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BIOLOGICAL CONTROL OF *BOTRYTIS CINEREA* IN LETTUCE & STRAWBERRY CROPS

A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy at Lincoln University, Canterbury, New Zealand

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

BIOLOGICAL CONTROL OF *BOTRYTIS CINEREA* IN LETTUCE & STRAWBERRY CROPS

by Stuart Douglas Card

Two preliminary biological control screens were developed, using lettuce & strawberry as model systems, to test potential biocontrol micro-organisms for their ability to control *Botrytis cinerea,* the causal agent of grey mould.

The first screen evaluated a range of saprophytic yeasts, bacteria & fungi for their ability to inhibit *Botrytis* lesions on whole lettuce plants. In a controlled environment (18 -22°C, 85-90% relative humidity & 12 h photoperiod), lettuce leaves were spray inoculated with antagonists (10^7 fungal spores mL^{-1} or 10^8 bacterial cells mL^{-1}), allowed to dry & challenged by spray inoculation with the pathogen (10^6 conidia mL^{-1}). After 7 days *Pseudomonas fluorescens* LU1001 & LU1003, *P. synxantha* LU1004 & *P. veronii* LU1002 had significantly reduced lesion areas by an average of 79% on whole lettuce plants. An *Ulocladium* sp. LU865 & *Epicoccum purpurascens* LU148 reduced lesion areas by 94% & 78%, respectively.

The second screen evaluated selected saprophytic bacteria & filamentous fungi, with previously identified antagonistic properties (against a range of plant pathogens), for the ability to inhibit *B. cinerea* on detached strawberry tissues & whole strawberry plants. The most successful isolates, *E. purpurascens* LU148 & *Trichoderma atroviride* LU132, inhibited lesion development by up to 32% & suppressed sporulation on detached strawberry leaves by up to 87%. They were further evaluated on detached strawberry flowers & whole strawberry plants in a controlled environment (18°C & 85-95% relative humidity. The most consistently effective antagonist was *T. atroviride* LU132, which inhibited *B. cinerea* in all experiments, significantly ($P \leq 0.05$) suppressing *B. cinerea* sporulation on detached strawberry flowers & leaf discs

removed from inoculated whole plants by 100% & 81%, respectively. This was similar to the level of disease suppression given by the commercial biocontrol product, Prestop & the fungicide, fenhexamid.

The mechanism of action of *T. atroviride* LU132 was investigated. Competition for glucose & sucrose occurred on detached strawberry leaf pieces & on polytetrafluoroethylene (PTFE) membranes, significantly inhibiting *B. cinerea* germ tube length at low sugar concentrations. The isolate also produced non-volatile inhibitory substances in minimal medium that inhibited *B. cinerea* germ tube elongation & in dual culture, *T. atroviride* LU132 was found to directly parasitize *B. cinerea* hyphae leading to cell collapse.

The biological activity of *T. atroviride* LU132 against the grey mould pathogen was investigated on three cultivars of strawberry under field conditions. The study also examined the effect of commonly used botryticides on germination & growth of *T. atroviride* LU132 to allow selection of a fungicide for mixed application. The fungicide fenhexamid significantly increased conidial germination frequency by up to 34% & was later used in mixed tank applications. The field trials demonstrated that treatment with *T. atroviride* LU132, fenhexamid, & the tank-mix of both could all equally suppress *B. cinerea* sporulation, significantly, on strawberry leaves & stamens by 42-100%. These treatments increased yield of strawberry fruit by up to 36% but they did not reduce disease incidence in the fruit in a 7 day ambient storage trial.

This research has identified *T. atroviride* LU132 as a successful biological control agent (BCA) of *B. cinerea* in strawberry. The more obvious mechanisms of action such as antibiosis, competition for nutrients & mycoparasitism have been investigated & the use of the BCA within an integrated management strategy, with the fungicide fenhexamid, has been successfully implemented. Future work will focus on developing a suitable formulation for the BCA in order to increase its efficacy under field conditions.

Keywords: *Botrytis cinerea*, grey mould, strawberry, lettuce, biological control, fluorescent *Pseudomonas*, *Epicoccum purpurascens*, *Trichoderma atroviride*, competition, antibiosis, mycoparasitism

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LIST OF ABBREVIATIONS & SYMBOLS

a.i.	active ingredient
ascn#	accession number
ACVM	agriculture & veterinary medicines group
ANOVA	analysis of variance
bp	base pair
BCA	biological control agent
cm	centimetre
cv.	cultivar
CFU	colony forming unit
©	copyright
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DF	dry flowable
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetate
FLO	flowable
g	gram
Gr	Greek
h	hour
IMP	integrated pest management
ITS	internally transcribed spacer
kb	kilobase
kg	kilogram
L	litre
Ltd	Limited
LPCB	lactophenol cotton blue
LSD	least significant difference
m	metre

mg	milligram
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
M	molar
ng	nanogram
nm	nanometre
NA	nutrient agar
NB	nutrient broth
NUV	near ultraviolet
NZ	New Zealand
Pers	Persoon
pH	potential of hydrogen
pmoles	picomole
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
PTFE	polytetrafluoroethylene
rpm	revolutions per minute
RH	relative humidity
RFLP	restriction fragment length polymorphism
®	registered trade mark
s	second
sp.	species
spp.	species plural
syn.	synonym
SA	strawberry agar
SC	suspension concentrate
Tris	Tris (hydroxymethyl) aminomethane
TM	Trade mark
U	units
UP-PCR	universally primed polymerase chain reaction

U.K.	United Kingdom
US	United States
U.S.A.	United States of America
UV	ultraviolet
v/v	volume per volume
v/w	volume per weight
V	volts
w/v	weight per volume
WA	water agar
WDG	water dispersible granule
WG	wettable granule
WP	wettable powder
6PAP	six pentyl-alpha-pyrone
μg	microgram
μm	micrometer
μM	micromolar
μL	microlitre
1:1	one to one ratio
°C	degrees Celsius
%	percent
-1	per
$	dollars (New Zealand, unless otherwise stated)
≤	less than or equal to
≥	greater than or equal to
>	greater than
<	less than
=	equal to
±	plus & minus
&	and
× *g*	gravity, measured in metres per second

CHAPTER 1:

Introduction

1.1 THE GENUS *BOTRYTIS*

Botrytis species are among the most ubiquitous plant pathogens & saprophytes (Jarvis, 1977). Persoon (1801) validated the genus *Botrytis*, after the original proposal by Micheli in 1729 & recently the genus was critically revised by Hennebert (1973), who reduced the 380 taxa (many of which had been assigned in error) to a total of 22 species. Most of these species occur in all continents of the world, including subtropical countries like Egypt & temperate countries such as New Zealand, to cool temperate regions like Greenland & Alaska. The class to which *Botrytis* spp. belong contains many of the moulds of industrial importance, with this genus including serious plant pathogens that can cause significant economic crop losses with a worldwide distribution (Onions *et al.*, 1981). *Botrytis* spp. can be recognised primarily by the unique way in which their conidia are produced on pegs on the bulky apices of branched conidiophores (Figure 1). This is emphasised by its name, Gr. Botrys, a bunch of grapes. Most members of the genus are saprophytes, with a few, including *Botrytis cinerea* Pers., being important necrotrophic pathogens of many plants, including vegetables & soft fruit (Table 1).

Figure removed for copyright compliance

Figure 1: Conidiophore of *B. cinerea* bearing conidia (Jarvis, 1980)

Table 1: The species in the genus *Botrytis* (Backhouse *et al.*, 1984; Hennebert, 1973; Jarvis, 1977; Pennycook, 1989)

Species [a]	Host range
B. aclada (syn. *B. allii*)	*Allium* spp.
B. anthophila	*Trifolium pratense* (Red clover)
B. byssoidea	*Allium* spp.
B. calthae [b] (*Botryotinia calthae*)	*Caltha palustris* (Marsh marigold)
B. cinerea (*Botryotinia fuckeliana*)	Polyphagous
B. convoluta [b] (*Botryotinia convoluta*)	*Iris* spp.
B. croci [b]	*Crocus versicolor*
B. elliptica	*Lilium* spp., *Colchicum autumnale*, *Cyclamen indicum*, *Gladiolus* spp., *Polyanthus tuberosa*
B. fabae (*Botryotinia fabae*)	Legumes including *Vicia faba*, *V. cracca*, *V. sativa*, *Phaseolus vulgaris*, *Pisum sativum*
B. ficariarum [b] (*Botryotinia ficariarum*)	*Ficaria verna* Huds (Lesser celandine)
B. galanthina	*Galanthus* spp. (Snowdrop)
B. gladiolorum (*Botryotinia draytonii*)	*Crocus versicolor*, *Freesia refracta*, *Gladiolus* spp., *Ixia* spp., *Watsonia* sp.
B. globosa [b] (*Botryotinia globosa*)	*Allium* spp.
B. hyacinthi [b]	*Lilium* spp.
B. narcissicola (*Botryotinia narcissicola*)	*Narcissus* petals & bulbs
B. paeoniae [b]	*Paeonia* spp., *Allium* spp.
B. perlargonii [b] (*Botryotinia perlargonii*)	*Pelargonium* spp.
B. polyblastis [b]	*Iris* spp., *Narcissus pseudonarcissus*
B. porri [b] (*Botryotinia porri*)	*Allium sativum*
B. ranunculi [b]	*Ranunculus septentrionalis* Poir (Swamp buttercup)
B. sphaerosperma [b]	*Allium triquetrum*
B. squamosa (*Botryotinia squamosa*)	*Allium* spp.
B. tulipae	*Lilium* spp., *Tulipa* spp. & *Allium* spp.

[a] Species in brackets indicate teleomorph

[b] Species currently absent from New Zealand (26/08/2004)

1.2 *BOTRYTIS CINEREA*

1.2.1 Taxonomy

Kingdom	Fungi
Phylum	Ascomycota
Class	Ascomycete
Order	Helotiales
Family	Sclerotiniaceae
Genus	Botryotinia

Botrytis is a non-phialidic amerospored genus belonging to the Ascomycetes & is a member of the order Helotiales. There has been much confusion over the taxonomy of this genus (Ormrod & Jarvis, 1994) as many *Botrytis* species are asexual states of other sexual species. For example, some are the conidial state of *Botryotinia* (a cup Ascomycete), some have other teleomorphs, such as *Sclerotinia* species (Onions *et al.*, 1981), & some have no known teleomorph (Dixon, 1984). The type species, *B. cinerea* is the conidial or anamorphic state of *Botryotinia fuckeliana* (syn. *Sclerotinia fuckeliana*) with the anamorphic name being preferred (cited in Prins *et al.*, 2000). There is a great deal of genetic variation within the species, which is induced by frequent mutations, aneuploidy (Baraldi *et al.*, 2002) & heterokaryosis (Backhouse *et al.*, 1984). The latter arising from individual vegetative mycelial, sclerotial & conidial cells containing two or more genetically distinct types of nuclei (Hansen & Smith, 1932). Sexual reproduction was only discovered within the family in the early 1930s (Faretra *et al.*, 1988) & as the teleomorph has rarely been detected in the field, it was thought that this type of reproduction was not related to the high amount of genetic variation found within the species. However, with the aid of recent molecular advances, it is now believed that sexual reproduction may be an important source of genetic variation (Faretra *et al.*, 1988) & may occur more frequently than previously thought (Giraud *et al.*, 1998). Isolates are therefore, more accurately termed '*Botrytis* of the *cinerea* type' (Bessey, 1950; Scheffer, 1997). In the Champagne region of France, this diversity is so striking that two sibling cryptic populations have been described, named *B. vacuma* & *B. transposa*. These populations have been shown to not only have differences in their adaptation on grape but also a difference in their resistant characteristics towards certain fungicides (Albertini *et al.*, 2002).

1.2.2 Morphology

Primary identification of *B. cinerea* is from its atypical spore & macroconidiophore structure (Figure 1). This is a specialised structure that incorporates a terminal cluster of synchronously produced hydrophobic conidia, borne on a well-developed macroconidiogenous hypha (Willets, 1997), which resembles a bunch of grapes (Figure 1). These hyphae originate from a cushion-shaped stroma, which forms just beneath the host surface. Macroconidia are large, specialised blastoconidia (Samson *et al.*, 1984; Willets, 1997) measuring 8-14 × 6-9 μm (Domsch *et al.*, 1980). Macroconidia (Figure 2) are oval, dry & hydrophobic, being produced singly on lateral branches for easy dispersal. When mature, the conidia are easily detached from the ampullae by rain splash &/or air turbulence, which also serve to distribute them.

Primary cultures on potato dextrose agar (PDA) have a grey fluffy appearance, hyaline (light coloured) at first, later becoming grey to greyish-brown with dark walled erect septate hyphae that grow in a creeping manner (Domsch *et al.*, 1980). The dark colour is attributed to the mature conidiophores, which branch alternately (Jarvis, 1980), frequently, in an irregular, erect, dendroid arrangement (Gilman, 1957).

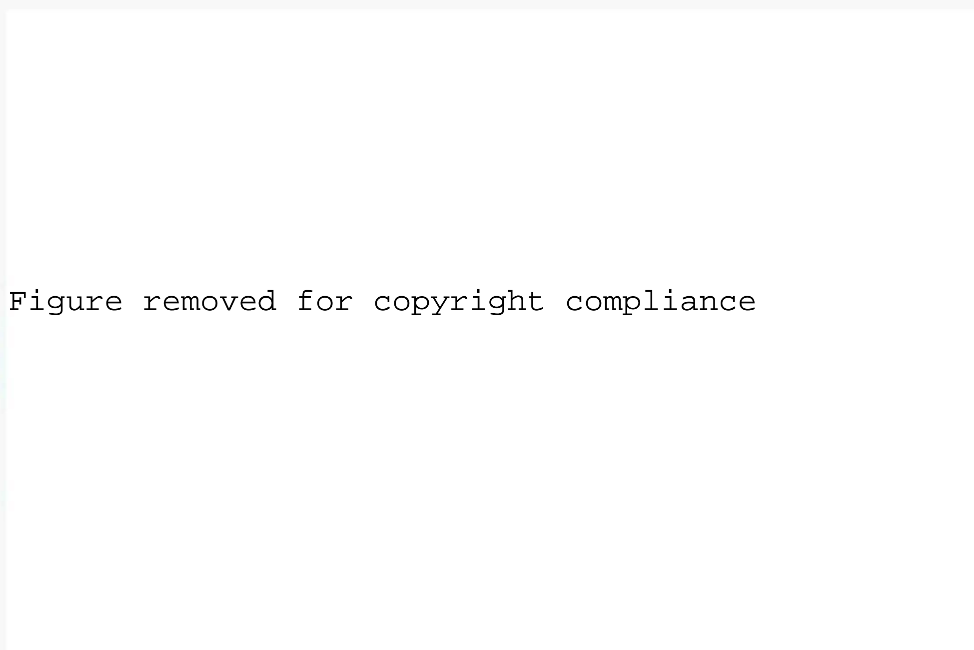

Figure 2: Conidia of *B. cinerea* showing nuclei (reproduced from the Crop Protection Compendium, 2004 Edition. © CABI, Wallingford, U.K.)

When conditions are unfavourable for growth, the fungus can form a secondary mycelial phase, a compact stone-like sclerotium (Figure 3), which comprises a dark mass of hyphae consisting of a medulla & a dark brown to black cortical layer of cells extremely variable in shape & size (Samson *et al.*, 1984). The evolution of sclerotia is proposed to have originated in the northern hemisphere & to have developed into effective resting structures towards the end of the ice age, when only those plants & their pathogens adapted to the harsh conditions survived (Willets, 1997). Human activity is then proposed to be the main mechanism by which these fungi were widely distributed around the world.

Figure removed for copyright compliance

Figure 3: Sclerotia of *B. cinerea* on tip of a necrotic highbush blueberry branch (reproduced from APSnet, 2004. © APS Press. U.S.A.)

Sclerotia are extremely effective resting structures, with many competitive advantages over other fungal structures as they are reserve-rich, thus having the ability to last for years within the soil (Fokkema, 1993). Sclerotia of *Botrytis* species are characterised by their planoconvexoid shape, which develop by lateral branching on the surface (Willets, 1997) or just beneath the cuticle of dead host tissue. They are flat & concave on the attachment surface with the hyphae interwoven, embedded within a hyaline flexible to gelatinous matrix (Jarvis, 1980). Sclerotia rarely germinate to form apothecia, with their

usual mode of development being through conidiophore production, which can continue for up to two months in the field (Jarvis, 1977).

1.2.3 Diseases

The majority of *Botrytis* spp. are seen as specialised pathogens as they are usually restricted to a few hosts. In many cases, *Botrytis* species can cause primary lesions in non-host plants but the lesions do not expand (Prins *et al.*, 2000). In contrast, *B. cinerea* is a ubiquitous pathogen (Elad *et al.*, 1996), with over 100 hosts cited in New Zealand (Pennycook, 1989) & over 230 hosts worldwide (Jarvis, 1977), & causes a variety of diseases. Pathogenic strains are typical necrotrophs although some parts of the actual infection cycle are still not clearly understood. Some strains of *B. cinerea* can grow on the phylloplane of plant surfaces without harming the healthy host tissue (Scheffer, 1997). Other strains, which differ in virulence, cause serious diseases on a wide range of nursery plants, vegetables, orchard crops, ornamental flowers & fruits within the field, & especially the glasshouse. Secondary rots caused by the pathogen are also a very serious problem within transit, storage & the market place. The pathogen can also create a latent or quiescent infection in which *B. cinerea* becomes active, causing typical symptoms visible only in ripe fruit. This characteristic can lead to particularly devastating losses in soft fruits like strawberry & kiwifruit.

Grey mould, caused by *B. cinerea* is the most common disease worldwide. This disease affects many herbaceous annuals & perennials, including houseplants, trees, shrubs, vegetable & small fruit plants (an extensive list was prepared by Schuster, 2004). It causes damage on flower parts, leaves, buds, shoots, seedlings, stems, & fruits. On some fruit species, grey mould may be found not only on ripening & harvested fruit, but also on young green fruit. In New Zealand, grey mould affects many important horticultural crops such as kiwifruit, strawberry, boysenberry, lettuce, grape & tomato. On grapevines worldwide, the fungus causes the disease Botrytis bunch rot (Figure 4) that reduces the quality & quantity of the crop (Bulit & Dubos, 1988). Losses result from rotting of berries on the vine & the detrimental effects on the subsequent wine quality. It can also occur on table grapes & post harvest grapes in storage or transit.

Figure 4: Botrytis bunch rot of grapes

'Botrytis blight' is a term that usually refers to diseases of flowers & stems, & it is particularly devastating in rose, carnation, geranium, gerbera, cyclamen, primula, strawberry & tomato. *B. cinerea* is termed 'dry eye rot' or 'blossom end rot' when affecting the calyx of pear & apple fruits, although these diseases are not deemed important within New Zealand. *B. cinerea* can also affect seedlings, especially of strawberry (Maas, 1984), causing the disease 'Botrytis crown rot'. This disease is mainly found within the glasshouse where environmental conditions often favour the pathogen's life cycle, & in the field if soil is contaminated with sclerotia (Agrios, 1978).

1.2.4 Life cycle on strawberry

The pathogen exists as a saprophyte on senescing or dead plant material & can over-winter as dormant mycelia in & around plant debris, & in Northern latitudes as sclerotia in the soil (Maas, 1984). Strawberry plants shed a large number of leaves throughout their normal growth, especially in perennial systems, with several seasons of cropping. These leaves can contribute significantly to the build up of *B. cinerea* inoculum. Under favourable conditions, the pathogen produces a primary inoculum on sources such as mummified fruit, straw mulch & weed residues. This inoculum consists of one or more large crops of conidia, which are considered the most important structures implicated in

the infection process (Gindro & Pezet, 2001). These conidia are dispersed mainly by water splash & air currents. Once these conidia have established on the phylloplane & if favourable conditions of moderate temperature (15-25ºC) with long periods of high humidity (or surface wetness during the flowering period) are met, the germinating fungus can infect fruit, flowers & leaves. Powelson (1960) was the first to show that latent infection of flower parts was the most important route leading to Botrytis fruit rot of strawberries, & it is now widely accepted that direct infection of fruit by airborne conidia is not significant (Jarvis, 1962). Supporting experiments undertaken in New Zealand found that in over 80% of infected berries, the infection was found at the stem end (Beever, 1970) & that this infection could be reduced by removing flower parts following pollination. Young strawberry leaves can become quiescently infected at any time during the growing season (Sosa-Alvarez *et al.*, 1995) & unlike a number of other fungal pathogens, *B. cinerea* does not cause symptoms in these green leaves (Sutton, 1990a). Following tissue senescence, the pathogen can colonise & sporulate (Legard *et al.*, 2002) if favourable conditions occur providing a source of secondary inoculum in a few days. The fungus exhibits its classic polycyclic nature as conidia continue to be produced throughout the season, spreading infection throughout the crop. Botrytis fruit rot (Figure 5) is chiefly controlled through intensive use of chemical fungicides, which are applied from blossoming onwards to protect flowers & the subsequent fruit.

Figure 5: Sporulation of *B. cinerea* on strawberry fruit

1.2.5 Economic importance

B. cinerea is a devastating necrotrophic pathogen causing millions of dollars of damage to a wide range of fruit, vegetable & ornamental crops worldwide. The pathogen causes both pre- & post-harvest diseases in economically important crops especially in glasshouse production (Elad, 1996). The pathogen has been isolated from healthy & senescing plants, as well as from the aerial surfaces of plants already infected by other pathogens (Blakeman, 1980). Worldwide, the economically important host crops are grape, strawberry, cabbage & lettuce (Domsch *et al.*, 1980) & within New Zealand the fungus can cause severe economic losses in the export & domestic markets for vegetables, fruit, wine & flowers. In 1999, total New Zealand horticultural exports exceeded $1.7 billion (HortResearch, 2000). Markets that are primarily affected by *B. cinerea* diseases include the berry fruit & floriculture markets, valued at $17 million & $55 million respectively. The wine industry export earnings for 1999 exceeded $114 million & the tomato industry has a retail value of about $75-$80 million (Ivicevich, 2004). In 2000 the annual losses attributed to this pathogen were in excess of $20 million in the kiwifruit industry alone (HortResearch, 1999). These industries are all affected by the pathogen in some way, either through direct crop loss or through the millions of dollars spent every year in controlling this ubiquitous pathogen. The value of the fungicide market for Botrytis diseases in the Far East & North America was approximately $28.6 million US (Hewitt, 1988).

In New Zealand, horticulture is driven by exports & not by domestic sales. In 2001, fresh berry fruit exports totalled $20 million, with strawberry production being the most dominant. New Zealand has a cool climate & strawberries are usually grown in areas with the highest humidity, such as in the Auckland region, which has accounted for over 70% of the national crop, resulting in high incidence of fruit rots.

1.2.6 Ecology/physiology

B. cinerea, like many fungi, is only pathogenic towards a host when certain environmental factors are met. If these factors are not met then the fungus will usually remain a saprophyte within the crop, living on senescing or dead tissue or in a state of quiescence. *B. cinerea* can be found in all continents of the world, but due to the

distribution of economic hosts, the diseases it causes are usually associated with cool-temperate & warm-temperate zones (Jarvis, 1977). Within the glasshouse, grey mould, is therefore, often found within the coldest regions where high humidity levels & water on the phylloplane remain for the longest periods (Fletcher, 1984). Palti (1981) stated that "*B. cinerea* diseases are favoured by high humidity levels especially in ageing crops with close leaf canopies".

1.2.6.1 Conidial dispersal

Conidia of *B. cinerea* are very commonly trapped from air & their high numbers increases the likelihood of landing on susceptible plant parts. Although probably the most obvious means of reaching the host, this is by no way the only means. Insects have also been observed to vector *B. cinerea* conidia to susceptible plant organs. The New Zealand flower thrip is seen as an important vector for conidia of the pathogen in grape (Marroni *et al.*, 2004). Adults & fruit-feeding larvae of *Amorbia emigratella* & *Cryptoblabes gnidiella* collected from infected fruit clusters of *Myrica faya*, in Hawaii were heavily infested with viable conidia of *B. cinerea* (Duffy & Gardner, 1994). Viable conidia of the pathogen have also been isolated from the internal digestive tract of *Drosophila melanogaster* (Louis *et al.*, 1996) & external bodies of adult fungus gnats (James *et al.*, 1995) with the latter insect living in glasshouses containing conifer seedlings, which are very susceptible to grey mould.

After conidia have landed on a suitable host & prior to germination, there are a number of vital interactions between the conidia & the host surface. One of these events is the production of cinerean, an extra- cellular β-glucan by germlings when in aqueous solutions. Cinerean coats the germlings in a slimy sheath & attaches them to their host. It is also believed to play two other roles: as nutrient storage for the germinating conidia & also to protect them from possible dehydration (Prins *et al.*, 2000).

1.2.6.2 Conidial germination

Germination of conidia is mainly dependent on the availability of free water (Figure 6), which occurs when atmospheric relative humidity (RH) reaches saturation (93-100%) (Snow, 1949). At levels lower than 93% RH, conidia take longer to germinate. High

RH also helps the conidia in the uptake of nutrients, which are also important for germination (Blakeman, 1980). The optimum temperature for germination is 20°C (Jarvis, 1977), which is the same temperature optimum for growth. It has also been hypothesised that gaseous compounds play a role in the germination of *B. cinerea* conidia (Prins *et al.*, 2000) especially the colourless alkene, ethylene. This compound is often produced in plant tissues that are weakening or senescing &, although some pathogenic fungi are reported to produce ethylene, it has been demonstrated that few *B. cinerea* isolates are able to do so (Elad, 1997). The association between ethylene & grey mould development is not yet clearly understood & some researchers have speculated that the compound might favour disease development by stimulating germination & subsequent hyphal development as well as weakening the host tissue (Prins *et al.*, 2000).

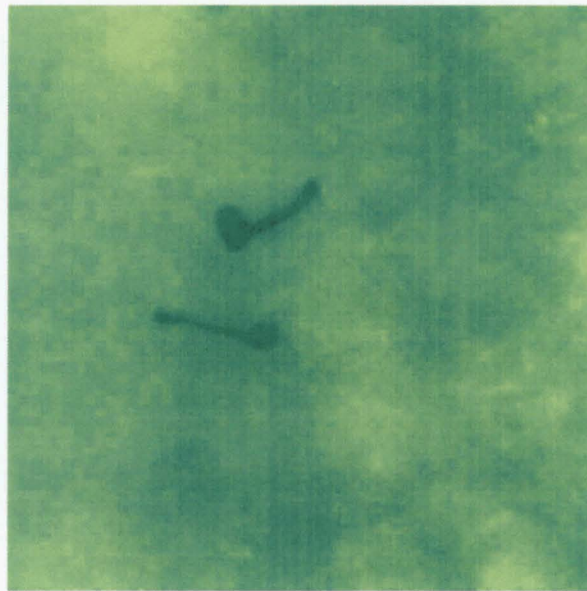

Figure 6: *B. cinerea* conidia germinating on strawberry leaves within a film of water

1.2.6.3 Infection

B. cinerea is able to penetrate host tissues directly by the use of enzymes including cutinases, pectinases & proteases, or by taking advantage of natural openings (such as via open stomata) or wounds. The infection process is favoured by an optimum temperature of 15°C with 12°C the minimum in the field (Domsch *et al.*, 1980), although Bulger *et al.*, (1987) found the optimum temperature for strawberry flower

infection to be approximately 20°C, with 100% infection at 24 h wetness. In strawberry fruit, infection originates with mycelium invading from already infected flowers (Braun & Sutton, 1988). Bristow (1986) found that once conidia had landed on the stigmas, they readily germinated in the stigmatic fluid but then required 4-6 weeks to grow down the styles to the receptacle. However it is now recognised that the most important route of infection is the mycelium that developed from germinated conidia growing down the stamens (Bristow *et al.*, 1986; Mertely *et al.*, 2002; Sutton, 1990b). Jersch *et al.* (1989) found that enzyme-inhibitory tannins, such as proanthocyanidins were largely responsible for the quiescence that followed.

1.2.6.4 Latency

The pathogen can cause a latent or quiescent infection, such as in young strawberry leaves & fruit receptacles (Legard *et al.*, 2000a). In strawberry leaves, microscopic observations by Braun & Sutton (1988) revealed that the fungus remained dormant in the epidermal cells as broad, lightly pigmented hyphae, which appeared to be an added survival mechanism. In strawberry fruit, the theory for quiescence is related to enzyme-inhibitory tannins such as proanthocyanidins, which are deposited in high concentrations within immature fruit (Jersch *et al.*, 1989). The conidia germinate, aided by the nutrients leached from pollen, & the hyphae start to make their way down to the receptacle. Here they are confronted with a variety of fungistatic & fungitoxic compounds, which inhibit the hyphal advance & this then, can lead to quiescence. The hyphae break into short fragments (Braun & Sutton, 1988) & fungal growth ceases (Prins *et al.*, 2000) until the fruit ripens, when levels of inhibitory compounds decrease & sugar levels increase. This latent phase is a common precursor to disease in many crop flowers including; pear, strawberry, raspberry, blackcurrant, apple, grape, eggplant & *Macadamia* (Jarvis, 1977) & can be devastating in the transit or storage of produce, in which no symptoms were visible at harvest.

1.2.6.5 Sporulation

B. cinerea can sporulate on all host plants (Prins *et al.*, 2000), usually profusely, with the process being largely dependant on irradiation, RH, temperature & wind speed (Willets, 1997). The process has a minimum requirement of 95% RH, with shorter macroconidiophores bearing many macroconidia being produced at lower RH levels

compared to longer conidiophores bearing few macroconidia being produced at near saturated atmospheres (Jarvis, 1977). On strawberry leaves (Figure 7), optimum temperatures for sporulation were found to be between 17 & 18°C at all tested wetness durations with decreased sporulation at 25°C & no sporulation at 30°C (Sosa-Alvarez *et al.*, 1995). Both the type & the intensity of light are important, with irradiation for 12 h inducing sporulation, followed by a further 8 h of darkness (Willets, 1997). In culture, sporulation can be induced by increasing the osmotic concentration within the growth media (Willets, 1997) &/or exposure to near ultraviolet light (NUV), wavelengths 290-400 nm (Tan & Epton, 1974) &/or the addition of NH_4NO_3 or asparagine (Domsch *et al.*, 1980). Once optimum conditions are met the fungus is able to exhibit its classic polycyclic nature & one infection cycle can be completed in 3-4 days (Prins *et al.*, 2000).

