

**THE INHIBITION OF *FUSARIUM OXYSPORUM* F. SP.
CUBENSE RACE 4 BY *BURKHOLDERIA CEPACIA***

by

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DECLARATION

I hereby declare that these studies represent original work by the author and have not been submitted in any form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.

A handwritten signature in black ink, appearing to read "Manjing Pan", is written over a horizontal line.

Manjing Pan

December 1997

PREFACE

The experimental work described in this thesis was conducted in the Department of Genetics, University of Natal, Pietermaritzburg, South Africa, under the supervision of Professor JW Hastings.

This thesis consists of five chapters. Chapter two was published in **Journal of Phytopathology**, 1997 Vol.145: 479-486.

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ABSTRACT

Inhibition of *Fusarium oxysporum* f. sp. *cupense* race 4 by *Burkholderia cepacia* was evident when grown on various media (TSA, PDA, PSA, YM, KMB, PPM, NYGA, LA) with different carbon sources and under various pH and temperature conditions. In addition, *B. cepacia* was able to inhibit several fungal pathogens *in vitro*.

Antagonism of *B. cepacia* against *F. oxysporum* f. sp. *cupense* occurred at high levels of Fe³⁺, which may suggest that antagonism by *B. cepacia* did not involve siderophore production. Thin layer chromatogram (TLC) examination showed that *B. cepacia* produced several substances, one of which had similar R_f value to that described for pyrrolnitrin. Cell-free supernatant of a 4-day culture of *B. cepacia* was applied to an Amberlite XAD-2 column and inhibitory activity co-eluted with the 95% methanol (pH 9.5) fraction. The concentrated activated fractions showed inhibitory activity against *F. oxysporum* f. sp. *cupense*.

A GC-MS chromatogram indicated numerous components in the antifungal extracts. The only compound identified in the Wiley 138 library, was 1,2-Benzenedicarboxylic acid, bis (2-Ethylhexyl) ester.

Observations by light microscopy indicated that *B. cepacia* inhibited spore germination in *F. oxysporum* f. sp. *cupense* race 4 and retarded the mycelial growth. The interaction between the endophytic bacterium, *B. cepacia* and *F. oxysporum* f. sp. *cupense* race 4 was investigated with aid of scanning and transmission electron microscopy. This demonstrated that the bacterium was able to colonize the surface of hypha and macrospore of *F. oxysporum* f. sp. *cupense*. Mycelial deformation, terminal and/or intercalary swelling were evident. At later stages, hyphae of *F. oxysporum* f. sp. *cupense*, colonized by *B. cepacia*,

were found to have collapsed. Further studies *in vivo* confirmed that *B. cepacia* colonized the hypha of *F. oxysporum* f. sp. *ubense* which had invaded banana roots. TEM observation showed that in the banana plant *B. cepacia* was closely associated with the healthy banana roots and a matrix was frequently found to be present between the bacterium and the plant surface. In addition, *B. cepacia* exists mainly in the intercellular space of the banana roots.

UV irradiation treatment of *B. cepacia* resulted in a mutant that had lost inhibitory activity against *F. oxysporum* f. sp. *ubense* on TSA agar.

Transposon mutagenesis of *B. cepacia* was performed by Tn5 insertion. Six mutants which had lost or had reduced inhibitory activity against *F. oxysporum* f. sp. *ubense* were generated. These mutants showed no inhibitory zones on TSA medium in the presence of the fungus. It was observed that one mutant *B. cepacia* :: Tn5-188 appeared to lose the ability to colonize the fungal hypha, whilst a different mutant *B. cepacia* :: Tn5 - 217 was still able to colonize the fungal hyphae. TLC analyses showed that there was a decrease in antibiotic production in mutants *B. cepacia* :: Tn5 - 217 and *B. cepacia* - UV - 34, compared with the wild type. GC- MS analyses showed that there was no evidence of the peaks at 14.62 minutes, 20.0 minutes and 20.46 minutes in both chromatograms of mutants *B. cepacia* :: Tn5 -217 and *B. cepacia* -UV - 34, compared with the wild type *B. cepacia*.

No PCR products were detected using primers that were developed from sequences within the biosynthetic loci for Phl of *P. fluorescens* Q2-87 (GenBank accession no. U41818) and PCA of *P. fluorescens* 2-79 (GeneBank no. L48616). Colony hybridization suggested that genomic DNA from *B. cepacia* could contain both Phl- and PCA probes. It was found that hybridization of genomic DNA digested with Cla-I of *B. cepaca* with Phl2a probe only occurred at low stringency. A hybridization signal was detected from a Cla-I fragment of approximately 2800bp.

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1. LITERATURE REVIEW

1.1 Inhibition of *Fusarium oxysporum* by *Pseudomonas / Burkholderia* spp.

1.1.1 Introduction

Fusarium wilts are caused by pathogenic *Fusarium oxysporum*, which may result in significant losses in several crops. There is a high level of specificity between susceptible plants and pathogenic *F. oxysporum* strains. This specificity is characterized by formae specialis and races to which *F. oxysporum* belongs (Armstrong & Armstrong, 1981). The life cycle of *F. oxysporum* includes a saprophytic and a parasitic phase (Nelson, 1981; Beckman, 1989). In the absence of a plant host, *F. oxysporum* is able to survive by formation of chlamydospores (Schippers & Van Eck, 1981) and to grow on organic matter from plant debris (Beckman, 1989). In the presence of a plant host, release of root exudates allow germination of chlamydospores and mycelial growth (Schroth & Hildebrand, 1964). When the nutrient supply is adequate, the mycelium may reach the roots of the plant. If the host plant is susceptible to the particular *F. oxysporum* strain, then the fungus will successfully infect its host and colonize its vascular system (Beckman, 1989). This pathogenic phase ends with the death of the diseased plant and the saprophytic phase starts again with dissemination of the fungus from the dead plant.

Banana fusarial wilt caused by *Fusarium oxysporum* f. sp. *cubense* is a devastating plant disease and a serious constraint to banana production world-wide. *F. oxysporum*

f. sp. *cubense*, like other forms of the *F. oxysporum*, can produce chlamydospores which act as survival units in the soil. The pathogen inhabits roots more often than soil. Banana fusarial wilt disease is a soil-borne disease and difficult to eliminate in the soil.

Until the early 1980s, *Burkholderia cepacia* (previously *Pseudomonas cepacia*, *Pseudomonas multivorans*, *Pseudomonas kingii* and eugonic-oxidizer group 1) was first described by Burkholder (Burkholder, 1950) and thought to be the cause of soft rot of onion bulbs (Latin *cepa*). In 1992, on the basis of 16S rRNA sequences, DNA-DNA homology values, cellular lipid, fatty acid composition, and phenotypic characteristics, Yabuuchi *et al* (1992) proposed that *P. cepacia* and other members of RNA homology group II be transferred to the new genus *Burkholderia cepacia*, with *B. cepacia* as the type species. This proposal was subsequently validated (Burns, 1993). Currently, the genus *Burkholderia* comprises *B. cepacia*, *B. gladioli*, *B. mallei*, *B. pseudomallei*, *B. carophylli*, with *B. plantarii*, *B. glumae*, *B. vandii*, *B. cocovenenans*, and *B. vietnamiensis* being recently added to the group.

1.1.2 Control of plant diseases caused by *Fusarium oxysporum*

F. oxysporum is able to produce chlamydospores, which permit pathogenic forms of fusaria to persist in most soils long after a susceptible crop has been removed. It is therefore difficult to control these notoriously "stubborn" soil-borne diseases. Previous observations and experiments have indicated that some measures, including agricultural practices and the use of resistant varieties, may reduce the severity of diseases and increase yields.

1.1.2.1 Screening and application of resistant cultivars

Currently, resistant cultivars are commonly used and may be an effective approach to control crop diseases caused by *F. oxysporum*. Some cultivars e.g. cotton and tomato, are found to have multiple-gene resistance and/or single-gene resistance (Beckman & Roberts, 1995). Methods and procedures for breeding and screening for resistance on a large scale, producing hybrids as well as the incorporation of resistance from wild-type into cultivated varieties are well set up and developed.

Multiple-gene resistance however, segregates and varies substantially within field plantings, so some wilt occurs regularly in such plants. On the other hand, the problem with single-dominant gene resistance is that the pathogen is genetically malleable and frequently produces new races that can cause problems in large scale planting of single-dominant gene cultivars. There is therefore a constant need for the incorporation of resistance to new races of *F. oxysporum*. Breeding for resistance must be a continuous and large-scale effort. Moreover, in some regions, the problems occur when a crop, such as tomato or banana, has several major disease challenges.

1.1.2.2 Altering the soil nutrients

Soil pH and nutrient amendment affects *F. oxysporum* wilt in tomato (Walker, 1971). Raising the soil pH from 6.0 to 7.5 by liming soils acts as indicates an effective control of *F. oxysporum* in tomato (Walker, 1971). Previous studies however also demonstrated that altering of soil pH and nutrients might also result in occurrence of virulent races of the pathogen or other pathogens. It is thus imperative to determine a rational combination of control factors, soil pH, soil nutrients and to make the

measures effective in reducing both the number and virulence of fusaria propagules in soils.

1.1.2.3 Chemical fumigation/liming for reducing the inoculum quantity in soils

Some chemicals, such as D-D (a mixture of 1,3-dichloropropene, 1,2-dichloropropane and related hydrocarbons), chloropicrin and methyl bromide, when used as fumigants, can reduce the appearance of symptoms caused by *F. oxysporum* in tomato and other crops (Jones & Overman, 1971a,b; 1978). Jones and Overman (1976a,b) also noted that soil treatment with these broad-spectrum fumigants and the nematocidal agent carbobofuran, plus the adjustment of soil pH, can achieve the best control for tomato fusaria wilt and the gross yields were increased by more than 200%.

Other chemicals e.g. carbendazim and captan, have also been tested their efficacy in reducing *F. oxysporum* propagules in naturally infested soils (Dwivedi & Pathak, 1981; Jaworski *et al.*, 1981). While these authors reported that the chemicals tested can yield effective protection of crops from *F. oxysporum* infection, Maraite and Meyer (1973) failed to obtain significant reductions in infection or symptom development following treatment of soils with the fumigants chloropicrin or methyl bromide. These discrepancies may be caused by differences in methods, rates or timing of chemical application (Weststeijn, 1973). Another problem with soil fumigants is that it is not cost-effective in many regions.

1.1.2.4 Agricultural practices to reduce the inoculum load in soils

Some agricultural practices, such as destroying infected plant materials (debris) in fields, can decrease the infection pressure by *F. oxysporum* exerted upon a crop. Some diseases caused by *F. oxysporum* (e.g cotton wilt) can be transmitted via seeds, plants or infected soils. Plant quarantine to prevent dissemination of the pathogen from a disease-free region could therefore be of great importance. It was also found that ammonium and higher phosphorus and magnesium levels in soils are favourable to fusaria wilt disease development. In practice one needs to avoid excessive use of phosphorus and magnesium soil amendments and use nitrate rather than ammonium (Beckman & Roberts, 1995; Sarhan & Kiraly, 1981; Jones & Woltz, 1981). Recently soil solarization was also found to have great potential to control fusaria wilt diseases on crops (Beckman & Roberts, 1995).

1.1.2.5 Development of systemic chemical fungicides

Systemic chemical fungicides for control of *F. oxysporum* are currently being developed. Davis and Dimond (1952) proposed that three possible actions could make such chemicals effective: they could a) counteract toxins produced by the fungus, b) enhance host resistance against *F. oxysporum* attack and/or c) kill or suppress the pathogen directly within the host plant.

Early work by Davis and Dimond on fusaria wilt of tomato (1952) demonstrated that several chemicals, acting through the host metabolism, reduced symptom expression. Buchenauer (1971) showed that several growth inhibitors reduced fusaria wilt of tomato.

Among the chemicals tested so far, the systemic fungicide benomyl proved successful in controlling of fusaria wilt of crops (Biehn & Dimond, 1969, Fuchs *et al*, 1970, Dacallone & Meyer, 1972). Benomyl was found to be able to inhibit spore germination and mycelial growth, decrease RNA synthesis (Decallone & Meyer, 1972) and reduce vascular colonization and foliar symptoms (Channon & Thomson, 1973). However fungal resistance to benomyl has also been reported in different countries (Sozzi & Gessler, 1980; Erwin, 1981; Thanassoulopoulos *et al.*, 1971). Another problem with the use of benomyl for control of *F. oxysporum* is economic. Because of its strong adsorption in the soils and its low solubility, it should be applied to soils at high concentration and this increases expense.

1.1.2.6 Use of biological control

Selection, development and maintenance of suppressive soils for control of *F. oxysporum* was proposed (Beckman & Roberts, 1995). Plants can also be "immunized" or "cross-protected" by inoculation with other nonpathogenic microorganisms, including fusaria (Davis, 1967, 1968; Homma *et al.*, 1978; Langton, 1968; Ouf *et al.*, 1981; Turhan, 1981). There has been considerable progress in the last decade in isolating microorganisms from soil suppressive to a number of diseases and employing them directly in the control of the diseases. Most of these antagonists proved effective in glasshouse studies, and a few like the fluorescent pseudomonads have reduced relevant diseases to a significant degree in small field experiments. Table 1.1 gives examples of *Burkholderia* and/or *Pseudomonas* spp applied in biological control of soil-borne pathogen diseases.

There is therefore much optimism that these microorganisms may be harnessed for biological control. The use of antagonistic microorganisms to control *Fusarium*

which have received most attention in recent years will be dealt with next in more detail.

1.1.3 Biocontrol of *Fusarium* wilts

Biological control is becoming an urgently needed component in agriculture. Chemical pesticides have been the object of substantial criticism in recent years, due to the adverse environmental effects causing health hazards to humans and other nontarget organisms, including beneficial natural enemies. Therefore, it is important to develop safer and environmentally feasible control alternatives, mainly by the use of existing living organisms in their natural habitat. These organisms are able to provide protection against a range of plant pathogenic fungi, without damage to the ecological system.

Pseudomonads are considered favourable organisms to select as biocontrol agents for soil-borne diseases, especially the two fluorescent species or groups, *Pseudomonas fluorescens* and *Pseudomonas putida*. They are normal inhabitants of the soil and especially the root surfaces of plants. They grow and colonize well when introduced artificially (Weller, 1983).

There are many ways in which an antagonistic organism could operate: rapid colonization in advance of the pathogen or subsequent competition or combat which may lead to niche exclusion. Antibiotics may be produced, which could result in the lysis of the pathogen. In addition some micro-organisms may act simply by making the plant grow better, so that even if the disease is not cured its symptoms are at least partly masked.

Table 1.1 Examples of *Burkholderia* (*Pseudomonas*) spp. applied in biological control of soilborne plant pathogens

Biocontrol organism	Crop	Pathogen or disease	Site	References
<i>Pseudomonas putida</i>	Flax, radish, cucumber	Fusarium wilt	Field	Scher & Baker, 1982
<i>Pseudomonas putida</i>	Beans	<i>Fusarium solani</i>	Field	Anderson & Gurra, 1985
Fluorescent pseudomonads	Radish	Nonspecific pathogens	Field	Kloepper & Schroth, 1981
Fluorescent pseudomonads	Potatoes	Nonspecific pathogens	Field	Kloepper <i>et al.</i> , 1980a Burr <i>et al.</i> , 1978 Kloepper <i>et al.</i> , 1991
<i>Pseudomonas</i> spp.	Douglas fir	<i>Fusarium oxysporum</i>	CE	Reddy <i>et al.</i> , 1994
<i>Pseudomonas aeruginosa</i> S-7	Adzuki bean	<i>Fusarium oxysporum</i> f. sp. <i>adzukicola</i>	Glasshouse	Hasegawa <i>et al.</i> , 1991
<i>Burkholderia cepacia</i> AMMD	Pea	<i>Aphanomyces euteiches</i> f. sp. <i>pisi</i>	Glasshouse	Parke <i>et al.</i> , 1991
<i>Burkholderia cepacia</i> B-17	Adzuki bean	<i>Fusarium oxysporum</i> f. sp. <i>adzukicola</i>	Glasshouse	Hasegawa <i>et al.</i> , 1991
<i>Burkholderia cepacia</i>	Protea	<i>Phytophthora cinnamomi</i>	Glasshouse	Turnbull <i>et al.</i> , 1992
	Cucumber	<i>Pythium ultimum</i>	Glasshouse	Fridlender <i>et al.</i> , 1993
	Cotton	<i>Rhizoctonia solani</i>	Glasshouse	Fridlender <i>et al.</i> , 1993
	Bean	<i>Sclerotium rolfsii</i>	Glasshouse	Fridlender <i>et al.</i> , 1993
<i>Burkholderia cepacia</i> J82rif	Sunflower	<i>Sclerotinia sclerotiorum</i>	Field	McLoughlin <i>et al.</i> , 1992
<i>Burkholderia cepacia</i> N24	Sunflower	<i>Sclerotium rolfsii</i>	Field	Hebbar <i>et al.</i> , 1991 1992
<i>Burkholderia cepacia</i> UPR5C	Common bean	<i>Macrophomina phaseolina</i>	Glasshouse	Sánchez <i>et al.</i> , 1994

CE: Controlled environment facility

Cook and Baker (1983) suggested that the soil micro flora results in two types of mechanisms of suppression, one is called general, the other specific. General suppression is achieved by the whole micro flora; the higher the microbial biomass and activity, the stronger the suppressiveness. With a background of general suppression, some microbial populations exert specific antagonist activities against pathogenic *F. oxysporum*. An effective biological disease-control organism was thought to do some of the following: (a) rapidly colonize the root zone; (b) producing antibiotics to antagonize pathogenic microorganisms; (c) producing high-affinity iron-chelating compounds called siderophores that make iron less available to the pathogens; (d) compete for substrates that are essential for growth of the pathogens; (e) compete with the pathogen for infection sites and (f) produce plant-growth-promoting compounds, such as giberellin-like substance or indolyl-3-acetic acid.

1.1.3.1 Colonization

Colonization of the host plant rhizosphere by the biocontrol bacteria was believed to be necessary for disease suppression (Suslow, 1982; Weller, 1988; Bowen, 1991; Parker, 1991). It seemed logical that a biological agent should grow and persist, or colonize the surface of the plant it protects. Colonization is widely believed to be essential for biocontrol (Weller, 1983; de Weger *et al.*, 1987; Parker, 1991). It was generally agreed that the process of colonization includes at least the following: bacterial migration towards plant root attachment, distribution along the elongating root, and proliferation on the root. *Pseudomonas* strains have been shown to migrate actively towards soybean seeds in soil (Scher *et al.*, 1985), but no correlation between chemotactic ability and root colonization was observed when the bacterial strains were directly inoculated into seeds (Scher *et al.*, 1988). In a study showing the suppression of damping-off of peas by *Burkholderia cepacia*, there was significant relationship between population size of the biocontrol agent and the degree of disease suppression (Parker, 1990). Also, suppression of take-all of wheat was correlated with colonization of roots by a *P. fluorescens* strain (Bull *et al.*, 1991). In addition to the colonization of plant roots, the colonization of fungal hyphae

could be an important mechanism for maintaining a close association between antagonistic bacteria and fungal pathogens.

1.1.3.2 Competition

Competition occurs when two (or more) organisms require the same resource and the use of this by one reduces the amount available to the other. Micro-organisms may compete for nutrients: such as carbon and nitrogen sources. Competition is also possible for oxygen, space and in the case of autotrophs light.

1.1.3.2.1 Nutrient competition

Competition for nutrients is generally believed to have a role in biological control. Most nutrients in the rhizosphere come from plant exudates. Some compounds serve not only as nutrients, but also as signals to initiate interaction between plants and microorganisms. Most fungal pathogens exist in a state of dormancy in the soil (Lockwood, 1977). Infection occurs only after dormancy is broken in the presence of signals or stimulants from the plant host. A biocontrol agent could provide plant protection by efficient removal of such signal or stimulants from the rhizosphere (Elad & Chet, 1987; Nelson & Craft, 1991; Paulitz & Loper, 1991).

Among the different models of action of microbial antagonism, nutrient competition was shown to play a major role in soil suppressiveness (Alabouvette *et al.*, 1985; Scher & Baker, 1982; Lemanceau, 1989). Demonstration of this was achieved by following the principle proposed by Baker (1968). Soil was supplemented with the supposed limiting nutrient and the effects on growth of the pathogen and on disease severity were then recorded. The results strongly implicated competition for carbon and for iron in soil suppressiveness to fusarium wilts. Increase of carbon or iron availability in either suppressive or conducive soil, by introduction of glucose or FeEDTA respectively, could increase growth of the pathogen and the severity of the disease (Kloepper *et al.*, 1980a; Scher & Baker, 1982; Alabouvette *et al.*, 1985; Elad & Baker, 1985a,b). In contrast, reduction of iron availability by introduction

of strong iron chelators to soils, showed that growth of the pathogen and disease severity could be decreased (Scher & Baker, 1982). In the same way, when iron availability was increased by lowering the soil pH, the natural suppressiveness of soil was reduced (Scher & Baker, 1980; Elad & Baker, 1985b). It was likely that significant modification of disease severity required greater variation of carbon or iron availability in suppressive than in conducive soil. Carbon and iron competition interacts to determine the level of fusarium wilt severity (Lemanceau, 1989), lowering the concentration of available iron in soil by introduction of EDDHA, minimized the increase of disease severity due to addition of glucose to the soil.

1.1.3.2.2 Iron competition

There could be competition for ferric iron by the production of special iron-chelating compounds called siderophores in iron-limited environments. Iron competition was associated with the physico-characteristics of soils, such as pH and CaO content, and with the presence of fluorescent pseudomonads (Baker *et al.*, 1986). In normally aerated soils, iron is present mainly in its oxidized form, Fe^{3+} . The higher the soil pH the lower the concentration of Fe^{3+} in solution. Indeed, based on a solubility product of 10^{-38} for $\text{Fe}(\text{OH})_3$, the calculated concentration of Fe^{3+} in a solution at pH 7.7 would only be $10^{-19} \text{ mol}^{-1}$ (Lindsay & Schwab, 1981). This concentration would be even lower in soils due to the possible adsorption of Fe^{3+} to colloids or its precipitation with other irons (Segalen, 1964) and as such would limit the germination of *Fusarium* chlamydospores. Germination of *F. oxysporum* spores was inhibited by *P. putida* A12 when the Fe^{3+} concentration was equal to or lower than $10^{-19} \text{ mol}^{-1}$ (Simeoni *et al.*, 1987). These authors also evaluated the minimum amount of iron required for optimal germination of chlamydospores of *F. oxysporum* f. sp. *cucumerinum* alters to be 0.6 ng per chlamydospore. Most organisms have systems for the specific chelation and transport of Fe (-III) into the cell. Plants and associated microbes have different, interacting strategies for obtaining iron from the soil. To obtain iron (Fe^{3+}) for metabolism, aerobic microorganisms have developed an uptake strategy most frequently based on the synthesis of siderophores and protein membrane receptors (Neilands, 1973, 1982). Synthesis only occurs under iron stress.

