SUPPRESSION OF FUSARIUM ROOT AND STEM ROT OF GREENHOUSE CUCUMBER USING COMPOSTS CONTAINING FLUORESCENT PSEUDOMONADS

by

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BSc. University of British Columbia, 2004

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ABSTRACT

Three composts (Ball, dairy, and greenhouse) were tested for their ability to suppress development of fusarium root and stem rot of greenhouse cucumber, caused by *Fusarium oxysporum* f. sp*. radicis-cucumeriunum* (*Forc*). Dairy and greenhouse compost significantly reduced disease (P≤0.05), Ball compost had no effect. Microbial assessment of the composts showed a correlation between disease suppression and pseudomonad levels. *Pseudomonas* species were isolated using dilution plating. Strains showing the greatest *in vitro* antagonism were identified as *P. aeruginosa.* Growth room trials were conducted to test *P. aeruginosa* P23, the non-antagonistic *P. maculicola* and two strains of *P. fluorescens* for *Forc* suppression. Cucumbers grown in compost with P23 had reduced disease severity compared to controls. The locus for biosynthesis of the antibiotic 2,4-diacetylphloroglucinol (DAPG) was detected by Southern blot and confirmed by PCR; DAPG production by *P. aeruginosa* in liquid culture was detected by thin layer chromatography.

Keywords: Biological control, compost, *Fusarium oxysporum* f.sp. *radicis-cucumerinum*, *Pseudomonas aeruginosa*, antibiotics, 2,4-diacetylphloroglucinol

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LIST OF ABBREVIATIONS

AHL – N-acyl homoserine lactone
ANOVA – Analysis of variation
BCA – Biological control agent
CFU – Colony forming units
CTAB - Cetyl trimethylammonium bromide
DAPG – 2,4-diacetylphloroglucinol
DSI – Disease severity index
FSPA – fluorescent pseudomonad selective agar
Forc – *Fusarium oxysporum* f.sp*. radicis-cucumerinum*
Gac – global antibiotic and cyanide control
HCN – Hydrogen Cyanide
ISR – Induced systemic resistance
KMB – King et al's medium B
PAL – Phenylalanine ammonia lyase
PDA – Potato dextrose agar
PHL – Phloroglucinol
PLT – Pyoluteorin
PRN – Pyrrolnitrin

CHAPTER 1: INTRODUCTION

British Columbia Greenhouse industry and cucumber production

The Fraser Valley of British Columbia (B.C.) is one of the major regions of greenhouse crop production in Canada, with approximately 220 ha (BCMAFF, 2003) in cultivation. The annual harvest from greenhouses in B.C. is valued at over $240 million. Tomato, bell pepper, and Long English cucumber make up the majority of B.C. greenhouse production. The Canadian greenhouse industry is one of the most advanced in the world, utilizing many modern practices and technologies that result in high yields of high value crops. Up to three crops of cucumbers may be harvested per year, with annual fruit production of up to 160 cucumbers/m^2 (BCMAFF 2003).

Greenhouse cucumber plants are propagated in large rockwool blocks from hybrid seed. Plants are placed in bags of sawdust or other soil-less media in a semi-hydroponic system with drip irrigation and recycling of nutrient solution. This system is highly efficient but also can facilitate the spread of soil-borne pathogens. In addition, the semi-sterile nature of the growth media can allow rapid multiplication of invading microbes due to the lack of competing microorganisms.

Among the diseases of greenhouse cucumber, the most economically important in B.C. are root and stem rot caused by *Fusarium oxysporum* f.sp. *radicis-cucumerinum*, powdery mildew caused by *Podosphaera xanthii* and gummy stem blight caused by *Didymella bryonae* (Howard et al. 1994). While there are several other pathogens of greenhouse cucumbers in Canada, they are generally

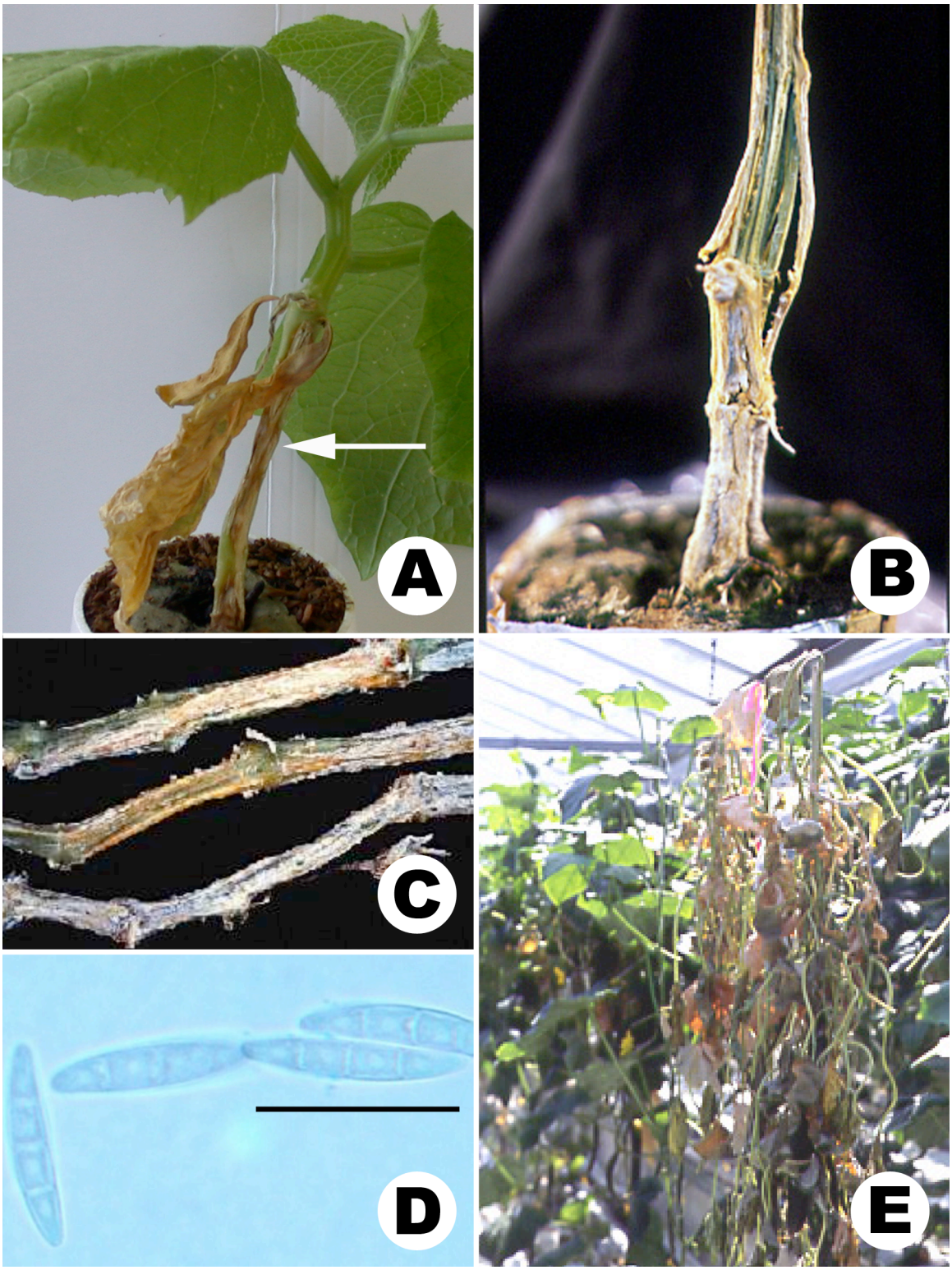
problematic only under poor cultural conditions or are of little economic consequence (Howard et al. 1994).

Fusarium root and stem rot

Fusarium root and stem rot, caused by the fungal pathogen *Fusarium oxysporum* f. sp. *radicis-cucumerinum* (*Forc*), was first observed in cucumber greenhouses in British Columbia in 1995 (Punja & Parker 2000). Prior to its introduction in the province, the pathogen had previously only been reported in Crete, Greece (Vakalounakis 1996). The disease has since spread to several other provinces within Canada and has been reported to occur on greenhouse cucumbers in other countries including Israel, France, and the USA (Z.K. Punja, unpublished).

Diagnostic disease symptoms include a brown discolouration at the crown and vertical stem lesions that can extend up to 30 cm from the crown (Punja and Parker, 2000; Vakalounakis, 1996). White mycelial growth and orange spore masses are often observed on diseased tissues at advanced stages of infection, especially under high humidity conditions. Seedlings may occasionally show symptoms but the most distinctive symptoms generally appear after fruit set or following periods of drought stress at which time plants begin to wilt rapidly (Punja and Parker, 2000; Vakalounakis, 1996). An examination of the root system reveals brown discolouration and cortical rot. Figure 1.1 presents some of the characteristic symptoms of RSR.

Figure 1.1: Typical symptoms of fusarium root and stem rot of greenhouse cucumber. A) Early symptoms on a cucumber seedling showing discolouration and lesion formation, arrow indicates location of lesion on stem. B) Advanced lesion at crown of mature plant showing white and orange mycelial masses. C) Several infected cucumber stems showing large quantities of mycelium and conidia. D) Light micrograph of *Forc* macro-conidia, bar represents approximately 15 µm. E) Wilted cucumber plant showing advanced infection


A
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E

No fungicides are presently available to control root and stem rot, and disease control strategies are limited to sanitation and use of resistant cultivars (Rose & Punja 2004). While use of resistant cultivars can provide protection against *Forc,* cultivars that are resistant to *Forc* tend to be more susceptible to powdery mildew (and vice-versa), and are not optimal for growing in all seasons in B.C. (Ken Ng, personal communication).

Research objectives

The purpose of this research was to investigate the effectiveness of compost-based biological control of *Forc* in order to provide B.C. greenhouse cucumber growers with increased knowledge and tools for its control. The three main objectives for this research were:

1. To identify composts suppressive to *Forc*
2. To isolate and identify microbial species from disease-suppressive composts responsible for reduction in disease severity
3. To determine the disease suppression mechanism(s) employed by antagonistic microbial isolates

CHAPTER 2: LITERATURE REVIEW

Control of fungal disease using composts

Compost production

Composting is the biological process of breaking down organic materials through microbial activity. This process mineralizes complex molecules found in the organic material providing useable nutrients such as nitrogen, phosphorous and a range of minerals. Composts also provide physical structure to the soil in which they are incorporated (de Bertoldi, Vallini, and Pera 1983). In addition to providing the benefits of fertilization and soil improvement, a great deal of research has demonstrated the ability of compost amendments to effectively suppress plant diseases (Hoitink et al. 1997; Litterick et al. 2004; Noble & Coventry 2005; Zinati 2005).

Composts for use in horticultural systems are made from a diverse range of organic materials (referred to as feedstock) which include: biosolids; livestock manure; municipal solid wastes such as household organic waste and yard trimmings etc.; and agricultural/horticultural wastes (such as greenhouse waste) (Goldstein 2001). The biological degradation of the feedstock during composting is due to the action of microbes, particularly aerobic bacteria, but also actinomycetes and fungi, and requires the presence of O_2 within the organic matter (Day & Shaw 2001).

Several types of systems are commonly used in compost production but the general biological processes that break down the feedstock are essentially the same in all of them. Windrow composting is the most common type of large-scale compost production; it includes long narrow piles of materials, composted in the open. Aeration is usually maintained by turning of the pile and passive diffusion of O_2. In-vessel composting is a general term for various types of composting done in containers. There are several types of in-vessel composters, which use a variety of methods to maintain aeration during the composting process. These include silos, rotating drums, aerated containers and agitating beds; aeration is maintained by physical mixing and/or forced air circulation in these methods (Rynk & Richard 2001). These containers allow for increased control of the composting environment but also increase the complexity and cost of equipment

The decomposition process during composting produces CO_2 and NH_3 as well as heat. The heating cycle of composting has an important role in the determination of disease suppressive ability of the compost (Scheuerell & Mahaffee 2005). The process of composting follows a predictable sequence of temperature change over time, with specific types of microbes associated with each phase of composting (Day & Shaw 2001). In the initial phase, temperatures in the compost pile are low and the compost contains high concentrations of mesophilic bacteria (those that grow at 25-45˚C), including *Bacillus* spp., *Pseudomonas* spp., *Streptococcus* spp., plus numerous others as well as actinomycetes and fungi (Day & Shaw 2001). As the composting process progresses, temperatures within the compost begin to increase due to microbial oxidation of carbon-containing molecules such as sugars and cellulose, eventually reaching peak heats of approximately 60-

70˚C. During this period, thermophilic bacteria become the dominant organisms, while mesophilic organisms, including plant pathogens are killed by the sustained high temperatures (Hoitink et al. 1997; Day & Shaw 2001; Zinati 2005). Bacterial species diversity during this period drops significantly as does total bacteria number. Strom (1985) found that during this period, up to 87% of surviving bacterial species in the compost belonged to the genus *Bacillus*. This finding was later confirmed using modern PCR-based methods by Ishii et al. (2000).

Following the peak-heating period, there is a cooling stage during which the compost returns to a temperature that can be re-colonized by mesophilic bacteria. These organisms re-enter the compost from the outer, cooler parts of the pile or by introduction from an environmental source (Hoitink et. al 1997). This is of particular importance for disease control because it is during this period that biocontrol bacteria must establish themselves at high concentrations in order for disease suppression to later occur. Factors governing re-colonization by potential biocontrol agents include moisture content and pH of the maturing compost. Moisture content greater than 40% and pH greater than 5 will favour re-colonization by bacteria while lower moisture will result in an increase in fungal colonization (Hoitink et. al 1997). Increased colonization by biocontrol agents (those organisms capable of suppressing disease) and thus increased consistency of biological control by composts can be ensured by inoculating composts with a biological control organism immediately after the peak-heating phase, before other organisms have the opportunity to colonize the compost (Hoitink et al. 1997; Postma et al. 2003; Scheuerell & Mahaffee 2005).

