BIOLOGICAL CONTROL OF *PYTHIUM* DAMPING-OFF AND CROWN ROT OF GREENHOUSE CUCUMBERS USING ANTAGONISTIC BACTERIA

by

Maria McCullagh

B.Sc. (Agr.) McGill University

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF PEST MANAGEMENT

in the Department

of

Biological Sciences

© Maria McCullagh 1993

SIMON FRASER UNIVERSITY

December 1993

All rights reserved. This work may not be
reproduced in whole or in part, by photocopy or other means,
without permission of the author.

APPROVAL

Name: **MARIA MCCULLAGH**

Degree: **Master of Pest Management**

Title of Thesis:

BIOLOGICAL CONTROL OF PYTHIUM DAMPING-OFF AND CROWN ROT OF GREENHOUSE CUCUMBERS USING ANTAGONISTIC BACTERIA

Examining Committee:

Chair: Dr. D. L. Baillie, Professor

Dr. Z. K. Punja, Associate Professor, Senior Supervisor,
Department of Biological Sciences, SFU

Dr. M. Mackauer, Professor,
Department of Biological Sciences, SFU

Dr. R. Utkhede, Research Scientist
Agriculture Canada, Research Station, Summerland, B.C.

Dr. J. E. Rahe, Professor
Department of Biological Sciences, SFU
Public Examiner

Date Approved Dec. 17, 1993

PARTIAL COPYRIGHT LICENSE

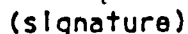
I hereby grant to Simon Fraser University the right to lend my thesis, project or extended essay (the title of which is shown below) to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users. I further agree that permission for multiple copying of this work for scholarly purposes may be granted by me or the Dean of Graduate Studies. It is understood that copying or publication of this work for financial gain shall not be allowed without my written permission.

Title of Thesis/Project/Extended Essay

Biological Control of Pythium Damping-Off

and Crown Rot of Greenhouse Cucumbers

Using Antagonistic Bacteria

Author: 

(signature)

Maria McCullagh

(name)

Dec 12, 1993

(date)

ABSTRACT

The effect of cucumber (*Cucumis sativus* L. cv. Fidelio) plant age on infection by *Pythium aphanidermatum* was evaluated. Seedlings were most susceptible to inoculation between 0 and 15 days after planting, after which they appeared to escape damping-off. Twenty-eight strains of bacteria belonging to the genera *Bacillus, Enterobacter* and *Pseudomonas* were screened for *in vitro* antagonism against *P. aphanidermatum* and *Pythium ultimum*, the major causal agents of damping-off of seedlings and crown rot of greenhouse cucumbers in British Columbia. Only *E. aerogenes* B 8 produced an inhibition zone, which was 11.6 mm in diameter against *P. ultimum* and 6.4 mm against *P. aphanidermatum*. Four bacterial strains (*E. aerogenes* B 8, *B. subtilis* AB 8, and *Ps. fluorescens* strains 63-28 and 63-49) were evaluated further in growth chamber studies for biocontrol efficacy on cucumber plants. Seeds were planted in soil infested with *P. aphanidermatum* (2500 cfu per ml of soil) and the soil was drenched with a bacterial suspension (10^8 cells/ml) either 48 hr prior to or at the time of seeding. Of the plants receiving *Ps. fluorescens* 63-28 48 hr prior to and at time of seeding, 62.5 % and 50 % survived, respectively, while no plants survived in the untreated controls. Of the plants inoculated with *Ps. fluorescens* 63-49 48 hr prior to and at the time of seeding 62.5 % and 37.5 % survived, respectively, while none of the plants treated with *Pythium* alone survived. In a second trial, 48.3 % and 55.2 % of plants inoculated with strains 63-28 and 63-49 48 hr prior to seeding survived. Of the control plants (*Pythium* alone), only 20.7 % survived damping-off. Neither *E. aerogenes* nor *B. subtilis* provided any protection against *Pythium*

infection. On hydroponically grown cucumbers, seed treatment with *Ps. fluorescens* strains 63-28 and 63-49 increased the height of 29-day-old plants by 13 and 27 %, respectively, when compared to the control. Plants treated with strain 63-49 were significantly (P=0.04) taller than the untreated control. Average dry weight of plants treated with 63-49 and *Pythium* harvested 69 days after seeding was increased significantly (P=0.05) compared to the *Pythium*-treated plants. Studies were conducted to determine the optimum temperatures for growth of *P. aphanidermatum* and *P. ultimum*, as well as *Ps. fluorescens* strains 63-28 and 63-49, *E. aerogenes* B 8, and *B. subtilis* AB 8. Based on measurements of radial growth on agar, *P. aphanidermatum* grew slowly at 15°C and optimally at 35°C, while growth of *P. ultimum* was slow at 15°C, most rapid at 30°C, and nil at 35°C. In nutrient broth, growth was optimal for *Ps. fluorescens* 63-28 at 30°C, for *Ps. fluorescens* 63-49 and *E. aerogenes* B 8 at 25°C, and for *B. subtilis* AB 8 at 35°C. These results illustrate the potential for using strains of *Pseudomonas fluorescens* to reduce *Pythium* damping-off and crown rot of cucumbers at temperatures at or below those required for disease development.

DEDICATION

To Jean-Pierre,

Helena,

Gerard and Greta.

ACKNOWLEDGMENTS

I would like to express sincere thanks to my senior supervisor, Dr. Zamir Punja, who was always available, and provided good advice. I would also like to thank Dr. Raj Utkhede for allowing me to work in his lab and for providing helpful advice, and Dr. Manfred Mackauer for advice and help with statistical analyses. I am very grateful to Mr. Emmerson Smith and Ms. Paula Haag for excellent technical assistance, generosity with regard to space and equipment, encouragement, and patience.

My thanks are also due to the Greenhouse Vegetable Research Council and the Science Council of B.C. for providing financial support for this project through a G.R.E.A.T. Award, and in particular to Mr. Brian Mauza for invaluable advice concerning crown rot and the culture of hydroponic cucumbers.

I thank all the people at the Summerland Research station who helped me at one time or another including Mr. Mark Gardiner, Dr. Gary Judd, Dr. Peter Sholberg, Mr. John Kuck, Ms. Heather McBrien, and Mr. Jeff Bechard.

I would like to thank all the people in Dr. Punja's lab for making me feel a part of the lab, even though I was working elsewhere. My thanks are also due to Ms. Marlene Nguyen for help with administrative matters.

I am very grateful to John Hall from the Vancouver Agriculture Canada Research Station and Francois Bellavance from Simon Fraser University for help with statistics.

I thank Dr. Jim Menzies of Agriculture Canada, Agassiz Research Station for supplying

me with cultures of *Pythium* and for advice about hydroponic cucumbers and Dr. M.S. Reddy of Esso Chemical Canada, Saskatoon, for providing the two strains of *Pseudomonas fluorescens* used in this project. I am very grateful to Deruiter Seeds (Columbus, Ohio) for providing me with high quality, hybrid cucumber seeds free of charge.