Figure 7: *B. cinerea* sporulation on strawberry leaf discs

1.2.6.6 Sclerotial formation

Sclerotia are the most efficient structures for long term survival of the fungus (Gindro & Pezet, 2001), with formation being favoured by low pH. The minimum RH for vegetative growth lies at 93% & mycelium has been found to survive for a year or more

at 90-100% RH at 0°C, whereas sclerotia can last over a year but not at these high humidity levels (van den Berg & Lentz, 1968). Temperatures that favour mycelial production depress sclerotial production & *vice versa.*

1.3 CONTROL MEASURES

1.3.1 Cultural control

There are many cultural steps that can be taken to suppress diseases caused by *B. cinerea.* Strategies aim to suppress initial infection, sporulation & survival of the pathogen.

1.3.1.1 Suppression of initial infection

This strategy is concerned with suppressing levels of primary inoculum entering the cropping system via either conidia or diseased planting material. Before planting a crop the disease history of the cropping field or glasshouse should be established. The fungus could be dormant in the soil or weeds or even in the crevasses in the actual glasshouse structure. Even if the specific cropping area is free from potential inoculum, neighbouring areas could have a high disease pressure & planting a crop in such a location should be avoided. Consideration should be given to the many alternate hosts of *B. cinerea,* & the risk that different species of neighbouring crops could pose as a primary inoculum source for the pathogen. When the crop is to be planted, pathogen-free seed & propagating material is essential & a simple seed sterilisation procedure before sowing is recommended (Jones, 1987). Sterilising the soil, to a temperature of 55°C for 15 min has been shown to be lethal to *Botrytis* spp. (McGovern & McSorley, 1997). This procedure can be effective for a small cropping area, but it has not been adopted by many commercial growers due to the high expense, a poor understanding of the technique & it's perceived impracticality.

1.3.1.2 Suppression of pathogen sporulation & survival

This strategy is possibly the most important as it is virtually impossible to eliminate the pathogen completely from the cropping area. Jarvis (1977) recorded 2×10^4 conidia m^{-3} at a height of 1 m above the ground & conidial velocity has been calculated at a rate of

0.22-0.45 cm s^{-1} (Domsch *et al.*, 1980). The numerous conidia produced therefore, have an enormous potential to be dispersed great distances. The pathogen also has many survival strategies that allow it to remain in the cropping system, either in the soil as sclerotia, as quiescent infections within plant leaves or flowers or as a saprophyte on dead tissues. To realistically reduce disease, it therefore seems logical to incorporate techniques that reduce sporulation by the pathogen within the crop & thereby, suppress production of secondary inocula. It is imperative that strict crop hygiene is applied at all times of the year to all areas of the cropping system including outbuildings such as the glasshouse or tool shed. All diseased plants & weeds, & plant debris, should be carefully removed from the crop & destroyed (Jones, 1987). Burning infective material is an effective method of reducing pathogen populations (Fry, 1982) but open fires are banned in various parts of the world & therefore, cultivating it into the ground is more acceptable & takes advantage of degradation by natural soil microorganisms. McGovern & McSorley (1997) also found that composting plant material for a period of 10-12 weeks at a temperature between 40-60°C eliminated *B. cinerea.*

The most important environmental factors, which promote infection by the pathogen are low vapour pressure deficit (high RH), free moisture on plant surfaces & cool weather (Elad *et al.*, 1996). Any technique that reduces RH &, therefore, the period of leaf & fruit wetness should be applied, together with techniques that increase air circulation & light penetration. Glasshouse environments are obviously easier to manipulate than crops grown in a field, although some of the following techniques can be applied to both glasshouse & field crops.

Techniques that reduce high relative humidity:

- Avoid over watering. Crops should be watered in the mornings to allow drying throughout the day & thus reduce damp conditions overnight.
- On strawberry crops, some growers use drip instead of overhead irrigation, which has proved worthwhile not only in reducing the period of leaf wetness but also in preventing splash dispersal of *B. cinerea* conidia (Legard *et al.*, 2000a).
- Air circulation & light penetration should be promoted within glasshouses. Heaters & ventilators should be used (Jones, 1987) in order to keep RH below the recommended threshold of 85%.

- Under field conditions, sowing or planting the crop in rows parallel to the direction of the prevailing wind is recommended (Palti, 1981). Selecting an appropriate site that is open &, therefore, exposed to sunlight & winds is more favourable than one that is enclosed & low-lying.
- Consideration should also be given to providing adequate plant spacing in order to avoid excessive foliage density that can lead to increased levels of RH (Palti, 1981).
- Pruning can promote ventilation & light penetration within the canopy of a crop. For example, in grapevines, exposing grape clusters to the sun by removing leaves around the fruit is recommended (Bulit & Dubos, 1988).

There are a number of other cultural techniques aimed at suppressing the pathogen, which are specific to certain crops. On strawberry & raspberry crops, polyethylene sheets used to provide barriers between the low lying plants has been successful in preventing disease caused by soil borne inoculum (Palti, 1981). In crops such as grape, selection of the variety can be important, as those with tighter clusters & thinner skins are more susceptible to disease (Bulit & Dubos, 1988). In a glasshouse crop of cucumber & tomatoes, grey mould was reduced by up to 20% when UV light (300-390 nm) was filtered out of sunlight (Fry, 1982) thus reducing the pathogen's ability to sporulate. The use of fertilisers can also be beneficial in suppressing disease. Potassium has been shown to promote wound healing in grapevines & the use of calcium has been shown to make host cell walls more resistant to penetration by *B. cinerea* (Palti, 1981).

1.3.2 Chemical control

Undoubtedly, the main method of controlling Botrytis diseases throughout the world is through the frequent use of chemical fungicides (Köhl *et al.*, 1995b). The value of the fungicide market for Botrytis diseases in the Far East & North America was approximately $28.6 million US (Hewitt, 1988), which included glasshouse & field crops as well as for protection against post-harvest rots in transit & storage.

The main botryticides can be categorised according to their biochemical mode of action & their market introduction date. There have been three important advancements in fungicide development over the last 80 years. Firstly, there was a move away from the crude, highly toxic, inorganic chemicals such as sulphur, copper & mercury, towards

more selective organic fungicides. The first of these families was the broad-spectrum dithiocarbamates developed for use as foliar, soil & seed treatments for fruit species. Table 2 shows the main multi-site fungicides used for controlling *Botrytis* spp., which generally work by affecting respiration, & includes thiram, one of the first organic sulphur fungicides (Ware, 2000). These broad-spectrum or multi-site fungicides although effective, required uniform application & only protected the plant where they covered the leaf, flower &/or fruit surface. To overcome this deficiency, the second important advancement came in the late 1960s, with the release of the systemic fungicides. These were much more specific than their protectant predecessors & constituted the "perfect method of disease control" (Ware, 2000). The plants could absorb the applied fungicides & therefore, total spray coverage, required by the older chemicals to cause maximum effect was not needed.

Table 2: The main broad-spectrum protectant fungicides with activity against *Botrytis* spp.

Chemical groups	Common names	Introduced	Trade names
Dithiocarbamates	Thiram	1931	Thiram®
Phthalimides	Captan	1952	Captan® Flo
Triazines	Anilazine	1955	Botrysan®
Sulphamides	Dichlofluanid	1965	Euparen® DF,
	Tolylfluanid	1972	Euparen® Multi 500 WG
Chlorophenyls	Chlorothalonil	1965	Chlorocarb® [a], Greenguard® [a]

[a] Fungicides that include chemicals from more than one chemical group

These chemicals worked by attacking the pathogen at its site of entry or activity & were more effective at lower doses than the protectants, allaying environmental concerns on the high amounts of pesticides used in horticulture. These newer fungicides were more selective & only targeted a single metabolic reaction. The benzimidazoles (Table 3) operated as antimicrotubule toxicants & paved the way for the newer systemic fungicides. Thiophanate methyl, although belonging to the group thiophanates, is converted to a benzimidazole by the host plant & the fungus through their metabolism (Ware, 2000).

Table 3: Systemic antimicrotubule toxicants that affect *B. cinerea*

Chemical groups	Common names	Introduced	Trade names
Benzimidazoles	Benomyl	1968	Benlate®
	Thiophanate methyl	1969	Topsin® M-4 A, Greenguard [a]
	Carbendazim	1972	Bavistin® DF, Chlorocarb® [a]
Phenylcarbamates	Diethofencarb	1988	Sumico®

[a] Fungicides that include chemicals from more than one chemical group

The dicarboximides (Table 4) were the second predominant group of systemics used against *Botrytis* diseases & their biochemical mode of action centred on lipid peroxidation. *B. cinerea* is a highly variable heterokaryotic pathogen & is perhaps the most important pathogen of protected crops where there is a problem, or potential problem, of resistance (Fletcher, 1984). The systemic fungicides (Table 3 & 4) were highly effective, efficient fungicides, which gave a high degree of control against *B. cinerea*. Unfortunately, because they were very specific, only affecting single metabolic targets, & were widely & intensively used on many crops, they were prone to serious fungicide resistance problems (Delp, 1995).

Table 4: The dicarboximides recommended for use against *Botrytis* spp.

Chemical group	Common names	Introduced	Trade names
Dicarboximides	Procymidone	1969	Sumisclex® 25 Flo
	Iprodione	1970	Rovral® WP
	Vinclozolin	1975	Ronilan®

As *Botrytis* species produce vast numbers of conidia, resistant mutants soon dominate pathogen populations if the same fungicide is routinely used. For example, resistance of *B. cinerea* to benzimidazole & dicarboximide fungicides is now widespread (Dik & Elad, 1999; Köhl *et al.*, 1998; Sutton *et al.*, 1997). In New Zealand, *B. cinerea* strains have been found that are resistant to both the benzimidazole & dicarboximide groups (New Zealand Agrichemical Manual, 2003). To help combat resistance, the third main advance in fungicides came in the 1990s with the introduction of the anilinopyrimidines, the phenylpyrrol & hydroxyanilide groups of fungicides (Table 5). These have all shown excellent activity against *B. cinerea* (Rosslenbroich & Stuebler, 2000).

Table 5: Newly introduced botryticides

Chemical group	Common names	Introduced	Trade names
Anilinopyrimidines	Mepanipyrim	1990	Frupica®
	Pyrimethanil	1992	Scala®
	Cyprodinil	1994	Switch® [a]
Phenylpyrroles	Fludioxonil	1990	Switch® [a], Maxim®
Hydroxyanilide	Fenhexamid	1998	Elevate® 50, Decree® 50 WDG, Teldor® 500 SC

[a] Fungicides that include chemicals from more than one chemical group

1.3.3 Biological control with commercially available products

Over the past century, growers have relied heavily on the use of chemicals to control diseases caused by *B. cinerea*. Fungicide resistance coupled with current public concern for both the environment & pesticide residues in food (Mehrotra *et al.*, 1997), has highlighted the need for alternative methods for disease control & biological control is one such approach. There are differing opinions of the role that biological control plays in the overall management of plant diseases. Many growers & chemical companies completely ignore the concept whilst others view it as a complete pesticide replacement. Irrespective of opinion it must be noted that there is a worldwide concern over the environmental drawbacks of many current chemicals employed to control plant diseases (Fokkema, 1993). In addition, the cost of developing new fungicides is high as shown by the fact that in the U.S.A. in 1993 it was nearly 40 times as expensive to register a synthetic pesticide than an indigenous, non-manipulated micro-organism (Hewitt, 1988). Agrochemical companies should, therefore, view natural antagonists as not only aiding in the discovery of potentially new biochemical modes of action, but also as key components to be used in anti-resistance management strategies that could increase the sales life of their products. Growers should also be aware of this last point & take advantage of the fact that certain biocontrol agents can be used in integrated pest management (IPM) strategies, which incorporate cultural, chemical & biological controls, therefore reducing the amount of chemicals used & delaying development of resistance towards these chemicals by the pathogen.

Few biological control agents have been commercialised, especially for use as foliar treatments (Elad *et al.*, 1996) & even fewer have had any real impact in crop protection (Hewitt, 1988). Compared to the number of successful agents reported to be screened in the literature, there are only a limited number of biological products commercially available (Table 6) recommended for use against *B. cinerea* diseases. Powell & Faull (1989) stated that "there is obviously a serious gap between scientific observations in the laboratory & the practicalities of mass production & field usage of biological control agents". From a pathogen control viewpoint, potential agents must have a high speed of action relative to the pathogen. They must persist for a required period of time in order to control the pathogen & at the same time be environmentally tolerant to fluctuating temperatures, UV light, moisture & pH levels.

Table 6: Commercially available biological control agents with activity against *B. cinerea*

Trade name	Manufacturer	Active ingredient
Trichodex®	Makhteshim Chemical Works Ltd, Israel	*Trichoderma harzianum* T39
Serenade®	AgraQuest Inc., U.S.A.	*Bacillus subtilis* QST713
Polyversum®	Remeslo SSRO, Czech Republic	*Pythium oligandrum* ATCC38472
Biosave®	Village Farms, U.S.A.	*Pseudomonas syringae* ESC-10
Prestop®	Kemira Agro Oy, Finland	*Gliocladium catenulatum* Xi, R43
Sentinel®	Agrimm Technologies Ltd, New Zealand	*Trichoderma atroviride*
BotryZen®	BotryZen Ltd, New Zealand	*Ulocladium oudemansii*

Then from a commercial viewpoint, there are many formulation & application problems that need to be overcome in order to maximise the effectiveness of the biocontrol agent. These may be far more complex than the systems used for chemicals, as biocontrol agents are living organisms. "The limiting factors of biological control products has been their efficacy, reliability & activity when compared to chemical pesticides" (Mehrotra *et al.*, 1997). The commercialisation process can be complicated further with registration & patent laws that can delay or even halt the process.

Trichodex® is probably the most widely used biocontrol agent that is effective against grey mould & has successfully been registered in a number of countries including Israel, Australia, Bulgaria, Chile, Greece, Hungary & Romania. It is currently being registered in the U.S.A., South Africa, throughout Europe & Latin America. Trichodex® comes as a wettable powder, which can be used as a foliar spray & applied with conventional chemical spray equipment. The agent is registered in Israel for use on grapes, tomatoes & cucumbers & recommended at a rate of 2-4 kg $hectare^{-1}$. The mode of action of *Trichoderma harzianum* T39, the active ingredient of Trichodex®, has been found to include competition for nutrients, inhibition of pathogenicity enzymes & induction of locally induced systemic resistance (Elad, 2000a, 2000b).

Trichodex® gave similar control to that of fungicides when used in alternation with a fungicide, therefore reducing chemical use by 50% (Elad *et al.*, 1996). Trichodex® was also effective in IPM strategies on grape & cucumber & was shown to spread to non-treated parts in the glasshouse (Elad *et al.*, 1996). Serenade® is a broad-spectrum preventative product recommended for the control of many plant diseases. It is currently registered in the U.S.A. to control *B. cinerea* in grape, cherry & pepper (Serenade fact sheet, 2004). The product is also recommended for use by conventional, sustainable & organic growers & is compatible with many fungicides & silicon surfactants (Serenade fact sheet, 2004). Polyversum® is registered as a plant growth stimulator & not a biological control agent. The product is, however, recommended for control of a variety of fungal pathogens including *Botrytis* spp. on many crops. On cucurbits, garlic, onion, pepper & tomato, the product is applied as a seed dressing & on grape, as a root spray at a rate of 250 g L^{-1} $hectare^{-1}$ (Polyversum data sheet, 2002). Biosave® is registered in the U.S.A. as a post harvest bioprotectant. The product is recommended for controlling *Penicillium expansum, Mucor piriformis* & *B. cinerea* of apples & pears, *P. expansum* & *B. cinerea* of cherries & *Fusarium sambucinum* of potatoes (Biosave 10 LP specimen label, 2004). Mycostop® is primarily targeted to *Fusarium* control (Backman *et al.*, 1997) & has been shown to reduce disease incidence of *B. cinerea* on lettuce, while also significantly increasing yield (White *et al.*, 1990). The product has shown inhibition of *B. cinerea* on dual culture plates with the mechanism of action attributed to antibiosis, although the biocontrol product has not been registered for the control of the grey mould, "...suppression of *Botrytis* can be mentioned in the label as a possible side effect" (Mohammadi, personal

communication). The same company has now developed a new product, named Prestop® WP, which is based on the filamentous fungus, *Gliocladium catenulatum*. "We have successfully tested the product for the control of grey mould in horticultural seedlings & strawberry plants" (Mohammadi, personal communication). In New Zealand, research by HortResearch has led to the commercialisation of BotryZen®, a preparation of *Ulocladium oudemansii* spores. BotryZen® is registered to control *B. cinerea* on grapes, with the mechanism of action determined to be competition for bunch trash & therefore exclusion of the pathogen from the senescent tissue (Graves, 2001).

The fact that these biocontrol agents have been commercialised & registered in many countries worldwide, shows that biological control agents are of significant importance in the future control of crop diseases. As more biological control products reach the market place & receive more public attention, this will pave the way for development of newer products to control *B. cinerea* & other fungal pathogens, together with the recent developments in molecular biology, also offer opportunities for the genetic modification of biocontrol agents. Techniques such as hybridisation can produce mutant strains that are resistant to certain chemicals & the use of UV radiation has been shown to produce strains of biocontrol organisms that are more effective in disease suppression than the wild types (Mehrotra *et al.*, 1997).

The most recent strategy for controlling *B. cinerea* & slowing down its resistance to chemical fungicides is integrated pest management (IPM), which selects the most effective technologies from cultural, chemical & biological control systems. "Integration of chemical & biological control agents may provide an opportunity not only to reduce chemical use but also to cope more effectively with the development of pathogen populations resistant to common fungicides" (Shtienberg & Elad, 1997). Realistically, biological control agents will not replace chemicals for the control of fungal disease in the foreseeable future, but will complement them & allow the development of improved integrated control measures (Jutsum, 1988).

1.4 RESEARCH INTO BIOLOGICAL CONTROL

In its widest sense biological control is a means of controlling disease or reducing the effects of pathogens by relying on biological mechanisms or organisms other than man (Campbell, 1989). In Morocco for example, Arras *et al.* (1995) successfully used the oil extract from *Thymus capatus* to reduce grey mould on stored mandarin fruits. Observations showed that the oil (formulated & applied as a vapour) reduced fungal growth significantly, compared to the control, by injuring the fungal hyphae. Oil extracts have also been assessed in New Zealand for the inhibition of *B. cinerea.* Antonov *et al.* (1997) evaluated 21 oil extracts at varying concentrations for their effects on conidial germination, germ tube elongation & subsequent mycelial growth of the pathogen. Extracts of thyme (*T. capitatus*), clove (*Syzygium aromaticum*) & Australian minttree (*Prostanthera rotundifolia*) were found to reduce conidial germination & germ tube length significantly at a concentration of 0.1%. Bryophyte extracts have also been studied for controlling *B. cinerea.* In Ethiopia, ethanol extracts from tested species significantly reduced mycelial growth of *B. cinerea in vivo* & gave a 15-23% reduction in grey mould severity when used to protect green pepper plants, compared to the pathogen only control (Tadesse *et al.*, 2003).

Andrews (1992) defined biological control as simply being "applied ecology" & in relation to controlling plant pathogens, biocontrol is often the artificial introduction of antagonistic microorganisms into the environment. "Microorganisms rank among our best allies in the control of plant disease" (Sutton & Peng, 1993b). Wood (1951) pioneered the early work, where a variety of fungi, actinomycetes & bacteria were screened for control of grey mould on lettuce, both *in vitro* & *in vivo.* Since then, there has been inconsistent success in the field (Sutton & Peng, 1993b), largely due to lack of knowledge of the complex interactions between the host plant, the biocontrol agent, the pathogen & the natural microflora as depicted by Figure 8. Due to this lack of knowledge & understanding, widespread commercial use is currently unlikely. A realistic short-term goal is the integration of biocontrol agents with fungicides, this combination has been shown to give little or no loss in disease control (Edwards *et al.*, 1994). A wide range of bacteria (Table 7) & fungi (Table 8) together with a variety of yeasts (Table 9) have shown control or an antagonistic potential towards *B. cinerea*, either *in vitro* or on a range of glasshouse & field crops.

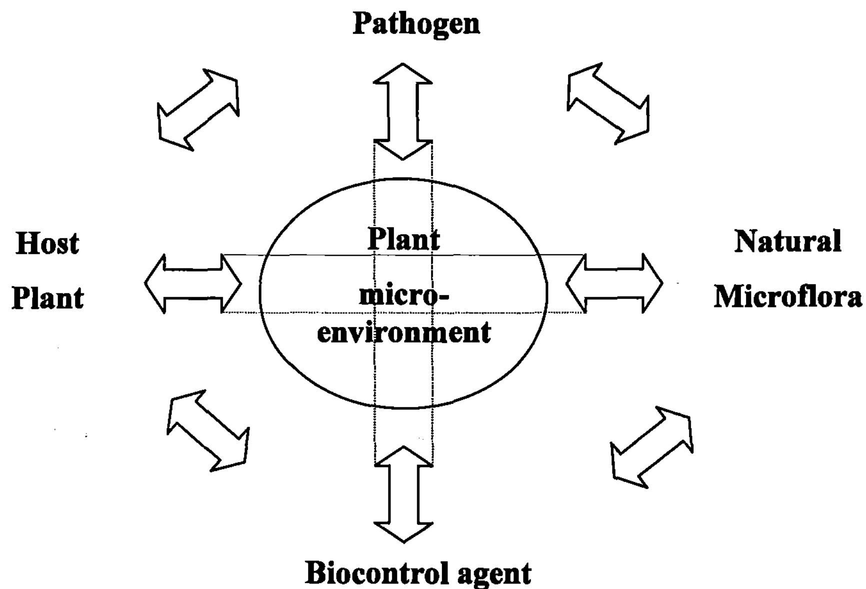

Figure 8: The complex interactions involved in biological control (taken from Edwards *et al.*, 1994)

"All three groups of biocontrol agents require development of appropriate formulations to enhance application & survival, & all require additional study to elucidate the various modes of action against the fungal pathogens" (Punja, 1997). Research must now aim to include molecular techniques to characterise &/or modify strains to evaluate their ecological competence & aim to enhance their survival (Punja, 1997). Sutton & Peng (1993b) also highlighted the fact that successful biological control depends largely on methods & strategies for introducing & maintaining these organisms within the cropping system.

1.5 MECHANISMS OF ACTION

Biocontrol agents (BCAs) should be selected such that their known mechanisms of action are able to be effective at one of the vulnerable periods of the pathogen's life cycle. There are three strategies with respect to the biological control of foliar pathogens, such as *B. cinerea* (Fokkema, 1993):

- *Microbial suppression of infection.* This includes the stages from conidia arriving on the host tissue, through germination & penetration of host tissue by the germ tube.

- *Reduction of sporulation.* This is of particular importance in the control of *B. cinerea* as the pathogen can undergo multiple infection & sporulation cycles within a single season within the same crop.
- *Suppression of the survival of the pathogen.* This strategy focuses on the dormant states of the pathogen, such as sclerotia & vegetative mycelia.

The ability of specific biocontrol microorganisms to deliver on these strategies is however related to their interaction(s) with the target pathogen(s). The recognised mechanisms of action are competition, antibiosis, mycoparasitism & host induced resistance (which is increasingly being recognised as important). Microorganisms may incorporate one or more of these modes of action in their antagonism towards *B. cinerea*.

1.5.1 Competition

Antagonism by competition is brought about when there are insufficient resources for the populations of microorganisms on the host surface, therefore causing them to compete for these resources in order to multiply/grow & survive. Campbell (1989) defined competition as being "when two (or more) organisms require the same thing & the use of this by one reduces the amount available for the other". Only those factors, which are limiting can be competed over, for instance carbohydrates, space, amino acids, oxygen, sugars, &, with particular reference to *B. cinerea*, necrotic tissue. Water though, cannot be competed over, as water is either available or not available, but the space where water is most obtainable can be competed over (Campbell, 1989). Competition is viewed by some as the most important model for the antagonism of *B. cinerea* (Cook *et al.*, 1997).

Fokkema (1993) suggested that biocontrol of *B. cinerea* diseases could be achieved by the suppression of pathogen infection & in particular the initial germination of *B. cinerea* conidia. It has been well reported that the conidia require external nutrients for germination (Elad, 1996) & growth before infection (Redmond *et al.*, 1987). At this susceptible stage of growth *B. cinerea* conidia have been reported to leach nutrients into surrounding water & thus, this induced nutrient stress can result in competition for the pathogen's own leachates.

Table 7: Bacteria that have shown recent biological control activity against *B. cinerea*

Species	System & reference	Proposed mechanism of action [b]
Bacillus sp.	Apple [a] (Sholberg *et al.*, 1995)	Unknown
	Apple [a] (Touré *et al.*, 2004) & ginseng [a] (Chung *et al.*, 1998)	Possibly A
	Grape (Paul *et al.*, 1998)	ISR & possibly A
	Tomato (Tsomlexoglou *et al.*, 2001)	A & possible CS & CN
B. licheniformis	*In vitro* (Walker *et al.*, 1998)	A
B. mycoides	Strawberry (Guetsky *et al.*, 2002b)	A
B. pumilus	Strawberry (Swadling & Jeffries, 1998)	A
B. subtilis	*Astilbe* micro plants (Leifert *et al.*, 1995) & *in vitro* (Walker *et al.*, 1998)	A
	Rose petals (Tatagiba *et al.*, 1998)	Unknown
Brevibacillus brevis	Chinese cabbage (Edwards *et al.*, 1994)	A, using gramicidin-S, C &
	Tomato & lettuce (McHugh & Seddon, 2001)	changing leaf surface wettability
Enterobacteriaceae spp.	Strawberry fruit (Guinebretiere *et al.*, 2000)	CN
Paenibacillus polymyxa	Strawberry (Helbig, 2001) & *in vitro* (Walker *et al.*, 1998)	A &/or CN &/or CWDE
Pseudomonas aeruginosa	Bean & tomato (Audenaert *et al.*, 2001)	ISR
Ps. antimicrobica	*In vitro* (Walker *et al.*, 2001)	A
Ps. cepacia	Apple & pear [a] (Janisiewicz & Roitman, 1988)	A, using pyrrolnitrin
Ps. fluorescens	Lettuce (Card *et al.*, 2002)	Unknown
	Petunia (Gould *et al.*, 1996)	A & possibly C
	Strawberry (Swadling & Jeffries, 1998)	A

Ps. fluorescens (continued)	White cabbage [a] (Leifert *et al.*, 1993)	Possibly A, CN
Ps. marginalis	*In vitro* (Cook *et al.*, 1997)	Possibly C
Ps. putida	*In vitro* (Moline *et al.*, 1999)	Possibly A, CN & M
Ps. synxantha	Lettuce (Card *et al.*, 2002)	Unknown
Ps. veronii	Lettuce (Card *et al.*, 2002)	Unknown
Serratia liquefaciens	Grape leaves (Whiteman & Stewart, 1998)	Possibly A
	White cabbage [a] (Leifert *et al.*, 1993)	Possibly A, CN
S. plymuthica	White cabbage [a] (Leifert *et al.*, 1993)	Possibly A, CN

[a] post harvest control.

[b] A=antibiosis, C=competition, CN=competition for nutrients, CS=competition for space, CWDE=cell wall degrading enzymes, ISR=induced systemic resistance, M=mycoparasitism

Table 8: Fungi that have shown recent biological control activity against *B. cinerea*

Fungi	System & reference	Proposed mechanism of action [b]
Acremonium breve	Apple [a] (Janisiewicz, 1988)	Unknown
A. cephalosporium	Grape (Zahavi *et al.*, 2000)	Unknown
Aureobasidium pullulans	Cucumber (Dik *et al.*, 1999) & strawberry [a] (Lima *et al.*, 1997)	CN
Colletotrichum gloeosporioides	Strawberry (Peng & Sutton, 1991)	Unknown
Cladosporium oxysporum	Rose flowers (Tatagiba *et al.*, 1998)	C for substrate
Cl. cladosporioides	Tomato (Eden *et al.*, 1996)	C
Clonostachys rosea (syn. *Gliocladium roseum*)	Chickpea (Burgess *et al.*, 1997), *Eucalyptus* seedlings (Stowasser & Ferreira, 1997) & black spruce seedlings (Zhang *et al.*, 1996a)	C for seed exudates & colonisation of seed surface

Clo. rosea (continued)	Glasshouse flowers (Sutton *et al.*, 1997)	Unknown
	Raspberry (Yu & Sutton, 1998)	CN
	Rose (Morandi *et al.*, 2003; Nobre *et al.*, 2005)	C for senescent rose tissue
	Strawberry (Nobre *et al.*, 2005; Sutton & Peng, 1993a)	A & CN & ISR & M
	Detached strawberry flowers (Card *et al.*, 2004b)	Unkown
	Tomato (Nobre *et al.*, 2005; Sutton *et al.*, 2002)	C for substrate & possibly ISR
Epicoccum purpurascens	Bean (Hannusch & Boland, 1996)	C & possibly A
	Detached grape leaves (Stewart *et al.*, 1998) & detached strawberry leaves (Card *et al.*, 2003)	unknown
Gliocladium catenulatum	Bean & tomato (Elad *et al.*, 1994)	CN & ISR
	Detached strawberry flowers (Card *et al.*, 2004b)	Unknown
Penicillium sp.	Strawberry leaves (Peng & Sutton, 1991)	C
Pythium citrinum	*in vitro* (Paul, 2004)	Unknown
Py. paroecandrum	detached grape leaf (Abdelghani *et al.*, 2004)	Possibly M
Py. periplocum	Grape (Paul, 1999a)	M
Py. radiosum	Grape (Paul, 1999b)	M
Trichoderma atroviride	Strawberry (Card *et al.*, 2003, 2004a; Hjeljord *et al.*, 2001)	C & A
T. hamatum	Strawberry *in vitro* (Elad *et al.*, 2001)	Possibly M
T. harzianum	Apple (Batta, 2004) & grape (Harman *et al.*, 1996)	Unknown
	Bean, lettuce, pepper, tobacco, tomato (Elad *et al.*, 1996; Meyer *et al.*, 1998) & strawberry (Freeman *et al.*, 2004)	A & CN & ISR & M

T. koningii	Strawberry (Tehrani & Alizadeh, 2000)	A & M
T. longibrachiatum	Strawberry (Freeman *et al.*, 2004)	NOT through A & M
T. viride	Grape cell suspension (Calderon *et al.*, 1993)	Possibly ISR
	Strawberry leaves (Sutton & Peng, 1993a)	CN
Ulocladium atrum	Cyclamen (Köhl *et al.*, 1998), onion, strawberry (Köhl & Fokkema, 1998) & dead lily leaves (Köhl *et al.*, 1995b)	C for substrate
	Roses (Yohalem, 2000)	Unknown
Ulocladium sp.	Grape (Stewart *et al.*, 1998)	Unknown

[a] post harvest control.