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Mechanisms of disease control by composts

Both biotic and abiotic factors are important in suppression of diseases by composts. Abiotic factors such as the physical makeup of the compost including pH, nutrient composition and the presence of materials like clays, all influence the ability of the compost to suppress disease (Coventry et al. 2005; Zinati 2005; Borrero et al. 2009). Other abiotic factors can also play a role in disease suppression, such as isothiocyanates produced in the breakdown of plant material or the level of phenolic compounds in the mature composts (Erhart et al. 1999; Coventry et al. 2005)

While these abiotic factors are important in suppression of plant diseases, the majority of reports of compost-mediated disease suppression are attributed to the action of micro-organisms found within the compost rather than the physio-chemical properties of the compost itself.

Biotic disease suppression is often separated into two broad categories: general biotic suppression and specific suppression (Hoitink et. al 1997; Zinati 2005). General suppression is defined as indirect disease inhibition based on the abundant microflora of the compost out-competing invading pathogens for nutrients and/or niches for colonization. This type of suppression is common for pathogens such as *Pythium* spp. and *Phytophthora* spp., which are not generally killed, but rather prevented from germinating due to the lack of resources imposed by the activity of compost microflora (Hoitink et. al 1997; Zinati 2005). Predictive assays for the ability of a compost to suppress *Pythium* and *Phytophthora* diseases by general suppression have been developed based on determination of total microbial activity by measures such as rate of degradation of fluorescein diacetate (Chen et al. 1988; Boehm & Hoitink 1992).

Specific suppression occurs when one or more microbes in the compost antagonize the pathogen through mechanisms such as hyperparasitism or production of antibiotics or siderophores (Hoitink et. al 1997; Zinati 2005). Specific suppression generally results in reduced survival of pathogen propagules in the compost. In addition to reducing survival of pathogens, certain beneficial micro-organisms in disease-suppressive composts can reduce disease by inducing systemic resistance in the host plant (Zhang et al. 1996; Hoitink et al. 1997; Lievens 2001; Kavroulakis et al. 2005; Noble & Coventry 2005; Paplomatas et al. 2005; Zinati 2005; Kavroulakis et al. 2006). The mechanisms of specific suppression by the microorganisms in composts are the same as those of individual, isolated biological control agents and will be discussed shortly.

The incorporation of composts made from various different feedstocks into potting mixes and soils has been shown to reduce many different plant diseases. Table 2.1 provides examples of previous studies that have demonstrated suppression of soil-borne diseases by composts of various compositions. Disease suppression by compost has also been reviewed thoroughly by Litterick et al. (2004) and Noble & Coventry (2005).

The antagonistic microflora of disease-suppressive composts and soils are a major source of biological control agents. Many of the biocontrol bacteria listed in Table 2.2 were originally isolated from such sources. The mechanisms, identification and use of these bacteria are discussed in the following sections.

Biological control of plant pathogens

That certain microbial species, particularly pseudomonads, can act as biological control agents (BCA) of plant diseases has been known for decades (Weller 2007). There has been a continual interest in the study of these organisms due to growing concerns over chemical use in agricultural production and the effects of these chemicals on the environment and human health. In Canada, stringent regulations have limited the number of registered biological control agents. However, in the USA and Europe, numerous biocontrol agents (both bacterial and fungal in origin) are registered for use (Fravel 2005).

The greatest limitation to biological control of plant diseases has been variability in disease suppression. This is because the effectiveness of a BCA is influenced by the host plant, rhizosphere ecology, soil chemistry, fertilizer application, and a host of abiotic factors. Knowledge of how these, and other, factors affect BCAs can be used to increase the effectiveness and consistency of biocontrol.

Table 2.1: Examples of disease suppression by composts from various sources

Compost source	Pathogen	Host plant	Reference
Cattle manure	*Fusarium oxysporum* f.sp. *radicis-cucumerinum*	Cucumber	Kannangara et al. (2000)
Greenhouse waste, dairy waste	*Fusarium oxysporum* f.sp. *radicis-cucumerinum*	Cucumber	Rose, Parker, and Punja (2003)
Hardwood bark, biowastes	*Pythium ultimum*	Cucumber	Chen et al. (1987)
Spruce or pine bark	*Pythium ultimum*	Cucumber	Zhang et al. (1996)
Chitin-manure-shrimp	*Phytophthora fragariae* var. *rubi* *Pythium ultimum*	Cucumber	Labrie et al. (2001)
	Fusarium oxysporum f.sp. *lycopersici*	Tomato	Szczech (1999)
Horticultural waste	*Fusarium oxysporum* f.sp. *dianthi* *Rhizoctonia solani*	Carnation Sugar beet, potato	Postma et al. (2003)
Vegetable waste	*Verticillium dahliae*	Potato	Entry et al. (2005)
	Sclerotium cepivorum	Onion	Coventry et al. (2005)
Greenwaste and Greenwaste mixtures	*Gaeumannomyces graminis* var. *tritici* *Pseudocercosporella herpotrichoides*	Winter wheat	Tilston et al. (2002)
	Phoma medicaginis var. *pinodella*	Garden pea	
	Plasmodiophora brassicae	Chinese cabbage	

Numerous genera of rhizosphere bacteria have been shown to reduce diseases by antibiotic production, including *Agrobacterium, Bacillus, Enterobacter, Pseudomonas, Rhizobium* and *Streptomyces*. Among these genera, the pseudomonads have garnered the greatest attention from researchers because of a number of useful characteristics: easy cultivation in the lab; proven disease suppression in naturally suppressive soils; high production of secondary metabolites including a diverse group of antibiotics; ability to grow on a wide range of carbon sources with minimal nutritional requirements including the ability to colonize the rhizosphere and internal plant tissues; and easy genetic transformation (Haas & Defago 2005; Weller 2007). Disease suppression by *Pseudomonas* spp. has been documented for many different plant pathogens on many different plant hosts; examples can be found in Table 2.2.

Bacterial biocontrol agents

Identification and assessment of bacterial biocontrol agents

The traditional method of identifying biological control agents has been a scattergun approach using dilution-plating and *in vitro* antagonism tests. This approach can be exceptionally time-consuming and labour-intensive. For example, Fravel (1988) cites an example in which 3500 bacterial strains were screened and 40% were shown to be antagonistic *in vitro*. Of the *in vitro* antagonists, only 4% were found to be effective as BCAs in the soil. At the same time, some of the non-antagonists were shown to be effective in the soil. This highlights another problem for identifying BCAs; there is frequently little correlation between *in vitro* antagonism and *in situ* effectiveness as a biocontrol agent. One of the suggested strategies for increased implementation of biological control is to develop many strains, each

suited to a particular combination of plant host, pathogen, and soil type (Cook 1993). The difficulty with this strategy is the incredible amount of work involved in isolating and testing bacteria which is multiplied for each host species. Implementing such a strategy requires development of new methods of identifying BCAs that are much less labour intensive and time consuming.

One of the most basic strategies to minimize the amount of work required to find new biocontrol strains is to isolate bacteria from naturally suppressive soils such as take-all (*Gaeumannomyces graminis* var. *tritici*) and *Thelaviopsis basicola* suppressive soils (Weller et al. 2002). Similarly, to increase the odds of a bacterial species being a good colonizer of the target plant, a requirement for biological control, the bacteria can be isolated from the rhizosphere of the plant species on which they are to be used (Cook 1993; Duffy & Defago 1999).

More advanced methods for simplifying the search for BCAs have involved identifying common traits of biocontrol bacterial strains. Ellis et al. (2000) used RFLP ribotyping, metabolic profiles and fatty acid profiles of a library of pseudomonads with known biocontrol ability and found a correlation between the ability to produce HCN and accumulation of C17:0 cyclopropane fatty acid (CFA). To date, there has been no evidence that C17:0 CFA can be used as an indicator of antibiotic production outside of the pseudomonads.

Table 2.2: Diseases suppressed by *Pseudomonas* biocontrol species

Strain	Host	Pathogen	Reference
P. fluorescens Pf-5	Cotton	*Rhizoctonia solani*	Howell & Stipanovic (1979)
		Pythium ultimum	Howell and Stipanovic (1980)
	Cucumber	*Pythium ultimum*	Kraus & Loper (1992)
	Turfgrass	*Sclerotinia homeocarpa*	Rodriguez & Pfender (1997)
		Drechslera poae	
P. fluorescens 2-79	Wheat	*Gaeumannomyces graminis* var. *tritici*	Thomashow & Weller (1988)
P. fluorescens CHA0	Tobacco	*Thelaviopsis bassicola*	Stutz et al. (1986)
P. aeruginosa 7NSK2	Bean	*Botrytis cinerea*	De Meyer & Hofte (1997)
	Rice	*Magnaportha grisea*	De Vleesschauwer et al. (2006)
	Wheat	*Blumeria graminis* f.sp. *tritici*	Muyanga et al. (2005)
		Cochliobolus sativus	
P. fluorescens 89B-27	Cucumber	CMV	Raupach et al. (1996)
P. aeruginosa FP10	Banana	*Fusarium oxysporum* f.sp *cubense.*	Ayyadurai et al. (2006)
P. aeruginosa PNA1	Chickpea	*Fusarium* spp.	Anjaiah et al. (1998)
		Pythium spp.	
P. chlororaphis Tx-1	Bell pepper	*Pythium spp.*	Chatterton et al. (2004)
P. chlororaphis PCL1391	Tomato	*F. oxysporum* f.sp. radicis-lycopersici	Chin-A-Woeng et al. (1998)
P. chlororaphis PA-23	Canola	*Sclerotinia sclerotiorum*	Fernando et al. (2007)
P. cepacia	Maize	*Fusarium moniliforme*	Hebbar et al. (1992)
P. aureofaciens	Soybean	*Rhizoctonia solani*	Jung et al. (2007)

Using *a priori* knowledge that production of antibiotics is important for disease control by many species of bacteria, searches for biocontrol agents can also be done by specifically looking for strains that produce antibiotics (Raaijmakers et al. 1997; Ellis et al. 2000; Giacomodonato et al. 2001; McSpadden Gardener et al. 2001). The genes for production of several pseudomonad antibiotics are highly conserved both between and within genera (Raaijmakers et al. 1997; Hammer et al. 1999). Using antibiotic biosythetic locus-specific PCR primers and/or hybridization probes, colonies of bacteria can be screened for their ability to produce a given antibiotic (Raaijmakers et al. 1997; McSpadden Gardener et al. 2001). Giacomodonato et al. (2001) used PCR screening to identify *Bacillus* isolates that produced antifungal metabolites. When the PCR positive isolates were tested, most of them were found to be inhibitory to the plant pathogen *Sclerotinia sclerotiorum*, demonstrating the effectiveness of such a strategy for screening biocontrol agents of other species in addition to *Pseudomonas* spp..

The most efficient use of methods like those described above will likely be as screens of composts, soils, or other media previously proven to suppress disease. PCR screens of total rhizosphere/soil/compost DNA to determine whether strains producing antifungal products are present, followed by colony hybridization to identify the bacteria within the soil which contain antibiotic genes could save considerable amounts of time compared to traditional scattergun screening approaches by reducing the number of strains that must be screened for *in situ* biocontrol ability (Giacomodonato et al. 2001). The biggest drawback of these methods is that by their nature they are very specific in their targets; a consequence of this specificity is that many bacteria involved in disease suppression may be missed. For example, any organisms using methods such as competition or ISR

may not show up in these screens. Because of this, there is still a need to develop further methods for easily identifying biocontrol agents.

Finally, colony hybridization, PCR or other methods that can monitor biocontrol species *in situ* are tools that can be used to determine which strains are the most competent in the soil (Raaijmakers et al. 1997), helping to further improve selection of good BCAs.

Mechanisms of biological control of soil-borne pathogens by *Pseudomonas* spp.

Several methods of disease suppression have been implicated in biological control by *Pseudomonas* spp. The most frequently cited of these are competition, iron limitation through siderophore production, antibiosis, and induced systemic resistance (Weller 1988; Buchenauer 1998; Haas & Defago 2005). While there are examples of BCAs that utilize one specific method of control, these factors often work in combination. For example, Thomashow and Weller (1988) showed that while *Pseudomonas fluorescens* 2,4-diacetylphloroglucinol (DAPG) knockout mutants were drastically decreased in their ability to control take-all of wheat (*Gaeumannomyces graminis* var. *tritici*) compared to wild-type *P. fluorescens,* they still exhibited biocontrol ability at a lowered level.

Antibiotic production

One of the advantages of using fluorescent pseudomonads as biocontrol agents is their ability to produce numerous secondary metabolites. Many of these metabolites are potent anti-microbial allelochemicals (Fravel 1988; Raaijmakers et.al 2002). The production of antibiotics by *Pseudomonas* species has been determined

to be the primary mechanism of disease suppression in classic suppressive soils, such as Take-all decline (Weller et al. 2003). Antibiotics produced by pseudomonads are often broad-spectrum in activity against microbes and even nematodes and may be also be involved in triggering induced systemic resistance (Raaijmakers et al. 2002; Bakker et al. 2007). Raaijmakers et al. (1997), showed that in suppressive soils worldwide, antibiotic producers make up a considerable proportion of the total microflora (approximately 3 -11%). This same study showed that genes for DAPG production are strongly conserved both globally within species and between *Pseudomonas* species. Antibiotic production by biocontrol *Pseudomonas* strains will be discussed in detail in further sections.

Siderophore production

The earliest study to show strong evidence of siderophore production as a mechanism of disease suppression was that of Kloepper et al. (1980), which demonstrated increased yield and suppression of *Erwinia carotovora* in potato, sugarbeet and radish.

Siderophores are iron-chelating molecules produced by microorganisms in response to low environmental iron concentrations. These molecules help the microorganism to acquire iron, which is often biologically unavailable in the environment, and create iron-limiting conditions for competitors, including pathogenic microorganisms (Meyer & Stintzi 1998). The primary siderophore molecule produced by fluorescent pseudomonads is pyoverdine, the yellow-green pigment that gives them their characteristic fluorescence under UV light. Secondary siderophores include pyochelin and salicylic acid (Meyer et al. 1992). In addition to producing their own siderophores, beneficial pseudomonads can further compete for

iron through the uptake of xeno-siderophores, produced by other species (Poole & McKay 2003; Compant et al. 2005).

