phoma sp* (FF9), *Fusarium sp* (FO30) and a pink yeast (FB3) differed greatly in their induction of inhibition zones with *B.cinerea* on different media (Table 7). The antagonists were overgrown by *B.cinerea* in all media at the range of pH 3.8-6.3 during the time periods and temperatures used (Table 7). Bhatt & Vaughan (1962) indicated, that *Cladosporium herbarum* and *Aureobasidium pullulans* inhibited the germination and the growth of *B.cinerea* on tomato and strawberries because of low pH (pH 4.0).

The antagonism on KFJ 1:20 and 1:30, indicated that FF9 and FO30 preferred nutrient substrate with higher acidity as a battle field against *B.cinerea* than lower acidity. This can lead to an opportunity on having the kiwifruit as a source for antagonism *in vivo* test at low temperatures.

The growth rate of the antagonists varied considerably even at optimum temperatures. Incubation temperatures had a greater effect than the choice of medium, thus the temperature at which the antagonism is tested is clearly critical.

The behaviour of the *B.cinerea* mycelium in the vicinity of the antagonist varied. The longer the time period allowed for antagonism, the more *B.cinerea* adapted to the antibiotics produced by the antagonists and was able to overgrow them. The main inhibitory effect appears due to the production of antibiotic substances and not to the pH of the medium.

Although isolates FF9 and FO30 showed some antagonistic activity to *B.cinerea* on PDA medium at pH 5.6 and 6.2 at 0°C, *B.cinerea* was not successfully controlled by any antagonist at pH 4.5, 5.0, 5.6, and 6.2 at different temperatures (Table 8). Newhook (1951) indicated that pH may have a direct effect on the growth of *B.cinerea*, and also an indirect effect by altering the activity of pectinase produced. PH of the media had little effect on inhibition of *B.cinerea* by the antagonists but it is known to affect the pathogenesis by decreasing the actions of pectinase at high pH.

Dependency and the growth of *B.cinerea* on a specific nutrient-medium could be related to the increasing of the age of *B.cinerea* spores at which the need of aged spores to nutrient is most required for spore swelling, germination and growth increasing.

INHIBITION OF B.cinerea SPORE GERMINATIO IN VITRO

CHAPTER SIX

IN VITRO TESTING INHIBITION OF *B.cinerea* SPORE GERMINATION

6.1 INTRODUCTION

Spores of all postharvest pathogens require high humidity or free water for a number of hours for successful spore germination (Sommer, 1985). Antibiotics or inhibitory substances produced by spores or cells of a potential antagonist could affect *B.cinerea* spore germination. Control of *B.cinerea* presumably depended on the inhibition of spore germination or retardation of its subsequent growth (Wood, 1951). Glass slide examination could reveal morphological effect of the antagonist on the pathogen propagules such as inhibition of spore germination and abnormal germ tube growth (Blakeman & Fokkema, 1982).

In this study, a mixture of aqueous suspension of the test pathogen (*B.cinerea*) and each of the antagonists FB3, FF9, FO30 were cultured on agar petri dish to investigate the antagonism *in vitro*.

6.2 OBJECTIVES

To investigate the germination and the growth of *B.cinerea* in the presence of biocontrol agents *in vitro*.

6.3 MATERIALS AND METHODS

Spore suspensions of *B.cinerea* and of the antagonists (FB3, FF9, FO30) were prepared from 10 days old cultures on PDA by removing the spore-bearing mat, shaking well in SDW and straining through sterile glass wool to remove the large hyphal fragments.

The *B.cinerea* control spore concentration was 1x10^6 spore/ml. Equal volumes of a *B.cinerea* (2x10^6 spore/ml) and of each of the antagonists FB3, FF9, FO30 (2x10^7 spore/ml) were used as inoculum.
The spore concentrations were determined using a haemocytometer. A drop of 10µl containing 10.000 spores was used in this test.
Germination tests were made by placing a drop of spore suspension on V.8 medium. Spore suspension tests were conducted on 2.5% V.8 medium (2.5ml of mixed vegetable juices/100ml dist.water + 1.5g agar/100ml dist.water). Discs (2cm diam) of V.8 medium were cut using a cork borer and 2 discs were placed on each slide using a needle. After inoculation, the slides were incubated in small humid chambers at 0 or 20°C for 24, 48, 72hr or for 4, 8, 24hr respectively. The discs after incubation periods were mounted with a drop of Lactophenol acid fuchsin stain and were covered with a coverslip.
Assessment of sporulation, germination of *B.cinerea* and antagonist spores were determined arbitrary under 1mm^2 counting grid (x100), with the aid of a compound microscope.

Because of the high variation in germinated spore lengths, the twenty longest germ tubes were measured with an eyepiece micrometer (100 divisions, each division equals 2.5µm), (Table 9). A spore was considered germinated when the germ tube was half the width of the spore.
The percentage of spore germination of *B.cinerea* and the antagonists were assessed at 0 and 20°C for different incubation periods (Table 9).

6.4 RESULTS

The percentage of *B.cinerea* germination at 20°C for 4 and 8hr was slightly higher than at 0°C. More spores of *B.cinerea* germinated after 72hr at 0°C than after 24hr at 20°C.

The germination of *B.cinerea* was not affected at all by the presence of the isolate FB3 or FF9 at 0°C and 20°C.

The percentage germination of *B.cinerea* in the presence of isolate FO30 was greatly reduced at 0°C, with 1% germination of the isolate itself, but at 20°C the germination of *B.cinerea* and the isolate FO30 gradually increased with time, especially after 24hr of incubation (Table 9).

The germ tube elongation of *B.cinerea* was greater when *B.cinerea* spore suspension was inoculated alone or with any spore suspension of FB3 or FF9 simultaneously at 0°C and 20°C than with FO30.

The inhibition of *B.cinerea* spore germination in the presence of FO30 at 0•C and 20°C is illustrated in Fig 6 and 7.

Table 9. Percentage germination of *B.cinerea* and maximum germ tube lengths(μm) on 2.5% V.8 agar discs inoculated with spore suspensions of *B.cinerea* or *B.cinerea*/antagonists mixtures after incubation at 0°C or 20°C.

	0°C				20°C			
spore suspension	hr	% germination of *B.cinerea*	% germination of Antagonist	Max.g.tube length(μm) of *Botrytis*	hr	% germination of *B.cinerea*	% germination of Antagonist	Max.g.tube length (μm) of *Botrytis*
B.cinerea	24	40.0		67.5	4	72.6		52.5
B+FB3	24	27.9	0.0	32.5	4	74.6	1.0	50.0
B+FF9	24	37.8	0.0	32.5	4	72.9	0.0	45.0
B+FO30	24	1.5	1.0	10.0	4	0.0	1.0	0.0
B.cinerea	48	95.6		95.0	8	96.2		165.5
B+FB3	48	82.7	0.0	72.5	8	88.9	1.0	175.5
B+FF9	48	94.8	0.0	125.0	8	95.7	0.0	75.0
B+FO30	48	8.0	1.0	37.5	8	12.5	64.0	27.5
B.cinerea	72	96.7		200.0	24	83.5		225.5
B+FB3	72	96.4	0.0	120.0	24	52.5	1.0	262.5
B+FF9	72	94.9	0.0	117.5	24	92.8	0.0	237.5
B+FO30	72	7.9	0.0	37.5	24	63.1	100.0	55.0

Fig 6. B.cinerea spore germination at 0°C without (a, b, c) or with (d, e, f) the presence of FO30 for 24, 48 and 72hr.

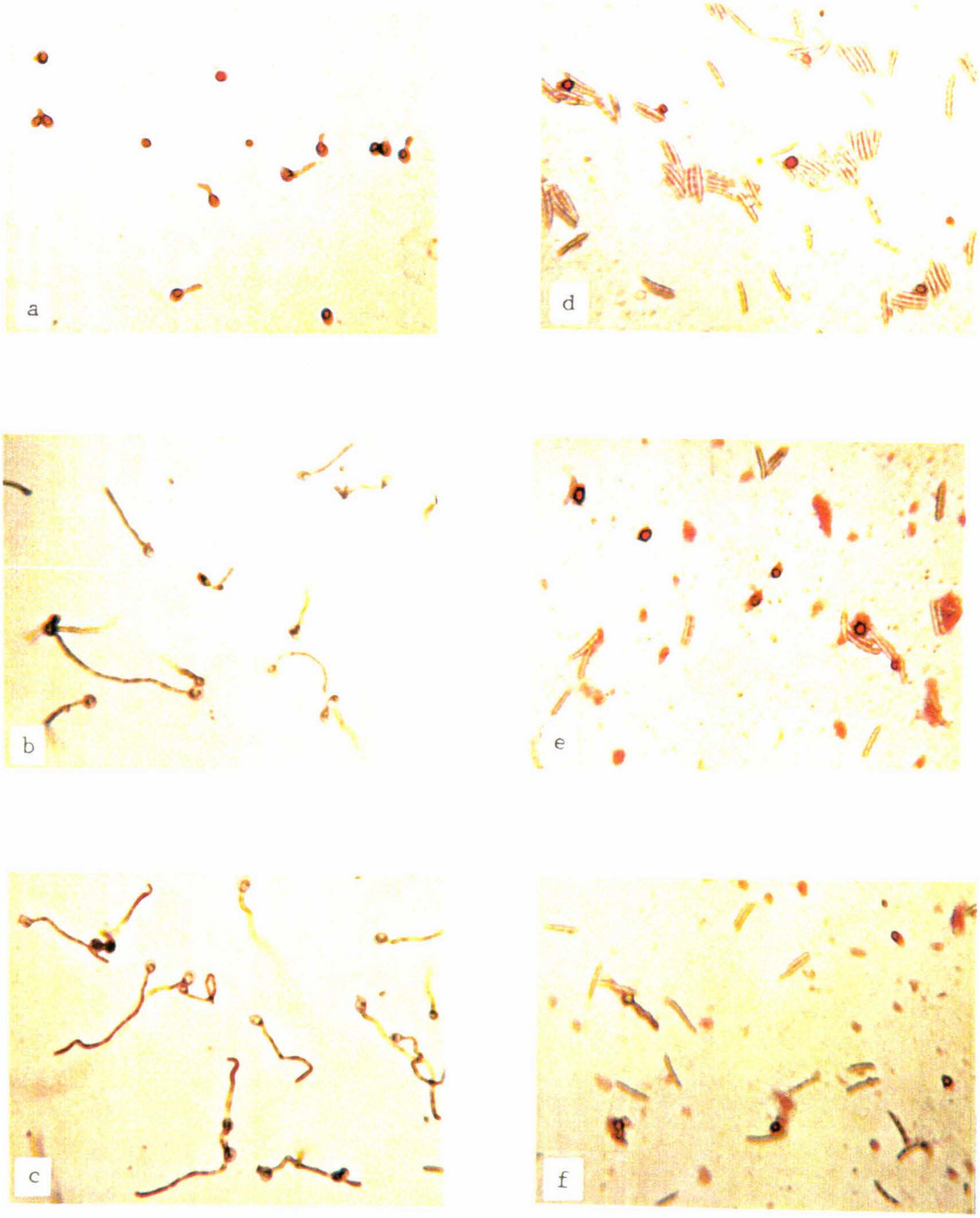
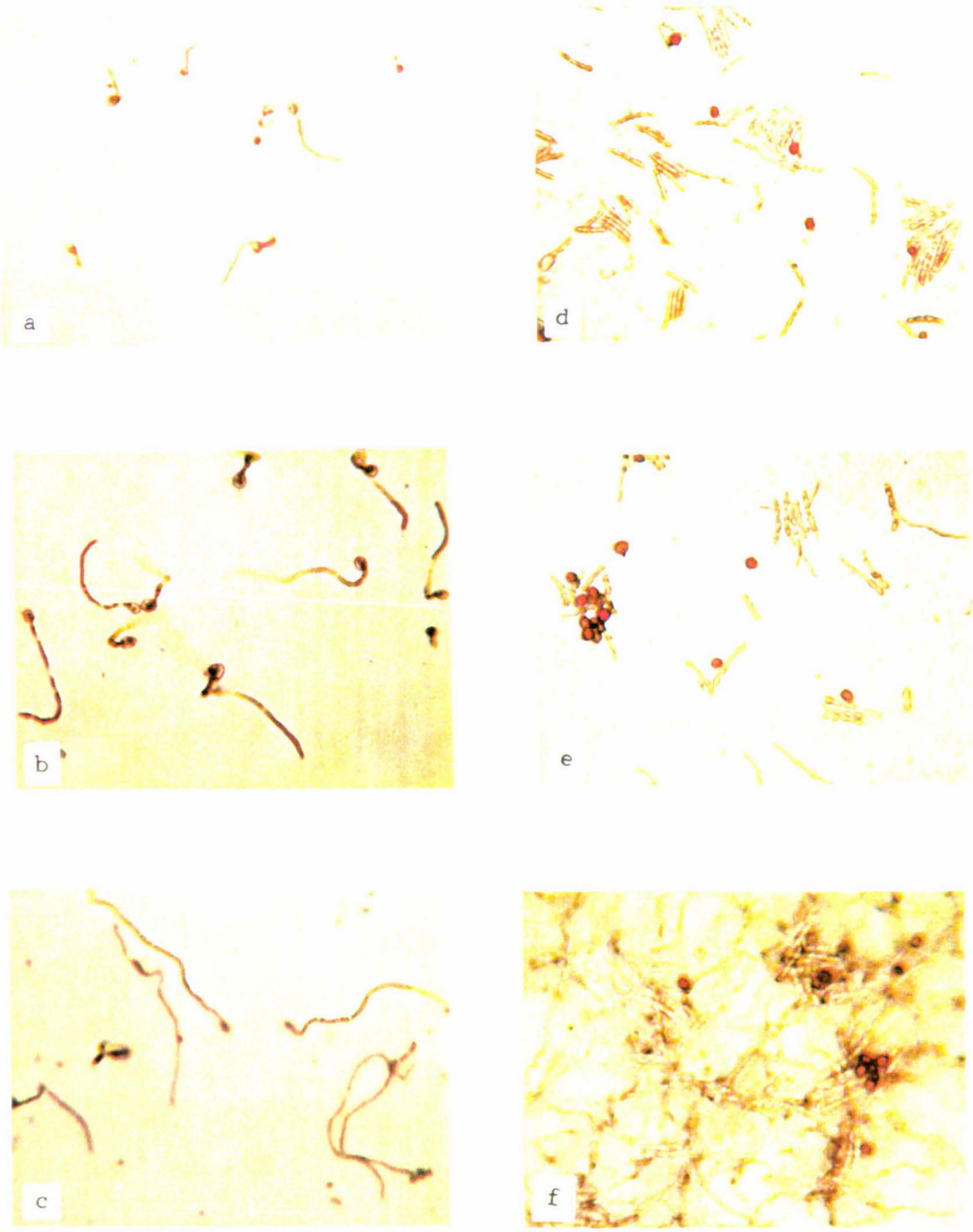


Fig 7. *B.cinerea* spore germination at 20°C without (a, b, c) or with (a, b, c) the presence of FO30 for 4, 8 and 24hr.



6.5 DISCUSSION

Fusarium isolate FO30 has shown its ability to inhibit spore germination and germ tube length of *B.cinerea* in aqueous spore suspensions of B+FO30 *in vitro* (Table 9).

The failure of *B.cinerea* spore germination could be by the accumulation of inhibitory substances in the presence of FO30, or the absence of essential substances required.

However *in vivo*, at or soon after germination, *Botrytis* spores become very sensitive to nutrient conditions and may suffer from competition (Campbell, 1989).

The information of inhibitory substances by the host, however remains a possible mechanism of antagonism when interactions on wound are concerned (Fokkema, 1976). Therefore reasons for biocontrol could be, by inhibitory substances and reduction in nutrient supply.

Examples of inhibitory substances;

In vitro, Penicillium, Aureobasidium and *Cladosporium* inhibited *B.cinerea* on agar showing inhibition zones of 3.5, 1, and 0mm respectively.

B.cinerea was inhibited on buffered media by *Cephalosporium, Fusarium* or *Phoma* which produced antibiotic substances, and saprophytic antagonism was responsible for some of the natural control of *B.cinerea* in the field (Newhook, 1951).

In vivo, the production of antibiotics as the mode of action of some bacteria like *Bacillus subtilis* and *Pseudomonas cepacia* have been granted to be used in Japan on fresh commodities. *B.subtilis* produced antibiotic (Liturin A) was found to be active against *B.cinerea* (Wilson & Wisniewski, 1989).

Examples of reduction in nutrient supply;

In vitro, a selection procedure based on nutritional characteristics of the desired microbial component, e.g chitin agar to enrich actinomycetes (Hsu & Lockwood, 1975).

In vivo, the ability of *Debaryomyces hansensii* to rapidly colonize and proliferate on wound sites of fruit, producing an extracelluar polysaccarides and compete *B.cinerea* for nutrient. The antagonistic yeast *D.hansenii* US-7 applied to the wound site have successfully compete with *P.digitatum* on grape fruit for space and nutrient (Droby, *et al.* 1991).

The mechanism of inhibition may involve in the continuous production of an inhibitors of FO30. Antibiotics or inhibitory substances produced by a potential antagonist like FO30 *in vitro* could retard the germination and spore swelling of *B.cinerea*. Moisture absorption is an initial stage of germination and is usually accompanied by spore swelling (Sommer, 1985). *Botrytis* could have been absorbed less moisture in the presence of FO30. The presence of FO30 spores could lead to a loss of endogenous nutrients from *B.cinerea* spores, and reduced spore germination, subsequent growth and reproductive capacity of *B.cinerea*. If spores of the pathogen are surrounded by sufficient numbers of saprophytes which are strong nutrient competitors, loss of endogenous nutrients from such spores will occur, thereby the germination will be reduced or prevented (Brodie & Blakeman, 1976).

In vivo some loss of endogenous nutrient reserves leaked from *Botrytis* germinating spores under some conditions (e.g reduction in the amount of exogenous nutrients available to *B.cinerea*), will either prevent its germination or germinating poorly (Brodie & Blakeman, 1976).

The spore germination and the length of germ tubes of *B.cinerea* could be stimulated by the presence of the yeast isolate FB3. The isolates FB3 and FF9 neither reduced the percentage of germination nor the length of *B.cinerea* germ tubes, because those isolates could produce ineffective substances which have not affect *B.cinerea* growth, and the wall layer of *B.cinerea* becomes apparent during the germination of its spores (Gull & Trinci, 1971).

No direct evidence was indicated that nutrient competition was involved in the antagonism process on 2.5% V.8 agar discs. The inhibition of *Botrytis* spores at 0°C with the presence of isolate FO30 on 2.5% V.8 agar medium could have the same impact within the fruit itself, and such test would be required in coolstore.

CHAPTER SEVEN

EFFECT OF BIOCONTROL AGENTS ON INFECTION OF KIWIFRUIT BY *B.cinerea* IN STORAGE

7.1 INTRODUCTION

Most postharvest infection of fruits and vegetables occur through wounds on surface tissue. The picking wound is the preferred entry point for *Botrytis* into kiwifruit (Sharrock & Hallett, 1992).