Table of Contents

Approval ii

Abstract iii

Dedication v

Acknowledgments vi

Table of Contents viii

List of Tables x

List of Figures xi

List of Plates xiii

I. Introduction 1

1.1 Greenhouse Cucumber Production 1

1.2 *Pythium* Damping-off and Crown Rot of Greenhouse Cucumbers 2

1.3 Biological Control of Diseases Caused by *Pythium* spp 6

II. Materials and Methods 12

2.1 Media Preparation 12

2.2 *In-vitro* Antagonism Trials 12

2.3 Temperature-Growth Studies 14

2.3.1 *Pythium* spp 14

2.3.2 Bacteria 15

2.4 Evaluation of Effect of Cucumber Age on Infection by *Pythium* 15

2.4.1 Preparation of Inoculum 15

2.4.2 Planting 16

2.5 Biocontrol of *Pythium* Damping-off of Cucumbers 17

2.5.1 Preparation of Bacterial Inoculum 17

2.5.2 Treatments 17

2.6 Biocontrol of Crown Rot on Cucumbers in Hydroponic Culture 20

III. Results 22

3.1 *In-vitro* Antagonism Trials 22

3.2 Temperature-Growth Studies 22

3.2.1 *Pythium* spp 22

3.2.2 Bacteria 35

3.3 Evaluation of Effect of Cucumber Age on Infection by *Pythium* 41

3.4 Biocontrol of *Pythium* Damping-off of Cucumbers 41

3.5 Biocontrol of Crown Rot of Cucumbers in Hydroponic Culture 53

IV. Discussion 58

References 69

Appendix I 74

Appendix II 84

List of Tables

Table 1. Reports on biological control of *Pythium* spp. (1952-1992) 4

Table 2. Parameters of four trials testing the biocontrol ability of bacterial antagonists to protect cucumber plants from damping-off caused by *Pythium aphanidermatum* 18

Table 3. Treatments used to test the ability of bacterial antagonists to protect cucumber plants from damping-off caused by *Pythium aphanidermatum* 19

Table 4. Effect of age on survival of cucumber plants transplanted into sandy loam soil infested with *Pythium aphanidermatum* 44

Table 5. Growth of hydroponically grown cucumber plants seed-treated and inoculated with *Pseudomonas fluorescens* strains 63-28 and 63-49 on days 9 and 35, and inoculated with *Pythium aphanidermatum* on days 18 and 35 56

Table 6. Fresh weight, fruit weight, and dry weight of hydroponically grown cucumber plants seed-treated and inoculated with *Pseudomonas fluorescens* strains 63-28 and 63-49 on days 9 and 35 and inoculated with *Pythium aphanidermatum* on days 18 and 35 57

List of Figures

Figure 1a. Radial growth of *Pythium aphanidermatum* at 15-35°C 23

Figure 1b. Mycelial dry weight of *Pythium aphanidermatum* at 15-35°C 23

Figure 2. Radial growth of *Pythium aphanidermatum* at 31-35°C25

Figure 3. Relationship between radial growth of *Pythium aphanidermatum* and temperature 27

Figure 4a. Radial growth of *Pythium ultimum* at 15-35°C 29

Figure 4b. Mycelial dry weight of *Pythium ultimum* at 15-35°C 29

Figure 5. Radial growth of *Pythium ultimum* at 31-35°C 31

Figure 6. Relationship between radial growth of *Pythium ultimum* and temperature 33

Figure 7a. Growth of *Pseudomonas fluorescens* 63-28 in nutrient broth at 15-35°C 37

Figure 7b. Growth of *Pseudomonas fluorescens* 63-49 in nutrient broth at 15-35°C 37

Figure 8a. Growth of *Bacillus subtilis* AB 8 at 15-35°C 39

Figure 8b. Growth of *Bacillus subtilis* AB 8 at 15-35°C 39

Figure 9. Effect of plant age on mortality of cucumber plants caused by *Pythium aphanidermatum* 42

Figure 10. Relationship between arcsin of the proportion survival of cucumber plants inoculated with *Pythium aphanidermatum*

and plant age at the time of inoculation 45

Figure 11. Influence of bacterial treatment on survival of cucumber plants inoculated with *Pythium aphanidermatum*. Results from trials 1 and 2 47

Figure 12. Results from trial 3 49

Figure 13. Results from trial 4 51

List of Plates

Plate 1. Growth promoting effect of *Pseudomonas fluorescens* strains 63-28 and 63-49 on 34-day old cucumber seedlings growing in rockwool and inoculated with *Pythium aphanidermatum* 54

Chapter 1

Introduction

1.1 Greenhouse Cucumber Production

Long English cucumber (*Cucumis sativus* L.) is an important crop in the greenhouse vegetable industry in British Columbia. About 13.5 out of a total of 60 hectares presently under glass are used to produce cucumbers, which provided an annual revenue of $9.4 million in 1992. British Columbia greenhouse growers produce a high quality product which is exported to markets in Canada and the United States (B. Mauza, personal communication)[1].

Cucumber plants are grown hydroponically in rockwool blocks or in 10-litre plastic bags filled with sawdust. In early January, seeds are planted in wooden flats or small pots containing a soilless propagation mix comprised of peat and perlite amended with vermiculite, sand and/or sawdust (Favrin, 1987). The seedlings are transplanted in late January into rockwool slabs or sawdust bags, with one or two plants in each container. Plants are supplied with a complete nutrient solution (Anonymous, 1988) via a system of polyethylene tubing from a central tank. Plants come into production 8-12 weeks after seeding, and each plant may yield approximately 50-60 cucumbers by the end of the growing season in October (B. Mauza, personal communication).

---

[1]Greenhouse Specialist, Western Greenhouse Growers Co-op, Surrey,B.C.

1.2 *Pythium* Damping-Off and Crown Rot of Greenhouse Cucumbers

a) Prevalence

Crown rot of greenhouse cucumbers is a disease that is relatively new to the B.C. industry, having first appeared with the establishment of hydroponic culture in the last 10-15 years. Favrin (1987) described the causal agents of crown rot on cucumber in British Columbia to be *Pythium aphanidermatum* (Edson) Fitzp., *P. irregulare* Buisman, and *Pythium* sp. Group G Van der Plaats-Niterink, with *P. aphanidermatum* being the most important (Favrin et al., 1988). *Pythium aphanidermatum* and *P. ultimum* Trow are also the causal agents of crown rot elsewhere in Canada (Paulitz et al., 1992). In Quebec, crown rot is the main limiting factor in the production of cucumbers and has lead to the abandonment of this crop by all but one of the larger new entreprises in that province (Carrier, 1989).

b) Symptoms

The most typical symptoms of crown rot include rotting of the basal stem and crown of the plant, accompanied by wilting and reduced growth and fruit production. If infection is severe, death of the plant can result. Infection is thought to occur at the seedling propagation stage, and *Pythium* spp. have been isolated from most peat-based propagation mixes used in British Columbia greenhouses (Favrin, 1987, Favrin et al., 1988). The pathogen can also be introduced by infested water (Paulitz et al., 1992). Symptoms generally develop at early fruit set (4-8 weeks after transplant) or during hot weather when the plants are water-stressed.

c) Infection Process

Several *Pythium* species are known to cause seed rots, pre- and post-emergence damping-off, and root and crown rots of various important horticultural crops. Damping-off is a common

disease of cucumber seedlings. Units of inoculum of *Pythium* (sporangia, zoospores, mycelia, and oospores) are attracted to soluble carbohydrates which are exuded from the germinating seed (Keeling, 1974) and by vitamins, phytohormones, and sugars exuded by the roots and stems of seedlings (Barton, 1957). The pathogen infects host cells by producing a penetration peg, which extends from an appressorium or hyphal swelling. Entry can occur through the seed coat, the root-cap cells, root hairs, and the region of root elongation. Penetration requires mechanical pressure or a combination of pressure and enzymatic action (Endo and Colt, 1974). Penetration may also occur through natural openings without the need for appressoria, and hyphae can also enter through wounds (Agrios, 1988).