[b] A=antibiosis, C=competition, CN=competition for nutrients, CS=competition for space, ISR=induced systemic resistance, M=mycoparasitism

Table 9: Yeasts that have shown recent biological control activity against *B. cinerea*

Yeast	System	Proposed mechanism of action [b]
Candida sp.	Strawberry fruit (Guinebretiere *et al.*, 2000)	CN
C. guilliermondii	Grape (Zahavi *et al.*, 2000)	Unknown
C. oleophila	Grape [a] (El-Neshawy & El-Morsy, 2003) & strawberry [a] (Lima *et al.*, 1996)	Unknown
Cryptococcus albidus	Apple [a] (Tian *et al.*, 2002), cucumber, tomato (Dik *et al.*, 1999) & strawberry (Helbig, 2002)	CN
Cr. laurentii	Apple [a] (Roberts, 1990) & pear [a] (Zhang *et al.*, 2005)	CN
	Sweet cherry [a] (Qin *et al.*, 2004)	Unknown
Exophiala jeanselmei	Rose (Redmond *et al.*, 1987)	CN
Metschnikowia sp.	Grape (Schena *et al.*, 2000)	CN & CS

M. fructicola	Strawberry (Karabulut *et al.*, 2004)	CN & CS
Pichia anomala	Grape (Masih *et al.*, 2000)	Secretion of CWDE
P. guilliermondii	Apple fruit (Wisniewski *et al.*, 1991)	Tenacious attachment & CWDE
	Strawberry (Guetsky *et al.*, 2002b)	CN
P. membranifaciens	Grape (Masih *et al.*, 2001)	Secretion of CWDE
	Sweet cherry [a] (Qin *et al.*, 2004)	Unknown
	Apple fruit [a] (Santos *et al.*, 2004)	Antibiosis through killer toxins
Rhodotorula glutinis	Apple (Sansone *et al.*, 2005), cucumber, grape, tomato (Kalogiannis *et al.*, 2001), sweet cherry [a] (Qin *et al.*, 2004) & Geranium (Buck, 2004a, 2004b)	Unknown, possibly CN
Trichosporon sp.	Apple [a] (Tian *et al.*, 2002)	CN
T. pullulans	Sweet cherry [a] (Qin *et al.*, 2004)	Unknown

[a] post harvest control.

[b] A=antibiosis, C=competition, CN=competition for nutrients, CS=competition for space, CWDE=cell wall degrading enzymes, ISR=induced systemic resistance, M=mycoparasitism

Brodie & Blakeman (1975) showed that in the first few minutes of a suspension of dry conidia entering water there followed a leakage of previously labelled ^{14}C in the range of 2.5-20%. This was thought to be due to the reorganisation of membranes on hydration. Thus, microorganisms that can take advantage of this situation could suppress pathogen infection &, therefore, be successful candidates for biocontrol. In early studies on chrysanthemum leaves, Blakeman & Fraser (1971) demonstrated the inhibition of germinating *B. cinerea* conidia by the presence of foliar bacteria. They showed that conidia on water drops leached nutrients into the immediate vicinity & that within these droplets there was a higher foliar, bacterial population compared to water droplets without *B. cinerea* conidia. They also demonstrated that the presence of these bacteria significantly inhibited the germination of *B. cinerea* conidia.

The importance of amino acids in the germination of *B. cinerea* conidia has also been confirmed. In studies using ^{14}C- labelled mixed amino acids added to water droplets on leaves, it was shown that over a period of 24 h the bacterial population had risen & taken up nearly 80% of the label. At this point, germinating *B. cinerea* conidia added to the droplets were strongly inhibited (Blakeman, 1993). A particular *Pseudomonas* sp. was found to rapidly utilise the amino acids (removing 80% in 5 h) & therefore, markedly inhibited the pathogen's germination.

Guinebretiere *et al.*, (2000) found that isolates from the *Enterobacteriaceae & Candida* spp. actively competed for nutrients on post harvest wounds of strawberry fruits. The antagonists were observed surrounding germinating conidia of *B. cinerea in vitro* & could have been utilising leached nutrients. Fokkema (1993) concluded that all yeasts on the phylloplane generally operate through nutrient competition. He also stated that "necrotrophic pathogens, which utilise exogenous nutrients can be antagonised by almost all microorganisms which are capable of colonising the phyllosphere". Examples of such saprophytic fungi include *Clonostachys* (Figure 9), *Gliocladium*, *Trichoderma* & *Ulocladium* species, which have shown great potential as biocontrol agents of *B. cinerea* as they are able to rapidly colonise the phyllosphere, especially necrotic tissue, & thus outcompete the pathogen. *Clonostachys* (syn. *Gliocladium*) *roseum* (Schroers *et al.*, 1999) "colonised & exploited" senescent tissue much more rapidly than *B. cinerea* on black spruce seedlings (Zhang *et al.*, 1996b) & leaves of strawberry, raspberry, cyclamen & begonia (Sutton *et al.*, 1997). The biocontrol agent

also reduced the sporulation of the pathogen by reducing available necrotic tissue which *B. cinerea* uses for secondary spread within the crop (Sutton & Peng, 1993a).

Figure 9: An isolate of *Clonostachys* sp. colonising senescing strawberry stamens

The saprophytic fungus *U. atrum* has also been found to work by a similar mechanism. It suppressed sporulation of *Botrytis* spp. on strawberry, onion, cyclamen & geranium (Köhl & Fokkema, 1998) by competitively excluding the pathogen from colonising necrotic leaves (Köhl *et al.*, 2000). In New Zealand, an *Ulocladium* isolate was found to consistently suppress *B. cinerea* sporulation on grape (Stewart *et al.*, 1998) & uppressed the pathogen by up to 90% in field trials under conducive conditions.

A possibly unique method of competition has been observed with the antagonistic bacterium *Brevibacillus brevis*, which was found to cause water droplets to spread & dry on the phylloplane of Chinese cabbage making the environment unsuitable for germination of *B. cinerea* conidia (Edwards *et al.*, 1992). Elad (1996) stated that "this phenomenon is probably widespread but has been neglected in our search for potent mechanisms of biocontrol".

1.5.2 Antibiosis

Fravel (1988) defined antibiosis as "antagonism mediated by specific or non-specific metabolites of microbial origin, by lytic agents, enzymes, volatile compounds or other toxic substances".

Bacillus spp. generally antagonise by antibiosis (Edwards *et al.*, 1994). Examples of antibiosis directed towards *B. cinerea* include a strain of *Bacillus subtilis* that was effective *in vivo* micro-plants of *Astilbe* spp. (Leifert *et al.*, 1995) & a strain of *Br. brevis* that produced the peptide antibiotic Gramicidin S (Edwards *et al.*, 1992). Swadling & Jeffries, (1998) have also demonstrated that a strain of *B. pumilus* produced an anti-fungal agent that was fungicidal towards germinating *B. cinerea* conidia, even when cell-free extracts were diluted by 1:4.

Species of *Pseudomonas* may also control *B. cinerea* through antibiosis. For example, *Pseudomonas putida* exhibited fungistatic effects towards conidia & fungicidal effects towards grey mould on stored fruit (Moline *et al.*, 1999). Janisiewicz & Roitman (1988) established that the powerful antibiotic pyrrolnitrin was the active metabolite produced by *P. cepacia*, which inhibited *B. cinerea in vitro* at concentrations below 1 mg L^{-1}. A strain of *P. fluorescens* was also found to produce a compound that was fungistatic towards conidia *in vitro* (Swadling & Jeffries, 1998). Similarly anti-fungal metabolites from *P. antimicrobica* were found to reduce the percentage of conidial germination on strawberry leaves (Walker *et al.*, 1996) & later showed a significant reduction in conidial germination, germ tube extension & a high rate of abnormal germination *in vitro* (Walker *et al.*, 2001).

There are a number of fungi that are known to include antibiosis as a mechanism of action, although there remains a great deal of controversy over the role that these metabolites play in biological control on the phylloplane. Pfender (1996) stated that there was still no definitive experiment able to show antibiosis in general, as the major mechanism for the biocontrol of fungi on necrotic/senescent aerial plant tissue. The role of antibiotics in biocontrol can only be clearly established by testing antibiotic deficient mutants & comparing them in dual cultures with the parent strains.

Species of the genera *Gliocladium* & *Trichoderma* are known to produce metabolites that are antagonistic towards *B. cinerea*. One, namely gliotoxin, was reported to inhibit conidial germination of *B. cinerea* in *vitro* (Pietro *et al.*, 1993), although, Howell & Stipanovic (1995) reported that gliotoxin deficient mutants gave equal biocontrol efficacy to that of the parent strains. *C. roseum* is also known to produce anti-fungal metabolites (Köhl *et al.*, 1998; Sutton *et al.*, 1997) although it has also been reported

that they play no role in biocontrol (Sutton *et al.*, 1997). There are many experiments involving dual plate cultures with *B. cinerea* where distinct zones of inhibition have been observed. Antibiotics are presumed responsible but it is also known that antibiotics are produced most abundantly in nutrient rich media (Campbell, 1989) & that the phyllosphere is often nutrient limiting (Andrews, 1992). Thus, the phyllosphere is thought to rarely have enough nutrients to support the production of these compounds (Pfender, 1996).

1.5.3 Mycoparasitism

Mycoparasites can produce enzymes like proteinase, mananase & chitinase to dissolve host cell walls, before penetrating the cells (Elad *et al.*, 2001). Mycoparasitism of *B. cinerea* can follow two of the strategies put forward by Fokkema, (1993). Antagonists, such as the yeast *Pichia guilliermondii*, have been observed tenaciously attaching to hyphae of the pathogen while secreting cell wall degrading enzymes (Wisniewski *et al.*, 1991). *Pythium oligandrum* is also a well-known mycoparasite & when in contact with *B. cinerea*, it was observed penetrating host hyphae causing lysis after 21 min on water agar (Laing & Deacon, 1991). Other well known parasites include *Trichoderma* & *Gliocladium* spp. (Elad *et al.*, 1996), these antagonists employ the strategies mentioned above as well as degrading the pathogen's survival structures, sclerotia. *C. roseum* has been reported to parasitize hyphae, conidia & sclerotia of *B. cinerea* (Sutton *et al.*, 1997). This mode of action seems to have positive implications, although according to Elad (1996), the role of mycoparasitism in control of *B. cinerea in vivo* is doubtful. His observations indicated that the process was too slow compared to the fast germination & host penetration rates of the pathogen.

1.5.4 Induced resistance

This mechanism has only recently been recognised as important in the control of diseases caused by *B. cinerea* (Elad, 1996). The general principle is that certain microorganisms can work as elicitors of the plants own biochemical defence mechanisms & therefore, restricts pathogen development within the plant (Agrios, 1978). Host induced resistance can be local, when the elicitor is applied to the pathogen infected site, or induced systemically, whereby the elicitor is applied at a location separated from the pathogen infected site. Elad *et al.* (1994) demonstrated this locally

induced resistance by adding dead cells of antagonists to whole bean plants. This reduced grey mould disease severity significantly. The antagonists that caused this response, namely a *Pseudomonas* sp. & *G. catenulatum* were applied a short distance from the *B. cinerea* infection site. It was concluded that a locally induced resistance mechanism was stimulated within the host plant as no inhibitory compounds were found to be produced by the antagonists (Elad *et al.*, 1994).

Induced systemic resistance was highlighted by Meyer *et al.* (1998) who showed that *T. harzianum* T39 (the active ingredient of Trichodex®) caused the induction of systemic induced resistance in a whole range of plants including tomato, lettuce, pepper, bean & tobacco. Here the antagonist was applied as a soil treatment & considered responsible for the successful control of *B. cinerea* on the leaves of the plants. Induced resistance is often associated with the production of fungitoxic substances around the site of an injury & include mainly phenolic compounds (Agrios, 1978). An *in vitro* example of this host response was observed when cellulases from *T. viride* were applied to a suspension of grapevine cell culture. This induced plant defence reactions including a hypersensitive response & phytoalexin production (Meyer *et al.*, 1998). The use of yeast cell wall extracts has also been shown to elicit phytoalexin production (Reglinski *et al.*, 1995) to protect soyabean cotyledons & lettuce plants from *B. cinerea* infection. Jeandet *et al.* (1998) stated that "the use of elicitors of natural defence responses offers novel opportunities for biological control of crop protection". Elad (Elad, 1996) concluded that induced resistance overall is a credible mechanism by which suppression of *B. cinerea* can be achieved.

It is apparent that in many systems more than one mode of action is responsible for biocontrol (Elad, 1996). This can only aid in the effectiveness of the antagonist as a good biological control agent should be capable of inhibiting the pathogen during one or more key stages of its life cycle, as previously listed by Fokkema (1993). The challenge now comes in converting decades of phyllosphere research into effective & dependable biological control strategies (Andrews, 1992). The critical areas of research are focused on microbial ecology of plant microbe interactions, the factors that influence the dominance of these organisms & the development of suitable formulations & application systems for individual BCAs.

1.6 AIMS & OBJECTIVES OF THIS THESIS

In this study lettuce & strawberry crops were used as initial model systems to screen & evaluate potential BCAs for their antagonism towards *B. cinerea*. Lettuce & strawberry are key vegetable & fruit crops that suffer significant worldwide losses to diseases caused by *B. cinerea*. Once a successful screening model was developed & implemented, & a successful BCA identified, further work investigated the biocontrol activity exhibited under field conditions.

The key research objectives were to:

1. Develop an understanding of *B. cinerea* & its control by undertaking an in depth review of the current literature (Chapter 1).
2. Identify a suitable model for *B. cinerea* biocontrol of a vegetable or fruit crop with relevance to the New Zealand horticultural industry by:
    - the development of preliminary screens on whole lettuce plants (Chapter 2) & strawberry leaf tissues (Chapter 3).
    - selecting promising isolates for *B. cinerea* control potential (Chapters 2 & 3).
3. Investigate the mechanisms of action of the most successful isolate by using assays that would determine competition for nutrients, antibiotic production & mycoparasitism (Chapter 4).
4. Determine the compatibility of the most successful biocontrol isolate with fungicides to allow selection of a fungicide for mixed application (Chapter 5)
5. Assess the biological control activity of the most successful isolate on a model crop under field conditions (Chapter 5).

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CHAPTER 2:

Evaluation of microorganisms for biocontrol of grey mould of lettuce

2.1 FOREWORD

The research conducted at the start of this thesis focussed on two model systems directed at screening microorganisms for biocontrol of *Botrytis cinerea*. This chapter discusses the development & implementation of a screen using lettuce as the model system, with a strategy of inhibition of pathogen conidial germination.

The research presented in Chapter 2 was conducted by the first author with supervision from the other listed authors. The research has been published in New Zealand Plant Protection, (2002) Volume 55 Pages 197-201. This chapter presents an extended version of the original manuscript, which has been updated for thesis publication.

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2.2 ABSTRACT

A range of micro-organisms, including saprophytic yeasts, bacteria & fungi, were evaluated for control of *Botrytis* grey mould on whole lettuce plants. In a controlled environment (18-22°C, 85-90% relative humidity & 12 h photoperiod), lettuce leaves were spray inoculated with antagonists (10^7 fungal spores mL^{-1} or 10^8 bacterial cells mL^{-1}), allowed to dry & challenged by spray inoculation with the pathogen (10^6 spores mL^{-1}). After 7 days, the mean *Botrytis* lesion areas were assessed. *Pseudomonas fluorescens* (LU1001 & LU1003), *P. synxantha* (LU1004) & *P. veronii* (LU1002) significantly reduced lesion areas by an average of 79% on whole lettuce plants in two experiments. An *Ulocladium* sp. (LU865) & *Epicoccum purpurascens* (LU148) reduced lesion areas by 94% & 78%, respectively in one of two experiments.

Keywords: *Botrytis cinerea*, *Epicoccum*, *Ulocladium*, *Pseudomonas,* antagonist.

2.3 INTRODUCTION

The causal agent of grey mould, *Botrytis cinerea* Pers., is a ubiquitous necrotrophic pathogen & can infect almost every plant part on a wide range of plants worldwide (Rosslenbroich & Stuebler, 2000). In New Zealand, the fungus can cause significant economic losses on grape, strawberry, tomato, lettuce & kiwifruit. Current control relies heavily on the use of chemical fungicides but due to the pathogen's highly variable heterokaryotic nature, there is a high risk of resistance developing (Fletcher, 1984). Fungicides used to control the pathogen are rapidly losing their efficacy since resistant strains are now widespread throughout the world, including New Zealand. This coupled with the current public concern over pesticide residues on foodstuffs & the expanding demand for organically grown vegetables & fruits, highlights the need for an alternative to chemical control. Biological control of this ubiquitous fungal pathogen has been shown to be effective in many crops (Elad *et al.*, 1996) & can offer an attractive alternative to chemical control.

In vitro biological control screens are generally poor predictors of the performance of micro-organisms in the field (Andrews, 1992) &, therefore, the objective of this study was to develop a standardised, efficient screen that would evaluate the biocontrol potential of micro-organisms in the whole plant environment. Lettuce was chosen as the model system since *B. cinerea* can cause significant loss to the crop both pre & post harvest.

2.4 MATERIALS & METHODS

Test micro-organisms

There were two separate trials of different test micro-organisms. Each trial consisted of two identical experiments, the second experiment being carried out 10 days after the first one. Each experiment was a randomised complete block design, with 14 treatments in Trial 1 & 10 treatments in Trial 2. All treatments were replicated four times. In all experiments, the pathogen only control was sprayed with aqueous Tween 80 solution (1 drop per 1000 mL) instead of test micro-organism or fungicide.

In Trial 1, seven fungal antagonists & three bacterial antagonists with known biocontrol activity against other fungal pathogens were selected from The National Centre for Advanced Bio-Protection Technologies microbial culture collection (Lincoln University, New Zealand). These were *Cladosporium* spp. LU173 & LU172 (previously ER3 & YNAZF1 respectively) from *Camellia japonica* flowers (van Toor, 2002), *Trichoderma atroviride* LU132 (previously *T. harzianum* C52) & *T. hamatum* LU593 (previously *T. longipile* 6SR4) from lettuce (Rabeendran *et al.*, 1998), *Ulocladium* spp. LU865 & LU866 (previously U13 & U16 respectively) & *Epicoccum purpurascens* LU148 (previously E21) from grape leaf (Stewart *et al.*, 1998), *Bacillus subtilis* LU1241 (previously PT69) & *Paenibacillus polymyxa* LU1133 (previously 18-25) from soil (Wakelin, 2001). The performance of the selected micro-organisms was compared to two commercial biocontrol products & a chemical fungicide, recommended for control of *Botrytis*. The commercial product Trichodex® (provided by Makhteshim Agan Chemical works Ltd., Be'er Sheva, Israel), containing over 5×10^8 Colony Forming Units (CFU) g^{-1} of *T. harzianum* T39, was applied at the recommended rate of 4 g L^{-1}. The commercial product Prestop® WP (provided by Kemira Agro Oy, Porkkalankatu, Helsinki, Finland), containing over 10^7 CFU g^{-1} of *Gliocladium catenulatum* Xi, R43, was applied at the recommended rate of 10 g L^{-1}. The fungicide iprodione was applied at the recommended rate of 0.25g a.i. L^{-1} (Rovral FLO 15 mL L^{-1}). The second trial (Trial 2) tested one unidentified white yeast (PK10), three unidentified pink yeasts (Y44, Y46 & Y48) & four unidentified fluorescent *Pseudomonas* spp. (LU1001, LU1002, LU1003 & LU1004) isolated from strawberry fruit.

2.4.1 Identification of *Pseudomonas* isolates

Pseudomonas isolates were identified through their morphological characteristics, biochemical tests & partial sequencing of the internally transcribed spacer (ITS) regions.

Extraction of bacterial DNA

Bacteria were cultured in 50 mL of nutrient broth (NA, Oxoid Ltd, Hampshire, England) in 250 mL Erlenmeyer flasks at room temperature on an orbital shaker at 160 rpm for 24 h. Cells were collected by centrifugation of 1 mL bacterial suspension for 2

min at 7, 000 × *g*. Cells were resuspended in 0.1 mL TE buffer (Appendix 1.1), 0.5 mL GES lysis solution (Appendix 1.2) was added to the suspension & incubated at 60°C. After 15 min incubation, 0.25 mL of 7.5 M ammonium acetate was added to the suspension & gently mixed. After 2 min, the solution was immediately put on ice for 10 min before adding 0.5 mL chloroform: isoamyl alcohol (24:1 ratio). The resulting solution was centrifuged at 12, 000 × *g* for 10 min before the top phase was removed to a new tube. DNA was precipitated by the addition of 0.54 volumes of 100% isopropanol. The DNA was removed using a bent tip, washed three times in 70% ethanol & centrifuged for 2 min at 7, 000 × *g*. The resulting pellet was air dried, resuspended in 0.5 mL H_2O & refrigerated at 4°C.

DNA Quantification

To quantify genomic DNA, 2 μL aliquots mixed with 3 μL 6 × loading buffer (appendix 1.3) & 13 μL water were loaded into individual wells of 1% agarose gel (appendix 1.4). A 2 μL aliquot of DNA High Mass ™ ladder (Invitrogen Corporation, Carlsbad, U.S.A.) prepared in the same manner was loaded into an adjacent well. DNA was separated by gel electrophoresis at a constant electrical voltage (12.5 V cm^{-1}) for approximately 50 min. The gel was stained by submerging in 0.5 μg mL^{-1} ethidium bromide for 30 min. DNA was visualised using a Versadoc 3000 (Bio-rad Laboratories, Hercules, U.S.A.). DNA was quantified by visual comparison of sample DNA intensity with that of the DNA Mass ™ ladder.

Amplification of ITS region

Amplification of the ITS region was performed using the previously described primers F27 (5'-AGA GTT TGA TCM TGG CTC AG-3') & R1494 (5'-CTA CGG YTA CCT TGT TAC GAC-3') (Kane *et al.*, 1993; Weisberg *et al.*, 1991). Each 25 μL reaction contained 10 mM Tris-HCL pH 8.0, 50 mM KCL, 1.5 mM $MgCl_2$, 200 μM each of dATP, dGTP, dCTP & dTTP (Roche Molecular Biochemicals, Auckland, New Zealand), 10 pmoles of each primer, 1.25 U Hotmaster Taq (Eppendorf Hamburg, Germany) & 10 ng template DNA. The PCR amplification conditions were 5 min at 94°C then 30 cycles of 94°C for 30 s, 55°C for 30 s & 65°C for 1 min, with a final extension time of 7 min at 65°C. All reactions were carried out using an Eppendorf Mastercycler® Gradient (Eppendorf).

PCR product size determination

To estimate molecular weight of PCR products, 5 µL aliquots were mixed with 1 µL 6 × loading buffer & separated by agarose gel electrophoresis as described above. A 5 µL aliquot of a 1 kb Plus DNA ladder ™ (Invitrogen Corporation) was separated in an adjacent well. PCR product size was estimated by position in the gel relative to bans of known molecular weight in the ladder.

ITS Sequence analysis

The PCR product was diluted to a final concentration of 15 ng μL^{-1}. This was then sequenced (DNA sequencing, The Centre for Gene Technology, The University of Auckland) from both directions using the F27 & R1494 primers. Identity to other ITS regions was analysed using GenBank's Blastn function (www.ncbi.nlm.nih.gov). Homologous sequences or those with high similarity were aligned & homology trees constructed using DNAman™ (Version 4.0a, Lynnon Biosoft, Quebec, Canada).

2.4.2 Inoculum production

B. cinerea LU829 was isolated from an infected commercial lettuce crop (Marshlands, Christchurch, New Zealand) & maintained on potato dextrose agar (PDA, Oxoid Ltd, Hampshire, England) at 20°C. Inoculum was produced in the same way as the antagonistic fungi. Filamentous fungi, yeasts & bacteria were stored on PDA, malt extract agar (MEA, Difco Laboratories, Detroit, U.S.A.) & nutrient agar (NA, Difco) respectively at -4°C. Filamentous fungi were cultured on PDA at 20°C for 10-29 days & yeasts for 3 days on MEA plates. Sporulation of the fungi was initiated by cultivating under fluorescent light with a 12 h photoperiod regime. All fungal spore suspensions were prepared by flooding individual cultures with 10 mL aqueous Tween 80 solution (1 drop in 1000 mL sterile water) & scraping gently with a sterile loop. The resulting crude suspension was filtered through a layer of Miracloth (Calbiochem, Germany) to remove mycelial fragments. Bacteria were cultured in 50 mL of nutrient broth (Difco) in 250 mL Erlenmeyer flasks at room temperature on an orbital shaker at 160 rpm for 24 h. Cells were collected by centrifugation for 10 min at 1000 × *g* & re-suspended in aqueous Tween-80 solution (1 drop in 1000 mL sterile water). Inoculum concentration was estimated using a haemocytometer & adjusted to 1×10^6 conidia mL^{-1} for *B. cinerea*, 1×10^7 spores mL^{-1} for other fungi & 1×10^8 cells mL^{-1} for bacteria.

2.4.3 Treatment & assessment of plants

Plastic pots (2 litre) were filled with potting mix consisting of 80% composted pine bark, 20% WAP5 washed crusher dust & Osmocote Plus fertiliser at 5 kg m^{-2}. Lettuce seeds of the Marksman type (Yates, Australia), a variety of iceberg lettuce partially resistant to powdery mildew, were planted four to a pot with a coverage of no more than 5 mm potting mix. They were grown in a glasshouse with temperatures of 15-25°C (monitored using a Tinytag® temperature data logger (Orion Components Ltd., Chichester, England), with irrigation as needed, but without additional lighting. After 3 weeks, they were thinned to one per pot & transferred to a controlled environment (18-22°C, 85-90% relative humidity & 12 h photoperiod) for 3 weeks to adapt to these conditions.

At the start of the experiments, small wounds (3 mm diameter) were made on the centre lower section of three medium sized leaves per lettuce plant (1 wound per leaf) using an electric powered soldering iron. Freshly wounded leaves were sprayed with a suspension of antagonist, aqueous Tween 80 solution (pathogen only control) or fungicide till near run off (leaves were uniformly covered) using a handheld sprayer. After 10 min, a suspension of *B. cinerea* was applied to each plant, one spray per wound, using the hand held sprayer. Disease progress was assessed after 7 days by measuring the length & width of disease lesions (mm) spreading from the wound site using a digimatic calliper (Mitutoyo Corporation, Japan). Total lesion area was calculated & analysis of variance (ANOVA) was used to determine differences between means. Fisher's protected Least Significant Difference (LSD) test was used to examine differences between treatment means at $P \leq 0.05$. Figure 10 shows lettuce plants arranged in the controlled environment chamber near assessment time.

Figure 10: Whole lettuce plants arranged in a controlled environment

2.5 RESULTS

2.5.1 Identification of *Pseudomonas* isolates

All four isolates were gram-negative, aerobic, motile rods which were able to grow above 42ºC. All the isolates showed strong green fluorescence under UV light when grown on King's B medium (King *et al.*, 1954). All isolates produced arginine dihydrolase & were oxidase positive (Kelman & Dickey, 1980). Isolate LU1002 was able to formulate levan (Kelman & Dickey, 1980). Using the primers F27 & R1494, the ribosomal DNA of the ITS region was successfully amplified which yielded approximately 1500 bp DNA fragment. The ITS sequences of LU1001 & LU1003 were identical & shared 100% identity to *Pseudomonas fluorescens* deposited in GenBank (ascn# AF336349) (Figure 11). The ITS sequence of LU1002 shared 100% identity to *P. veronii* deposited in GenBank (ascn# AB056120). The ITS sequence of LU1004 shared 100% identity to *P. synxantha* deposited in GenBank (ascn# AF267911) (Figure 11).

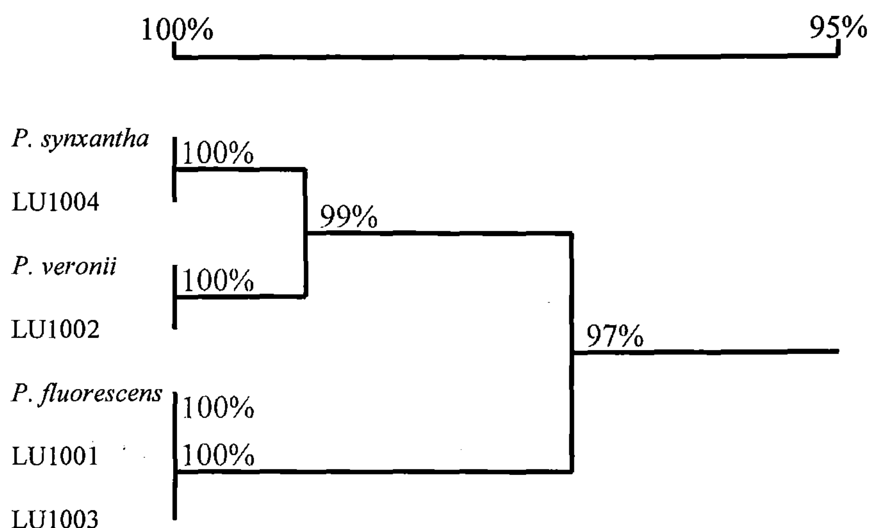

Figure 11: Homology tree based on ITS sequence alignment constructed using DNAman™ (Lynnon Biosoft)

2.5.2 Trial 1

In Experiment 1, iprodione & *Ulocladium* (LU865) significantly reduced lesion development compared to the pathogen-only control ($P<0.05$). These treatments were not different from each other ($P<0.05$). Percentage reductions in lesion area were 95% & 94% respectively (Table 10). Overall, *E. purpurascens* (LU148) reduced mean lesion area by 78%, although due to high variability between the replicates this was not significant at the 0.05 level of significance. Other isolates, namely *Paenibacillus polymyxa* (LU1133), *Ulocladium* sp. (LU866), *Trichoderma* spp. (LU132 & LU593) & the commercial product Prestop® WP gave 56% to 74% reductions in lesion area but these were not significantly different from the pathogen control (Table 10). In Experiment 2, no treatment gave a significant reduction in lesion development compared to the pathogen-only control, although *E. purpurascens* (LU148) & *Ulocladium* (LU865) gave the greatest reduction in lesion area of 78% & 41%, respectively (Table 10).