Evidence for siderophore-mediated biocontrol taking place is reduced disease suppression when iron concentrations are increased, an increase in disease suppression when purified siderophore or other chelating agent (eg. EDTA) is added to the soil, or decreased effectiveness of a siderophore knock-out mutant (Loper & Buyer 1991). It is worth noting that siderophore-mediated biocontrol is strongly influenced by the bio-availability of iron, which varies with pH, and may not be a major factor in disease suppression as there are many fluorescent pseudomonads that produce pyoverdine but do not control plant pathogens (Haas & Defago 2005)

Induced Systemic Resistance

Induced systemic resistance (ISR) is a mechanism of disease suppression that occurs as a plant response to colonization by certain beneficial microbes, which results in a preparedness of the plant's defense responses so that if a pathogen is subsequently encountered, the plant's response is swifter and more robust (Conrath et al. 2006). Colonization by the inducing BCA does not result in altered expression of defense genes or accumulation of defense gene products in the plant, but rather potentiates these genes so that in the presence of a pathogen they are rapidly expressed (Shoresh et al. 2005; Conrath et al. 2006; van Hulten et al. 2006; Bakker et al. 2007). A variety of genes are involved in the defense response during ISR. These genes include: peroxidases, chitinases, phenylalanine ammonia-lyases, glucanases, subtilisin-like genes and others (Chen et al. 2000; Ongena et al. 2000; Muyanga et al. 2005; Shoresh et al. 2005; van Loon et al. 2006; Jung et al. 2007).

In plants colonized by an ISR-stimulating microbe in the absence of a pathogen, defense gene expression is not altered, however, genes involved in signalling for ISR do undergo an increase in transcription. Previous studies have shown that inoculation with beneficial microbes triggers ISR through the jasmonic acid/ethylene signalling pathway (Shoresh et al 2005; Ahn et al. 2007). The genes involved in formation and perception of these hormones, such as lipoxygenase, *Etr1*, and *Ctr1* are involved in ISR signalling in cucumber (Shoresh et al. 2005).

In order for ISR to occur, the colonizing bacteria must reach a minimum threshold population (approximately 10^5 CFU/g of root tissue) and the plant must detect the presence of the colonizing microorganism or one of its metabolites (Han et al. 2006; Bakker et al. 2007). In the case of fluorescent pseudomonads, a wide range of molecules have been implicated as determinants of ISR, including pyoverdine, salicylic acid, 2,4-diacetylphloroglucinol, flagellins and lipopolysaccharides (De Meyer & Hofte 1997; Ongena et al. 2000; De Vleesschauwer et al. 2006; Bakker et al. 2007).

Antibiotic production by *Pseudomonas spp.*

Classes of Antibiotics

Pseudomonad species have been shown to produce a wide range of antibiotic compounds. These tend to be low molecular weight metabolites. For six general categories of antibiotic including phenazines, phloroglucinols, pyoluteorin (PLT), pyrrolnitrin (PRN), cyclic lipopeptides and hydrogen cyanide (HCN) there is

significant experimental evidence confirming their role in disease suppression. (Raaijmakers et al. 2002; Haas & Defago 2005). All of these antibiotics are diffusible molecules with the exception of HCN, which is a volatile compound. Many antibiotics produced by *Pseudomonas* spp. have broad-spectrum activity.

Most of the antibiotics produced by pseudomonads are broad-spectrum antibiotics and the modes of action for most of these antibiotics have been elucidated. Phenazines are electron transport chain inhibitors and also produce hydroxyl radicals in the presence of ferripyochelin (conjugated iron-pyochelin complex); phloroglucinols cause extensive membrane damage, and similarly, cyclic lipopeptides insert into membranes thereby disrupting their function; HCN acts by inhibiting metalloenzymes in particular cytochrome-oxidase c; pyrrolnitrins are inhibitory to fungal respiratory chains and are the most specific of these antibiotics; the mode of action for pyoluteorin is currently unknown (Haas & Défago, 2005).

Factors influencing antibiotic production

Various biotic and abiotic factors in the rhizosphere can affect the production of antibiotics by rhizobacteria. Physical properties of the environment such as temperature, moisture, and pH can influence antibiotic production and activity (Slininger et al. 2000; Raaijmakers et al. 2002). Nutritional factors including carbon source, mineral availability and host plant also greatly impact antibiotic production. Finally, there are a host of endogenous signals that regulate the production of various antibiotics.

Nutritional Factors

The sources of carbon and nitrogen, mineral nutrition and host plant all play important roles in regulating antibiotic production by pseudomonads. The majority of nutrients used by rhizosphere bacteria originate from root exudates; therefore, the host plant is a major determinant of the nutrients available to rhizobacteria. Numerous examples show differential production of antibiotics on different host plants. For example, *P. fluorescens* CHA0 differentially produced pyoluteorin when grown on cress compared to cucumber (Maurhofer et al. 1994). To overcome host effects, the simplest solution is to isolate bacteria from the plant on which they are intended for use. It has even been suggested that using knowledge of important nutritional factors it may be possible to breed plants to be specifically amenable to colonization by certain bacterial biocontrol agents (Raaijmakers, Vlami & de Souza, 2002).

One of the nutritional factors that plays the biggest role in regulating antibiotic production is the carbon source. For example, Duffy & Defago (1999) found that *P. fluorescens* CHA0 phloroglucinol production was stimulated by glucose, while pyoluteorin production was inhibited by glucose but stimulated by glycerol. Similarly, pyrrolnitrin production was increased five-fold when fructose or mannitol was supplied as a carbon source.

Another nutritional factor that influences antibiotic production is concentration of minerals. For example, $ZnSO_4$ stimulated production of pyoluteorin, phenazines and phloroglucinols in a wide range of *Pseudomonas* spp. (Slininger et al. 1998; Duffy & Defago 1999). Knowledge of the mineral nutrients that are required for optimal antibiotic production could provide approaches to increase biocontrol effectiveness. Application of mineral amendments to soils may be a simple and in

expensive way to increase the effectiveness of BCA through enhanced antibiotic production. Adding the mineral supplement directly to the BCA formulation has been suggested as a method to minimize non-target effects of mineral application, such as phytotoxicity, while at the same time efficiently delivering the supplement to the BCA (Duffy & Défago, 1999).

Minerals are also potential repressors of antibiotic production. In particular, increasing concentration of inorganic phosphate has been shown to significantly decrease the production of phloroglucinol and to a lesser extent pyoluteorin (Milner et al. 1996; Duffy & Defago 1999). Antibiotic production occurs primarily when there is limitation on growth of the bacteria. Hence, addition of phosphate may relieve nutritional stress, thereby stimulating bacterial growth and reducing production of secondary metabolites. This suggests that other agricultural practices, such as use of phosphate fertilizers must be taken into account when working with biocontrol agents (Duffy & Défago, 1999). Other soil attributes, such as pH, temperature, moisture, clay composition, etc. can also affect the efficiency of biological controls. Understanding how these factors impact BCA strains can be very important for their effective use. Predictive systems based on these types of factors have been successful in identifying field sites where biocontrols have the greatest potential to be effective (Duffy & Defago 1999).

Nitrogen source is a less important factor in antibiotic production. It affects the initial time required to synthesize antibiotics but does not change overall accumulation (Slininger et al. 1998).

Regulatory Systems

GacS/GacA

A signalling system that is particularly important in regulation of antibiotic production is the GacS/GacA (global antibiotic and cyanide control) signal cascade. All of the different antibiotics produced by pseudomonads with demonstrated function in biocontrol are produced only when the Gac system is active (Haas & Keel 2003). In this two-component system, GacS is a sensor kinase and GacA is a response regulator. Production of the GacS/GacA gene products themselves is favoured by conditions of restricted bacterial growth, helping to trigger the production of antibiotics and other secondary metabolites during periods of high competition (Laville et al. 1992). This is likely an adaptation for increased competitiveness at times of high stress and lowered metabolic costs when stress is low. The Gacs/GacA sensor system is part of an extremely complex signalling network. It works by up-regulating production of small RNAs that remove translational suppressors from mRNA coding for downstream proteins (Haas & Keel, 2003). One of the major effects of activation of the GacS/GacA system is activation of genes involved in quorum sensing (Haas et al. 2000).

Quorum Sensing

Quorum sensing is used by many species of bacteria as a density dependant signal for secondary metabolite production and fluorescent pseudomonads are no exception. Positive feedback loops mediated by the quorum signal molecules N-acyl homoserine lactones (AHL) have been identified which trigger production of antibiotics in *Pseudomonas* and other bacteria species (Pierson et al. 1998). These are generally two-component systems comprised of an AHL synthase and an AHL

receptor. The receptor is frequently a transcription factor that, in the presence of the AHL, activates transcription of antibiotic biosynthetic genes (Pierson et al. 1998). One of the first quorum sensing systems identified in *Pseudomonas* was the *phzR*/*phzI* system in *P.aureofaciens,* which controls production of phenazine in this bacterium (Chin-A-Woeng et al. 1998). In this system the *phz*I gene encodes an AHL synthase, and *phz*R a receptor for AHL. In the presence of the appropriate AHL phzR upregulates the genes for phenazines synthesis, *phz*I, and itself, inducing a positive feedback loop. The *phz*I/R genes are closely linked to the Phz biosynthetic locus.

Manipulation of quorum sensing systems has been suggested as a way to increase the effectiveness of *Pseudomonas* biocontrol activity. Chin-A-Woeng *et al.* (2001) showed that increasing the copy number of *phzI* and *phzR*, the respective producer and receptor genes for AHL in *P. chlororaphis,* resulted in increased production of Phz. This type of genetic manipulation could be a fairly simple way to increase the efficiency of a biocontrol strain. Another tactic to use the quorum sensing system to improve biological control could be to incorporate and constitutively express AHL synthases into the host plant. This should stimulate earlier and larger quantity antibiotic production by the bacteria. Raaijmakers et al. (2002) successfully expressed AHL synthases in tobacco restoring disease suppression to AHL defective *P. aureofaciens*. Identification of AHLs important in antibiotic production and determining their regulatory genes and signalling network is a crucial first step in using modification of quorum sensing to increase the effectiveness of BCA.

Another important consideration for BCA that use AHL-triggered antibiotic production is that the presence of AHL-degrading bacteria in the soil can disrupt quorum sensing and antibiotic production and their presence could be detrimental to biological control (Haas & Defago 2005).

Antibiotic Autoinduction and Mutual Repression

A further example of positive-feedback mechanisms employed by antibiotic-producing bacteria is the capacity of antibiotics to positively regulate their own production. Exogenous application of antibiotics or the presence of two genotypically different, antibiotic producing *P. fluorescens* strains has been shown to increase production of both DAPG and pyoluteorin (Schnider-Keel et al. 2000; Baehler et al. 2005). Through autoinduction, DAPG can act as a signalling molecule, stimulating further production of DAPG *in vitro* and in the rhizosphere of wheat (Maurhofer et al. 2004). In addition to positive regulation of their own production, DAPG and pyoluteorin also act as mutual repressors (Baehler et al. 2005).

Autoinduction and mutual repression regulatory mechanisms of PLT and DAPG allow antibiotic-producing bacteria to quickly respond to changes in the environment by adjusting the concentrations of antibiotics and likely is important in preventing over-production of antibiotics *(Baehler et al. 2005)*. Understanding the autoinduction and mutual repression of PHL and PLT is important for optimizing application of multiple strains of antibiotic-producing *Pseudomonas.*

By combining regulatory systems such as GacS/GacA, quorum sensing, and antibiotic autoinduction and mutual repression the bacteria is able to quickly make fine adjustments to their metabolism to adapt to changes in environmental conditions

both biotic and abiotic. Understanding how antibiotic production is regulated is of great practical importance for effective use of BCAs and is critical if we wish to use genetic engineering techniques to improve upon biocontrol strains.

CHAPTER 3: COMPOSTS CONTAINING FLUORESCENT PSEUDOMONADS SUPPRESS FUSARIUM ROOT AND STEM ROT DEVELOPMENT ON GREENHOUSE CUCUMBER

Introduction

Fusarium root and stem rot, caused by the fungal pathogen *Fusarium oxysporum* f. sp. *radicis-cucumerinum*, is a major pathogen of greenhouse cucumbers in B.C. There are no fungicides presently available to control root and stem rot, and disease control strategies are limited to sanitation and use of select resistant cultivars (Rose & Punja 2004). Composts have been shown to suppress development of *F. oxysporum* on a number of hosts, including cucumber (Chef et al. 1983; Trillas-Gay et al. 1986; J. Pera & Calvet 1989; Szczech 1999; Kannangara et al. 2000; Rose et al. 2003). The presence of a diverse, antagonistic microflora (including bacteria, fungi, and actinomycetes) is one of the mechanisms through which composts can suppress the development of root diseases (Hoitink & Boehm 1999). Since most greenhouse cucumber growers utilize rockwool, an inert, almost sterile substrate for plant propagation, the establishment of a microbial population antagonistic to *Fusarium* could provide a unique opportunity to utilize composts for disease management. The objective of this study was to evaluate the potential of three different compost amendments added to rockwool medium to reduce the development of root and stem rot under greenhouse conditions, and to determine the mechanisms by which disease suppression may be occurring.

MATERIALS AND METHODS

Plant and microbial materials

Cucumber (*Cucumis sativus* L.) plants used in this work were *Fusarium*-susceptible cultivars Mystica and Ladner (provided by Rjick-Zwaan Inc., De Lier, the Netherlands). *Fusarium oxysporum* f.sp. *radicis-cucumerinum* (*Forc)* was recovered from the crown and roots of diseased cucumber plants in several British Columbia greenhouses and grown as described by Rose et al. (2003). *Forc* cultures were maintained on potato dextrose agar (PDA) and transferred to V-8 liquid medium with constant agitation (150 rpm) for the production of spores. Bacteria used were compost-derived strains isolated as described below, plus *P. fluorescens* strains Pf-5 and 2-79 (supplied by Dr. L. Thomashow USDA, Pullman, Washington) and were maintained on King's (1964) medium B (KMB) (see Table 3.1). Freezer stocks of each culture were stored in nutrient glucose broth (20% glycerol) at -80˚C.