Storage of fruits and vegetables for weeks or months prior to marketing may create disease problems, therefore the physiological and physical conditions of horticultural commodities are of great importance (Sommer, 1985). Complete loss of the commodity occurs when one or a few pathogens invade and breakdown the tissues, this initial attack is rapidly followed by a broad spectrum of weak pathogens (*Fusarium*, *Trichoderma*, *Penicillium*) which magnify the damage on kiwifruit caused by the primary pathogen (*Botrytis*), (Wills, *et al.* 1981).
kiwifruit is well known for its longevity in storage and this is one of the reason for its remarkable success as a fresh fruit export (Hewett, 1979). A single tray is the preferred tray for stored kiwifruit or for kiwifruit shipment to minimize nesting.

In this study, the first step was to isolate and identify the micro-organisms that have potential activity for disease suppression *in vitro*, and the antagonists were selected on the basis of their pronounced antagonism in pure culture. A look for antagonists that are well adapted to survive and grow on fruit and wound under storage conditions are most likely to be successful agents but their behaviour on an agar culture might not reflect their action in a natural substrate, particularly where antagonism for available nutrient is the cause of inhibition of germination of a particular pathogen.

Isolates of *Phoma sp* (FF9) from fruit surface, *Fusarium sp* (FO30) from old leaf and pink yeast (FB3) from young bud collected from kiwifruit orchard were used as biocontrol agents against *B.cinerea in vivo* test.
The aim of this experiment was to determine the effect of each biocontrol agent on *B.cinerea* growth on kiwifruits, and to find out whether the incubation temperature and the interval periods between harvesting and inoculation affected the incidence of storage soft rot caused by *B.cinerea*.

the interval periods between harvesting and inoculation affected the incidence of storage soft rot caused by *B.cinerea.*

7.2 OBJECTIVES

To determine the ability of selected antagonists to control *Botrytis* rot on kiwifruits in storage conditions.

7.3 MATERIALS AND METHODS

7.3.1 Source of fruit

Kiwifruit (*Actinidia deliciosa var.deliciosa* [A.Chev]. Liang & Ferguson, cv. Hayward) from a Wanganui orchard (six year old vines, unsprayed) were harvested on the morning of 23.May 1990 when the R.H was 88% and the fruit at a commercial stage of maturity for harvest (total soluble solids 8.8 Brix as determined by a refractometer).

7.3.2 Spore suspension

A conidial suspension $2x10^6$ spore/ml was prepared from a 2 week old culture of *B.cinerea* on PDA.

The washed spores were adjusted with SDW to get the desired concentration as calculated from haemocytometer readings.

The spores of FF9, FO30 and the cells of FB3 were prepared from 2 week old culture for FF9, FO30 and 10 days for FB3 on PDA petri dish.

Botrytis spore suspension $2x10^6$ spore/ml was mixed with each of the isolate FB3, FF9, FO30 at a concentration of $2x10^7$ spore/ml and was applied simultaneously in the ratio of 1:10 to inoculate the picking wound of the fruit.

An advantage of FO30 over other antagonists, was the formation of many macroconidia which were easily harvested and measured.

Botrytis alone spore suspension was diluted to $1x10^6$ spore/ml since it was not diluted by addition of antagonist spore suspension.

The inoculum levels used for the inoculation of the stem scar were :

1. Untreated fruits (Control).
2. 10µl droplet of water (Inoculum level 1).
3. 10µl droplet of a *B.cinerea* spore suspension 1x10^6 spores/ml as (Inoculum level 2).
4. 10µl droplet of spore suspension containing 1x10^6 spores/ml of *B.cinerea* and 1x10^7 cells/ml of FB3 (this was prepared by mixing equal proportions of spore suspensions containing 2x10^6 spores/ml and 2x10^7 cells/ml respectively), as (Inoculum level 3).
5. 10µl droplet of spore suspension containing 1x10^6 spores/ml of *B.cinerea* and 1x10^7 spores/ml of FF9 (this was prepared by mixing equal proportions of spore suspensions containing 2x10^6 spores/ml and 2x10^7 spores/ml respectively), as (Inoculum level 4).
6. 10µl droplet of spore suspension containing 1x10^6 spores/ml of *B.cinerea* and 1x10^7 spores/ml of FO30 (this was prepared by mixing equal proportions of spore suspensions containing 2x10^6 spores/ml and 2x10^7 spores/ml respectively), as (Inoculum level 5).

Fruit were inoculated 7hr after harvest and were subjected to different curing periods before storage at 0°C.

The curing periods were:

1. Stem scar inoculated immediately after harvest and stored at 0°C on the same day (CP1).

2. Stem scar inoculated immediately, cured 2 days at ambient temperature (8 - 12°C), and stored at 0°C (CP2).

3. Stem scar cured 2 days at ambient temperature, inoculated and stored at 0°C (CP3).

4. Stem scar cured 2 days at ambient temperature, inoculated and cured for a further 2 days and stored at 0°C (CP4).

The suspensions were left to dry on the stem scar for 30min, then the fruit was cured for 6hr or 2 days at ambient temperature (8-12•C) before storage at 0°C. All fruit were stored over 13 weeks at 0°C with a relative humidity of 87-90% in the plant growth unit storage at Massey University. A tray of fruit was served as a replicate in a randomized complete block design.

To minimise temperature and humidity variation, each block of (24 trays) was randomly arranged in the storage every 2wks. Individual fruit was examined in each tray for *Botrytis* storage rot at 2 week intervals starting from week 5 after inoculation. Lesion length was measured after weeks 5, 7, 9, 11 and 13 of fruit incubation at 0°C.

7.3.4 Statistical methods

Two kinds of data were available from this study :

1. Number of infected fruit.
2. Lesion size.

General linear models (least squares means) and analysis of variance procedures were used to examine the effects of inoculum levels and curing periods on the number of infected fruits and lesion size respectively (Steel & Torrie, 1981).

The computations were carried out using the generalized linear models computing programme (SAS) held at the Massey University computer centre.

7.4 **RESULTS**

Through *in vitro* selection, it was possible to choose antagonists that adapt to low temperatures and consequently are effective biocontrol agents.

The treatment comparisons were based on inoculum level and curing period and their relationship with the number of infected fruit and the diameter of lesion development.

The incubation temperature and the interval between harvesting and inoculation with a mixture of *B.cinerea* with three antagonists individually were varied in the effect of biocontrol agents on *Botrytis* development. The inoculation of a picking wound is shown in Fig 8.

The development of the number of infected fruit/99 fruit (in 3 trays) using different inocula and various curing periods over 13wks at 0°C is shown in Table 10. When the fruits were examined after the inoculation over 13 weeks, the untreated fruit, fruit with a drop of water and fruit with the inoculum level of B+FO30 have significantly reduced the number of infected fruit (Table 11). The GLM procedure showed that the inoculum level significantly affected the number of infected fruits (Table 11), and the GLM procedure also showed no interaction between inoculum levels and curing periods, except in week 9 when the interaction was significant at the 5% level. On the basis of percentage infection developing, the inoculum types could be divided into two groups: group 1 (untreated, drop of water, B+FO30) and group 2 (*Botrytis* suspension, B+FB3, B+FF9). There was no significant difference between treatments within a group but the two groups were significantly differed from each other (Table 11).

The untreated fruit which were stored immediately after harvest were significantly more likely to develop lesions than if they were allowed to heal 4 days at ambient temperature before storage (Table 12). The curing period had no significant affect on the number of infected fruit except in curing period four (Table 12). As the interval period between picking wound and inoculation with *B.cinerea* increased from 0 to 4 days , susceptibility of wounds to decay by *B.cinerea* decreased (Table 12). Leaving the fruit untreated or inoculated with a drop of water for 4 days curing period before storage at 0°C, gave better results than immediately or 2 days curing period before storage (Appendix 3:

Table 1a, b, c, d). Curing allows time for a natural defence against the high level of *Botrytis* inoculum. Therefore several days may required for an antagonist to grow and reproduce to a level that will prevent infection by a pathogen.

The regression line for the type of lesion size in all treatments was linear, and the lesions advanced in the same way in all treatments after the rots started (Table 13).

Secondary infections (nesting) were not observed in the 13 weeks period.

The proportion of fruit showing symptoms of disease over 13wks at 0°C after treatment with different inocula and subjected to curing period one and curing period two are shown in (Fig 9). The natural infection rate was 2-6% and was not increased by addition of a drop of water to the stem scar. Most infections had appeared by week 5 and few developed after week 9.

Botrytis inoculum of 10,000 spores increased infection from about 3 fruit/99 to 18 fruit/99. The number of infected fruit which approximates to the percentage of infection in four curing periods are shown in (Fig 10a, b, c and d). In my experiment using *Botrytis* liquid suspension on harvested fruit, the percentage of infected fruit was 17-23%.

Neither FB3 nor FF9 controlled *Botrytis* infections except FB3 on fruit cured for 4 days. FO30 gave good control of *Botrytis* in curing period two (Appendix 3: Table 1b). *Botrytis* infection was suppressed by curing for 2 and for 4 days.

Table 10. Number of infected fruit / 99 at various curing periods and different inoculum levels. Fruit were stored over 13 weeks at 0°C.

	CP1					CP2					CP3					CP4				
Inocul.level	5	7	9	11	13	5	7	9	11	13	5	7	9	11	13	5	7	9	11	13
Control	1.0	5.0	6.0	6.0	6.0	1.0	2.0	2.0	4.0	4.0	0.0	2.0	2.0	2.0	2.0	0.0	1.0	1.0	1.0	1.0
Drop.water	2.0	4.0	4.0	4.0	4.0	1.0	3.0	3.0	3.0	3.0	0.0	1.0	1.0	2.0	2.0	0.0	0.0	0.0	1.0	1.0
B.suspens.	15.0	18.0	19.0	19.0	19.0	18.0	20.0	22.0	22.0	23.0	9.0	15.0	17.0	18.0	19.0	6.0	9.0	11.0	11.0	11.0
B + FB3	11.0	15.0	18.0	18.0	19.0	13.0	14.0	16.0	17.0	18.0	9.0	13.0	13.0	13.0	13.0	1.0	2.0	3.0	3.0	3.0
B + FF9	8.0	11.0	11.0	11.0	11.0	10.0	12.0	13.0	14.0	15.0	7.0	10.0	12.0	12.0	12.0	9.0	11.0	12.0	13.0	13.0
B + FO30	2.0	2.0	2.0	2.0	2.0	1.0	1.0	1.0	1.0	2.0	2.0	4.0	4.0	4.0	4.0	1.0	3.0	3.0	3.0	3.0

B.suspens = *Botrytis* suspension. Inocul.level = Inoculum level
CP1 = Fruit immediately inoculated and stored at 0°C.
CP2 = Fruit immediately inoculated, cured 2d at ambient temperature (20 ± 1°C), and stored at 0°C.
CP3 = Fruit cured 2d at ambient temperature, inoculated and stored at 0°C.
CP4 = Fruit cured 2d at ambient temperature, inoculated, cured a further 2d at ambient temperature and stored at 0°C.

Fig 8. Kiwifruit inoculated with 10µl drop of inoculum on the picking wound.

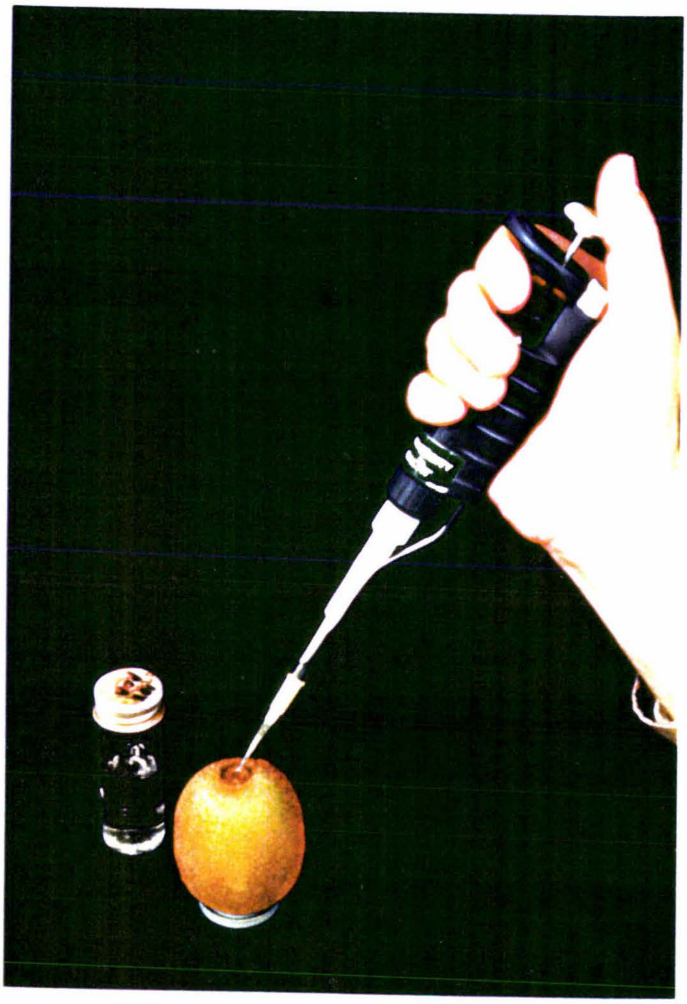
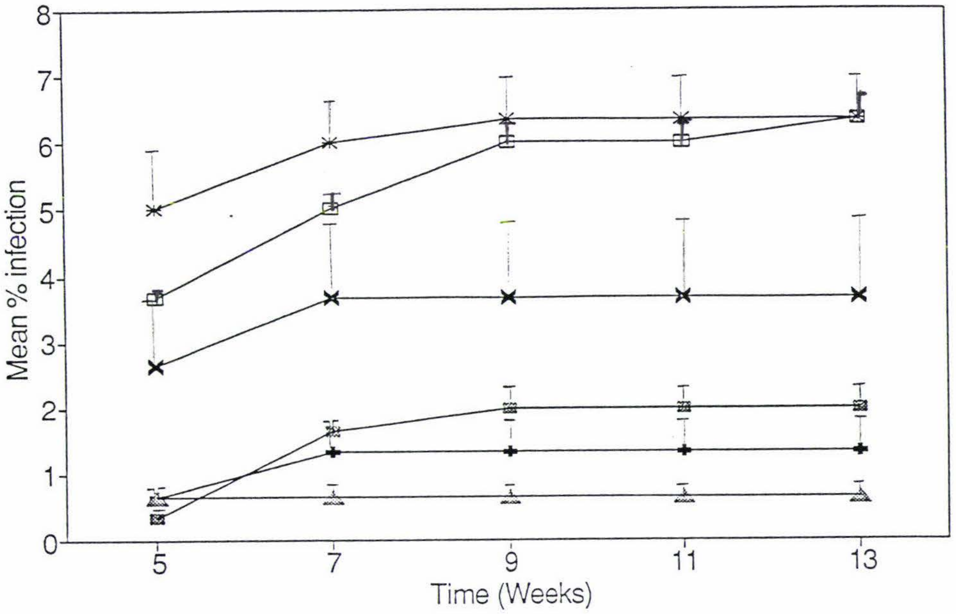


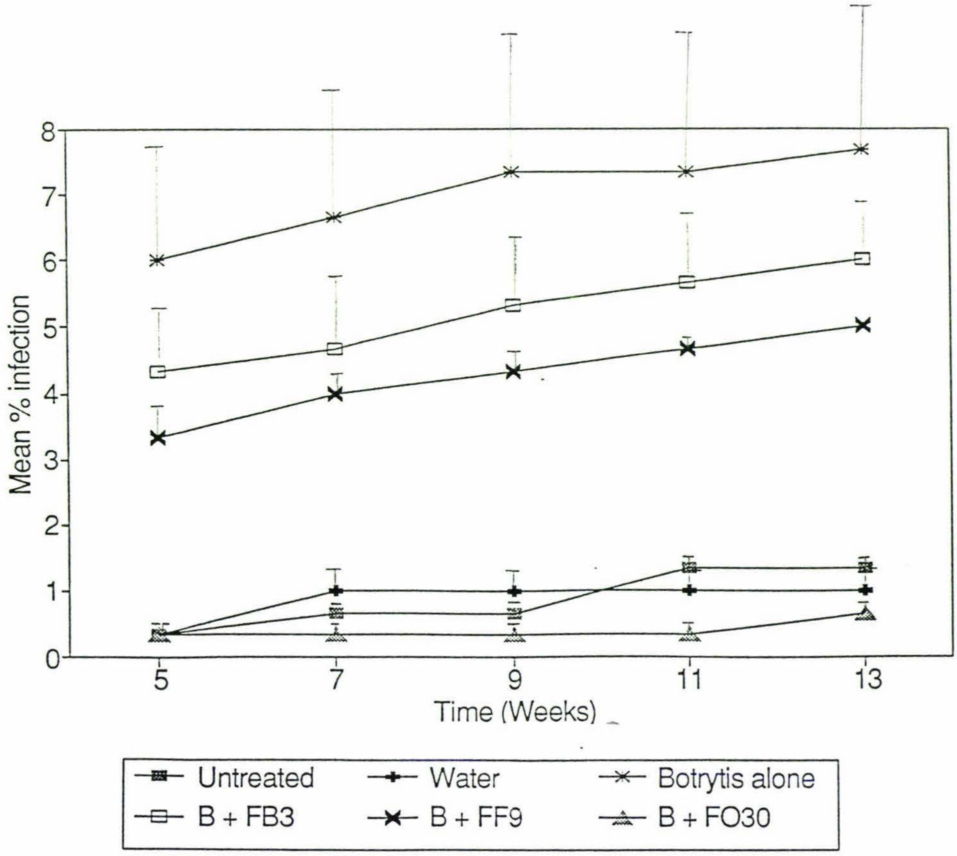
Fig 9. Healthy and diseased fruit in single trays after inoculation with various inoculum levels and subjected to (a) CP1 and (b) CP2 over 13wks storage at 0°C.

Fig 10. Number of infected fruit/99 (percentage of infection) with different inoculum levels at (a) CP1, (b) CP2, (c) CP3, (d) CP4, over 13wks at 0°C.

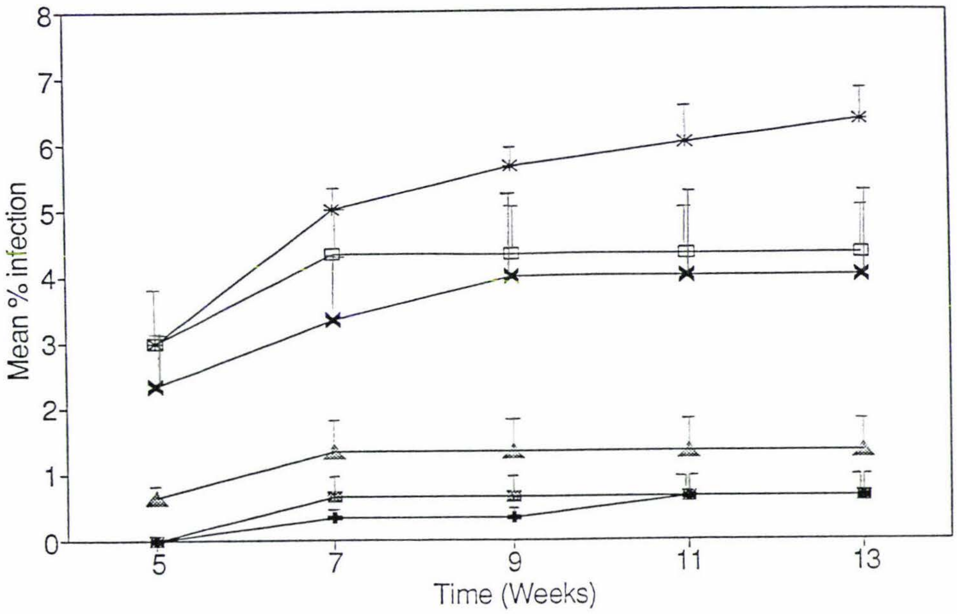
a. CP1: Fruit immediately inoculated and stored at 0°C.



b. CP2: Fruit immediately inoculated, cured 2d at ambient temperature (8-12°C) and stored at 0°C.



c. CP3: Fruit cured 2d at ambient temperature, inoculated and stored at 0°C.



d. CP4: Fruit cured 2d at ambient temperature, inoculated, cured a further 2d at ambient temperature before storage at 0°C.