Table 10: The size of *B. cinerea* lesions (mm^2) on lettuce after treatment with various micro-organisms. Results are presented for both experiments in Trial 1

Treatment	Experiment 1[z]		Experiment 2[z]	
Pathogen control	1990	a	514	a
Pseudomonas marginalis (LU1009)	1420	a	424	a
Paenibacillus polymyxa (LU1133)	520	a	750	a
B. subtilis (LU1241)	1500	a	873	a
E. purpurascens (LU148)	441	a	115	a
Ulocladium (LU865)	122	b	303	a
Ulocladium (LU866)	665	a	387	a
T. atroviride (LU132)	875	a	620	a
T. hamatum (LU593)	692	a	1090	a
Cladosporium (LU173)	761	a	1308	a
Cladosporium (LU172)	1340	a	855	a
Trichodex®	1180	a	1070	a
Prestop® WP	824	a	459	a
Iprodione	93	b	498	a

[z] Means in a column followed by the same letter are not significantly different at $P \leq 0.05$ according to Fisher's protected LSD test

2.5.3 Trial 2

P. fluorescens (LU1001 & LU1003), *P. synxantha* (LU1004) & *P. veronii* (LU1002) (Figure 12) gave a significant reduction in *Botrytis* lesion area compared to the pathogen control in both experiments. Lesion areas were reduced by 60% to 84% in Experiment 1 & by 62% to 96% in Experiment 2 (Table 11). *P. fluorescens* LU1003 showed greatest suppression with lesion areas ranging from 159 to 513 mm^2 which was equivalent to a lesion area reduction of 76% & 96%, respectively compared to the pathogen control (Table 11).

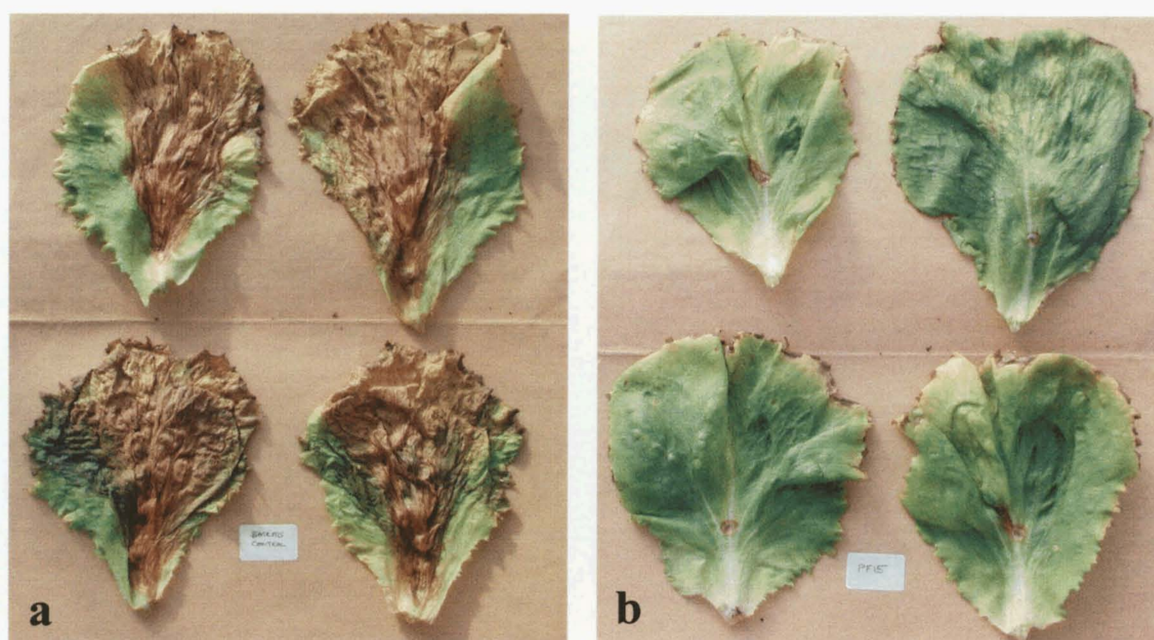

Figure 12: Lettuce leaves taken from pathogen-only control plant (a) & leaves taken from *P. veronii* LU1002 treated plant (b)

Table 11: The size of *B. cinerea* lesions (mm^2) on lettuce after treatment with various micro-organisms. Results are presented for both experiments in Trial 2

Treatment	Experiment 1 [z]		Experiment 2 [z]	
Pathogen control	2120	a	3810	a
Unidentified yeast (PK10)	1110	a	4010	a
Unidentified yeast (Y44)	1560	a	2830	a
Unidentified yeast (Y46)	1720	a	2620	a
Unidentified yeast (Y48)	1740	a	3890	a
Pseudomonas synxantha (LU1004)	601	b	371	b
P. fluorescens (LU1003)	512	b	159	b
P. fluorescens (LU1001)	349	b	1430	b
P. veronii (LU1002)	845	b	222	b

[z] Means in a column followed by the same letter are not significantly different at $P \leq 0.05$ according to Fisher's protected LSD test

2.6 DISCUSSION

This study evaluated 18 micro-organisms, two commercial biocontrol products & a fungicide for the suppression of *B. cinerea* lesions on whole lettuce plants in a

controlled environment. Four fluorescent *Pseudomonas* spp. significantly reduced *Botrytis* lesion development compared to the pathogen only control. None of these isolates were significantly better than the others. Fluorescent *Pseudomonas* spp. have been shown to antagonise a wide range of fungal & bacterial pathogens on a wide range of plants. For example, an isolate marketed as BlightBan has been commercialised for the control of bacterial pathogens on aerial plant surfaces (Wilson, 1997). Another *P. fluorescens* isolate (PB92BB10E) was found to suppress *B. cinerea* on whole petunia plants by an average of 77% across seven different trials (Gould *et al.*, 1996). Swadling & Jeffries (1996) identified a strain of *P. fluorescens* that was equally or more effective than the fungicide dichlofluanid for controlling grey mould in a strawberry field trial. This is the first time *P. synxantha* or *P. veronii* has been reported to control the plant pathogen *B. cinerea*. However, *P. veronii* has successfully controlled *Achlya klebsiana* & *Pythium spinosum*, which cause seedling diseases of rice (Adhikari *et al.*, 2001). There are no reports of *P. synxantha* being used in the control of plant diseases, although isolates from this species have been associated with the biocontrol of nematodes (Wechter *et al.*, 2002).

Past research has shown that antagonistic activity of fluorescent *Pseudomonas* spp. is based on fungistatic &/or germicidal activity (Gould *et al.*, 1996; Slininger *et al.*, 1996; Swadling & Jeffries, 1996). Leifert *et al.* (1993) showed that a number of *P. fluorescens* isolates produced large inhibition zones in dual culture with *Alternaria brassicicola* & *B. cinerea* on cabbage agar indicating that antibiosis was involved in the inhibition given by these bacteria. It is hypothesised that the *Pseudomonas* isolates tested in this study operated by this mechanism, because they showed clear zones of inhibition on dual culture plates with *B. cinerea* (data not shown). However, these inhibitory compounds have not been identified. Fluorescent *Pseudomonas* spp. are known to produce an array of antagonistic metabolites, including acetylphloroglucinols, pyoverdin, pyrrolnitrin & cyclic peptides as reviewed by Chin-A-Woeng *et al.* (2003). One of the most important groups of antagonistic metabolites produced by *Pseudomonas* spp. are the phenazines, which are known to be toxic to a wide range of organisms including bacteria, fungi & algae (Toohey et al., 1965 cited in Chin-A-Woeng *et al.*, 2003). These heterocyclic nitrogen containing metabolites have been shown to play a pivotal role in biocontrol (Slininger *et al.*, 1996).

E. purpurascens (LU148) reduced lesion areas by an average of 78% in both experiments compared to the pathogen only control, although this difference was not statistically significant. An *Epicoccum* sp. has previously been shown to suppress *B. cinerea* on bean (Hannusch & Boland, 1996). They reported that biocontrol efficacy was independent of the atmospheric environment when evaluated at temperatures ranging from 20–28°C & relative humidities ranging from 90% to 100%. Under these conditions, the isolate reduced grey mould by 100%. Mechanisms of action were attributed to competition for nutrients or antibiosis (Hannusch & Boland, 1996).

Ulocladium sp. (LU865) reduced lesion area significantly in Experiment 1 but not Experiment 2 of Trial 1. An *Ulocladium atrum* isolate suppressed the sporulation of *B. cinerea* on strawberry, onion, cyclamen & geranium (Köhl & Fokkema, 1998) by competitively excluding the pathogen from colonising senescent tissue. Both the *Epicoccum* & *Ulocladium* isolates used in this study had already been shown to suppress *B. cinerea* infection & sporulation on sterilised grape rachii (Fowler *et al.*, 1999). However, their mechanisms of action in antagonism towards *B. cinerea* are currently unknown.

Isolates belonging to the genera of *Ulocladium* & *Epicoccum* are primarily saprophytic &, therefore, are more likely to be successful in biocontrol of the necrotrophic pathogens infecting senescing plant material rather than healthy plants, such as the ones used in this study. Application of BCAs to healthy plant leaves is generally not very effective as the pathogen can rapidly penetrate the leaf & escape competition (Fokkema, 1993). *U. atrum* has previously been shown to be less effective in wound protection, but successful in suppressing sporulation of *B. cinerea* (Dik *et al.*, 1999).

Expression of antagonism by *E. purpurascens* (LU148) & *Ulocladium* sp. (LU865) towards *B. cinerea* would not occur immediately upon co-inoculation since their conidia require time to break dormancy & germinate. However, the *Pseudomonas* spp. tested in this study would have already been in an active metabolic state when inoculated onto lettuce leaves, thus there would have been no delay in the expressed antagonism by these bacteria. It is therefore hypothesised that the time difference in expressed antagonism by the bacteria & fungi, the phyllosphere arena of healthy lettuce

leaves & the assumed antibiotics produced by the bacteria are responsible for the success of the fluorescent *Pseudomonas* isolates screened in this study.

It is concluded that *E. purpurascens* (LU148), *Ulocladium* (LU865), *P. fluorescens* (LU1001 & LU1003), *P. synxantha* (LU1004) & *P. veronii* (LU1002) offer the most potential for the antagonism of *B. cinerea* in this system. Future research should assess these isolates for the control of *B. cinerea* on whole plants in a large-scale field trial. Research should also aim to identify the mode of action of the most successful isolate(s) as this information may help to optimise their biocontrol efficiency in the field.

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CHAPTER 3:

Targeted selection of antagonistic microorganisms for biocontrol of *Botrytis cinerea* of strawberry

3.1 FOREWORD

The assays developed in this chapter used strawberry as the model system & used a strategy of inhibition of pathogen sporulation to control the necrotrophic pathogen *Botrytis cinerea*. These assays were developed by the author & later used by HortResearch in the 'Sustainable disease management & control of strawberry fruit rots in New Zealand' research programme, which was funded by the MAF Sustainable Farming Fund (contract No. 18925).

The detached flower experiment presented in this chapter was carried out by Kirsty Boyd-Wilson, HortResearch, Canterbury. All other research within this chapter was conducted by the first author, with support from Professor Abraham Sztejnberg, who was on sabbatical leave from The Hebrew University of Jerusalem, Israel.

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3.2 ABSTRACT

A selected range of microorganisms, with previously identified antagonistic properties towards a variety of plant pathogens, were screened for their biological control activity against the foliar necrotroph, *Botrytis cinerea*. The most successful isolates, *Epicoccum purpurascens* LU148 & *Trichoderma atroviride* LU132 were able to inhibit *B. cinerea* lesion development & suppress sporulation on detached strawberry leaves. They were further evaluated on detached strawberry flowers & whole strawberry plants within a controlled environment (18°C & 85-95% relative humidity), for their ability to control grey mould. The most consistently effective antagonist was *T. atroviride* LU132, which inhibited *B. cinerea* in all experiments, reducing lesion expansion in detached strawberry leaves by up to 32% & significantly ($P \leq 0.05$) suppressing *B. cinerea* sporulation on detached strawberry leaves, flowers & leaf discs removed from inoculated whole plants by 87%, 100% & 81%, respectively. This was similar to the level of disease suppression given by the commercial biocontrol product, Prestop (with the active ingredient *Gliocladium catenulatum*) & the fungicide, fenhexamid.

3.3 INTRODUCTION

Grey mould, caused by the necrotrophic fungus, *Botrytis cinerea* Pers., is a most important disease, wherever strawberries are grown (Gullino *et al.*, 1989; Maas, 1984). Nearly all of the plant is susceptible to the pathogen including the leaves, flowers, fruits & crown (Sutton, 1990b). Serious yield losses of fruit can occur in the field, near to & during the harvesting period, in transport & at the market place. In the pathogen's disease cycle, senescent strawberry leaves can play an important role as an inoculum source (Braun & Sutton, 1987; Guetsky *et al.*, 2001). A quiescent infection frequently occurs if the dispersed conidia arrive on flower parts during blossoming, only expressing disease once the fruit matures (Powelson, 1960). Therefore, in many disease management programmes, protection of strawberry flowers with chemical fungicides has been the primary focus for successful control of *B. cinerea* (Mertely *et al.*, 2002). However, due to the development of fungicide resistant strains of the pathogen, & increasing public concern about the level of fungicide residues on strawberry fruit, an alternative control option is required. Biological control has been shown to be effective in controlling Botrytis diseases in many crops including strawberry (Peng & Sutton, 1991; Swadling & Jeffries, 1996; Tronsmo & Dennis, 1977), with strategies that include protecting strawberry flowers from infection & reducing the initial inoculum build up by reducing the availability of necrotic tissue (Köhl & Fokkema, 1998; Sutton & Peng, 1993a).

Although reports describing research into biological control of *Botrytis* diseases can be found as far back as the early 1950s (Newhook, 1951b; Wood, 1951), this area of plant pathology is still seen by many as a young science (Boland, 1990), & there are many different approaches with varied levels of success, especially at the initial selection & primary screening stage. Successful biological control agents (BCAs) are usually selected from a large number of isolates following a careful screening process (Elad *et al.*, 2001), which can be both expensive & labour-intensive. Andrews (1992) stated that "Designing suitable primary screens for controlling fungal pathogens is complicated by our limited knowledge of the phenotypic features that determine success of BCAs. Success involves many properties of an antagonist & biocontrol subsequently results from a sequence of events." The aim of the study described here was to select for a limited range of species on which to conduct primary screening trials. The selection

strategy comprised a two-stage process. Firstly, the current literature on the biological control of *B. cinerea* was reviewed & the species reported to give successful control of *B. cinerea* were identified. Secondly, isolates from these same species, which had given success in biocontrol research at Lincoln University were selected & evaluated for their ability to control *B. cinerea* in detached strawberry leaves. The most successful isolates were then evaluated in a detached flower assay & a whole plant bioassay, under *Botrytis*-conducive conditions.

3.4 MATERIALS & METHODS

Microorganisms & commercial products

Table 12 lists the antagonistic microorganisms selected for use in this study. The three *Botrytis cinerea* isolates used in this study were LU829, an isolate from an infected commercial lettuce crop (Marshlands, Christchurch, New Zealand), LU138 & LU139, the latter two isolates obtained from infected strawberry fruit grown at Lincoln, New Zealand.

The following biocontrol products & chemical fungicides were used; Trichodex®, containing conidia of *Trichoderma harzianum* T39, was supplied by Makhteshim Chemical Works, Israel. Prestop® WP, containing spores of *Gliocladium catenulatum* Xi R43, was supplied by Kemira Agro Oy, Finland. BotryZen®, containing spores of *Ulocladium oudemansii*, was supplied by BotryZen Ltd, New Zealand. Serenade®, containing *Bacillus subtilis*, QST713, was supplied by Elliot Chemicals Ltd, New Zealand.

The following chemical fungicides used is this study were; fenhexamid (Teldor® 500 SC, Bayer CropScience NZ Ltd), tolylfluanid (Euparen® Multi, Bayer CropScience NZ Ltd), trifloxystrobin (Flint®, Bayer CropScience NZ Ltd), captan (Captan® WG, Nufarm NZ Ltd), thiram (Thiram DF, Nufarm NZ Ltd), chlorothalonil (Elect® 750 SC, Nufarm NZ Ltd), mancozeb (Penncozeb® 750 DF Nufarm NZ Ltd) & cyprodinil & fludioxonil (Switch®, Syngenta Crop Protection Ltd, New Zealand). Application rates of fungicides are shown in Tables 13 & 14.

Table 12: Antagonistic microorganisms used in this study & their previous biocontrol trials

Antagonist with isolate number	Previous target pathogen	Host plant	Reference
Bacillus subtilis LU1241 (previously PT69) & *Paenibacillus polymyxa* LU1133 (previously 18-25)	*Aphanomyces euteiches*	Pea	Wakelin, 2001
Epicoccum purpurascens LU148 (previously E21)	*Botrytis cinerea*	Grape	Stewart *et al.*, 1998
Pseudomonas fluorescens LU1001 & LU1003 (previously PF14 & PF13 respectively), *Ps. veronii* LU1002 (previously PF15) & *Ps. synxantha* LU1004 (previously LC8)	*B. cinerea*	Lettuce	Card *et al.*, 2002
Ps. marginalis LU1009 (previously GA8 PS4)	*Ciborinia camelliae*	Camellia	van Toor, 2002
Trichoderma atroviride LU132 [a] (previously *T. harzianum* C52)	*Sclerotium cepivorum*	Onion	Kay & Stewart, 1994; McLean & Stewart, 2000
T. hamatum LU593 [b] (previously *T. longipile* 6SR4)	*Sclerotinia sclerotiorum* & *S. minor*	Lettuce	Rabeendran *et al.*, 1998

[a] Available in the commercial product Onion mate®, manufactured by Agrimm Technologies Ltd, Christchurch, New Zealand

[b] Available in the commercial products, Trichodry 6S® & Trichopel 6S®, manufactured by Agrimm Technologies Ltd

Inoculum production

All fungi & bacteria were stored on potato dextrose agar (PDA, Oxoid Ltd, Hampshire, England) & nutrient agar (NA, Difco Laboratories, Detroit, U.S.A.), respectively at 4°C. Fungi were cultured on PDA, except for *Epicoccum purpurascens* which was cultured on V-8 agar (according to Hannusch & Boland, 1996), for 10-29 days at 20°C, with a 12 h photoperiod regime that incorporated fluorescent light to encourage sporulation. All fungal spore suspensions were prepared by flooding individual cultures with 10 mL aqueous Tween-80 solution (1 drop in 1000 mL sterile water) & scraping gently with a sterile loop. The resulting crude suspension was filtered through a layer of sterile Miracloth (Calbiochem, Darmstadt, Germany) to remove mycelial fragments. Bacteria were cultured in 50 mL of nutrient broth (Difco) in 250 mL Erlenmeyer flasks shaken on an orbital shaker at 160 rpm at room temperature for 24 h. Cells were collected by centrifugation (1000 × *g*) for 10 min & re-suspended in aqueous Tween-80 solution. Inoculum concentration was estimated using a haemocytometer & adjusted to 1×10^7 spores mL^{-1} for fungi & 1×10^8 cells mL^{-1} for bacteria. Commercial products were prepared according to the manufacturer's instructions.

Strawberry plants

Strawberry plants (cv. Aptos, cv. Camerosa & cv. Seascape) were grown from runners in potting mix (80% composted pine bark, 20% WAP5 washed crusher dust & Osmocote Plus fertiliser at 5 kg m^{-2}) within 2 L plastic pots. They were grown in a glasshouse at 25-35°C with the temperature determined using a Tinytag® temperature data logger (Orion Components Ltd, Chichester, England).

3.4.1 Inhibition of lesion development on detached leaves

Healthy, fully expanded leaves (cv. Camerosa) were collected, broken into individual leaflets & surface sterilised by agitating in 1% sodium hypochlorite solution for 90 s & rinsing twice in tap water. Leaflets (henceforward referred to as leaves) were left to dry on paper towels on the laboratory bench & then sprayed on the upper surface with the BCA suspension (Table 13) or an aqueous Tween-80 solution (pathogen only control). The leaves were uniformly covered using a hand-held sprayer until just before run-off. They were then allowed to dry on the laboratory bench for approximately 20 min, before being centrally inoculated with a mycelial plug taken from a 4-day old

B. cinerea (LU829) culture. The leaves were then placed on plastic trays, each of which was housed in a small humidity chamber, which consisted of a 170×240×80 mm plastic box, containing 500 mL tap water & closed with a tightly fitting lid. Treatments were allocated to separate chambers to avoid cross contamination. There were four replicate chambers per treatment, each containing three leaves. The humidity chambers were placed in an incubator at 18-20°C, with a 12 h photoperiod. After 7 days, disease progress was assessed by measuring the length & width of diseased lesions that spread beyond the mycelial plugs using a digimatic calliper (Mitutoyo Corporation, Tokyo, Japan). The experiment was performed twice.

3.4.2 Inhibition of sporulation on detached leaves

Strawberry leaves (cv. Aptos) were surface sterilised as before, patted dry with paper towels & then washed for 30 s in DAS® herbicide (simazine 4.8 g L^{-1}, amitrole 1.5 g L^{-1}, 2,2-dichloropropionic acid 3.1 g L^{-1}) (Yates, Homebush, Australia). This treatment caused the leaves to senesce within 4 days & allowed the pathogen to quickly colonise & sporulate. Using a hand-held sprayer, leaves were then sprayed with a suspension of the designated BCA or aqueous Tween-80 solution (pathogen only control) until near run-off. The leaves were then left to dry for 1 h on the laboratory bench, before being challenge inoculated with a *B. cinerea* (LU189) conidial suspension (2×10^5 conidia mL^{-1}). The leaves were then placed in small humidity chambers , with replication, arrangement & incubation as mentioned in section 3.4.1. The six BCAs & two commercial biocontrol products evaluated in this experiment are shown in Table 13. Pathogen growth & subsequent sporulation was quantified after 6 days incubation, by estimating the percentage leaf area covered by *B. cinerea* conidiophores using a 1 to 5 scale, corresponding to conidiophore coverage of 0-20%, 21-40%, 41-60%, 61-80% & 81-100%, respectively. The experiment was performed twice.

3.4.3 Inhibition of sporulation on whole plants

The experiment comprised a randomised complete block design with 11 treatments, each treatment replicated six times. Four week-old plants (cv. Aptos) grown in 2 L plastic pots were placed in a controlled, walk-in growth chamber (Controlled Environments Ltd, Manitoba, Canada) set at 18°C, >85% RH & 12 h photoperiod, &

left to acclimatise for 7 days (Figure 13). All above ground foliage of the plants was then uniformly sprayed with suspensions of the designated BCA (Table 13) or aqueous Tween-80 solution (pathogen only control) until near run-off. The spray regime was repeated 7 days later to increase the populations of BCAs on the phylloplane. Three BCAs, one commercial biocontrol product & one chemical fungicide, were evaluated (Table 13). Plants were then challenge inoculated with a conidial suspension of *B. cinerea* (LU829 at 2×10^5 conidia mL^{-1}). After 24 h, one compound leaf per plant was removed & a 3 cm disc cut from each of the three leaflets using a flamed cork borer. Discs were then immediately placed on a herbicide-amended agar medium (adapted from Peng & Sutton, 1991) to hasten senescence. The medium contained 4.8 g agar & 0.16 g chloramphenicol, which were dissolved in 800 mL tap water & autoclaved, after which 9.6 g DAS® herbicide was added to the cooled mixture. Pathogen growth & subsequent sporulation was quantified after 6 days incubation, by estimating the percentage leaf area covered by *B. cinerea* conidiophores using a 1 to 5 scale as before. This experiment was repeated four times with the treatments being applied every 7 days & the pathogen applied every 14 days. There were four assessments, with new leaves sampled from the same plants, & the experiment was performed twice.

Figure 13: Set up of whole strawberry plant sporulation screen arranged in walk-in growth chamber

3.4.4 Inhibition of sporulation on detached flowers

Strawberry flowers (cv. Seascape) were collected from the field at the fully opened petal stage & their stems inserted into flower oasis (Eden Floral Foam, Horticom Ltd, Auckland, New Zealand) cubes ($105\times75\times20$ mm^2) which had been soaked overnight in tap water. Each cube represented a replicate & held six flowers inserted equidistantly apart. Flowers were sprayed with the treatments (Table 14) to just before run-off using an airbrush (model 250-2, Badger Air-Brush Company, Illinois, U.S.A.) & propellant (1,1-difluroethane & butane mix, Badger Air-Brush Company). They were left to dry for 1 h & then challenge inoculated with a mixed *B. cinerea* conidial suspension (LU138 & LU139 in equal amounts) at 2×10^5 conidia mL^{-1}. After inoculation the cubes were placed on a surface sterilised plastic tray & enclosed in a plastic bag to increase humidity. Trays were then placed on the laboratory bench at room temperature (21±3°C). After 6 & 8 days incubation, each flower was assessed for incidence of *B. cinerea* sporulation on stamens & styles. Two BCAs, two commercial biocontrol products & nine chemical fungicides were evaluated in this experiment (Table 14). The experiment was performed twice & as results from the repeat experiment followed a similar trend, the results from only one experiment are presented.

3.4.5 Statistical analysis

For the assay on inhibition of *B. cinerea* lesion development, areas per leaf were calculated & data analysed by analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) test to determine significant ($P\leq0.05$) differences between treatment means, compared to the pathogen only control. For the assays on inhibition of *Botrytis* sporulation on detached leaves & leaves taken from whole plants, statistical tests were performed using the Kruskal-Wallis test followed by the multiple comparisons test ($P\leq0.05$) in order to compare treatment means to the pathogen-only control. For presentation purposes, percentage values were derived from the conidiophore categories by taking the category mid-point value of each treatment mean & transforming it to a percentage reduction of the control mean. Statistical analysis was performed using the statistical package Minitab ™ 13.1, Pennsylvania, U.S.A.

3.5 RESULTS

The most successful isolates were *P. veronii* LU1002, *E. purpurascens* LU148, *T. atroviride* LU132 &, which inhibited *B. cinerea* lesion expansion in the first experiment by 12%, 20%, & 25% respectively & by 40%, 11% & 32%, respectively, in the second experiment (Table 13) compared to the pathogen control. This inhibition was significant only for *T. atroviride* LU132 in Experiment 2 (Figure 14).

Figure 14: Leaves taken from the mycelial inoculated leaves, showing lesions spreading from the mycelial plug. Top row, the pathogen-only control & bottom row, the *T. atroviride* LU132 treated leaves

The five most effective isolates were then further tested in the leaf sporulation assay with the commercial biocontrol products Prestop® & Trichodex®. In Experiment 1, only *E. purpurascens* LU148, *T. atroviride* LU132 & Prestop® significantly suppressed *B. cinerea* sporulation on detached leaves by 74%, 83% & 83%, respectively (Table 13). In Experiment 2, these three isolates & *P. veronii*, LU1002 significantly suppressed *B. cinerea* sporulation, with *T. atroviride* LU132 giving the highest level of control, 87%, equal to that of the commercial biocontrol preparation, Prestop®. The four most effective isolates were then further tested on whole strawberry plants in a controlled environment, with Prestop® & the chemical fungicide, fenhexamid.

Table 13: Percentage inhibition of grey mould leaf lesion area (mycelial inoculation of detached leaves) & *B. cinerea* sporulation (conidial inoculation of detached leaves & whole plants) due to treatments, compared to pathogen only controls

Treatment	Leaf Lesion [a]		Leaf sporulation [b]		Whole plant sporulation [b]	
	Exp.1	Exp.2	Exp.1	Exp.2	Exp.1	Exp.2
Bacillus subtilis, LU1241	0	0	-	-	-	-
Epicoccum purpurascens, LU148	20	11	74*	38*	41	79*
Paenibacillus polymyxa, LU1133	0	0	-	-	-	-
Pseudomonas fluorescens, LU1001	0	25	0	34	-	-
Ps. veronii, LU1002	12	40	48	85*	34	35
Ps. fluorescens, LU1003	0	0	-	-	-	-
Ps. synxantha, LU1004	6	25	7	54	-	-
Ps. marginalis, LU1009	0	0	-	-	-	-
Trichoderma atroviride, LU132	25	32*	83*	87*	81*	81*
T. hamatum, LU593	0	1	-	-	-	-
Trichodex® at 4 g L^{-1}	-	-	47	28	-	-
Prestop® at 10 g L^{-1}	-	-	83*	87*	82*	81*
Fenhexamid at 1.5 mL L^{-1}	-	-	-	-	83*	81*

- not tested

[a] Lesion areas were analysed by ANOVA & Fisher's protected LSD test ($P \leq 0.05$)

[b] Sporulation area categories analysed by Kruskal Wallis multiple comparisons test ($P \leq 0.05$). Data presented as category midpoint values, with control group =100%

*Significantly different to pathogen-only control at ($P \leq 0.05$)

In Experiment 1, *T. atroviride* LU132 (Figure 15), Prestop® & fenhexamid significantly ($P \leq 0.05$) reduced *B. cinerea* sporulation compared to the pathogen only control with reductions of 81%, 82% & 83%, respectively. In Experiment 2, the same treatments significantly ($P \leq 0.05$) reduced *B. cinerea* sporulation by 81%.