Compost evaluation

Three composts were selected to evaluate disease suppressive activity, two of which were obtained from local sources. Greenhouse compost (compost derived from tomato and pepper plant waste using an in-vessel method, provided by South Alder Greenhouse Ltd., Delta, BC), and dairy compost (compost derived from solid material separated from dairy farm liquid manure, using a windrow composting method (provided by R. Utkhede, Agriculture and Agri-Food Canada, Agassiz, BC), were compared to Ball compost (compost derived from pine bark using a windrow

composting method, provided by Ball Horticulture Co., Chicago, IL). Microbial species diversity in each compost was analyzed by BBC Laboratories Inc. (Tempe, AZ) using standard plating methods on selective agar media. Two replicate samples were included from two different compost batches prepared at different times.

An experimental system was used to simulate growing conditions during propagation. Cucumber seeds (Mystica) were placed in seeding cavities of rockwool blocks (10 cm^2) contained in individual plastic bags. The seeding cavities were then filled with 15 cm^3 of compost medium or vermiculite (control). Inoculum of *Forc* containing 10^6 spores/ml was added to the seeding cavity of the rockwool block 48 hr after seeding, at a rate of 3 ml per block. Plants were maintained at ambient temperatures (20-23˚C) and a 16 hr photoperiod under 400 watt high-pressure sodium lamps (light intensity of 100±15 $\mu mol \cdot m^{-2} \cdot sec^{-1}$). Plants received a second application of 15 cm^3 of compost or vermiculite 13 days after seeding, and two days later, a second application of inoculum was made to each plant (3 ml of 10^6 spores/ml). Plant height and disease incidence were recorded after 8 weeks. Plants were rated for disease symptoms using a scale of 0 to 5, where 0 = no visible symptoms, 1 = minor crown discolouration, 2 = a small (< 10 mm) stem lesion, 3 = a moderate (10 to 20 mm) crown lesion, 4 = a severe (> 20 mm) crown lesion, and 5 = dead plant. Height of surviving plants was also measured at the end of the trial. A disease severity index (DSI) was used to assess the effect of the treatments. The index was calculated from the following formula: disease rating (as above from 0 to 5) + 5[1- (height of survivors/average height of control plants)] +5[1- (weight of survivors/average weight of control plants)]. The final disease severity index score has a range from 0 – 15 with plants scoring 0 being healthy and dead plants having

a score of 15. Plants receiving no inoculum were included as a control, with and without compost. The experiment was conducted as a randomized block design with 4 blocks and 4 replicate plants of each treatment per block. The experiment was conducted three times.

Pathogen survival in compost

Each of the three composts was evaluated for effect on survival of *Forc* in the absence of a host. Spore inoculum was added to 200 cm^3 of compost in a plastic container to achieve an estimated concentration of 10^4 spores/cm^3 of compost. Sterile distilled water was added to each cup to bring the total weight of the contents to 90 g (representing 67% moisture holding capacity), and the cups were sealed with Saran wrap. The cups were incubated at 20-23˚C, weighed periodically and moisture added as required. Samples (1g) of each type of compost were taken at weekly intervals for up to 8 weeks from two replicate cups, diluted to 10^{-2} or 10^{-3} in sterile distilled water and 1 ml was plated onto each of two Petri dishes containing *F. oxysporum* selective medium (Komada 1975). Dishes were left to dry in a flowhood for 1 hour and then sealed and incubated at room temperature (20-23˚C). Colonies were counted after 4 days.

Recovery of *Pseudomonas* species and antagonism tests

Samples of greenhouse compost were diluted in sterile distilled water to 10^{-6} or 10^{-7} and 0.1 ml was plated onto both potato dextrose agar (PDA) and fluorescent pseudomonad selective agar medium (FPSA) (Hildebrand et al. 1988). Yellowish-

pigmented colonies on PDA and colonies that stained dark purple on FPSA were transferred onto King et al.'s (1954) medium B (KMB) to assess the production of fluorescent pigments when viewed under UV light. In addition, *Forc*-infected cucumber roots were wrapped in fine nylon mesh and buried in greenhouse compost for either 7 or 14 days to recover bacteria competent in colonizing roots. Buried roots were removed and shaken in sterile distilled water for 30 min. The water was filtered through a 3µm syringe to remove fungal spores and plated onto PDA. Morphologically distinct colonies were transferred to KMB agar plates.

In-vitro assays were conducted to determine if any of the recovered isolates were antagonistic to *Forc*. Approximately 130 isolates obtained using the procedures above were each streaked onto PDA and 48 hr later, a 0.5 cm^2 plug of *Forc* was placed on the dish 3 cm away from the bacterium. Dishes were monitored regularly for the presence of an inhibition zone. All antagonistic bacterial isolates and all isolates recovered on pseudomonad-selective medium (approximately 30 isolates in total) were identified to genus and species using a Biolog® system (Biolog Inc., Hayward, CA).

Effect of bacteria on pathogen survival and disease development

The isolate of *P. aeruginosa* that produced the largest inhibition zone against *Forc*, and a *P. maculicola* isolate that showed no antagonism, were selected and added to sterilized greenhouse compost (autoclaved 2 times with 48 hr between autoclave cycles) to determine if they could render the compost suppressive to *Forc*. Isolates were grown in nutrient broth for 24 hr at 27˚C, shaken at 120 rpm. The broth was centrifuged at 2500 x g for 15 min and the pellet was re-suspended in phosphate buffer consisting of 7 g K_2HPO_4, 3g KH_2PO_4, and 0.2g anhydrous $MgSO_4$

per liter of water. Bacterial density was estimated using a spectrophotometer at 590 nm and adjusted to an optical density of 0.3, corresponding to $5x10^8$ cells/ml, based on a previously established standard curve. Bacterial concentration was confirmed by dilution plating onto KMB. The two isolates (*P. aeruginosa* strain P23 and *P. maculicola* strain P15) were added to sterilized greenhouse compost at an initial concentration of 10^8 CFU/g and incubated at 20-23˚ C. Bacteria were allowed to incubate in the compost at room temperature in sealed containers for 2 weeks prior to use in experiments. Bacterial survival was monitored weekly by dilution plating of 1 g samples onto King's B medium. *Forc* inoculm was added to bacteria amended compost in a separate experiment and pathogen survival was monitored weekly by dilution plating on Komada's medium.

To assess the effectiveness of bacteria for disease suppression, cucumber seeds were planted in the cavities of rockwool blocks with bacteria-amended compost as described above. *Pseudomonas fluorescens* strains Pf-5 and 2-79 were also included. Initial levels of bacteria were determined by dilution plating and bacterial survival was monitored. Pathogen inoculation and growing conditions were as described above. Autoclaved greenhouse compost with and without *Forc* were included as controls. Disease severity was assessed after 8 weeks as previously described. The experiment was repeated twice.

Following assessment of disease severity, cucumber stem pieces were surface-sterilized for 60 sec in 70% ethanol, followed by 60 seconds in 10% bleach solution (0.525% NaOCl) and 0.5 cm cross-sections were plated onto Komada's medium to assess the extent of internal colonization by *Forc*. Stem sections were taken at 10 cm intervals beginning at the crown of the plant up to a height of 90 cm

and presence or absence of *Forc* in the sections was determined after 5 days. Plants that died prior to termination of the experiment were considered to be colonized along the entire stem.

Statistical analyses

Disease suppression experiments were conducted in a randomized block design as described. The experiments were repeated 2 times. Data was analyzed by analysis of variation (ANOVA) using JMP 7.0 software (SAS Institue Inc.). Significant differences between treatments ($P < 0.05$) were determined using Student's t-test.

Presence of bacterial antibiotic genes

P. aeruginosa strain P23 was screened for the presence of antibiotic biosynthetic loci by Southern blot hybridization and gene-specific PCR amplification. The two strains of *P. fluorescens* (Pf5 and 2-79) were used as positive controls for the appropriate genes. Loci screened were those for the production of the antibiotics pyrrolnitrin (PRN), pyoluteorin (PLT), 2,4-diacetylphloroglucinol (DAPG), phenazine-1-carboxylic acid (PCA), and hydrogen cyanide (HCN) (see Table 3.2). Bacterial genomic DNA was isolated using CTAB extraction and digested for 12 hrs using *Hin*d*III* restriction endonuclease (Promega). Duplicate 10 µg samples of the restriction products of the genomic DNA of *Pseudomonas* strains P23, P15, Pf5 and 2-79 were run on a 1% agarose 0.5x TBE gel at 20 volts for 15 hr. Electrophoresed DNA was transferred to Hybond-XL nylon membranes (GE Healthcare) using alkaline capillary transfer for 18 hr (0.4 M NaOH transfer buffer) and UV crosslinked

(Sambrook et al. 1989). Nucleotide probes for each biosynthetic locus were prepared using PCR amplicons from the positive controls (Pf5 and 2-79) using the gene-specific primers listed in Table 3.2. PCR samples to be ^{32}P dCTP labeled were purified using a QIAquick PCR cleanup kit (Qiagen Inc.) and labeled with a Prime-a-Gene labeling system (Promega) following the manufacturer's protocol. Membranes were pre-hybridized at 55˚C for 2 hr using EKONO^{TM} hybridization buffer (Research Products International Corp, Mt. Prospect, IL, USA), followed by hybridization for 12 hr at 65˚C. Following hybridization, blots were washed twice in 2x SSC, 0.1% (w/v) SDS for 10 min at 65˚C, twice in 1x SSC, 0.1% SDS for 15 min (Sambrook *et al.* 1989). Hybridized probes were detected by exposure to X-ray film for 1-7 days at -80˚ C.

PCR reactions for confirmation of the presence of the antibiotic genes were carried out using the published primer pairs shown in Table 3.2.

Colony PCR was performed with the reference strains and strain P23 using EconoTaq polymerase (Lucigen, Middleton, WI) using the manufacturer's protocol. PCR reactions were carried out using a BioRad MJ Mini Gradient thermal cycler following previously published reaction conditions (Raaijmakers et al. 1997; Ramette et al. 2003; de Souza et al. 2003). PCR products and GeneRuler DNA ladder (Fermentas) were electrophoresed for 45 min at 80 V on a 1% agarose gel in 0.5% TBE and stained with ethidium bromide for visualization under UV light

Phloroglucinol production

Production of 2,4-DAPG in culture was detected using thin layer chromatography. Bacteria were grown in 200 ml of King's B broth or Pigment

Producing Medium (Ayyadurai et al. 2006) in 1 L Erlenmeyer flasks at 27˚C and 200 rpm for 5 days. The resulting cultures were centrifuged and the supernatant separated from the bacterial pellet. Extraction of antibiotics was conducted as described by Raaijmakers & Weller (2001) and Ayyadurai et al. (2006). The supernatant was adjusted to pH 2.0 with HCl followed by extraction with an equal volume of ethyl acetate. The organic fraction was separated by centrifugation, removed and evaporated to dryness. The resulting extract was dissolved in methanol and syringe filtered (0.2 µm, Millipore Corp). TLC was conducted using 10 x 20 cm aluminum-backed silica gel plates ($60F_{254}$). Each plate was spotted with 5 x 10µl of extract from each of strains P23, P15, and the known antibiotic producers *P. fluorescens* Pf5 and 2-79, allowing the spots to dry completely between applications. Plates were developed with chloroform:methanol (9:1 v/v) and DAPG visualized by treatment with *p*-anisaldehyde (0.5 ml in 5 ml H_2SO_4 and 95 ml 95% ethanol).

Table 3.1: Bacterial strains used in this study

Bacteria	Characteristics	Source
Pseudomonas aeruginosa P23	DAPG, siderophore production	Compost isolate
Pseudomonas syringae pv. *maculicola* P15	Siderophore production	Compost isolate
Pseudomonas fluorescens Pf5	DAPG, Pyoluteorin, pyrrolnitrin, siderophore production	L. Thomashow, USDA
Pseudomonas fluorescens 2-79	Phenazine-1-carboxylic acid production, siderophore production	L. Thomashow, USDA

Table 3.2: Nucleotide primers used for oligonucleotide probe synthesis and PCR analysis of antibiotic biosynthetic loci

Antibiotic	Primer Sequence	Reference
Pyrrolnitrin	PRND1 5`-GGGGCGGGCCGTGGTGATGGA-3` PRND2 5`-YCCCGCSGCCTGYCTGGTCTG-3`	DeSouza &Raaijmakers, (2003)
Pyoluteorin	PLTC1 5`-AACAGATCGCCCCGGTACAGAACG-3` PLTC2 5`-AGGCCCGGACACTCAAGAAACTCG-3`	DeSouza &Raaijmakers, (2003)
2,4-DAPG	Phl2a 5`-GAGGACGTCGAAGACCACCA-3` Phl2b 5`-ACCGCAGCATCGTGTATGAG-3`	Raaijmakers et al. (1997)
PCA	PCA2a 5`-TTGCCAAGCCTCGCTCCAAC-3` PCA3b 5`-CCGCGTTGTTCCTCGTTCAAT-3`	Raaijmakers et al, (1997)
HCN	HCN ACa 5`-ACTGCCAGGGGCGGATGTGC-3` HCN ACb 5`-ACGATGTGCTCGGCGTAC-3`	Ramette et al, (2003)

Root colonization and survival of DAPG-producing *P. aeruginosa*

The ability of *P. aeruginosa* strain P23 to colonize the roots of cucumber plants and to survive at levels adequate to provide biological control was assessed using the growing system previously described for growth-room trials. Compost was amended as described above and plants receiving P23-inoculated compost and control plants receiving sterile compost were grown for 4 weeks. Approximately 1 g of root tissue was taken from the distal roots of each plant. This tissue was macerated in a bead-beater for 30 sec and extent of colonization was determined by dilution plating. Plating was done using antibiotic-supplemented agar medium on which strain P23 was shown to be capable of growing (KMB supplemented with 200 mg/L cylohexamide, 100 mg/L rifampicin, 9 mg/ L basic fuchin, 23 mg/L naldixic acid and10 mg/L nitrofurantoin). Colonies were counted after 3 days using 5 plants (with 3 replicate samples from each) grown in each of the P23 treated and control composts. Colonization experiments were performed twice.