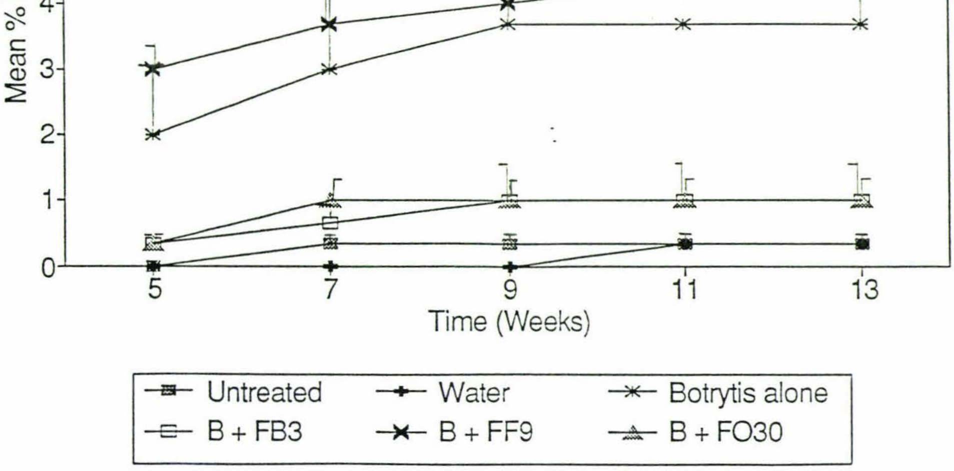


Table 11. Lsmeans and probability of significant differences in treatments with different inoculum levels on kiwifruit using four curing periods over 13 weeks in storage at 0°C.

	Inoculum levels					
Inf.fruit(wks)	Untreated	D.water	B.suspens.	B+FB3	B+FF9	B+FO30
RF5	20.3^{a}	22.2^{a}	50.7^{b}	48.5^{b}	49.2^{b}	28.0^{a}
RF7	24.0^{a}	21.5^{a}	52.0^{b}	47.2^{b}	49.5^{b}	24.5^{a}
RF9	24.3^{a}	20.4^{a}	53.6^{b}	48.0^{b}	49.1^{b}	23.3^{a}
RF11	25.2^{a}	21.8^{a}	53.2^{b}	47.7^{b}	48.7^{b}	22.1^{a}
RF13	24.8^{a}	21.5^{a}	53.3^{b}	48.2^{b}	48.2^{b}	22.8^{a}

D.water = Drop of water, B.suspens = *Botrytis* suspension.
RF5, RF7, RF9, RF11, RF13 = Rank for variables F5, F7, F9, F11, F13.
B+FB3, B+FF9, B+FO30 = *Botrytis* + either of antagonist suspension.
^{a,b} means with the same superscript are not significantly different at the 5% level.

Table 12. Lsmeans and probability of significant differences in treatments with different curing periods on kiwifruit using different inoculum levels over 13 weeks in storage at 0°C.

	Curing periods			
Inf.fruit(wks)	CP1	CP2	CP3	CP4
RF5	41.6^{b}	40.0^{b}	36.0^{b}	28.3^{a}
RF7	42.5^{b}	37.7^{b}	38.0^{b}	27.7^{a}
RF9	42.6^{b}	37.4^{b}	37.3^{b}	28.7^{a}
RF11	41.5^{b}	38.3^{b}	37.3^{b}	28.8^{a}
RF13	41.2^{b}	39.3^{b}	37.0^{b}	28.4^{a}

^{a,b} means with the same superscript are not significantly different at the 5% level.

Table 13. Lesion development (mm) of kiwifruits inoculated with different inoculum levels over 13 weeks in storage at 0°C. Fruit were separated into 3 groups based on the week in which the disease lesion was fruit detected.

	Week 5						Week 7						Week 9					
	Ctrl	In.1	In.2	In.3	In.4	In.5	Ctrl	In.1	In.2	In.3	In.4	In.5	Ctrl	In.1	In.2	In.3	In.4	In.5
Curing period 1																		
Lesion size	3.5	6.0	10.0	9.0	9.5	7.0	12.5	8.5	14.0	13.5	10.5		18.5		14.5	12.5		
Intercept	-31.4	-24.2	-18.5	-18.5	-17.0	-16.2	-35.5	-32.7	-26.5	-22.1	-30.0		-44.5		-41.4	-34.1		
SE	6.7	4.3	2.2	2.2	2.4	2.2	10.3	6.6	3.0	4.4	3.8		3.0		3.8	4.4		
Slope	7.0	6.1	5.7	5.5	5.3	4.7	6.9	5.9	5.8	5.1	5.8		7.0		6.2	5.2		
SE	0.7	0.4	0.2	0.2	0.2	0.2	1.0	0.6	0.3	0.4	0.3		0.3		0.3	0.4		
Curing period 2																		
Lesion size	3.5	10.0	11.0	12.5	10.0	4.0	21.0	14.0	9.0	8.5	17.5				18.0	14.0	11.5	
Intercept	25.9	-19.0	-17.6	-16.4	-18.2	-20.0	-24.4	-33.7	-33.5	-35.4	-25.9				-25.8	-45.3	-32.9	
SE	12.2	2.1	1.3	2.5	2.3	4.7	2.9	6.7	7.9	4.8	0.5				23.7	9.0	4.6	
Slope	6.0	5.8	5.7	5.8	5.7	4.8	6.6	6.8	6.1	6.3	6.2				4.9	6.6	5.1	
SE	1.3	0.2	0.1	0.2	0.2	0.5	0.3	0.6	0.7	0.5	0.1				2.1	0.8	0.4	
Curing period 3																		
Lesion size			7.0	8.5	8.0	10.0		8.5	15.5	8.5	9.0	9.0			15.0	26.5	24.0	
Intercept			-19.7	-19.7	-19.7	-19.0		-40.3	-25.1	-33.3	-33.4	-37.6			-38.1	-37.9	-39.6	
SE			2.6	3.2	1.9	4.2		6.3	7.3	3.6	3.4	5.8			22.7	1.7	21.5	
Slope			5.4	5.7	5.6	5.8		7.0	5.8	6.0	6.1	6.7			5.9	7.2	6.2	
SE			0.2	0.3	0.2	0.4		0.6	0.7	0.3	0.3	0.5			2.0	0.1	1.9	
Curing period 4																		
Lesion size			9.5	5.0	11.0	4.5	13.5		7.0	15.0	10.0	8.5			18.5	9.0	8.5	
Intercept			-16.9	-19.1	-16.8	-24.7	-32.7		-37.7	-29.5	-27.6	-30.3			-35.2	-48.6	-48.1	
SE			4.1	4.0	2.3	2.4	4.9		3.6	6.4	5.8	7.9			7.6	3.3	2.2	
Slope			5.3	4.9	5.6	5.9	6.6		6.4	6.0	5.4	5.6			6.0	6.4	6.3	
SE			0.4	0.4	0.2	0.2	0.5		0.3	0.6	0.5	0.7			0.7	0.3	0.2	

Ctrl = Control. In. = Inoculum level. SE = Standard error
The regresion line is linear and responded to the equation Y = Bo + B1X
Y = Lesion size, Bo = Intercept, B1 = Slope, X = Start week

7.5 DISCUSSION

Curing reduces infection of kiwifruit. This could partly due to reduced spore germination and penetration especially if fewer nutrients are available on the surface of the stem scar. The formation of protective barriers usually prevents penetration by germinating fungus spores. Physical barriers alone, are unlikely to be the sole explanation for reduced germination and penetration. The phenolic substances may be incorporated into lignified cell walls during wound healing and these substances could be toxic to the pathogen (Lakshminarayana, *et al.* 1987). The restricted hyphal growth in living stem end zone of kiwifruit tissues, could be explained by cell or cell wall components that inhibit fungal growth (Poole & Mcleod, 1992). Enhanced production of phenolic substances near wounded tissue is known to be a common response to wounding and infection. Curing may alter the environment of the stem scar so that it becomes relatively more favourable for growth of antagonists.

As kiwifruit approach maturity, they are increasingly susceptible to attack by *B.cinerea* in refrigerated storage. To achieve fruit storage life, it is essential that fruit be harvested at the correct stage of maturity and must be stored under the optimum conditions of temperature and humidity (Harman, 1979). Storage condition are planned to slow down metabolic rate of the fruit and thus extend crop life (Derbyshire, 1973). The growth of *Botrytis* and the antagonist would depend very much on climatic condition (temperature, Humidity) following inoculation and in storage conditions.

To control wound invading pathogens, effective antagonists must be able to colonize wounds and inhibit the pathogen, thus wound sites would become the battle ground for pathogens and antagonists. Many factors have to be considered in deciding whether a biological system is feasible for the control of a particular pathogen. There may be competition to establish on fresh resource, survive and produce in the presence of other organisms, beside the antagonist spore concentration could have impact on biocontrol effectiveness (Janisiewicz, 1987).

Understanding the antagonist's behaviour in different storage environments will be possible to manipulate these environments to favour the activities of the antagonist, therefore biocontrol of *B.cinerea* could be achieved or enhanced in storage by manipulating both the antagonist and the environmental parameters (Wilson & Pusey, 1985).

Evidence indicated that microbial antagonists on the stem scar may limit the success of *B.cinerea* in becoming established (Sharrock & Hallett, 1992), but it is important to emphasize that once *B.cinerea* is established, it begins to invade the healthy tissues rapidly and gain a marked competitive advantage over microorganisms limited to these healthy tissues (Wood, 1951).

The lesion development on fruit surface was similar with all inoculum levels used, which could be explained by the weakness of the antagonist activity or defeat of the antagonist by *B.cinerea* in a particular stage of fruit maturity (Table 13). A large proportion of fruit that carried *Botrytis* spores on their stem scars did not develop stem-end rots, and complete inhibition of soft rot did not occur at all.

The presence of water together with nutrient exuded within the fruit may have caused the germination of FO30.
Of the three biocontrol isolates tested against *B.cinerea*, only one have reduced significantly the percentage of infected fruit (Table 11). The performed *Fusarium* Isolate FO30 *in vitro* have shown its ability to reduce the percentage of *Botrytis* infection *in vivo*, and this study demonstrated that an isolate of *Fusarium sp* (FO30) got the potential as a biocontrol agent to *Botrytis* storage rot of kiwifruit. By using biocontrol agent ***Fusarium*** isolate FO30, the severity of soft rot of kiwifruit caused by *B.cinerea* have reduced by 8%-21% during storage at 0°C over 13 weeks using various curing periods (Table 10, Appendix 3: Table 1a, b, c, d).
The mode of FO30 activity on fruit could be toward *Botrytis* spore germination or early germ tube development with less minimal effect on subsequent fungal growth. Therefore, it can be concluded that a biocontrol agent can be used to reduce fruit rot on kiwifruit, and *Botrytis* can evidently be inhibited and competed by other microorganisms present on the picking wounds when incubated at room temperature and storage. Control of *Botrytis* rot with the isolate FO30 was demonstrated to be a function of challenge spore concentration of *B.cinerea* and *Fusarium* spore concentration, thus increasing the FO30 spore concentration could improve biocontrol of *B.cinerea*.

Although *Phoma* isolate FF9 was highly antagonistic to *B.cinerea* on agar petri dish, it showed no antagonism at all on the fruit surface. The yeast isolate FB3 showed little promise in either the *in vivo* tests or *in vitro* tests.
It appears therefore, that the *in vitro* tests is not a good guide to the efficiency of an antagonist *in vivo*.

Staining *Botrytis* and antagonists

CHAPTER EIGHT

STAINING SPORES AND MYCELIUM OF *B.cinerea* ANTAGONISTS ON AGAR MEDIA AND IN FRUIT TISSUES

8.1 INTRODUCTION

Microscopically, *Botrytis* septate mycelium with its branches and spores on PDA medium and kiwifruit tissue are well developed and well coloured.
Stained microscopic objects (plant tissue/micro-organisms) are more clearly visible than unstained (Conn's, 1940). Some dyes are selective in their staining of different structures, so it is possible to differentially stain different structures or organisms.
Combinations of stains have been employed for highly specialized purposes, such as staining fungi in tissues, and numerous dyes such as thionine, acid fuchsin, orange G, carmine and others have been tested for such purposes (Conn's, 1940).
As a general rule, the thicker the sections, the more general the stain, and the more intense stain, the more difficult to see details. Different objects in a preparation may take up different amount of the same dye, and different dyes (for instance: a typical basic or a typical acid dye) may attach themselves differently to the same object, therefore it can be said, one dye may dye the object deeply, another slightly or not at all (Baker, 1958).

In this study, several stains were used on 5% V.8 medium and on fruit tissue to evaluate the best stain that coloured clearly and homogenously *B.cinerea* and antagonists propagules.

8.2 OBJECTIVES

To observe and to distinguish the best stain for *B.cinerea* and the antagonists on medium, kiwifruit tissue, cross and on longitudinal section of the stem scar.

8.3 MATERIALS AND METHODS

8.3.1 Stain on fruit tissue

A $2cm^2$ portion of a 2 week old *B.cinerea* culture on PDA was shaken in 10ml SDW in a McCartney bottle for 2 minutes, filtered through sterilized glass wool, measured and adjusted to $1x10^6$ spore/ml by using haemocytometer. Colonies of 2 week old culture of each of isolates FF9 (*Phoma sp*), FO30 (*Fusarium merismoides*), and FB3 (*Rhodotorula sp*) on PDA were shaken in the same way as the *Botrytis* culture, measured and adjusted to $1x10^6$ spore/ml for FF9 and $4x10^6$ spore/ml for FO30 and FB3.
Twenty slides within humid glass plates were sterilized for 15min at 15 lb in an autoclave. A very thin section of outer pericarp was taken from the surface sterilized kiwifruit with 20% dilution of janola for 20min. Two sections of the tissue were placed on each slide, and slides were placed in pairs on bent class tube in a sterilized glass plate. A 10µl drop of *B.cinerea* suspension containing 10.000 spores of ($1x10^6$ spore/ml) was used in the inoculation of each of 10 tissue sections. A mixture of 10µl of *Botrytis* and one of each isolate suspensions was used in the inoculation of each of 10 tissue sections. After the inoculation of 40 tissue sections on glass slides in the sterilized glass plates, the glass plates were incubated at ambient temperature (20°C ± 1°C) for 3 days. The tissue sections were then mounted with 10 different stains.

The stains were :
Sudan 1V, 0.1% trypan blue in lactophenol, 0.1% thionine in 5% phenol, lactophenol acid fuchsin, gram's iodine, trypan blue in 0.85% saline, 0.5% phloxine in 0.8% NaCl, luxol brilliant green BL, acridine orange 0.01g/10ml, lactophenol cotton blue.

The tissue sections and the coloured mycelium with spores/cells of *B.cinerea* and the antagonists FF9, FO30, FB3, were observed under a compound microscope.

8.3.2 Stain on cross and longitudinal fruit stem scar section

kiwifruits (40 fruits) from a Wanganui orchard stored for two months at 5°C were used in this test. A concentration of *Botrytis* spore suspension, measured and adjusted to $2x10^6$ spore/ml was used in the inoculation of stem scar fresh surface.

8.3.2.1 Cross section

Fruits with fresh stem scars were inoculated immediately with *B.cinerea* spore suspension and left for 2, 24 or 72hr.
Two thin layers of the new healing surface cut with sterilized scalpel were placed on a slide.

8.3.2.2 Longitudinal section

Fruits with fresh stem scars inoculated immediately with *B.cinerea* spore suspension were left for 24 or 72hr.

The picking scar including underlying tissue was excised and halved longitudinally to about 1mm thick sections using a sterilized scalpel. The cross and longitudinal layers were mounted with one of five biological stains (sudan 4, lactophenol acid fuchsin, 0.5% phloxine in 0.8% NaCl, lactophenol cotton blue, and chlorazol black).

For chlorazol black stain, the layer of kiwifruit tissue was stained overnight in 0.1% chorazol black in chloral hydrate, rapidly washed in 50% ethanol until the black colour was removed as seen with the naked eye. The scar section was cleared for 24hr in saturated chloral hydrate. This was poured off and the section washed 3 times with 70% chloral hydrate. It was left in 70% chloral hydrate overnight.

Cross and longitudinal sections bearing *B.cinerea* spores were observed under the compound microscope and were photographed.

B.cinerea and the antagonists FB3, FF9, FO30 were also stained on 5% V.8 medium using the 10 stains listed above.

8.4 RESULTS

Spores were observed microscopically in stem end scar that have been inoculated after healing for different periods, and the spores were laid freely in the scar. No germination was found after 2hr and 72hr of the inoculation of *Botrytis* suspension on picking wound, but it was clear with distinctive mycelium and vigorous growth on outer pericarp fruit tissue.

Many stains have coloured the fungal hyphae and the spores clearly, beside the ability of the stain to prevent the growth of the spores. The distinctive and coloured spores with the mycelium on the pericarp tissue were shown in (Fig 14).

On 5% V.8 medium, the stains 0.1% thionine in 5% phenol and Lactophenol acid fuchsin were better than on tissue section (Table 14, and Fig 12c, Fig 13c). However, Lactophenol acid fuchsin was not bad on longitudinal section (Fig 13c). Thionine coloured yeast cells and *Fusarium* macroconidia on 5% V.8 medium discs with a light colour and their mycelium with a very light colour (Fig 11d, 11e). The stain 0.5% Phloxine in 0.8 NaCl stained *B.cinerea*, FB3 and FO30 on the 5% V.8 medium or on tissue surface slightly, but did not stain FF9 (Table 14). Sudan 1V gave some staining of the fungi on agar medium but only lightly stained them on kiwifruit tissue (Table 14). The spores and mycelium of *B.cinerea*, FB3, FF9 and FO30 were clearly stained with Lactophenol cotton blue on 5% V.8 medium, cross and longitudinal sections and were able to be seen under the compound microscope as circular, green coloured spores (Fig 1b, Fig 2b, Fig 3b, Fig 11c, Fig 14b, Table 14). Chlorazol black stained the spores and mycelium of *B.cinerea* on the cross, longitudinal sections and on tissue better than other stains (Fig 12a, 12b, Fig 13a, 13b, and Fig 14a).

Generally, stains such as gram's iodine, trypan blue in 0.85% saline, luxol brilliant green blue and acridine orange 0.01g/10ml (0.1%) did not shown any promise because of lightly colour of gram's iodine and sharply colour of the others, which referred to tissue-constituents response to stain's ions (Fig 11a, 11b, and Table 14).