For the detached flower trial, on day 6, *T. atroviride* LU132 gave equal or better control of Botrytis flower infections as shown by the degree of sporulation than all the tested fungicides, including fenhexamid, cyprodinil/fludioxonil & pyrimethanil. On day 8, *T. atroviride* LU132 significantly inhibited *B. cinerea* sporulation to a higher degree than any other treatment tested (Table 14), giving a reduction of 100%, compared to the pathogen-only control.

Figure 15: Leaf discs taken from whole strawberry sporulation screen, showing pathogen-only control on left hand side & *T. atroviride* LU132 treated leaves on the right hand side

Table 14: Percentage inhibition of *B. cinerea* sporulation on detached strawberry flowers due to treatments compared to the pathogen only control after 6 & 8 days incubation at room temperature

Treatment	Flower bioassay			
	6 days		8 days	
Trichoderma atroviride, LU132	100	***	100	***
Fenhexamid at 1.5 mL L^{-1}	100	***	95.8	***
Cyprodinil/fludioxonil at 0.8 g L^{-1}	100	***	93.8	***
Pyrimethanil at 2 mL L^{-1}	89.6	***	85.4	***
Tolylfluanid at 2 g L^{-1}	95.8	***	54.2	***
Trifloxystrobin at 0.1 g L^{-1}	95.8	***	54.2	***
Thiram at 2 g L^{-1}	85.4	***	52	***
Serenade at 9 g L^{-1}	68.8	***	43.7	**
Chlorothalonil at 2.3 mL L^{-1}	81.2	***	33.3	**
Epicoccum purpurascens, LU148	41.7	**	33.3	
Captan at 1.5 g L^{-1}	50	***	22.9	
BotryZen at 14 g L^{-1}	31.2	*	12.5	
Mancozeb at 2.1 g L^{-1}	8.3		4.2	

*, **, *** Significantly different to pathogen-only control at $P \leq 0.05$, $P \leq 0.01$ & $P \leq 0.001$, respectively

3.6 DISCUSSION

The design & development of suitable techniques for screening potential BCAs is a crucial factor in the detection of bioactive microorganisms (Swadling & Jeffries, 1996). Many of the initial primary screens used in the past, which are designed using artificial nutrient media, have been severely criticised as they do not closely resemble the final phyllosphere arena where biocontrol ultimately takes place. Andrews (1992) stated that "BCAs should be screened by a protocol that is both efficient & a reliable predictor of field performance." The screens described here were based on the strategies devised by Peng & Sutton (1991). They were easily reproducible, with high amounts of disease found in the pathogen only controls, indicating that the conditions set in each experiment were highly conducive for *B. cinerea* development. This is important as the evaluation of potential BCAs in environmental conditions that are marginal for disease can overestimate their efficacy in subsequent field environments

(Hannusch & Boland, 1996). The first screen investigated the inhibition of mycelial infection of strawberry leaves, & only four isolates inhibited *B. cinerea* in both experiments (Table 13). However, since significant grey mould damage on crops usually involves secondary disease cycles, conidial infection & subsequent sporulation were also investigated. Clearly, an antagonist that can affect more than one stage of the disease development process, such as inhibition of infection & subsequent sporulation, should have a greater chance of success (Punja, 1997).

The most successful isolate from all screens was *T. atroviride* LU132. The isolate was able to inhibit *B. cinerea* lesion development on strawberry leaves by 25% & 32% (Table 13) in a bioassay where the pathogen was already established, & was able to significantly suppress pathogen sporulation on detached leaves, flowers & leaves from treated whole plants by 87%, 100% & 81%, respectively (Tables 13 & 14). This was similar to the level of disease control given by the commercial biocontrol product, Prestop® & the chemical fungicide, fenhexamid. This suppression was absolute as the BCA was seen to completely colonise all inoculated tissues (data not shown). The second most successful isolate was *E. purpurascens* LU148, which inhibited *B. cinerea* lesion development from mycelial inoculation on strawberry leaves by 20% & 11% in two experiments. The isolate also suppressed *B. cinerea* sporulation significantly in detached leaves by an average of 74% & 38% in two experiments & on whole strawberry plants, in one experiment, by 79%. This isolate, however, failed to suppress *B. cinerea* sporulation on detached strawberry flowers (Table 14).

Similar screens to the ones used in this study have been conducted by other researchers to test microorganisms for the biocontrol of *Botrytis* on strawberry. Peng & Sutton (1991) evaluated over 200 fungi, yeasts & bacteria for the suppression of *B. cinerea* sporulation & found that an isolate of *G. roseum*, at a concentration of 10^7 spores mL^{-1}, was able to completely suppress sporulation on a range of strawberry tissues, including leaves, stamens & petals. After further biocontrol tests in four field trials, they concluded that the methods developed in the laboratory & growth rooms provided a strong indication of the biosuppressive activity of microorganisms in the field (Peng & Sutton, 1991). Helbig (2002) tested the antagonistic yeast *Cryptococcus albidus* for the biocontrol activity against *B. cinerea* on detached strawberry leaves &

flowers. The isolate was found to significantly reduce the incidence & conidiophore density of *B. cinerea* conidiophores when applied at a concentration of 1×10^6 cells mL^{-1} but could not reduce the incidence of *B. cinerea* on detached flowers, even when the cell density was increased to 1×10^7 cells mL^{-1}. However, this did not influence the biocontrol activity of the isolate under field conditions, where the BCA reduced incidence of grey mould on ripe strawberry fruits after harvest by 33%, 28% & 21%, respectively, in three years of field trials (Helbig, 2002). Swadling & Jeffries (1996) showed that isolates of *P. fluorescens* & *B. pumilus* could control *B. cinerea* in a range of *in vitro* screens, including a detached strawberry leaf disc assay. Their results also conclude that their preliminary screens were a valuable precursor for selecting isolates for testing under field conditions. Elad *et al.* (2001) also reported results that showed that suppression of *B. cinerea* on whole strawberry plants was correlated to disease control on detached plant parts rather than antagonism on sterile growth media or the isolate's ability to produce cell wall hydrolysing enzymes.

A factor that may influence the activity of BCAs is the timing of their application in relation to the pathogen. It is well known that the success of many BCAs is dependent on the antagonists being applied prior to the pathogen. The screens used in this study & those by other researchers investigating biocontrol of *B. cinerea* in strawberry, inoculated the antagonists several hours before the application of *B. cinerea* (Peng & Sutton, 1991; Swadling & Jeffries, 1996) & this could have given the BCA an advantage over the pathogen. Future work could investigate different application times in relation to the BCA & the pathogen, such as applying the pathogen before the antagonist or at the same time. Szandala & Backhouse (2001) investigated the suppression of *B. cinerea* sporulation on bean leaf discs after pathogen infection. They found that an isolate of *G. roseum* was able to significantly reduce sporulation when applied up to 120 h after the pathogen & that it was effective at all the concentrations tested.

It should also be noted that of the commercial biocontrol products tested, only Trichodex & Prestop are recommended for the control of *B. cinerea* on strawberry. This could explain the low activity demonstrated here by Serenade & BotryZen, which are primarily targeted to control Botrytis bunch rot of grape, indicating that the active antagonists of these products may not be suited to strawberry flowers. A

previous report by Hjeljord *et al.* (2000) also showed a lack of disease control by Trichodex against *B. cinerea* in strawberry & concluded that the environment of the greenhouse & conidia formulation were contributing factors & that the dosage was not responsible. In the experiments reported here, the commercial product Prestop was successful in suppressing *B. cinerea* sporulation (Table 13) on strawberry leaves. The mechanism of action of the active ingredient of this product, *G. catenulatum* strain Xi R43, is currently unknown but observations from this study showed that the fungus was able to quickly colonise senescent strawberry leaves, perhaps excluding *B. cinerea* from the niche with which it is so commonly associated. Similar effects were observed with *T. atroviride* LU132 on strawberry leaves & flowers, indicating that competition for nutrients may play an important role in the biocontrol activities of these two fungi.

There was a wide variation in the control given by the different fungicides tested in this study. The fungicides that gave the best control on strawberry flowers, with more that 93% inhibition of *B. cinerea* sporulation after 8 days, included cyprodinil/fludioxonil & fenhexamid. These fungicides both have multi site action, inhibiting spore germination & mycelial growth. The fungicides that gave unsatisfactory control, giving less than 23% inhibition of *B. cinerea* sporulation on strawberry flowers, were captan & mancozeb. These fungicides require excellent coverage & drying before they can give satisfactory disease control (New Zealand Agrichemical Manual, 2004). They also break down rapidly in the environment (captan for example having a half life of 1 day in the soil) & so should be applied every 7 days to give adequate disease control. Therefore, inadequate coverage & the fact that these fungicides were only applied once to strawberry flowers at the start of the experiment could be responsible for their lack of efficacy.

A number of *Trichoderma* species have previously been found to control *B. cinerea* development on a variety of crops. The best known of these isolates & products involve phyllosphere applications for the biocontrol of *Botrytis* species that frequently invade senescing or dead plant tissue, which the pathogen may use as a nutritive base in order to invade healthy tissue (Hjeljord & Tronsmo, 1998). Early work by Tronsmo & Dennis (1977) on strawberry, showed that the presence of *Trichoderma* isolates can reduce the incidence of *B. cinerea* on stored fruit to a level similar to that provided by

the fungicide, dichlofluanid. In later research work, Peng & Sutton (1991) found that under controlled conditions, an isolate of *T. viride* was highly suppressive in the majority of studies on grey mould of strawberry. In field studies, the same isolate of *T. viride* significantly reduced the incidence of *B. cinerea* on petals, stamens & fruits compared to the pathogen only control & reduced its incidence to a similar level as did the fungicide, captan. There has, however, also been unsatisfactory & inconsistent control when *Trichoderma* spp. were used to protect strawberry crops from the grey mould pathogen (Gullino *et al.*, 1989; Tronsmo, 1986). This could be explained by the inhospitable environment of the phyllosphere under field conditions causing poor colonisation by a microorganism whose natural habitat is the soil. In addition, the use of existing spray applicators that are usually employed for applying chemical treatments may also have contributed to their failure.

Although protection of strawberry flowers is of critical importance in grey mould management, the relationship between *B. cinerea* & strawberry leaves is also of critical importance in the development of disease epidemics (Sutton, 1990b). *B. cinerea* does infect green leaves, but any biocontrol strategy that aimed to reduce infection was proposed to be ineffective (Fokkema, 1993), owing to the rapid germination & subsequent host penetration exhibited by the pathogen (Elad, 1996). After infection, *B. cinerea* remains quiescent until the leaves senesce (Sutton, 1990b) & so a biocontrol strategy that aims to suppress the subsequent pathogen sporulation has the advantage of a long interaction period, targeting the polycyclic nature of *B. cinerea* (Fokkema, 1993). The strategy for suppression of *Botrytis* sporulation has now been used successfully in many host systems including strawberry, tomato, onion, rose & lily (Köhl *et al.*, 1995a; Köhl *et al.*, 1995b; Marcelo *et al.*, 2003; Peng & Sutton, 1991; Sutton *et al.*, 2002). Clearly, a biological control strategy needs to be based on the ecology of both the pathogen & the antagonist (Köhl *et al.*, 1995a) if it is to have maximum effect.

The research in this chapter has identified *T. atroviride* LU132 as a potential BCA of *B. cinerea* in strawberry. *T. atroviride* LU132 inhibited lesion formation in leaves & suppressed pathogen sporulation on leaves & flowers, two tissues critical to the development of the disease cycle. The general observations from this study indicated that *T. atroviride* LU132 restricted pathogen growth through competition for limited

resources, because once leaves/flowers senesced, *T. atroviride* LU132 rapidly colonised dead or dying material & sporulated abundantly, thereby excluding the pathogen from its saprophytic niche.

The research conducted here showed that some biocontrol traits are not necessarily specific to the target pathogen or host system, allowing some BCAs to be selected from previous biocontrol programmes. *T. atroviride* LU132 was previously found to protect onions from *Sclerotium cepivorum* (Kay & Stewart, 1994; McLean & Stewart, 2000) & in this study was shown to suppress *B. cinerea* in strawberry. The underlying mechanism of action of LU132 may be complex & involve competition for nutrients, production of inhibitory compounds, mycoparasitism & induced resistance, & it is apparent that in many systems more than one mechanism of action may be responsible for the biocontrol activity (Elad, 1996). It is therefore plausible that many BCAs could possibly control multiple unrelated pathogens on different host plants which would be advantageous (Punja, 1997), as the success of a commercial biocontrol product will depend on its marketability &, therefore, its ability to control a number of diseases on a variety of crops (Swadling & Jeffries, 1998). This is true for one of the most successful commercially available biocontrol products, Trichodex® (active ingredient *T. harzianum* T39), which has been shown to control a range of unrelated foliar pathogens on a range of crops. These include *B. cinerea* (grey mould) in various greenhouse crops & in vineyards, *Cladosporium fulvum* (leaf mould) in tomato (Elad, 2000b), *Colletotrichum acutatum* (anthracnose) in strawberry (Freeman *et al.*, 2004), *Sclerotinia sclerotiorum* (white mould) in various greenhouse field crops, *Pseuperonospora cubensis* (downy mildew) & *Sphaerotheca fusca* (powdery mildew) of cucumber (Elad, 2000a).

Further research should now evaluate *T. atroviride* LU132 under field conditions, which can be described as the ultimate test for BCAs & often, their frequent downfall (Pusey, 1990 cited in Swadling & Jeffries, 1996). Under field conditions, the antagonist is exposed to many abiotic & biotic factors, including large fluctuations in temperature, humidity, surface wetness & ultraviolet light, interference by established resident microorganisms & the type, amount & availability of nutrients, which may reduce their efficacy. However, the efficacy of *T. atroviride* LU132 in these trials & the fact that many researchers have found a good correlation between suppression of

B. cinerea on detached plant parts & satisfactory disease control in the field (Helbig, 2002; Peng & Sutton, 1991; Swadling & Jeffries, 1996) indicates the potential for success.

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CHAPTER 4:

Mechanisms of action employed by *Trichoderma atroviride* LU132 in the control of grey mould in strawberry

4.1 FOREWORD

The previous two chapters were concerned with the development & implementation of bioassays to screen microorganisms for the biocontrol of *Botrytis cinerea.* Both the lettuce & strawberry systems were successfully developed & proved to be easily reproducible. It was decided that the remainder of the research programme would focus on the most successful biocontrol microorganism from Chapter 3, *Trichoderma atroviride* LU132.

The work was carried out by the first author with supervision from the other listed authors.

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4.2 ABSTRACT

The mechanisms of action by the biological control agent *Trichoderma atroviride* LU132 against the foliar pathogen *Botrytis cinerea* were studied. Competition for sugars on strawberry leaf surfaces & polytetrafluoroethylene (PTFE) membranes, production of inhibitory substances & direct parasitism were examined as possible mechanisms of action. *T. atroviride* LU132 was found to compete for glucose & sucrose on detached strawberry leaf pieces & on PTFE membranes, significantly inhibiting *B. cinerea* germ tube length at low concentrations. The isolate also produced non-volatile inhibitory substances in minimal media that inhibited *B. cinerea* germ tube elongation &, when mycelial plugs of both microorganisms were placed opposing each other on water agar, *T. atroviride* LU132 was found to directly parasitize *B. cinerea* hyphae leading to cell collapse. This work provides evidence to demonstrate that multiple mechanisms of action may operate in the biocontrol of *B. cinerea* in strawberry by *T. atroviride* LU132.

4.3 INTRODUCTION

Plant disease control provided through the introduction of antagonistic microorganisms is highly inconsistent compared to chemical control. This may be partially due to a lack of understanding of the underlying mechanisms of action by which antagonistic microorganisms operate (Boland, 1990; Punja, 1997). Such information could provide for modification of the application methods, which could not only reduce the inconsistency & variability found commonly in many biocontrol systems, but could also enhance their efficacy (Guetsky *et al.*, 2002b). This information may also provide a better understanding of the antagonist's limitations (Howell, 2003) & aid in the interpretation of reasons for success or failure in any given biocontrol system (Gullino, 1992). In order to achieve widespread use & acceptance of biological control agents (BCAs), this understanding is crucial (Fravel, 1988). Suppression of plant disease by BCAs can be achieved through many mechanisms including, (but not limited to) antibiosis, competition for nutrients & space, systemic induced resistance & direct parasitism.

The methodologies currently available for studying antagonistic mechanisms, exhibited by foliar BCAs, are often insensitive & many mechanisms of action exhibited by BCAs are often not fully clarified. This is due to constraints brought about by factors such as the type of crop & tissue under study, the interference by resident microflora that are already established in that particular niche, the type, amount & availability of nutrients as well as the inter- & intra- species variation found in antagonistic activity expressed by BCAs (Elad *et al.*, 2001). These variations can all contribute to the difficulty experienced by many researchers in attempting to follow & expand on previously reported work. Establishing the actual mechanisms expressed by BCAs under natural conditions is a formidable task as laboratory-based assays can only prove the ability of the BCA to act by certain mechanisms within the confines of the conditions set by the experimenter (Tronsmo & Hjeljord, 1997). In addition, when one or more mechanisms fail to be effective (whether it be due to lack of nutrients or a change in environmental conditions) other mechanisms may compensate, adding further complexity to the subject.

Trichoderma species are soil-inhabiting microorganisms that are not naturally adapted to life in the phyllosphere. However, isolates from this genus have been widely investigated for the control of foliar diseases (Gullino, 1992) & a number of commercial products (aimed for use in the phyllosphere) are based on isolates from this genus (Hjeljord *et al.*, 2000; Wilson, 1997).

In earlier reports (Card *et al.*, 2003; 2004a), a high level of biocontrol activity by *Trichoderma atroviride* LU132 was demonstrated against the necrotrophic pathogen *Botrytis cinerea,* which causes grey mould in strawberry. *T. atroviride* LU132 inhibited lesion formation in leaves & suppressed pathogen sporulation on leaves & flowers, two tissues critical to the development of the disease cycle. The general observations from that study indicated that *T. atroviride* LU132 restricted pathogen growth through competition for limited resources, because once leaves/flowers senesced, *T. atroviride* LU132 rapidly colonised dead or dying material & sporulated abundantly, thereby excluding the pathogen from its saprophytic niche. This paper reports on further studies on the mechanism of biocontrol action by *T. atroviride* isolate LU132.

4.4 MATERIALS & METHODS

Inoculum production

Botrytis cinerea (LU829), an isolate from an infected commercial lettuce crop (Card *et al.*, 2002) & *Trichoderma atroviride* LU132 (previously *T. harzianum* C52), which was originally isolated from soil (McLean & Stewart, 2000), were cultured on potato dextrose agar (PDA, Oxoid Ltd, Hampshire, England) at 18°C for 10-14 days, under a 12:8 h (light: dark) photo regime. Conidial suspensions of the two fungi were prepared by flooding individual cultures with 10 mL aqueous Tween 80 solution (1 drop in 1000 mL sterile water) & scraping gently with a sterile loop. The resulting crude suspension was then filtered through a layer of Miracloth (Calbiochem, Darmstadt, Germany) to remove mycelial fragments. Inoculum concentration was estimated using a haemocytometer & adjusted to the desired concentrations.

4.4.1 Competition for nutrients & space

Two experiments were performed to determine possible competition for nutrients by the BCA. The first, on detached strawberry leaves, was adapted from Guetsky *et al.* (2002a). The second, using polytetrafluoroethylene (PFTE) membranes in tissue culture cell plates, was adapted from Janisiewicz *et al.* (2000), & had the advantage of being able to eliminate competition for space as a potential mechanism. For both experiments, conidial suspensions were prepared in double distilled water at concentrations of 2×10^5 conidia mL^{-1} for *B. cinerea* & 1×10^7 conidia mL^{-1} for *T. atroviride* LU132. Suspensions were centrifuged (3000 rpm for 3 min) & the resulting pellets supplemented with the sugars, glucose, sucrose or fructose, to give final concentrations of 0.05%, 0.1% & 0.2% (w/v).

(i) Competition on detached strawberry leaves

Healthy, mature strawberry leaves (cv. Camerosa) were surface sterilised by agitating in 1% sodium hypochlorite solution for 90 s, rinsing twice in tap water & cut into quarters. The conidial suspensions for inoculation contained *B. cinerea* (LU829) mixed (1:1 v/v) with *T. atroviride* LU132 in their matching sugar concentrations, or with matching sugar solutions only (pathogen-only control), & a 30 µL drop of each treatment was placed on each leaf quarter. These were then randomly assigned to small humidity chambers, which consisted of a 170×240×80 mm plastic box (with a lid) that contained 500 mL tap water. Leaves were placed on a plastic tray which was suspended above the water. There were five leaf quarters per humidity chamber & six replicate chambers per treatment.

(ii) Competition in tissue culture plates

Flat-bottomed cell culture clusters, each with 24 wells (Corning Inc., Corning, U.S.A.) & 12 mm diameter Millicell-CM culture plate inserts (Millipore Corp., Bedford, U.S.A.), were used (Figure 16). Each culture plate insert was constructed from a polystyrene cylinder & a polytetrafluoroethylene (PTFE) membrane with 0.4 µm pores, which allowed the free movement of liquids through the membrane. For each well, 600 µL of the *T. atroviride* LU132 suspensions or sugar only (controls) was firstly dispensed into the well of culture plate, the cylinder insert was then placed into the well & 300 µL of the pathogen in the matching sugar & concentration was

dispensed into the cylinder. This method kept the two fungi physically separated but allowed competition for the sugars in which they were suspended. Culture plates were then assigned to small humidity chambers (as mentioned previously) with four replicates per treatment. Incubation was at 20°C in the dark for 18 h, for both experiments.

The microorganisms from both experiments were fixed in 2.5% (v/v) glutaraldehyde solution & kept at 4°C (for a maximum of 5 days) until examination.

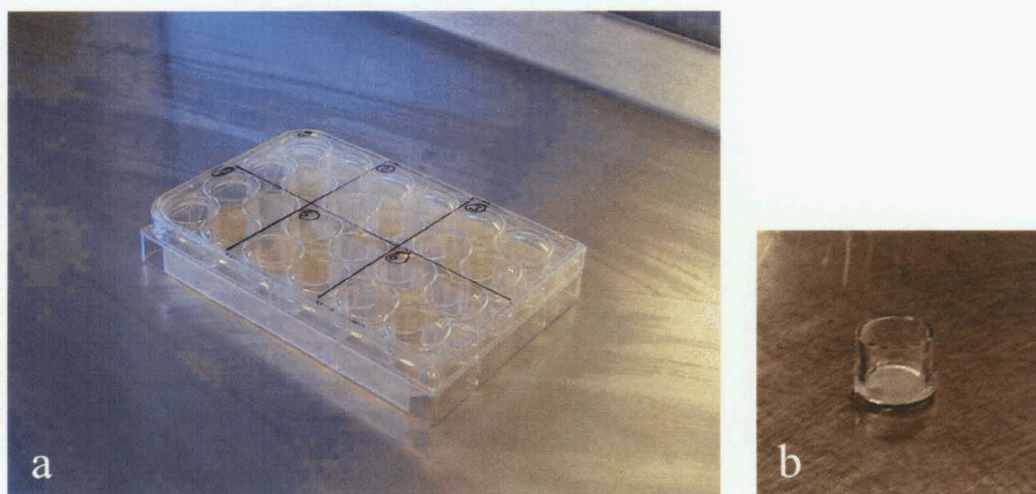

Figure 16: (a) Flat-bottomed cell culture clusters, & (b) Millicell-CM culture plate inserts used in competition studies

Leaf quarters were each placed on a glass slide, dyed with half strength lactophenol cotton blue (LPCB) & a cover slip placed over the top. Each cylinder insert was removed from its well, blotted onto a paper towel, inverted & placed onto a glass slide. For all treatments in both experiments, the germination frequency of 50 *B. cinerea* conidia & the average length of germ tubes was assessed from four replicates. Results were recorded using a DP12 digital camera system connected to a light microscope (BX51, Olympus New Zealand Ltd., Auckland, New Zealand) connected to the computer driven software Analysis® (Soft Imaging System GmbH, Münster, Germany). Analysis of variance (ANOVA) was used to determine significant differences ($P \leq 0.05$) between nutrient concentrations, & Fishers protected LSD test was used to rank concentrations in order of effectiveness. Paired t tests were used to determine significant ($P \leq 0.05$) differences between treatments. Statistical analysis

was performed using the package GenStat® 6.1 (Rothamsted Experimental Station, Hertfordshire, U.K.).

4.4.2 Volatile anti-fungal metabolite bioassay

The production of volatile antibiotics was examined using the method of Kexiang *et al.* (2002). *B. cinerea* & *T. atroviride* LU132 were grown on PDA at 20°C under a 12 h photoperiod for 6 & 4 days, respectively. There were two experiments, one performed on a high nutrient medium, PDA, & the other on a defined minimal medium, 1% (w/v) Czapek dox liquid medium (Oxoid), plus 20 g L^{-1} bacteriological agar (Germantown Company, Auckland, New Zealand). The Petri plates containing 15 mL agar each were centrally inoculated with an agar plug from an actively growing culture of *B. cinerea*. The lid of each Petri plate (also containing 15 mL of agar) was centrally inoculated with an agar plug from an actively growing culture of *T. atroviride* LU132. In control plates, the agar in the lids was not inoculated. The plates were then immediately sealed with Parafilm®, inverted & incubated for 3 d at 20°C. There were seven replicates per treatment. The colony diameters (mm) were measured after 24 h, 48 h & 72 h using a digimatic calliper (Mitutoyo Corporation, Tokyo, Japan). Treatment means were compared using paired t tests. Statistical analysis was performed using the package GenStat® 6.1. Both experiments were performed twice, & as results from repeated experiments followed a similar trend, the results from only one experiment are presented.

4.4.3 Non volatile anti-fungal metabolite bioassay

To test the hypothesis that inhibitory compounds produced by *T. atroviride* LU132 were stress induced, three treatments were prepared, *T. atroviride* LU132 alone, *B. cinerea* alone, & a mixed conidial suspension of both the fungi. Cell free extracts from these three treatments were prepared in flat-bottomed cell culture clusters with 24 wells (as mentioned previously). The conidial suspensions were centrifuged at 3000 rpm for 3 min, the supernatant removed & the resulting pelleted cells resuspended in 1% (w/v) Czapek dox liquid medium. The *T. atroviride* LU132 inoculum was prepared at a final concentration of 5×10^6 conidia mL^{-1}, while the inocula containing a mix of *T. atroviride* LU132 & *B. cinerea* in a 1:1 ratio (v/v) & the *B. cinerea* (LU829) alone were prepared to concentrations of 1×10^5 conidia mL^{-1}.

For all conidial suspensions, 300 μL was inoculated into individual wells & the clusters incubated at 20°C for 72 h. Suspensions were pH tested before & after incubation & then filter sterilised through 0.22 μm sterile Millex-GS filter units (Millipore, Malsheim, France) & the resulting liquid was freeze dried using a MicroModulyo 230 connected to an FDP120 oil vacuum pump & a spin freezer 96 (Thermo Electron Corporation, Milford, U.S.A.). The dried product was then suspended in 1% Czapek dox broth containing a fresh *B. cinerea* conidial suspension (1×10^5 conidia mL^{-1}). The 30 μL drops of the various treatments were then dispensed onto individual surface sterilised strawberry leaf quarters (cv. Camerosa) & placed in humidity chambers. Leaf quarters were incubated at 20°C for 18 h & then fixed in 2.5% (v/v) glutaraldehyde solution. Germination frequency & the average length of germ tubes was assessed under a light microscope, as described for the competition assays. Analysis of variance (ANOVA) followed by Fishers protected LSD ($P\leq0.05$) test were used to analyse results & rank concentrations in order of effectiveness. Statistical analysis was performed using the package GenStat® 6.1. The experiment was performed twice & as results from the repeated experiment followed a similar trend, the results from only one experiment are presented.

4.4.4 Hyphal interactions

Hyphal interactions between the pathogen & antagonist were studied on a high nutrient medium (PDFA) that resembled the nutrient status of newly opened strawberry flowers, as described by Hjeljord & Strømeng (2004) & on a low nutrient medium (1% Czapek dox agar, Oxoid). Plates were dual inoculated with mixed conidial suspensions of the two fungi, 2×10^5 conidia mL^{-1} for *B. cinerea* & 1×10^7 conidia mL^{-1} for *T. atroviride* LU132, & interactions observed daily over a period of 7 days using a light microscope. As coiling of the antagonist hyphae around the pathogen was observed on both media, a further experiment was set up to study the interaction more closely. Dual cultures of *B. cinerea* & *T. atroviride* LU132 were prepared inside Petri dishes by mounting microscope slides onto glass elbows. Tap water agar (3.4 g L^{-1} bacteriological agar), which gave the sparse mycelial growth needed for ease of observation, was then poured thinly onto slides & allowed to set. Small (3 mm diameter) plugs cut from each of the actively growing cultures of the two fungi were then inoculated at opposite ends of the agar-coated slide. The lids of

the Petri dishes were replaced & they were incubated at 20°C for approximately 12 h, or until an interaction zone could be seen. The agar containing the interaction zone was then removed from the slide & examined using a Leica 440 scanning electron microscope (SEM) (Leica Micro-Systems Inc., Illinois, U.S.A.) with an Oxford Cryo specimen preparation system (Oxford Cryosystems Ltd, Oxford, U.K.).

4.5 RESULTS

4.5.1 Competition for nutrients & space

After 18 h incubation, there were no significant differences ($P>0.05$) in the percentage germination of *B. cinerea* conidia between the control & LU132 treatments in any of the three tested sugars, on either the detached strawberry leaves or the PTFE membranes. However, differences were observed in the length of *B. cinerea* germ tubes. Increasing concentrations of glucose counteracted the inhibitory effect given by the germinating conidia of *T. atroviride* LU132 (Table 15). For example, on detached strawberry leaves, in the presence of glucose at 0.05%, *B. cinerea* germ tubes from the control & LU132 treatments were significantly different with mean lengths of 46.7 µm & 24.7 µm respectively, an inhibition of 47%. When the glucose concentration was increased to 0.2%, *B. cinerea* germ tubes from the control & LU132 treatment measured 41.8 µm & 38.07 µm respectively, an inhibition of only 9% (not significant). This trend was also observed on PTFE membranes (Table 15). This trend was observed in the presence of fructose on strawberry leaf pieces, but not on PFTE membranes (Table 15). In the presence of sucrose, however, there was a continuous, significant, inhibitory effect by *T. atroviride* LU132 at all tested concentrations of the sugar on strawberry leaf pieces & PFTE membranes (Table 15).