Defense-related gene expression analysis

Northern blot analysis of several defense-related and ISR signalling genes was conducted to determine if *P. aeruginosa* strain P23 is capable of inducing ISR in cucumber. Details can be found in the Appendix.

RESULTS

Compost evaluation

Among the three composts evaluated, greenhouse compost provided the highest degree of disease suppression, as seen by the significantly lower disease index compared to the treatment with *Forc* alone (Figure 3.1). The disease severity index of plants receiving dairy compost was also significantly reduced, while Ball compost provided no significant protection from disease development compared to control plants receiving *Forc* alone (Figure 3.1). Similarly, plants receiving autoclaved compost showed no disease suppression.

An analysis of the composts for microbial content (presented as diversity and total counts of specific microbe groups) indicated that yeasts and molds were highest in the Ball compost and lowest in the dairy compost (Figure 3.2). In contrast, levels of total pseudomonads were lowest in the Ball compost (1×10^2 CFU/g) and highest in the greenhouse and dairy composts (1×10^8 CFU/g). For other groups of microbes, there were no consistent trends between composts. The results from two replicate samples and two batches of each compost showed a similar results, with total *Pseudomonas* levels reaching 1×10^8 CFU/g each time, and yeasts and molds $<10^5$ CFU/g in the suppressive compost types.

In both the greenhouse and dairy composts, the population levels of added *Forc* showed a dramatic decline during the first 3 weeks of the 8-week trial. In contrast, the Ball compost showed a much more gradual decline, reaching 50% of the initial population after 8 weeks (Figure 3.3).

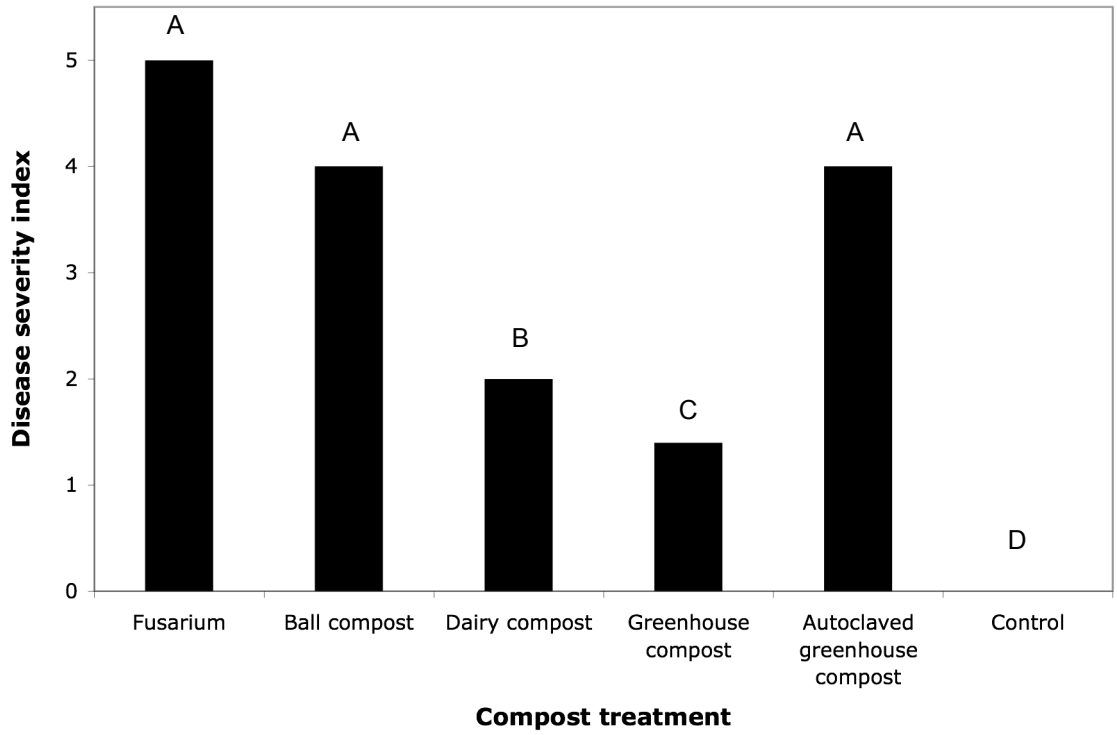

Figure 3.1 :Effect of greenhouse, dairy and Ball composts on disease development due to *F. oxysporum* f.sp.*radicis-cucumerinum* on greenhouse cucumber. Disease severity was rated after 40 days of growth. Bars with the same letter are not significantly different according to one-way ANOVA and Fischer's T test ($P \leq 0.05$)

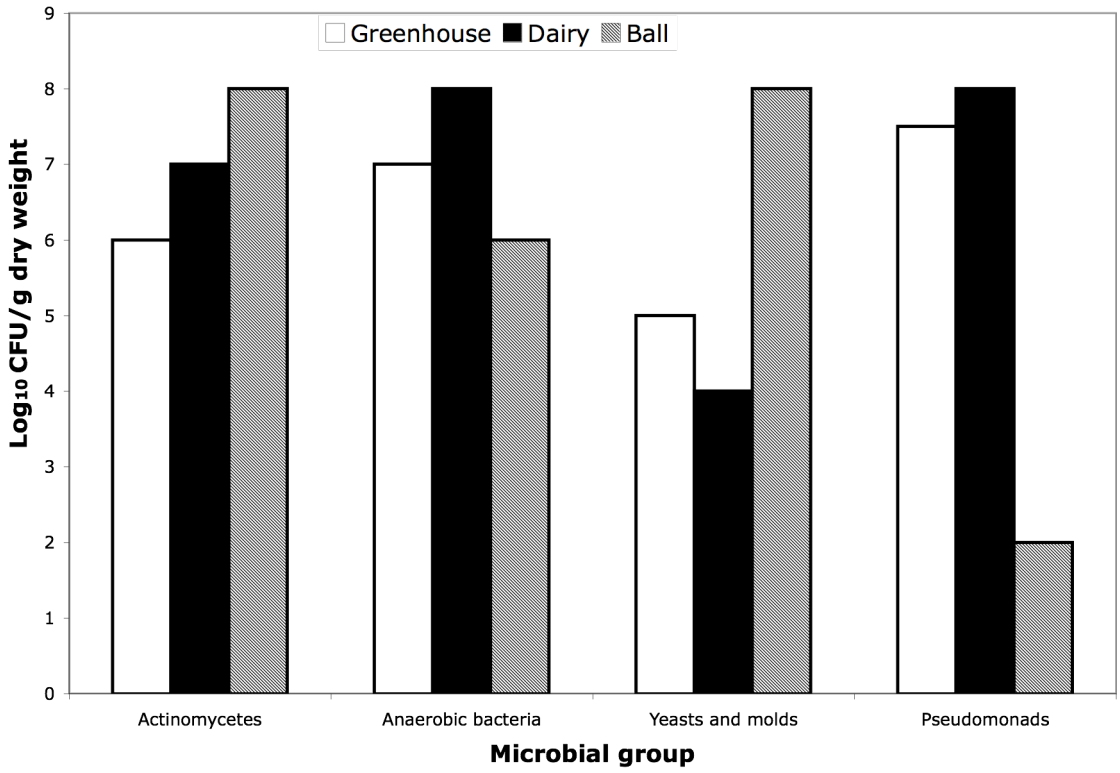

Figure 3.2: Microbial diversity in 3 composts used in this study. Samples were analyzed by BBC Labs, Tempe AZ. Data are from two replicate samples. The analysis was conducted twice.

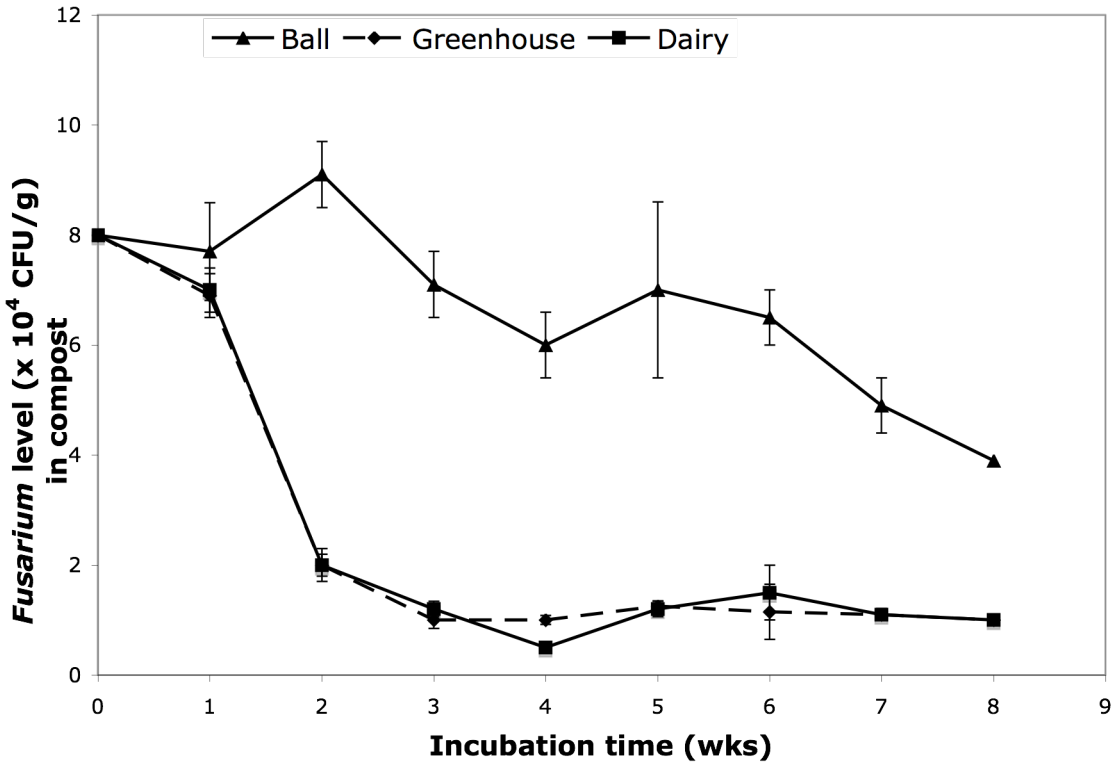

Figure 3.3: Effect of greenhouse, dairy and Ball composts on survival of *Fusarium.* Inoculum was added to each compost and incubated at 20-23˚C and populations levels were measured weekly by plating onto Komada's medium. Error bars represent standard error of the mean (n=4).

Recovery of *Pseudomonas* and antagonism tests

Bacteria isolated using dilution plating were tested for antagonism to *Forc* on PDA. Antagonism tests showed that putative *Pseudomonas* isolates fell into two groups, those with antagonistic ability and those without. Subsequent Biolog® identification revealed that all strains with antagonistic ability were *P. aeruginosa* and those lacking antagonism ability were *P. syringae* pv. *maculicola* (to be refered to here-in as *P. maculicola*). Biolog® identification of antagonistic strains recovered by root-baiting showed that antagonistic strains included various *Enterobacter* species, of which *E. nimipressuralis* showed the greatest *in vitro* inhibition to *Forc* and so was selected for further testing. However, preliminary growth-room trials demonstrated that this strain did not suppress *Forc in situ*, and therefore, only *P.aeruginosa* was included in subsequent *experiments.*

Effect of *Pseudomonas* on disease development and stem colonization

Growth of *Forc*- susceptible cucumber plants in the presence of *Forc* inoculum in composts treated with the bacteria isolates as described above, showed that compost receiving *P. aeruginosa* P23 significantly reduced disease severity compared to sterilized control compost (Figure 3.4), and plants treated with amended compost had an average DSI that was not significantly different from the control receiving no *Fusarium.* Composts amended with *P. maculicola* P15 did not have any effect on reducing DSI and disease was not significantly different from *Fusarium* treated controls. Both *P. fluorescens* strains reduced disease severity and

strain 2-79 was the more effective of the two; however neither was as effective as *P. aeruginosa* P23.

Assessment of cucumber stems for *Forc* colonization showed that a high percentage of plants were colonized at the crown regardless of bacterial treatment (Figure 3.5). However, plants grown in compost receiving *P. aeruginosa* and *P. fluorescens* had significantly lower colonization at increasing distance from the crown compared with plants grown in *P. maculicola*-treated compost and un-amended compost. Survival of *Forc* in compost receiving

bacteria was dramatically lower than in un-amended, autoclaved compost (Figure 3.6).

Colonization of the roots of cucumber plants by *P. aeruginosa* P23 was demonstrated in growth room trials. Dilution plating showed that strain P23 survived on cucumber roots at 1.9±(0.73) x 10^6 CFU/g of root tissue after 4 weeks.

Screening of bacterial antibiotic genes and detection of antibiotics

The biosynthetic locus for PHL was detected in *P. aeruginosa* strain P23 by Southern blot analysis and confirmed by colony PCR using the gene-specific primers shown in Table 3.2. PCR produced an amplified band of 745 bp corresponding to the expected amplicon size for the *Phl*D locus in strain P23 and the positive control strain Pf5. Southern blot hybridization and PCR amplification of PCA, PRN, PLT and HCN biosynthetic genes were seen only in control strains and was not detected in strains P23 and P15 (data not shown) indicating that these biosynthetic loci are not carried by either of strains P23 or P15.

Production of PHL by strain P23 was confirmed by TLC analysis. Both strain P23 and the positive control Pf5 showed an orange band with an R_f of approximately 0.77, matching the colour and R_f values for PHL (see Figure 3.7) (Yuan et al. 1998; Ayyadurai et al. 2006). Also, visualization of TLC plates with reagents for detection of PRN, PLT and PCA (eg. Van Urk's reagent and Fast Blue RR) showed the production of these antibiotics only in the control *P. fluorescens* strains, confirming the Southern blot and PCR results.