Fig 11. (a) *B.cinerea* and (b) *Phoma* FF9 stained with Gram's iodine, (c) *B.cinerea* stained with Lactophenol cotton blue, (d) Yeast FB3 and (e) *Fusarium* FO30 stained with Thionine in 5% phenol, on 5% V.8 medium disc.

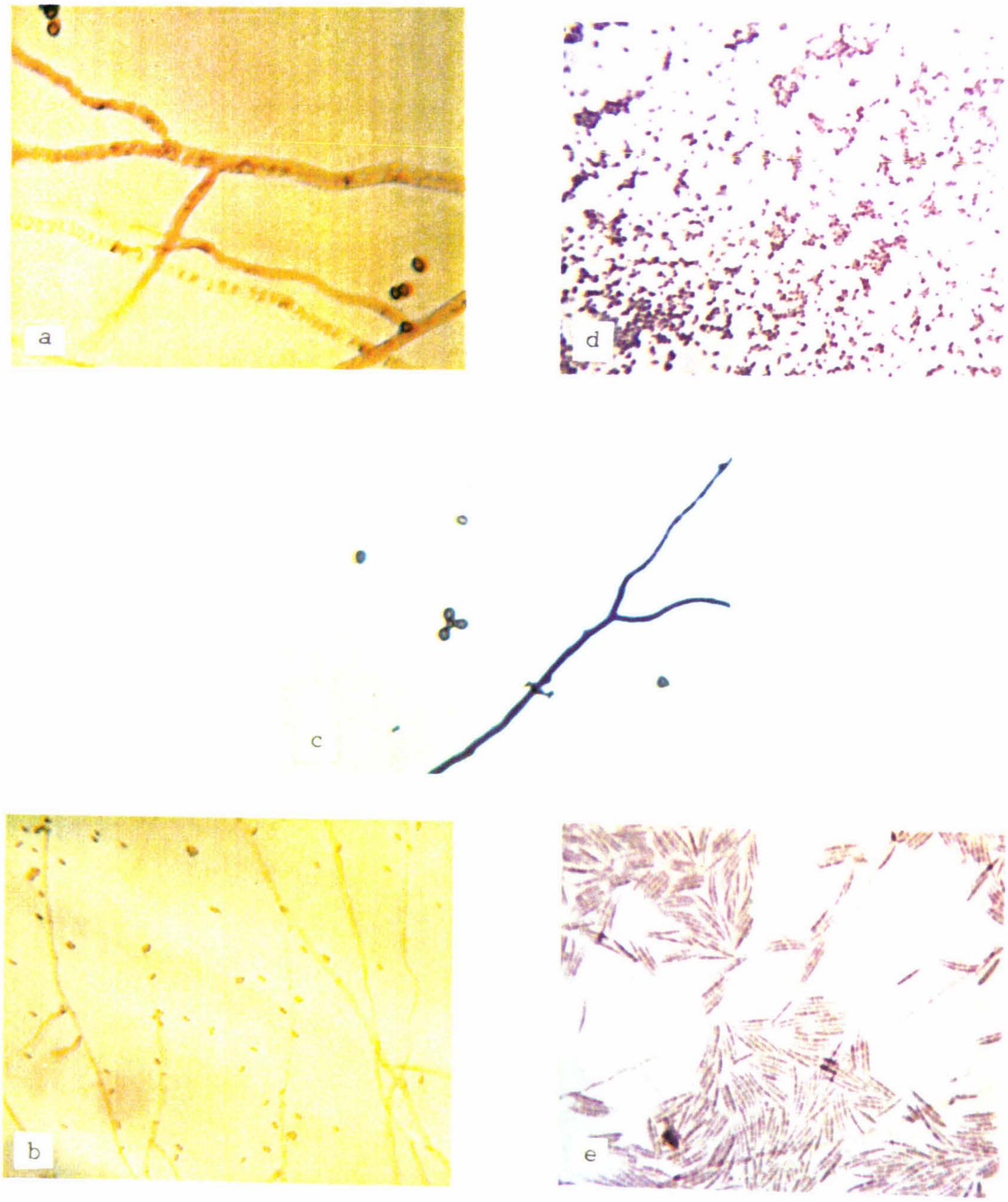


Fig 12. *B.cinerea*: cross section of fruit stem scar showing spores stained with (a) Chlorazol black after clearing with chloral hydrate, 24hr, (b) Chlorazol black after clearing with chloral hydrate, 72hr, and (c) Lactophenol acid fuchsin, 24hr, after the inoculation with *B.cinerea* suspension.

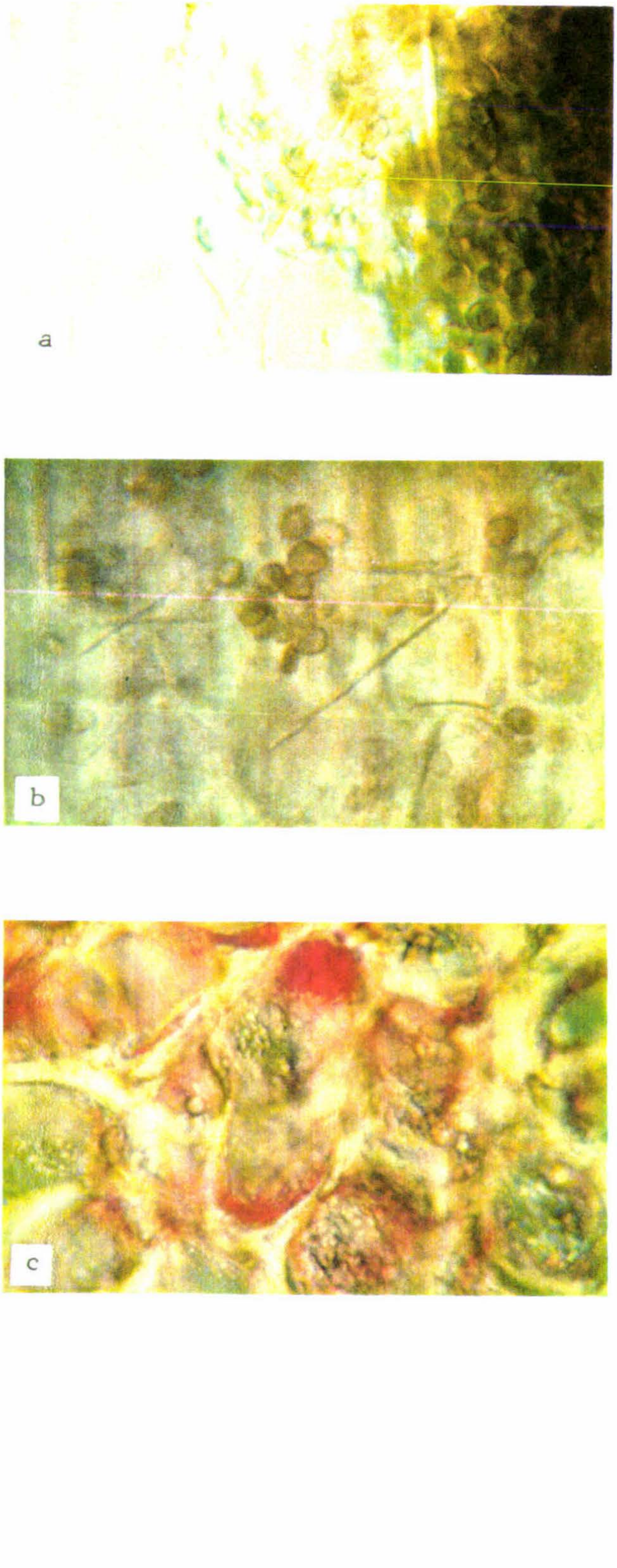


Fig 13. *B.cinerea*: longitudinal section of fruit stem scar showing spores stained with (a) Chlorazol black after clearing with chloral hydrate, 2hr, (decayed fruit), (b) Chlorazol black after clearing with chloral hydrate, 1 month, (healthy fruit), and (c) Lactophenol acid fuchsin, 72hr, after the inoculation with *B.cinerea* suspension.

Fig 14. *B.cinerea*: cross tissue section of fruit outer pericarp showing spores and mycelium stained with (a) Chlorazol black after clearing with chloral hydrate, 72hr, and (b) Lactophenol cotton blue, 24hr, after the inoculation with *B.cinerea* suspension.

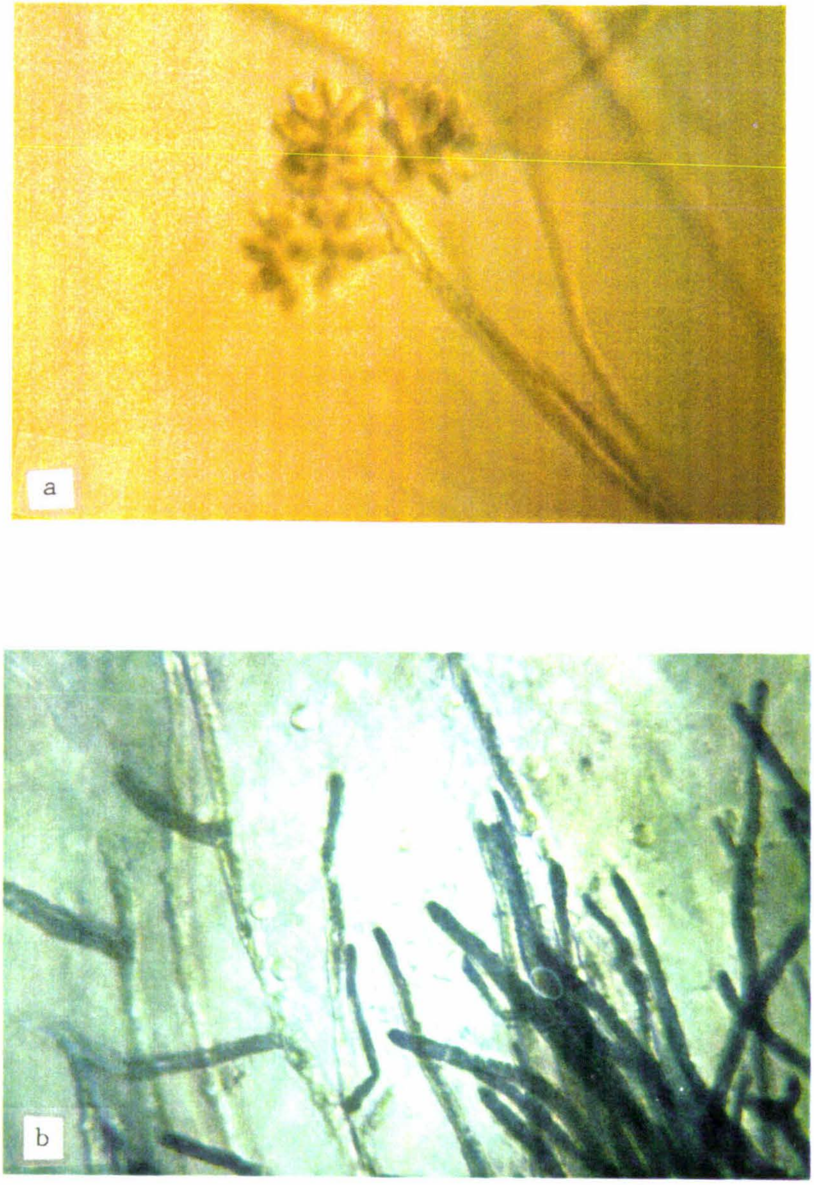


Table 14. Clarity of *B.cinerea* and the antagonist spores on 5% V.8 medium and within kiwifruit tissues after treatment with various stains.

Stains	B.Suspens.		FB3 suspens.		FF9 suspens.		FO30 suspens.	
	media	tissue	media	tissue	media	tissue	media	tissue
1. Sudan 1V	2	2	4	4	4	4	4	4
2. 0.1% trypan blue in lactoph.	2	2	2	3	2	3	3	3
3. 0.1% thionine in 5% phenol.	2	3	2	4	1	4	2	3
4. Lactophenol acid fuchsin.	2	2	4	3	2	3	3	4
5. Gram's iodine	3	3	2	4	3	4	3	4
6. Trypan blue in 0.85% saline	2	4	4	4	4	4	4	4
7. 0.5% phloxine in 0.8% NaCl	4	2	4	3	4	4	4	3
8. Luxol brilliant green BL	4	4	3	2	4	4	4	3
9. Acridine orange 0.01g/10ml	3	4	4	4	4	4	4	3
10. Lactophenol cotton blue	2	2	1	2	1	3	2	3

B.suspen. = Botrytis suspension. FB3 suspens. = FB3 suspension

Scale 1=excellent
2=good
3=moderate
4=poor

8.5 DISCUSSION

The stain on germinating spores of *B.cinerea* and the antagonists were visible on medium but difficult to observe on the tissue because of sharp staining of the tissue-constituents and the thickness of the sections.
Intensity of the staining depends on the chemical affinity between the object and the dye, the density of the object, and the permeability of the object to the dye. The matter of coloration is complicated because these factors (affinity, density, permeability) may either act together or antagonize one another. The distribution of the dye depends on the ability of the dye to penetrate the plant tissues.
There may be chemical or physical reasons for the stronger coloration of particular object, and the object may be dyed strongly either because it possesses many chemical group capable of reacting with the particular dye used, or because there is a lot of colourable matter in it per unit volume, or because it is easily permeable by the dye used, while other tissue-constituents are more difficult to penetrate (Baker, 1958).

Dyes such as Chlorazol black and Lactophenol cotton blue were satisfactory for staining the spores in stem scar sections than some dyes such as Gram's iodine and Trypan blue that were not useful for this purpose, because of the combination of *Botrytis* spores or the well-coloured tissue-constituents with the coloured ions of the dye. Dyes and tissue-constituents are electrically charged, therefore it is natural that they react with one another. The acidic constituents have an affinity for basic dye ions, while the basic constituents attract acid dye ions. The negative charged substances are generally dyed by acid dyes (Baker, 1958). Conn's (1940) indicated, that cotton blue is a strongly acidic dye and is successfully used in staining unfixed tissues. And because spores were well stained by acidic dye such as lactophenol cotton blue on tissues and moderate stained by other acidic dyes such as lactophenol acid fuchsin, and phloxine, but slightly stained by a weak acidic dye such as sudan 1V, then it can be said that *Botrytis* spores could have been negative charged, but the spores were well stained on 5% V.8 medium by a strongly basic dye such as thionine and poorly stained by other basic dye such as luxol brilliant green. As a general rule, acid dyes are used to stain cytoplasm, and basic dyes to stain chromatin. However, cytoplasm can be stained with basic dyes if time is allowed, and acid dyes can stain chromatin unless it has been prestained by a basic dye (Baker, 1955).

Chlorazol black gave promise as an excellent spore stain. The kiwifruit tissue sections can be progressively destained by immersion in chloral hydrate and by one month were colourless, but spores could be seen clearly (Fig 13b). Washed material of tissue sections kept more than 2 days in 70% alcohol, become brittle.
Since most tissue-constituents will eventually be coloured to some extent by any dye, it may be desirable to limit the period of dyeing, thus some of them will appear colourless when others stained quite strongly (Baker, 1958). Therefore destaining should be carefully timed in overstaining procedure. Too little destaining leave the entire section too dark with little differences between

structures. Too much destaining and all structures including spores and hyphae lose their stain and become colourless.

CHAPTER NINE

GENERAL CONCLUSIONS

The nature of the pathogen is probably the principal key factor determining the development of successful biocontrol. Ways in which biocontrol agents work are antibiosis, mycoparasitism and competition. The condition for maximum disease reduction vary for each individual antagonist, and a combination of two or more modes of action may be involved in the control of a certain disease. Success in the future will probably result from the use of a combination of antagonists that could effectively inhibit the target organism during different phases of its life cycle and changing environmental conditions (Schroth & Hancock, 1981). But it is unlikely that a perfect antagonist for the control of a particular pathogen will be found in nature (Blakeman & Fokkema, 1982). In the field, saprophytic colonization of host substrate by biocontrol agents artificially introduced into the environment could be very difficult. The problem is to find out how, when and under what conditions the selected antagonists work, therefore the application of a biocontrol agent should have to be very carefully timed (Campbell, 1989). By understanding the mode of action, manipulating the agent and the environment to the maximum benefit, biocontrol processes should be elucidated (Scher & Castagno, 1986).

The agar plate test is useful to observe antibiosis and mycoparasitism and the range of temperatures that biocontrol agent can grow and reproduce, but the medium itself can not be considered the most suitable substrate to study biocontrol agent behaviour. Natural biocontrol agents against *B.cinerea* were obtained from the field but not from stored fruit. All the potential biocontrol agents, among them the tested isolates FF9, FO30, and FB3, produced antibiotics that retarded the growth of ***B.cinerea*** at some stage *in vitro*. However, an organism showing strong antagonism against another on agar does not necessarily behave similarly under natural conditions because of environmental, nutritional, and chemical changes which stimulate nutrient competition and production of antibiotics differently.

The antagonists FB3, FF9 and FO30 did not stop the growth of *B.cinerea* on different media or on the same medium with different pH (4.5, 5.0, 5.6, 6.2), and this indicated that *B.cinerea* grew independently of medium pH (Table 7, 8). Newhook (1951), in his work indicated that when medium pH reached 8-8.5, it become inhibitory to growth of ***B.cinerea*** and to the activity of pectinase production. Also it can be suggested that production of antibiotic substances by the antagonist could be either insufficient or not active against *B.cinerea* growth. Incubation temperature had a greater effect than choice of medium because temperature affects growth rate and survival of hyphae and propagules (Stott, 1971) more than what medium do.

The spore germination test is useful to observe the germination of the test pathogen and biocontrol agent together on agar. *Fusarium* isolate FO30 reduced the percentage spore germination of *B.cinerea* on 2.5% V.8 medium by 90% at 0°C for 72hr (Table 9). Therefore *Fusarium* have proved as an effective antagonist to *B.cinerea* by producing antibiotics and reducing the germination of *B.cinerea* spores *in vitro*. Although there was no direct evidence that antibiotics played a role *in vivo*, antibiotic production was considered to be the main cause of antagonism *in vitro* (Fokkema, 1976), because *in vitro*-antibiotic can be detected and can be observed the growth of *B.cinerea* and antagonist-specific activity in reducing germination and sporulation of *B.cinerea*, but *in vivo* the microbial antibiotics are difficult to detect and are possibly broken down by the enzymes of the plant host.

Inoculum of antagonist FO30 (*Fusarium merismoides*) on stem scars reduced the percentage of stem end rot caused by *B.cinerea* by 17-21% (Table 10 and Fig 10). Therefore it can be said that *Fusarium* could have synthesized materials that are inhibitory to *Botrytis*.

Not one of the inoculum level of biocontrol agent have stopped *B.cinerea* from its incidence on the stored fruit completely, however effectiveness of biocontrol agents is closely related to *Botrytis* spore concentration, the sequence of application and the number of spores (e.g *Fusarium sp*) applied to the picking wound which could be important factors in suppression *Botrytis* storage rot in the detached wound site of kiwifruit. Therefore the feasibility of using the biocontrol agent FO30 to control *B.cinerea* on kiwifruit stored at low temperature needs further work.