Table 15: Effect of increasing concentration of three sugars on the length of *B. cinerea* germ tubes after 18 h incubation in the presence of *T. atroviride* LU132 germinating conidia on both strawberry leaf pieces & PTFE membranes

		Mean *B. cinerea* germ tube length (µm)[a] on strawberry leaf pieces			Mean *B. cinerea* germ tube length (µm)[a] on PTFE membranes		
Sugar	Concentration %	Control	LU132	P value[b]	Control	LU132	P value[b]
Glucose	0.05	46.7	24.7	0.007	100.9	75.5	0.001
	0.1	48.9	31.8	0.002	89.6	80.1	0.088
	0.2	41.8	38.1	0.202	68.2	66.9	0.420
Sucrose	0.05	45.3	22.5	0.007	80.0	44.4	0.009
	0.1	43.2	24.9	0.055	70.4	50.7	0.038
	0.2	41.9	30.0	0.027	80.0	55.1	0.008
Fructose	0.05	37.5	27.0	0.001	61.5	47.7	0.046
	0.1	44.7	34.8	0.061	60.7	49.6	0.101
	0.2	48.5	36.0	0.105	70.3	53.2	0.001

[a] Mean calculated from a total of 200 germ tubes from four replicates

[b] P value taken from paired t test determined from germ tube lengths between control & LU132 treatments

4.5.2 Volatile anti-fungal metabolite bioassay

On PDA, inhibition of *B. cinerea* colony growth was observed in the presence of *T. atroviride* LU132 suggesting the production of anti-fungal volatiles under high availability of nutrients. The differences between the two treatments were not significant (P>0.05) after 24 h, but were significant after 48 h & 72 h, with inhibition of 20% & 27%, respectively (Table 16). However, under conditions of lower nutrient levels (1% Czapek dox agar), colony diameters of *B. cinerea* from the control & LU132 treated plates were not significantly different at any time period tested (Table 16).

Table 16: The percentage inhibitory effect of volatile inhibitory compounds produced by *T. atroviride* LU132 on colony expansion of *B. cinerea* when both were grown on the same plates on PDA & 1% Czapek dox agar

Time (hours)	% inhibition of *B. cinerea* on PDA	% inhibition of *B. cinerea* on 1% Czapek dox agar
24	4	6
48	20 *	0
72	27 **	2

* Colony diameters significantly different (P≤0.05) compared to control according to paired t-test

** Highly significant at P≤0.001

4.5.3 Non volatile anti-fungal metabolite bioassay

Cell free extracts from the treatments, *B. cinerea* alone, *T. atroviride* LU132 alone & a mixture of both microorganisms, were pH tested before being freeze dried. All treatments recorded small pH changes of pH ±0.4. When the freeze dried product was resuspended in Czapek dox broth, *T. atroviride* LU132 alone, & the mixed treatments significantly (P≤0.05) inhibited *B. cinerea* germ tube length from 98 μm in the control treatment to 42 μm & 58 μm, respectively (Figure 17).

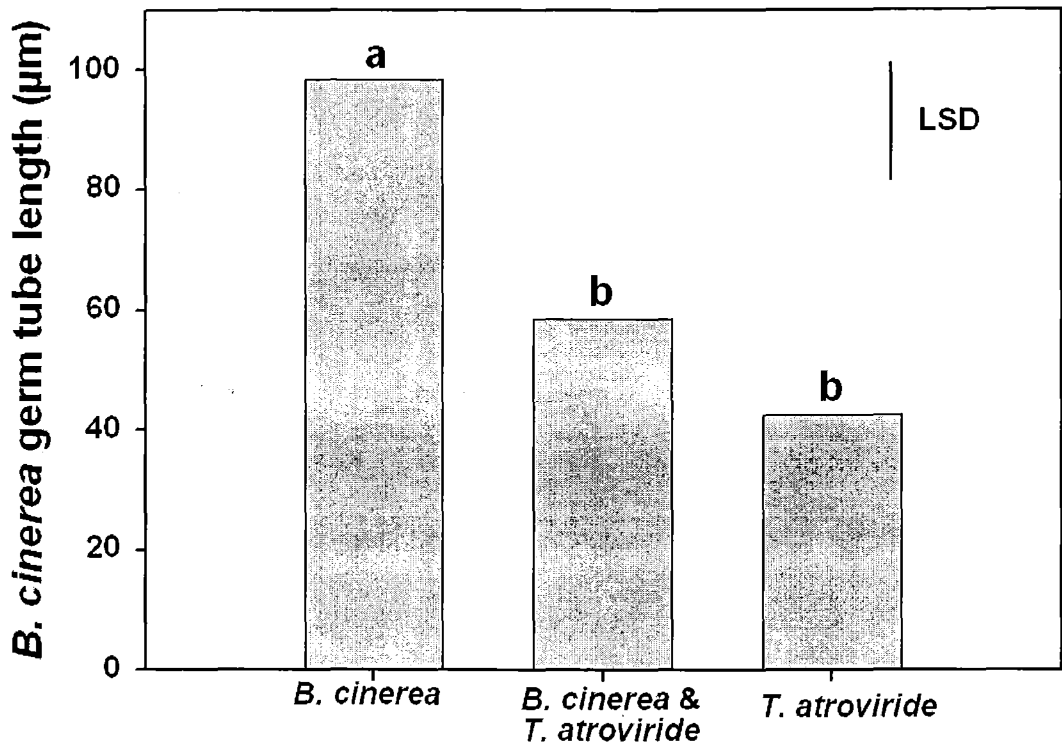

Figure 17: The effect of non-volatile inhibitory compounds produced by *T. atroviride* LU132 alone, or in mixed Czapek dox liquid culture with *B. cinerea*, on germ tube length of *B. cinerea* conidia. Columns followed by the same letter are not significantly different according to Fisher's protected LSD test ($P \leq 0.05$)

4.5.4 Hyphal interactions

Microscopic observations revealed that *T. atroviride* LU132 was able to prevent growth of *B. cinerea* when dual inoculated as mycelial plugs & when the conidia were mixed & co-inoculated on all media tested. Light micrograph studies showed that *T. atroviride* LU132 grew alongside & frequently coiled tightly around hyphae of *B. cinerea* (Figure 18). Although penetration was not observed, after the initial interaction, *B. cinerea* hyphae were seen to rapidly collapse & die in all tested situations within 4 days following inoculation.

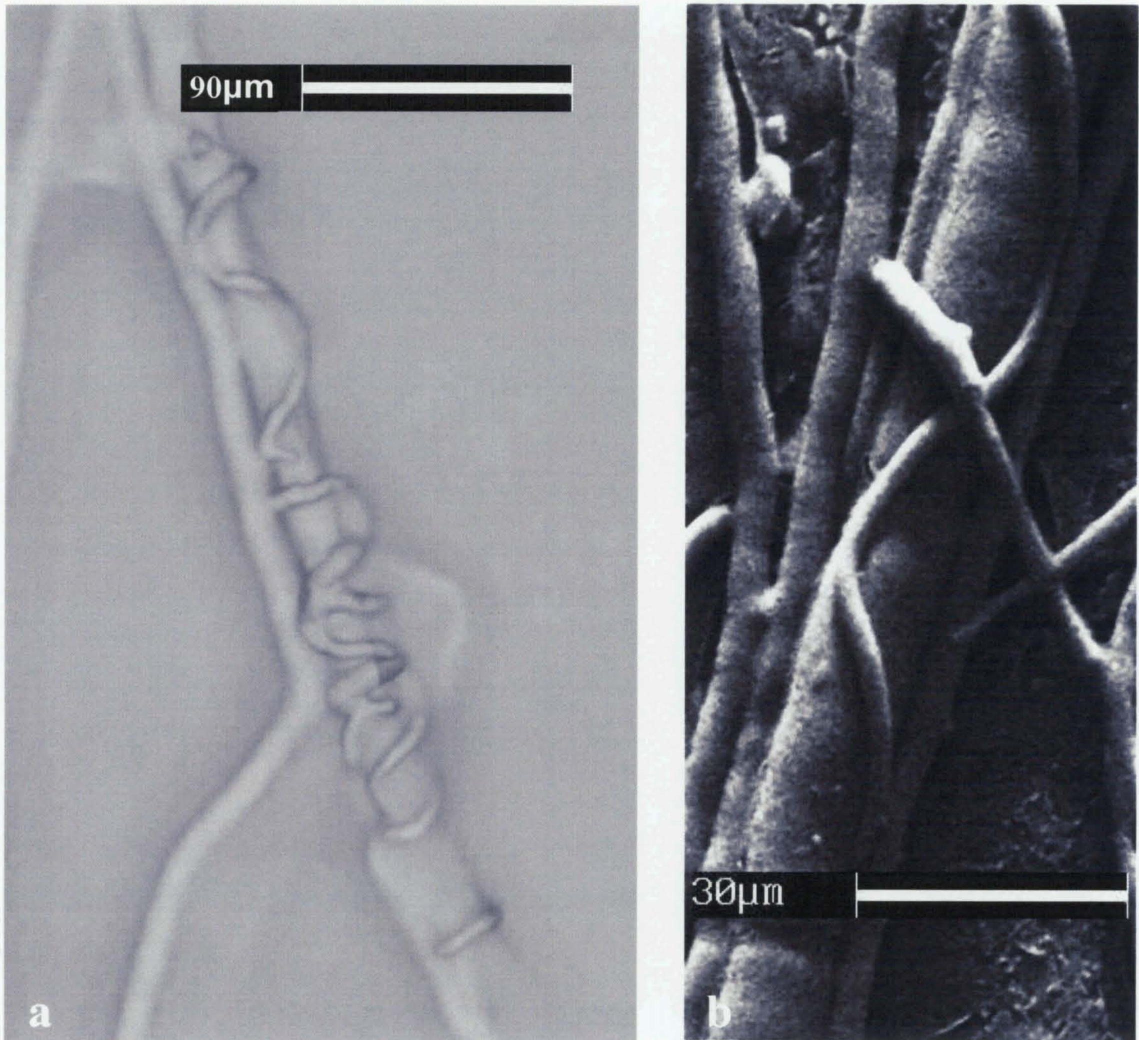

Figure 18: *T. atroviride* LU132 coiled tightly around hyphae of *B. cinerea* (a=the light micrograph & b the scanning electron micrograph)

4.6 DISCUSSION

Most studies on the mechanisms of action of BCAs have focused on soil borne pathogens, with few studies on foliar pathogens (Janisiewicz *et al.*, 2000) as described here. From this study, it appears that *T. atroviride* LU132 inhibits *B. cinerea* on strawberry leaves through competition for glucose, fructose & sucrose, through the production of non-volatile & volatile inhibitory compounds, &, with as yet, an undefined level of inhibition through direct parasitism. Preliminary experiments inoculating live or dead conidia of LU132 on roots of young strawberry plants failed to protect the foliage from *B. cinerea* infection, indicating that the antagonist did not elicit a systemic induced response following root inoculation (Appendix 4). It is clear that in

the presence of sucrose all tested concentrations of the sugar were limiting as at the highest concentration of 0.2% sucrose, the length of *B. cinerea* germ tubes was still significantly shorter than that of the control, on strawberry leaf pieces & PFTE membranes. This suggests that sucrose is competed for at a greater level than either glucose or fructose or that inhibitory substances were produced by *T. atroviride* LU132 in the presence of the sugar.

The major sugars on plant surfaces are glucose, sucrose & fructose & these carbohydrates originate in the phyllosphere endogenously through direct leaching from plant leaves (Mercier & Lindow, 2000; Tukey, 1971) or exogenously from sources such as dead microorganisms, insect & bird excrement, pollen & dust (Andrews, 1992). Competition for these sugars & other nutrients has been shown to be an effective mechanism in biocontrol of necrotrophic pathogens as many, including *B. cinerea,* require exogenous nutrients for germination & initiation of the infection process (Elad, 1996).

The sugar levels on strawberry leaves were not measured & can be assumed to vary according to many factors including cultivar, time of year, region, & how much pollen they accumulate. Blakeman (1975) noted that beetroot leaf surfaces contained carbohydrate levels of 50 $\mu g\ mL^{-1}$ or below & agreed with the belief of many researchers that microorganisms growing on plant surfaces would be competing for a limited amount of nutrients (Andrews, 1992; Blakeman, 1993; Mercier & Lindow, 2000). Sugar levels on strawberry leaves could, therefore, be assumed to be limiting to phyllosphere organisms, including foliar pathogens, at most times of the year. The sugar content of newly opened strawberry flowers collected from a glasshouse was measured at 0.5-0.6 mg each of glucose & fructose per flower, while older flowers had up to twice this level (Hjeljord, personal communication). However, in the field environment this level would probably be lower as factors such as rain, relative humidity & insects can all influence these sugar concentrations. The results from this study indicate that competition for sugars may play more of a major role in the antagonism of *B. cinerea* by LU132 on strawberry leaves than on strawberry flowers where at certain stages of flower development there are greater levels of sugars from nectar, & these can help to support the pathogen & other microorganisms present.

Guetsky *et al.*, (2002b) reported similar results to this study for the BCA *Pichia guilliermondii*. This yeast competed with *B. cinerea* conidia, inhibiting their germination in glucose & sucrose solutions of 0.03%, but when the concentrations of the solutions were increased ten fold, the biocontrol effect was lost. In this study, *B. cinerea* germinated very well on PTFE membranes & to our knowledge this is the first report of using this method for determining mechanisms of action for this pathogen. The results of the glucose & sucrose treatments followed a similar trend on PFTE membranes to those observed on strawberry leaf pieces. However, in the presence of fructose the trend on PFTE membranes was different to that on strawberry leaf pieces. Both the methods used in this study to measure competition for nutrients demonstrated reduced *B. cinerea* germ tube length as an outcome of competition. A more detailed investigation could involve measuring & comparing the actual rates of sugar uptake by the antagonist & the pathogen using ^{14}C-labelled sugars. This technique has been reported by Filonow (1998) who found that the yeasts, *Cryptococcus laurentii* & *Sporobolomyces roseus* had a higher uptake of ^{14}C-labelled fructose, sucrose & glucose than *B. cinerea in vitro* & in post-harvest apple wounds. This subsequently decreased germination of pathogen conidia compared to the pathogen-only controls. Other mechanisms were evidently at work with these yeasts, since *Saccharomyces cerevisiae* (which was used in the same study) was capable of utilising the sugars as well or better than *C. laurentii* & *S. roseus*, but it was found to be a poor BCA. This method, therefore, has limitations as a screening method for BCAs.

Previous researchers, who investigated competition for nutrients, recorded an inhibition of *B. cinerea* germination by BCAs (Brodie & Blakeman, 1975; Filonow, 1998; Guetsky *et al.*, 2002b), whereas in this study, the only effect observed for *T. atroviride* LU132 was that it inhibited germ tube elongation & no effect was found on germination frequency. However, the earlier investigations used bacteria or yeasts as BCAs, unlike the filamentous fungal isolate used in this study. In addition, many *Trichoderma* species have been found to require 1.5 - 4 days to germinate at 18°C (Hjeljord *et al.*, 2000) compared to the *B. cinerea* isolate used in this study, which germinated in less than 18 hours at 20°C. Therefore, it is hypothesised that *T. atroviride* LU132 was not at the optimum metabolic activity needed to bring about inhibition of pathogen germination under the conditions tested, as shown for the bacteria & yeast BCAs reported by other researchers.

Nutrients are frequently limiting on the phylloplane (Andrews, 1992) & it is well known that the most important factor governing the production of antibiotics in *Trichoderma* species is the growth substrate (Howell, 1998). *T. atroviride* LU132 produced non-volatile compounds in 1% Czapek dox broth, which significantly inhibited germ tube elongation of *B. cinerea* conidia. The inhibitory compound(s) have not been identified as yet, but were shown to not alter the pH of the medium significantly. Tronsmo & Dennis (1977) proposed that on strawberry flowers & fruit, antagonism by non-volatiles was the most important mechanism exhibited by *Trichoderma* isolates in the control of *B. cinerea.* Similarly, in screens by Prokkola (1992) (cited in Belanger *et al.*, 1995), it was noted that the most successful *Trichoderma* BCAs were the ones that produced non-volatile antibiotics. The production of U.V. mutants of the BCA that cannot produce the compound(s) would facilitate comparative studies to clarify the role of these antibiotics in biocontrol (Elad, 1996).

Most antibiotics are produced by soil inhabiting microorganisms & there is now sufficient evidence to indicate that antibiotics may play an active role in biological control in nature (Fravel, 1988). The most widely studied antibiotic produced by many *Trichoderma* species is the volatile polyketide, 6-n-pentyl-2H-pyran-2-one (6PAP) which possesses a characteristic coconut-like aroma. As this aroma was strongly emitted by cultures of LU132, it is possible that this volatile compound was partly responsible for antagonism on the high nutrient medium, PDA. Recent biochemical analysis has confirmed this (Stewart, personal communication).

Trichoderma species are the most widely studied fungi with respect to mycoparasitism (Punja & Utkhede, 2003) & this phenomenon has been well documented for many years. The results shown here concur with the original conclusion drawn about the modes of action for *Trichoderma* spp. reported by Weindling (1932), since *T. atroviride* LU132 was observed to coil around pathogen hyphae regardless of the external nutrients supplied to the host or antagonist. Although *Trichoderma* isolates have been observed to be mycoparasitic on a range of plant pathogens (Benhamou & Chet, 1993; Dennis & Webster, 1971; Gupta *et al.*, 1999; Pisi *et al.*, 2001), the role this mechanism plays in biological control remains uncertain due to the scarcity of recorded observations *in vivo* (Punja & Utkhede, 2003) & the slow rate at which *Trichoderma*

spp. operate when compared to the fast germination & subsequent host penetration exhibited by the pathogen (Elad, 1996). The observations made in this study are similar to those found by Hjeljord *et al.* (2001) who observed mycoparasitism of *B. cinerea* by *T. atroviride* P1 *in vitro* but not under field conditions.

T. atroviride LU132, as with several other *Trichoderma* BCAs has been shown to employ more than one mode of action, & it seems likely that this optimises its effectiveness against *B. cinerea* on strawberry as well as reducing the risk of the pathogen developing resistance to fungicides.

Further research with *T. atroviride* LU132 will investigate competition using ^{14}C-labelled sugars, characterise the antibiotics produced by LU132 & define the level of mycoparasitism expressed by LU132 towards *B. cinerea* on the strawberry phylloplane *in situ.* Once the roles of these mechanisms have been fully defined, research could investigate the less obvious mechanisms, such as changing the wettability of plant surfaces, attachment of the BCA to the pathogen's polysaccharide extracellular matrix, production of cell wall degrading enzymes or reducing the pathogenicity of *B. cinerea* enzymes, all of which have been implicated as being important in the control of *Botrytis* diseases (Elad & Stewart, 2004).

4.7 ACKNOWLEDGEMENTS

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CHAPTER 5:

Biological & integrated control of *Botrytis cinerea* of strawberry with *Trichoderma atroviride* LU132

5.1 FOREWORD

This chapter evaluated the antagonistic fungus *Trichoderma atroviride* LU132 for the biocontrol of *Botrytis cinerea* under field conditions.

All the work was conducted by the first author with supervision from the other listed authors.

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5.2 ABSTRACT

Field trials were carried out to evaluate the ability of the biological control agent (BCA), *Trichoderma atroviride* LU132 to control *Botrytis cinerea* in three cultivars of strawberry. The study also investigated the effect of commonly used botryticides on germination & growth of the BCA, to determine potential compatibility for mixed application. The fungicide fenhexamid significantly increased *T. atroviride* LU132 conidial germination frequency by up to 34% in fungicide sensitivity assays & was later used in mixed tank applications. These field trials demonstrated that treatment with *T. atroviride* LU132 & fenhexamid alone, & the tank-mix of both could significantly suppress *B. cinerea* sporulation on strawberry leaves & stamens by 42-100%. These treatments increased yield of strawberry fruit similarly by up to 36% more than the control, but they did not reduce disease incidence in the fruit in a 7 day ambient storage trial.

5.3 INTRODUCTION

Botrytis cinerea Pers, is a necrotrophic pathogen that causes grey mould, an important disease of strawberry that can be found wherever the crop is grown (Gullino *et al.*, 1989; Maas, 1984). In strawberry fruit, the disease often begins with conidial infection of strawberry flowers during blossoming. These infections can remain quiescent in floral parts as mycelium until the fruit ripens when the disease is expressed (Bristow *et al.*, 1986; Jarvis, 1962; Legard *et al.*, 2000b; Powelson, 1960). Control currently relies on the heavy use of chemical fungicides. However, because *B. cinerea* is a highly variable heterokaryotic pathogen, there is a problem, or potential problem, of resistance developing to many of the systemic botryticides (Fletcher, 1984). For example, strains of *B. cinerea* resistant to the benzimidazole & dicarboximide groups of fungicides are now widespread (Dik & Elad, 1999; Köhl *et al.*, 1998; Sutton *et al.*, 1997). In recent years, problems with fungicide resistance & concerns from consumer groups over the levels of chemical residues on the fruit has added intensity towards the search for microbial antagonists for plant protection that can offer an effective alternative to chemical control (Helbig, 2002). Many microbial isolates have been screened for the biocontrol of Botrytis diseases under *in vitro* conditions where some have shown promise. However, the ultimate test & frequent downfall of many potential biological control agents (BCAs) is to test them under field conditions (Pusey, 1990 cited in Swadling & Jeffries, 1996). Strategies used to overcome such variable efficacy include integration with fungicides (Shtienberg & Elad, 1997) or resistant crop cultivars (Elad *et al.*, 1996) or using mixtures of two or more BCAs, which have different ecological niches &/or different modes of action (Elad & Stewart, 2004; Ippolito & Nigro, 2000).

Integration of chemical fungicides with a BCA may improve the efficacy of the BCA under the sub-optimal conditions often experienced in the phyllosphere, & therefore, aid in the germination & subsequent colonisation by the antagonist. The advantages of integrating BCAs & fungicides also include the potential to lower the amounts of fungicide applied to the cropping area, decreasing the chances of the pathogen developing fungicide resistance, & an improved ability to meet consumer expectations by lowering the level of chemical residues on marketed products & within the environment (Ippolito & Nigro, 2000). This approach has been used successfully in cucurbit powdery mildew which was controlled with mixtures of the commercial BCA,

AQ10, whose active ingredient is *Ampelomyces quisqualis*, & the fungicide myclobutanil (McGrath & Shishkoff, 1996).

Previous work has shown that the BCA, *Trichoderma atroviride* LU132 can inhibit lesion formation on detached strawberry leaves & significantly suppress *B. cinerea* sporulation on detached strawberry leaves & flowers (Card *et al.*, 2004a; Card *et al.*, 2004b). In the current study, the sensitivity of *T. atroviride* LU132 to a range of commonly used chemical botryticides was determined & the most compatible fungicide tested for its efficacy in field trials with the BCA, either alone or mixed.

5.4 MATERIALS & METHODS

Pathogen inoculum

Botrytis cinerea LU829, an isolate from an infected commercial lettuce crop (Card *et al.*, 2002), was maintained at 4°C on strawberry agar (SA), which was prepared according to Peng & Sutton (1991). Cultures used for inoculation were grown on SA at 20°C under a 12:8 h (light:dark) photo regime for 14 days or until they sporulated profusely. To maintain pathogenicity on strawberry, the isolate was regularly inoculated onto autoclaved strawberry leaves & then reisolated. Conidial suspensions were prepared by flooding cultures with aqueous Tween 80 solution (1 drop in 1000 mL sterile water) & filtering the resulting suspensions through sterile Miracloth (Calbiochem, Darmstadt, Germany) to remove mycelial fragments.

Antagonist inoculum

Trichoderma atroviride LU132 (previously named *T. harzianum* C52) (McLean & Stewart, 2000), was cultured on potato dextrose agar (PDA, Oxoid Ltd, Hampshire, England) at 18°C for 10 days under a 12:8 h (light:dark) photo regime. For *in vitro* experiments, conidial suspensions were prepared as for *B. cinerea*, whereas for field trials they were produced from sterilised wheat grain cultures. The high-grade wheat grain (200 g) & 62.5 mg chloramphenicol were added to 1 L Erlenmeyer flask & made up to 500 mL with tap water. The flask was heated until boiling & then left to stand for 10 min before draining through two layers of cheesecloth. The grain was washed three times in tap water, drained & autoclaved in the flask at 121°C for 15 min. After 24 h, the cooled grain was again washed in tap water, drained & autoclaved at 121°C for 15

min. *T. atroviride* LU132 was inoculated onto the wheat grain using mycelial plugs taken from an actively growing culture & incubated at 20°C, with a 12 h diurnal photoperiod that incorporated fluorescent light to encourage colonisation. The flask was shaken vigorously every day to avoid clumping. After 6 days, the grain was dispensed into Petri dishes & incubated at 20°C for a further 7 days or until profuse sporulation was evident. One Petri plate of grain was then poured into a sterile 1 L Erlenmeyer flask with 500 mL aqueous Tween-80 solution & shaken vigorously to release conidia. The resulting suspension was filtered twice through sterile Miracloth to remove mycelial fragments.

5.4.1 Sensitivity of *T. atroviride* LU132 to commercial fungicides

Effect on conidial germination

The most common method for determining fungicide compatibility is to measure the effect on development of the BCA when grown in a nutrient medium containing the fungicide (Punja, 1997). In this trial, Czapek dox liquid medium (Oxoid Ltd, Hampshire, England) was amended with a range of fungicides (Table 17).

Table 17: Fungicides screened for their effect on development of *T. atroviride* LU132

Fungicide	New Zealand supplier	Application rate per 100 L water
Tolylfluanid (Euparen® Multi DF)	Bayer NZ Ltd, Christchurch	200 g
Iprodione (Rovral® Flo)	Bayer NZ Ltd, Christchurch	200 mL
Procymidone (Sumisclex® 25 Flo)	Crop Care Holdings Ltd, Nelson	200 mL
Cyprodinil & Fludioxonil (Switch®)	Syngenta Crop Protection Ltd, Auckland	80 g
Fenhexamid (Teldor® 500 SC)	Bayer NZ Ltd, Christchurch	150 mL
Thiram (Thiram®)	Nufarm Ltd, Auckland	150 mL

Trade names are given in brackets

The fungicides were added to the medium at four different rates, 25, 50, 75 & 100% (of the recommended field rate). Tubes containing 20 mL of the Czapek dox-amended fungicide solutions were each inoculated with 1 mL of a conidial suspension of *T. atroviride* LU132 (1×10^7 conidia mL^{-1}) & shaken vigorously for 10 min using a flask shaker (Griffin & George Ltd, England). From these tubes, 30 µL drops were dispensed onto glass microscope slides, four replicate slides per treatment arranged in a randomised block design. The slides were placed onto a plastic tray which was housed in a small humidity chamber consisting of a 170×240×80 mm plastic box that contained 500 mL tap water & closed with a tightly fitting lid. The chambers were incubated for 18 h at 20°C in the dark & then stained with half strength lactophenol cotton blue (LPCB). Slides were maintained at 4°C until needed. On each slide, in a randomly selected position, the germination frequency of 50 conidia & the average length of their germ tubes were recorded. Results were recorded using a DP12 digital camera system connected to a light microscope (BX51, Olympus New Zealand Ltd., Auckland, New Zealand) connected to the computer driven software Analysis® (Soft Imaging System GmbH, Münster, Germany).

Effect on mycelial growth

Czapek dox (modified) agar (Oxoid Ltd) was amended with the same fungicides & concentrations as before. Petri plates containing the amended agars were each inoculated with a 6 mm culture plug taken from an actively growing culture of *T. atroviride* LU132. The control treatment consisted of an inoculated Petri plate containing only Czapek dox agar (Oxoid Ltd) & no fungicide. Petri plates were incubated for 5 days at 20°C. The diameters of the colonies were measured using a digimatic calliper (Mitutoyo Corporation, Tokyo, Japan) & the mean colony area was calculated from 10 replicate plates per fungicide treatment.

5.4.2 Strawberry field trial 2003

Treatments

The effectiveness of *T. atroviride* LU132 at concentrations of 1×10^6 & 1×10^7 conidia mL^{-1} & the fenhexamid, Teldor® 500 SC (Bayer New Zealand Ltd) at the recommended concentration of 1.5 mL L^{-1} was investigated. All concentrations of the fungicide fenhexamid significantly increased conidial germination & subsequent germ tube

length of *T. atroviride* LU132 (Table 18). Therefore, preliminary trials using an integrated strategy were set up with the fungicide at half the recommended concentration (0.75 mL L^{-1}) & *T. atroviride* LU132 at a final concentration of 5×10^5 conidia mL^{-1}. The pathogen only control treatment was sprayed with aqueous Tween-80 solution.

Experimental design

Two separate field trials were carried out from April to May 2003, using an annual crop (cv. Yolo), planted in early October 2002, & a three-year old perennial crop (cv. Aptos). Plants of both cultivars were treated in the same way. They were spaced 80 cm apart between rows & 30 cm apart within rows, with buffer plants at each end of the rows & between treatments within rows. During the trial, plants were trickle irrigated in hot weather & grown without fertiliser or pesticide applications (other than the treatments) with weeding being done manually. To promote pathogen infection, cloches (Figure 19) were placed over plants, which were also spray irrigated by an automatic system for 1 h every evening installed to increase humidity inside the cloches.