1.1.3.3 Production of antagonistic substances

1.1.3.3.1 Siderophores

Siderophores are low molecular weight compounds that are produced under iron-limiting conditions, chelating the ferric ion Fe^{3+} with a high specific activity, and serve as vehicles for the transport of Fe (III) into a microbial cell (Neilands, 1981). In recent years, a great deal of attention in rhizosphere studies has been focused on the production of siderophores. It has been recognized for a long time that bacteria produce a range of siderophores (Table 1.2). The fluorescent pseudomonads are characterized by the production of yellow-green pigments that fluoresce under ultraviolet light and function as siderophores. The fluorescent siderophores, termed pyoverdines, pyoverdins or pseudobactins, represent only one class of siderophores produced by the fluorescent pseudomonads (Demange *et al.*, 1987; Teintze *et al.*, 1981). The structure of pseudobactins produced by different strain of fluorescent pseudomonads has been characterized (Teintze *et al.*, 1981). Pyoverdines have a common chromophore, which is derived from 2,3-diamino-6,7-dihydroxyquinoline (Demange *et al.*, 1987; Teintze *et al.*, 1981), linked to a small peptide, which differs among strains by the number and composition of amino acids. The pyoverdines have three bidentate chelating groups that bind iron (III):

Table 1.2 Partial list of siderophores from bacterial species. Adopted from Lynch (1990)

Organism	Siderophores
<i>Pseudomonas</i> spp.	Pyochelin, pyoverdine, pseudobactin, ferribactin
<i>Enteric</i> spp.	Enterobactin, aerobactin
<i>Paracoccus denitrificans</i>	Parabactin
<i>Vibrio cholerae</i>	Vibriobactin
<i>Bacillus megaterium</i>	Schizokinen
<i>Anabaena</i> spp.	Schizokinen
<i>Azotobacter vinelandii</i>	α e -bis-2,3-dihydroxybenzoyllysine
<i>Actinomyces</i> spp.	Ferrioxamines
<i>Mycobacteria</i>	Mycobactins

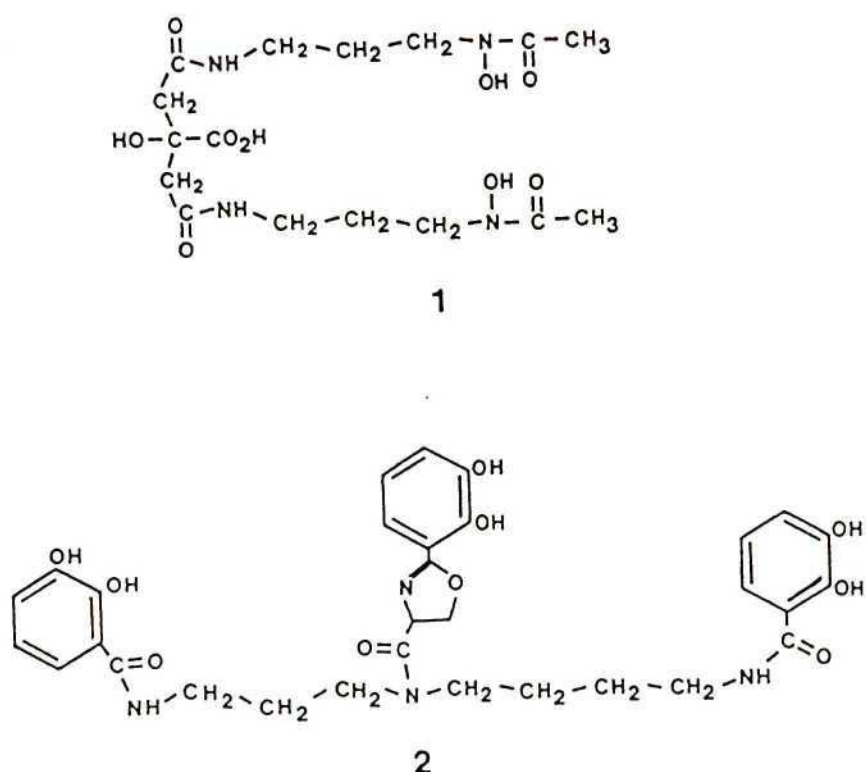


Figure 1.1: Several typical siderophores produced by microorganisms (Lynch 1990): (1) A hydroxamate siderophore, schizokinen; and (2) A typical catechol siderophore, agrobactin.

a) a catecholate group from the chromophore, b) a hydroxamate group from a N^δ-hydroxyornithine of the peptide chain, and c) either an α-hydroxyacid from a β-hydroxyaspartic acid or another hydroxamate group from a second N^δ-hydroxyornithine. Pyoverdines are intermediate between the usually strict catechol or hydroxamate siderophores found in a majority of microorganisms.

Pseudobactins are water soluble and made up of a small peptide chain of amino acids alternatively L- and D-linked to a yellow-green chromophore group and to a succinamide group. Several typical siderophores produced by microorganisms are shown in Figure 1.1. Different studies have associated the bacterial antagonism to pseudobactin synthesis (Kloepper *et al.*, 1980b; Sneh *et al.*, 1984; Elad & Baker 1985a,b; Baker *et al.*, 1986; Meyer *et al.*, 1987; Lopper, 1988; Lemanceau *et al.*, 1992). Reduction of fungal growth *in vitro* by different strains of pseudomonads

occurred when pseudobactins were produced (Kloepper *et al.*, 1980b). In the presence of iron however both pseudobactin synthesis and antagonism were suppressed. Repression of siderophore synthesis by pseudomonads is due to siderophore-mediated iron competition.

1.1.3.3.2 Antibiotics

Biocontrol is often attributed to antibiosis. Antibiotics are generally considered to be metabolites that can inhibit microbial growth. In many biocontrol systems that have been studied, one or more antibiotics have been showed to play a role in disease suppression.

Many of the antibiotic producing organisms have been isolated from soil, leaves and other plant parts. There are a large number of bacterial biocontrol agents which produce antibiotics (Table 1.3) and different antibiotics appear to be important for the control of different fungal pathogens. The antibiotics that are detected *in vitro* are very diverse and may be specific for particular target organisms or have a wide spectrum of activity. A number of highly effective disease-suppressive agents are found among the fluorescent pseudomonads, making this group of bacteria the most widely studied group of antibiotics producer in the rhizosphere. The production of antibiotic metabolites, such as phenazine carboxylic acid (Thomashow & Weller, 1988), pyoluteorin (Howell & Stipanovic, 1980), pyrrolnitrin [3-chloro-4(2'-nitro-3'-chlorophenyl)-pyrrole] (Homma *et al.*, 1989; Howell & Stipanovic, 1979), 2,4-diacetylphloroglucinol (Keel *et al.*, 1990; Vincent *et al.*, 1991), oomycin A (Howie & Suslow, 1991) and hydrogen cyanide (Voisard *et al.*, 1989), has been shown to be involved in the suppression of fungal pathogens. The first antibiotics described in biocontrol fluorescent pseudomonads were the phenazine derivatives, that contribute to disease suppression by *P. fluorescens* and *P. aureofaciens*, and control take-all of wheat (Weller & Cook, 1983; Brisbane & Rovira, 1988). Furthermore,

antibiotic is produced on roots grown in soil (Mazzola *et al.*, 1992). *P. fluorescens* produces hydrogen cyanide, 2,4-diacetylphloroglucinol, and pyoluteorin, which directly interfere with growth of various pathogens and contribute to disease suppression (Voisard *et al.*, 1989; Keel *et al.*, 1990; Maurhofer *et al.*, 1994). Further investigation demonstrated that the production of a phenazine antibiotics was responsible for suppression for take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici*, and they provided evidence for the role of antibiotics in biological control (Thomashow *et al.*, 1990). Recently, *B. cepacia* has become a potential biological control agent. Antibiotics, including cepacin A, cepacin B (Parker *et al.*, 1984), altericidins (Kirinuki *et al.*, 1977), 2-[2-heptenyl]-3-methyl-4-quinolinol (Hashimoto & Hattori, 1967), pyrrolnitrin [3-chloro-4-(2'-nitro-3'-chlorophenyl)-pyrrole (Fernandez 1990; Homma *et al.*, 1989) and 2-[2-nonenyl]-3-methyl-4quinolinol (Homma *et al.*, 1989), were isolated from *B. cepacia*. In addition, there are also gaseous products like ethene (ethylene), ammonia, and hydrogen cyanide which could affect microbial growth, and could be active at the low concentrations, but are not generally considered as antibiotics.

Antibiotics generally could reduce of growth or sporulation of pathogenic fungi, or reduce germination of pathogenic fungal spores. This may be accompanied by various distortions of the hyphae of an affected fungus, changes in branching patterns of colonies, the production of specialized growth forms such as pseudoparenchymatous tissues. If the antibiotics causes death then endolysis of cell also occur. Some antibiotics which are produced by pseudomonads are shown in Figure 1. 2 and Figure 1. 3.

1.1.3.3.3 Lytic enzymes

Besides production of siderophores and antibiotics, bacterial synthesis of lytic enzymes is a possible mechanism of microbial antagonism. This mode of action does not seem to play a major role in natural soil suppressiveness to fusarium wilts since introduced pathogenic strains survive as well in suppressive

Table 1.3 Examples of antibiotics or antifungal metabolites produced by bacteria which may be involved in disease control

Antibiotic	Bacterium	Pathogen/host	Reference
2,4-diacetyl phoroglucinaol	<i>Pseudomonas</i> sp. F113	<i>Pythium ultimum</i> on sugar beet.	Fenton <i>et al.</i> , 1992 Shanahan <i>et al.</i> , 1992
	<i>Pseudomonas aureofaciens</i> Q2-87	<i>Gaeumannomeces graminis</i> var. <i>tritici</i> on wheat.	Vincent <i>et al.</i> , 1991
	<i>Pseudomonas fluorescens</i> CHAO	<i>G. graminis</i> var. <i>tritici</i> on wheat.	Keel <i>et al.</i> , 1992
HCN	<i>Pseudomonas fluorescens</i> CHAO	<i>Thielaviopsis basicola</i> on tobacco.	Keel <i>et al.</i> , 1992
		<i>Thielaviopsis basicola</i> on tobacco.	Voisard <i>et al.</i> , 1989
Monochloroamin o-pyrro-nil-trin	<i>Burkholderia cepacia</i> J82	<i>Sclerotinia sclerotiorum</i> on sunflower.	McLoughlin <i>et al.</i> , 1992
OomycinA	<i>Pseudomonas fluorescens</i> HV37a	<i>Pythium ultimum</i> on cotton.	Gutterson ,1990 Howie & Suslow, 1991
		<i>G. graminis</i> var. <i>tritici</i> on wheat.	Thomashow & Weller, 1988
Phenazine-1-carboxylic acid	<i>Pseudomonas fluorescens</i> 2-79	<i>G. graminis</i> var. <i>tritici</i> on wheat.	Thomashow <i>et al.</i> , 1990;
	<i>Pseudomonas fluorescens</i> 30-84	<i>G. graminis</i> var. <i>tritici</i> on wheat.	
Pyoluteorin	<i>Pseudomonas fluorescens</i> CHAO	<i>Pythium ultimum</i> on cress.	Maurhofer <i>et al.</i> , 1994
	<i>Pseudomonas fluorescens</i> Pf-5	<i>Pythium ultimum</i> on cress.	Kraus & Lopez, 1995
Pyrrolnitrin	<i>Burkholderia cepacia</i> B37W	<i>Fusarium sambucum</i> dry rot of potato.	Burkhead <i>et al.</i> , 1994
	<i>Burkholderia cepacia</i> J82	<i>Sclerotinia sclerotiorum</i> on sunflower.	McLoughlin <i>et al.</i> , 1992
	<i>Burkholderia cepacia</i> RB425		Homma <i>et al.</i> , 1989
Unknown antibiotic	<i>Pseudomonas fluorescens</i>	<i>Rhizoctonia solani</i> on radish.	Hill <i>et al.</i> , 1994
		<i>Rhizoctonia solani</i> on cotton.	Carruthers <i>et al.</i> , 1995
	<i>Pseudomonas aureofaciens</i> PA147-2	<i>Phytophthora megasperma</i> root rot of asparagus	

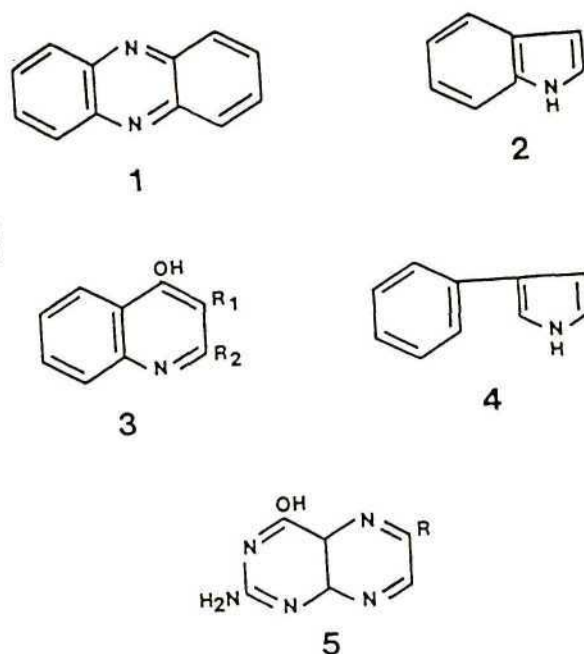


Figure 1. 2: Some of the more important classes of secondary metabolites produced by fluorescent pseudomonads (Handelsman & Stabb, 1996): (1) Phenazine. (2) Indoles. (3) Pyo compounds. (4) Phenylpyrroles. (5) Pterines.

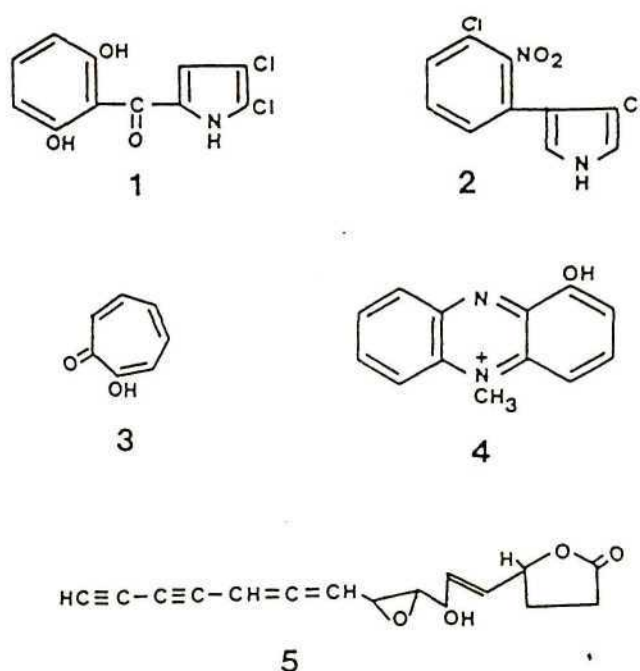


Figure 1.3: Representative antibiotics that are produced by pseudomonads (Lynch, 1990): (1) Pyoluteorin. (2) Pyrrolnitrin. (3) Tropolone. (4) Pyocyanin. (5) Cepacin A.

as in conducive soils. Sneh *et al.* (1984) however evaluated the possible implication of fungal lysis as a mechanism of biological control fusarium wilts by bacteria and actinomycetes from the suppressive soil of Salinas Valley. Strains were tested *in vitro* for their ability to lyse germ tubes of *F. oxysporum* or to produce either chitinase or fluorescent siderophores. Most of the strains that produced chitinase were able to lyse fungal germ tubes, but some isolates producing chitinase were not able to lyse germ tubes. Isolates determined to be germ-tube lytic *in vitro* did not lyse germ tubes of germinating chlamydospores in soil. It seemed that there was greater efficacy of the siderophore producers to suppress fusarium wilt of cucumber. Sneh *et al.* (1984) concluded their study as follows "no evidence was obtained indicating that lysis of germ tubes of chlamydospores was induced in soil by any of the microorganisms exhibiting this attribute *in vitro*." After having cloned chitinase-encoding genes from *Serratia marcescens*, Sundhein (1992) made a fluorescent pseudomonad transconjugant that expressed chitinase activity and then reported that the growth of *F. oxysporum* f. sp. *redolens* was inhibited and fusarium wilt of radishes in sand was also suppressed by this bacterial construct.

1.1.3.4 Induced systemic resistance in plants

Systemic acquired resistance (SAR) refers to a distinct signal transduction pathway that plays an important role in the ability of plants to defend themselves against pathogens. The acquisition of systemic resistance to a variety of pathogens by plants previously inoculated either with a pathogen or with inducing chemical compounds has been documented in numerous systems (Sequeira, 1983; Kuc, 1987; Ryals *et al.*, 1991) and progress has been made at understanding plant response at the molecular level (Ward *et al.*, 1991). Pseudomonads may be effective biological control agents because they antagonize a pathogen directly, by competition for nutrient or antibiosis - or indirectly, by activating plant defence mechanisms.

Kempe and Sequeira (1983) conducted a study in which they attempted to induce resistance in potato to a highly virulent strain of *Pseudomonas solanacearum* by prior inoculation of potato tubers with various pseudomonads, including two

P. fluorescens strains. Interestingly, by dipping potato eyepieces in suspensions of some of the pseudomonads, including the *P. fluorescens* isolates, disease severity was reduced when plants were challenged 3–4 weeks later with stem inoculations. Colonization with beneficial isolates of *P. putida* increased the defensive potential of bean plants against *F. solani* f. sp. *phaseoli*, and it was also noted that lignin increased in root tissue in response to *P. putida* colonization. This notion was further supported by the observation that the colonization of bean roots by *P. putida* enhanced root surface peroxidase activity (Albert & Anderson, 1987).

The production of HCN by *P. fluorescens* CHAO has been suggested as another potential inducer of plant defence mechanisms by generating of cyanide stress in root tissue (Défago *et al.*, 1990). Van Peer *et al.*, (1991) examined whether the biocontrol of *Fusarium* wilt of carnation by *Pseudomonas* sp. strain WCS417r involved an induced resistance mechanism. When root-bacterized carnations were challenged 1 week later by stem inoculation with *F. oxysporum* (*i.e.*, pathogen and antagonist were separated spatially to rule out competition for nutrients) a significant reduction in *Fusarium* wilt was found. It was also noted that the disease suppression was relative to that seen either when plants were not bacterized with the *Pseudomonas* strain or when bacterization was simultaneous with the *Fusarium* stem inoculation. Phytoalexin accumulation in the stem was implicated, but did not occur in the absence of the pathogen. The authors suggested that bacterization of the roots with the pseudomonad generated signal that induced sensitization of the stem, ultimately leading to higher phytoalexin accumulation when the fungal challenge occurred. Taking all of these together observations suggests that certain root-colonization bacteria may induce systemic resistance in plants.

1.2 The molecular basis of antagonism by *Burkholderia/Pseudomonas* spp.

Certain strains of beneficial *Pseudomonas* spp. could suppress a variety of soil-borne plant diseases, enhancing the growth and yield of agricultural crops (Weller, 1988; Cook, 1993). The inhibition of root pathogens by production of antimicrobial and

iron-chelating metabolites is considered to be a primary mechanism of disease suppression by these bacteria. A role in disease suppression has been demonstrated for phenazine-carboxylic acid, hydrogen cyanide, 2,4-diacetylphloroglucinol (PhI), oomycin A, pyoluteorin (Plt), and pyrrolnitrin (O'Sullivan & O' Gara, 1992; Keel & Défago, 1996; Thomashow & Weller, 1996). Recently, genetic analysis of several *Pseudomonas* strains has established a correlation between antibiotic production and disease suppression.

Transposon-mediated mutagenesis is one of the most powerful techniques available for genetic analysis in bacteria. To understand the genetic system of organism, the development of genetic techniques such as mutagenesis, transformation, transduction and conjugation are needed. Tn5 mutagenesis is an efficient strategy for evaluating the possible implications of bacterial metabolites in the suppression of soil-borne disease. With mutants affected in the production of some metabolites and reduced suppressive ability. Use of transposon mutagenesis to investigate a role of secondary metabolites in diseases suppression has received most attention in recent years.

1.2.1 Genetic analysis of phenazine antibiotic in disease suppression

P. fluorescens 2-79, which has been showed to control take-all of wheat (Weller & Cook, 1983), produces phenazine-1-carboxylic acid (PCA) (Gurusiddaiah *et al.*, 1986; Brisbane *et al.*, 1987). It has been demonstrated that this compound inhibits *G. graminis* var. *tritici* *in vitro* at a concentration of less than 1 ug/ml. It was also produced in the rhizosphere of wheat plants inoculated with strain 2-79 (Thomashow & Pierson, 1991). Thomashow & Weller (1988) isolated mutants of strain 2-79 that were defective in production of PCA (Phz⁻) by Tn5 transposon mutagenesis. Six independent prototrophic Phz⁻ mutants were tested. Each was no longer inhibitory to *G. graminis* var. *tritici* and provided significantly less control of take-all than the wild type strain on wheat seeding. It was found that the sequences containing Tn5 in the mutants are required for phenazine synthesis, and it strongly supports the importance of the phenazine antibiotic in biological control of take-all

of wheat. Phenazine could be isolated from roots of wheat inoculated with Phz⁺ strain but not from those inoculated with Phz⁻ strain. The mutants were complemented to Phz⁺ by the plasmid pDWE108A. This plasmid, when transferred into other *Pseudomonas* strains that normally did not produce phenazine and did not inhibit *G. graminis* var. *tritici* *in vitro*, the resulted in the transconjugants that had restored ability to produce both the antibiotics and inhibit the pathogen (Thomashow, 1991). Similar results were also obtained with *P. aureofaciens* strain 30-84 (Thomashow & Pierson, 1991).

1.2.2 Genomic locus required for synthesis of 2,4-diacetylphloroglucinol (PhI)

To examine the role of 2,4-diacetylphloroglucinol (PhI^r) in disease control, Keel *et al.* (1990, 1992) isolated a Tn5 mutant of strain CHAO that no longer produced the compound PhI^r. These mutants showed lower inhibition of *Thielaviopsis basicola* and *G. graminis* var. *tritici* *in vitro* (colonizing the rhizosphere as well as the wild type parent), and provided lower disease control. Complementation of the mutant to PhI^r also restored fungal inhibition *in vitro* and disease control, and the compound was recovered from the rhizosphere of wheat inoculated with the wild type and the complemented strains (PhI^r), but not from that inoculated with the PhI^r mutant. That indicated that the sequences containing Tn5 in the mutants were required for PhI synthesis as well as disease suppression. Gene(s) involved in the biosynthesis of PhI by *Pseudomonas* sp. strain F113 were identified and were also assessed for their ability to confer PhI production to other *Pseudomonas* strains (Fenton, *et al.*, 1992). Tn5 mutagenesis and complemetation analysis showed that a 6-kb fragment was required for biosynthesis of 2,4-diacetylphloroglucinol (PhI). The 6-kb wild-type fragment from F113 was expressed in one of eight other non-producing pseudomonads. The transgenic strain produced PhI and was significantly more suppressive than F113 to *Pythium ultimum* on sugar beet (Fenton *et al.*, 1992). Two cosmid clones of 25 or 37kb containing the PhI synthetic locus from strain Q2-87 were also expressed in the nonproducers, *P. fluorescens* 2-79 and *Pseudomonas* strain 5097 (Vincent *et al.*, 1991). Bangera & Thomashow (1996)

further characterized the locus described by Vincent *et al.* (1991), which was required for synthesis of PhI by *P. fluorescens* Q2-87. Keel *et al.* (1996) demonstrated that the region is conserved in all of the strains in their collections known to produce PhI by using a cloned from the PhI biosynthetic region of *P. fluorescens* Q2-87 (Vincent *et al.*, 1991; Bangera & Thomashow, 1996; GenBank accession no. U41818). They also found there is considerable diversity among PhI-producing pseudomonads in soils originating from different geographic locations.

1.2.3 A genomic region involved in the synthesis of pyrrolnitrin (Prn)

Pyrrolnitrin [3-chloro-4-(2'-nitro-3'-chlorophenyl)-pyrrole] (Prn) has been shown to be involved in the suppression of fungal pathogens (Homma *et al.*, 1989; Howell *et al.*, 1979). *P. fluorescens* strain BL915 produced pyrrolnitrin (Prn), which may be an effective component in biological control agents. Genetic analysis of the role of Prn synthesis in disease control with *P. fluorescens* BL915 was investigated by Hill *et al.* (1994). A Prn⁻ mutant, which was generated by exposure to NTG (N-methyl-N-nitro-N-nitrosoguanidine), was unable to produce Prn, and had greatly reduced ability to control *Rhizoctonia solani* seeding disease of cotton. A gene region which has a role in Prn synthesis was isolated from strain BL915 and it was found that a 15kb BamHI fragment, 11kb EcoRI fragment, and 13kb HindIII-BamHI fragment were all capable of complementing the Prn⁻ phenotype of mutant 2-1. The 4.9-kb HindIII-EcoRI fragment did not appear to be of sufficient size to code for all of the enzymes involved in Prn synthesis. This 4.9-Kb fragment may only contain a portion of the genes required for Prn synthesis or may contain a regulatory gene. Similar results were obtained with *P. fluorescens* Pf-5 (Pfender *et al.*, 1993). A Tn5 mutant of Pf-5 that did not produce detectable levels of pyrrolnitrin also did not inhibit *Pyrenophora tritici-repentis* on a culture medium or on wheat straw. A genomic region of Pf-5 was cloned and able to restore the pyrrolnitrin deficient Tn5 mutant to pyrrolnitrin production and subsequent antagonization of *P. tritici-repentis* in culture.