Defense-related gene expression analysis

Northern blots of defense-related genes in cucumber plants treated with *P. aeruginosa* strain P23 showed no expression patterns that suggest that P23 is inducing systemic resistance response (see appendix figure A.1)

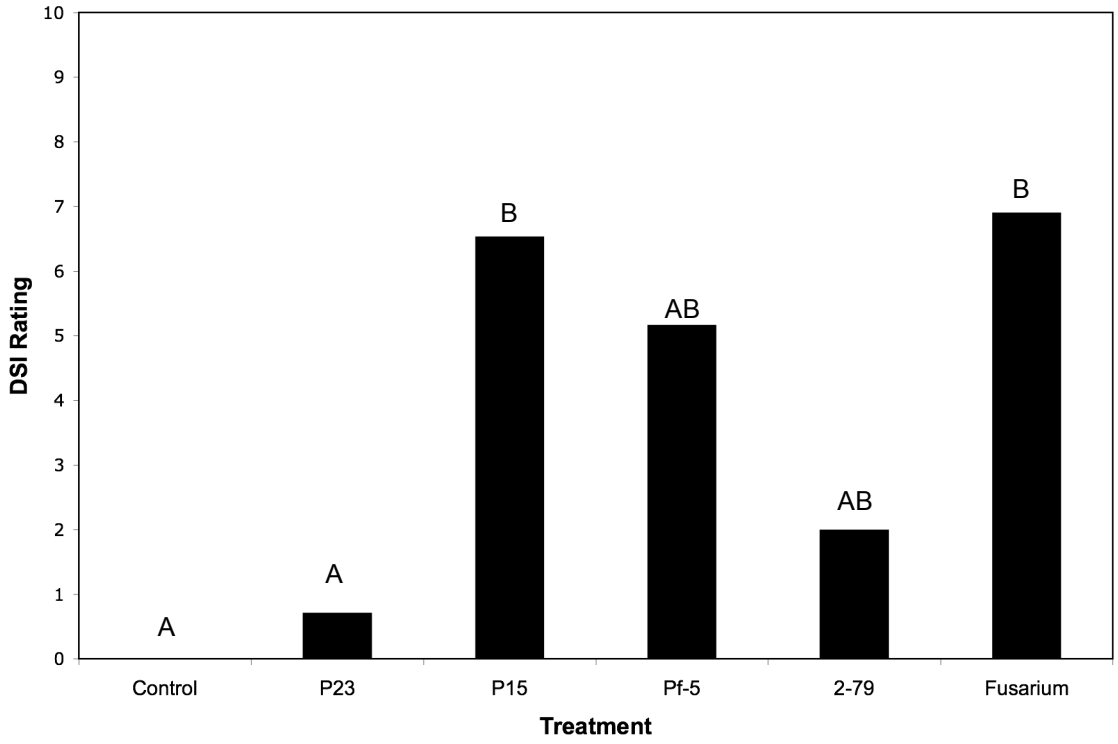

Figure 3.4: Effect of bacteria-amended greenhouse compost on disease development due to *F. oxysporum* f.sp. *radicis-cucumerinum* on greenhouse cucumber. Disease severity was rated after 8 weeks of growth. P23 = *P. aeruginosa* P15 = *P. maculicola* Pf-5 and 2-79 = *P. fluorescens* strains. Bars with same letter are not significantly different according to one-way ANOVA and Fischer's T test (P≤ 0.02)

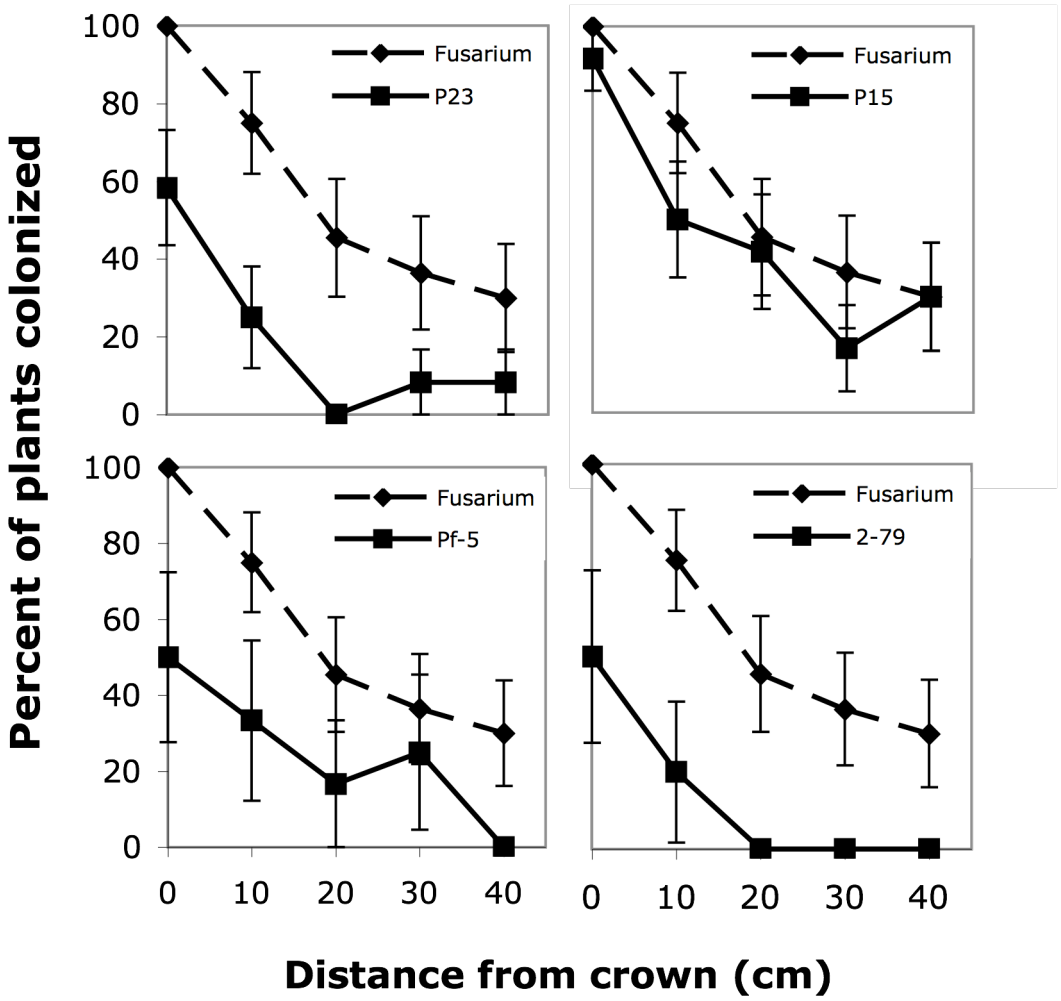

Figure 3.5: Extent of colonization of cucumber stems by *Fusarium* in plants grown in bacteria amended compost compared to non-amended sterilized compost. Percent of plants colonized was determined at 10 cm increments by plating surface-sterilized stem segments onto Komada's medium. Plants dead at time of DSI rating were scored as being colonized at all points. Bars indicate SEM (n=6). P23 = *P.aeruginosa*, P15 = *P. maculicola*, Pf-5 and 2-79= *P. fluorescens* strains, Fusarium = plants receiving no bacterial treatment.

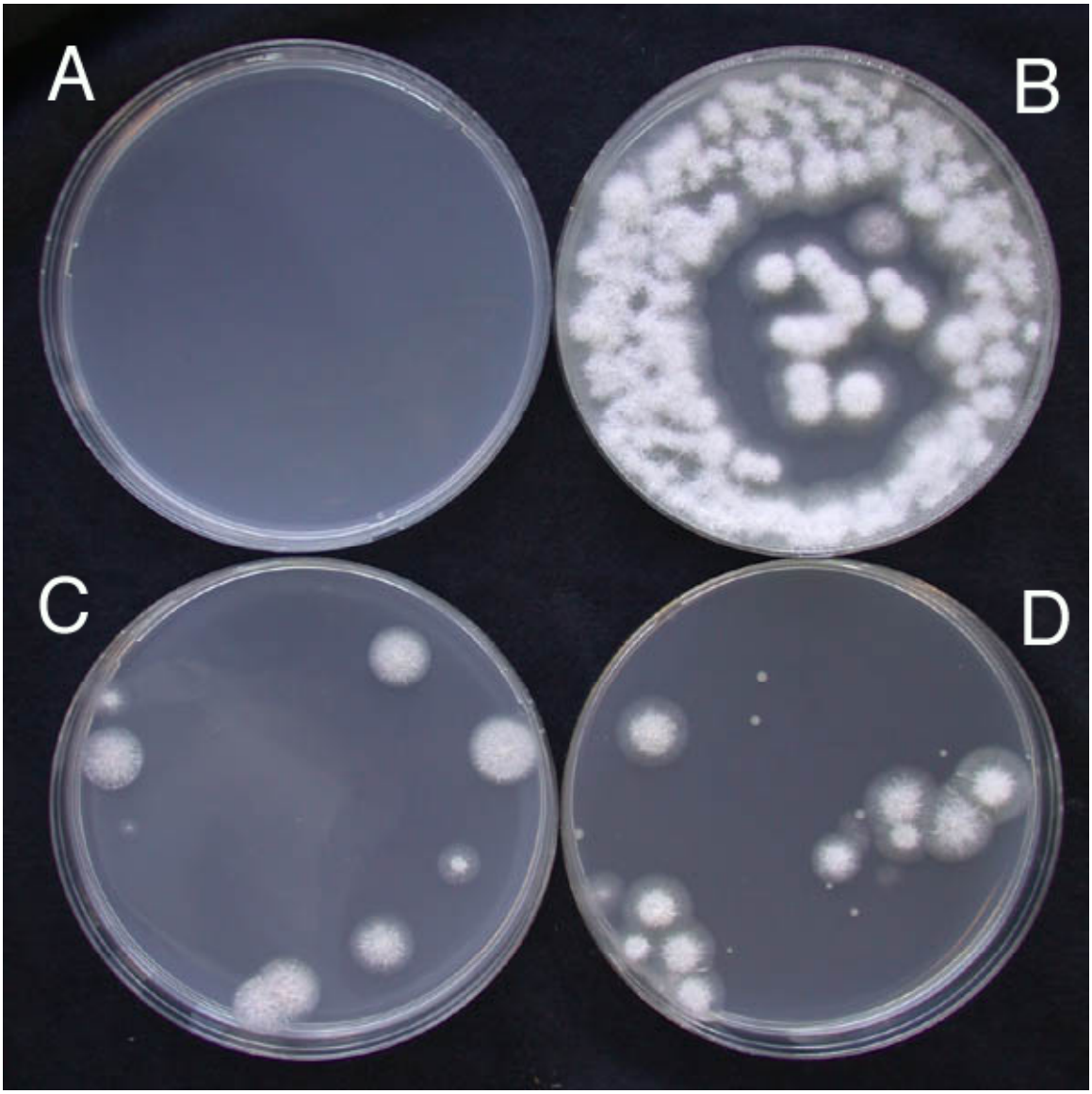

Figure 3.6: Recovery of colonies of *Fusarium* on Komada's medium following addition of fungal inoculum (10^6 spores/cm^3) to greenhouse compost (A), autoclaved greenhouse compost (B), and autoclaved greenhouse compost amended with *P. aeruginosa* strains P22 and P23 (C and D respectively). Samples were taken after 3 weeks and diluted to 10^{-4} before plating. Photographs were taken after 4 days of incubation at room temperature.

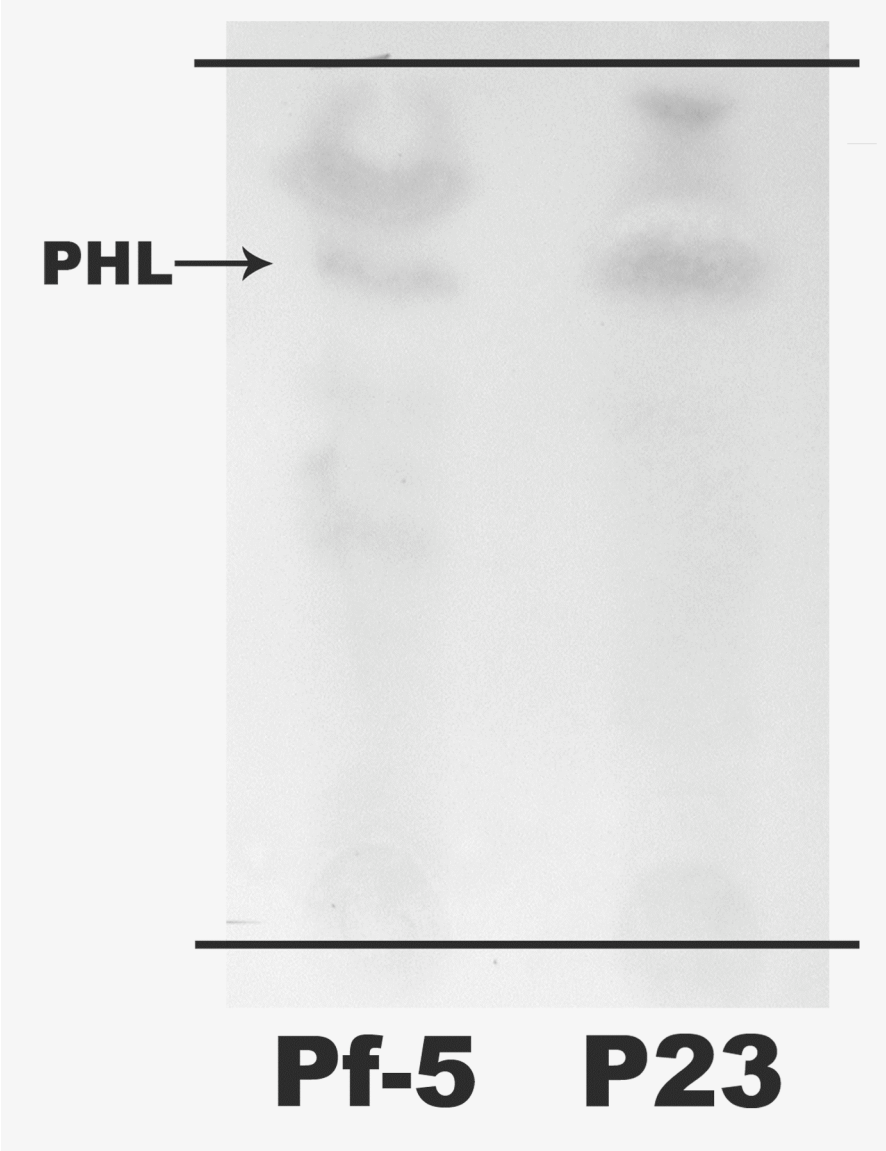

Figure 3.7: Thin layer chromatograph indicating production of 2,4-DAPG by *P. aeruginosa* P23 and the positive control *P. fluorescens* Pf-5. Extracts were developed in chloroform:methanol (9:1 v/v) and stained with *p*-anisaldehyde. Spot containing 2,4, DAPG is indicated. R_f = 0.76 for Pf-5 and 0.77 for P23. Top and bottom bars indicate solvent front and loading position respectively.