The use of *Fusarium*, or *F.merismoides* isolated from kiwifruit old leaves for biocontrol of *B.cinerea* was first reported in this work, although many reports have mentioned the use of *Fusarium* as potential antagonist against *B.cinerea* on other horticultural products. Wood (1951), indicated that *Fusarium spp* isolate no.1 have reduced *Botrytis* rot caused by *B.cinerea* on lettuce leaves at 5°C significantly by 80% when the inoculum was used simultaneously (*Fusarium* + *Botrytis*), and by 100% when *Fusarium spp* was 3 days preinoculated, and in both situations the sporulation was completely inhibited. In glasshouse conditions, an unidentified species of *Fusarium* applied with other fungi such as *Phoma*, *Trichoderma* and *Penicillium* to lettuce leaf lesion has prevented colonization of *Botrytis*, and *Fusarium* was the most effective fungal antagonist when applied together with nutrient before *Botrytis* on leaf material (Fokkema, 1976). Also under glasshouse conditions, an unidentified species of *Fusarium* among several fungi (*Aureobasidium pullulans, Alternaria tenussima, Cladosporium, Phoma*) have reduced the number of *Alternaria zinniae* (Heuvel, 1970).

Campbell (1989), indicated that inoculation of plant host prior to planting with avirulent *Fusarium oxysporum* have reduce the amount of wilt at later stage of growth, which could be linked to competition or with the triggering of the host defence mechanisms. Carter & Price (1974), showed that *Eutypa armeniacae* was controlled on apricot trees in south Australia by inoculating the fresh wounds with a spore suspension of *Fusarium lateritium* in 0.3ppm of benomyl. The benomyl inhibited *Eutypa* until the *Fusarium* which is insensitive to benomyl was sufficiently established to provide long-term protection. *Fusarium* wilt of sweet potato controlled in Japan by inoculating cuttings prior to planting with a non-pathogenic *F.oxysporum* originally isolated from a healthy plant (Baker, 1987). *Fusarium* was recorded as pathogenic on particular hosts (Dickinson, 1976), and *Fusarium* are known to be little affected by antibiotics (Campbell, 1989). However, in order to have success in using the biocontrol agent, the antagonist or the hyperparasite should be placed on the infection site before or with the pathogen.

In vivo test indicated that the physiology/chemistry of the fruit could play a major role in the fruit defence mechanism, therefore it is more preferable to encourage the breeding and propagation of kiwifruit plants to get fruit with highly effective defence mechanisms to disease than to look for a method to control the disease or decrease it.

Biological control is making a good start, but it needs more research in development programmes. Biocontrol should take less time and cost less money than the present sophisticated chemical control systems to achieve acceptable commercial control of disease.
From the scientific viewpoint, it should be a move ecologically sound process which results in less contamination of the environment by toxic chemicals.

For staining procedures, Chlorazol black in chloral hydrate gave the best result in staining *B.cinerea* on cross and longitudinal sections of the stem scar and pericarp tissue of the fruit (Fig 12a,b, 13a,b, 14a). This conclusion can be confirmed by the work of Sharrock and Hallett (1992), in which they successfully stained *B.cinerea* spores in kiwifruit tissues using the same stain, but in my work the germ tubes or hyphae were not clearly stained, possibly because of the thickness of the tissue sections.

FUTURE WORK

CHAPTER TEN

FUTURE WORK

This study concluded that *Fusarium merismoides* isolate FO30 reduced *Botrytis* rot on kiwifruit in coolstore by 21%.

In the immediate term the following research is required:

1. Repeat research on using isolate FO30 to ascertain the effect of various temperatures and relative humidities during the curing period before placing the fruit in the coolstore at 0°C, and inoculation of the fruit by biocontrol agent before or after the curing period to maximize biocontrol process.

2. Biocontrol reduction of *Botrytis* rot by inoculation of the fruit stem end with a suspension of isolate FO30 at different concentrations with various aqueous concentrations of inorganic salt solutions such as $CaCl_2$, KCL, CaCo3, $Ca(No3)_2$, individually or mixed together. Such a process may strengthen the potential of the antagonist and improve the efficacy of the biocontrol method. Mclaughlin, *et al* (1990), have used such inorganic salt to enhance *Candida sp.* to control *B.cinerea* and *P.expansum* on harvested apples.

3. The isolate BF21 of bacterium *Xanthomonas sp.* has given potential antagonistic activity to *B.cinerea* on PDA medium at 0°C, therefore it could be worth testing *in vivo*.

4. To avoid antibiotic accumulation in fruit, it is preferable to search for biocontrol agent that compete for nutrients on/in fruit rather than those produce antibiotic, and such work will give a crucial advance in biocontrol of *B.cinerea* on kiwifruit.

In the long term, the following research is required:

Enhancement of *F.merismoides* isolate FO30 or *Xanthomonas sp.* isolate BF21, by recombination of the genetical structures using gene manipulation procedures, UV light or chemicals *in vitro* and *in vivo*.

GLOSSARY

cf.(Latin confer), means compare the definition of the word indicated with the definition being considered.
q.v.(Latin quod vide), means that the word is defined elsewhere in the glossary.

antagonist (hence **antagonism**) An organism exerting a damaging effect another: e.g. by the production of lytic enzymes (*cf.* lysis) or antibiotics *q.v.*, or by competition *q.v.* *cf.* specific and general antagonism.
antibiotic A substance produced by a micro-organism which is damaging to another at low concentrations (µg/ml). *cf.* toxin, bacteriocin.
avirulence The lack of ability to cause disease. *cf.* virulence. A strain *q.v.* of a pathogen *q.v.* may be avirulent, even though the species or genus as a whole are pathogens.
biotroph A pathogen that requires living host tissue for its nutrition. *cf.* necrotroph.
canker An area of necrotic *q.v.* tissue, especially in a stem, caused by a pathogen. There may be disturbance of the growth of the stem producing swelling around the dead tissue.
community The micro-organisms, of several different taxa, which live together in a habitat *q.v.* A collection of populations *q.v.*
competition An interaction between organisms which both need a limited resource e.g. a food supply, so that the preferential use of it by one organism harms the other or reduces its growth rate.
competitive saprophytic (saprotrophic) ability The capacity of a pathogen to compete with saprotrophs *q.v.* during a stage in its life cycle when it is not being pathogenic.
cultivar A subdivision of a species, usually of a plant, which has been produced by an artificial breeding programme on a plant in cultivation. *cf.* variety.
ecosystem Ecological system formed by the interaction of coating organisms and their environment.
environment All the biotic and abiotic factors which make up the surroundings of an organism, hence **micro-environment.**
epidemiology The study of the spread of disease, particularly of animals. The spread of a plant disease should be called an epiphytotic (equivalent to epidemic), but the terminology is little used.
formulation The mixture, and means of preparing it, by which an antagonist (or chemical) is packaged, stored and delivered to the point of use. It may include a food base, wetting agents, inert diluting agents, water binding or desiccating agents, etc. as well as the active ingredient or actual micro-organisms.
fungicide A chemical which kills or harms a fungus. *cf.* pesticide.
fungistasis The prevention of fungal growth, mainly by carbon limitation. The production of germination inhibitors by other organisms, or by the organism itself, may also be included.

genetic engineering The artificial manipulation of the genome of an organism to produce strains *q.v.* or cultivars *q.v.* with characters not previously occurring or not occurring in that combination before. It especially refers to the recent ability to change genomes by the transfer of DNA.

habitat The place where an organism lives. *cf.* niche.

hyperparasitism See mycoparasitism.

immune Complete freedom from infection by a pathogen because of some characteristic of the host, hence **immunity**. *cf.* resistance.

in vitro A test or procedure carried out (literally 'in glass') in laboratory conditions: e.g. testing an antagonist in a petri dish against a culture of the pathogen.

in vivo A test or procedure carried out with live host material: e.g. testing an antagonist against a pathogen that is growing on or in its living host.

induced resistance A form of cross-protection *q.v.* in which the increased resistance of the host is caused by stimulation of the host defence systems after inoculation with an isolate of the pathogen which is avirulent *q.v.* or which does not cause disease on that host: e.g. a different forma specialise *q.v.*

inoculate Introduce a living organism (**the inoculum**) into a culture medium or the environment.

inoculum (inoculant) An organism introduced into an environment, usually after being produced artificially in the laboratory, to carry out some special function: e.g. disease control, plant growth promotion, straw decomposition, etc.

integrated control A combination of chemical, biological and agricultural practices which together control a disease. An overall approach to disease control.

lysis The rupture or dissolution of a cell, hence **lytic**. **Endolysis** (= autolysis) is the breakdown of the cell by its own enzymes following death which may be caused by starvation, antibiotics etc. **Exolysis** (= heterolysis) is the destruction of the cell by enzymes of another organism: e.g. in mycoparasitism *q.v.*

microclimate The climate in a very small habitat or area.

microhabitat A small habitat *q.v.*, often only micrometres in dimensions, where a micro-organism lives.

mycoparasitism The parasitism of one fungus by another (= hyperparasitism).

mycotoxins Toxins *q.v.* produced by fungi, usually growing on seeds and fruits. Animals which subsequently eat the seeds are affected by the toxins.

necrotroph A pathogen which kills host cells and then lives on the dead tissue. *cf.* biotroph.

niche The sum of all the physiological properties of an organism, of the environment and its exploitable resources which together define what a microbe does in that habitat *q.v.* **Fundamental niche**: the niche which an organism is inherently capable of utilizing. **Realized niche**: the actual niche available because of biotic or environmental limitations.

nutrient limitation The normal condition in most natural habitats (soil, leaves, plant stems, etc) in which microbial growth is restricted or stopped by a lack of available nutrients.

parasite An organism living on or in another living organism (the host) from which it obtains its food. If it harms the host in the process (cause a disease) it is a pathogen *q.v.*, but not all parasites are pathogens. See symbiosis.

Pathogen An organism capable of causing disease in a plant, animal or micro-organism. *cf.* parasite. It may apply in general to genera, species or strains, though particular isolates of the species or strain may vary in virulence *q.v.*

pathogenicity The tendency of member of a group of organisms known to be pathogens *q.v.* to actually cause disease. *cf.* virulence, resistance.

phyllosphere The environment around a leaf which is influenced by that leaf. Hence **phylloplane,** the actual leaf surface. *cf.* rhizosphere.

population A group of similar organisms, usually a single species if this can be defined, that live in the same habitat (= a colony). Many different populations make up a community *q.v.*

predation A form of symbiosis *q.v.* in which one organism eats another, hence **predator.**

resistance To disease: the ability of a host to overcome, to some extent anyway, the invasion of or symptoms produced by a pathogen *q.v.* *cf.* virulence, immunity.

saprophyte An organism (plant or microbe) which lives on dead organic material.

saprotroph An organism which depends on non-living material for its nutrients.

sclerotium A resting structure of fungi composed of a mass of hyphae, usually surrounded by a waterproof layer with thickened hyphal walls.

siderophore A chemical, produced by an organism, which binds cations, especially Fe^{3+} and helps to transport it into the organism in iron limited environments.

specific antagonism Reduction in disease or in pathogen inoculum due to the activity of a particular species or strain of micro-organism. *cf.* general antagonism.

strain A group of similar, characterized, isolates *q.v.* of a micro-organism, essentially this applies to laboratory isolates, cultures or selections. *cf.* race, variety, forma specialise.

strategy The general way in which a micro-organism lives; the strategy may be to produce many spores so that there is always one ready should a food source arrive, or to use a food that no other organism has the enzymes to degrade etc. *cf.* r-strategist, k-strategist, ruderal, stress tolerant, combative.

stress tolerant Organisms which persist in stressed environments: e.g. low food, low water, high temperature.

symbiosis The living together of two or more organisms, regardless of the nature of the interdependence, hence **symbiotic.** *cf.* mutualism, amensalism, commensalism, neutralism, parasite, pathogen, predator, synergism.

toxin Any substance, usually organic, which is harmful to an organism, a poison. *cf.* antibiotic.

virulence The relative capacity of a pathogen to attack a host. A pathogen *q.v.* is virulent if it causes disease and overcomes host resistance *q.v.* *cf.* avirulent.

wild type The organism as growing in the natural environment, i.e. before laboratory culture, selection, mutation, etc.

REFERENCES

References

Adams, P. B. 1990: The potential of mycoparasites for biological control of plants diseases. *Annual review of phytopathology 28*:59-72.

Ale-Agha, N., Dubos, B., Grosclaude, C., and Ricard, J. L. 1974: Antagonism between nongerminated spores of *Trichoderma viride* and *Botrytis cinerea, Monilia laxa, M.fructigena,* and *Phomopsis viticola. Plant disease reporter 58*:915-917.

Andrew, J. H. 1985: Strategies for selecting antagonistic microorganisms from the phylloplane *in*: *Biological control on the phylloplane*, Windels, C. E. and Lindow, S. E., eds. st Paul: The American phytopathology society. PP 31-44.

Aronson, J. M. 1981: Cell wall chemistry, ultrastructure and metabolism *in*: *Biology of conidial fungi*, Vol.2, Cole, G. T. and Kendrick, B., eds. PP 459-507.

Baker, J. R. 1955: staining *in*: *Cytological technique*. PP 128-172.

Baker, J. R. 1958: Principles of biological microtechnique. PP 187-228.

Baker, K. F. 1987: Evolving concepts of biological control of plant pathogens. *Annual review of phytopathology 25*:67-5.

Baker, K. F., and Cook, R. J. 1982: Biological control of plant pathogens. American phytopathological society. St. Paul, MN. PP 433.

Baker, R. 1968: Mechanisms of biological control of soil-borne pathogens. *Annual review of phytopathology 6*:263-294.

Baker, R. T. 1985: Biological control of plant pathogens: Definitions *in*: *Biological control in agriculture* IPM system, Hoy, M. A. and Herzog, D. C., eds. Florida: Academic press. PP 25-39

Barnett, H. L., and Lilly, V. G. 1962: A destructive mycoparasite, *Gliocladium roseum. Mycologia 54*:72-77.

Barnett, J. A., Payne, R. W., and Yarrow, D. 1990: Yeasts: characteristics and identification, Chapter 5, 6, 7, 2nd ed.

Beever, D. J., McGrath, H. J. W., Clarke, D. L., and Todd, M. 1984: Field application and residues of fungicides for the control of *Botrytis* storage rot of kiwifruit. *New Zealand journal of experimental agriculture 12*:339-346.

Bhatt, D. D., and Vaughan, E, K. 1962: Preliminary investigations on biological control of grey mold (*Botrytis cinerea*) of strawberries. *Plant disease reporter 46(5)*:342-345.

Bisiach, M., Minervini, G., Vercesi, A., and Zerbetto, F. 1985: Research on protection against *Botrytis* in viticulture using microbial competitors. *Difesa delle piante 8*:429-439.

Blakeman, J. P. 1972: Effect of plant age on inhibition of *Botrytis cinerea* spores by bacteria on beetroot leaves. *Physiological plant pathology 2*:143-152.

Blakeman, J. P. 1975: Germination of *Botrytis cinerea* conidia in relation to nutrient conditions on leaf surfaces. *Transactions of the british mycological society 65*:239-247.

Blakeman, J. P. 1978: Microbial competition for nutrients and germination of fungal spores. *Annals of applied biology 89*:151-155.

Blakeman, J. P. 1980: Behaviour of conidia on aerial plant surfaces *in*: *The biology of Botrytis*, Coley-Smith, J. R., Verhoeff, K. and Jarvis, W. R., eds. PP 115-150.

Blackman, J. P. 1981: Microbial ecology of the phylloplane. London: Academic. PP 502.

Blakeman, J. P. 1985: Ecological succession of leaf surface microorganisms in relation to biological control *in*: *Biological control on the phylloplane*, Windels, C. E., and Lindow, S. E., eds. PP 6-30.

Blakeman, J. P., and Brodie, I. D. S. 1977: Competition for nutrients between epiphytic microorganisms and germination of spores of plant pathogens on beetroot leaves. *Physiological plant pathology 10*:29-42.

Blakeman, J. P., and Fokkema, N. J. 1982: Potential for biological control of plant diseases on the phylloplane. *Annual review of phytopathology 20*:167-192.

Blakeman, J. P., and Fraser, A. K. 1971: Inhibition of *Botrytis cinerea* spores by bacteria on the surface of chrysanthemum leaves. *Physiological plant pathology 1*:45-54.

Blakeman, J. P., and Sztejnberg, A. 1973: Effect of surface of wax on inhibition of germination of *Botrytis cinerea* spores on beetroot leaves. *Physiological plant pathology 3*:269-278.

Blakeman, V. H., and Welsford, E. J. 1916: Studies in the physiology of parasitism. Infection by *Botrytis cinerea*. *Annals of Botany 30(119)*:389-397.

Booth, C. 1971: The genus *Fusarium*. PP 32-72

Bradbury, J. F. 1970: Isolation and preliminary study of bacteria from plants. *Review of plant pathology 49(5)*:213-217.

Brodie, I. D. S., and Blakeman, J. P. 1976: Competition for exogenous substrate *in vitro* by leaf surface micro-organisms and germination of conidia of *Botrytis cinerea*. *Physiological plant pathology 9*:227-239.

Brooks, F. T. 1908: Observation on the biology of *Botrytis cinerea*. *Annals of botany 22(87)*:479-487.

Burchill, R. T., and Maude, R. B. 1986: Microbial deterioration in stored fresh fruit and vegetables. *Outlook on agriculture 15(4)*:160-166.

Burrage, S. W. 1976: Aerial microclimate around plant surfaces *in*: *Microbiology of aerial plant surfaces*, Dickinson, C. H., and Preece, T. F., eds. London: Academic. PP 173-184.

Campbell, R. 1989: Introduction to plant pathology and microbial ecology *in*: *Biological control of microbial plant pathogens*. PP 1-65.

Cappellini, R. A., and Ceponis, M. J. 1985: Postharvest losses in fresh fruits and vegetables *in*: *Postharvest technology of horticultural crops*, Kader, *et al.*, eds. PP 24-28.

Carter, M. V., and Price, T. V. 1974: Biological control of *Eutypa armeniacae* & *Fusarium lateritium* and their relative sensitivities o benzimidazole chemicals. *Australian journal of agricultural research 25(1)*:105-119.

Chalutz, E., Ben-Arie, R., Droby, S., Cohen, L., Weiss, B., and Wilson, C. L. 1988: Yeasts as biocontrol agents of postharvest diseases of fruits. *Phytoparasitica 16*:69.

Chalutz, E., and Wilson, C. L. 1990: Postharvest biocontrol of green, blue mold and sour rot of citrus fruit by *Debaryomyces hansenii*. *Plant disease 74(2)*:134-137.

Chalutz, L. W., and Wisniewski, M. E. 1989: Biological control of postharvest diseases of fruits and vegetables: An emerging technology. *Annual review of phytopathology 27*:425-441.

Cheah, L. H., De silva, N., Irving., D. E., Hunt, A. W., and Tate, K. G. 1992: Hot water dips for control of *Botrytis* storage rot in kiwifruit. (in press), *Agricultural research centre*, Hastings - New Zealand.

Chet, I., and Henis, Y. 1985: *Trichoderma* as a biocontrol agent against soilborne root pathogens *in*: *Ecology and management of soilborne plant pathogens*. Parker, *et al.*, eds. St Paul, Minnesota, American phytopathological society. PP 110-112.

Chiang, M.-Y., Van Dyke, C. G., and Leonard, K. J. 1989: Evaluation of endemic foliar fungi for potential biological control of johnsongrass (*Sorghaum halepense*): Screening and host range tests. *Plant disease 73*:459-464.