1.2.4 A global regulator of secondary metabolites

The global regulator GacA (global activator) of strain CHAO regulates the expression of several secondary metabolites such as HCN, PhI and Plt (Laville *et al.*, 1992), the enzymes tryptophan side-chain oxidase (Laville *et al.*, 1992), protease and phospholipase C (Sacherer *et al.*, 1994). The response-regulator GacA and the corresponding sensor LemA are components of a highly conserved signal pathway within the genus *Pseudomonas* (Gaffney *et al.*, 1994; Rich *et al.*, 1994; Corbell *et al.*, 1995). GacA is a response regulator in the Fix-DegU family of two-component regulatory systems (Laville *et al.*, 1992; Gaffney *et al.*, 1994) and a conserved regulator of virulence factors in *Pseudomonas aeruginosa* (Rahme *et al.*, 1995). In the current report (Schmidli-Sacherer *et al.*, 1997) a derivative of CHAO with a mutation in the global regulator gene *gacA* (GacA), which was unable to produce PhI, Plt and HCN, failed to protect the dicotyledonous plants cress and cucumber against damping-off caused by *P. ultimum*. In contrast, the GacA⁻ mutant could still protect the wheat and maize against damping-off mediated by the same strain of *P. ultimum*, and wheat against take-all caused by *G. graminis*. The GacA⁻ mutant overproduced Pch and Pvd. The GacA⁻ Pvd⁻ double mutant overproduced Pch and Sal compared with the wildtype and protected wheat against *P. ultimum* and *G. graminis*, whereas cress and cucumber were not protected. The authors suggested that a functional *gacA* gene was necessary for the protection of dicotyledons against root diseases, but not for that of *Gramineae*, and Pch and/or Sal were not involved in the ability of the GacA⁻ Pvd⁻ mutant to suppress root disease in *Gramineae*.

A similar result was obtained with the *apdA* mutant of strain *P. fluorescens* Pf-5 (Corbell *et al.*, 1995). The authors noted that mutant in the *apdA* gene of *P. fluorescens* Pf-5 pleiotropically abolish the production of an array of antibiotics, including pyrrolnitrin, pyoluteorin, and 2,4-diacetylphloroglucinol, as well as the production of tryptophan side chain oxidase, hydrogen cyanide, and an extracellular protease. The lack of production of metabolites by *apdA*⁻ mutants was correlated with the loss of inhibition of the phytopathogenic fungus *Rhizoctonia solani* in culture. Sequencing of the *apdA* region identified an open reading frame of 2,751bp, and the predicated amino

acid sequence of the *apdA* gene contained conserved domains of histidine kinase that serves as a sensor component of prokaryotic two-component regulatory systems. The *apdA* nucleotide and predicted amino acid sequences were strikingly similar to the sequences of *lemA* and *repA* genes encoding putative sensor kinase which are required for the pathogenicity of *Pseudomonas syringae* pv. *syringae* and *Pseudomonas viridiflava*, respectively. Introduction of the cloned *apdA*⁺ gene restored the wild type phenotype to both *lemA*⁻ mutants of *P. syringae* and *apdA*⁻ mutants of Pf-5. The 101-kDa Apd protein reacted with an anti-*lemA* antiserum, further demonstrating the similarity of ApdA to LemA. The authors suggested that *apdA* may encode a putative sensor kinase component of a classical two-component regulatory system that could be required for secondary-metabolite production by *P. fluorescens* Pf-5.

1.2.5 Genetic control of other disease suppressing activities

1.2.5.1 Siderophores

A pyoverdine-negative mutant of *P. Fluorescens* strain CHA0 has been described (Keel *et al.*, 1989; Haas *et al.*, 1991; Keel & Défago, 1991), which was not impaired in its ability to protect wheat from diseases caused by *P. ultimum* or *G. graminis* var. *tritici*. This was true in the wheat/*Pythium* system even under conditions of iron deficiency. The authors noted that iron competition could still play some role in disease suppression of *P. fluorescens* CHA0 synthesis of siderophore(s) and pyoverdine. Paulitz and Loper (1991) isolated Tn 5 mutants of *P. putida* strain N1R which were deficient in pyoverdine production (Pvd⁻ phenotype). The Pvd⁻ derivatives showed no reduction in ability to protect cucumber from *Pythium* damping-off in three soil types. Mutants of *P. putida* WCS358 defective in the biosynthesis of the fluorescent siderophore were also generated by transposon-mediated mutagenesis (Marugg *et al.*, 1985). Complementation of these mutants with the cosmid clones of a genomic library of WCS358 resulted in the identification of five separate gene clusters involved in siderophore biosynthesis. The different cosmid groups represent distinct genes or gene, which were coding enzymes with

functions involved in biosynthesis or excretion of the siderophore. Further investigation (Marugg *et al.*, 1988) indicated that a minimum length of 33.5kb and at least five transcription units were involved in the biosynthesis and transport of pseudobactin 358. The authors found that the iron-dependent expression of at least two genes within this cluster appears to be regulated at the level of transcription.

1.2.5.2 Hydrogen cyanide (HCN)

The *P. fluorescens* strain CHA0 produces a number of secondary metabolites, one of which was hydrogen cyanide (HCN). To determine the role of HCN in disease suppression, Voisard *et al.* (1989) constructed a cyanide-negative (*hcn*⁻) mutant, CHA5 (with an -Hg insertion in the *hcn* genes) and tested its disease control efficacy against black root rot. Strain CHA5 provided reduced disease suppression when compared with strain CHA0 wild type, but was better than the control. Introduction of a plasmid carrying the *hcn*⁺ genes (pME3013) into strain CHA5 restored HCN production (to higher than the wild-type level) and disease control efficacy. The *hcn*⁺ genes, when inserted into strain *P. fluorescens* P3 which was naturally *hcn*⁻ and provided poor plant protection, conferred upon the recombinant the ability to produce HCN and provided improved plant protection (Voisard *et al.*, 1989) and strain CHA5 did not protect wheat against *G. graminis* var. *tritici* (Défago *et al.*, 1990). Interestingly, another *hcn*-mutant, CHA77 (a deletion mutant), protected wheat against *G. graminis* var. *tritici* at wild-type levels, though it behaved similar to strain CHA5 in the tobacco system (Haas *et al.*, 1991). These results indicated that the role of HCN might have different effects in different host-pathogen systems.

1.2.5.3 Colonization

It was accepted that bacterial surface molecules may aid in attachment to plant root and enhanced colonization, and agglutinin has been correlated with colonization and disease suppression (Chao & Chang, 1988; Anderson *et al.*, 1988; Tari & Anderson, 1988). The Tn5 mutants, which were agglutinin producing-negative, adhered

to cucumber at lower levels, colonized less, and provided less protection against *F. oxysporum*, compared to the parent strain (Tari & Anderson, 1988). A genomic fragment, which corresponded to the site of Tn5 insertion in Agg⁻ mutant 5123, has been isolated (Buell & Anderson, 1991). This fragment complemented the Agg⁻ phenotype in 5123 but not other Agg⁻ mutants, which indicated the likelihood that may be involved more than one genetic locus of agglutination. The role of motility in colonization was examined by several groups. In two studies (Howie *et al.*, 1987; Scher *et al.*, 1988), the nonmotile mutants were able to colonize as well as the parents, and de Weger *et al.*, (1987) found that the nonmotile mutants were impaired in their ability to colonize lower parts of the plant roots.

1.3 Conclusion

Fluorescent pseudomonads are efficient biocontrol agents of soilborne diseases. (Schroth & Hancock, 1982; Schipper *et al.*, 1987; Weller, 1988; Lemanceau *et al.*, 1992). There has been an increase in the number of biocontrol agents for control of *Fusarium* wilt diseases (Table 1.1). The future of biological control of root disease such as *Fusarium* wilts could be promising. It has been shown that antibiosis and the production of siderophores by the biocontrol organism are two of the major mechanisms in the biological control of diseases. There exists indirect evidence that lytic enzymes, colonization and the induction of systemic resistance, all have a role in biological disease control, as discussed in section 1.2.

Genetic manipulation of biocontrol bacteria has the potential both to improve their effectiveness and to increase the range of crops and soil. Molecular biology has enabled target screens to be developed, searching for further antagonists. A mutant of Hv37a that produced oomycin A was shown to improve control of *Pythium* infections (Gutterson, 1990). The production of pyoluteorin (Plt) and 2,4-diacetylphloroglucinol (Phl) increased when the cosmid pME3090 from strain CHAO was reintroduced into the CHAO background. The resulting strain, CHAO/pME3090 provided improved disease suppression on cucumber but showed a toxic effect on cress and sweet corn. Both Plt and Phl were to have phytotoxic effects, but cucumber was less

sensitive to PhI than the other plants (Maurhofer *et al.*, 1992). These results indicated that higher amount of antibiotics is not always better. The overall effect depends on the host-pathogen system, and has to be determined empirically. Several DNA regions have been isolated which, when introduced into unrelated recipient bacteria, confer the ability to produce antibiotics that were previously undetected in the recipient (Hill *et al.*, 1990; Thomashow, 1991; Vincent *et al.*, 1991). Improvement in disease suppression was observed in one case where the recipient was not normally antagonistic (Hill *et al.*, 1990). No improvement was observed in another case where the recipient strains already had good disease suppressive activity (Vincent *et al.*, 1991).

In the field, crop losses are frequently due to the presence of more than one pathogen. As a result, bacterial strains that have been shown to provide good protection against specific fungal pathogens in the green house often fail to perform in the field. Biocontrol strains with broad disease control spectrum are required. The *P. fluorescens* strain CHA0, for example, produces a variety of antagonistic compounds, each of which may contribute differently to the suppression of different pathogens (Défago *et al.*, 1990; Haas *et al.*, 1991). Even in the suppression of a single pathogen, multiple control mechanisms are the rule rather than the exception. Most antibiotic negative mutants, for example, still retain significant disease control activity (Thomashow & Weller, 1988; Voisard *et al.*, 1989; Howie & Suslow, 1991; Keel *et al.*, 1992). Further research into the following areas of biological control of plant root diseases may therefore include: (a) elucidation of biological control mechanisms in a variety of different soil types; (b) identification of environmental parameters that affect root colonization; (c) development of improved strain-selection producer and carrier systems; and (d) identification of key genes coding for siderophore production, antibiotic production and root colonization. This research will serve to increase the scope of the biological control of root disease. Attempts to improve biocontrol strain by genetic modification are in their infancy.

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2. ULTRASTRUCTURAL STUDIES ON THE COLONIZATION OF BANANA TISSUE AND *FUSARIUM OXYSPORUM* F. SP. *CUBENSE* RACE 4 BY THE ENDOPHYTIC BACTERIUM *BURKHOLDERIA CEPACIA*

2.1 Introduction

The use of rhizosphere bacteria, specifically rhizobacteria (root-colonizing bacteria), for biological control of soil-borne plant pathogens has been well documented (Kloepper, 1993; Schippers, 1988; Weller, 1988). In spite of renewed interest in exploiting bacterial endophytes as a method for controlling plant diseases, the exact mechanisms by which these microorganisms confer increased plant protection have not been fully investigated (Bull *et al.*, 1991).

Numerous hypotheses for protection have been proposed, including production of siderophores, accumulation of antifungal metabolites, nutrient competition, and niche exclusion (Chen *et al.*, 1995). In a recent report, Benhamou *et al.* (1996) provided evidence that root colonization by the endophytic bacterium *Pseudomonas fluorescens*, involved a sequence of events that included bacterial attachment to the plant roots, proliferation along the elongation root, and local penetration of the epidermis. It seemed logical that a biological agent should grow and persist, or "colonize", the surface of plant that it protects. Colonization is widely believed to be essential for biocontrol (Weller, 1983; de Weger *et al.*, 1987; Parke, 1991). In a study showing the suppression of damping-off of peas by *Burkholderia cepacia*, there was a significant relationship between population

size of the biocontrol agent and the degree of disease suppression (Parke, 1990). Also, suppression of take-all of wheat was correlated with colonization of roots by a *P. fluorescens* strain (Bull *et al.*, 1991).

Fusarium wilts are caused by pathogenic *Fusarium oxysporum*. There is a high level of specificity between susceptible host plants and pathogenic *F. oxysporum* strains. This specificity is characterized by formae specialis and races to which *F. oxysporum* belongs (Armstrong & Armstrong, 1981). The life cycle of these fungi includes a saprophytic and a parasitic phase (Nelson, 1981; Beckman, 1989). In the absence of a host plant, *F. oxysporum* is able to survive by formation of chlamydospores (Schippers & Van Eck, 1981) and to grow on organic matter from plant debris (Beckman, 1989). In the presence of a host plant, release of root exudate allows germination of chlamydospores and mycelial growth (Schroth & Hildebrand, 1964). When the nutrient supply is high enough the mycelium may reach the roots of the plant (Lockwood, 1990). Banana wilt disease caused by *Fusarium oxysporum* f. sp. *cubense* is a serious constraint to banana production world-wide. Fusarium wilt disease is a soil-borne disease and is difficult to eliminate in the soil.

This study was part of a programme to evaluate the potential of *B. cepacia*, isolated from asparagus, as a biological agent to control Fusarium wilt in banana. As a first step, we investigated, the interaction of the bacterium with the fungal pathogen using microscopy techniques. Secondly, colonization of banana roots and the fungus by the bacterium was investigated. Colonization is a vital prerequisite for the successful biological control of a plant pathogen.

The objective of this study is to understand the colonization mechanism of *B.*

cepacia at the ultrastructural level, particularly on banana roots and the hyphae of *F. oxysporum* f. sp. *cupense* race 4.

2.2 Materials and methods

2.2.1 Strains, plants and inoculation

Banana plants of the *Musa* cultivar "Williams" belonging to the Cavendish group of banana (AAA), the banana wilt disease pathogen, *F. oxysporum* f. sp. *cupense* race 4 and the bacterium *B. cepacia* in this study were supplied by AECL's Plant Biotechnology Section (Modderfontein, South Africa). *B. cepacia* was isolated from *in vitro* grown asparagus plants (*Asparagus officinalis* L.; cultivar Calet). This cultivar is known to have increased resistance to Fusarium.

For isolation of *B. cepacia*, *in vitro* grown tissue culture plants were sampled and homogenized in peptone water and dilutions of the homogenate were plated onto GSP agar for selection of pseudomonads. Colonies found on the GSP agar were identified using the API 20 NE identification system. The most prominent organism present was identified to be *Pseudomonas cepacia*, recently reclassified as *Burkholderia cepacia*.

The bacterium *B. cepacia*, and plant pathogen *F. oxysporum* f. sp. *cupense* race 4 were cultured on Tryptone Soya Agar (TSA) and/or Trypton Soya Broth (TSB) and YM (0.3% Yeast Extract, 0.3% Malt Extract, 0.5% Peptone, 1% Glucose) at 26°C. *F. oxysporum* f. sp. *cupense* was cultured overnight in TSA and YM Broth, and then over-night cultured suspension of *B. cepacia* were added at 8% (V/V). After 24, 48, 72, 96, 120 h, the fungal mycelium was processed for

scanning and transmission electron microscopy.

The banana plant, cultivar Williams, was cultured on 0.8% agar medium, supplemented with 2% sucrose. The young banana plants in the medium were inoculated with a 0.5 ml spore suspension (1×10^5 spores/ml) of *F. oxysporum* f. sp. *cubense* plus 0.5ml bacterial solution (1×10^5 cells/ml) of *B. cepacia*, by infiltrating the growth medium. The plant roots were sampled daily up to 7 days post-inoculation and processed further for scanning and transmission electron microscopy.

2.2.2 Scanning electron microscopy (SEM)

Samples were cut into 2×2 mm squares and fixed in 3% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2, for 24 h, washed twice in the buffer, post-fixed for 2 h in 2% osmium tetroxide in the buffer, washed twice in the buffer, and dehydrated through an ethanol series (30, 50, 70, 80, 90, 100% for 10 min each) followed by a duplicate absolute ethanol wash for 30 minutes. After dehydration, the specimens were then critical point dried with carbon dioxide as a transition fluid and were mounted on copper stubs. Specimens were coated with gold-palladium in a Polaron sputter coater and examined with a S-570 scanning electron microscope at 8.0 or 10 kV. After freeze-fracturing in an EM Scope SP 2000 cryo unit, freshly harvested material was viewed with the SEM at 8.0 kV.

2.2.3 Transmission electron microscopy(TEM)

Pieces of samples were cut into 2×2 mm squares and were fixed for 24 hours

PLATE 1

Figure 1: Colonization of hyphae of *Fusarium oxysporum* f. sp. *ubense* race 4 by *Burkholderia cepacia* indicating the collapse of fungal hypha as indicated by arrow. Bar = 5 μ m. B, the bacterium; H, hyphae; S, septum.

Figure 2: Macroconidia of *Fusarium oxysporum* f. sp. *ubense* showing colonization by *Burkholderia cepacia*. Bar = 2 μ m. B, the bacterium; MAS, macroconidia.

Figure 3: Microconidia of *Fusarium oxysporum* f. sp. *ubense* colonized by *Burkholderia cepacia*. Bar = 2 μ m. B, the bacterium; MIS, microconidia.

Figure 4: Collapsed hyphae of *Fusarium oxysporum* f. sp. *ubense* (arrow) after treatment with an inoculum of *Burkholderia cepacia*. Bar = 10 μ m. H, hyphae.

Figure 5: Control test. Normal hyphae of *Fusarium oxysporum* f. sp. *ubense*. Bar = 10 μ m. H, hyphae.

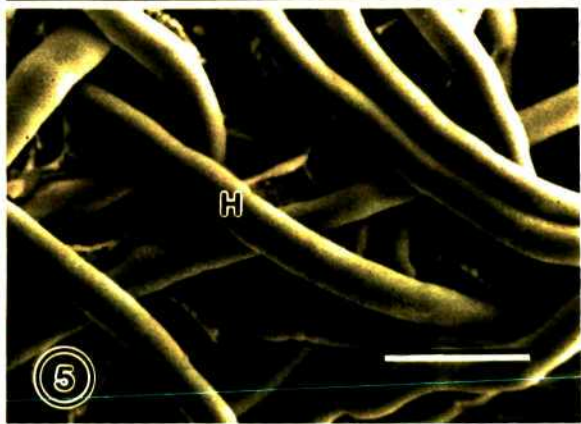
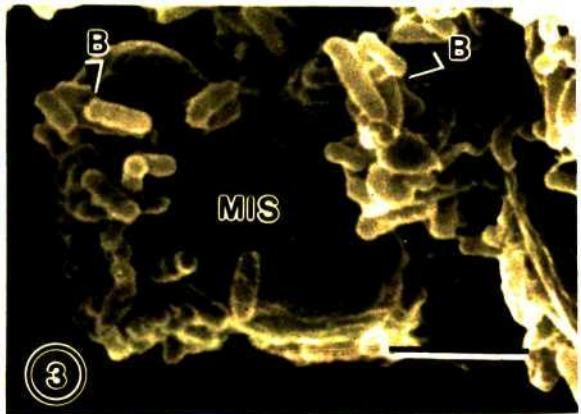
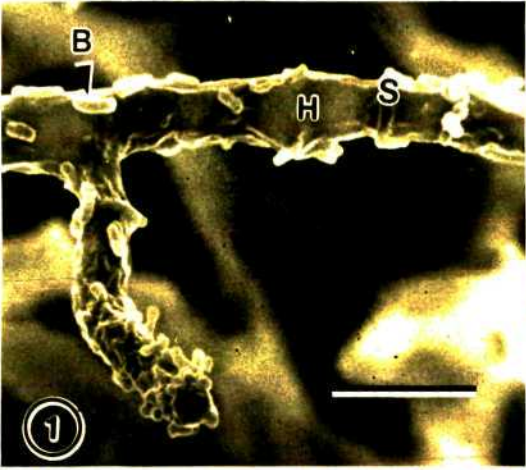
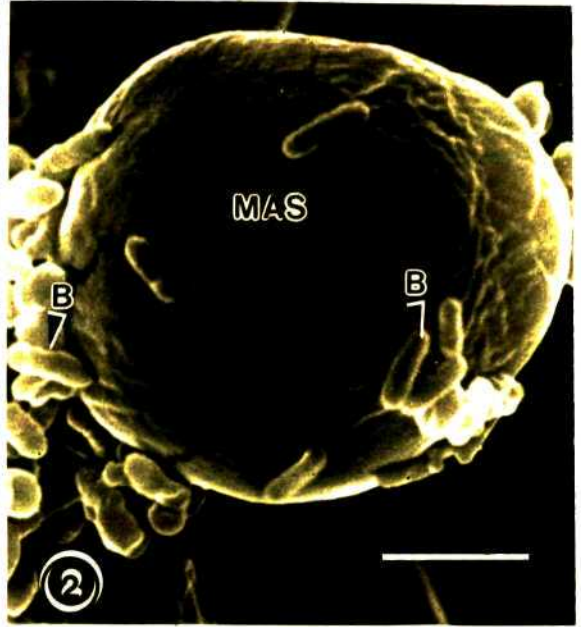
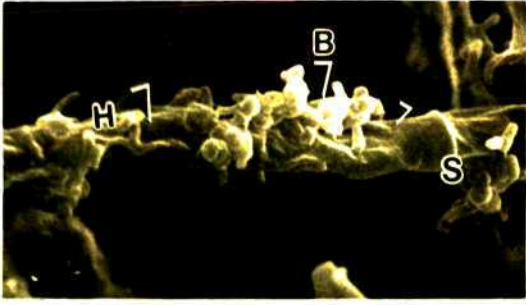


PLATE 2

Figure 6: Control test. Normal hyphae of *Fusarium oxysporum* f. sp. *cubense*. Bar = 15 μ m. H, the hyphae.

Figure 7: Association of *Burkholderia cepacia* with *Fusarium oxysporum* f. sp. *cubense*, *in vitro*, showing interaction of the bacterium with the fungus. Bar = 15 μ m. H, hyphae; B, the bacterium.

Figure 8: Hyphae of *Fusarium oxysporum* f. sp. *cubense* growing with *Burkholderia cepacia* showing the thickening of the fungal cell wall and the attachment to the cell wall of the hyphae. Bar = 48 μ m. H, hyphae; CW, cell wall.