DISCUSSION

In this study, two composts (greenhouse and dairy) that were able to suppress fusarium root and stem rot development on cucumber were identified, demonstrating that the application of composts at seeding may be a useful method to reduce this disease. Suppression of *Fusarium* by greenhouse and dairy composts in this study supports other research findings that demonstrated that composts reduced diseases of greenhouse cucumber, including root rot and damping off caused by *Pythium ultimum* and *P. aphanidermatum*, anthracnose caused by *Colletotrichum orbiculare*, as well as cucumber mosaic virus and powdery mildew (Raupach et al. 1996; W. Zhang et al. 1996; Vogt & Buchenauer 1997; Lievens 2001). Our findings showed that the disease suppressive ability of the two composts in our study was associated with high *Pseudomonas* levels in these composts. Fluorescent pseudomonads are among the best-characterized and well-studied biological control agents (Stockwell & Stack 2007; Weller 2007). They have been shown to provide protection against *Fusarium* species infecting numerous crops (de Boer et al. 1999; Anjaiah et al. 2003; Rose et al. 2003).

Previous work has demonstrated that *Pseudomonas* species may show chemotactic attraction to plant roots infected by *F. oxysporum* (de Weert et al. 2004). This suggests that *Fusarium*-infected roots could be used as a bait to isolate potential biocontrol pseudomonads from the composts of interest. Using both baiting and dilution plating methods, two species of *Pseudomonas* were isolated from suppressive composts, namely *P. aeruginosa* and *P. maculicola*. *In vitro* antagonism tests revealed that *P. aeruginosa* was capable of inhibiting the growth of

Forc up to a distance of 1.5 cm from the bacterial colony, suggesting the presence of a diffusible inhibitory agent. Further studies indicated that *P. aeruginosa* strain P23 not only carries the genes for production of DAPG but also produces it in culture, as indicated by presence of a distinct red-orange band present on TLC of liquid culture extracts, and by the characteristic red pigmentation of colonies grown on specific agar media. In addition, the *P. aeruginosa* P23 was able to colonize the root system of host plants and survive at or above threshold population levels of $5x10^5$ CFU/g of root tissue reported in other studies where DAPG production has been demonstrated to be the primary mechanism of biological control (Raaijmakers et al. 1997). Several factors known to influence DAPG production can be easily manipulated by growers, such as pH, temperature and phosphate concentrations (Raaijmakers et al. 2002), leading to a potential increase in consistency of antibiotic production and biological control.

The *P. aeruginosa* P23 isolate used in growth room trials in this study was equally or more effective than *P. fluorescens* strains Pf-5 and 2-79 in reducing disease severity. However, despite a significant reduction in disease severity, these plants still had some internal colonization by *Forc*, particularly at the crown (Figure 3.5) indicating that pathogen invasion was occurring without symptom expression. We hypothesize that the fungal pathogen may be able to invade the host but has reduced spread within the plant tissue in the presence of the antibiotic producing bacteria, which were shown to colonize the root system and also found in stem tissue (data not shown).

All of the *Pseudomonas* strains used in this work (both those that provided disease suppression and those that did not) showed siderophore production on

chrome azurol-S medium (data not shown). Thus, the production of siderophores by these bacteria is unlikely to be involved in disease suppression. Additionally, both the nutrient solution used for cucumber production and the compost used in this study contain high available iron levels, reducing the likelihood of disease suppression through iron limitation by siderophore production.

Previous studies have shown that treatment with a beneficial microorganism that induces systemic resistance in a plant host does not lead to immediate changes in defense gene expression; rather, these genes are 'primed' so that their expression occurs more rapidly in the presence of a pathogen (Conrath et al. 2006). If ISR was occurring in P23-treated cucumber plants we would expect to see no alteration of expression of these genes in plants receiving only P23. In plants receiving both P23 and *Forc,* we would expect to see a more rapid and intense expression of defense-related genes.

The signalling pathway in the plant leading to ISR is the jasmonic acid/ethylene signalling pathway (Shoresh et al., 2005). Previous studies have demonstrated that in contrast to defense genes, genes related in JA/ethylene signalling such as lipoxygenase (involved in JA synthesis) and *Etr1* (involved in ethylene signal perception and transduction) are upregulated during ISR, prior to pathogen encounter.

While other reports have described ISR in cucumber as a mechanism of biological control (eg. Ongena et al. 2000; Kim et al. 2004; Shoresh et al. 2005), repeated studies of expression of several genes involved in ISR (as indicated in Shoresh et al. (2005)) plus the defense-related genes phenylalanine ammonia-lyase and glucanase revealed no altered gene expression patterns in plants pre-treated

with P23 prior to pathogen challenge relative to controls at various time intervals from 0 to 72 hr (see appendix, Figure A.1).

Previous studies have shown *P. aeruginosa* strains to be effective biological control agents against a wide range of plant pathogens, including powdery mildew of wheat (Muyanga et al. 2005), *Pythium* and *Fusarium* in tomato (Buysens et al. 1996), *Fusarium* wilt of pigeonpea and chickpea (Anjaiah et al. 2003), *F. oxysporum* and *Cylindrocladium spp.* in banana (Ayyadurai et al. 2006) and *Botrytis cinerea* on bean (De Meyer & Hofte 1997). The ability to control such a broad spectrum of diseases is likely due to the wide range of biocontrol mechanisms displayed by *P.aeruginosa* strains. The various strains used in the above studies were reported to induce systemic resistance (Muyanga et al. 2005), produce antibiotics including phenazines and phloroglucinol (Anjaiah et al. 2003; Ayyadurai et al. 2006), and engage in siderophore-based iron competition (Buysens et al. 1996: De Meyer & Hofte 1997; Manwar et al. 2004). These strains originated from a range of sources such plant rhizosphere, compost, and seawater (Anjaiah et al. 1998; Manwar et al. 2004; Ayyadurai et al. 2006). These previous successes in use of *P. aeruginosa* against various soil-borne pathogens support our findings that *P. aeruginosa* strains from compost are competent colonizers of the rhizosphere and plant root system, which likely contributes to biological control activity.

Scheuerell and Mahaffee (2005) describe the “microbial void” after the peak heating of composts during the composting process; heat during the thermophilic stage of composting eliminates the majority of microbes in the starting material, including those capable of suppressing disease. Re-colonization of the compost is influenced by factors including pH and moisture, with pseudomonads re-colonizing

composts with high moisture content shortly after peak heating (24-48 hr) (Hoitink, Stone, and Han 1997; Zinati 2005). Since *P. aeruginosa* is a ubiquitous bacterial species (demonstrated by the wide range of sources of origin of *P. aeruginosa* discussed above), its presence in the composts used in this study is likely from an environmental source such as soil or untreated water. Once introduced, its ability to produce both antibiotics and siderophores may allow it to colonize these composts at high levels. Artificial introduction of disease suppressive *P. aeruginosa* strains into peak-heated composts prior to colonization by other microbes (as previously suggested for other biological control organisms (Postma et al. 2003; Scheuerell & Mahaffee 2005) should allow for increased colonization by *P.aeruginosa* and may provide composts which control *Fusarium* diseases.

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

Improving selection and use of biological control agents

The last several decades have seen a consistent increase in public awareness and concern over the use of chemical pesticides in agriculture. The use of alternative methods of disease control, such as changes in cultural technique and use of beneficial bacterial species, could lead to significant reductions in the amount of chemical fungicides and pesticides required. Despite this, there has been only a very gradual increase in commercial use of biological control agents for plant diseases. The major stumbling block hampering widespread implementation of BCAs to date is the lack of consistency of biocontrols compared to chemical alternatives. There are significant up-front costs associated with the development and registration of a biopesticide; companies will not develop a product of inconsistent nature for a small market crop, such as greenhouse cucumber, for which they will not likely recoup these costs (Paulitz & Belanger 2001). This combined with the stringent regulations for the registration of any pesticide, either chemical or biological in nature, is the reason few BCAs are available in Canada. The regulatory system for pesticides for use on minor crops in North America has undergone simplification in the past decade, including implementation of joint registration between Canada and the USA (Paulitz & Belanger 2001), as well as the introduction in 2002 of the Minor Use Pesticide Program by Health Canada's Pest Management Regulatory Agency (Agriculture and Agri-Foods Canada 2003). As

regulations controlling the use of biological control agents continue to become less of a hurdle to implementation of biological control, minimizing inconsistencies in effectiveness to the point where growers are willing to switch to BCAs from chemical pesticides will become the key to encouraging their widespread use. In proven biocontrol systems such as *Bacillus thuringiensis* (*Bt*) for control of various insects, biocontrol has dramatically reduced chemical control use (Sanchis 2008). Another example of this is the use of *Agrobacterium*-84 (marketed under various trade names such as Dygol and Galtrol), which has been successfully used to control crown gall (caused by *Agrobacterium tumefaciens* on susceptible plants) since 1979 (Fravel 2005). Agrobacterium-84 is a non-pathogenic strain of *A. radiobacter* that carries plasmids containing the genes for production of the antibiotic Agrocin-84 and resistance to this antibiotic. Agrocin-84 prevents growth of pathogenic *Agrobacterium* thereby preventing disease. When applied properly this biocontrol shows efficacy equal to chemical control (Kerr 1980) and has little breakdown in control over time. The long-term success of this product highlights the tremendous potential that antibiotic-producing bacteria have as biocontrol agents. The success of both *Bt* and Agrobacterium-84 is due to their consistent, long-term effectiveness. Current research on the mechanisms of biological control and the factors regulating these mechanisms is important because it can help us understand why biological control of plant pathogens is effective in some situations and not others. Applying knowledge of the regulation of disease control mechanisms will lead to increases in biocontrol consistency that will in turn lead to increased marketability of BCAs, ultimately increasing the amount of effort that companies are willing to expend in getting BCAs registered for use.

Because of the complexity of interactions between plant, pathogen, biocontrol agent and environment, an advocated strategy for development of BCAs is to specifically tailor them for their intended plant-pathogen system to help ensure their effectiveness (Cook 1993). The problem with this is that identification of BCAs is labour intensive and the requirement to identify different biocontrol agents for every crop multiplies the amount of work required. To streamline development of biological control agents, we must first find identifying characteristics of good biological control agents. As discussed in previous chapters, research aimed at doing so has identified such markers as specific cyclopropane fatty acids and the presence of antibiotic biosynthetic loci. The production of antibiotics by bacterial biocontrol agents has been proven to be a primary factor in the suppression of many plant diseases. Through application of this knowledge and high-throughput screening methods, such as colony hybridization to identify carriers of these loci, identification of antibiotic producing bacteria could greatly minimize the labour required for BCA development.

Improved understanding of the regulatory systems in biocontrol bacteria, particularly antibiotic producers is the second requirement for increasing the consistency of biocontrol. Our understanding of these regulatory systems has greatly increased in the last decade but much work is still needed in this area as there are still important regulatory systems that we do not fully understand, such as GacA/GacS post-transcriptional regulation. Understanding how and when antibiotic production is activated in BCA is important to optimizing their ability to suppress disease.

Previous studies have shown that application of *Pseudomonas* biocontrol strains in combination can enhance the effectiveness of biocontrol (de Boer et al. 1999; Roberts et al. 2005). The antibiotics DAPG and PLT have been shown to positively regulate their own respective production (Maurhofer *et al.* 2004). Inter-population signalling between strains of bacteria using the molecule DAPG has been shown to enhance expression of DAPG genes both *in vitro*, and on the roots of wheat plants (Maurhofer *et al.* 2004). This phenomenon provides one potential explanation for the observation that combinations of biocontrol strains are often more effective than individual strains. In addition to being autoinducers, DAPG and PLT are also mutual repressors (Schnider-Keel et al. 2000); this complicates the task of devising combinations of antibiotic producing strains and must be taken into account to provide optimal antibiotic production and maximized biological control.

Genetic engineering of BCAs is another tool that could be used to enhance disease suppression. For example, Chin-A-Woeng et al. (2001) successfully showed that insertion of multiple copies of the regulatory genes *phzI* and *phzR* resulted in increased antibiotic production in *P. chlororaphis*. Genetically modified biocontrol agents, specifically the *A. tumefaciens* strain K1026, a modified strain of Agrobacterium-84, have been registered and used in several countries including Canada (Penyalver et al. 2000; Fravel 2005).

Application of research and future directions

The research conducted for this thesis identified composts suppressive to *Forc* on greenhouse cucumber both *in vitro* and in growth room trials and identified a strain of *Pseudomonas aeruginosa* (P23) that was responsible for this disease suppression. These results should prove to be of use to B.C. greenhouse cucumber

growers. This knowledge provides for a simple predictive screening tool that can be used to identify *Forc*-suppressive composts for use in cultivation of cucumber. It was also demonstrated that re-introduction of *P. aeruginosa* strain P23 into sterilized compost restored its disease suppressive ability. This suggests that suppressive compost could be produced by introducing P23 inoculum, such as either suppressive compost or bacterial culture, following peak-heating during the composting process. Testing and optimization of this process is a logical next step for incorporating use of *Pseudomonas*-containing composts into a management strategy for *Forc* in B.C.