Clark, C. A., and Lorbeer, J. W. 1977: Comparative nutrient dependency of *Botrytis squamosa* and *B.cinerea* for germination of conidia and pathogenicity on onion leaves. *Phytopathology 67*:212-218.

Coffey, M. 1990: Biological control of citrus postharvest diseases. *California grower 14(1)*:14-16.

Coley-Smith, J. R., Verhoeff, K., and Jarvis, W. R. 1980: Sclerotia and other structures in survival *in*: *The biology of Botrytis*. PP 85-114.

Conn's, H. J. 1940: Biological stains. PP 1-147.

Conway, W. S. 1985: Preharvest factors affecting postharvest losses from disease *in*: *Postharvest technology of horticultural crops*, Kader, *et al.*, eds. PP 11-15.

Cook, R. J. 1988: Management of the environment for the control of plants in: Biological control of pest, pathogens and weeds. *Philosophical transactions of the royal society of London 318*:171-182.

Cook, R. J., and Baker, K. F. 1983: The nature and practice of biological control of plant pathogens. st Paul: The American phytopathological society. PP 60.

Cork, A. T. K. 1978: Federation of British plant pathologists chairman's address. Microbial antagonisms affecting tree diseases. *Annals of applied biology 89*:89-114.

Davenport, R. R. 1981: Yeast and yeast-like organisms *in*: *Smith's introduction to industrial mycology*, Onion's, A. H. S., Allsopp, D. and Eggins, H. O. W., eds. 7th ed. PP 65-92.

Dekker, J. 1963: Antibiotics in the control of plant diseases. *Annual review of microbiology 17*:243-262.

Derbyshire, D. M. 1973: Postharvest deterioration of vegetables. *Chemistry and industry 17*:1052-1057.

Dickinson, C. H. 1976: Fungi on the aerial surfaces of higher plants *in*: *Microbiology of aerial plant surfaces*, Dickinson, C. H., and Preece, T. F., eds. Academic press.

Droby. S., Chalutz, E., and Wilson, C. L. 1991: Antagonistic microorganisms as biological control agents of postharvest disease of fruits and vegetables. *Postharvest news and information 2(3)*:169-173.

Dubos. B. 1984: Biocontrol of *Botrytis cinerea* on grapevines by an antagonistic strain of *Trichoderma harzianum in*: *Current perspectives in microbial ecology*, Klug, M. J., and Reddy, G. A., eds. PP 370-373.

Eckert, J. W. 1978: Postharvest diseases of citrus fruits. *Outlook on agriculture 9(5)*:225-232.

Fokkema. N. J. 1973: The role of saprophytic fungi in antagonism against *Drechslera sorokiniana* (*Helminthosporium sativum*) on agar plates and on rye leaves with pollen. *Physiological plant pathology 3*:195-205

Fokkema, N. J. 1976: Antagonism between fungal saprophytes and pathogens on aerial plant surfaces *in*: *Microbiology of aerial surfaces*, Dickinson C. H., and Preece, T. F., eds. Academic press. London. PP 487-506.

Fokkema, N. J. 1981: Fungal leaf saprophytes, beneficial or detrimental? PP 433-454.

Fokkema, N. J., and Schippers, B. 1986: Phyllosphere versus rhizosphere as environments for saprophytic colonization *in*: *Microbiology of the phyllosphere*, Fokkema, N. J., and Heuvel, J. Van den., eds. PP 137-160.

Fravel, D. R. 1988: Role of antibiosis in the biocontrol of plant diseases. *Annual review of phytopathology 26*:75-91.

Freeman, T.E. 1981: Use of conidial fungi in biological control *in*: *Biology of conidial fungi*. Vol.2, Cole, G. T., and Kendrick, B., eds. PP 143-166.

Godfrey, B. E. S. 1974: Phylloplane mycoflora of bracken, *Pteridium aquilinum*. *Transactions of the british mycological society 62*:305-311.

Groves, J. W. and Drayton, F. L. 1939: The perfect stage of *Botrytis cinerea*. *Mycologia 31*:485-489.

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

Gull, K., and Trinci, A. P. J. 1971: Fine structure of spore germination in *Botrytis cinerea. Journal of general microbiology 68*:207-220.

Hammer, P. E., and Marois, J. J. 1989: Nonchemical methods for postharvest control of *Botrytis cinerea* on cut roses. *Journal of the american society for horticultural science 114(1)*:100-106.

Harman, J. E. 1979: Maturity and storage of kiwifruit. *Proceedings of kiwifruit seminar held at Tauranga-New Zealand.*

Harvey, I. C. 1988: Biological control of foliar fungal pathogens. *5th international congress of plant pathology, Kyoto, international conference hall, Japan.*

Harvey, J. M. 1978: Reduction of losses in fresh market fruits and vegetables. *Annual review of phytopathology 16*:321-341.

Heuvel, J. van den. 1970: Antagonistic effect of epiphytic micro-organisms on infection of dwarf bean leaves by *Alternaria zinniae. Thesis, Utrecht. Mededelingen phytopathologische Laboratorium. Willie Commelin Schlten no.84.*

Heuvel, J. van den. 1971: Antagonism between pathogenic and saprophytic *Alternaria* species on bean leaves *in*: *Ecology of leaf surface micro-organism.* Preece, T. F., and Dickinson, C. H., eds. Academic press, London. PP 537-544.

Hewett, E. W. 1979: Low temperature injury to kiwifruit. *Proceedings of kiwifruit seminar held at Tauranga-New Zealand.*

Hopkirk, G., and Clark, C. 1990: All out effect on rot and soft fruit. *New zealand kiwifruit, april* 5-6.

Hsu, S. C., and Lockwood, J. L. 1969: Mechanisms of inhibition of fungi in agar by *Streptomycetes. Journal of general microbiology 57*:149-158.

Hsu, S. C., and Lockwood, J. L. 1975: Powdered chitin as a selective medium for enumeration of actinomycetes in water and soil. *Applied microbiology 29*:422-426.

Janisiewicz, W. J. 1987: Postharvest biological control of blue mold on apples. *Phytopathology 77(3)*:481-485.

Janisiewicz, W. J. 1988: Biocontrol of postharvest diseases of apples with antagonist mixtures. *Phytopathology 78(2)*:194-198.

Janisiewicz, W. J., and Roitman, J. 1988: Biological control of blue mold and grey mold on apple and pear with *Pseudomonas cepacia. Phytopathology 78(12)*:1697-1700.

Johnson, L. F., and Curl. E. A. 1972: Methods for research on the ecology of soil-borne plant pathogens. PP 139-177.

Jutsum, A. R. 1988: Commercial application of biological control: Status and prospects in: Biological control of pest, pathogens and weeds. *Philosophical transactions of the royal society of London 318*:357-373.

Ko, W. H., and Lockwood, J. L. 1967: soil fungistasis: relation to fungal nutrition. *Phytopathology 57*:494.

Kusuge, T., and Hewitt, W. B. 1964: Exudates of grapes berries and their effect on germination of conidia of *B.cinerea. Phytopathology 54*:167-172.

Lakshminarayana, S., Sommer, N. F., Polito, V., and Fortlage, R. J. 1987: Development of resistance to infection by *Botrytis cinerea* and *Penicillium expansum* in wounds of mature apple fruits. *Phytopathology 77*:1674-1678.

Leben, C. 1964: Influence of bacteria isolated from healthy cucumber leaves on two leaf diseases of cucumber. *Phytopathology 54*:405-408.

Leben, C. 1965: Epiphytic microorganisms in relation to plant disease. *Annual review of phytopathology 3*:209-230.

Leben, C. 1985: Introductory remark: Biological control strategies in the phylloplane *in: Biological control on the phylloplane,* Windels, C. E., and Lindow, S. E., eds. PP 1-5.

Leong, J. 1986: Siderophores: Their biochemistry and possible role in the biocontrol of plant pathogens. *Annual review of phytopathology 24*:187-209.

Louis, D. 1963: Les modalites de la penetration du *Botrytis cinerea* Pers. Dans des plantes. *Annales des epiphyties 14(1)*:57-72.

Mansfield, J. W. 1980: Mechanisms of resistance to *Botrytis in: The biology of Botrytis*, Coley-Smith, J. R., Verhoeff, K., and Jarvis, W. R., eds. PP 181-218.

Matta, A. 1971: Microbial penetration and immunization of uncongenial host plants. *Annual review of phytopathology 9*:387-410.

McLaughlin, R. J., Wisniewski, M. E., Wilson, C. L., and Chalutz, E. 1990: Effect of inoculum concentration and salt solutions on biological control of postharvest diseases of apple with *Candida sp. Phytopathology 80(5)*:456-461.

Moore-Landecker, E. 1982: Class discomycetes *in*: *Fundamentals of the fungi*. 2nd ed, PP 154-170.

Morris, C. E., and Rouse, D. I. 1985: Role of nutrients in regulating epiphytic bacterial populations. PP 63-82.

Nelson, K. E. 1951: Effect of humidity on infection of table grapes by *Botrytis cinerea. Phytopathology 41(10)*:859-864.

Nelson, M. E., and Powelson, M. L. 1988: Biological control of grey mold of snap beans by *Trichoderma hamatum. Plant disease 72*:727-729.

Newhook, F. J. 1951: Microbiological control of *Botrytis cinerea* Pers. a. The role of pH changes and bacterial antagonism. b. Antagonism by fungi and actinomycetes. *Annals of applied biology 38*:169-202.

Newhook, F. J. 1957: The relationship of saprophytic antagonism to control of *Botrytis cinerea* Pers. on tomatoes. *Journal of science and technology 38(5)*:473-481.

Onions, A. H. S., Allsopp, D., and Eggins, H. O. W. 1981: *Botrytis* Persoon ex Fries *in*: *Smith's introduction to industrial mycology*. 7th ed, PP 123-124.

Papavizas, G. C., and Lumsden, R. D. 1980: Biological control of soilborne fungal propagules. *Annual review of phytopathology 18*:389-413.

Parry, D. W. 1990: Biological control *in*: *Plant pathology in agriculture*. PP 145-151.

Pennycook, S. R. 1984: Spraying kiwifruit for control of *Botrytis* storage rot. *The orchardist of new zealand 57*:33-34.

Pennycook, S. R. 1985: Fungal fruit rots of *Actinidia deliciosa* (Kiwifruit). *New zealand journal of experimental agriculture 13*:289-299.

Pennycook, S. R. 1986: Biological factors. *National kiwifruit coolstore seminar*. Tauranga-New Zealand.

Pennycook, S. R. 1990: Biological control of *Armillaria. New zealand kiwifruit marketing board. National research conference*. PP 20-22.

Pennycook, S. R. 1990: *Botrytis* control. *New zealand kiwifruit marketing board. National research conference*. PP 36.

Pitt, J. I., and Hocking, A. D. 1985: Genus *Phoma* Sacc *in*: *Fungi and food spoilage*. Academic press, sydney. PP 127-129.

Poole, P. R., and McLeod, L. C. 1992: Inhibition of *Botrytis cinerea* infection in kiwifruit tissues. *Acta horticulturae* (in press).

Porter, C. L. 1924: Concerning the characters of certain fungi as exhibited by their growth in the presence of other fungi. *American journal of botany 11*:168-188.

Powelson, R. L. 1960: Initiation of strawberry fruit rot caused by *Botrytis cinerea. Phytopathology 50*:491-494.

Prasad, K., and Stadelbacker, G. J. 1973: Control of postharvest decay of fresh raspberries by acetaldehyde vapour. *Plant disease reporter 57*:795-797.

Pusey, P. L., and Wilson, C. L. 1984: Postharvest biological control of stone fruit brown rot by *Bacillus subtilis*. *Plant disease 68(9)*:753-756.

Redmond, J. C., Marois, J. J., and MacDonalds, J. D. 1987: Biological control of *Botrytis cinerea* on roses with epiphytic microorganisms. *Plant disease 71*:799-802.

Rishbeth, J. 1988: Biological control of air-borne pathogens in: Biological control of pests, pathogens and weeds. *Philosophical transactions of the royal society of london 318*:265-281.

Roberts, R. G. 1990: Postharvest biological control of grey mold of apple by *Cryptococcus laurentii. Phytopathology 80*:526-530.

Ruinen, J. 1961: The phyllosphere. 1. An ecologically neglected milieu. *Plant and soil 15*:81-109.

Schaad, N. W. 1988: Laboratory guide for identification of plant pathogenic bacteria, 2nd ed. PP 1-5

Scher, F. M., and Castagno, J. R. 1986: Biocontrol: A review from industry.*Canadian journal of plant pathology 8*:222-224.

Schroth, M. N., and Hancock, J. G. 1981: Selected topics in biological control. *Annual review of microbiology 35*:453-476.

Sharrock, K. R., and Hallett, I. C. 1992: Physiology of *Botrytis* infection in kiwifruit. *Acta horticulturae* (in press).

Sholberg, P., Meheriuk, M., and McPhee, W. 1989: Pressure inoculation as a technique for postharvest studies on fungal pathogens of pome fruits. *Plant disease 73*:260-262.

Skerman, V. B. D. 1967: A guide to the identification of the genera of bacteria, 2nd ed. PP 18-113.

Skidmore, A. M. 1976: Secondary spore production amongst phylloplane fungi. *Transactions of the british mycological society 66*:161-163.

Smilanick, J. 1990: Natural decay control. *California fruit grower 67(1)*:10-12.

Snow, D. 1949: Germination of mould spores at controlled humidities. *Annals of applied biology 36*:1-3.

Sommer, N. F. 1982: Postharvest handling practices and postharvest diseases of fruit. *Plant disease 66(5)*:357-364.

Sommer, N. F. 1985: Principles of disease suppression by handling practices *in*: *Postharvest technology of horticultural crops*, Kader, *et al.*, eds. PP 72-82.

Sommer, N. F. 1985: Strategies for control of postharvest diseases of selected commodities *in*: *Postharvest technology of horticultural crops*, Kader, *et al.*, eds. PP 83-85.

Sommer, N. F., Fortlage, R. J., and Edwards, D. C. 1983: Minimizing postharvest diseases of kiwifruit. *California agriculture 37(1,2)*:16-18.

Steel, R. G. D., and Torrie, J. H. 1981: Principles and procedures of statistics, A biometrical approach. McGraw-Hill International book company, 2nd ed. PP 239-377.

Stessel, G. J., Leben, C., and Keitt, G. W. 1953: Screening tests designed to discover antibiotics suitable for plant disease control. *Mycologia 45*:325-334.

Stevens, C., Khan, V., Lu, J. Y., and Tang, A. Y. 1988: The effect of ultraviolet radiation on resistance of sweet potato to storage rots. PP 424.

Stott, A. M. 1971: Studies on the physiology of some leaf saprophytes *in*: *Ecology of leaf surface micro-organisms*, Preece, T. F., and Dickinson, C. H., eds. Academic press, London. PP 203-210.

Sutton, B. C. 1980: The coelomycetes *in*: *Fungi imperfecti with pycnidia, acervuli and stromata*. Key, Surrey: Commonwealth mycological institute. PP 696.

Swinburne, T. R. 1978: Post-infection antifungal compounds in quiescent or latent infections. *Annals of applied biology 89*:322-324.

Tribe, H. T. 1957: On the parasitism of *Sclerotinia trifoliorum* by *Coniothyrium minitans*. *Transactions of the british mycological society 40*:489-499.

Tronsmo, A. 1978: Effect of temperature on antagonistic properties of *Trichoderma* species. *Transactions of the british mycological society 65*:239-247.

Tronsmo, A. 1986: Use of *Trichoderma spp.* in biological control of necrotrophic pathogens *in*: *Microbiology of the phyllosphere*, Fokkema, N. J., and Heuvel, J. Van den., eds. PP 348-362.

Tronsmo, A., and Dennis, C. 1983: The use of *Trichoderma* species to control strawberry fruit rots. *Netherlands journal of plant pathology 83*:449-455.

Tronsmo, A. M., and Raa, J. 1977: Cytology and biochemistry of pathogenic growth of *Botrytis cinerea* Pers. in apple fruit. *Phytopathologische zeitschrift 89*:208-215.

Tronsmo, A., and Raa, J. 1977: Antagonistic action of *Trichoderma pseudokoningii* against the apple pathogen *Botrytis cinerea*. *Phytopathologische zeitschrift 89*:216-220.

Tronsmo, A., and Ystaas, J. 1980: Biological control of *Botrytis cinerea* on apple. *Plant disease 64*:1009.

Tuite, J. 1969: Plant pathological methods (fungi and bacteria). PP 95.

United states department of agriculture. 1989: Kearneysville, where new ideas come to fruit. *Agricultural research service 37(11)*:4-8.

United states department of agriculture. 1990: Natural agents fight fruit spoilage. *Agricultural research service 38(4)*:15-17.

Vanbreuseghem, R., De Vroey, CH., and Takashio, M. 1978: Practical guide to medical and veterinary mycology, 7 ed. PP 251.

Waksman, S. A. 1947: What is an antibiotic or an antibiotic substance. *Mycologia 39*:565-569.

Webster, J. 1970: Ascomycotina *in*: *Inhibition to fungi*. PP 155-278.

Weindling, R. 1932: *Trichoderma lignorum* as a parasite of other soil fungi. *Phytopathology* 22:837-845.

Weller, D. M., Zhang, B. X., and Cook, R. J. 1985: Application of a rapid screening test for selection of bacteria suppressive to take-all of wheat. *Plant disease 69*:710-713.

Wills, R. H. H., Lee, T. H., Graham, W. B., McGlasson, W. B., and Hall, E. G. 1981: Pathology *in*: *Postharvest: An introduction to the physiology and handling of fruit and vegetables*. PP 97-108.

Wilson, C. L., and Pusey, P. L. 1985: Potential for biological control of postharvest plant diseases. *Plant disease 69(5)*:375-378.

Wilson, C. L., and Wisniewski, M. E. 1989: Biological control of postharvest disease of fruits and vegetables: An emerging technology. *Annual review of phytopathology* 27:425-441.

Wisniewski, M., Wilson, C., Chalutz, E., and Hershberger, W. 1988: Biological control of postharvest diseases of fruit: Inhibition of *Botrytis* rot on apple by an antagonistic yeast. *Proceedings of the 46th Annual meeting of the electron microscopy society of America 46*:290-291.

Wood, R. K. S. 1951: The control of diseases of lettuce by the use of antagonistic organisms. 1. The control of *Botrytis cinerea* Pers. *Annual of applied biology 38*:203-216.

Wright, B. 1989: Gene-spliced pesticide uncorked in Australia. *New scientist 4(march)*:23.

END OF THESIS

LIST OF APPENDICES

APPENDIX 1

Table 1. Inhibition zones around mycelial plugs of antagonists on plates of PDA seeded with conidia of *B.cinerea*. Antagonists obtained from isolation plates of collection one were incubated at 20°C. Data is given for the width of inhibition zones (mm).