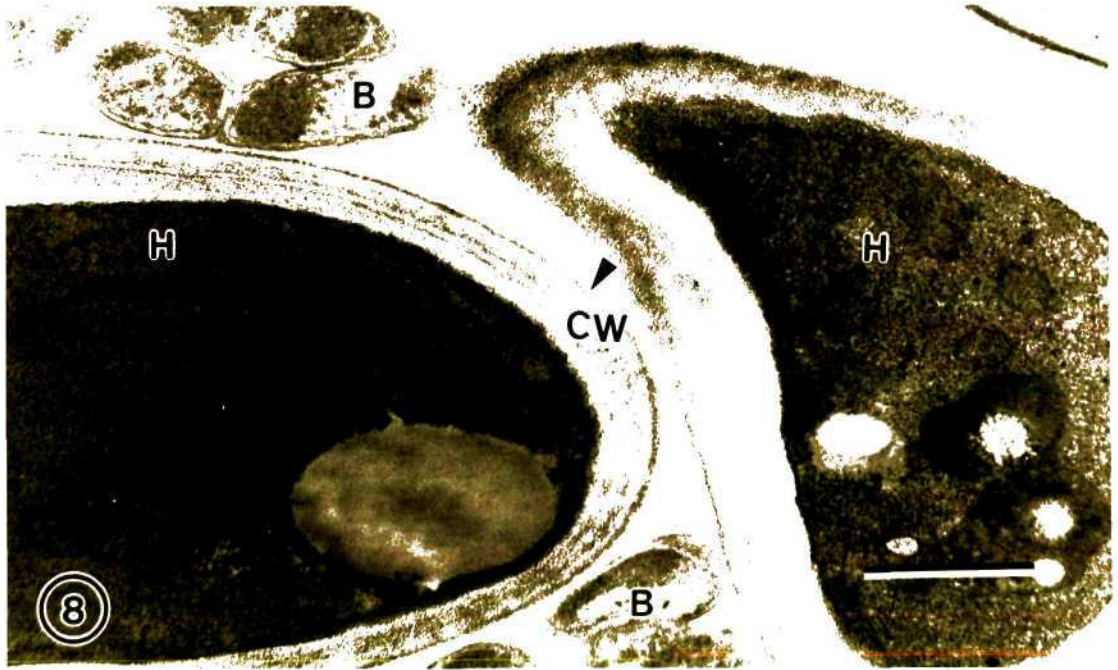
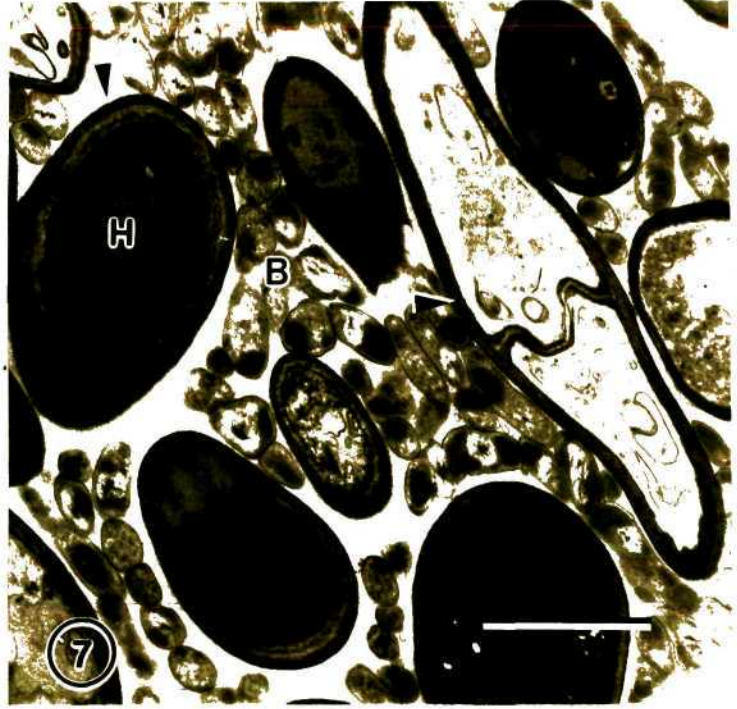
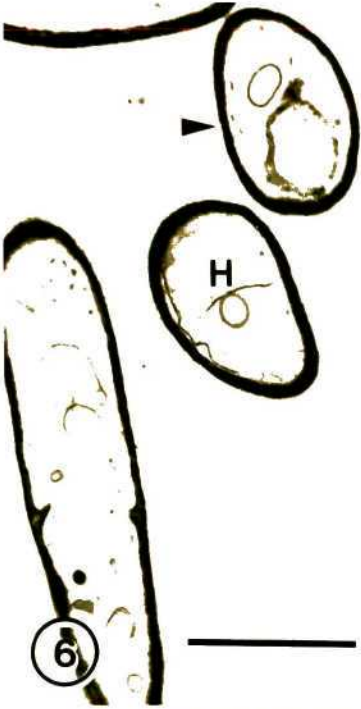


PLATE 3

Figure 9: *Burkholderia cepacia* accumulating on the root surface of banana.
Bar = 30 μm . RS, the root surface; RH, the root hair; B, the bacterium.

Figure 10: *Burkholderia cepacia* accumulating on the root surface of the banana showing torpedo-shaped bacteria aggregating on the banana root surface. A fibril-like extracellular matrix is associated with the bacteria. Bar = 3 μm . RS, the root surface; B, the bacterium.

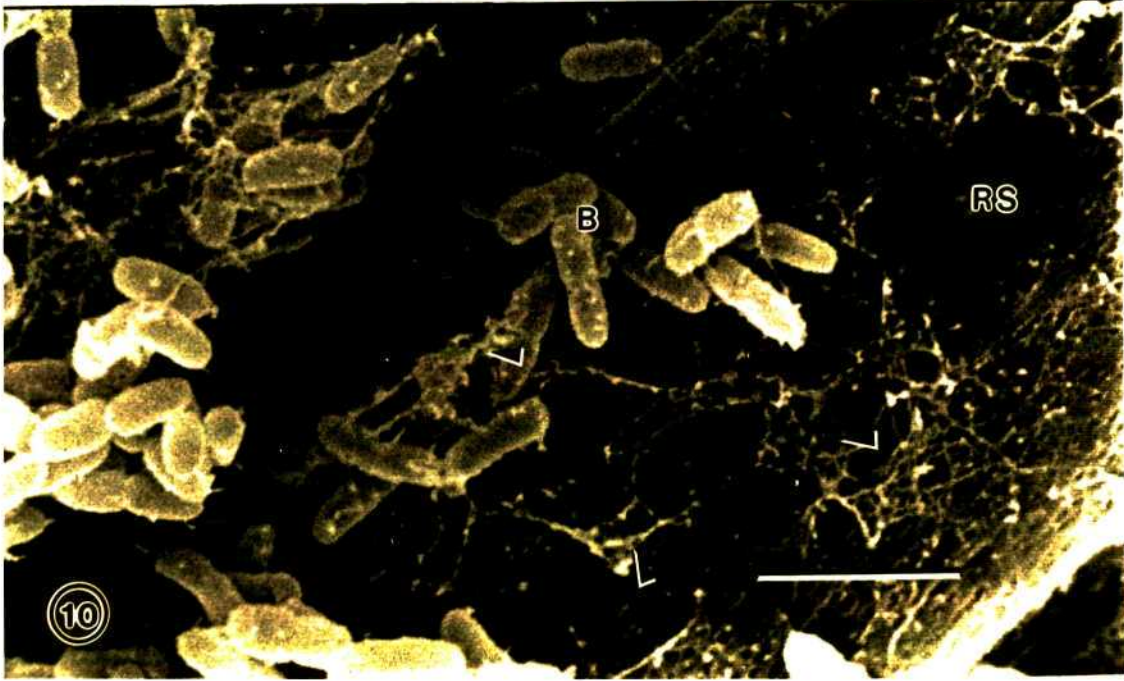
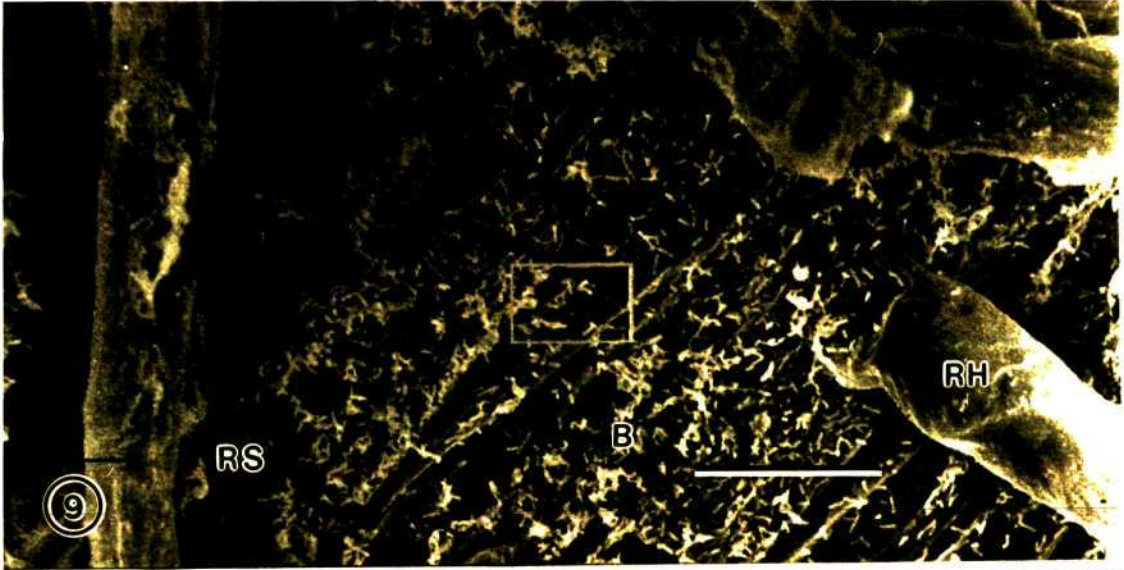


PLATE 4

Figure 11: Hyphae of *Fusarium oxysporum* f. sp. *cubense* multiply in the intercellular space of the banana root tissue. Showing the fungus invading into the cell from the intercellular space. An electron-densed extracellular matrix is discernible (arrow). Bar = 15 μm . H, hyphae; HC, the host cell; IS, intercellular space.

Figure 12: Segments of *Fusarium oxysporum* f. sp. *cubense* in the banana root tissue cells showing the host cytoplasm appeared disorganized and the fungus penetrating into the adjoining host cell. Bar = 15 μm . H, hyphae; HC, the host cell.

Figure 13: The hyphae of *Fusarium oxysporum* f. sp. *cubense* outside of the root surface of banana are surrounded by *Burkholderia cepacia*. Bar = 8 μm . H, hyphae; B, the bacterium; RS, the root surface.

Figure 14: *Burkholderia cepacia* attached to the hypha of *Fusarium oxysporum* f. sp. *cubense*. The hyphal surface appears to be partially deformed by the bacteria. Bar = 7 μm . H, hyphae; B, the bacterium; RS, the root surface.

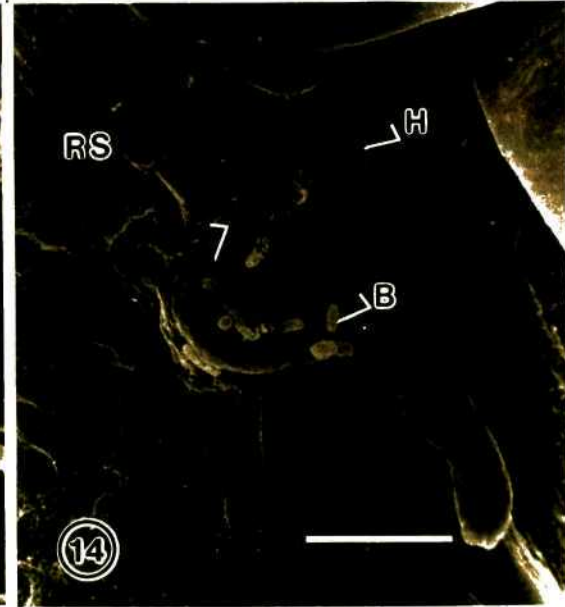
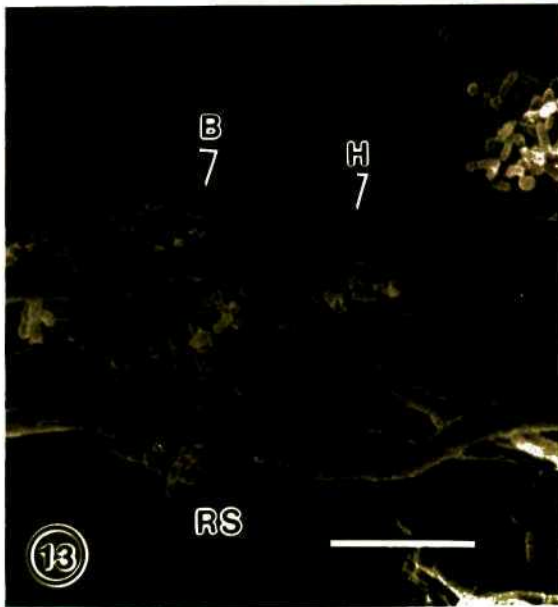
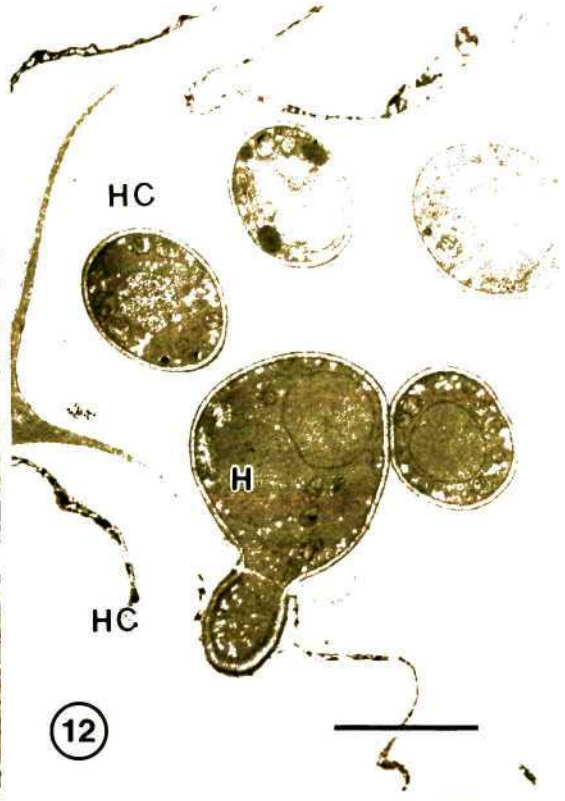
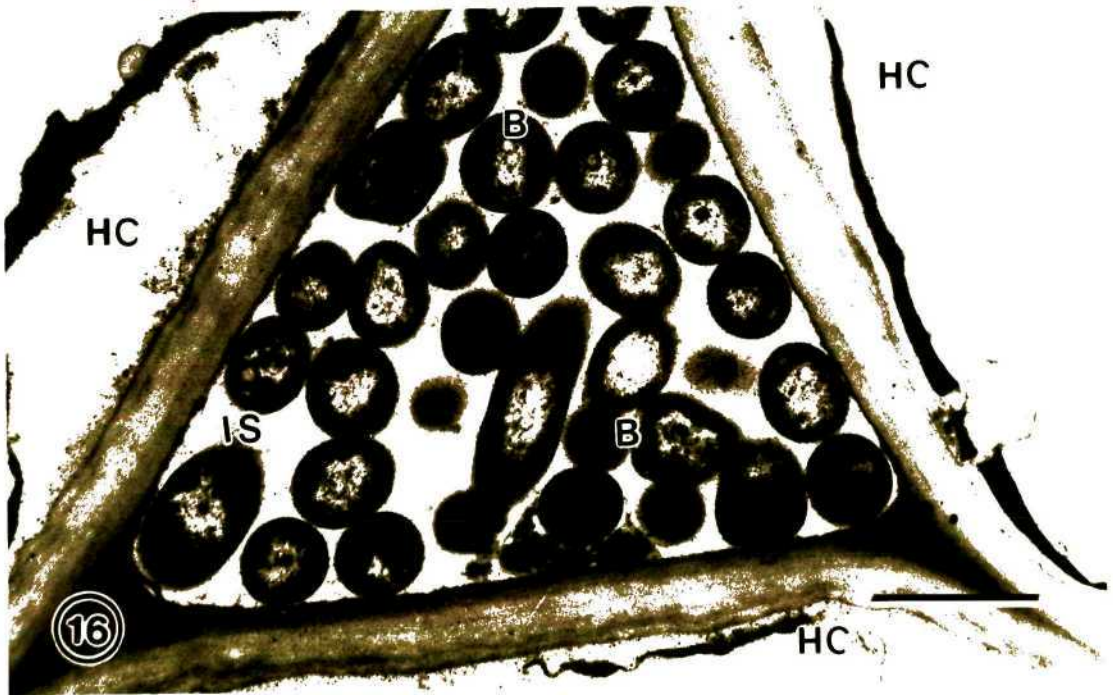
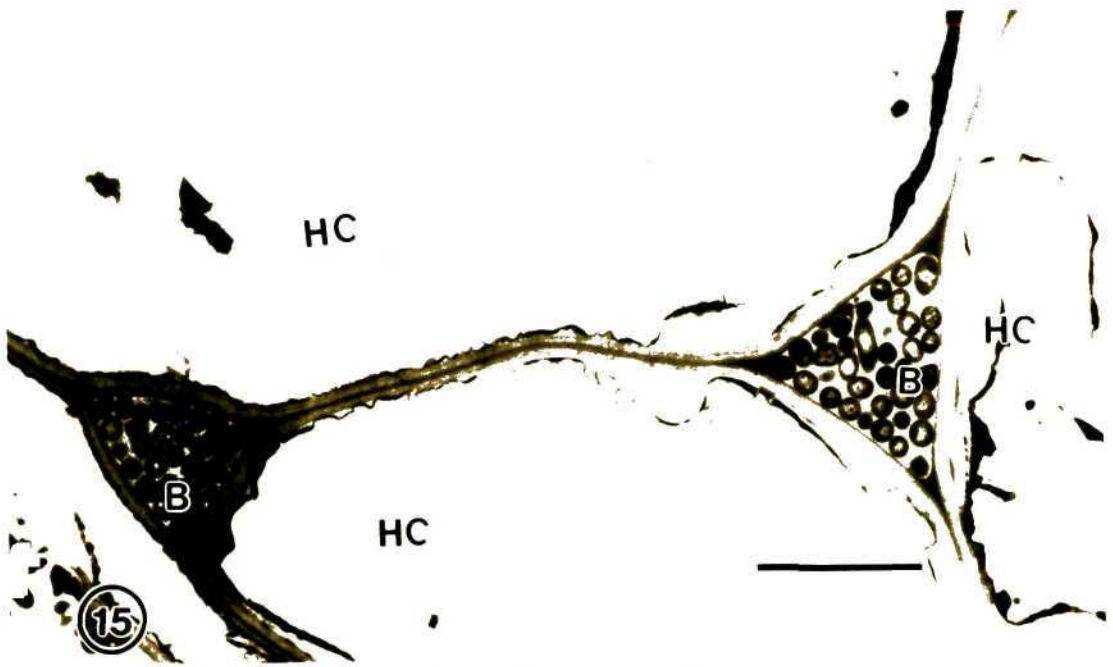


PLATE 5

Figure 15: *Burkholderia cepacia* accumulating in the intercellular space of banana root tissue, 7 days post inoculation. Bar = 10 μm . HC, the host cell; IS, intercellular space; B, the bacterium.

Figure 16: *Burkholderia cepacia* accumulating in the intercellular space of banana root tissue, 7 days post inoculation. 15. Bar = 48 μm . HC, the host cell; B, the bacterium.



in 3% glutaraldehyde in a 0.05 M sodium cacodylate buffer (pH 7.2). The specimens were rinsed twice in the sodium cacodylate buffer for 30 min per rinse, and post-fixed for 3 hours in 2% osmium tetroxide in the buffer at room temperature. The samples were then rinsed twice in a 0.05M sodium cacodylate buffer (PH 7.2). Samples were dehydrated in an ethanol series (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%) followed by absolute ethanol twice for 30 min per step, and embedded in Spurr's resin (Spurr, 1969). Ultrathin sections were cut using glass knives and thin sections were stained with 2% uranyl acetate in double-distilled water for 15 minutes, washed in double-distilled water, and post-stained in lead citrate for 15 minutes, washed in double-distilled water, and examined with a Jeol 100 C transmission electron microscope.

2.3 Results

On the different media (TSB, YM), after 3-4 days of cultivation, *F. oxysporum* f. sp. *ubense* grew rapidly and many macrospores of *F. oxysporum* f. sp. *ubense* were produced.

Scanning electron microscopy (SEM) demonstrated that when *F. oxysporum* f. sp. *ubense* was co-inoculated with *B. cepacia* the bacterium was able to colonize the surface of hyphae (Figure 1) of *F. oxysporum* f. sp. *ubense* and fungal macrospores (Figure 2) and microspores (Figure 3). Mycelial deformation (Figure 1, arrow), terminal and/or intercalary swelling (Figure 4, arrow) of *F. oxysporum* f. sp. *ubense* were evident, as compared with the control (Figure 5). At a later stage, the hyphae of *F. oxysporum* f. sp. *ubense*, colonized by *B. cepacia*, were observed to collapse (Figure 1, Figure 3, arrow). Transmission Electron Microscopy (TEM) confirmed that, compared with the control (Figure 6),

B. cepacia was closely associated with the pathogenic fungus, *F. oxysporum* f. sp. *cubense* (Figure 7). The cell wall of the fungus apparently thickened (Figure 8), after the bacteria was co-inoculated with the fungus for 7 days. Banana roots that were inoculated *in vivo* with *B. cepacia* showed that the bacterium was found to be closely associated with the healthy banana roots (Figure 9), and a matrix between the bacterium and the host are frequently present on the root surface (Figure 10). Transmission electron microscopy (TEM) indicated that, after 96 h post inoculation with the fungus *F. oxysporum* f. sp. *cubense*, the fungus invaded the intercellular space and entered the cell cytoplasm in the host tissues (Figure 11). The fungus then ramified throughout the host root tissues (Figure 12). However, when the bacterium and fungus were co-inoculated on to the banana roots, scanning electron microscopy (SEM) showed that, after 72 h post inoculation, the bacterium colonized the fungal hyphae which was present on the surface of the banana roots (Figure 13). The portion of the hypha, which was colonized, was deformed (Figure 14), and very few fungal segments of *F. oxysporum* f. sp. *cubense* were found in the intercellular space of the root tissues cell and in the cell cytoplasm. Moreover, transmission electron microscopy (TEM) also showed that, *in vivo*, after 7 days post inoculation on the banana roots, *B. cepacia* was present mainly in the intercellular space of the host tissues (Figure 15, Figure 16), and there was no evidence of *B. cepacia* elsewhere.

2.4 Discussion

Owing to enhanced environmental awareness, research and development efforts on the use of biological alternatives for chemical pesticides to control crop disease have increased. One focus of biological control is the use of introduced

antagonists; a major group of natural antagonists currently being investigated is plant-associated, including phylloplane and rhizosphere bacteria. A number of reports demonstrated that bacteria naturally inhabit healthy plant tissues, including tubers, stems, and roots, but very little information on the colonization of roots and pathogenic hyphae by *B. cepacia*.

In this study, we found that, when the bacteria was co-cultured with the fungus, the bacterium *B. cepacia* could colonize the surface hyphae of *F. oxysporum* f. sp. *cubeense*. Colonization might prevent the mycelium from both growth and infection of the banana roots and might further inhibit pathogen fungal macrospores from germinating.

With transmission electron microscopy (TEM), we further found that, after 96 hours post inoculation, the fungus *F. oxysporum* f. sp. *cubeense* invaded the intercellular space and cell cytoplasm in the host tissues of the banana plant. However after inoculation with both *F. oxysporum* f. sp. *cubeense* and *B. cepacia* there was little fungal segments being present in the intercellular space and cell cytoplasm in the host tissues. This may indicate that infection on the host by the pathogen was inhibited by *B. cepacia*. Final collapse of the hyphae might have been caused by antifungal compounds (Jayaswal *et al.*, 1993) and our preliminary studies, which investigated the antagonistic interaction via antifungal production in more detail also indicates this possibility.

In this study, *B. cepacia* was observed to be closely associated with healthy banana roots and a matrix between the bacterium and the host banana is present on the root surface. The function of matrix from the bacterium is unknown. Sequeira, *et al.* (1977) previously reported that non-pathogenic and

avirulent strains of *Pseudomonas solanacearum* became strongly attached to the host cell walls. The host cells responded initially by producing a matrix that enveloped and entrapped the bacterium cells, which subsequently became disorganized. If the matrix, which is present in this study, plays a similar role, it requires further investigation. Some biocontrol agents induce a sustained change in the plant, increasing its tolerance to infection by a pathogen. Induction of host plant resistance against other fungal plant pathogens by *P. fluorescens* and *Pseudomonas putida* has been suggested (Anderson & Guerra, 1985; Voisard *et al.*, 1989). Van Peer *et al.* (1991) reported experimental evidence for the implication of bacterial induction of host plant resistance against fusarium wilt. In our study whether or not *B. cepacia* induces resistance in the banana requires further investigation.

Endophytic bacteria have mainly been isolated from surface-disinfected plant tissues (Misaghi & Donndelinger, 1990; McInroy & Kloepper, 1995a, 1995b). Bacterial endophytes have been detected on the root surface (Old & Nicolson, 1978). The bacterial cells of the endophytes were found in intercellular spaces of the root. This was found with other endophytes in different plant species (Patriquin & Döbereiner, 1978; Gagné *et al.*, 1989). The results of our investigation showed that *B. cepacia* was present in the intercellular spaces of the host (banana) root tissues. This provided evidence that *B. cepacia* inhabits in the healthy banana plant tissues. It still remains unknown however whether this bacterium could enter the vascular tissue by colonizing the undifferentiated root-tip tissues, and whether single cells move through the conducting elements of the plant. Antagonistic bacteria are thought to protect against root pathogens more effectively if they have a strong ability to colonize the root system (Suslow, 1982, Weller, 1988). In addition to the colonization of plant roots, the

colonization of fungal hyphae could be an important mechanism for maintaining a close association between antagonistic bacteria and fungal pathogens.

In conclusion, both scanning and transmission electron microscopy showed that *B. cepacia*, even though isolated from asparagus, was shown to be closely associated with banana roots as well as with the fusarium wilt pathogen, *F. oxysporum* f. sp. *cubense*. In addition, *B. cepacia* interacted with the fungal pathogen, on the surface of the banana and in the banana tissues. This endophyte appears to have useful colonization properties that may render it effective as a biological control agent in bananas. Further studies are required to determine the role of secondary metabolites produced by *B. cepacia* in antagonism and deformation of the fungal pathogen.