Pectinolytic enzymes secreted by *Pythium* spp. break down the middle lamellae which hold the cells together. Proteolytic enzymes, which cause the protoplasm to become granular and collapse, may also be produced within host cells (Agrios, 1988). Infected seedlings become constricted at the stem and eventually wilt, fall over, and die. Plants may become resistant to infection by *Pythium* spp. after a certain species-specific age, i.e., entry may occur, but invasion is reduced by the cell wall which thickens and undergoes physiological changes, such as formation of lignin and conversion of pectin to calcium pectate (Endo and Colt, 1974). Rootlets appear to be infected regardless of the age of the plant. Although *Pythium* spp. enter young tissue and kill the rootlets, spread in older tissue is usually confined to the cortex of the root (Agrios, 1988). Infection results in reduced growth and vigour of the plant and ultimately to reduced yield.

d) Control

In the greenhouse industry, attempts to control damping-off due to *Pythium* spp. include the

Table 1: Reports on Biological Control of *Pythium* spp. (1952-1991)

Biocontrol Agent	**Host**	***Pythium* species**	**Reference**
Spicaria, Penicillium Aspergillus	corn	*P. arrhenomanes*	Johnson, 1952
Trichoderma* *viride ***Penicillium* *frequentens***	table beet seeds	*P. ultimum*	Liu and Vaughan, 1965
Arthrobacter spp.	tomato	*P. debaryanum*	Mitchell and Hurwitz, 1965
Chaetomium* *globosum	squash	*P. ultimum* *P. aphanidermatum*	Harman et al., 1978
T. hamatum	radish	*Pythium* spp.	Harman et al., 1980
P. oxalicum ***T. hamatum***	pea seeds	*P. ultimum*	Kommedahl et al., 1981 Harman et al., 1980
P. fluorescens	cotton seedlings	*P. ultimum*	Howell and Stipanovic, 1980
Enterobacter cloacae	cucumbers	*P. ultimum*	Hadar et al., 1983
Corticium spp. (***Laetisaria arvalis***)	table beets	*P. ultimum*	Martin et al., 1983
Pythium nunn	cucumbers	*P. ultimum*	Paulitz and Baker, 1987
Pseudomonas spp.	wheat, tomato, cucumber	*P. aphanidermatum*	Elad and Chet, 1987
Pseudomonas cepacia AMMD	pea seeds	*P. ultimum* *P. sylvaticum*	Parke, 1990
Pseudomonas putida	soybean; bean	*P. ultimum*	Paulitz, 1991

Table 1 (cont'd)

Biocontrol Agent	**Host**	***Pythium* species**	**Reference**
enteric bacteria *Pseudomonas* spp. *Enterobacter cloacae*	turfgrass	*P. aphanidermatum*	Nelson and Craft, 1992

use of fungicide drenches, eg: captan (N-Trichloromethylthio-4-cyclohexene-1,2-dicarboximide) and thiram (bis-dimethylthiocarbamoyl-disulfide) at the planting stage (B. Mauza, personal communication), in addition to treatment of commercial seeds with captan or thiram before packaging. If symptoms of crown rot are observed later in the season, growers will often replant, or alternatively, apply a fungicide drench. However, there are no chemicals registered for use on cucumbers grown in a hydroponic system, and fungicides may also be phytotoxic in the absence of soil. Thus, effective methods of disease control are currently not available. Since losses due to this disease can approach 25-30 %, there is considerable incentive to develop strategies which are reliable, economical, and safe to use. In light of the general movement away from chemicals in the management of horticultural diseases and the problems inherent in their use, biological control would seem to be a desirable alternative. The controlled environment in modern greenhouses permits the use of biological agents against several insect pests. It thus seems possible that a similar approach could be developed for control of crown rot.

1.3 Biological Control of Diseases Caused by *Pythium* spp.

A number of different organisms have been shown to be antagonist to plant pathogenic *Pythium* spp. (see Table 1). In the 1930's, the antagonistic nature of *Trichoderma* spp. was recognized (Allen and Haenseler, 1935). Work conducted in the 1940's and 1950's revealed a wide array of microorganisms that were antagonistic to *Pythium*, including species of *Spicaria*, *Penicillium* and *Aspergillus* (Johnson, 1952). Using *Arthrobacter* spp. as biocontrol agents, Mitchell and Hurwitz (1965) were able to protect young tomato seedlings against damping-off

caused by *Pythium debaryanum*. These workers attributed efficacy to the production of glucanase and protease by the bacteria which lysed the mycelia of the pathogen. At about the same time, Liu and Vaughan (1965) obtained good protection of beet seedlings against pre-emergence damping-off caused by *P. ultimum* using *Trichoderma viride* Fr. and *Penicillium frequentans* Westling. Howell and Stipanovic (1980) showed that treatment of cotton seed with *Pseudomonas fluorescens* (strain Pf-5) prior to planting in *P. ultimum*-infested soil resulted in 71 % seedling survival as compared to 28 % in the control. Harman et al. (1980) treated pea and radish seeds with *Trichoderma hamatum* and achieved control of damping-off caused by *Pythium* spp. that was comparable to that obtained with various fungicide treatments. This was accomplished in spite of soil conditions that were unfavourable for the biocontrol agent (i.e., an alkaline pH). In a study conducted by Hadar et al. (1983), *Enterobacter cloacae* (Jordan) Hornaeche and Edwards greatly reduced *Pythium* rots of pea, beet and cucumber seeds. *Laetisaria arvalis* Burdsall was found to be effective as a biocontrol agent against low-temperature *Pythium* spp. (eg., *P. ultimum*) in beets planted in both natural and pasteurized field soils. The fungus suppressed reproduction of the pathogen (Martin et al., 1983). Elad and Chet (1987) reported that germination of oospores of *P. aphanidermatum* in the rhizospheres of wheat, tomato, cucumber, melon and cotton was reduced by two strains of *Pseudomonas putida* (Trev.) Migula and three strains of *Ps. cepacia* Burk. These bacteria also reduced cucumber damping-off caused by *P. ultimum* by up to 94 %. Parke (1990) observed a 47 % reduction in pre-emergence damping-off caused by *P. ultimum* and *P. sylvaticum* when *Ps. cepacia* (strain AMMD) was applied to pea seeds.

The three mechanisms through which biocontrol is believed to occur are 1) antibiosis,

parasitism, and 3) competition. These mechanisms are described in more detail below.

1) Antibiosis

Antagonism achieved through antibiosis involves the production of compounds which are deleterious to the pathogen. *Gliocladium virens* Miller et al. produces gliovirin, a compound believed to be responsible for control of *P. ultimum*. Howell and Stipanovic (1983) used ultraviolet light to generate gliovirin-negative and superproducing mutants of *G. virens*. The former did not control the pathogen, whereas the latter gave control comparable to the parental strain.