		Isolates																
Temp	Days	FB2-3	FB3	FF1	FF3-3	FF4	FF7	FF9	FF11	FT1-1	FT3	FT4-1	FT4-2	FT5-1	FT6	FT7	FO1	FO2-1
0°C	8	5.5	8.0	8.5	4.5	9.0	6.0	18.0	4.5	7.0	5.5	7.0	5.5	9.5	8.0	13.0	13.0	7.0
	12	S	2.5	1.0	In	2.5	4.0	16.0	In	3.0	2.0	In	In	3.0	3.0	11.0	In	3.0
	16	In	1.5	S	In	2.5	4.0	15.0	In	3.0	2.0	In	In	2.0	2.0	11.0	In	3.0
	20	In	0.5	S	In	1.5	3.0	12.0	In	1.0	1.0	In	In	1.0	1.0	8.0	In	2.0
	24	In	S	In	In	1.0	S	9.0	In	S	1.0	In	In	S	In	7.0	In	1.5
	28	In	In	In	In	1.0	S	8.0	In	S	1.0	In	In	S	In	6.0	In	1.0
	32	In	In	In	In	1.0	In	5.0	In	S	1.0	In	In	S	In	3.0	In	1.0
	36	In	In	In	In	S	In	5.0	In	In	In	In	In	In	In	3.0	In	0.5
	40	In	In	In	In	In	In	2.5	In	In	In	In	In	In	In	1.5	In	0.5
	44	In	In	In	In	In	In	2.0	In	In	In	In	In	In	In	S	In	S
	48	In	In	In	In	In	In	S	In	In	In	In	In	In	In	In	In	In
5°C	8	9.0	11.0	9.5	11.0	11.5	7.5	11.5	6.5	11.0	12.0	7.0	12.0	7.0	7.0	7.0	10.0	12.0
	12	1.0	2.5	3.0	1.5	4.0	0.5	6.0	S	5.0	4.0	1.0	8.5	0.5	2.5	S	S	1.5
	16	1.0	S	1.0	0.5	4.0	0.2	4.0	S	3.0	S	In	7.0	0.2	S	S	In	0.5
	20	0.5	In	0.5	0.5	3.0	S	4.0	In	S	In	In	3.5	0.2	In	In	In	0.5
	24	0.5	In	S	In	3.0	In	4.0	In	In	In	In	3.0	In	In	In	In	0.2
	28	In	In	In	In	2.5	In	1.0	In	In	In	In	1.0	In	In	In	In	0.2
	32	In	In	In	In	In	In	0.5	In	In	In	In	1.0	In	In	In	In	In
	36	In	In	In	In	In	In	0.5	In	In	In	In	In	In	In	In	In	In
	40	In	In	In	In	In	In	0.5	In	In	In	In	In	In	In	In	In	In
	44	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In
10°C	4	9.0	9.0	16.0	16.0	9.0	17.0	18.0	3.0	17.0	16.0	8.0	14.5	12.0	12.0	12.0	3.0	7.0
	8	2.0	In	1.0	3.5	1.0	4.0	4.5	In	5.0	4.0	In	10.5	In	1.0	0.5	In	3.5
	12	1.0	In	In	S	1.0	1.0	3.5	In	In	In	In	10.0	In	In	In	In	2.0
	20	0.5	In	In	In	0.5	In	2.5	In	In	In	In	8.5	In	In	In	In	1.0
	24	0.2	In	In	In	S	In	1.0	In	In	In	In	8.0	In	In	In	In	1.0
	28	In	In	In	In	In	In	1.0	In	In	In	In	7.0	In	In	In	In	0.5
	32	In	In	In	In	In	In	1.0	In	In	In	In	6.5	In	In	In	In	0.5
	36	In	In	In	In	In	In	1.0	In	In	In	In	5.5	In	In	In	In	0.5
	40	In	In	In	In	In	In	S	In	In	In	In	5.5	In	In	In	In	S
	44	In	In	In	In	In	In	In	In	In	In	In	5.5	In	In	In	In	In
	48	In	In	In	In	In	In	In	In	In	In	In	S	In	In	In	In	In
15°C	4	In	S	In	2.0	S	4.0	5.0	In	5.0	S	In	2.0	S	In	In	In	In
	8	In	In	In	In	S	S	S	In	In	In	In	In	In	In	In	In	In

In - Isolate overgrown by *Botrytis*. S - *Botrytis* overgrowth commencing.
Isolates of *Phoma* were FF7, FF9, F02-1. Isolate of pink yeast was FB3. Isolate of *Epicoccum* was FF4,FT7.
Isolate of *Alternaria* was FT5-1.

Table 2. Inhibition zones (mm) around mycelial plugs of antagonists on petri dishes of PDA seeded with conidia of *B.cinerea*. Antagonists obtained from isolation petri dishes of collection one were incubated at 5°C.

		Isolates					
Temp	Days	F1T-1	F1T-2	F1F-2	F1B-1	F1B-2	F1Y1
0°C	16	4.5	7.0	4.5	4.5	7.5	8.0
	20	1.0	2.0	1.0	S	S	2.5
	24	1.0	2.0	1.0	In	In	2.5
	28	0.5	2.0	1.0	In	In	0.5
	32	0.5	2.0	S	In	In	0.5
5°C	20	1.0	1.5	S	S	S	1.0
	24	In	0.5	In	In	In	1.0
	28	In	S	In	In	In	S
	32	In	In	In	In	In	In
10°C	8	11.0	9.0	8.0	5.0	12.0	9.5
	12	In	0.5	0.2	In	In	In
	16	In	In	In	In	In	In
15°C	8	S	In	In	In	In	In
	12	In	In	In	In	In	In

Table 3. Inhibition zones (mm) around bacterial streaks of antagonists on petri dishes of PDA seeded with conidia of *B.cinerea*. Antagonists obtained from isolation petri dishes of collection one were incubated at 20°C.

		Isolates														
Temp	Days	B1Y-1	B1Y-2	B1Y-3	B1O-1	B1O-2	B1O-3	B1S-1	B1S-2	B1T-1	B1T-2	B1T-3	B1B-1	B1B-2	B1F-1	B1F-2
0°C	12	T	T	T	T	9.0	11.0	9.5	12.0	2.0	5.0	9.0	7.0	8.5	8.0	9.0
	16	8.0	6.0	5.0	7.0	2.0	4.5	3.0	5.5	In	S	1.0	4.5	5.0	0.5	4.0
	20	2.0	1.0	0.5	1.0	In	In	In	S	In	In	In	4.5	4.5	In	2.5
	24	In	In	In	In	In	In	In	In	In	In	In	1.5	4.5	In	S
	28	In	In	In	In	In	In	In	In	In	In	In	S	2.5	In	S
	32	In	In	In	In	In	In	In	In	In	In	In	In	1.0	In	In
	36	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In
15°C	4	5.0	2.0	0.5	2.0	2.0	5.0	7.0	4.0	6.0	2.5	4.0	1.5	In	In	3.0
	8	In	In	In	In	In	4.0	In	In	In	In	In	In	In	In	In
	12	In	In	In	In	In	4.0	In	In	In	In	In	In	In	In	In

T = Placed too far from the centre

Table 4. Inhibition zones (mm) around mycelial plugs of antagonists on petri dishes of PDA seeded with conidia of *B.cinerea*. Antagonists obtained from isolation petri dishes of collection two were incubated at 20°C.

		Isolates																	
Temp	Days	FF10	FF20	FF30	FF40	FO20	FO30	FY10	FY20	FY30	FY50	FY60	FT10	FT20	FT30	FT40	FT50	FT60	FB10
0°C	12	9.0	15.0	13.0	13.0	n.gr	13.0	10.0	10.0	9.5	13.0	13.0	10.0	8.0	10.0	8.5	10.0	11.0	5.0
	16	3.0	12.0	7.0	12.0		12.0	4.0	6.5	3.0	12.5	4.5	6.0	3.5	5.0	2.0	5.5	9.0	S
	20	0.5	9.5	5.5	7.5		11.0	S	5.5	In	10.0	2.0	2.5	3.0	S	In	3.0	4.0	In
	24	In	7.0	3.0	3.5		9.0	In	5.5	In	8.0	1.5	1.0	3.0	In	In	3.0	In	In
	28	In	3.5	In	In		8.0	In	5.5	In	6.0	1.5	0.5	3.0	In	In	2.5	In	In
	32	In	0.5	In	In		6.0	In	5.5	In	4.0	1.5	0.2	2.0	In	In	2.5	In	In
	36	In	In	In	In		5.0	In	3.0	In	4.0	1.5	In	2.0	In	In	2.0	In	In
	40	In	In	In	In		4.0	In	3.0	In	4.0	In	In	1.0	In	In	2.0	In	In
	44	In	In	In	In		2.0	In	3.0	In	3.5	In	In	1.0	In	In	2.0	In	In
	48	In	In	In	In		1.0	In	2.0	In	2.0	In	In	In	In	In	2.0	In	In
	52	In	In	In	In		1.0	In	1.5	In	2.0	In	In	In	In	In	0.5	In	In
	56	In	In	In	In		1.0	In	1.0	In	2.0	In	In	In	In	In	S	In	In
	60	In	In	In	In		In	In	0.5	In	1.0	In	In	In	In	In	In	In	In
	64	In	In	In	In		In	In	0.5	In	1.0	In	In	In	In	In	In	In	In
	68	In	In	In	In		In	In	0.5	In	1.0	In	In	In	In	In	In	In	In
	72	In	In	In	In		In	In	0.5	In	1.0	In	In	In	In	In	In	In	In
	76	In	In	In	In		In	In	S	In	1.0	In	In	In	In	In	In	In	In
5°C	8	4.0	8.5	6.5	8.5	6.0	5.0	1.0	1.0	In	In	In	2.5	3.0	In	In	2.5	In	In
	12	3.5	7.0	2.0	5.0	3.0	5.0	In	1.0	In	In	In	In	3.0	In	In	2.0	In	In
	16	3.0	5.0	In	3.0	1.5	4.0	In	1.0	In	In	In	In	2.5	In	In	2.0	In	In
	20	3.0	5.0	In	3.0	S	4.0	In	1.0	In	In	In	In	2.5	In	In	2.0	In	In
	24	1.5	3.0	In	0.5	In	3.5	In	1.0	In	In	In	In	2.0	In	In	2.0	In	In
	28	S	2.5	In	In	In	3.0	In	1.0	In	In	In	In	2.0	In	In	2.0	In	In
	32	In	2.0	In	In	In	2.0	In	1.0	In	In	In	In	2.0	In	In	1.5	In	In
	36	In	1.0	In	In	In	In	In	1.0	In	In	In	In	2.0	In	In	0.5	In	In
	40	In	1.0	In	In	In	In	In	0.5	In	In	In	In	0.5	In	In	S	In	In
	44	In	1.0	In	In	In	In	In	0.5	In	In	In	In	0.5	In	In	S	In	In
	48	In	0.5	In	In	In	In	In	0.5	In	In	In	In	S	In	In	S	In	In
	52	In	0.5	In	In	In	In	In	0.5	In	In	In	In	S	In	In	S	In	In
10°C	4	In	8.0	In	3.0	In	8.0	In	1.0	In	In	In	2.0	2.0	In	In	2.0	In	In
	8	In	4.5	In	In	In	5.5	In	0.5	In	In	In	1.0	1.0	In	In	2.0	In	In
	12	In	3.5	In	In	In	4.5	In	0.5	In	In	In	In	1.0	In	In	0.5	In	In
	16	In	3.5	In	In	In	3.0	In	S	In	In	In	In	0.5	In	In	S	In	In
	20	In	3.5	In	In	In	1.0	In	In	In	In	In	In	In	In	In	In	In	In
	24	In	3.0	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In
	28	In	3.0	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In
	32	In	1.5	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In
15°C	4	In	3.0	In	1.0	In	10.0	In	0.5	In	In	In	In	In	In	In	In	In	In
	8	In	1.5	In	In	In	4.0	In	In	In	In	In	In	In	In	In	In	In	In
	12	In	In	In	In	In	S	In	In	In	In	In	In	In	In	In	In	In	In

n.gr = no growth

Table 4, continues..

Temp	Days	FB20	FB30	FB40	FB50	FB60	FY40	FT70
0°C	12	6.5	5.0	2.0	10.0	5.0	8.0	11.0
	16	5.0	In	In	9.5	S	5.0	9.0
	20	5.0	In	In	8.0	In	S	4.0
	24	5.0	In	In	6.5	In	In	In
	28	5.0	In	In	5.0	In	In	In
	32	4.0	In	In	5.0	In	In	In
	36	4.0	In	In	5.0	In	In	In
	40	4.0	In	In	3.0	In	In	In
	44	4.0	In	In	3.0	In	In	In
	48	4.0	In	In	2.0	In	In	In
	52	3.0	In	In	1.0	In	In	In
	56	3.0	In	In	1.0	In	In	In
	60	2.0	In	In	0.5	In	In	In
	64	1.5	In	In	0.5	In	In	In
	72	S	In	In	S	In	In	In
5°C	8	4.5	In	In	In	In	In	In
	12	4.0	In	In	In	In	In	In
	16	3.5	In	In	In	In	In	In
	20	3.5	In	In	In	In	In	In
	24	3.5	In	In	In	In	In	In
	28	3.5	In	In	In	In	In	In
	32	3.5	In	In	In	In	In	In
	36	3.5	In	In	In	In	In	In
	40	1.0	In	In	In	In	In	In
	44	1.0	In	In	In	In	In	In
	48	1.0	In	In	In	In	In	In
	52	1.0	In	In	In	In	In	In
	56	0.5	In	In	In	In	In	In
	60	In	In	In	In	In	In	In
10°C	4	0.5	In	In	In	In	In	In
	8	In	In	In	In	In	In	In
15°C	4	In	In	In	In	In	In	In

Isolates of *Alternaria* were FB20, FB50, FY50. Isolates of *Epicoccum* were FY20, FY60, FT20, FT50.
Isolate of *Fusarium* was FO30.

Table 5. Inhibition zones (mm) around mycelial plugs of antagonists on plates of PDA seeded with conidia of B.cinerea. Antagonists obtained from isolation plates of collection two were incubated at 5°C.

		Isolates															
Temp	Days	FT-1	FT-2	FF-1	FF-2	FF-3	FD-1	FD-2	FD-3	FO-1	FO-2	FO-3	FB-2	FB-3	FY-2	FY-3	FY-4
0°C	16	4.0	3.0	6.0	7.0	4.5	7.5	4.0	1.5	3.5	4.5	2.0	S	1.0	2.0	3.0	2.5
	20	0.5	In	4.0	4.0	1.5	5.0	2.5	In	S	1.5	0.5	In	In	In	In	In
	24	In	In	1.0	1.5	1.0	1.5	2.0	In	In	In	S	In	In	In	In	In
	28	In	In	0.2	0.5	1.0	S	2.0	In	In	In	In	In	In	In	In	In
	32	In	In	In	0.5	S	In	1.5	In	In	In	In	In	In	In	In	In
	36	In	In	In	0.5	In	In	1.5	In	In	In	In	In	In	In	In	In
	40	In	In	In	In	In	In	1.5	In	In	In	In	In	In	In	In	In
	44	In	In	In	In	In	In	1.5	In	In	In	In	In	In	In	In	In
	48	In	In	In	In	In	In	1.0	In	In	In	In	In	In	In	In	In
	52	In	In	In	In	In	In	S	In	In	In	In	In	In	In	In	In
5°C	8	4.0	5.0	3.0	3.5	2.0	5.5	7.5	2.0	5.0	4.5	4.5	2.0	2.0	2.0	4.0	0.5
	12	In	In	In	In	In	In	4.0	In	In	In	1.5	S	1.5	In	In	In
	16	In	In	In	In	In	In	3.5	In	In	In	1.5	In	0.5	In	In	In
	20	In	In	In	In	In	In	3.0	In	In	In	1.0	In	In	In	In	In
	24	In	In	In	In	In	In	2.5	In	In	In	0.5	In	In	In	In	In
	28	In	In	In	In	In	In	1.5	In	In	In	0.5	In	In	In	In	In
	32	In	In	In	In	In	In	1.5	In	In	In	S	In	In	In	In	In
	36	In	In	In	In	In	In	0.5	In	In	In	In	In	In	In	In	In
	40	In	In	In	In	In	In	0.5	In	In	In	In	In	In	In	In	In
	44	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In
10°C	4	In	2.5	1.5	6.0	In	2.0	6.0	In	4.5	In	6.0	3.0	In	0.5	In	In
	8	In	In	In	4.0	In	In	1.5	In	In	In	1.5	1.5	In	In	In	In
	12	In	In	In	2.5	In	In	1.0	In	In	In	1.0	1.0	In	In	In	In
	16	In	In	In	1.5	In	In	0.2	In	In	In	0.2	0.5	In	In	In	In
	20	In	In	In	S	In	In	In	In	In	In	In	In	In	In	In	In
15°C	4	In	In	In	In	In	In	0.5	In	In	In	In	S	In	In	In	In
	8	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In

Isolates of *Phoma* were FT-1, FF-2, FD-2, FO-2, FO-3.
Isolate of *Epicoccum* was FF-3. Isolate of *Fusarium* was FF-1.

Table 6. Inhibition zones (mm) around bacterial streaks of antagonists on petri dishes of PDA seeded with conidia of *B.cinerea*. Antagonists obtained from isolation petri dishes of collection two were incubated at 20°C.

		Isolates							
Temp	Days	BT-01	BD-01	BY-01	BO-01	BO-02	BO-03	BO-04	BO-05
0°C	16	T	T	T	S	2.5	S	2.5	T
	20	8.0	7.0	8.0	In	0.2	In	0.5	7.5
	24	2.0	S	3.0	In	In	In	In	S
	28	S	In	1.0	In	In	In	In	In
	32	In	In	S	In	In	In	In	In
5°C	8	In	3.0	4.0	2.0	4.0	3.0	1.0	1.0
	12	In	In	In	In	In	In	In	In
10°C	4	In	In	In	In	4.0	In	2.5	In
	8	In	In	In	In	In	In	In	In
15°C	4	In	In	In	In	In	In	In	In

T= Placed too far from the centre.

Table 7. Inhibition zones (mm) around mycelial plugs of antagonists on petri dishes of PDA seeded with conidia of *B.cinerea*. Antagonists obtained from isolation petri dishes of collection two were incubated at 5°C.

		Isolates								
Temp	Days	F1Y-10	F1Y-20	F1F-10	F1F-20	F1F-30	F1S-10	F1S-20	F1B-10	F1O-10
0°C	12	8.5	8.0	8.0	10.5	6.0	12.0	8.0	9.0	9.0
	16	3.5	5.0	4.0	5.0	2.0	7.0	5.0	4.0	4.5
	20	In	1.5	3.5	2.0	In	3.5	4.5	1.0	1.5
	24	In	0.5	3.0	0.5	In	2.0	4.5	In	1.0
	28	In	In	3.0	In	In	2.0	3.0	In	S
	32	In	In	2.0	In	In	S	2.5	In	In
	36	In	In	2.0	In	In	S	1.5	In	In
	40	In	In	1.5	In	In	S	S	In	In
	44	In	In	1.5	In	In	In	In	In	In
	48	In	In	1.5	In	In	In	In	In	In
	52	In	In	1.5	In	In	In	In	In	In
	56	In	In	1.5	In	In	In	In	In	In
5°C	8	11.5	13.0	11.0	13.0	10.0	16.0	13.0	14.0	13.0
	12	3.0	6.0	7.0	5.5	3.5	8.0	5.0	7.5	4.0
	16	In	In	5.0	1.5	In	In	3.5	In	In
	20	In	In	4.5	1.5	In	In	3.5	In	In
	24	In	In	4.0	1.0	In	In	2.0	In	In
	28	In	In	4.0	0.5	In	In	2.0	In	In
	32	In	In	4.0	In	In	In	1.0	In	In
	36	In	In	4.0	In	In	In	0.5	In	In
	40	In	In	3.5	In	In	In	S	In	In
	44	In	In	3.0	In	In	In	In	In	In
	46	In	In	2.5	In	In	In	In	In	In
	50	In	In	2.0	In	In	In	In	In	In
	52	In	In	1.0	In	In	In	In	In	In
	56	In	In	1.0	In	In	In	In	In	In
	60	In	In	0.5	In	In	In	In	In	In
	64	In	In	S	In	In	In	In	In	In
10°C	8	7.0	5.5	9.0	6.5	5.0	10.5	6.0	9.0	7.0
	12	In	In	3.0	In	In	In	In	In	In
	16	In	In	2.5	In	In	In	In	In	In
	20	In	In	1.5	In	In	In	In	In	In
	24	In	In	0.5	In	In	In	In	In	In
	28	In	In	0.5	In	In	In	In	In	In
	32	In	In	In	In	In	In	In	In	In
15°C	8	In	In	0.2	In	In	In	In	In	In
	12	In	In	In	In	In	In	In	In	In

Isolates of *Phoma* were F1F-10, F1F-30.