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3. THE INHIBITORY ACTIVITIES OF ENDOPHYTIC BACTERIUM *BURKHOLDERIA CEPACIA*

3.1 Introduction

Fusarium wilts are caused by pathogenic *Fusarium oxysporum*, which may result in significant losses to crops. *F. oxysporum* can produce chlamydospores, which permit pathogenic forms of fusaria to persist in most soil long after the susceptible crop has been removed. It is difficult to control these soil-borne diseases. Previous observations and experiments have indicated that some measures, including chemical pesticides, agricultural practices, and use of resistant varieties may reduce the severity of diseases and increase the crop yields.

Biocontrol involves harnessing disease-suppressive microorganism to improve plant health. There are many ways in which an antagonistic organism could operate rapid colonization in advance of the pathogen or subsequent competition or combat may lead to niche exclusion, antibiotics may be produced or there may be mycoparasitism or the lysis of the pathogen (Elad, 1986). In many biocontrol systems that have been studied, one or more antibiotics have been shown to play a significant role in disease suppression (Thomashow & Weller, 1988; Keel *et al.*, 1990). Antibiotics are generally considered to be metabolites that can inhibit microbial growth. Often these are secondary metabolites produced by antagonists when nutrients become limiting and are frequently of relatively low molecular weight. The production of antibiotic metabolites, such as phenazine carboxylic acid (Thomashow & Weller, 1988), pyrolnitrin (Howell & Stipanovic, 1980), pyrrolnitrin[3-chloro-4 (2'-nitro-3'-

chlorophenyl)-pyrrole] (Homma *et al.*, 1989; Howell & Stipanovic, 1979), 2,4-diacetylphloroglucinol (Keel *et al.*, 1990; Vincent *et al.*, 1991), oomycin A (Howie & Suslow, 1991), and cyanide (Voisard *et al.*, 1989), has been shown to be involved in the suppression of fungal pathogens. The first antibiotics described were the phenazines produced by *Pseudomonas fluorescens* and *Pseudomonas aureofaciens*, which controlled take-all of wheat (Weller & Cook, 1983; Brisbane & Rovira, 1988). *P. fluorescens* can produce hydrogen cyanide, 2,4-diacetylphloroglucinol, and pyoluteorin, which directly interfere with growth of various pathogens and contribute to disease suppression (Voisard *et al.*, 1989; Keel *et al.*, 1990; Maurhofer *et al.*, 1994). It was also reported (Thomashow *et al.*, 1990) that *Burkholderia cepacia* (*Pseudomonas cepacia*) produced a phenazine antibiotic, and was implicated in the suppression of take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici*. In addition, there have been other antibiotics isolated from *B. cepacia*, such as cepacin A, cepacin B (Parke *et al.*, 1984), altericidins (Kirinuki *et al.*, 1977), 2-[2-heptenyl]-3-methyl-4-quinolinol (Hashimoto & Hattori, 1967), pyrrolnitrin [3-chloro-4-(2'-nitro-3'-chlorophenyl)-pyrrole] (Fernandez, 1990; Homma *et al.*, 1989) and 2-[2-nonenyl]-3-methyl-4-quinolinol (Homma *et al.*, 1989). It is thought that antagonism of *B. cepacia* against phytopathogens is mainly due to production of the antifungal substances, which may be antimicrobial peptide/enzymes or secondary metabolites.

In this study, the antifungal activity of the endophytic bacterium *B. cepacia* was described, and preliminary characterization of antifungal substances produced by *B. cepacia* was reported.

3.2 Materials and methods

3.2.1 Strains and growth conditions

The banana endophytic bacterium *B. cepacia* used in this study was isolated by AECl's Plant Biotechnology Section (Modderfontein, South Africa) from grown asparagus (*Asparagus officinalis* L.) plants grown *in vitro*. This cultivar is known to have increased resistance to *Fusarium* (*Asparagus officinalis* L.; cultivar Calet). For isolation of *B. cepacia*, tissue culture plants grown *in vitro* were sampled and homogenized in peptone water and dilutions of the homogenate were plated onto GSP agar for selection of pseudomonads. Colonies found on GSP agar were identified using the API 20 NE identification system. The most prominent organism present was identified to be *Pseudomonas cepacia*, recently reclassified as *Burkholderia cepacia*. (Isolation of *B. cepacia* in this study was conducted by AECl's Plant Biotechnology Section, Modderfontein, South Africa.)

The pathogens used in this study were cultured on Potato Dextrose Agar (PDA, Appendix I) at 26°C. *Fusarium oxysporum* f. sp. *ubense* race 4, *Mycosphaella fijiensis* and *Mycosphaella musicola* were provided by AECl's Plant Biotechnology Section, South Africa; *Phytophthora nicotianae*, *Botrytis cinerea*, *Rhizoctonia solani* spp. and *Sclerotinia* spp. were provided by Dr. G Hu, Department of Microbiology and Plant Pathology, University of Natal, South Africa.

3.2.2 Assay of antagonism of *Burkholderia cepacia*

3.2.2.1 Assay of antifungal activity of *Burkholderia cepacia* against fungal pathogens

Fungal inhibition was investigated *in vitro* by inoculating the fungal pathogens on the centre of Potato Dextrose Agar plates (PDA, Appendix I). Bacteria were then inoculated onto the agar at a distance of 10-15 mm from the centre. The plates were incubated at 26 °C for 3-5 days. To ascertain the involvement of siderophores in antagonism, Tryptone Soya Agar (TSA, Appendix I) medium was supplemented with 0, 50, 100 and 150 µM FeCl₃ and then inoculated with *F. oxysporum* f. sp. *ubense* and *B. cepacia*.

3.2.2.2 Effect of different media, pH and temperature on the antifungal activity of *Burkholderia cepacia*

Fungal inhibition was investigated *in vitro* by inoculating *F. oxysporum* f. sp. *ubense* on the centre of agar plates of the following media: TSA, YM, PDA, LA, PSA, KMB, PPM, NYGA agars (Appendix I). Bacterial cells of *B. cepacia* were inoculated onto the agars at a distance of 10-15 mm from the centre. Individual plates of each medium were incubated at 15, 20, 26, 32, 37, and 42 °C respectively. Fungal inhibition was also investigated *in vitro* by inoculating *F. oxysporum* f. sp. *ubense* and *B. cepacia* on TSA agars with pH 3.5, 4.5, 5.5, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and the plates were incubated at 26 °C for 3-5 days.

3.2.2.3 Assay of inhibition of the fungal spore germination of *Fusarium oxysporum* f. sp. *ubense* by *Burkholderia cepacia*

Mycelium of *F. oxysporum* f. sp. *ubense* was grown in YM broth and the fungal spores were harvested after 3-5 days incubation. To obtain a spore suspension, cultures were filtered with sterilized glasswool. The spores were treated with *B. cepacia* supernatant, following by incubation for 6-9 hours at 26°C. The spores were examined by light microscopy under 100X and 400X magnification.

3.2.3 Scanning electron microscopy (SEM)

All fungi were co-cultured with *B. cepacia* in petri plates on TSA agar. Small pieces of agar with mycelia were cut from the margins of the colonies which were adjacent to the bacterial colony. Samples were fixed and stored in glutaraldehyde (3%, v/v) in 0.1 µM sodium cacodylate pH7.2, and the samples processed further for scanning electron microscopy as described previously (Chapter 2, 2.2.2).

3.2.4 Preparation and purification of putative antimicrobial peptides produced by *Burkholderia cepacia*

B. cepacia was grown in 100 ml Tryptone Soya Broth (TSB) for four days on a rotary shaker at 26 °C. The fermentation broth was centrifuged at 8000 g for 10 minutes in Beckman JA-10 centrifuge and the supernatant was collected and loaded onto an Amberlite XAD-2 column. The column was washed three times with 50% methanol (300ml). Active fractions were eluted (1.65 ml/min) with 95% methanol pH 9.5. Active fractions were collected and concentrated by

freeze drying. The biological activity of the concentrated fractions was tested by spotting onto TSA medium cultured with the fungus *F. oxysporum* f. sp. *cubense*.

3.2.5 Extraction and isolation of anti-fungal compounds

B. cepacia was grown in 50 ml TSB for 4 days on a rotary shaker at 26°C. The fermentation broth was centrifuged at 8000 g for 7 min in a Beckman JA-10 centrifuge and the supernatant was collected, acidified to pH2 with 1N HCl and extracted with an equal volume of ethyl acetate. The ethyl acetate extracts were reduced to dryness under vacuum. The residue was dissolved in methanol.

B. cepacia was spread on the surface of TSA agar, and incubated at 26°C for four days. Cells on the agar surface of each plate were suspended in sterile deionized water and collected by centrifugation. Antibiotic compounds were extracted from pellet cells with 5ml of 80% acetone. The acetone extracted was reduced to dryness under vacuum. The residue was dissolved in methanol. The residues from the ethyl acetate extracts and the acetone extracts were combined and processed further for TLC (thin layer chromatography) analyses. Samples (10 ml) were separated on reverse-phase glass TLC plates (MERCK, Silica Gel 60), developed in the developing solvents and visualized by short wavelength ultraviolet light (280nm).

3.2.6 GC- MS analyses

The samples , which were extracted from culture of *B. cepacia* with ethyl acetate and 80% acetone, were dissolved in methanol (2ml) and filtered through a small plug of glass wool. One microliter of each sample was injected (splitless) into a

PLATE 6

Figure 3.1: Inhibition zones on the mycelial growth of *Fusarium oxysporum* f. sp. *cupense* around colonies of *Burkholderia cepacia*.

Figure 3.2: A: Growth of *Fusarium oxysporum* f. sp. *cupense*, co-inoculated with *Burkholderia cepacia* in TSB broth. B: Normal growth of *Fusarium oxysporum* f. sp. *cupense* in TSB broth (Control).

Figure 3.3: Normal hyphae of *Phytophthora nicotianae*. Bar = 4 μ M. H, hyphae.

Figure 3.4: Collapsed hyphae of *Phytophthora nicotianae* (arrow) after treatment with an inoculum of *Burkholderia cepacia*. Bar = 4 μ m. H, hyphae.

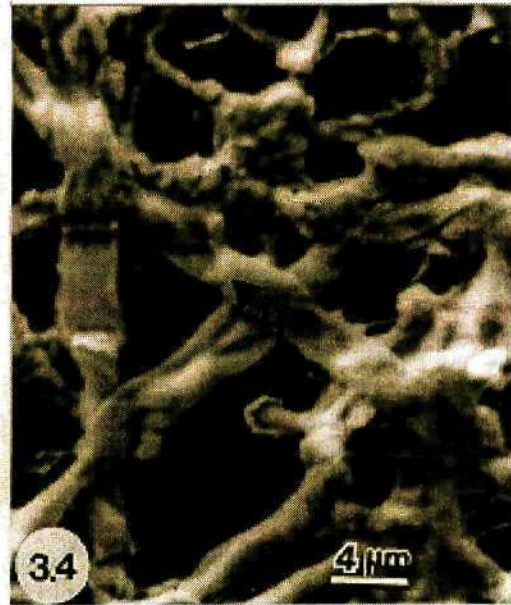
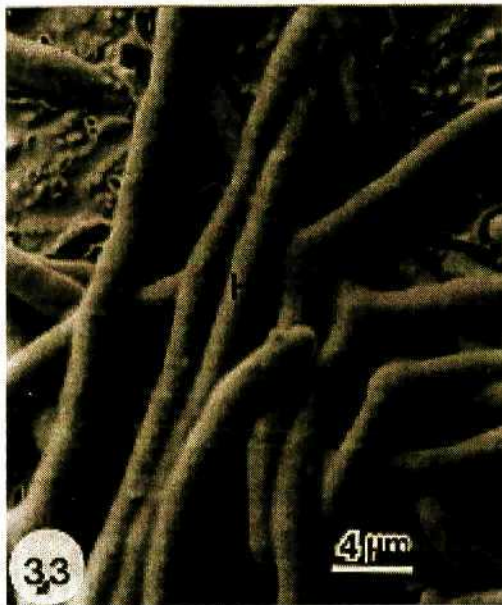
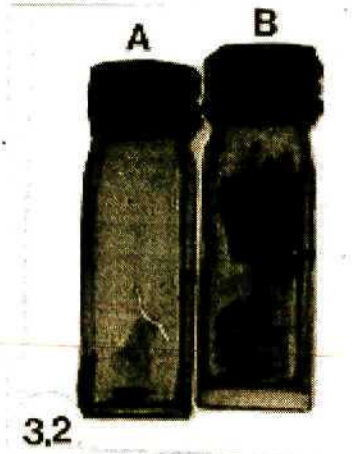
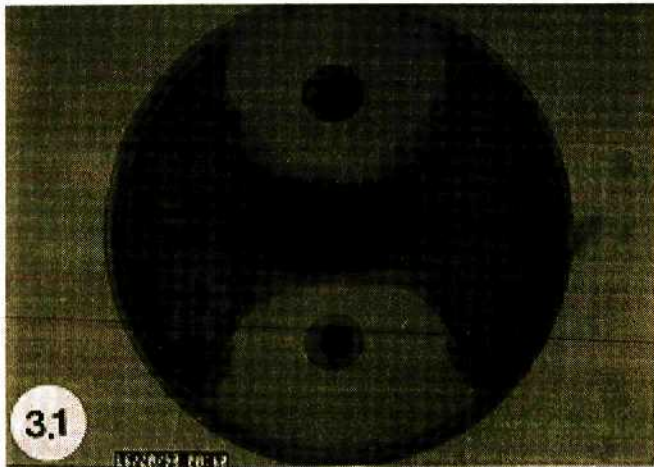


PLATE 7

Figure 3.5: Normal hyphae with spores of *Botrytis cinerea*. Bar = 4µm. S: spore; H: hyphae.

Figure 3.6: Collapsed hyphae and spore of *Botrytis cinerea* (arrow) after treatment with an inoculum of *Burkholderia cepacia*. Bar = 4µm; S, spore; H, hyphae.

Figure 3.7: Normal hyphae of *Mycospherella musicola*. Bar = 6 µm. H, hyphae.

Figure 3.8: Collapsed hyphae of *Mycospherella musicola* (arrow) after treatment with an inoculum of *Burkholderia cepacia*. Bar =6 µm. H, hyphae.

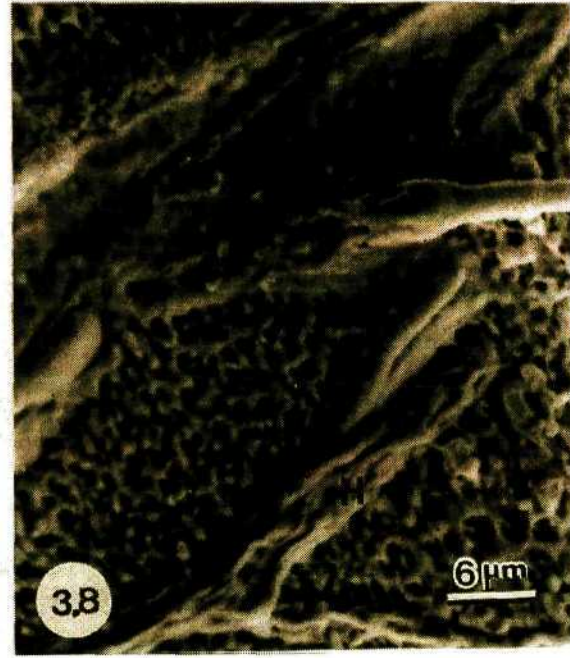
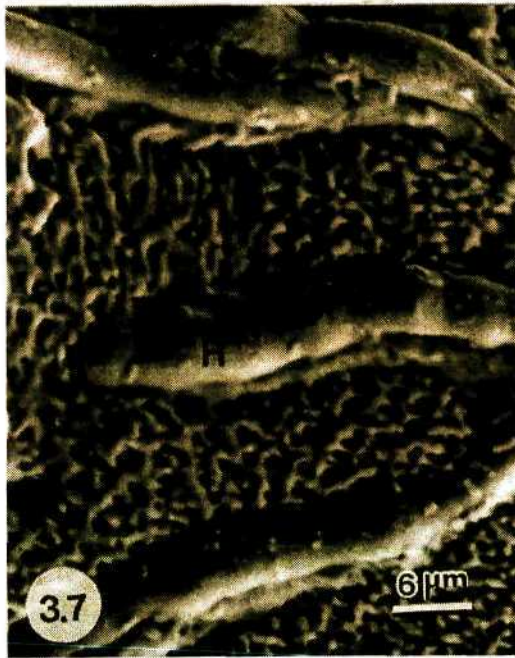
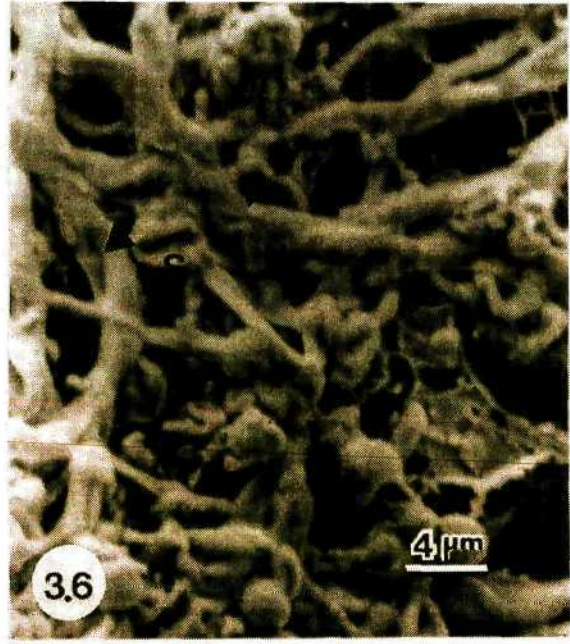
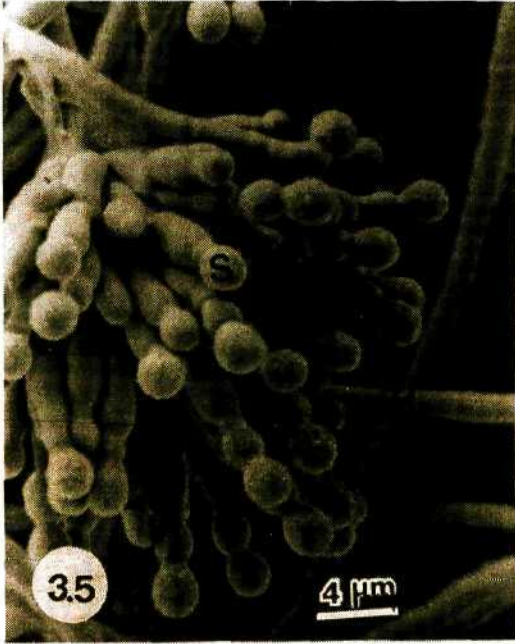


PLATE 8

Figure 3.9: Early fungal growth of the spores of *Fusarium oxysporum* f. sp. *ubense*, showing normal growth. Bar = 50 μ M.

Figure3.10: Growth of fungal spores of *F. oxysporum* f. sp. *ubense* after treatment with the supernatant of wild type *Burkholderia cepacia*. Bar = 50 μ M.

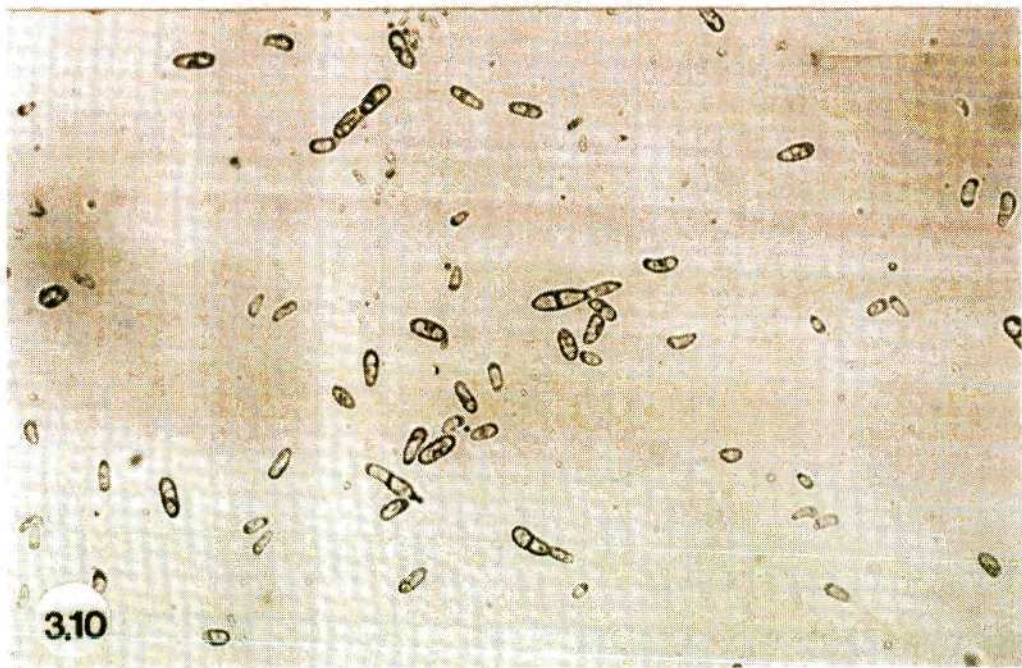
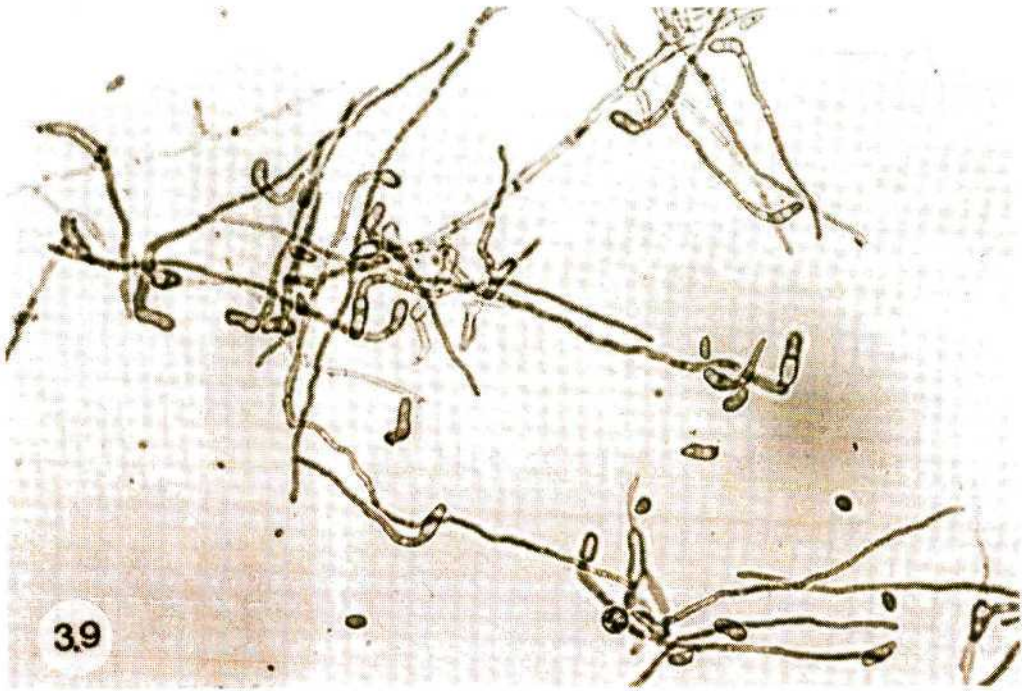


PLATE 9

Figure 3.11: Reduced inhibition zone around a *Burkholderia cepacia* colony (▲) when treated with proteinase K, as compared with control (bottom colony).

Figure 3.12: Growth of *Fusarium oxysporum* f. sp. *cubense* was slightly inhibited by the concentrated fraction from the supernatant of *Burkholderia cepacia* indicated by the arrow.