This research also found that production of 2,4-diacetylphloroglucinol (DAPG) was the primary mechanism of *Forc* suppression by *P. aeruginosa* P23. As previously discussed, knowledge of the regulation of antibiotic production is critical to making the most effective use of biological control bacteria. Identification of environmental factors that influence antibiotic production by P23, such as pH, temperature, and nutritional factors will help to increase the effectiveness and consistency of P23 for suppression of *Forc.*

There are several potential concerns with the use of antibiotic producing bacteria. One of these is horizontal gene transfer into other soil bacteria; particularly transfer of antibiotic biosynthesis or resistance genes, which could result in breakdown of biocontrol and build-up of antibiotic resistance in wild bacteria strains (Troxler et al. 1997). This is less of a concern under greenhouse conditions but becomes important with the use of antibiotic producing bacterial biocontrol agents in field settings. Previous work on the genetic diversity of antibiotic genes in *P. fluorescens* showed that for the *phlD* gene there has been little occurrence of horizontal gene transfer in the past suggesting that such transfer is unlikely to occur

from a biocontrol strain into rhizosphere bacteria (Mavrodi et al. 2001). In addition, the likelihood of transfer of this and other associated DAPG biosynthetic genes is reduced compared to plasmid-borne genes as they are borne chromosomally (Bangera & Thomashow 1999). A second concern is the possibility of development of resistance to DAPG by the targeted pathogens. Once again taking the examples of *Bt* and Agrobacterium-84 it can be seen that when used appropriately with the proper resistance management strategies biocontrols can be used effectively over long periods with little breakdown in control (Penyalver et al. 2000; Sanchis 2008). A major concern in the use of an antibiotic-producing BCA is build-up of resistance to said antibiotic. Two factors greatly reduce the likelihood of build-up of resistance to DAPG in *Forc. Fusarium oxysporum* has a wide range of susceptibility to DAPG and several defence mechanisms are involved in tolerance of DAPG. These mechanisms are: degradation of DAPG; efflux pumps; and production of fusaric acid, which results in lowered production of DAPG by *Pseudomonas* (Schouten et al. 2004). Contrary to resistance to Bt toxin which is the result of a mutation of a receptor protein in the pest, acquisition of these mechanisms of resistance all require the acquisition of one or more genes, a much less likely occurrence than mutation of an existing gene. The second factor that reduces the likelihood of build-up of resistance is sanitation practices used in greenhouses between crops; thorough cleaning and sterilization of the greenhouse can eliminate any pathogen that has managed to acquire resistance to DAPG and preventing future generations of DAPG-resistant pathogen.

The bacterium *P.aeruginosa* P23*,* isolated in this work, has advantages and disadvantages for use as a study organism and also as a biological control agent.

Some of the benefits include those that are common with other *Pseudomonas* spp., such as the production of numerous secondary metabolites, ease of culture and competence at colonizing plant roots. Another benefit for the use of *P. aeruginosa* as a study organism in particular is that the genome of several *P. aeruginosa* strains have been sequenced, the first of these was *P. aeruginosa* PA01 (Stover et al. 2000). This sequence data is a powerful tool for future studies investigating genetic and molecular aspects of the biology and biological control by this species.

One important factor that could limit the use of *P. aeruginosa* strains such as P23 for biological control of plant diseases is the possibility of opportunistic human pathogenicity. Previous work has showed that soil isolates of *P. aeruginosa* can have the potential to be opportunistic pathogens as they often carry the same virulence factors as clinical isolates (Alonso et al. 1999), however, the risk of human disease from use of *P. aeruginosa* in composts for plant disease suppression is likely low. *P. aeruginosa* is classified as a Risk Group 2 organism by the Public Health Agency of Canada (2001); these are defined as having has low community health risk and moderate risk to individuals handling them. However, infections of healthy individuals by *P. aeruginosa* extremely rare, it is almost exclusively pathogenic only on immuno-compromised individuals (Lyczak et al. 2000). The P23 strain in particular was isolated from compost in which it naturally occurred at population levels similar to those used in experiments, and inoculated compost is likely no more an inoculum source than natural compost. Additionally, the risk of human exposure to *P. aeruginosa* found in composts for plant disease suppression is likely to be low given the localized use in greenhouses at seeding time. The availability of the complete genome sequence of strain PA01 (Stover et al. 2000)

should allow for comparative studies between antagonistic strains used for disease control and opportunistic pathogens to determine if it is possible to identify *P. aeruginosa* strains that are not pathogenic to humans. This evaluation of the risk of human pathogenicity would be absolutely necessary before wide-spread implementation of *P. aeruginosa* biocontrol is possible.

Another difficulty in the use of *Pseudomonas* spp. as biological control agents is that they do not survive as well as other species such as *Bacillus* in long-term storage in formulations such as powders and clay pellets (Walsh et al. 2001; Weller 2007). *Bacillus* is able to survive for long periods by forming endospores, whereas *Pseudomonas* spp. do not form such resistant forms, leading to decline of *Pseudomonas* populations much sooner in storage than *Bacillus*. As a result, the formulation and application of *Pseudomonas* BCAs becomes important for their use. This research showed that addition of *Pseudomonas* to compost was an easy and effective method of application for control of a soil-borne pathogen. In addition the *Pseudomonas* spp used in this work survived at high levels for at least 6 weeks in compost, suggesting that composts may be an efficient way to store and handle *Pseudomonas* spp. The first four weeks following seeding are when cucumber plants are the most susceptible to *Forc* infection (Punja & Parker 2000); applying suppressive compost at seeding provides increased protection against the pathogen at the point in the disease cycle that is most critical to preventing disease.

Finally, the work conducted for this thesis was done under conditions designed to simulate greenhouse cultivation of cucumber in a growth room and significant disease reduction was seen in plants grown in composts containing *P. aeruginosa* P23 under these conditions. Before commercial implementation of this

strain is possible, the effectiveness of *Pseudomonas*-containing compost in commercial greenhouses needs to be assessed, including determination of what, if any, effect there is on yield. In addition, because of the potential for human disease caused by *P.aeruginosa,* the extent of colonization of stem tissue and fruit under commercial cultivation conditions by strain P23 must be determined. Preliminary *in vitro* antagonism tests showed that in addition to suppression of *Forc,* P23 was also highly antagonistic towards *Botrytis, Alternaria* and *Phomopsis* (A. DiCarlo, personal communication). This breadth of disease suppression suggests that it may be of potential use for control of other important diseases of cucumber, such as gummy stem blight, and warrants further investigation. A biological control agent suppressive towards multiple pathogens would be a particularly useful tool for greenhouse growers.

The potential of biological control is very high but as Fravel (1988) comments, "If biological control is to be useful, we must be aware not only of its strengths but also its limitations." As we look to improving biological control, we must bear in mind that because of its living nature, biocontrol of diseases will rarely be as totally effective as chemical treatment. This does not reduce the merit of biological control. It simply means that we need to move away from the idea of totally replacing chemical control with biological control. Instead, we need to begin to think about using biological controls in an integrated management system that includes appropriate use of all the tools at the disposal of the grower including chemical controls at reduced levels. Like other biological control applications, such as insect control, the most effective use of bacterial biocontrol will likely be in greenhouse environments where conditions are highly managed. Nevertheless, the

effectiveness of suppressive soils and *Agrobacterium* strain 84 show that biological control agents have strong potential in many cropping systems.

APPENDIX: DEFENSE-RELATED GENE EXPRESSION ANALYSIS

The ability of *P. aeruginosa* strain P23 to induce systemic resistance in the host plant was tested using an *in vitro* growth system with a combination of several *Forc* and *Pseudomonas* treatments. Change in expression of the defense related genes glucanase (*Glu*), peroxidase (*Pox*), phenyalanine-ammonia lyase (*Pal*), and the ethylene signalling genes *Etr1*, *Ctr1* and jasmonic acid signalling gene lipoxygenase (*Lox1*) in plants grown in the *in vitro* system described below was analyzed by Northern blot. The experiment was performed twice.

Cucumber plants cv. 'Ladner' were grown from seeds in sterile Magenta boxes containing 100 mL of nutrient solution (0.6 g 7-11-27 soluble fertilizer and 0.39 g $CaNO_3$ per L) with 3 seeds per box. Each box received treatment with either *P. aeruginosa* strain P23 to a final concentration of 10^6 CFU/mL of nutrient solution (P) or mock-inoculation with sterile water (C) 10 days after planting. Three days after the first treatment half of the boxes were treated with 10^6 CFU of *Forc* conidia suspension (F+) while the other half received mock-inoculation with 3mL sterile water (F-).

Plant tissue was harvested at 0, 6, 12, 24, 48, and 72 hours after *Forc* inoculation. Two Magenta boxes (a total of 6 plants) were harvested for each of the 4 treatments (PF+, PF-, CF+, CF-) at each time-point. At harvest, leaf, stem and root tissues for each plant were separated. All samples were immediately frozen in liquid nitrogen and stored at -80˚C. Total RNA was collected using Guanidinium

thiocyanate mono-phasic extraction (Chomczynski & Sacchi 1987) and the 6 samples of each tissue type at each time-point were pooled. Subsequently, all samples were concentrated by ethanolic precipitation and stored at -80˚C.

Probes for Northern blots were prepared from cucumber RNA extracted using an Aurum Total RNA Mini Kit (Bio-Rad Laboratories Inc.). Reverse transcription was then performed with SuperScript II RT (Invitrogen) on 500 ng of total RNA for each gene specific primer following the manufacture's protocol. Probes were prepared by PCR from the resulting cDNA. Reverse transcription and PCR were performed using the gene specific primers described in Table A.1. The cycling parameters for PCR amplification were: an initial four min at 94˚C, followed by 30 cycles of one min at 94˚C, one minute at 54˚C, and one min at 72˚C, and a final extension cycle of 10 min at 72˚C. Probes were ^{32}P labeled using the Prime-a-Gene labeling kit (Promega) as per the manufacturer's protocol.

Sample RNA was separated by agarose gel electrophoresis (1% agarose w/v) and Northern blots were prepared using HyBond –XL nylon membranes (GE Healthcare) following standard procedures (Sambrook et al. 1989). Hybridizations were performed overnight at 65˚C with Ekono Hybridization Buffer (Research Products International Corp.) and washed at high stringency as described above (Sambrook et al. 1989) and hybridization detected by exposure to x-ray film for 2-7 days. Representative results for glucanse, PAL and Etr1 are shown in Figure A.1. Blots for other genes all showed similar results.

Table A.1: Primer sequences used in amplification of probe fragments for Northern blot hybridization

Gene Amplified	Function	Primer Sequence	Accession number/ Reference
Actin (461 bp)	Housekeeping gene	5`-TGGTATCGTGCTGGATTCTG-3` 5`-GAATCTCTCAGCTCCGATGG-3`	DQ115883
Glucanase (265 bp)	Defense gene – fungal cell wall degradation	5`-TCGTTAGTTGAAGATGGGGAGT-3` 5`-TTCTCATCAGACATAGCAAACACA-3`	AB010922
Peroxidase (912 bp)	Defense gene – ROS production, lignin synthesis	5`-CACAGGCACCAACACCATTA-3` 5`-CTAATTTCCCCTTGGCTTCC-3`	M91374
PAL (468 bp)	Defense gene – Phenolic compound synthesis	5`-AGTTGCATGAAATGGATCCTC-3` 5`-TTATGTTGCTCGGCACTTTG-3`	AF529240
ETR1 (1001 bp)	Ethylene signalling	5`-ACAAATTGCAAGCCCAAGAC-3` 5`-ATCGGGCCGTAAAAATATCC-3`	AB026498
CTR1 (547 bp)	Ethylene signaling	5`-TACCGTGGTGATTGGCATGG-3` 5`-CCCACAAAATGACCCCAAAG-3`	Shoresh et al. 2005
Lipoxygenase (930 bp)	Jasmonic acid synthesis	5`-TGTTCAAAGCACTCGTGAGG-3` 5`-TGGCTGACCAAATGTCAAGA-3`	X92890

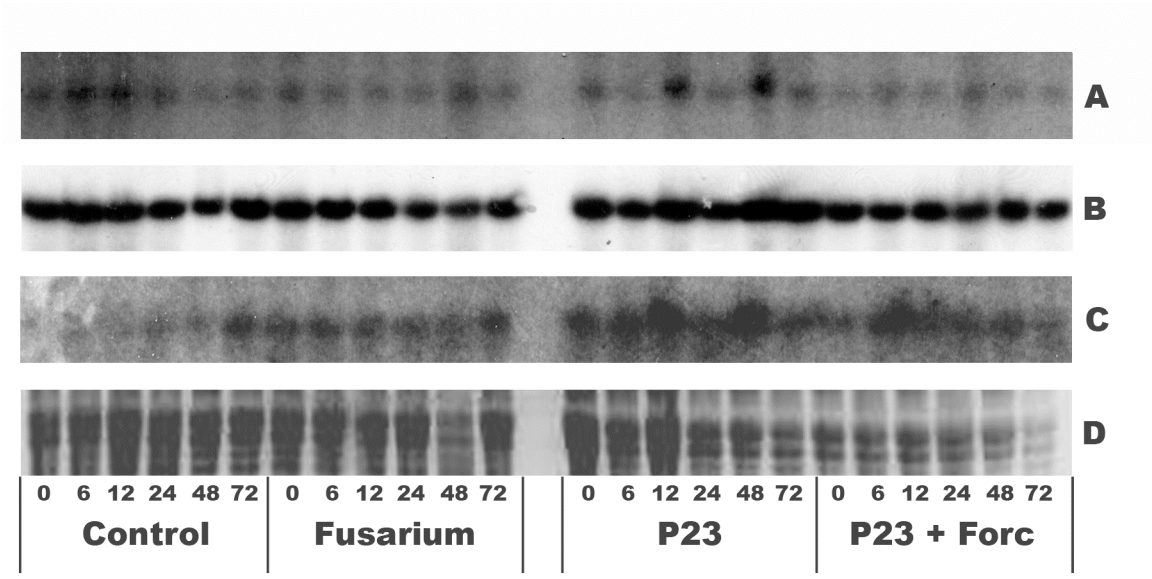

Figure A.0.1: Northern blots showing expression of ISR related genes in cucumber. Treatments as indicated are CF-, CF+, PF- and PF+ as described in text. Samples were taken at 0, 6, 12, 24, 48 and 72 hours after inoculation with Forc. Blots are: A) Glucanase, B) PAL, C) *etr1* and D) Ethidium bromide stained rRNA to show gel loading

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