Recently, much information about the role of antibiosis has been elucidated through the use of molecular techniques. Several antibiotic-producing pseudomonads, including various strains of *Pseudomonas fluorescens*, are known to be inhibitory to *Pythium* spp. Gutterson et al. (1986) showed that a minimum of five genes is involved in the antibiotic-mediated inhibition of *P. ultimum* by *Ps. fluorescens* HV37a in the rhizosphere of cotton. This conclusion was based on the recognition of three groups of mutants which lacked the ability to inhibit the fungus. After mapping by transposon mutagenesis, two of the cosmids (artificial plasmids that are used as a cloning vehicle) containing these loci were found to contain at least two genes. Howell and Stipanovic (1980) studied strain Pf-5 of *Ps. fluorescens*, which is antagonistic to *P. ultimum* and *Rhizoctonia solani*. They isolated the antibiotics pyoluteorin and pyrrolnitrin from cultures of the bacterium. When cotton seed was treated with pyoluteorin and planted in *P. ultimum*-infested soil, seedling survival was increased from 33 % to 65 %. Pyrrolnitrin was inhibitory to *R. solani* but not to *P. ultimum*. Kraus and Loper (1992) examined these substances, an uncharacterized antibiotic as well as pyoverdine, another antifungal metabolite

produced by *Ps. fluorescens* strain Pf-5. Tn5 insertion mutants of Pf-5 that exhibited an inability to produce pyoluteorin or pyoverdine were identified, in addition to mutants which produced greater quantities of pyoluteorin than the wild-type. The pyoluteorin and pyoverdine-deficient mutants did not inhibit *P. ultimum in-vitro*, while the mutants which synthesized increased amounts of pyoluteorin produced larger inhibition zones than those of the wild type Pf-5. However, all mutants showed a level of control of *Pythium* damping-off on cucumber that was comparable to the parental strain. Based on these results, the authors suggested that physical exclusion or competition for nutrients were acting to suppress *Pythium* in the cucumber rhizosphere. It is possible, however, that a combination of all the above mechanisms is involved in the biocontrol of *Pythium* by strain Pf-5.

2) Parasitism

Antagonism achieved through parasitism involves physical invasion of the pathogen by the biocontrol agent. One example is the interaction of *Trichoderma hamatum* and *P. ultimum*, in which the parasite grows toward and coils around the hyphae of the pathogen, and then produces appressoria-like structures which, aided by enzymes, are capable of penetrating hyphal cell walls. By separating the antagonist and the pathogen during parasitism, a series of pits and holes along the hyphae of the pathogen were seen (Harman and Hadar, 1983). Thus, *T. hamatum* utilizes a combination of antibiosis and parasitism in the exploitation of its host. Another example of parasitism is seen in the bacterium *Enterobacter cloacae,* which forms a sheath around the hyphae of *P. ultimum* on agar, and causes lysis of the hyphal tips (Harman and Hadar, 1983).

3) Competition

Antagonists and pathogens may compete for the same nutrients and space in the rhizosphere. Ko and Lockwood (1967) demonstrated that the inability of fungal spores to germinate in soil is a consequence of nutrient deprivation resulting from microbial activity. Elad and Chet (1987) proposed that bacteria compete with oospores of *Pythium* for carbon and nitrogen which are required for germination. In this way, the incidence of damping-off was greatly reduced. Kao and Ko (1986) suggested that suppression of cucumber damping-off caused by *Pythium splendens* Braun in a Hawaiin soil was due to a combination of high microbial population and high Ca content. Calcium may enhance activity of soil microorganisms that compete with *Pythium*, or increase resistance of the host plant to this pathogen. In a study conducted by Chen et al. (1988), the importance of microbial activity in suppression of *Pythium* damping-off was apparent, since a negative correlation between disease incidence and general microbial activity was observed.

In soils with high pH, iron may be rendered unavailable to *Pythium*. Fluorescent pseudomonad species are capable of producing siderophores, which are low molecular weight microbial transport molecules that chelate iron, making it unavailable to other organisms. Becker and Cook (1988) isolated several strains of bacteria that inhibited *Pythium* on agar or on wheat roots in a silt loam soil. Six fluorescent pseudomonads which were inhibitory to *Pythium* on King's medium B (KMB), or on KMB amended with ethylene-diamine-di-o-hydroxyphenylacetic acid (EDDA), were not inhibitory on KMB amended with EDDA + $FeCl_3$ at 50 ug/ml or $FeCl_3$ at 10 ug/ml. Five siderophore-negative mutants of one antagonist were found to have no inhibitory ability on KMB or in soil. These findings illustrate the importance

of siderophores in the ability of these bacteria to inhibit *Pythium* both *in vivo* and *in vitro*.

Several studies on cucumber have demonstrated the ability of antagonistic microorganisms to protect against seed rots and damping-off (Elad and Chet, 1987; Hadar et al., 1983; Paulitz and Baker, 1987). However, little research has been conducted to determine the potential of bacterial antagonists as biocontrol agents on mature cucumbers grown in a hydroponic system. The objectives of the present research were to screen various bacterial antagonists for inhibition of *P. aphanidermatum* and *P. ultimum in vitro* and to evaluate the performance of four selected bacteria *in planta*. The critical age at which cucumber plants inoculated with the pathogen show reduced susceptibility to damping-off was also determined. During the course of the research, hydroponically-grown plants were inoculated after this critical age and monitored for symptoms of *Pythium*-induced crown rot, and the protective ability of two species of pseudomonads in this system was assessed. Growth studies of the two species of *Pythium* and four bacterial antagonists were conducted at different temperatures to determine the optimal range. This information is particularly important in the selection of antagonists that perform at the same temperatures as the pathogen and under conditions found in a hydroponic culture system.

Chapter 2

Materials and Methods

2.1 Media Preparation

Corn meal agar (CMA), potato dextrose agar (PDA) and nutrient agar (NA), Pseudomonas Agar F (PAF), potato dextrose broth (PDB), and nutrient broth (NB) were obtained from Difco Laboratories (Detroit, Michigan) and prepared according to label directions. Agar was obtained from Anachemia (Mississauga, Ontario). All media were sterilized for 15 min at 250°C.

2.2 *In-Vitro* Antagonism Tests

The *Pythium aphanidermatum* strain used in this project was initially isolated in February, 1991 from diseased cucumbers in Gipaanda Greenhouses (David and Sarah Ryall) and was supplied by J. Menzies, Agriculture Canada Research Station, Agassiz, British Columbia (B.C.) The isolate of *Pythium ultimum* was supplied by A. Levesque, Agriculture Canada Research Station, Vancouver, B.C. Both isolates of *P. aphanidermatum* and *P. ultimum* were maintained on water agar (WA) at 10°C. Two strains of *Pseudomonas fluorescens* (Migula) that were isolated from canola roots were obtained from M.S. Reddy , Esso Chemical Canada, Saskatoon, Saskatchewan and were maintained on PAF. Stock cultures were stored at -70°C in nutrient broth and 10% glycerol. *Enterobacter aerogenes* (Kruse) Hornaeche and Edwards strain B 8 was isolated from an orchard soil in Summerland, B.C. by R. Utkhede, Agriculture Canada Research Station, Summerland, and grown on nutrient agar (NA). Strains of *Bacillus*

subtilis (Mendelson) were isolated from sclerotia of *Sclerotium cepivorum* originating from seven sources: *B. subtilis* isolate Hb 2 was obtained from P. van der Meer, Institute of Horticultural Research, Wageningen, The Netherlands; NZB 1 from B. Hawthorne, Lincoln Research, Private Bag, Christchurch, New Zealand; AB 1, 2, 3, 6, 7, 8 and 9 from I. D. Geard, Department of Agriculture, New Town Research Laboratories, New Town 7008, Tasmania, Australia; EBW 1, 2, 3, 4, and 6 from J.R. Coley Smith, University of Hull, U.K.; BACT 1, 2, 4, 8, and X from naturally infested soils from the Fraser Valley, B.C.; BACT K, ARB 1, 2, and 10 from mineral soil near Kelowna, B.C.; and BACT O from mineral soil near Olympia, WA, U.S.A. All isolates were grown on PDA. The strains of *Bacillus subtilis* and *Enterobacter aerogenes* B 8 were supplied by R. Utkhede, Agriculture Canada Research Station, Summerland, B.C.