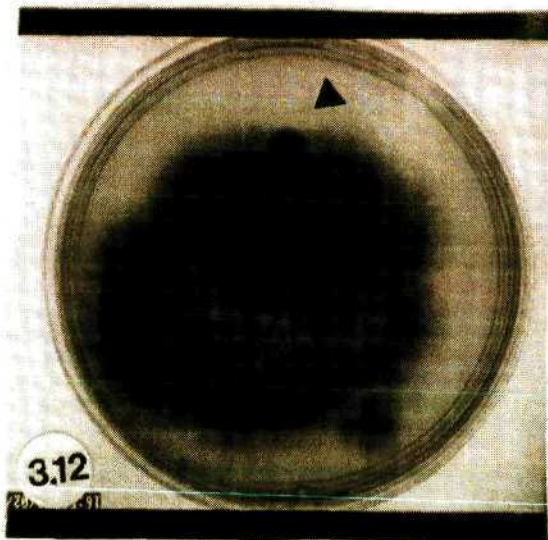
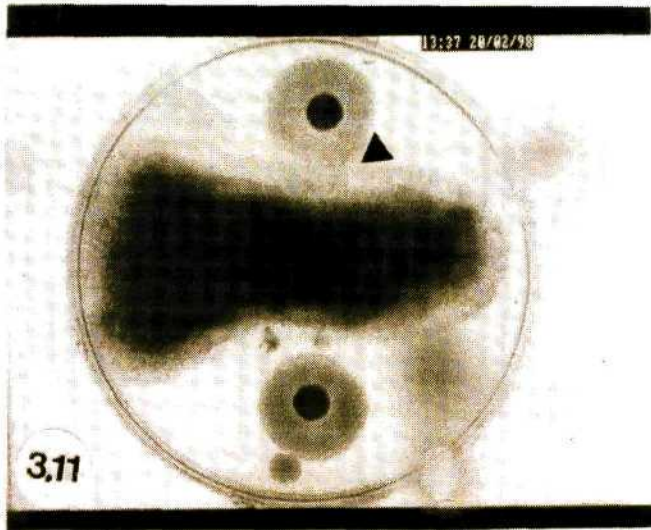
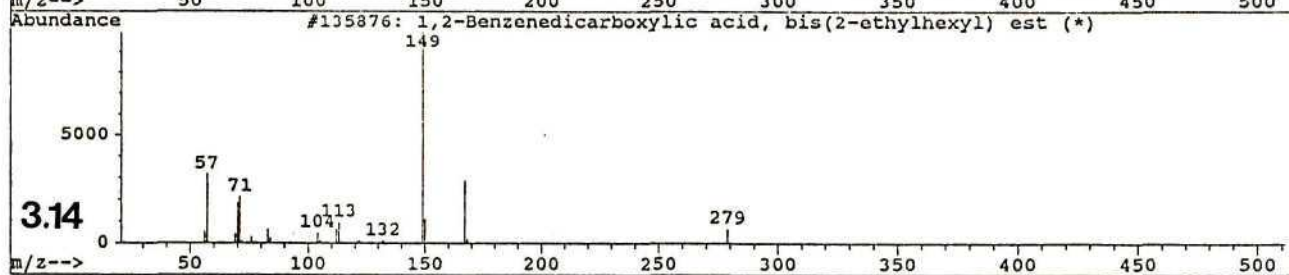
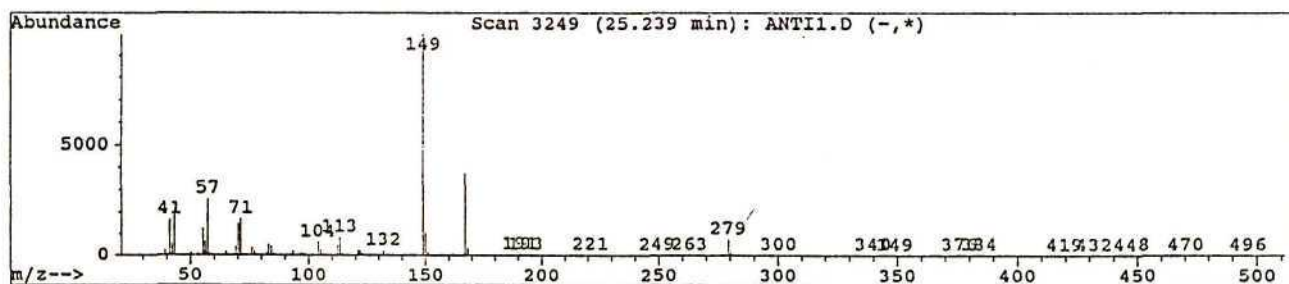
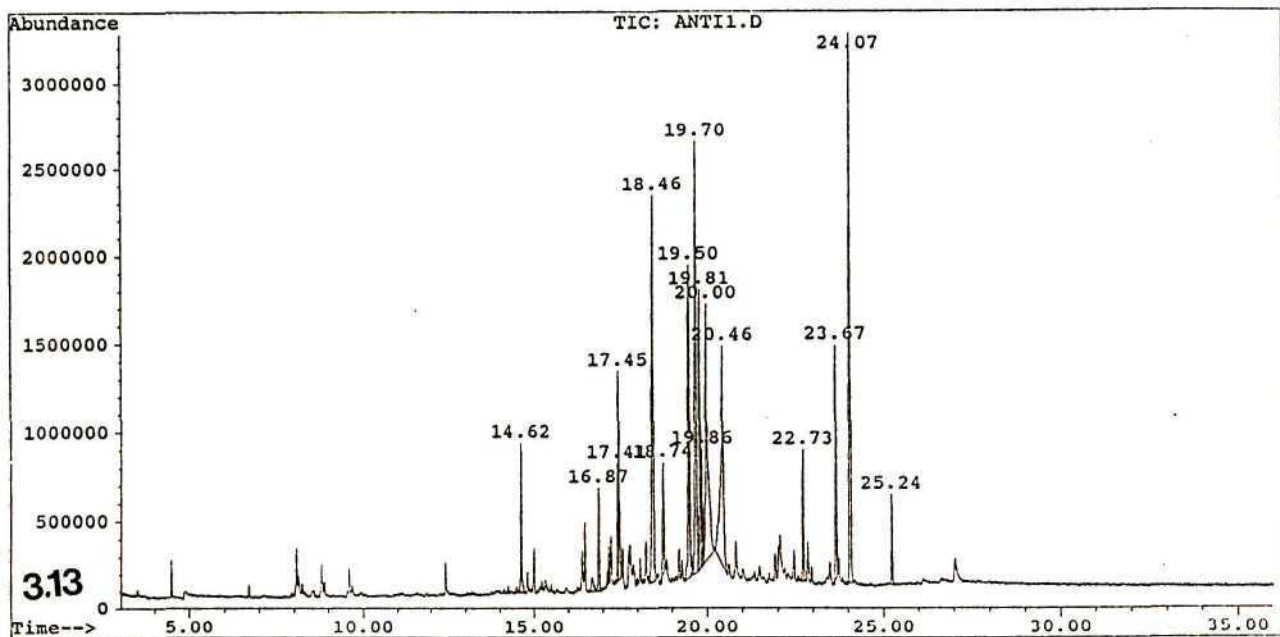


PLATE 10

Figure 3.13: Gas chromatography of extracts from 4-days culture of the wild type of *Burkholderia cepacia* indicated numerous components in the antifungal substance extraction.

Figure 3.14: Mass spectrum of the identified compound from gas chromatography of extracts from 4-days culture of the wild type of *Burkholderia cepacia*.



Hewlett Packard Gas Chromatograph (5890) interfaced to a mass selective (5970). Chromatographic separation was achieved using a DB column (J&W Scientific) of dimensions 30 m x 0.25 mm x 0.25 mm. An injector temperature of 260 degrees was used and an oven temperature program of 40 degrees for 1 minute, followed by a ramp of 25 degree/minute to a final temperature of 290 degrees and held for 5 minutes. EI spectra were collected at 70 eV and peak identification was achieved using the Wiley 138 spectral library (These analyses were conducted by Mr. J Wilson, Umgeni Water, Pietermaritzburg).

3.3 Results

The antagonistic properties of *B. cepacia* against various important fungal phytopathogens were detected on PDA agar plates. *B. cepacia* was able to inhibit the growth of several fungal pathogens, as shown in Table 3.1. The antagonistic effect of *B. cepacia* was most pronounced against *F. oxysporum* f. sp. *ubense*, *Mycosphaella musicola*, *Mycosphaella fijiensis*, *Phytophthora nicotianae*, *Botrytis* spp. and *Rhizoctonia solani*. *B. cepacia* strongly inhibited the growth of *F. oxysporum* f. sp. *ubense*, *M. musicola*, *P. nicotianae* and *Botrytis cinerea*. The inhibition of *F. oxysporum* f. sp. *ubense* by *B. cepacia* occurred with a clear inhibitory zones (Figure 3.1). It was also found that, when *F. oxysporum* f. sp. *ubense* was co-inoculated with *B. cepacia*, the density of the fungal mycelia was decreased (Figure 3.2 A), compared with the control (Figure 3.2 B).

The mycelia from the edge of the colonies towards the bacteria on the PDA agar were examined under an electron microscopy aid with a Cry-SEM (scanning electron microscopy). Deformities in the hyphae of the pathogens were noted.

The fungal hypha and/or spores appeared to have collapsed (Figure 3.4, 3. 6, 3.8), compared with the control (Figure 3.3, 3.5, 3.7).

Table 3.1 Antifungal activities of *Burkholderia cepacia* against various pathogens in the petri plate assay

Pathogen	Inhibition
<i>Fusarium oxysporum</i> f.sp. <i>cubense</i>	++
<i>Mycospharella fijiensis</i>	++
<i>Mycospharella musicola</i>	++
<i>Phytophthora nicotianae</i>	++
<i>Botrytis cinerea</i>	++
<i>Rhizoctonia solani</i>	+
<i>Sclerotinia</i> spp.	+

++, good inhibition; + poor inhibition; - no inhibition.

On the different media (TSA YM broth), after 3-4 days of cultivation, *F. oxysporum* f. sp. *cubense* grows rapidly and many macrospores of *F. oxysporum* f. sp. *cubense* were produced. Light microscopy demonstrated that, when macrospores of *F. oxysporum* f. sp. *cubense* were treated with the supernatant of *B. cepacia* cells, there was little germination of *F. oxysporum* f. sp. *cubense* (Figure 3. 9), compared with the untreated control (Figure 3.10).

Examination of plates containing different media, incubated with *B. cepacia* and *F. oxysporum* f. sp. *cubense*, showed that the inhibition of the fungus occurred on the different media (Table 3.2). It appeared that *F. oxysporum* f. sp. *cubense* was inhibited in YM medium slightly more than the other media. This indicated that antagonistic activity of the bacterium against *F. oxysporum* f. sp. *cubense* was not greatly influenced by nutritional substrate.

Table 3.2 Antifungal activities of *Burkholderia cepacia* against *F.oxysporum* f. sp. *cubense* in the petri assay

Media	Inhibitory zone (mm)
TSA	4.5
YM	5.0
PDA	4.0
LA	3.0
PSA	3.5
KMB	3.5
PPM	4.5
NYGA	4.0

To test the effect of temperature on the inhibition of *F. oxysporum* f. sp. *cubense* by *B. cepacia*, both the bacteria and the fungus were incubated at the following temperatures: 15, 20, 26, 32, 37 and 42°C. *B. cepacia* was able to inhibit the fungal growth at 20, 26 and 32°C, and it was found that the optimum temperature for the inhibition of *F. oxysporum* f. sp. *cubense* by *B. cepacia* was at 26°C, at which temperature *B. cepacia* showed strong inhibitory activity to the fungus on the petri assay plate. At 15°C and 37°C *B. cepacia* grew well, but *F. oxysporum* f. sp. *cubense* did not grow well. Neither *B. cepacia* nor *F. oxysporum* f. sp. *cubense* grew at 42°C.

Antifungal activity by *B. cepacia* was investigated over the pH range 3.5-9.0 in TSA agar. *B. cepacia* and *F. oxysporum* f. sp. *cubense* did not grow at pH 3.5 and 9.0 respectively. It was shown that antagonism was greater in the acidic range (4.5-7.5), antagonism occurred at pH 4.5 and increased to maximum at pH 7.0, above pH 7.5 the antagonism by *B. cepacia* decreased and the fungus grew slowly. At pH 8.0 and 8.5 there was no antagonism even though *B. cepacia* grew well and the fungus did not grow well. These results indicated that antagonism of *B. cepacia* against *F. oxysporum* f. sp. *cubense* is affected by pH

of the medium, and acidic pH appeared to favour the expression of the antagonistic compounds by *B. cepacia*.

It was observed that *B. cepacia* was able to inhibit the growth of the fungi on TSA agar supplemented with concentrations of iron between 0-150 μ M. The inhibitory activity of *B. cepacia* was not affected by the level of iron. This suggests that siderophores may not be involved in antagonism. In addition, when proteinase was added to the inhibition zone between the bacterial cells and the fungal mycelia, it was found that, after 3-5 days incubation, the inhibitory activity was slightly reduced and the inhibition zone appeared to be partly covered with the new fungal growth (Figure 3.11). In addition, when the concentrated fractions from the supernatant of *B. cepacia* were spotted onto TSA agar, which was cultured with the fungus *F. oxysporum* f. sp. *cupense*, it was found that the fungal growth was inhibited (Figure 3.12). Furthermore the antifungal activity was not as strong as when the bacterial living cells spotted. This indicated that proteins/peptides could contribute to the antifungal activity of *B. cepacia*.

The possibility of the production of antibiotic substances by *B. cepacia* was investigated. Crude extracts from 4-day-old culture of *B. cepacia* grown on TSA plates was partially characterized by thin layer chromatography (TLC). TLC was performed by using extracts from the supernatant and cells of *B. cepacia*. Table 3.3 showed that extracts from *B. cepacia* were separated in several developing solvents. It was found that among the several solvent systems used, benzene-ethyl acetate (1:1) appeared to be most effective in separating the active spot from the extracts. In this solvent system, there was an absorbed UV spot which appeared as a bright spot at R_f of 0.24; 0.45; 0.60; 0.81. Pyrrolnitrin has been

described as having a R_f of 0.59 (Burkhead *et al.*, 1994). In acetonitril-methanol-water (1:1:1) solvent system, there were absorbed UV bright spots at R_f values of 0.80; 0.90; and an extending streak from R_f 0.20-0.50. In chloroform-ethyl acetate (1:1) solvent system, there were absorbed bright spots at R_f values of 0.25; 0.55, and an extending streak from R_f 0.70-0.90. In chloroform-ethyl acetate-formic acid (5:4:1) solvent system, there was an absorbed UV bright spot at an R_f value of 0.55, and two extending streaks from 0.10-30; and from 0.70-0.90. In the chloroform and chloroform-acetone (9:1) solvent systems, only an extending streak from R_f 0.00-0.90 was found.

Table 3. 3 Migration of antifungal compounds produced by *Burkholderia cepacia* on silica gel thin layer chromatophy

Solvent system	R_f
Acetonitril-mathanol-water (1:1:1)	0.80; 0.90; 0.2-0.5 0.24; 0.45; 0.60; 0.81
Benene-ethyl acetate (1:1)	0.25; 0.55;.0.70-0.90
chloroform-ethyl acetate (1:1)	0.10-0.30; 0.55; 0.70-
chloroform-ethyl acetate-formic acid (5:4:1)	0.90;
Chloroform	NC
Chloroform-acetone (9:1)	NC

NC: separation was not clear

GC-MS analyses showed numerous components in the antifungal substance extraction and the retention times of those with a high abundance are indicated on the chromatogram (Figure 3.13). Most of these compounds were not present in the Wiley 138 spectral library. Only 1,2-Benzenedicarboxylic acid, bis(2-Ethylhexyl) Ester was identified (Figure 3.14). The mass spectra of the remaining unidentified components are provided in the appendices (Appendix II).

3.4 Discussion

In this study, the results showed that *B. cepacia* was able to inhibit the growth of several pathogens including *F. oxysporum* f. sp. *cubense*, *M. fijiensis*, *M. musicoia*, *P. nicotianae* and *Botrytis cinerea*. The investigation of antifungal activity with Cryo-SEM demonstrated that *B. cepacia* caused deformation of the hyphae of several pathogenic fungi. The broad antifungal activity spectrum of *B. cepacia* indicated that *B. cepacia* could be a potential biocontrol agent as crop losses are frequently due to the presence of more than one pathogens in the field. In addition, it was observed that fungal spore germination of *F. oxysporum* f. sp. *cubense* was inhibited by the supernatant of *B. cepacia* cultures. The inhibition of fungal spore germination might prevent fungal mycelia growth and subsequent infection of the host plant.

In the present study, the growth and production of antifungal activity by *B. cepacia* occurred on different media. This suggests that *B. cepacia* could be one of the most nutritionally versatile bacteria, and capable of using different carbohydrates as a carbon source to produce antagonism. In addition, *B. cepacia* was able to grow at temperature range 15 to 37° C, and the inhibition of *F. oxysporum* f. sp. *cubense* by *B. cepacia* occurred at 20 to 37° C. It was also found that *B. cepacia* was able to grow at pH range 3.5 to 8.5, although there was no antagonism shown by the bacterium *B. cepacia* at pH 8.0 or above, and the antifungal activity by *B. cepacia* was greater at acidic than at alkaline pHs. It is likely that acidic pH favoured the expression of the antagonistic compounds by *B. cepacia*. Environmental pH may structurally modify the antifungal activity and/or it may affect the antibiotic production of *B. cepacia* and alter its ability to produce the antagonistic compounds. This is a noteworthy observation for the

potential use of *B. cepacia* as a biological control. Natural soil environments with acidic pH will probably favour expression of *B. cepacia* antagonism. This could contribute to the success of the biocontrol system.

In this study, *B. cepacia* was able to inhibit the fungal growth of *F. oxysporum* f. sp. *ubense* at the concentration of FeCl_3 between 0-150 μM . This suggests that siderophores were not involved in the antagonism of *B. cepacia*.

The synthesis of an antimicrobial peptide is also a possible mechanism of microbial antagonism. It was reported that *B. cepacia* produced antifungal peptides such as altericidin A; altericidin B; altericidin C (Kirinuki *et al.*, 1977; 1984); and bacteriocins (Gonzalez & Vidaver, 1979). In this study, it was observed that the inhibitory activity of *B. cepacia* was affected by treatment with proteinase. The bioassay of the concentrated fractions from the supernatant of *B. cepacia* showed that the concentrated fractions were able to inhibit the fungal growth, although the antifungal activity of the concentrated fractions were not as strong as the bacterial living cells. This suggested that proteins/peptides could contribute to the antifungal activity of *B. cepacia*, but not sufficiently for full antagonism of *B. cepacia*.

Metabolites could be responsible for the antifungal activity of *B. cepacia*. In this study, antibiotic production was investigated by isolating them from crude cell extracts. Examination of the extracts of *B. cepacia* by TLC showed that *B. cepacia* produced a substance that had a R_f value of 0.60. Pyrrolnitrin was described as having a R_f value of 0.59 (Burkhead *et al.*, 1994). Pyrrolnitrin was first isolated in 1964 (Arima *et al.*, 1964) from a new species of pseudomonad, *Pseudomonas pyrocinia* (Imanaka *et al.*, 1965), and has also been isolated

from several *Pseudomonas/ Burkholderia* spp. (Elander *et al.* 1968; Lambert *et al.* 1987). It exhibited activity against *Aspergillus niger*, *Candida albicans*, *Bacillus subtilis*, *Proteus vulgaris* and *Staphylococcus aureus* (Nishida *et al.*, 1965). It has been reported that *Fusarium* species were inhibited by pyrrolnitrin or *B. cepacia* (Homma *et al.*, 1989; Kawamoto & Lorbeer, 1976; Lambert *et al.*, 1987). In addition to pyrrolnitrin, other antibiotic metabolites were suggested to be produced by various strains of *B. cepacia*. These metabolites include the phenylpyrroles (Roitman *et al.*, 1990a, b); acetylenic antibacterial compounds cepacin A and cepacin B (Parke *et al.*, 1984); pseudanes (Homma *et al.*, 1989) and unidentified metabolites (Jayaswal *et al.*, 1990). In the present study, an appropriate control could not be used in the TLC examination since pyrrolnitrin is not available as a commercial product. Identification of antifungal compounds analysed by GC-MS showed that there are numerous components in the substance extraction of *B. cepacia*, one of them was identified as 1,2-Benzenedicarboxylic acid, but most of these compounds were not present in the Wiley 138 library which was used.

In conclusion, this study showed that the bacterium *B. cepacia* was able to inhibit the growth of several pathogens *in vitro*, and may produce active antifungal substances. This indicated that *B. cepacia* could be a potential biological agent. Although *in vitro* agar studies may not reflect the environmental or microbiological conditions in the field, *in vitro* studies could provide useful data on growth parameters of antagonists and these may help with development procedure for a biological control agent. Therefore, *in vitro* bioassays and *in vitro* study are of key importance in developing successful biocontrol agents. A requirement to investigate the ability of *B. cepacia* to suppress disease in greenhouse and field conditions is obvious. Further purification, identification

and classification of antifungal substances, and the role of these metabolites in disease suppression also needs to be investigated.

3.5 References

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4. PRELIMINARY GENETIC ANALYSIS OF THE INHIBITORY ACTIVITY OF *BURKHOLDERIA CEPACIA*

4.1 Introduction

Certain strains of beneficial *Pseudomonas* spp. were able to suppress a variety of soil-borne plant diseases and enhance the growth and yield of agricultural crops (Weller, 1988; Cook, 1993). It was accepted that the production of siderophores, hydrogen cyanide (HCN), antibiotics and colonization have been shown to play a role in disease suppression (Weller, 1988; Cook, 1993). The inhibition of root pathogens by production of antimicrobial and iron-chelating metabolites is considered to be a primary mechanism of disease suppression by these bacteria.

Phloroglucinol antibiotics are phenolic bacterial and plant metabolites with antifungal, antibacterial, antiviral, anthelmintic, and phytotoxic properties (Thomashow & Weller, 1996). The broad-spectrum antibiotic 2,4-diacetylphloroglucinol (PhI) is a major determinant in the biocontrol activity of *Pseudomonas fluorescens* CHAO (Keel *et al.*, 1992). The antibiotics phenazine-1-carboxylic acid (PCA), 2,4-diacetylphloroglucinol (PhI), pyoluteorin (Plt) and pyrrolnitrin (Prn) are currently a major focus of research on biological control.

Support for the role of antibiotics in biological control has come mainly from studies that showed correlations between bacterial inhibition *in vitro* and disease suppression in the soil. Recently, genetic analysis of several *Pseudomonas* strains has established a correlation between antibiotic production and disease

suppression (Thomashow & Weller 1988; Keel *et al.*, 1992). Thomashow and Weller (1988) reported that a Tn5 mutant of *P. fluorescens* 2-79, deficient in the production of phenazine-1-carboxylic acid, was less suppressive to take-all (*Gaeumannomyces graminis* var. *tritici*) than the parental strain. Keel *et al.* (1992) showed that a Tn5 mutant of *P. fluorescens* CHAO, defective in the production of 2,4-diacetylphloroglucinol (Phl), exhibited reduced suppression to black root rot on tobacco (*Thielaviopsis basicola*) in a gnotobiotic system.

The biosynthetic loci for Plt, Prn, PCA, and Phl have been cloned, and the genes involved in PCA and Phl production have been sequenced (Banger & Thomashow, 1996; Boronin *et al.*, 1995; Hammer *et al.*, 1995; Pierson *et al.*, 1995). The availability of cloned and sequenced antibiotic-biosynthetic genes has facilitated the development of specific primers and probes that can be used to detect antibiotic-producing genes.

Recently, it has been demonstrated that *Burkholderia cepacia* can protect different plants from root disease caused by a variety of pathogenic fungi (Jayaswal *et al.*, 1993). Several secondary metabolites produced by *B. cepacia* have been shown to play a role in disease suppression (Homma *et al.*, 1989; Thomashow *et al.*, 1990).

The objective of this present study was to generate mutants of *B. cepacia* deficient in antifungal activity by UV and transposon mutagenesis and compared the wild type and mutants for inhibition of the fungus *Fusarium oxysporum* f. sp. *cubense* race 4. Phl and PCA specific primers were used to detect the presence of these antibiotic genes in the strain *B. cepacia*.

4.2 Methods and materials

4.2.1 Strains and growth media

The banana wilt pathogen, *Fusarium oxysporum* f. sp. *cubense* race 4 and the banana endophytic bacterium *B. cepacia* used in this study are described in a previous chapter (Chapter 2, 2.2.1). Strains were cultured on Tryptone Soya Agar (TSA) or Tryptone Soya Broth (TSB) at 26 °C. *Escherichia coli* W3110/pGL221 was provided by Professor G Défago (Institute of Plant Sciences/Phytopathology Group, Swiss Federal Institute of Technology (ETH), CH-1015 Lausanne, Switzerland), and was cultured on LA (Appendix I) at 37 °C.