Figure 19: Cloches used in 2003 to increase humidity around strawberry plants

The experimental layout was completely randomised with six replicate micro-plots per treatment with each micro-plot containing three plants. Cloches were placed over entire

rows. The treatment suspensions were sprayed onto the plants until run-off using a 5 L pressure sprayer (Butlers Ltd, Auckland, NZ). The applications were made at 7 day intervals on the evenings of warm sunny days starting on 25 March, & finishing on the 13 May. Control plots were treated with aqueous Tween-80 solution. A *B. cinerea* conidium suspension was sprayed onto the plants until run-off using a 5 L pressure sprayer 24 h after application of the treatments on all dates, except on 14 May when there were adequate levels of natural inoculum. Approximately 48 h later, one compound leaf (three leaflets) per plant was removed from the site & taken back to the laboratory for assessment. In addition, randomly selected flowers of the cultivar Yolo (12 flowers per replicate) were removed for assessment on 17 April & 14 May to determine the level of control achieved by the treatments.

Strawberry leaves

A 3-cm disc was cut from each of the leaflets using a flamed cork borer. The discs were then immediately placed on a herbicide-amended agar medium (adapted from Peng & Sutton, 1991) to hasten senescence. The medium was prepared with 4.8 g agar & 0.16 g chloramphenicol which were dissolved in 800 mL tap water & autoclaved, after which 9.6 g DAS® herbicide (simazine 4.8g L^{-1}, amitrole 1.5g L^{-1}, 2,2-dichloropropionic acid 3.1g L^{-1}) (Yates, Homebush, Australia) was added to the cooled mixture. Pathogen growth & subsequent sporulation was quantified after 7 days incubation, at 20°C, by estimating the percentage leaf area covered by *B. cinerea* conidiophores using a 1 to 5 scale that corresponded to conidiophore coverage of 0-20%, 21-40%, 61-80% & 81-100% respectively. This experiment was repeated four times with the treatments being applied every 7 days. There were four assessments, with new leaves sampled from the same plants.

Strawberry stamens

For each treatment & replicate, 20 stamens were removed from eight flowers (with no more than three stamens from the same flower) using a sterile scalpel & they were aseptically transferred onto two plates of PDA (Oxoid Ltd) that contained 2 mL L^{-1} Triton-X. This medium led to the development of small, discreet fungal colonies, which could be easily assessed under the stereomicroscope. Incidence & severity of *B. cinerea* sporulation was evaluated on stamens after 7 days incubation at 20°C. The severity score was determined by estimating the *B. cinerea* conidiophore density using a 1-6

scale (Helbig, 2001) where category 1= no conidiophores, 2=1-10, 3=11-20, 4=21-50, 5=51-100, 6=>100.

5.4.3 Strawberry field trial 2004

Treatments

Treatments were the same as for 2003, except that *T. atroviride* was only applied at the rate of 1×10^7 conidia mL^{-1}. This concentration was found to give a more consistent level of disease control than the lower concentration.

Experimental design

Two separate field trials were carried out from March to April 2004, using an annual crop (cv. Pajero), planted in early December 2003, & a one-year old crop (cv. Yolo) planted in October 2002 (Figure 20). The experimental layout was completely randomised for both cultivars. For cv. Pajero there were four replicate micro-plots per treatment with each micro-plot containing four plants. For cv. Yolo there were seven replicate micro-plots per treatment with each micro-plot containing three plants. Plants were trickle irrigated throughout the experiment & aphids were controlled in early October 2003 with two applications of fluvalinate (Mavrik®, Yates NZ Ltd). No other pesticides or fertilisers were applied to the site during the experiment, with weeding being done manually. On 19 February 2004, all flowers from both plots were removed to provide uniformity of flower development between plots. Plants were uniformly sprayed until run-off with suspensions of the treatments using a 2 L pressure sprayer (Butlers Ltd, New Zealand). Treatments were applied at 7 day intervals starting on 1 March & finishing on 12 April. As environmental conditions were conducive to *B. cinerea* development, natural pathogen inoculum levels were considered sufficient for infection.

Strawberry leaves

Strawberry leaves were collected & incubated as for 2003. As disease severity was found to be lower than in the previous year, pathogen sporulation was assessed by counting the number of *B. cinerea* conidiophores with the aid of a dissecting microscope.

Figure 20: Strawberry trial in 2004

Strawberry fruits

Plants of the cultivar Pajero were planted late in the season & failed to produce a uniform number of fruit per replicate. Therefore, only fruit from the cultivar Yolo were harvested & analysed. Fruit were harvested at the fully red stage on 22, 25 & 29 March, & on 1, 5, 13, 19 April, & taken back to the laboratory for disease assessment. To mimic normal storage conditions, fruit were placed well apart in 2.7 L plastic boxes lined with paper towels to prevent fruit surfaces coming into contact with the sides of the box. Boxes were sealed & stored at 4°C for 7 days, after which they were placed on a laboratory bench at room temperature. The fruits were inspected daily for presence of *B. cinerea*, & rotting fruit were discarded.

In both years, the amount of rainfall & mean daily temperature was recorded at 60 min intervals by an automatic weather station (Lincoln Broadfield, EWS) located in Lincoln, Canterbury.

Statistical analysis

Data of conidium germination frequency & mycelial area for *T. atroviride* LU132 were log transformed before analysis of the treatment effects with a two-way analysis of variance (ANOVA) followed by use of Fisher's protected least significance (LSD) test ($P \leq 0.05$). Treatments that completely inhibited conidial germination or mycelial growth were not included in the statistical analysis. Data from strawberry field trials were

analysed with the Kruskal Wallis multiple comparisons test (P≤0.05), for ordinal scaled data or ANOVA followed by Fisher's LSD (P≤0.05) for continuous data.

5.5 RESULTS

5.5.1 Sensitivity of *T. atroviride* LU132 to fungicides

The fungicides cyprodinil/fludioxonil, thiram & iprodione completely inhibited *T. atroviride* (LU132) germination & mycelial growth at all tested rates (data not presented). Procymidone completely inhibited conidial germination & significantly inhibited mycelial growth (P≤0.001) (Figure 21), while tolylfluanid significantly inhibited conidial germination (P≤0.05) & mycelial growth (P≤0.001) at all tested rates (Table 18 & Figure 21). The fungicide fenhexamid increased germination frequency at all tested rates compared to the control & germ tubes were also significantly (P<0.05) longer at all rates of fenhexamid (Table 18). Although fenhexamid significantly (P<0.05) inhibited mycelial growth compared to the control at all tested rates of the fungicide, overall this treatment was significantly less inhibitory to the BCA than the tolylfluanid & procymidone treatments (P<0.05) (Figure 21).

Table 18: Effect of fungicides, prepared in Czapek dox broth at four rates, on *T. atroviride* LU132 germination frequency & subsequent germ tube length

Treatment [c]	% rate	Conidial germination frequency (%) [a]	Conidial germ tube length (μm) [ab]
Control	-	66 a	19.9 a
Tolylfluanid	25	25 b	1.2 b
Fenhexamid	25	100 c	38.7 c
	50	93 d	29.8 c
	75	97 cd	38.8 c
	100	99 cd	34.7 c

[a] Values in a column followed by the same letter are not significantly different (P<0.05) according to Fisher's LSD test

[b] Data were subject to log transformed prior to ANOVA

[c] Treatments tested that completely inhibited conidial germination were not included in the statistical analysis & are not listed in the table

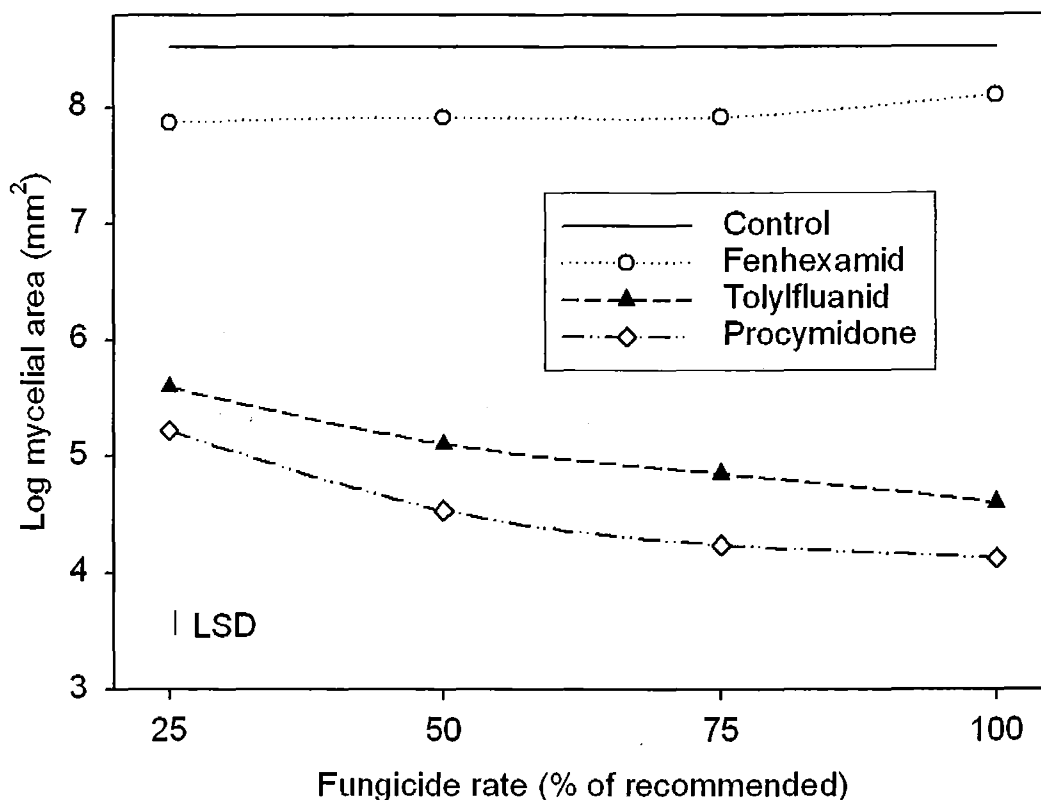

Figure 21: Mycelial area of *T. atroviride* LU132 grown on Czapek dox agar amended with fungicides, at four rates

5.5.2 Effect of *T. atroviride* LU132 on *B. cinerea* development in the field

Strawberry stamens

All treatments totally prevented *B. cinerea* sporulation on strawberry stamens with an average of 3.33 conidiophores from 10 strawberry stamens in the pathogen-only control. As no conidiophores developed, the treatments could not be ranked in order of effectiveness. On stamens treated with both concentrations of *T. atroviride* LU132, discreet colonies of *Trichoderma* spp. were observed growing from 90% of the stamens after 4 days incubation (Figure 22).

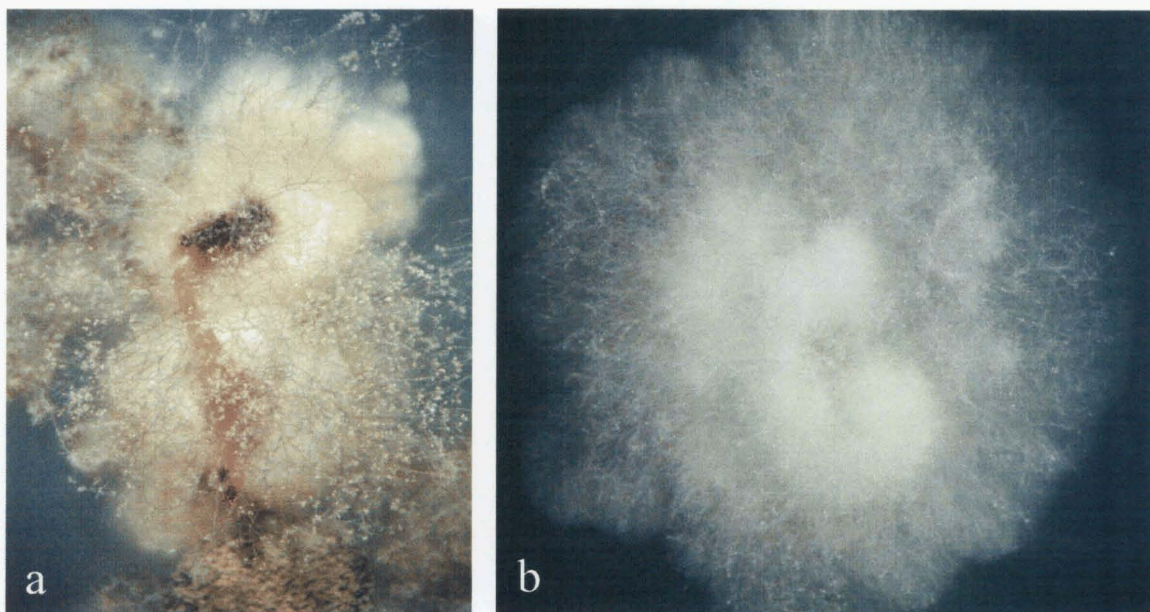

Figure 22: Strawberry stamens taken from pathogen only control (a) & *T. atroviride* LU132 treatments (b)

Strawberry leaves

Although fenhexamid significantly inhibited *T. atroviride* mycelial growth on Czapek dox agar, it was thought that the stimulatory effect that the fungicide had on conidial germination frequency & germ tube elongation after 18 hours was advantageous & therefore a tank mixture was prepared at 50% of its recommended rate (0.75 mL L^{-1}) & at a final concentration of 5×10^5 conidia mL^{-1} for *T. atroviride* LU132.

All treatments significantly ($P\leq0.05$) suppressed *B. cinerea* sporulation on strawberry leaves, for all cultivars. *T. atroviride* LU132, fenhexamid & the mixture all gave similar levels of control in all trials, for both years & across all assessments (Table 19).

Table 19: Effect of treatments, on *B. cinerea* sporulation on strawberry leaves collected from four separate trials. Mean values pooled from three assessments, except in 2003, cv. Yolo, where mean values were pooled from four assessments [a]

	Mean conidiophore coverage per leaf disc in 2003 [b]		Mean number of conidiophores per leaf disc in 2004	
Treatment	cv. Aptos	cv. Yolo	cv. Pajero	cv. Yolo
Water control	1.85 a	2.23 a	8.67 a	0.82 a
T. atroviride LU132 (1×10^6 mL^{-1})	1.05 b	1.04 b	-	-
T. atroviride LU132 (1×10^7 mL^{-1})	1.02 b	1.00 b	1.08 b	0.11 b
Fenhexamid (1.5 mL. L^{-1})	1.02 b	1.11 b	2.5 b	0.04 b
Mixture [c]	1.01 b	1.01 b	0 b	0.07 b

[a] Means in a column followed by the same letter are not significantly different (P≤0.05) according to Kruskal Wallis multiple comparisons test in 2003 & Fisher's LSD test in 2004

[b] Mean conidiophore coverage assessed using 1-5 scale

[c] The mixture consisted of fenhexamid (0.75 mL L^{-1}) & *T. atroviride* LU132 at 5×10^5 conidia mL^{-1}

- not tested

Table 20: Total yield of strawberry fruit from a strawberry field trial (cv. Yolo) in 2004, in which a range of treatments were applied at 7 day intervals

Treatment	Total number fruits [a]	Total fruit weight (g) [a]
Water control	86 a	1082 a
T. atroviride LU132 (1×10^7 mL^{-1})	117 a	1368 a
Fenhexamid (1.5 mL. L^{-1})	101 a	1205 a
Mixture [b]	103 a	1322 a

[a] Means in a column followed by the same letter are not significantly different at P≤0.05 according to Fisher's protected LSD test

[b] The mixture consisted of fenhexamid (0.75 mL L^{-1}) & *T. atroviride* LU132 at 5×10^5 conidia mL^{-1}

Strawberry fruit

Results from the field trial in 2004, showed that none of the treatments significantly affected the yield of strawberry fruit (Table 20). The lowest fruit yield & weight was found with the pathogen only control treatment with a total of 86 fruit, weighing 1088 g, & the highest yield & weight was with the BCA, *T. atroviride* LU132 treatment

where the yield was 117 fruit, weighing 1368 g (Table 20). None of the treatments applied in the field in 2004 significantly reduced the percentage of fruit that were diseased compared to the control (Figure 23). After 5 days incubation on the laboratory bench at room temperature, the most effective treatment was found to be the fungicide fenhexamid, for which 55% of fruit were infected with *B. cinerea* compared to 62% in the control. The least successful treatment was *T. atroviride* LU132, for which the percent of rotten fruit was increased by 2%. However, after 6 & 7 days incubation, the lowest amount of Botrytis rot was in the *T. atroviride* LU132 treatment, which had 71% & 74% respectively of rotten fruit compared to 81% & 82% in the control & 78% & 84% in the fenhexamid treatment (Figure 23). The mean longevity of fruit at room temperature was 6.4, 6.3, 5.5 & 5.2 days for *T. atroviride* LU132, the fungicide fenhexamid, the mixture & the water control treatments respectively.

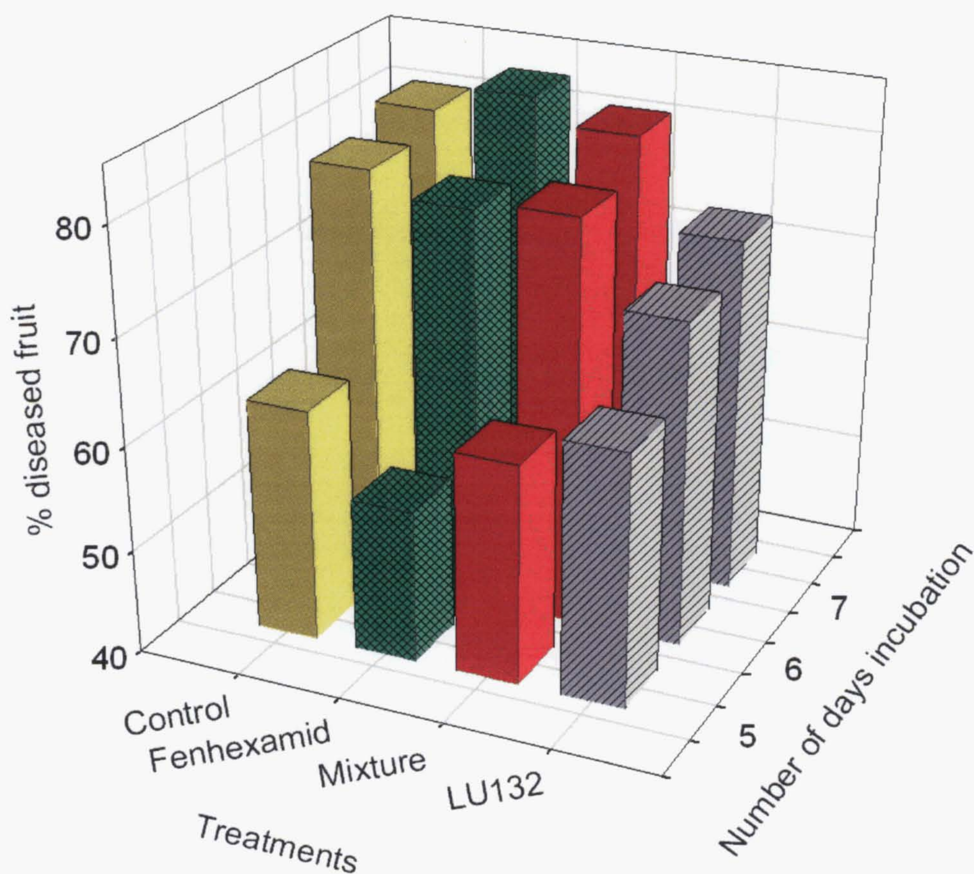

Figure 23: Effect of treatments on percent of harvested fruit from a field trial in 2004 that showed Botrytis fruit rot after 5, 6 & 7 days incubation on the laboratory bench at room temperature

Figure 24 shows the daily temperature & rainfall during the harvesting of the 2004 field trial.

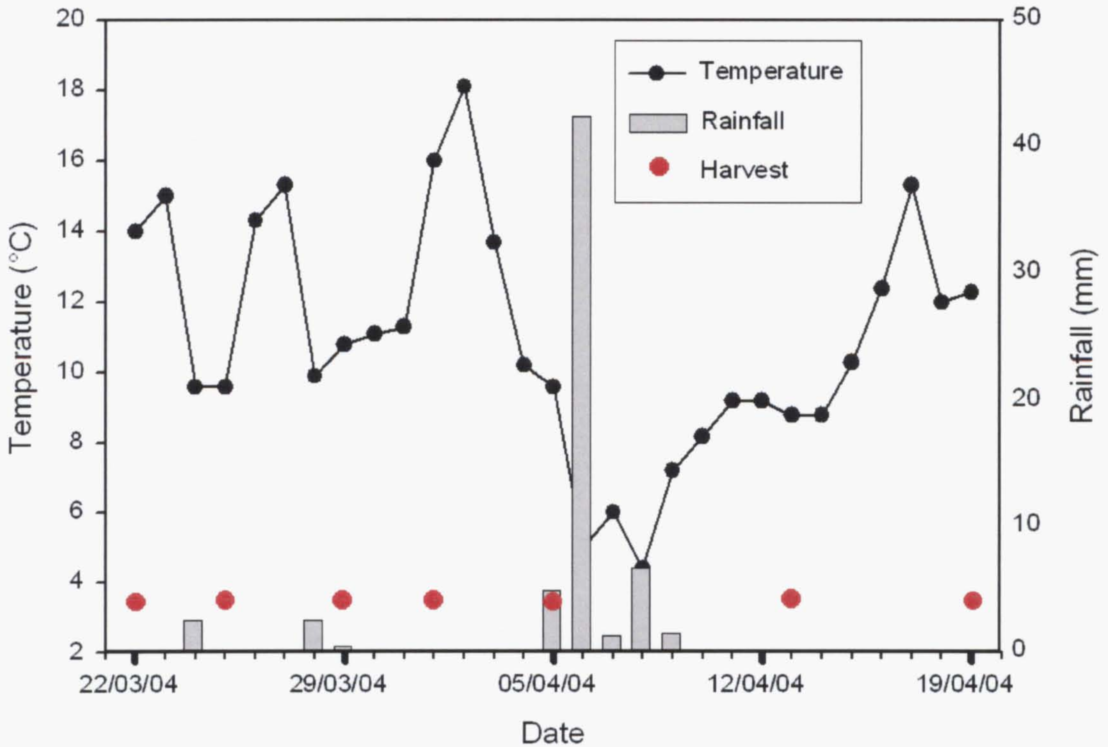

Figure 24: Weather data during the 2004 field trial

5.6 DISCUSSION

The effect of six fungicides on conidial germination frequency, subsequent germ tube elongation & mycelial growth of the BCA *T. atroviride* LU132 was investigated. These fungicides were chosen as they are all commonly used to control Botrytis rot in strawberry crops. The trials were undertaken to investigate the possibility of integrating use of a fungicide with a BCA & to provide an explanation for potential loss in efficacy if the BCA was used in a commercial setting where it was exposed to these fungicides. The fungicides cyprodinil/fludioxonil, thiram, iprodione & procymidone completely inhibited conidial germination by the BCA at all tested rates (Table 18) & therefore, could not be recommended for use in an integrated disease management strategy incorporating the BCA. Iprodione was also found by Harman *et al.*, (1996) to reduce the growth of the BCA, *T. atroviride* P1 when grown on PDA amended with the fungicide. In this study, the fungicide tolylfluanid could also not be recommended for

use with the BCA as it significantly reduced conidial germination (Table 18) & mycelial growth compared to the control at all concentrations tested (Figure 21). Sensitivity of this fungus to a high number of fungicides was not unexpected since Moyano *et al.*, (2003) found that many insecticides & fungicides commonly used in Spanish glasshouses typically inhibited germination of *T. harzianum* T39 conidia by more than 75%.

In this study, the chemical fungicide fenhexamid was found to stimulate *T. atroviride* LU132 conidial germination & subsequent germ tube elongation (Table 18). Fenhexamid was therefore considered suitable for field trials that incorporated a mixture of fenhexamid & the BCA. The inhibition caused by fenhexamid on mycelial growth of the BCA in Petri dishes was thought likely to be of minor effect since the fungicide would be found at a much lower concentration after application in the field. This is due to the fungicides short half-life in the environment (<1 day in soil) (National Registration Authority for Agricultural & Veterinary Compounds, 2001) & a short withholding period on strawberries (1 day) (New Zealand Agrichemical Manual, 2004). The field trials conducted in 2003 & 2004 showed that the BCA & the mix of both fenhexamid & the BCA inhibited *B. cinerea* sporulation to a similar level as the fungicide used alone, on strawberry leaves & stamens (Table 19). The results from the field trial in 2004 also found that the treatments had no significant effect on the total number & weight of harvested strawberry fruit (Table 20). In any new method for disease control it is imperative that the treatment does not decrease the yield or affect the quality of the crop harvested (Swadling & Jeffries, 1996). Therefore, the results for *T. atroviride* LU132 are encouraging as the treatment did not result in a significant change in crop yield or total weight of strawberry fruit (Table 20) with an equal level of disease control compared to the fungicide treatment. This was an important result since few BCAs have shown better or at least as effective control under field conditions as the existing chemical treatments (Swadling & Jeffries, 1996).

T. atroviride LU132 has been shown to inhibit *B. cinerea* lesion development on detached strawberry leaves & to suppress *B. cinerea* sporulation on detached leaves, flowers & leaves from treated whole plants (Chapter 3). In this study, this isolate was able to suppress *B. cinerea* sporulation on leaves & flowers from treated plants from the field & gave a similar level of control to the fungicide fenhexamid. Other researchers

have used similar concentrations of BCAs in the field to the ones used in this study in the biocontrol of *B. cinerea* in strawberry. Peng & Sutton (1991) used concentrations of 10^7 spores mL^{-1} for the application of *T. viride*, *Myrothecium verucaria* & *Gliocladium roseum*, all of which significantly suppressed incidence of *B. cinerea* on strawberry fruits. Helbig (2002) reported a 33% reduction in the incidence of grey mould on strawberry fruit after the application of the BCA *Cryptococcus albidus* at a concentration of 1×10^7 cells mL^{-1}. Boff (2001) tested the BCA *Ulocladium atrum* U385 at a concentration of 2×10^6 conidia mL^{-1} & reported an average reduction of 21% in disease incidence on ripe strawberry fruit. However, many researchers have reported disappointing results with *Trichoderma* spp. Tronsmo (1986) tested a variety of *Trichoderma* isolates at 10^7 spores mL^{-1}, all of which gave inconsistent control of *Botrytis* on strawberry, sometimes increasing the incidence of the disease. In trials conducted by Gullino *et al.* (1990), they reported that the use of *Trichoderma* BCA isolates did not offer a practical & realistic option to growers for control of *B. cinerea.* Their poor disease control was explained by the high disease pressure experienced at the time of the trial & the possibility that the isolates used had difficulties in colonising the strawberry canopy after inoculation.

The high effectiveness of a BCA at a low density is an important characteristic in respect to field applications, as mortality on the phylloplane is normally high & the lower the required density for disease suppression the higher the probability of the BCA being consistently effective in the field (Helbig, 2001). Therefore, the results obtained here were encouraging since the lower concentration of *T. atroviride* LU132 (1×10^6 conidia mL^{-1}) gave a similar level of disease control on strawberry leaves & stamens as the higher concentration (1×10^7 conidia mL^{-1}).

Disappointing results in the control of *B. cinerea* on strawberry fruits by *T. atroviride* LU132 was experienced in 2004. This season was particularly conducive to grey mould development with cool daily temperatures & high amounts of rainfall in early April (Figure 24). Hail was also experienced in mid April leading to severe damage of plants & fruit. The amount of disease observed in the fungicide control treatment was also high indicating the high disease pressure & possibly an inadequate coverage of the flowers during fungicide application. This lack of coverage has been experienced by other researchers. For example, Swadling & Jeffries (1996) concluded that their

bacterial BCAs, were probably unable to reduce grey mould of strawberry fruits under field conditions because of inadequate coverage of strawberry flowers during application &/or the frequency of BCA applications, rather than the BCAs inability to control the disease. Boff (2001) also reported that the only moderate control given by *U. atrum* was due to poor coverage of the strawberry stamens with spores of the BCA. Poor coverage of strawberry flowers could explain the lack of control given by LU132 on strawberry fruit when there was a high degree of control given by LU132 on strawberry leaves. Hjeljord *et al.* (2000) described reduced & inconsistent control of strawberry grey mould by a number of commercial *Trichoderma* products including Trichodex, Binab & Rootshield & the laboratory strain *T. atroviride* P1. However, this poor efficacy was put down to a lack of capacity to germinate on & effectively colonise nutrient-poor natural substrates, which was not the case for *T. atroviride* LU132 in this study (Chapters 2 & 4). There have also been reports that when disease incidence is low to medium application of *Trichoderma* spp. can give satisfactory control but once disease pressure is high these BCAs are often not effective on their own (Garabaldi *et al.*,1989 cited in Gullino, 1992).

Integrated control of a plant disease with a BCA & a fungicide becomes feasible when strains with high fungicide tolerance are available. Although field data suggests that combining *T. atroviride* & fenhexamid did not show an increased level of disease control, results indicated that the fungicide could be used at half the recommended rate with the BCA, with no loss in efficacy. The mode of action of fenhexamid may have been responsible for the tolerance of the BCA. Fenhexamid does not effect *B. cinerea* conidial germination, but inhibits germ tube elongation & mycelial growth (Debieu *et al.*, 2001). The chemical acts through a novel mechanism of action, different from that of all other botryticides (Rosslenbroich & Stuebler, 2000), by inhibiting the biosynthesis of ergosterol (Debieu *et al.*, 2001). Fenhexamid has a narrow spectrum of activity, being effective against *B. cinerea* & related pathogens such as *Monilinia* spp. & *Sclerotinia sclerotiorum* (Rosslenbroich *et al.*, 1998).

Integration of chemical fungicides & BCAs has been successful against Botrytis diseases in many crops. El-Neshawy & El-Morsy, (2003) used a combination of *Candida oleophila* & tolylfluanid against Botrytis rot of grape in Egypt. Tank mix

applications of *C. oleophila* with a low concentration of tolylfluanid, preharvest, gave significantly better control than either the BCA or tolylfluanid (at a high rate).