To evaluate antagonism of each bacterial isolate mentionned above, a 3-cm long streak was made close to the edge of a 60 X 15 mm petri plate containing CMA. A 5-mm-diameter disk from the periphery of an actively growing culture of *P. ultimum* or *P. aphanidermatum* was placed 35 mm from the bacterial streak 24 hr later. The control consisted of a *Pythium* disk placed alone on CMA. There were five replicates for each fungus/bacterium combination, and five controls were included for each species of *Pythium*. Colony diameter was measured after 72 hr of incubation at 23°C. Data from the five replicates were averaged. The experiment was repeated once.

2.3 Temperature-Growth Studies

2.3.1 *Pythium* Species

Radial Growth

A 5-mm-diameter mycelial disk of *P. aphanidermatum* or *P. ultimum* was transferred from the periphery of an actively growing 2-day old culture to the centre of a 100 X 15mm petri plate containing water agar. There were five replicate plates of each species for each temperature. The plates were wrapped in aluminum foil and placed in complete darkness in growth chambers set at 15, 20, 25, 30, 31, 32, 33, 34, and 35°C and 50 % relative humidity. Radial growth of the two species was measured every 12 hr by taking one measurement of colony diameter at a set point on each plate. The plates were returned immediately to their respective growth chambers and the experiment was continued to 60 hr or until the fungus had crossed the petri dish. An ANOVA with repeated measures was carried out to determine whether differences in growth at the various temperatures were significant at $P=0.05$.

Mycelial Dry Weight

A mycelial disk from an actively growing culture of *P. ultimum* or *P. aphanidermatum* was placed in 100 ml of potato dextrose broth (PDB) in an Erlenmeyer flask and incubated in continuous darkness in growth chambers set at 15, 20, 25, 30, and 35°C and 50 % relative humidity. There were five replicate flasks of each species for each temperature. After 2.5, 5, 7.5, and 10 days, the broth was suctionned through a Buchner funnel on pre-dried and weighed filter papers. The mycelial mats were dried at 60°C for at least 24 hr and weighed. Data from the five replicates were averaged. A two-way ANOVA was performed to determine if

differences between the various temperatures were significant at P=0.05. In addition, mean relative growth rates between 2.5 and 5 days were compared to determine the optimum temperatures for growth.

2.3.2 Bacteria

The effect of temperature on growth of four antagonistic bacterial isolates was studied. *E. aerogenes* B 8, *Ps. fluorescens* 63-28 and 63-49 were grown in nutrient broth (NB), while *B. subtilis* AB 8 was grown in PDB. One loopful from an actively growing culture on a petri dish was placed in 50 ml of the appropriate broth and incubated at 28°C at 150 rpm for 24 hr. One ml of this suspension was added to 50 ml (X 5 replicates) of broth and placed in the dark on a shaker at 100 rpm in growth chambers set at 15, 20, 25, 30 and 35°C. Growth was measured every 12 hr. Bacterial populations were estimated using optical density vs. population curves illustrated in Appendix 1. An ANOVA with repeated measures was carried out to determine if there were significant differences (P=0.05) in growth at the five different temperatures. Data recorded at 20°C were not included in the analysis since measurements were not made at 12 hr at this temperature. A separate test was conducted which included 20°C, but only took the last three times into account.

2.4 Effect of Cucumber Age on Infection by *Pythium*

2.4.1 Preparation of Inoculum

Inoculum of *P. aphanidermatum* and *P. ultimum* was prepared following a method similar to that described by Paulitz and Baker (1987). Five mycelial mats of each *Pythium* species were

grown for one wk in 100 ml PDB. Each mat was added to 100 ml of sterile distilled water (SDW) and blended for a total of 30 sec. The homogenized mats were added to 6 kg of sandy loam field soil (pH 6.8) obtained from the Summerland Research Station that had been amended with 1 % ground oatmeal and autoclaved twice at 250°C for 15 min.

The *Pythium*-infested soil was placed in autoclaved aluminum roasting pans and covered with aluminum foil and incubated under aseptic conditions. Inoculum of *P. ultimum* was incubated at 23°C and that of *P. aphanidermatum* at 27°C. The colony-forming units (CFU) of each species in the soil were estimated after two wk using the soil-drop method of Mircetich and Kraft (1973) and found to be approximately 2500 cfu/cc soil.

<u>2.4.2 Plants</u>

Cucumber seeds cv. Fidelio F1 (courtesy of Deruiter Seeds, Columbus, Ohio) were planted in styrofoam cups each containing approximately 160 g of autoclaved sandy loam soil, with one seed per cup. Five seeds were also planted directly into soil infested with *P. aphanidermatum* or *P. ultimum*. After 3, 6, 9, 12, 15, 18 and 21 days, germinating seeds or seedlings were transplanted into infested soil. To eliminate any effect due to physical damage incurred during transplanting, the control group (non-inoculated) was treated in the same manner but seedlings were transplanted into sterile soil. The cups were arranged in a completely randomized design in a growth chamber maintained at 24°C with a 16 hr photoperiod, with five replications for each transplant time. Each cup received 15 ml of distilled water (DW) initially, which was increased to 25 ml per day as the plants grew. After the seedlings had been transplanted into infested (or sterile) soil, they received 40 ml DW. Data representing the

proportion of plants that survived were transformed to their arcsin values and plotted against plant age. An ANOVA was performed on age to determine if increased age resulted in a measurable increase in survival.

2.5 Biocontrol of *Pythium* Damping-Off on Cucumbers

2.5.1 Preparation of Bacterial Inoculum

E. aerogenes B 8 and both strains of *Ps. fluorescens* were grown in 50 ml NB and *B. subtilis* in 50 ml of PDB for 24 hr at 28°C on a shaker at 150 rpm. One ml of each culture of *Ps. fluorescens* and 1 ml SDW were pipetted onto two plates of PAF. After 48 hr, bacterial suspensions were made by pouring a few ml of SDW onto the plates, loosening the bacteria with a sterile rubber scraper and adding the suspension to 150 ml SDW. Bacterial populations (cfu/ml suspension) averaged 4.5 X 10^8, 2.3 X 10^8, 3 X 10^8 and 3 X 10^6 for *Ps. fluorescens* 63-28 and 63-49, *E. aerogenes* and *B. subtilis*, respectively. Ten ml of these bacterial suspensions (diluted to 40 ml with SDW) were applied as a soil drench to each pot.