4.2.2 Fungal inhibition assay

B. cepacia cultures were grown overnight and 20 µl of culture was spotted on plates of TSA. *F. oxysporum* f. sp. *cubense* was inoculated in the centre of the agar plates. Plates were incubated at 26 °C. The inhibition zones were measured/ observed after 3-5 days. Thin layer chromatography (TLC) analyses was performed by the method described previously (Chapter 3, 3.2.5). Scanning electron microscopy (SEM) investigations were conducted by the methods described previously (Chapter 2, 2.2.2).

4.2.3 DNA manipulations

4.2.3.1 Isolation of DNA

Isolation of total DNA of *B. cepacia* was performed according to the modified

method of Gamper *et al.* (1992). After growth overnight at 26 °C, the cells were pelleted, washed with TE buffer (10mM Tris.Cl pH8.0; 1mM EDTA pH8.0), and resuspended in 600 µl 50 mM Tris.Cl pH8.0; 10 mM EDTA pH8.0; 100 µg/ml Rnase containing 20 µl lysozyme (20 mg/ml) and 88 µl of 10% sodium dodecyl sulfate (SDS), and incubated for 1 h at 37 °C. The total DNA was extracted with phenol/NaCl and re-extracted with phenol/NaCl: chloroform (1:1). The DNA was precipitated by KAC and ethanol, then resuspended in 20-30 µl ddH₂O.

In this study, heat-lysis bacterial suspensions used in PCR analysis were prepared according to Raaijmakers *et al.* (1997). Heat-lysis bacterial suspensions were extracted from cultures grown on TSA for 48 h at 26 °C. Two bacterial colonies (2-mm diameter) were suspended in 100 µl of lysis solution (0.05 M NaOH, 0.25% SDS) and incubated for 15 minutes at 100 °C. The suspension was centrifuged for 1 minutes at 12000 g and diluted 50-fold in sterile distilled water. Five micro litres of the diluted suspension was used in each reaction.

4.2.3.2 Primers and PCR analysis

The oligonucleotide primers list in Table 4.1 were developed from sequences within the biosynthetic loci for PhI of *Pseudomonas fluorescens* Q2-87 (Gen Bank accession no. U41818) and PCA of *P. fluorescens* 2-79 (Gen Bank accession no. L48616). Primers were synthesized at University of Cape Town (South Africa) using an Oligo 1000M DNA Synthesizer (Beckman Inc.). Primers PhI2a and PhI2b were developed from sequences with in *the phlD* gene, which predicts a protein of 349 amino acids that is homologous to chalcone synthase from plants (Bangera & Thomashow, 1996). Primer PCA2a and PCA3b were

developed from sequences within *phzC* and *phzD*, respectively. *PhzC* encodes a 400 amino acid with similarity to 2-keto-3-deoxyarabinoheptulosonate-7-phosphate (DAHP) synthase from *E. coli* and plants. The *phzD* gene encodes 207 amino acids with similarity to isochorismatase from *E. coli* (Boronin, *et al.*, 1995).

PCR amplification was carried out in a 25 µl reaction mixture which contained approximately 20 ng of total DNA, 1X GeneAmp PCR buffer (Promega, Madison), 200 µM each dATP, dTTP, dGTP, and dCTP (Promega, Madison), 20 pmol of each primer, and 2,0 U of AmpliTaq DNA polymerase (Promega, Madison). Each mixture was covered with 1 drop of mineral oil. Amplification cycles were conducted with a Perkin-Elmer thermal cycler 480. The PCR program consists of an initial denaturation at 94 °C for 2 minutes followed by 30 cycles of 94°C for 60s, 60 °C or 67° C for 45s, 72 °C for 60s. Samples (25 µl) of the PCR products were separated on a 1.3% agarose gel in 1 x TAE buffer (0.04M Tris-acetate acid, 0.001M EDTA) at 60V for 3h. The gel was stained with ethidium bromide for 30 minutes, and the PCR products were visualized with a UV transilluminator.

Table 4.1 Description of primers used for PCR analysis

Primer Position ^a	Sequence	G+C (%)	T _m	
Phl2a	5' GAGGACGTCGAAGACCACCA 3'	60	73	1915 ^a
Phl2b	5' ACCGCAGCATCGTGTATGAG 3'	55	72	2660
PCA2a	5' TTGCCAAGCCTCGCTCCAAC 3'	60	79	3191
PCA3b	5' CCGCGTTGTTCTCGTTCAT 3'	55	76	4341

^aPosition of the 5' and of the primer in the database sequence

4.2.4 Colony and Southern hybridization

4.2.4.1 Colony blotting

Colony blots were prepared by inoculating a broth culture of the wild type and mutant of *B. cepacia* onto nitrocellulose membrane (Amersham, Bucks). The membranes were placed for 5 minutes on two sheets of Whatman 3 MM paper which had been saturated with 0.5 M NaOH. The residual colony debris were removed by washing in 5XSSC (SSC is 0.3 M Na₃Citrate, 3 M NaCl [pH 7.0]) for 1 minute at room temperature.

4.2.4.2 Southern blotting

Total DNA of the wild type and the mutants *B. cepacia* were isolated by the method described previously. Total DNA was digested with ClaI at 37 °C for 2 h, and fractionated by agarose gel electrophoresis. Southern blots were then prepared on nitrocellulose membrane (Amersham, Bucks) using 20 X SSC as the transfer buffer (Maniatis *et al.*, 1982). The filters were air dried and baked at 80°C for approximately 2 h.

4.2.4.3 Hybridization

Both Southern and colony blotting membranes were prehybridized with ECL hybridization buffer containing 5% blocking agent (ECL kit, Amersham, Bucks) and 1 M NaCl at 42 °C for 1 h in HB 400 hybridization oven (HOEFER Sciences Instrument, San Francisco). Hybridization with specific DNA probes (PhI2a, PCA2a) was performed for 16 h at 42 °C in a hybridization buffer. The filters

were subsequently washed in stringency primary wash buffer of 0.5XSSC or 0.1XSSC at 42 °C and secondary wash buffer (0.1XSSC) at room temperature. The filters were exposed on Hyperfilm-ECL film in a film cassette at room temperature for 1 h. Hybridization probes were labelled with the enzyme horseradish peroxidase by the ECL direct nucleic acid labelling and detection systems PRN3000 (Amersham, Bucks).

4.2.5 Mutagenesis of *Burkholderia cepacia*

4.2.5.1 Tn5 transposon mutagenesis

For transposon mutagenesis, the donor *E.coli* W3110/pLG221 and recipient *B. cepacia* were grown separately in LB medium at 30 °C for 12-18h. Donor and recipient cultures (0.1 ml each) were mixed and grown overnight in 3 ml of LB medium. One and a half of ml of the mixed overnight culture was centrifuged at 13000 rpm for 30 seconds and resuspended well, spotted on a sterile nitrocellular filter disc, and the disc was plated onto LB agar and incubated at 32 °C for 16-20 h. Transconjugants were selected on LB agar containing kanamycin (300 µg/ml) and rifampicin (100 µg/ml) at 37 °C. In control experiments, either donor or recipient cells were plated directly on LB kanamycin-rifampicin plates. Transconjugants (kanamycin-rifampicin resistant Km^r Rif^r) were picked, and inoculated into 5 ml TSB Broth, and incubated with shaking at 26 °C for 24 h.

Transconjugants also were screened for the loss of their ability to inhibit fungal growth (*F. oxysporum* f. sp. *cupense*) on TSA agar by the petri plate assay described previously. The mutants were also used for further analyses of the inhibition of the fungal spore germination, production of the secondary metabolites and colonization ability.

4.2.5.2 UV (Ultraviolet light) mutagenesis

B. cepacia was grown on TSA agar for over-night, and treated with Ultraviolet light for 5, 10, 15 20 minutes, followed by incubated in dark at 26 °C. The frequency of the surviving cells was measured by irradiating cell suspensions, and the surviving cells (approximately 75% killing) were plated on TSA agar. The number of surviving cell at each UV treatment was determined by washing the cells off after irradiation and growth, and plating the cell on TSA agar after suitable dilution, following incubation at 26 °C for 2-3 days. The mutants were screened, isolated and tested by the methods described previously (4.2.5.1).

4.3 Results

4.3.1 Colony and Southern hybridization

Colony hybridizations were performed at high stringency. The results showed a strong hybridization signal from the wild type and mutants of *B. cepacia* which were able to hybridize with the probes PhI2a (Figure 4.1) and PCA 2a.

When Southern hybridization was preferred at high stringency, in which the primary wash buffer contained 0.1XSSC, there was no evidence that genomic DNA digested with *Cl*I of *B. cepacia* was hybridized with probes PhI2a and PCA2a. However when performed at low stringency (in which primary wash buffer contained 0.5XSSC), a hybridization signal from a band of about 2,800 bp was evident when the PhI2a probe was used (Figure 4.2A, 4.2B), but there was no evidence that *B. cepacia* could hybridized to the PCA2a probe.

PCR amplification was carried out with the primers PCA2a, PCA, and PhI2a, PhI 2b, the results showed that there were no PCR products amplified from the DNA

PLATE 11

Figure 4.1: Colony blot analysis to detect the presence of genes required for the production of 2,4-diacetylphloroglucinol (PhI) by *Burkholderia cepacia*. Culture of the wild type and the mutants of *Burkholderia cepacia* were inoculated onto nitrocellulose membranes. The DNA was denatured by NaOH and was hybridized with the ECL labelled specific probe PhI2a under high stringency conditions.

Lane 1. Control (*E. Coli* JM103).

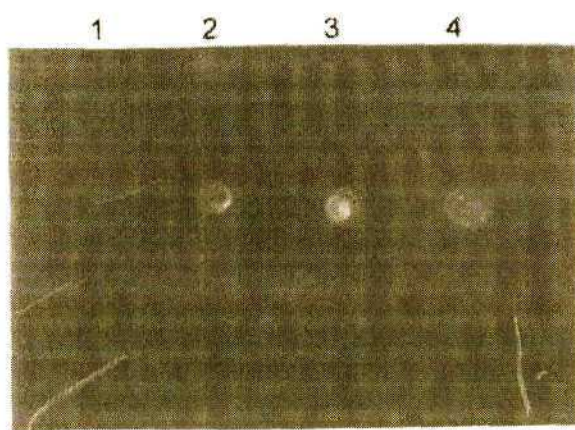
Lane 2. The wild type of *Burkholderia cepacia*.

Lane 3. The Tn5 mutant *Burkholderia cepacia* ::Tn5-217.

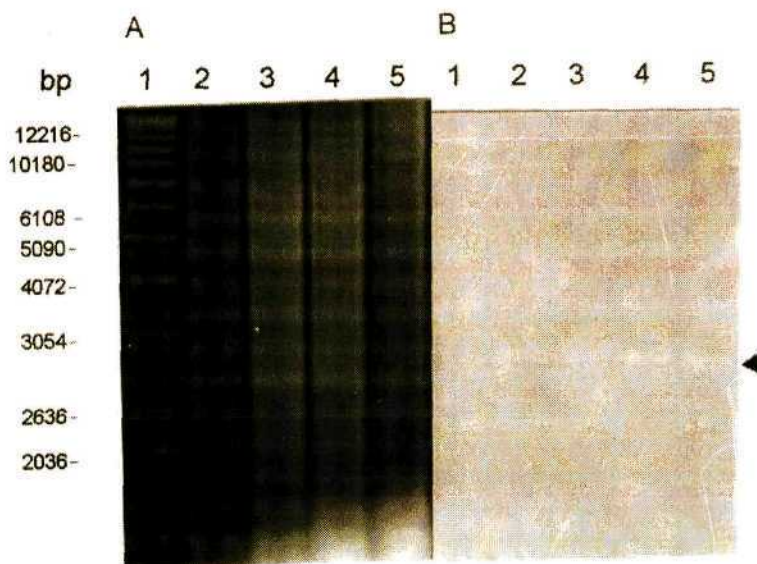
Lane 4. The UV mutant *Burkholderia cepacia*- UV-34.

Figure 4.2: Southern blot analysis to detect the presence of genes required for the production of 2,4-diacetylphloroglucinol (PhI) by *Burkholderia cepacia*. Total DNA of the wild type and the mutants *B. cepacia* (10µg/lane) were digested with *Cl*I. The digested DNA was then fractionated on a 1.1 % agarose gel and transferred to a nylon membrane. The DNA was hybridized with the ECL labelled specific probe PhI2a under low stringency conditions.

Gel A. Lane 1. DNA molecular weight marker X (control), Lane 2. The wild type *B. cepacia*, Lane 3. The Tn5 mutant *B. cepacia* :: Tn5-217, Lane 4. The Tn5 mutant *B. cepacia* :: Tn5-188, Lane 5. The UV mutant *B. cepacia* -UV-34. B. Southern Blotting, PhI2a was used as probe.



4.1



4.2

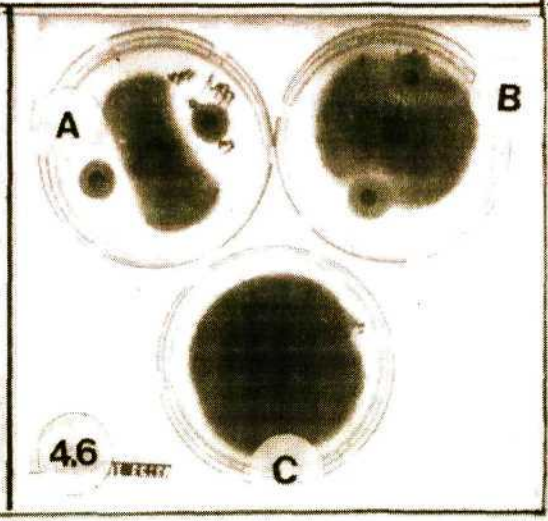
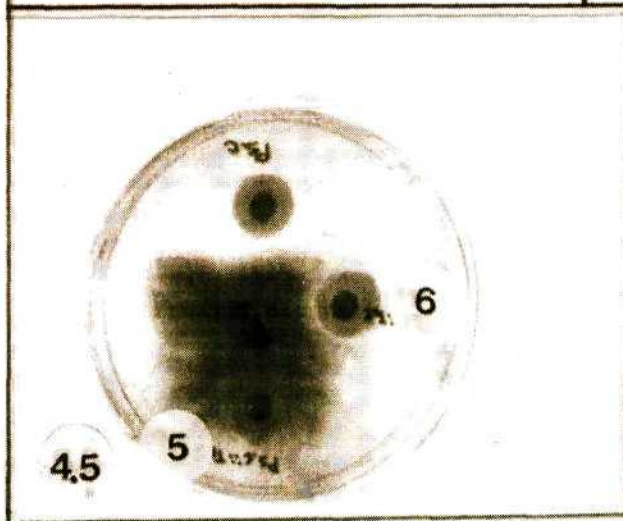
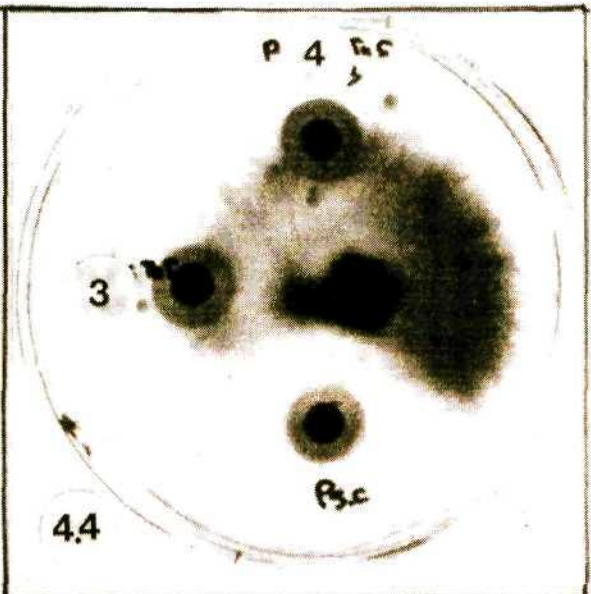
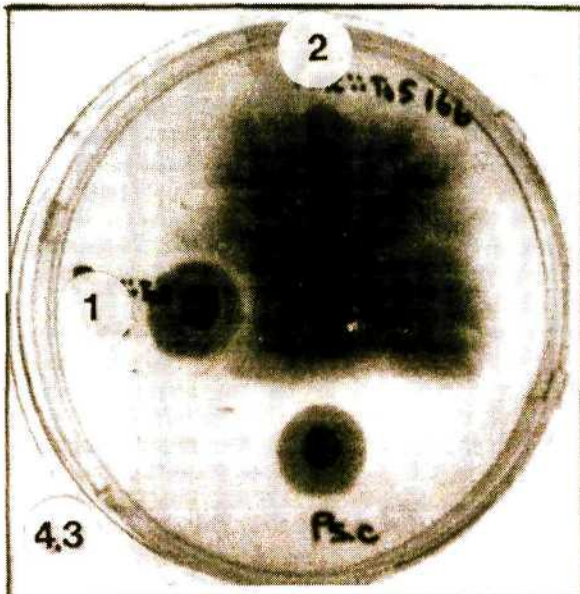
PLATE 12

Figure 4.3: Colonies of the wild type *Burkholderia cepacia* and the mutants *Burkholderia cepacia* :: Tn5-116 (1) and *Burkholderia cepacia* :: Tn5-166 (2) growing in the presence of *Fusarium oxysporum* f. sp. *cubense*.

Figure 4.4: Colonies of the wild type *Burkholderia cepacia* and the mutants *Burkholderia cepacia* :: Tn5-188 (3) and *Burkholderia cepacia* :: Tn5-253 (4) in the presence of *Fusarium oxysporum* f. sp. *cubense*.

Figure 4.5: Colonies of the wild type *Burkholderia cepacia* and the Tn5 mutants *Burkholderia cepacia* :: Tn5-217 (5) and *Burkholderia cepacia* :: Tn5-289 (6) in the presence of *Fusarium oxysporum* f. sp. *cubense*.

Figure 4.6: A: The inhibitory zone was shown around the wild type *Burkholderia cepacia* bacterial colonies on TSA agar. B: The inhibitory zone did not occur around the UV mutant *B. cepacia* - UV -34 bacterial colonies on TSA agar. C: Control test, normal growth of *Fusarium oxysporum* f. sp. *cubense* on TSA agar.



of *B. cepacia* under the present reaction conditions. It may be that the 3'-terminal of the primers were mismatched, resulting in blocking extension and annealing of the PCR reaction.

4.3.2 Screening and characterization of mutants

Several transposon delivery systems were tried for mutagenesis of *B. cepacia*. The most affective transposon donor was *E. coli* which carried the Colbdrd-1 based plasmid pLG221 (Collbdrd-1 cib::Tn5), this plasmid is a suicide plasmid for Tn5 and is transmissible to *B. cepacia*. The kanamycin-rifampicin-resistant transconjugants were recovered at approximately 10^{-9} per donor. Transposon insertion mutants were tested for their ability to cause antibiosis on TSA agar by the petri plate assay. Among 750 transconjugants, six were found that had reduced and/or lost inhibitory activities, when compared with the wild-type strain. Six of the mutants were not able to inhibited the growth of *F. oxysporum* f. sp. *cupense* on TSA medium (Figure 4.3, 4.4, 4.5,). For the UV mutagenesis of *B. cepacia* , the mutants were screened and tested for their inhibitory activities, it was found that one mutant lost inhibitory activity to the fungus (Figure 4.6).

Whether both UV mutant and Tn5 mutants had the same type of antagonism against *F. oxysporum* f. sp. *cupense* was tested. It was found that the Tn 5 mutant *B. cepacia* :: Tn5 - 217 failed to show antagonism on TSA medium, but the inhibitory activity was recovered on YM medium (Appendix I), and the UV mutant *B. cepacia* UV-34 mutant still failed to inhibit the fungal growth (Table 4.2) on YM agar. It is suggested that mutation site on the chromosome of the bacterium could be different in each case.

Table 4.2 Antifungal activities of the Tn5 mutant, the UV mutant and the wild type of *Burkholderia cepacia* against *Fusarium oxysporum* f. sp. *cupense* in the petri plate assay

Strains	Antifungal activity in ^a :	
	TSA	YM
<i>Burkholderia cepacia</i> :: Tn5 - 217	-	+
<i>Burkholderia cepacia</i> :: UV - 34	-	-
<i>Burkholderia cepacia</i> wild type	+	+

^a: + good inhibition; - no inhibition

TLC analyses was performed by the method described previously (Chapter3 , 3.2.5). TLC examination showed that in the benzene-ethyl acetate (1:1) developing solvent system, there were absorbed UV spots appearing as bright spots at R_f 0.24; 0.45; 0.60; 0.81 in the wild type of *B. cepacia*. In the both mutants of *B. cepacia* ::Tn5 -217 and *B. cepacia* -UV- 34, there were only bright spots only at R_f 0.24; 0.45. The spots with R_f 0.60 and 0.81 were hardly visible which may suggest that the amount of these substances produced decreased greatly in the mutants as compared to the wild type strain (Figure 4. 7).

GC-MS analyses was performed by the method described previously. It showed that there was differences in the chromatograms between the wild type and the mutants of *B. cepacia*. There was no evidence of peaks at 14.62 minutes, 20.0 minutes and 20.46 minutes in the chromatograms of mutants of *B.cepacia* ::Tn5 -217 and *B. cepacia* :: UV - 34, compared with the chromatogram of the wild type *B. cepacia* (Figure 4.8, 4.9, 4.10).

Three mutants were tested for their abilities to colonize the hyphae of *F. oxysporum* f. sp. *cupense* by SEM (Scanning Electron Microscopy)

PLATE 13

Figure 4.7: Chromatogram of thin layer chromatography (TLC) of extracts from 4-days cultures of the wild type *Burkholderia cepacia*, the Tn5 mutant *Burkholderia cepacia* ::Tn5 - 217, and the UV mutant *Burkholderia cepacia* -UV -34.

Lane 1: showing 4 spots at R_f of 0.81, 0.60, 0.45, 0.24 in the wild type *B. cepacia*.

Lane 2: showing 2 spots at R_f of 0.45, 0.24 in the mutant *B. cepacia*.

Lane 3: showing 2 spots at R_f of 0.45, 0.24 in the mutant *B. cepacia*.

Rf

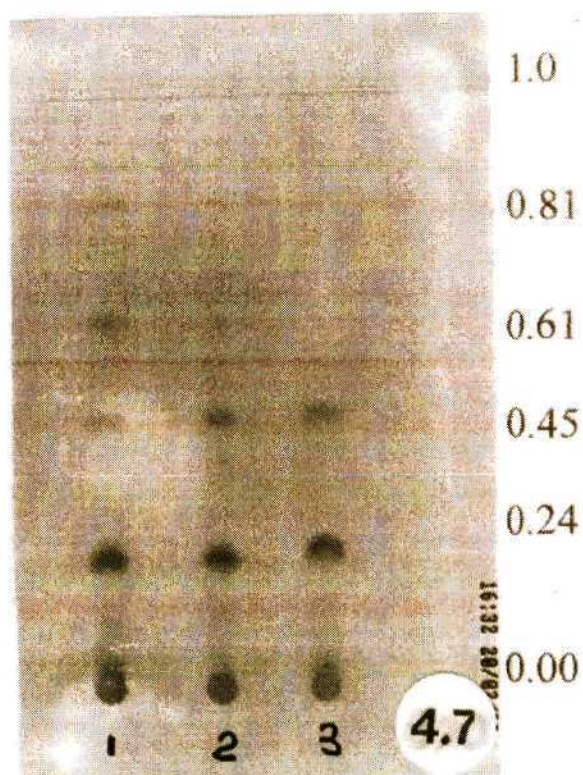


PLATE 14

Figure 4.8: Gas chromatogram of extracts from 4-days culture of the wild type *Burkholderia cepacia*, indicating numerous components in the antifungal substance extraction.

Figure 4.9: Gas chromatogram of extracts from 4-days culture of the mutant *Burkholderia cepacia* :: Tn5 - 217, showing the peaks at 14.62, 20.0 and 20.46 minutes are missing compared with the chromatogram of the wild type as indicated by arrow in Figure 4.8.

Figure 4.10: Gas chromatogram of extracts from 4-days culture of the mutant *Burkholderia cepacia* -UV - 34, showing the peaks at 14.62, 20.0 and 20.46 minutes are missing compared with the chromatogram of the wild type as indicated by arrow in Figure 4.8.