In Italy, research was conducted using the antagonistic yeasts *Rhodotorula glutinis*, *Cryptococcus laurentii* & *Aureobasidium pullulans* to reduce post harvest decay of apples. Results showed that combinations of the BCAs with a low dose of benomyl resulted in the same level of disease control to that of the fungicide alone at full dosage (Lima *et al.*, 2003). More recently, research also conducted with another isolate of the yeast *R. glutinis*, was reported to provide an improved level of control against *B. cinerea* damping-off of geranium seedlings when the BCA was combined with the fungicides azoxystrobin, trifloxystrobin & vinclozolin than when the yeast was applied alone (Buck, 2004a, 2004b). In contrast, trials using *T. harzianum* T-39, the active ingredient of the commercial biocontrol preparation Trichodex, found that mixing the BCA with vinclozolin or iprodione resulted in the same level of control against *B. cinerea* in cucumber as that achieved by the BCA or the fungicide alone (Elad *et al.*, 1993). However, later work on cucumber found that an alternation of Trichodex with iprodione was more effective against *B. cinerea* diseases overall, than iprodione alone (Dik & Elad, 1999). On strawberry & tomato, alternating application of *Trichoderma* spp. with a mixture of dicarboximide & thiram completely controlled *B. cinerea* diseases even in the presence of strains resistant to dicarboximides (Gullino, 1992).

The use of a BCA to improve efficacy & to extend the life of a fungicide can be of great value particularly to a small country such as New Zealand, where chemical companies are not always willing to register new products due to the small economic market. Integration of BCAs with protectant fungicides is a strategy that may have potential since fungicides are usually effective under climatic conditions or levels of disease pressure in which the BCA is less effective, while an active BCA may be able to compensate for any failures by prophylactically colonising wounds or senescing plant tissue (Hjeljord & Tronsmo, 1998).

T. atroviride LU132 has proved to be a potential BCA of *B. cinerea* in strawberry. The isolate has been shown to be as effective as the chemical fungicide fenhexamid, where it reduced *B. cinerea* development in three cultivars of strawberry on strawberry leaves & stamens. Although none of the treatments were able to significantly reduce the

incidence of disease on strawberry fruit, during a 7 day ambient storage trial, the results obtained are thought to be a promising basis for further investigation to improve the BCA's efficacy. Further work can now be directed at developing suitable formulations & integrated strategies with the chemical fungicide fenhexamid to provide a consistent & efficient means of managing *B. cinerea* in strawberry.

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CHAPTER 6:

GENERAL DISCUSSION

Biocontrol research with filamentous fungi has largely focused on the use of *Trichoderma* spp. (Swadling & Jeffries, 1996) & the majority of commercial biocontrol preparations registered for control of *Botrytis*-incited diseases incorporate isolates of this genus. Such wide use is indicative of environmental conditions tolerated by many *Trichoderma* isolates (Tronsmo & Hjeljord, 1997) & the wide range of substrates that they are able to metabolise (as summarised by Klein & Eveleigh, 1998).

Successful biological control agents (BCAs) are usually selected from a large number of isolates following a careful screening process (Elad *et al.*, 2001), designed to realistically test their ability to interfere with susceptible areas of the target pathogen's lifecycle. Primary screens should be customised for each host plant. In lettuce, for example, it is common for 8-10 outer leaves to normally die before hearting, making all plants susceptible to *B. cinerea* infection (Newhook, 1951a). After infecting the senescing tissue, the pathogen can then cause a fast-spreading soft rot that can destroy healthy tissue in less than 72 hours (Delon *et al.*, 1977). The strategy most appropriate for the protection of lettuce should, therefore, aim to protect the outer leaves from initial infection (Wood, 1951). In strawberry, it is important to protect healthy leaves in order to suppress *B. cinerea* sporulation on them when they senesce (Sutton & Peng, 1993a), thereby reducing levels of inoculum, which are likely to infect the strawberry flowers, eventually leading to Botrytis rot of the fruit. The research strategies outlined in Chapters 2 & 3 were developed to interfere with these susceptible stages in the lifecycle of *B. cinerea* in lettuce & strawberry, respectively. The results from these investigations highlight the importance of appropriate design for primary biocontrol screens.

In lettuce, conidia of the pathogen were inoculated into small wounds created in lettuce leaves which had previously been inoculated with a range of BCAs. Antagonists such as *Pseudomonas* spp. that inhibited pathogen conidial germination were the most successful, & it appeared that antibiosis was the most effective mechanism of action

(Chapter 2). In strawberry, the strategy for suppression of sporulation was used in primary screens. Subsequently, *Trichoderma atroviride* & *Epicoccum purpurascens* were shown to be the most successful antagonists as these saprophytic species could colonise senescent plant material more quickly & effectively than *B. cinerea*. The *Pseudomonas* isolates which were successful on lettuce did not reduce *B. cinerea* sporulation on detached strawberry leaves. This could indicate that their mechanisms of action were not effective within the strawberry bioassays developed.

Worldwide, strawberry is a more economically important crop than lettuce & therefore, there was greater need for development of a BCA for the control of grey mould in this crop. There are also many obstacles in the large scale use of gram negative bacteria, such as *Pseudomonas* species, including their formulation & storage (Emmert & Handelsman, 1999). In addition, staff of the National Centre for Advanced Bio-Protection Technologies have extensive expertise & knowledge on the development of *Trichoderma* species as biocontrol agents. *T. atroviride* LU132 was therefore selected for detailed investigation in Chapters 4 & 5 as a BCA in strawberry rather than attempting to find ways of using the fluorescent *Pseudomonas* isolates.

6.1 EFFECT OF ENVIRONMENTAL FACTORS ON BCA ACTIVITY

As living organisms, BCAs are dependent upon favourable environmental conditions for growth, survival & antagonistic activity. Evaluations of potential BCAs in environmental conditions that are marginal for development of the target disease can overestimate the BCA's efficacy in the field (Hannusch & Boland, 1996). Therefore in this study it was imperative that all primary screens, including those incorporating whole plants, be carried out under conditions conducive for development of grey mould. All screens described in Chapters 2 & 3 were carried out under these conditions (high relative humidity & temperatures of 18-20°C). Inoculum for all BCAs was also grown under these conditions & not in their optimum conditions to ensure their capacity for growth under normal challenge environments. It has been recognised that selecting *Trichoderma* strains that are actively antagonistic at the temperatures most conducive to *Botrytis*-incited diseases is of utmost importance (Dubos, 1987, cited in Hjeljord & Tronsmo, 1998). *T. atroviride* LU132 was found in Chapters 3 & 4 to be

antagonistic to *B. cinerea* at temperatures of 18-20°C & since it was originally selected from New Zealand soils (McLean & Stewart, 2000), it is likely to be well adapted to relatively cool temperatures. However, disappointing results in the control of *B. cinerea* on strawberry fruits by *T. atroviride* LU132 was experienced in 2004 (Chapter 5). This season was particularly conducive to grey mould development. The amount of disease observed in the fungicide control was also high (Chapter 5) suggesting a high disease pressure or inadequate coverage of the flowers during fungicide application.

6.2 METHODS TO IMPROVE BCA ACTIVITY

The survival of *Trichoderma* populations on the phylloplane is essential if they are to provide effective biocontrol (Elad *et al.*, 1993). The conidia of *Trichoderma* species are considered relatively delicate, having a limited lifespan in the phyllosphere, especially when exposed to harsh conditions such as long periods of drought & damaging ultraviolet light. They are also relatively intolerant of low moisture levels (Klein & Eveleigh, 1998) & require exogenous nutrients in order to germinate (Hjeljord *et al.*, 2001). To improve their competence in environmental conditions that might favour the pathogen, *Trichoderma* isolates can be nutrient-activated before being applied to the phyllosphere. When co-inoculated with *B. cinerea*, nutrient-activated conidia of *T. atroviride* P1 reduced *in vitro* germination of the pathogen by ≥87% at 12-25°C, a level of inhibition previously reported to be possible only at 25°C, which is optimal for *Trichoderma* spp. At 12°C, the nutrient activated conidia also performed significantly better than quiescent conidia in the suppression of *B. cinerea* sporulation on detached strawberry flowers (Hjeljord *et al.*, 2001). If the same technique was applied to LU132, by growing the isolate in 2.4% (w/v) potato dextrose broth, this could enhance the competitive ability of the antagonist under sub-optimal environmental conditions.

To ensure reliable performance, BCAs should have better stress tolerance levels than their target plant pathogens (Kredics *et al.*, 2004). When entomopathogenic fungal inoculum contained high levels of specific polyols, it was found to have improved field performance (Hallsworth & Magan, 1994). The authors found that accumulation of the endogenous compounds responsible could be enhanced by growing the entomopathogen on a medium of low water activity (a_w). Water availability is one of the most limiting factors for microbial growth in the phyllosphere (Pascual *et al.*, 2000)

& microbial cells can absorb water when their internal water potential is less than that of their environment. This technique can help the conidia to germinate faster under unfavourable conditions & may also increase the survival of the conidia during storage, since those spores with increased endogenous reserves are in a more dormant state than those without (Pascual *et al.*, 2000). This technique was used in the control of *Botrytis*-incited diseases with the BCA, *Ulocladium atrum*. The water stressed spores of the BCA were produced on a modified oatmeal agar & found to be significantly larger than unmodified spores. They contained a higher concentration of polyols, produced more germ tubes & had a higher germ tube extension rate than unmodified spores when inoculated under conditions of low water availability (Frey & Magan, 1998). Previous work by Jin *et al.*, (1991) increased the levels of trehalose in conidia of *T. harzianum* by lowering the medium water potential. Therefore, it is likely that this form of modification could be applied to *T. atroviride* LU132 to improve its desiccation tolerance on the phylloplane.

Trichoderma species are also able to produce chlamydospores (Figure 25), which are naturally less dependent on exogenous nutrients for germination, & better adapted to withstand unfavourable environmental conditions. They are larger than conidia & possess higher levels of solutes, but since production of chlamydospores in culture is more difficult the numbers produced are usually significantly less than for conidia (Whipps, personal communication). Throughout this research, conidia of *T. atroviride* LU132 were the chosen propagule for use in all primary biocontrol screens & in field trials. The isolate was found to produce large numbers of conidia on PDA & sterilised wheat grain, & thus experimentation with chlamydospores was not investigated. Further work could investigate the production of these propagules by LU132 on various types of media although there have been few reports of using chlamydospores in the biocontrol of plant diseases.

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Figure 25: Chlamydospores of *T. atroviride* (Samuels *et al.*, 2004)

6.3 METHODS TO IMPROVE BCA DELIVERY

The most common way to apply BCAs for controlling *Botrytis* diseases is to use the existing spray applicators that are also used for applying chemical treatments. In the biocontrol of *B. cinerea* in strawberry, this is often not an effective method in preventing later fruit rots as many of the flowers are likely to be closed at the time of spray application (Peng *et al.*, 1992).

An innovative, practical & environmentally friendly method of effectively delivering BCAs to glasshouse & field crops affected by flower infecting pathogens, is through the use of natural pollinators such as honey bees & bumblebees (Sutton *et al.*, 1997). In crops such as strawberry, many spray applicators fail to deliver the BCA to every flower, possibly due to the excessive foliage, which can smother newly opened flowers. Spray application of BCAs to strawberry flowers is often therefore inefficient, wasteful (Sutton & Peng, 1993b) & very labour-intensive. This method of application has now been investigated by a number of researchers with satisfactory & promising results. For example, *Clonostachys roseum* (formerly *Gliocladium roseum*) has been effectively

applied to strawberries in the field, where it significantly suppressed incidence of *B. cinerea* on petals, stamens & fruit, giving similar control to a spray application of the same BCA. Assessment after a spore-vectoring investigation found more than 6×10^3 CFU of *G. roseum* were recovered per flower in the field, a number as high or higher than for most spray-treated strawberries (Peng *et al.*, 1992). The *G. roseum* inoculum was prepared in a powder formulation & placed in a custom-made dispenser at the entrance to each hive. The bees were obliged to crawl through & collect the inoculum on their legs & bodies which they deposited as they visited flowers (Peng *et al.*, 1992).

T. harzianum has also been applied strawberry flowers using the same method. During a 4 year study, its delivery by bumble bees or honey bees gave better control of Botrytis diseases than the spray treatment & provided the same or a better level of control than with the commercial fungicides tested (Kovach *et al.*, 2000). This was despite the fact that flowers collected from the bee-treated plots usually had half the inoculum density of the spray-treated flowers. In addition, a study by Brownold (cited in Brimner & Boland, 2003) into the effect of BCAs on bee health also showed that honey bees exposed to *T. harzianum* T39 (the active ingredient of Trichodex) for 30 days showed no difference in lifespan, brood size or hive weight compared to unexposed bees. Research by Bilu *et al.*, (2004) is now investigating the use of a dispenser that loads inoculum onto the bees. To be effective, the dispenser needs to dispense a large quantity of inoculum onto a large number of bees over a long period of time.

Although the majority of strawberry crops in New Zealand are pollinated by natural insect vectors &/or wind currents, some growers have bee hives on their property for pollination of other crops (Langford, personal communication). Hives of bees could therefore be incorporated within strawberry cropping areas to vector *T. atroviride* LU132 to newly opened strawberry flowers. The inoculum would have to be formulated into a dry powder that is suitable for effective collection & delivery to flowers. This technique could be even more appropriate for many glasshouse-grown crops, where the usual mechanism of pollination is by bees. Since the bees are kept within an enclosed system, they can only visit the crop being grown, unlike the field where there is no guarantee the bees will visit the intended target crop, & thus, this method has a greater chance of being effective.

6.4 FURTHER MECHANISM OF ACTION STUDIES

The three main direct mechanisms of action for biological disease control are parasitism, antibiosis & competition for nutrients or space (Whipps, 1992), with probably few BCAs being able to act by a single type of mechanism (Andrews, 1992). Howell (2003) stated that "what we observe & define as biocontrol may be the culmination of different mechanisms working synergistically to achieve disease control". In order for biological control to be used successfully, the mode of antagonism must be able to operate in nature (Andrews, 1992). It may also be beneficial to know the mode of antagonism exhibited by the BCA so that it may be directed at the vulnerable stages of the pathogen's lifecycle in order to bring about control. Those BCAs with several mechanisms of action are better able to interfere with many stages of the disease cycle (Elad *et al.*, 1996), thereby having a greater chance of success. Competition for nutrients as a mode of action could inhibit conidial germination only under circumstances of very low nutrient supply or very high antagonist populations (Helbig, 2002). This mechanism has been discussed in detail by Fokkema (1993) who reported that it commonly acted at germination & initiation of the infection processes, during which *B. cinerea* requires exogenous nutrients (Elad, 1996). In this study, *T. atroviride* LU132 was shown to significantly reduce germ tube elongation on detached strawberry leaves when in low concentrations of glucose, sucrose & fructose (Chapter 4) indicating that competition for nutrients may be the mechanism by which it inhibits pathogen germination. In the biocontrol of necrotic pathogens with many infection cycles per season, suppression of sporulation may be an advantage as there is a long period of interaction available (Fokkema, 1993). In this study, *T. atroviride* LU132 successfully suppressed *B. cinerea* sporulation on detached strawberry leaves & flowers, on leaves removed from whole plants treated in the growth chamber &, on strawberry leaves inoculated in the field (Chapters 3 & 5). Thus *T. atroviride* LU132 meets Elad's (1996) criterion for a BCA, which is that it should be capable of inhibiting the pathogen during one or more of the key stages of its disease cycle.

Antibiotic production can require copious amounts of nutrients (Atlas & Bartha, 1993 cited in Pfender, 1996). However, *T. atroviride* LU132 produced non-volatile inhibitory compounds when grown in a minimal medium (Chapter 4). It is, therefore, possible that

this mechanism plays an active role on senescent & necrotic aerial plant tissue as it has a higher nutritional status than living leaves & therefore, antibiotic production is more likely to occur (Pfender, 1996). Tronsmo (1986) stated that on flowers & fruits of strawberry, non-volatile inhibitors & direct hyphal interactions are of greater importance than volatile metabolites, since the latter would not be able to accumulate under the variable environmental parameters experienced in the field. This agrees with the findings in Chapter 4, which show that *T. atroviride* LU132 produces volatile inhibitory compounds only when grown under conditions of high nutrients. *Trichoderma* spp. are capable of producing a whole range of secondary metabolites, of which many of the functions remain unknown. Many are antibiotics & include compounds such as cyclic peptides, isocyanide-containing metabolites, trichothecenes, sesquiterpenoids, & diketopiperazines (Klein & Eveleigh, 1998). However, the relative risk of pathogens developing resistance to antibiotic producing strains of BCAs cannot be fully judged until at least 5 years after their commercial release (Elad & Stewart, 2004). Discovering the full profile of the secondary metabolites produced by *T. atroviride* LU132 would be the next step in attempting to understand which antibiotics are of importance in the biocontrol of *B. cinerea* by the BCA.

The induction of systemic resistance is an increasingly important topic of research for control of *B. cinerea* in a range of crops. There has been no direct evidence for the involvement of *T. atroviride* LU132 with respect to this mechanism (Appendix 2). However, only root application was investigated in this study & other BCAs have been shown to induce a systemic response when applied to green strawberry fruit (Adikaram *et al.*, 2002). Further work could therefore investigate the application of *T. atroviride* LU132 at different stages of strawberry fruit development in relation to the incidence of grey mould. The mechanism of induced systemic resistance (ISR) could provide for development of a valuable strategy in the inhibition of *Botrytis* diseases, especially the latent infections commonly found in strawberry & grape, as attempts to reduce these infections by the direct methods of antagonism (competition for nutrients, antibiosis or mycoparasitism) may not be effective due to the lack of confrontation between the pathogen & the BCA. Therefore, a mechanism that utilises the plants natural defence system could be advantageous. Biochemical elicitors of disease resistance produced by *Trichoderma* species are now known (Harman *et al.*, 2004) & include proteins with enzymatic or other functions, homologues of proteins encoded by the avirulence (Avr)

genes & oligosaccharides. However, one of the problems with developing methods that depend on ISR mechanisms is the absence of specific molecular markers that correlate to the phenotypic expression of resistance (Reglinski, personal communication).

It is expected that in the future the most successful BCAs will be those with multiple mechanisms of action & increased competitive & survival capabilities. If they also had the ability to produce inhibitory bioactive compounds & to induce host plant resistance, they would be able to provide an all-round package for control of Botrytis diseases (Elad & Stewart, 2004).

6.5 FURTHER FIELD EVALUATIONS

The relative success of biological approaches to the control of diseases must be judged through the most rigorous type of screen, which is under field conditions (Swadling & Jeffries, 1996). This exposes the BCA to abiotic & biotic factors, including large fluctuations in temperature, humidity, nutrients, surface wetness & ultraviolet light, as well as the presence of other microorganisms, which may be present in varying numbers on the phylloplane.

The field evaluation of *T. atroviride* LU132 for the inhibition of *B. cinerea* on four cultivars of strawberry is discussed in Chapter 5. The trials were carried out over two years & showed that the BCA could significantly reduce disease on strawberry flowers & leaves & gave a level of inhibition on fruits similar to that of the fungicide treatment. This research also showed that *T. atroviride* LU132 could be effectively integrated into a management strategy with the chemical fungicide, fenhexamid, by using tank-mix applications applied with fenhexamid at half the recommended rate. Further work with *T. atroviride* LU132 could now be directed at field evaluations in the Auckland region where over 70% of the strawberry crop is currently grown. In this region, the climatic conditions are more conducive to disease due to the high relative humidity often experienced in the region.

Additives are essential ingredients in all commercial biocontrol formulations as they can improve the shelf life of the products & their efficacy in the field (Tang *et al.*, 2001). To be successful, a formulation needs to be economical to produce, safe, stable

in the phyllosphere, easily applied using existing agricultural equipment, & most importantly should enable the BCA to act effectively & consistently under fluctuating environmental conditions (Elad, 1996; Tronsmo & Hjeljord, 1997). These conditions vary between countries & regions & so may lead to the development of specific formulations. For example New Zealand experiences a harsh solar ultraviolet radiation climate (http://www.moh.govt.nz/uv.html, 2005) &, therefore, one of the most important additives would be an ultraviolet protector.

A number of formulations have been successfully trialled with conidia of *Trichoderma* species. Batta (2004) found that an invert emulsion based on coconut & soybean oils provided the most stable & effective formulation for *T. harzianum* conidia in the biocontrol of *B. cinerea* in apple. The formulation allowed the conidia to remain viable for 36 months at 21±1°C compared to 2.7 months for unformulated conidia. With conidia of *T. harzianum*, Tronsmo (1986) showed that soluble cellulose could improve their adherence to plants & this adjuvant was also found to serve as a nutrient supply for the germinating conidia in the biocontrol of *B. cinerea* diseases in apple. Development of new formulations could be crucial to the commercial success of a product based on *T. atroviride* LU132. The correct formulation can turn a potential BCA from a laboratory curiosity to a commercially successful product (Connick et al., 1990 cited in Tronsmo & Hjeljord, 1997).

Mass production of inoculum is one of the most important aspects for commercial development of a BCA (Frey & Magan, 1998), with the goal being to have a high spore yield & then to have a good shelf life (i.e. a high percentage of viable spores) (Tronsmo & Hjeljord, 1997). However, there are currently two major limitations to successful commercial biocontrol: one is the low tolerance of inocula to environmental stresses & the second is the lack of economical methods for producing ecologically competent propagules (Frey & Magan, 1998). Presently the main objective for *Trichoderma*-based products is mass production of conidia, however, Tang *et al.*, (2001) have reported that chlamydospores & mycelia may be used as BCA propagules.

Inocula of many antagonists has been produced using a wide range of different substrates, such as molasses, corn steep liquor, sulphite waste liquor, brewers yeast & cottonseed & soy flours, with the rationale being to use cheap waste products from the

agricultural industry (Jackson *et al.*, 1991). *Trichoderma* species can be cultured on a wide range of carbon & nitrogen sources (Hjeljord & Tronsmo, 1998) &, therefore, these by-products can be used as substrates, making mass production very economic. Worldwide, there is an extensive amount of experience in the liquid fermentation of microbial products such as antibiotics, organic acid & enzymes (Andrews, 1992). However, for production of *Trichoderma* conidia, use of semi-solid substrate fermentation is more effective than liquid culture. Because of their bulk, these substrates are difficult to handle on a large scale & so are not commercially favoured (Jackson *et al.*, 1991). The liquid fermentation facilities that are already in place in developed countries tend to be more economic (Churchill, 1982 cited in Lewis & Papavizas, 1991) & so will be the preferred approach taken by many commercial companies in the near future. In this study, use of sterilised wheat grain was employed in the production of *T. atroviride* LU132 conidia for field inoculations (Chapter 5). On such a small scale, this technique was successful, but for larger amounts & commercial viability, further experimentation will have to be undertaken to find ways of producing viable inoculum. Since New Zealand is dominated by agricultural industries, obtaining cheap waste products for the viable production of *T. atroviride* LU132 should not be a future obstacle for large scale commercial production.

6.6 FUTURE RESEARCH INCORPORATING BIOTECHNOLOGY

Biological researchers are starting to use innovative strategies in the management of foliar diseases. The BCAs of the past were applied to foliar surfaces in the same manner as chemical fungicides, by spraying them on to healthy leaves to prevent infection (Fokkema, 1993; Sutton & Peng, 1993b). For *B. cinerea*, the lifecycle is now fully understood on a range of crops, & so researchers are able to identify & target specific vulnerable points suitable for long term interactions with BCAs. Improvements in the understanding of other diseases will provide similar opportunities but the strategies employed will differ for each host, the disease encountered & the type of BCA that is applied.

New techniques in biotechnology may provide information on the survival & spread of *T. atroviride* LU132 under field conditions. Freeman *et al.*, (2004) have successfully

developed a molecular marker that uses a UP- PCR technique to track a BCA isolate of *Trichoderma* in the field & Dodd *et al.*, (2004) successfully employed a semi selective medium & an RFLP marker to monitor the survival & spread of *T. atroviride* C65 on leaves & flowers of kiwifruit in New Zealand. It was subsequently found that this isolate was able to survive & spread on the phylloplane & fructoplane of kiwifruit over an entire growing season. These techniques could also provide valuable information to improve strategies for timing & frequency of applications of *T. atroviride* LU132 that would maintain a viable level of inoculum on the phylloplane to bring about satisfactory disease control. A molecular marker for *T. atroviride* LU132 has been developed for studying the isolate within the rhizosphere (McLean, 2001) & together with colony morphology & selective sampling techniques, enabled the isolate to be distinguished from other *Trichoderma* isolates present in the soil. However, subsequent research using a range of *Trichoderma* isolates found that this marker was not isolate-specific (McLean, 2001) & further work would be needed to search for a marker that could be used to easily distinguish *T. atroviride* LU132 from other isolates within this species.

Genetic analysis & manipulation can improve understanding & so enhance use of BCAs (Boland, 1990). Methods such as transposon mutagenesis could be used to study the mechanisms of action (Lam et al., 1987 cited in Boland, 1990). For *T. atroviride* LU132, this method may provide information on different mechanisms by which *T. atroviride* LU132 brings about control & on those already demonstrated in this study (Chapter 4). Similar techniques may be used to study the activity of *T. atroviride* LU132 on surfaces of fruit in storage, where *B. cinerea* is known to be a significant problem (Helbig, 2002). Methods for studying *Trichoderma* isolates on leaf surfaces using transformed isolates with reporter genes have now been described by Freeman *et al.*, (2002). The first stage in applying this type of technology would be the production of a transgenic strain of *T. atroviride* LU132 that incorporated a marker gene, such as the GUS reporter gene or GFP-encoding gene. Inoculum from the transformed isolate could then enable further population & survival studies to be carried out.

In the search for a BCA strain with improved field tolerance, one can either select strains with the desired characteristics, such as tolerance of low vapour deficits, or mutate &/or engineer isolates to introduce the desired characteristics into existing

strains (Elad *et al.*, 1996). As many BCA isolates possess one or more important qualities, techniques such as hybridisation between different strains may now allow for the combination of beneficial traits (Howell, 2003). Many researchers believe that in the future, development of superior *Trichoderma* strains through transgenic techniques, such as protoplast fusion or genetic manipulation with cloned genes that have defined functions, will provide additional opportunities to improve BCAs (Gullino *et al.*, 1989; Harman & Bjorkman, 1998; Ippolito & Nigro, 2000; Migheli *et al.*, 1995; Shi-Wang *et al.*, 2004). *T. atroviride* LU132 possesses many important qualities that are critical in the control of *B. cinerea* in strawberry but there are other properties such as cold tolerance that may be found in other *Trichoderma* isolates that could be used to create a superior strain. However, according to Fokkema (1993), genetic modification of BCAs may serve as an experimental tool but should not supersede ecology & epidemiology in biocontrol research.

6.7 CONCLUSIONS

This study has identified *T. atroviride* LU132 as a potential BCA of *B. cinerea* in strawberry. *T. atroviride* LU132 was found to inhibit *B. cinerea* mycelial growth & significantly inhibit pathogen sporulation on senescent strawberry leaves (Chapter 3). However, as found by Boff (2001), suppression of pathogen inoculum production on senescent & dead strawberry leaves is not an effective strategy on its own for reducing Botrytis fruit rot in the field. Therefore a further screen was developed in order to assess the most successful microorganisms & a range of chemical fungicides for the protection of strawberry flowers, as this is usually a precursor to infection of the fruit. *T. atroviride* LU132 was found to significantly inhibit *B. cinerea* sporulation on strawberry flowers & consistently gave equal or better control than all the tested fungicides (Chapter 3). The isolate was later found to completely inhibit *B. cinerea* on strawberry stamens removed from inoculated flowers in the field (Chapter 5) & was observed to effectively colonise & sporulate on these tissues, which are critical in the infection of strawberry fruits by *B. cinerea.* Thus the combined strategies of suppression of *B. cinerea* sporulation & protection of strawberry flowers were used for the further field trial. Unfortunately, as the 2004 field trial was conducted late in the season, the weather conditions proved to be very conducive for grey mould development & the *Trichoderma* isolate failed to give satisfactory disease control.

However, poor disease control was also experienced with the fungicide control, fenhexamid. Further research, as discussed in this chapter can now be directed at improving this control efficacy. Chapter 5 also highlighted the possibility of integrating the BCA with the chemical fenhexamid but found that other chemicals, such as thiram, cyprodinil/fludioxonil, iprodione, procymidone & tolylfluanid, can significantly inhibit *T. atroviride* LU132 conidial germination &/or mycelial growth. Studies investigating this BCA's mechanisms of action (Chapter 4) concluded that *T. atroviride* LU132 can inhibit *B. cinerea* germ tube elongation by competing for the sugars glucose & sucrose on strawberry leaves. This has implications for the strawberry phylloplane as the environment is considered nutrient limiting & it is well known that germinating *B. cinerea* conidia are vulnerable to the absence of nutrients. Chapter 4 also showed that *T. atroviride* LU132 can produce volatile inhibitory substances when supplied with high amounts of nutrients, such as those found in newly opened strawberry flowers, & can also produce non-volatile inhibitory substances in minimal media, as found on the strawberry phylloplane.

T. atroviride LU132, which was first isolated from New Zealand soils (McLean & Stewart, 2000) has been used extensively in bio-protection research at Lincoln University where it was shown to be effective in the biocontrol of *Sclerotium cepivorum*, the causal agent of onion white rot (McLean, 2001). The product Onion mate ®, which is based on this isolate, is now commercially available (Agrimm Technologies, Ltd, Christchurch, New Zealand) for the control of *S. cepivorum*. In this study, the same isolate has given a similar level of control to the chemical fungicide fenhexamid in laboratory, growth chamber & field trials against Botrytis rot in strawberry (Chapters 3 & 5). Further work is now planned, in partnership with Agrimm Technologies Ltd, a Christchurch based bio-inoculant company, to evaluate the efficacy of a *T. atroviride* LU132 formulation in the control of strawberry grey mould in the Auckland region, where the majority of New Zealand's strawberry crops are grown.

CHAPTER 7:

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PUBLICATIONS/PRESENTATIONS FROM THIS THESIS

PAPERS:

Card, S., Jaspers, M.V., Walter, M. & Stewart, A. (2002). Evaluation of microorganisms for biocontrol of grey mould on lettuce. New Zealand Plant Protection. 55, 197-201.

POSTERS:

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