2.5.2 Treatments

The following treatments were tested in the first trial - 1) control, 2) B 8 alone, 3) AB 8 alone, 4) *Ps. fluorescens* 63-28 alone, 5) *Ps. fluorescens* 63-49 alone, 6) B 8 applied to cups containing soil infested with *P. aphanidermatum* (as described in section 2.4.1), 7) AB 8 and *P. aphanidermatum,* 8) *Ps. fluorescens* 63-28 and *P. aphanidermatum,* 9) *Ps. fluorescens* 63-49 and *P. aphanidermatum*, 10) B 8 and *P. aphanidermatum* with planting delayed by 48 hr, 11) AB 8 and *P. aphanidermatum* with delayed planting, 12) *Ps. fluorescens* 63-28 and *P.*

Table 2: Parameters of four trials testing the biocontrol ability of bacterial antagonists to protect cucumber seedlings from damping-off caused by *Pythium aphanidermatum*

Trial #	Medium	Antagonists tested	Inoculum level(cfu/cc)	No. of replicates per treatment
1	sandy loam soil	*P. fluorescens* strain 63-28 strain 63-49 *E. aerogenes* strain B 8 *B. subtilis* strain AB 8	5,400	5
2	sandy loam soil	*P. fluorescens* strain 63-28 strain 63-49	14,600	5
3	sand + vermiculite	*P. fluorescens* strain 63-28 strain 63-49	2,800	10
4	sand + vermiculite	*P. fluorescens* strain 63-28 strain 63-49	2,800	30

Table 3: Treatments used to test the ability of bacterial antagonists to protect cucumber plants from damping-off caused by *Pythium aphanidermatum*

TRIAL 1	
Treatment	
1	None
2	*E. aerogenes* B 8
3	*B. subtilis* AB 8
4	*P. fluorescens* 63-28
5	*P. fluorescens* 63-49
6	*E. aerogenes* B 8 + *P. aphanidermatum*
7	*B. subtilis* AB 8 + *P. aphanidermatum*
8	*P. fluorescens* 63-28 + *P. aphanidermatum*
9	*P. fluorescens* 63-49 + *P. aphanidermatum*
10	*E. aerogenes* B 8 + *P. aphanidermatum*; delayed planting
11	*B. subtilis* AB 8 + *P. aphaniderarum*; delayed planting
12	*P. fluorescens* 63-28 + *P. aphanidermatum*; delayed planting
13	*P. fluorescens* 63-49 + *P. aphanidermatum*; delayed planting
14	*P. aphanidermatum* alone

TRIALS 2-4	
Treatment	
1	None
2	*P. fluorescens* 63-28 + *P. aphanidermatum*
3	*P. fluorescens* 63-49 + *P. aphanidermatum*
4	*P. fluorescens* 63-28 + *P. aphanidermatum*; delayed planting
5	*P. fluorescens* 63-49 + *P. aphanidermatum*; delayed planting
6	*P. aphanidermatum* alone

aphanidermatum with delayed planting 13) *Ps. fluorescens* 63-49 and *P. aphanidermatum* with delayed planting and 14) *P. aphanidermatum* alone. These treatments are listed in Table 2. For each treatment, there were five replicate cups, each with one seed. The cups were arranged in a completely randomized design in a growth chamber in which day/night temperatures were 27/23°C. Bacterial treatments (40 ml of bacterial suspension) were applied as a drench as described in Section 2.4.1 at seeding, except for *Pythium*-infested soil (treatment 1) and the control, which received 40 ml of DW. The watering schedule was 15 ml DW per day, which was increased to 25 ml per day over time. In a second trial, six treatments were tested - treatments 1, 8, 9, 12, 13 and 14 above which were numbered treatments 1 through 6 (see Table 2). Data analysis was carried out using log-linear models fitted by maximum likelihood to determine if differences were consistent from trial 1 to trial 2; subsequently, the data from the two trials were pooled. A third and a fourth trial were conducted using sand and vermiculite instead of soil as a growth medium. The parameters of all of four trials are summarized in Table 3. Fisher's Exact Test was used to determine if differences existed between the treatments.

2.6 Biocontrol of Crown Rot in Hydroponic Culture

Seeds of cv. Fidelio were soaked in 50 ml of a suspension of each of the two *Pseudomonas* strains (10^8 cfu/ml) or in SDW for 7 min. The seeds were dried on filter paper in petri dishes in a laminar flow hood for 3 hr. Ten ml of SDW was added to the seeds and the petri dishes were placed in the greenhouse at 28°C with a 16-hr photoperiod. After the seeds had germinated, the seedlings were transferred to 10 X 6.5 X 10 cm rockwool cubes on plastic saucers. Each seedling was placed in vermiculite in a 2 cm well cut into the rockwool cube,

which was watered daily from below. After the cotyledons had fully expanded, the greenhouse temperature was lowered to 24°C and halogen lighting was provided 24 hr a day. On day 9, 40 ml of a bacterial suspension (10^8 cfu/ml) was added to half of the cubes. On day 18, the plants were inoculated with 10 ml of *P. aphanidermatum* inoculum which had been prepared from freshly harvested 9-day old fungal mats grown in V8-cholesterol broth (V8CB), rinsed twice with DW and blended for 10 sec in 20 ml DW. Control plants were drenched with an equal volume of DW. When the plants reached the four-leaf stage (35 days after seeding), they were transplanted into individual 5-litre plastic bags filled with sawdust and inoculated once more with the bacteria and *P. aphanidermatum* as described above. Each plant received the complete nutrient solution described in the production guide for commercial growers (Anonymous, 1988). Fertilizer applications were made eight times a day for 10 min and each plant received 0.87 L at each application or 7 L per day. The plants were supported using heavy twine that was attached to a horizontal wire 2 m from the ground. Fruit was removed from the first nine nodes from the ground. From the ninth node upward, only one fruit was left per node. Plant height was measured when plants were 29, 42, and 54 days old. Cucumber fruits were harvested and weighed when they had reached a diameter of 4.5 cm. Nine days after harvest had begun (69 days after seeding), fresh and dry weights of shoots of all plants were measured. There were nine replicate plants for each of the four treatments (untreated, strain 63-28 with *P. aphanidermatum*, strain 63-49 with *P. aphanidermatum*, and *P. aphanidermatum* alone). Data were subjected to an ANOVA and means were compared using Duncan's Multiple Range Test.

Chapter 3

Results

3.1 *In-Vitro* Antagonism Tests

None of the bacteria in this study, with the exception of *Enterobacter aerogenes* B 8, produced an inhibition zone when tested against either *Pythium ultimum* or *P. aphanidermatum* in dual culture on CMA. The inhibition zone produced by *E. aerogenes* against *P. ultimum* averaged 11.6 mm, and for *P. aphanidermatum* it averaged 6.4 mm. This represents 13.6 % and 7.5% inhibition for *P. ultimum* and *P. aphanidermatum*, respectively, when compared to the control. All of the other bacteria inhibited the growth of *P. ultimum* only at the point where the two organisms met. However, in the case of *P. aphanidermatum*, the fungus grew over the bacterial streak of all other isolates and reached the opposite side of the petri dish unobstructed.