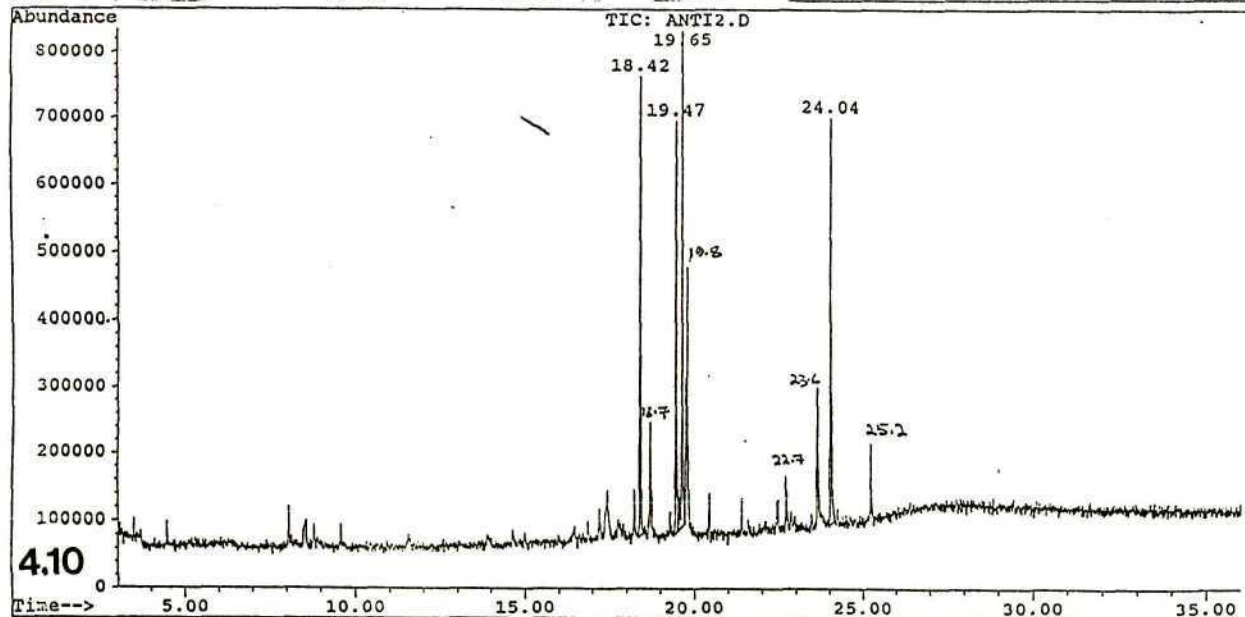
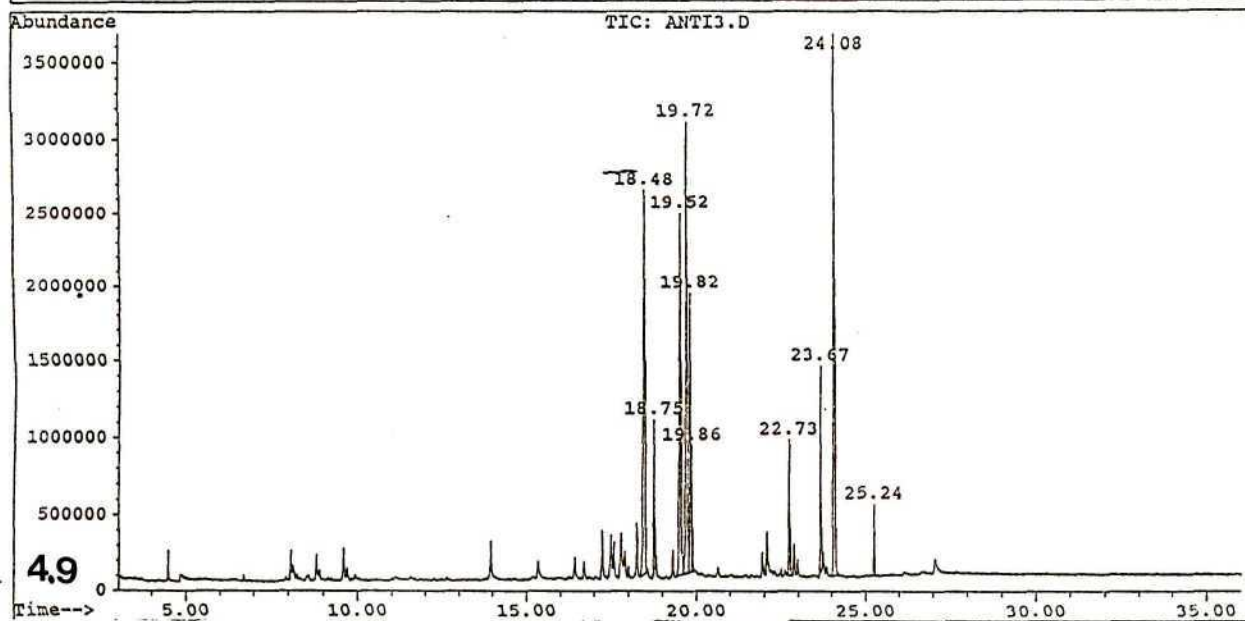
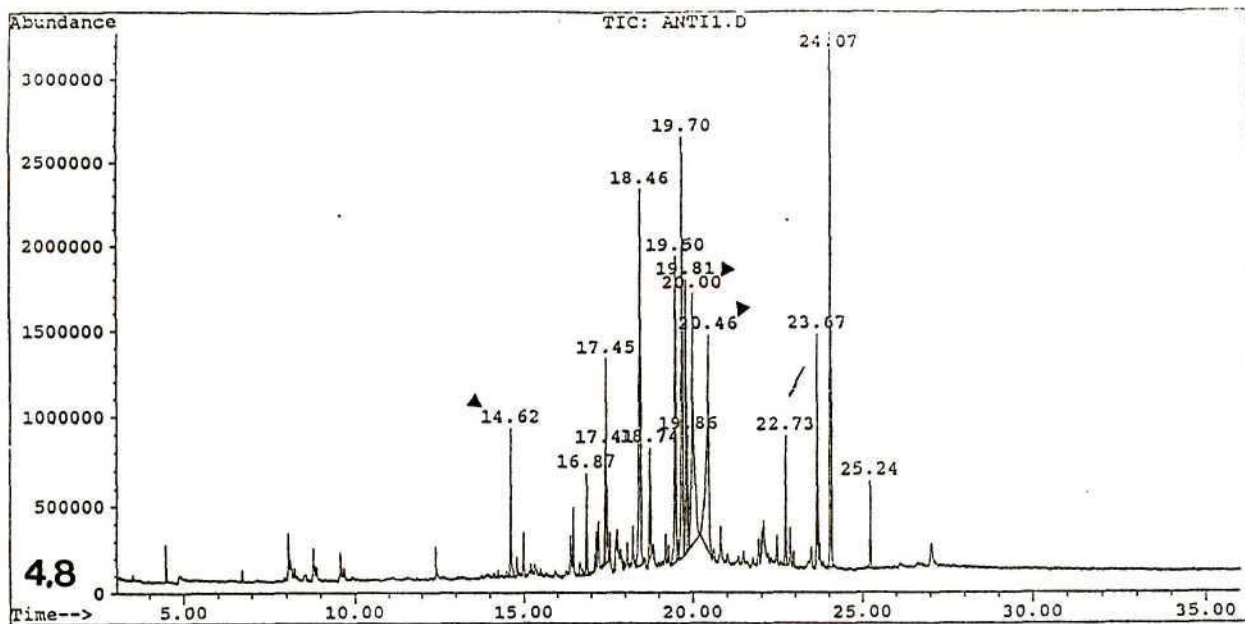


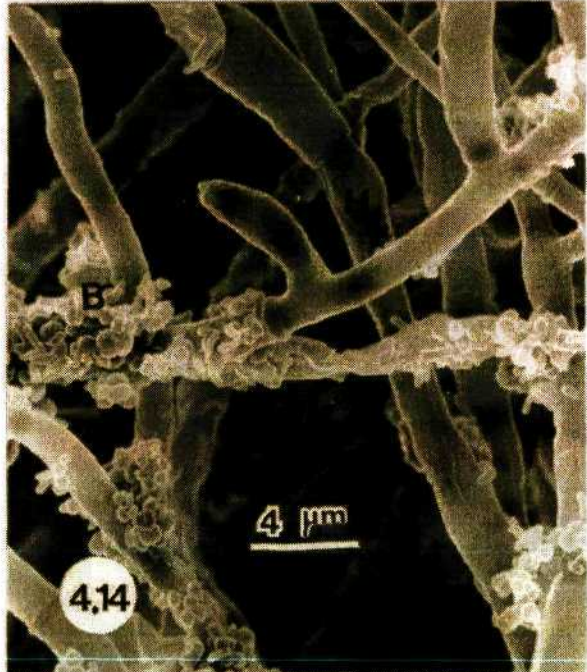
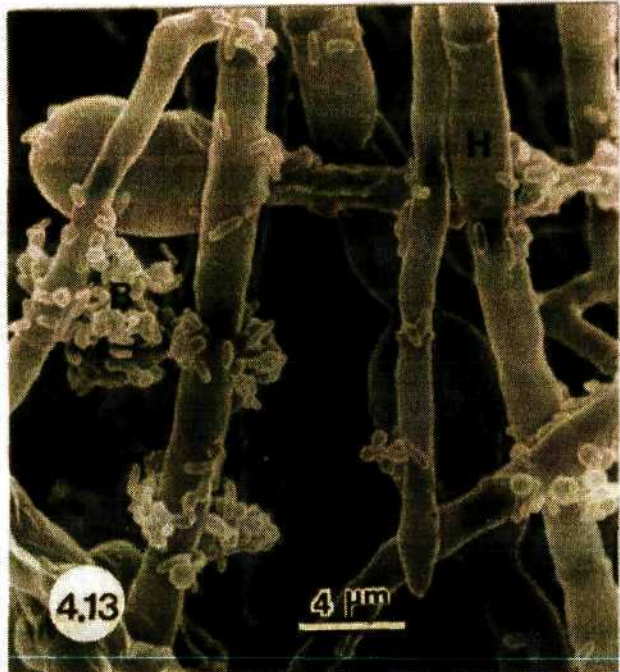
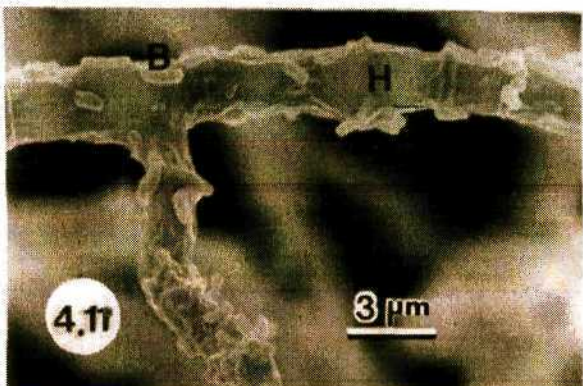
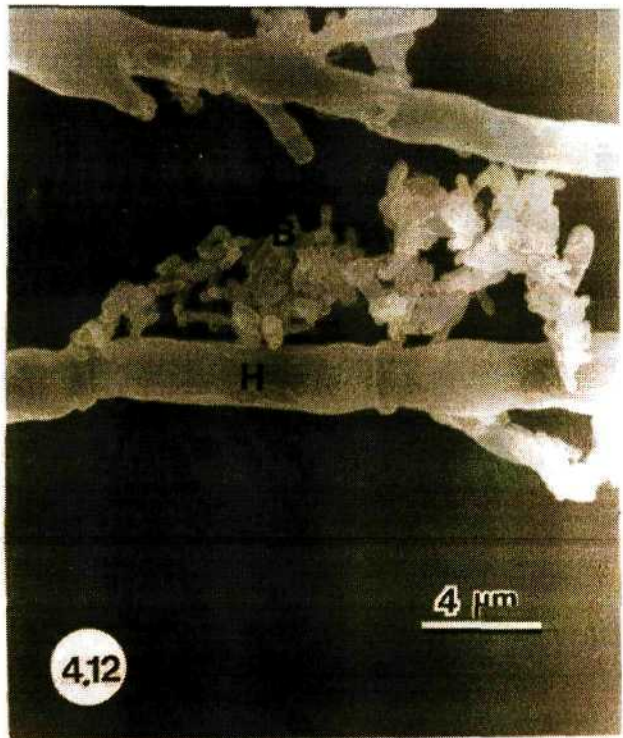
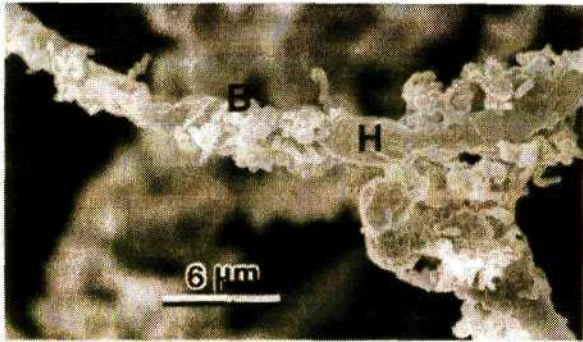
PLATE 15

Figure 4.11: Colonization of the hyphae of *Fusarium oxysporum* f. sp. *cupense* by the wild type *Burkholderia cepacia*, the hyphae appeared to have collapsed indicating by arrow. Bar = 5 μ m. B, bacteria; H, hyphae.

Figure 4.12: Showing the Tn5 mutant *Burkholderia cepacia* :: Tn5 -188 appeared not to colonize the hyphae of *Fusarium oxysporum* f. sp. *cupense*. Bar = 4 μ m. H, hyphae, B, bacteria.

Figure 4.13: Colonization of *Fusarium oxysporum* f. sp. *cupense* hyphae by the Tn5 mutant *Burkholderia cepacia* :: Tn5 -217. Bar = 4 μ m. B, bacteria; H, hyphae.

Figure 4.14: Colonization of *Fusarium oxysporum* f. sp. *cupense* hyphae by the UV mutant of *Burkholderia cepacia* -UV -34. Bar =4 μ m. B, bacteria; H, hyphae.



investigations (described previously, Chapter 2, 2.2.2). Compared with the wild type *B. cepacia* (Figure 4.11), *B. cepacia* :: Tn5-188 was not able to colonize the fungal hyphae (Figure 4.12); and *B. cepacia* :: Tn5 -217 was able to colonize on the fungal hyphae (Figure 4.13). It is therefore likely that the mutants colonization ability was reduced, compared with the wild type of *B. cepacia* (Figure 4.11). SEM also showed that the mutant *B. cepacia* -UV - 34 derived from UV mutagenesis of *B. cepacia* unchanged ability to colonize the fungal hyphae (Figure 4.14). This indicated that the differences between mutant phenotypes may be due to the insertion of Tn5 and/or random mutation.

4.4 Discussion

The antibiotic PCA and PhI are major determinants of the biological control ability of soilborne plant pathogens by strains of fluorescent *Pseudomonas* spp. (Thomashow & Weller, 1996). The PhI primers in this study were directed against *PhID*, one of the six biosynthetic genes in the PhI biosynthetic cluster of *P. fluorescens* Q2-87 (Bangera & Thomashow, 1996). PCA primers were directed against *PhzC* and *PhzD*, two of the nine genes in the PCA biosynthetic cluster of *P. fluorescens* 2-79 (Boronin *et al.*, 1995). These primers allow the detection of strains that could produce diacetylphloroglucinol (PhI) and phenazine (PCA) antibiotics. In this study, the primers enabled the specific detection of PhI and PCA production by *B. cepacia*. Colony hybridizations showed that *B. cepacia* was able to hybridize with the PhI2a and PCA2a probes. Hybridization occurred at high stringency (0.1XSSC; 45 °C), which suggested that homology could be present between the probes and the target DNA of *B. cepacia*, and *B. cepacia* may produce the antibiotics PhI and PCA, which are thought as one of the most important factors in disease suppression. In addition,

it was observed by Southern hybridization that genomic DNA of *B. cepacia* hybridized to the PhI probe, and the hybridization only occurred at low stringency (0.5XSSC; 45 °C). In this study, attempts to obtain PCR products were unsuccessful. There were no PCR products amplified from genomic DNA of *B. cepacia* with the primers PhI2a and PhI2b under the PCR reaction condition used. It is possible that the 3'-terminal of the primers may be mismatched, which could result in the blocking of extension in the PCR reaction. Further work on optimal PCR conditions and use of a polymerase with a strong 3'-5' exonuclease activity to correct mismatches may prove successful. Gene(s) involved in PhI synthesis might be present in the chromosome of *B. cepacia*.

It is known that gene probe techniques and PCR have been used extensively in environmental microbiology to detect pathogenic bacteria, fungi, and viruses, nitrogen-fixing-organisms, and soil bacteria that degrade xenobiotics (Sayer & Layton, 1990). Although in this study no PCR products amplified from the DNA of *B. cepacia* with these primers was found, this method could be used to detect PhI-producing strains which may present in natural environments. It could also be developed to analyse the phenotypic and genetic diversity of isolated antibiotic producing strains for biological control of soil born plant pathogens.

It is becoming more important to investigate the factors which could be involved in the inhibition of pathogenic fungi and disease suppression. Mutagenesis studies could reveal correlation between bacterial antagonism and pathogenic fungal inhibition. In this study, it was found that both mutants, *B. cepacia* ::Tn5-217 and *B. cepacia* UV-34, were not able to inhibit the fungal growth (*F. oxysporum* f. sp. *ubense*) on TSA agar, and the inhibitory activity of *B. cepacia* :: Tn5-217 was recovered on YM agar. This suggests that mutations in *B.*

cepacia occurred randomly and that there could be many factors involved in the inhibitory activity of *B. cepacia*, and that mutations may occur at different sites on the chromosome in the mutants.

TLC examination showed that production of the substances, with R_f values of 0.60 and 0.81, had decreased in the mutants of *B. cepacia* ::Tn5 -217 and *B. cepacia* UV-34. This suggested that in both mutants gene(s) could be involved in the synthesized and/or accumulation of these compounds.

GC-MS analyses showed that differences were present in the chromatograms between the wild type and the mutants of *B. cepacia*. There was no evidence of peaks at 14.62 minutes, 20.0 minutes and 20.46 minutes in the chromatograms of *B. cepacia* ::Tn5 -217 and *B. cepacia* -UV- 34, compared with the chromatogram of the wild type *B. cepacia*. This revealed that both mutants of *B. cepacia* were defective in production of the three compounds, which were responsible for the three peaks and could contribute to the inhibitory activity of *B. cepacia*. Further investigations to identify the three compounds is required.

All these results indicated that inhibitory activity of *B. cepacia* is controlled by multiple genes and that various factors could be responsible for antagonistic properties. Mutation generated by both UV and Tn5 occurred at random and the excision of mutated sites is required to be further investigated. It is presently unclear whether the mutants described here are impaired specifically in antibiotic synthesis. Biosynthesis of antifungal compounds could limit availability for antibiotic synthesis and that related pathways may dramatically influence antibiotic biosynthesis.

In this study, it was found that the colonization and inhibition of *F. oxysporum* f. sp. *cubense* by *B. cepacia* *in vitro* were disrupted by Tn5 insertion. The mutant *B. cepacia* :: Tn5 - 188 failed to colonize the fungal hyphae, but both of the mutants *B. cepacia* :: Tn5 - 217 and *B. cepacia* UV -34 were not affected in their ability to colonize the fungal hyphae. This suggests that colonization ability could be controlled by multiple genes. De Weger *et al.* (1987) reported that the loss of flagella in Tn5 mutants of *P. fluorescens* was associated with an inability to colonize potato roots. However, the mutants derived from chemical mutagenesis of three strains of *P. fluorescens* were not affected in their ability to colonize the wheat roots. In the present study, factors involved in colonization of *B. cepacia* and which factors within the mutants were defective, were not determined.

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5. GENERAL CONCLUSION

Attempts to use bacteria for plant disease control or yield improvement were started in the early part of this century. Interest in the area has increased steadily since the mid-1960s. Certain strains of beneficial *Pseudomonas* spp. could suppress a variety of soil-borne plant diseases, enhancing the growth and yield of the plants. More recently, research efforts have begun to move beyond system descriptions and towards mechanistic studies. A number of biological activities have been postulated to be involved in successful disease control. These include the abilities to colonize the appropriate host plant parts and to produce antagonistic compounds, such as antibiotics, siderophores, hydrogen cyanide and hydrolytic enzymes.

In the present study, *Burkholderia cepacia* was isolated from grown asparagus plants *in vitro* and the inhibition of *Fusarium oxysporum* f. sp. *ubense* race 4 by *B. cepacia* was described. This study showed that *B. cepacia* was able to inhibit several fungal pathogens *in vitro*. Although *in vitro* agar studies may not reflect the environmental or microbiological conditions in the field, *in vitro* studies could provide useful data on growth parameters of antagonists and these may help with development procedure for a biological agent.

In addition, inhibitory activity of *B. cepacia* was noted when grown on various media with different carbon sources and at various pH and temperature conditions. The present study also showed that antagonism by *B. cepacia* was not involved in siderophore production. TLC examination indicated that *B. cepacia* produced several substances which may play a role in antagonism of

B. cepacia. The synthesis of an antimicrobial peptide is a possible mechanism of microbial antagonism. In this study, the inhibitory activity of *B. cepacia* was affected by treatment with proteinase and the concentrated activated fractions were able to inhibit the fungal growth. That may suggest that proteins/peptides could contribute to the antifungal activity of *B. cepacia*. A GC-MS chromatogram indicated numerous components in the antifungal substances extraction, and 1,2-Benzenedicarboxylic acid, bis (2-Ethylhexyl) ester was identified. All these results suggests that production of several antibiotics and anti-microbial peptide may contribute to the antagonism of *B. cepacia*.

Biocontrol involves harnessing microorganisms that suppress diseases to improve plant health. Colonization of the plant and the pathogen may both be important factors in the suppression of disease. In the present study, both scanning and transmission electron microscopy showed that *B. cepacia* was shown to be closely associated with the fusarium wilt pathogen, *F. oxysporum* f. sp. *cubense* and interacted with the fungal pathogen, on the surface and in the banana root tissues. *B. cepacia* appears to have useful colonization properties that may render it effective as a biological control agent.

In this study, mutants of *B. cepacia* generated by UV and Tn5 transposon had lost or reduced inhibitory activity against *F. oxysporum* f. sp. *cubense*.. There was a decrease in antibiotic production in two mutants of *B. cepacia*. GC- MS analyses showed that there was absence of the peaks in the chromatograms of the mutants, which were present in the chromatogram of the wild type. This revealed that the inhibitory activity of *B. cepacia* could involve a variety of factors which are responsible for the antagonistic properties.

The antibiotics phenazine-1-carboxylic acid (PCA) and 2,4-diacetylphloroglucinol (Phl) could be major determinants in biocontrol. In this study, hybridization with the specific primers for these antibiotics showed that these antibiotics might be present in *B. cepacia*. Colony hybridization suggested that genomic DNA from *B. cepacia* could contain both Phl- and PCA genes. It was found that genomic DNA digested with Cla-I with hybridized to the Phl2a probe, however, only occurred at the low stringency. A signal was detected from a Cla-I fragment of approximately 2800bp. Additional experiments are necessary to clarify whether *B. cepacia* could produce both Phl and PCA antibiotics.

In conclusion, the results in this study showed that *B. cepacia* possesses antagonistic properties against several plant pathogens. The strain also demonstrates antifungal activity over a broad range of temperature and pH conditions, and appears to produce several antibiotics and therefore could be an attractive candidate for biocontrol of fungal plant pathogens.

APPENDIX I

Media for bacterial and fungal growth.

All quantities described are those needed to prepare 1 l of medium. Media are solidified by the addition of 15 g of agar per litre.

TSA: Tryptone Soya Agar (Difco)

PDA: Potato Dextrose Agar (Difco)

PPM: 20 g peptone
20 g glycerol
5 g NaCl
1 g KNO_3
pH 7.2

YM: 3 g yeast extract
3 g malt extract
5 g peptone
10 g glucose
pH 7.0

KMB: 20 g peptone
1.908 g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$
1.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
15 g glycerol
pH 7.0

NYGA: 5.0 g peptone
3.0 g yeast extract
20 g glycerol
pH 7.0

PSA: 10 g peptone
10 g sucrose
1.0 g sodium glutamate
pH 7.0

LA: 10 g tryptone
5.0 g yeast extract
5 g NaCl
1 g glucose
pH 7.0

APPENDIX II