Table 8. Inhibition zones (mm) around mycelial plugs of antagonists on petri dishes of PDA seeded with conidia of *B.cinerea*. Antagonists obtained from isolation petri dishes of collection three were incubated at 20°C.

Temp	Days	Isolates																			
		FF11	FF21	FF31	FF41	FD1	FD2	FD3	FD4	FD5	FD6	FD7	FT11	FT21	FT31	FT41	FY11	FY21	FY31	FY41	FO11
0°C	16	6.0	8.0	7.0	1.0	5.5	5.0	5.0	5.5	0.5	8.0	8.0	In	6.0	6.0	0.5	4.0	3.5	2.0	1.0	8.5
	20	6.0	6.0	6.5	0.5	1.0	2.0	1.0	5.0	0.5	6.0	5.0	In	S	3.5	0.5	0.5	In	In	0.5	8.5
	24	5.0	5.0	5.0	0.5	In	In	In	5.0	0.5	5.0	3.0	In	In	3.0	0.5	In	In	In	0.5	7.0
	28	4.0	3.5	4.0	S	In	In	In	3.0	In	5.0	3.0	In	In	1.0	S	In	In	In	S	5.0
	32	4.0	3.0	3.5	In	In	In	In	2.0	In	4.0	1.0	In	In	S	In	In	In	In	In	4.5
	36	3.0	3.0	3.0	In	In	In	In	1.0	In	3.0	S	In	In	In	In	In	In	In	In	4.0
	40	2.5	3.0	3.0	In	In	In	In	1.0	In	1.0	S	In	In	In	In	In	In	In	In	3.0
	44	2.0	2.0	2.5	In	In	In	In	1.0	In	S	In	In	In	In	In	In	In	In	In	2.5
	48	0.5	1.5	2.0	In	In	In	In	0.5	In	In	In	In	In	In	In	In	In	In	In	1.5
	52	In	1.5	2.0	In	In	In	In	0.5	In	In	In	In	In	In	In	In	In	In	In	0.5
	56	In	1.0	0.5	In	In	In	In	0.5	In	In	In	In	In	In	In	In	In	In	In	0.5
	60	In	1.0	S	In	In	In	In	S	In	In	In	In	In	In	In	In	In	In	In	0.5
	64	In	S	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In
5°C	8	5.0	5.0	4.0	S	3.0	3.5	6.0	6.0	In	3.0	4.0	4.5	8.0	7.0	3.0	4.0	S	2.5	In	8.0
	12	4.0	3.5	3.5	In	In	In	In	1.5	In	In	In	3.0	S	3.0	1.5	In	In	In	In	6.0
	16	4.0	3.0	3.5	In	In	In	In	1.5	In	In	In	2.5	In	2.5	In	In	In	In	In	4.0
	20	2.0	2.0	2.5	In	In	In	In	1.0	In	In	In	2.5	In	S	In	In	In	In	In	4.0
	24	1.5	0.5	2.0	In	In	In	In	S	In	In	In	2.0	In	In	In	In	In	In	In	2.5
	28	0.5	S	S	In	In	In	In	In	In	In	In	2.0	In	In	In	In	In	In	In	2.0
	32	In	In	S	In	In	In	In	In	In	In	In	2.0	In	In	In	In	In	In	In	1.0
	36	In	In	In	In	In	In	In	In	In	In	In	1.5	In	In	In	In	In	In	In	S
	40	In	In	In	In	In	In	In	In	In	In	In	1.5	In	In	In	In	In	In	In	In
	44	In	In	In	In	In	In	In	In	In	In	In	1.5	In	In	In	In	In	In	In	In
	48	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In
10°C	4	10.0	10.0	9.0	6.0	5.0	8.0	8.0	5.0	In	3.0	6.0	8.0	7.0	8.0	3.0	11.0	9.0	11.5	3.0	8.0
	8	1.5	1.0	0.5	In	In	In	In	2.0	In	In	In	3.0	In	In	In	In	In	1.0	In	1.5
	12	In	In	In	In	In	In	In	1.5	In	In	In	2.0	In	In	In	In	In	1.0	In	1.5
	16	In	In	In	In	In	In	In	1.0	In	In	In	1.5	In	In	In	In	In	1.0	In	S
	20	In	In	In	In	In	In	In	In	In	In	In	0.5	In	In	In	In	In	1.0	In	In
	24	In	In	In	In	In	In	In	In	In	In	In	0.5	In	In	In	In	In	1.0	In	In
	28	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	0.5	In	In
15°C	4	S	2.0	S	In	In	1.0	In	In	In	In	In	1.0	In	In	In	In	In	0.5	In	S
	8	In	S	In	In	In	S	In	In	In	In	In	In	In	In	In	In	In	0.5	In	In
	12	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	0.5	In	In
	16	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In

Table.8, continues..

		Isolates					
Temp	Days	FO21	FO31	FO41	FB11	FB21	FB31
0°C	16	9.0	8.0	5.0	7.0	11.0	4.0
	20	8.0	8.0	4.0	6.5	9.5	2.5
	24	6.0	6.0	3.0	5.0	6.5	2.0
	28	5.0	6.0	2.5	4.0	6.0	0.5
	32	3.5	4.0	2.0	2.5	4.0	S
	36	3.0	3.5	1.0	1.5	3.0	S
	40	1.5	3.5	1.0	1.0	2.5	S
	44	1.5	2.5	1.0	1.0	1.0	S
	48	1.0	2.5	0.5	0.5	S	In
	52	S	2.0	In	0.2	In	In
	56	In	0.5	In	In	In	In
	60	In	S	In	In	In	In
5°C	8	9.0	8.0	3.0	4.0	10.0	3.0
	12	6.5	5.0	2.0	3.0	8.0	1.0
	16	4.0	4.0	S	2.5	6.0	1.0
	20	2.0	2.5	S	1.5	3.0	0.5
	24	0.5	1.5	In	1.0	In	In
	28	In	1.0	In	0.5	In	In
	32	In	0.5	In	0.5	In	In
	36	In	In	In	S	In	In
10°C	4	15.0	11.0	4.5	4.5	11.0	10.5
	8	11.5	2.0	In	0.2	6.0	5.5
	12	6.0	S	In	In	2.0	3.0
	16	5.0	In	In	In	S	1.5
	20	3.0	In	In	In	In	S
	24	1.0	In	In	In	In	In
	28	0.5	In	In	In	In	In
	32	S	In	In	In	In	In
15°C	4	4.0	4.0	In	S	6.0	6.5
	8	S	2.0	In	In	1.0	3.0
	12	In	S	In	In	In	2.0
	16	In	In	In	In	In	In

Isolates of *Phoma* were FF21, FT11, FO21, FO31, FB21, FB31. Isolates of *Epicoccum* were FF11, FF31, FF41, FD4, FO11.
Isolate of *Alternaria* was FD7.

Table 9. Inhibition zones (mm) around bacterial streaks of antagonists on petri dishes of PDA seeded with conidia of *B.cinerea*. Antagonists obtained from isolation petri dishes of collection three were incubated at 20°C.

		Isolates													
Temp	Days	BD1	BD2	BY11	BY21	BY31	BB11	BB21	BB31	BO11	BO21	BF11	BF21	BF31	BS11
0°C	16	10.0	6.0	8.0	3.5	6.5	5.0	0.5	4.5	5.0	7.0	7.5	10.0	8.0	7.0
	20	7.0	4.0	3.0	In	0.5	0.5	In	In	0.5	0.5	6.0	9.0	5.5	5.5
	24	3.5	1.5	In	In	In	In	In	In	In	In	5.5	8.5	4.0	5.0
	28	In	In	In	In	In	In	In	In	In	In	2.0	7.0	1.5	2.0
	32	In	In	In	In	In	In	In	In	In	In	0.5	5.0	S	S
	36	In	In	In	In	In	In	In	In	In	In	In	3.5	In	In
	40	In	In	In	In	In	In	In	In	In	In	In	2.0	In	In
	44	In	In	In	In	In	In	In	In	In	In	In	1.0	In	In
	48	In	In	In	In	In	In	In	In	In	In	In	In	In	In
5°C	8	9.0	7.0	5.5	4.0	7.5	5.0	4.0	In	8.0	4.0	4.0	10.0	4.5	5.0
	12	2.0	4.0	In	In	In	In	In	In	In	In	In	8.0	In	In
	16	In	In	In	In	In	In	In	In	In	In	In	6.5	In	In
	20	In	In	In	In	In	In	In	In	In	In	In	3.0	In	In
	24	In	In	In	In	In	In	In	In	In	In	In	In	In	In
10°C	4	11.0	14.0	10.0	10.0	12.0	9.0	9.0	7.0	13.0	15.0	14.5	11.5	11.5	10.0
	8	In	6.0	In	In	In	In	In	In	In	In	In	3.0	In	In
	12	In	0.5	In	In	In	In	In	In	In	In	In	In	In	In
	16	In	In	In	In	In	In	In	In	In	In	In	In	In	In
15°C	4	In	4.0	In	In	In	In	In	In	In	In	In	In	In	In
	8	In	In	In	In	In	In	In	In	In	In	In	In	In	In

Table 10. Inhibition zones (mm) around mycelial plugs of antagonists on petri dishes of PDA seeded with conidia of *B.cinerea*. Antagonists obtained from isolation petri dishes of collection four were incubated at 20°C.

		Isolates																
Temp	Day	FF-21	FF-31	FD-11	FT-41	FY-11	FY-21	FY-31	FY-41	FY-51	FO-11	FB-11	FB-21	FB-31	FB-41	FB-51	FB-61	FB-71
0°C	20	In	5.5	4.0	In	In	In	In	1.0	In	In	In	In	In	In	In	In	In
	24	In	5.0	3.5	In	In	In	In	1.0	In	In	In	In	In	In	In	In	In
	28	In	1.0	1.5	In	In	In	In	In	In	In	In	In	In	In	In	In	In
	32	In	1.0	1.5	In	In	In	In	In	In	In	In	In	In	In	In	In	In
	36	In	1.0	1.0	In	In	In	In	In	In	In	In	In	In	In	In	In	In
	40	In	S	S	In	In	In	In	In	In	In	In	In	In	In	In	In	In
5°C	8	1.0	2.5	1.5	In	S	S	In	5.5	1.0	In	0.5	S	0.5	0.5	S	1.0	S
	12	In	1.5	0.5	In	In	In	In	3.0	In	In	In	In	In	In	In	In	In
	16	In	1.0	0.5	In	In	In	In	In	In	In	In	In	In	In	In	In	In
	20	In	S	S	In	In	In	In	In	In	In	In	In	In	In	In	In	In
10°C	8	0.5	0.2	0.5	In	1.0	2.0	2.0	1.0	S	1.0	S	S	0.5	S	1.0	2.0	S
	12	S	In	In	In	S	S	S	S	S	In	S	S	S	S	S	S	S
15°C	4	In	4.5	1.0	S	In	In	In	In	In	In	In	In	S	S	In	In	In
	8	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In	In

Isolate of *Trichoderma* was FY-41. Isiolate of *Phoma* was FF-31. Isolate of *Epicoccum* was FD-11.

Table 11. Inhibition zones (mm) around bacterial streaks of antagonists on petri dishes of PDA seeded with conidia of *B.cinerea*. Antagonists from isolation petri dishes of collection four were incubated on 20°C.

		Isolates							
Temp	Day	BO-11	BO-21	BT-11	BT-21	BB-11	BY-11	BS-11	BD-11
0°C	20	In	In	In	In	3.5	0.5	3.0	2.0
	24	In	In	In	In	1.5	In	In	In
	28	In	In	In	In	In	In	In	In
5°C	12	In	In	In	In	In	In	In	In
10°C	4	6.0	7.0	6.0	4.0	3.5	2.0	1.0	1.0
	8	In	In	In	In	In	In	In	In
15°C	4	In	In	In	In	In	In	In	In

Isolate of actinomycete was BB-11.

APPENDIX 2

Table 1. The effect of different temperatures on inhibition zones (mean and standard error) of *B.cinerea* caused by the antagonists FF9, FO30, FB3 on various media.

Temp	PDA		MA		V.8		CMA		KFJ (1:20)		KFJ (1:30)	
C	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE
0	23.1	3.2	22.6	2.8	23.1	3.2	24.3	3.1	19.0	3.1	22.9	3.8
5	18.6	3.7	20.5	3.2	19.3	3.7	17.8	3.7	20.2	3.6	20.6	3.9
10	24.8	3.7	23.3	3.2	21.9	3.7	23.3	3.6	24.6	3.6	20.3	3.9
15	20.4	4.6	23.4	3.9	20.1	4.6	22.1	4.5	21.8	4.4	23.7	4.8
20	18.8	4.6	16.0	3.9	22.3	4.6	18.1	4.5	23.5	4.4	19.7	4.8

Table 2. The effect of different isolates on inhibition zones (mean and standard error) of the *B.cinerea* caused by the antagonists FF9, FO30, FB3 on various media.

Iso	PDA		MA		V.8		CMA		KFJ (1:20)		KFJ (1:30)	
	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE
FF9	25.8	2.5	25.7	2.3	28.7	2.5	24.4	2.6	23.6	2.5	29.1	2.4
FO30	25.6	2.5	22.8	2.3	21.0	2.5	25.8	2.6	27.0	2.4	23.4	2.4
FB3	13.0	2.5	16.0	2.3	14.9	2.5	15.4	2.6	14.0	2.5	12.0	2.4

Table 3. The effect of time periods on inhibition zones (mean and standard error) of the *B.cinerea* caused by the antagonists FF9, FO30, FB3 on various media.

Days	PDA		MA		V.8		CMA		KFJ (1:20)		KFJ (1:30)	
	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE
5	26.1	4.5	23.4	4.0	23.8	4.5	27.3	4.5	28.7	4.0	27.0	4.7
9	19.0	3.6	21.4	3.3	22.2	3.7	18.9	3.7	21.9	3.3	20.7	3.4
14	27.8	4.5	25.6	4.0	21.3	4.5	26.7	4.5	27.5	4.1	23.5	4.7
26	19.8	3.6	20.4	3.3	23.3	3.7	20.4	3.7	20.4	3.3	19.8	3.8
36	20.3	4.5	19.8	4.0	23.5	4.5	18.7	4.5	17.0	4.1	21.1	4.7
49	22.8	6.3	21.8	5.7	13.5	6.3	19.3	6.4	14.0	5.8	24.2	6.6
65	13.0	6.3	16.0	5.7	13.5	6.3	18.1	6.4	14.0	5.8	12.0	6.6

APPENDIX 3

Table 1a. Number of infected fruit / tray with fruit immediately inoculated and stored over 13 weeks at 0°C.

Inoc.Level	REPLICATES			WEEKS		
		5	7	9	11	13
UNTREATED	1	0	0	1	1	1
	2	0	2	2	2	2
	3	1	3	3	3	3
DROP.WATER	1	1	3	3	3	3
	2	1	1	1	1	1
	3	0	0	0	0	0
B.suspension	1	2	4	4	4	4
	2	5	6	7	7	7
	3	8	8	8	8	8
B + FB3	1	4	6	8	8	8
	2	3	5	6	6	7
	3	4	4	4	4	4
B + FF9	1	1	2	2	2	2
	2	7	8	8	8	8
	3	0	1	1	1	1
B + FO30	1	0	0	0	0	0
	2	1	1	1	1	1
	3	1	1	1	1	1

B = *Botrytis*

Table 1b. Number of infected fruit / tray with fruit immediately inoculated, cured 2d at ambient temperature (8-12°C) and stored over 13 weeks at 0°C.

Inoc.Level	REPLICATES			WEEKS		
		5	7	9	11	13
UNTREATED	1	0	0	0	0	0
	2	0	1	1	2	2
	3	1	1	1	2	2
DROP.WATER	1	0	1	1	1	1
	2	1	2	2	2	2
	3	0	0	0	0	0
B.SUSPENSION	1	11	13	14	14	14
	2	7	7	8	8	9
	3	0	0	0	0	0
B + FB3	1	2	2	2	2	3
	2	3	3	5	6	6
	3	8	9	9	9	9
B + FF9	1	5	5	5	5	5
	2	2	4	5	5	5
	3	3	3	3	4	5
B + FO30	1	1	1	1	1	2
	2	0	0	0	0	0
	3	0	0	0	0	0

Table 1c. Number of infected fruit / tray with fruit cured 2d at ambient temperature, inoculated and stored over 13 weeks at 0°C.

Inoc.Level	REPLICATES	WEEKS				
		5	7	9	11	13
UNTREATED	1	0	0	0	0	0
	2	0	2	2	2	2
	3	0	0	0	0	0
DROP.WATER	1	0	0	0	0	0
	2	0	1	1	2	2
	3	0	0	0	0	0
B.SUSPENSION	1	3	4	5	5	6
	2	3	5	5	5	5
	3	3	6	7	8	8
B + FB3	1	6	7	7	7	7
	2	2	3	3	3	3
	3	1	3	3	3	3
B + FF9	1	2	2	2	2	2
	2	5	7	9	9	9
	3	0	1	1	1	1
B + FO30	1	1	3	3	3	3
	2	0	0	0	0	0
	3	1	1	1	1	1

Table 1d. Number of infected fruit / tray with fruit cured 2d at ambient temperature, inoculated, cured a further 2d at ambient temperature and stored over 13 weeks at 0°C.

Inoc.Level	REPLICATES	WEEKS				
		5	7	9	11	13
UNTREATED	1	0	1	1	1	1
	2	0	0	0	0	0
	3	0	0	0	0	0
DROP.WATER	1	0	0	0	1	1
	2	0	0	0	0	0
	3	0	0	0	0	0
B.SUSPENSION	1	6	8	8	8	8
	2	0	0	1	1	1
	3	0	1	2	2	2
B + FB3	1	0	0	0	0	0
	2	1	2	3	3	3
	3	0	0	0	0	0
B + FF9	1	3	4	5	5	5
	2	2	2	2	2	2
	3	4	5	5	6	6
B + FO30	1	0	1	1	1	1
	2	1	2	2	2	2
	3	0	0	0	0	0