3.2 Temperature-Growth Studies

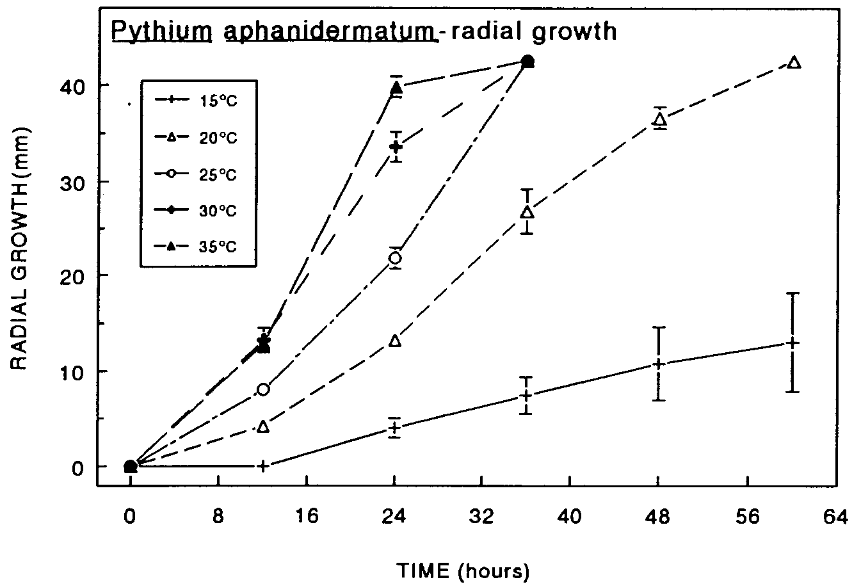
3.2.1 Fungi

Radial Growth

For *P. aphanidermatum* (Fig 1a), growth after 24 hr was significantly different at all temperatures between 15 to 35°C. Growth, however, was not correlated with temperature within the range of 31-35°C (Fig 2). Growth of *P. aphanidermatum* was maximal at 35°C, but was more rapid at 31 and 32°C than at 33 and 34°C. The fungus did not grow well at 15°C (Fig 1a). There was no significant difference ($P=0.05$) between growth at 31 and 32°C nor

Fig. 1a. Radial growth of *Pythium aphanidermatum* on water agar at 15-35°C.

Fig. 1b. Mycelial dry weight of *Pythium aphanidermatum* grown in potato dextrose broth at 15-35°C.


Pythium aphanidermatum-radial growth
15°C
20°C
25°C
30°C
35°C
RADIAL GROWTH (mm)
0
10
20
30
40
0
8
16
24
32
40
48
56
64
TIME (hours)

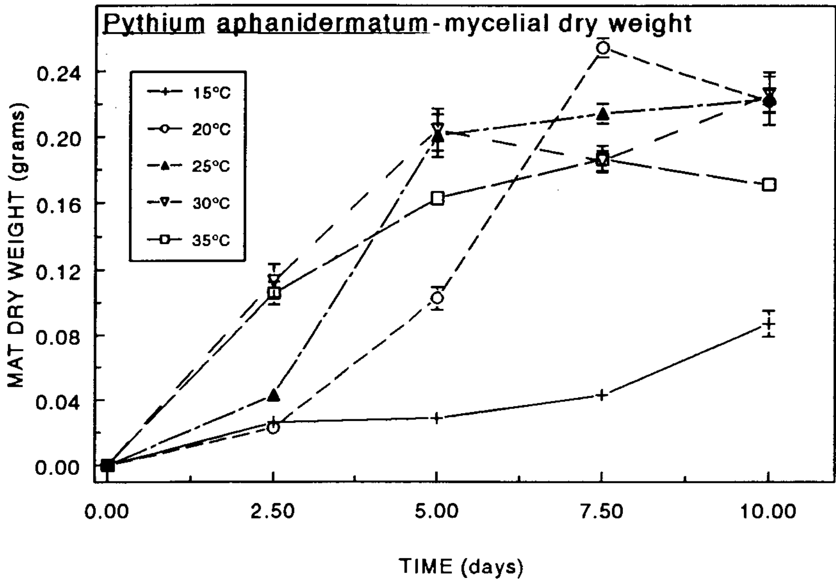

Pythium aphanidermatum-mycelial dry weight
15°C
20°C
25°C
30°C
35°C
MAT DRY WEIGHT (grams)
0.00
0.04
0.08
0.12
0.16
0.20
0.24
0.00
2.50
5.00
7.50
10.00
TIME (days)

Fig. 2. Radial growth of *Pythium aphanidermatum* on water agar at 31-35°C.

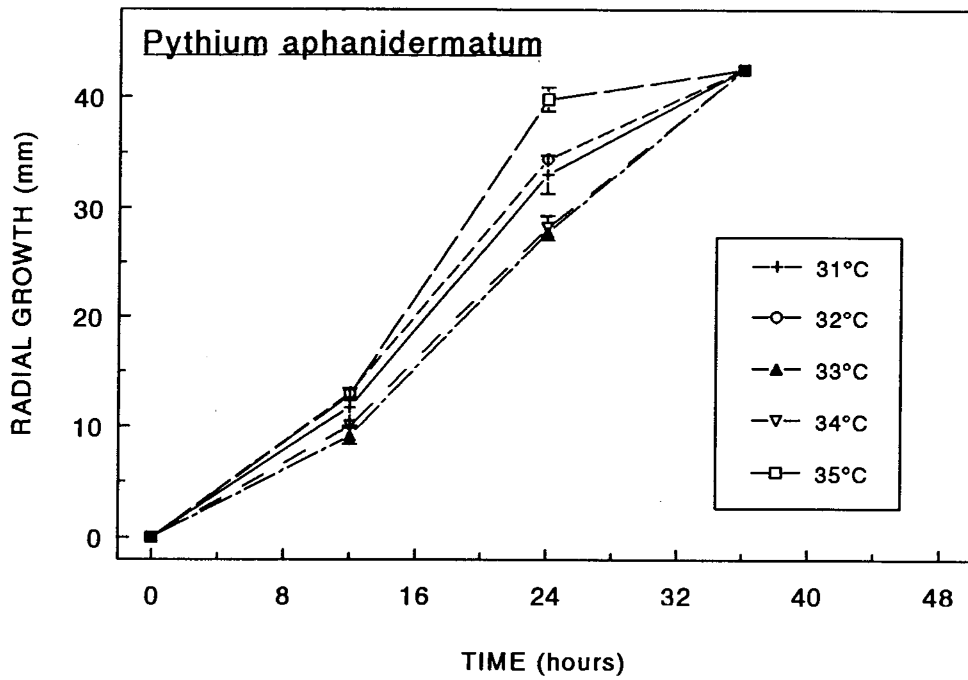

Pythium aphanidermatum
RADIAL GROWTH (mm)
TIME (hours)
0
10
20
30
40
0
8
16
24
32
40
48
31°C
32°C
33°C
34°C
35°C

Fig. 3. Relationship between radial growth of *Pythium aphanidermatum* and temperature. Regression line illustrates linear relationship from 15-30°C which deteriorates at higher temperatures.

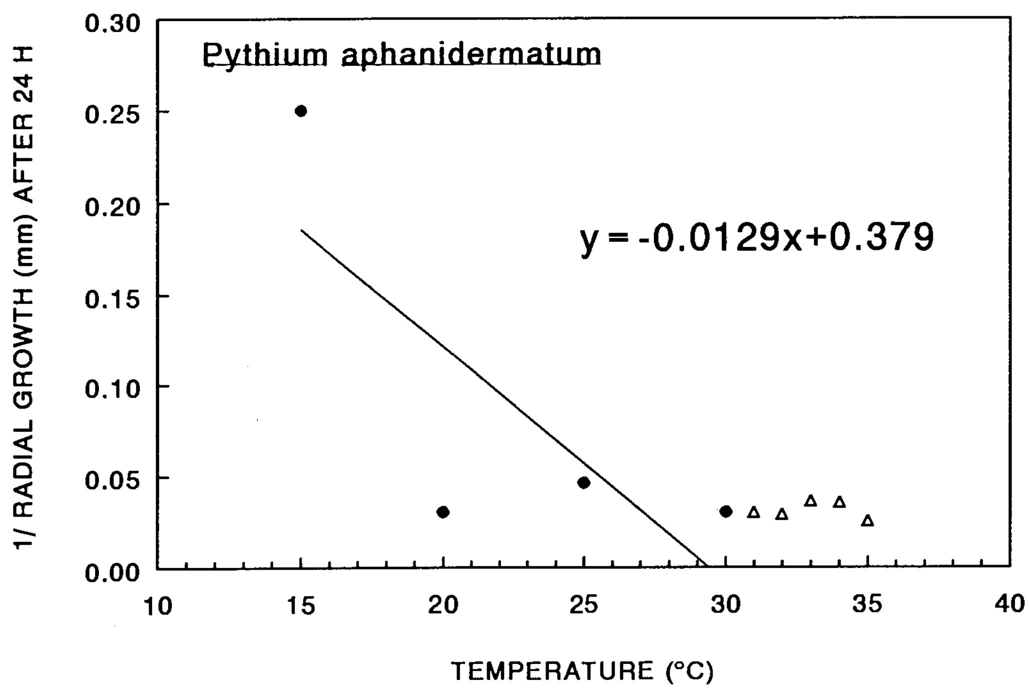
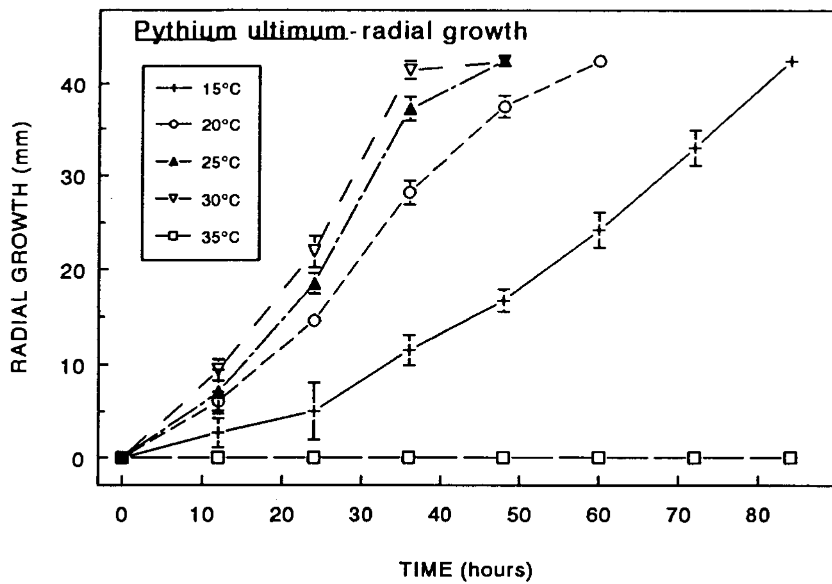

Pythium aphanidermatum
y = -0.0129x+0.379
1/ RADIAL GROWTH (mm) AFTER 24 H
0.30
0.25
0.20
0.15
0.10
0.05
0.00
10
15
20
25
30
35
40
TEMPERATURE (°C)

Fig. 4a. Radial growth of *Pythium ultimum* on water agar at 15-35°C.

Fig. 4b. Mycelial dry weight of *Pythium ultimum* grown in potato dextrose broth at 15-35°C.


Pythium ultimum-radial growth
15°C
20°C
25°C
30°C
35°C
RADIAL GROWTH (mm)
0
10
20
30
40
0
10
20
30
40
50
60
70
80
TIME (hours)

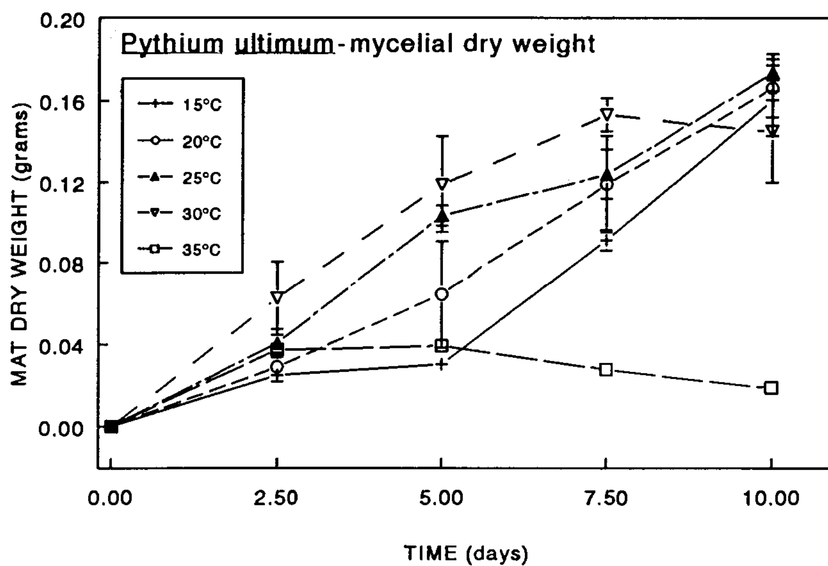

Pythium ultimum-mycelial dry weight
15°C
20°C
25°C
30°C
35°C
MAT DRY WEIGHT (grams)
0.00
0.04
0.08
0.12
0.16
0.20
0.00
2.50
5.00
7.50
10.00
TIME (days)

Fig. 5. Radial growth of *Pythium ultimum* on water agar at 31-35°C.

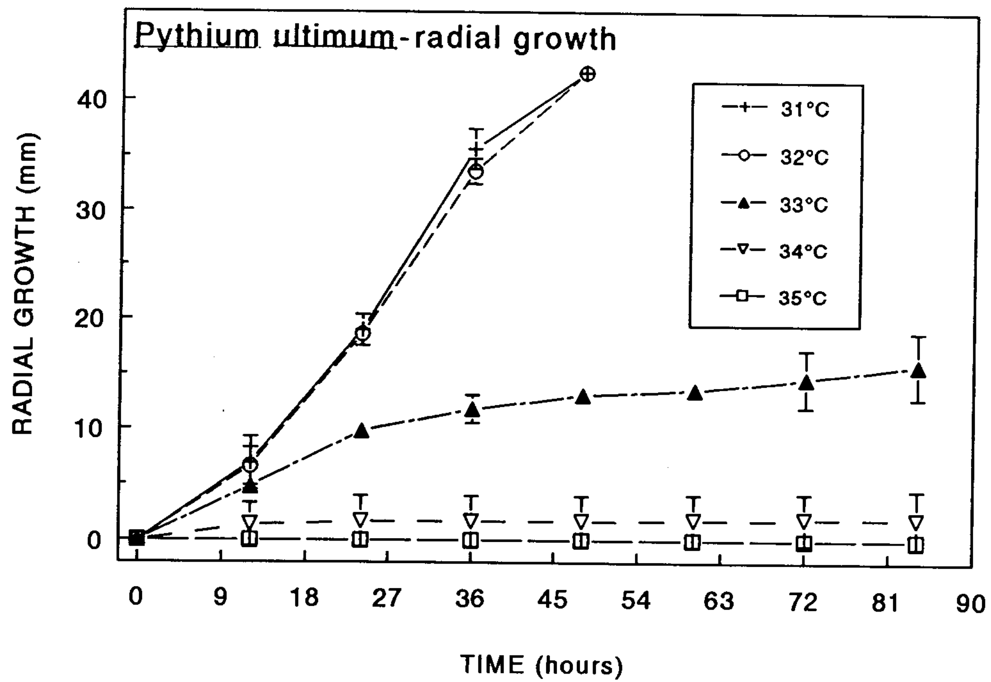
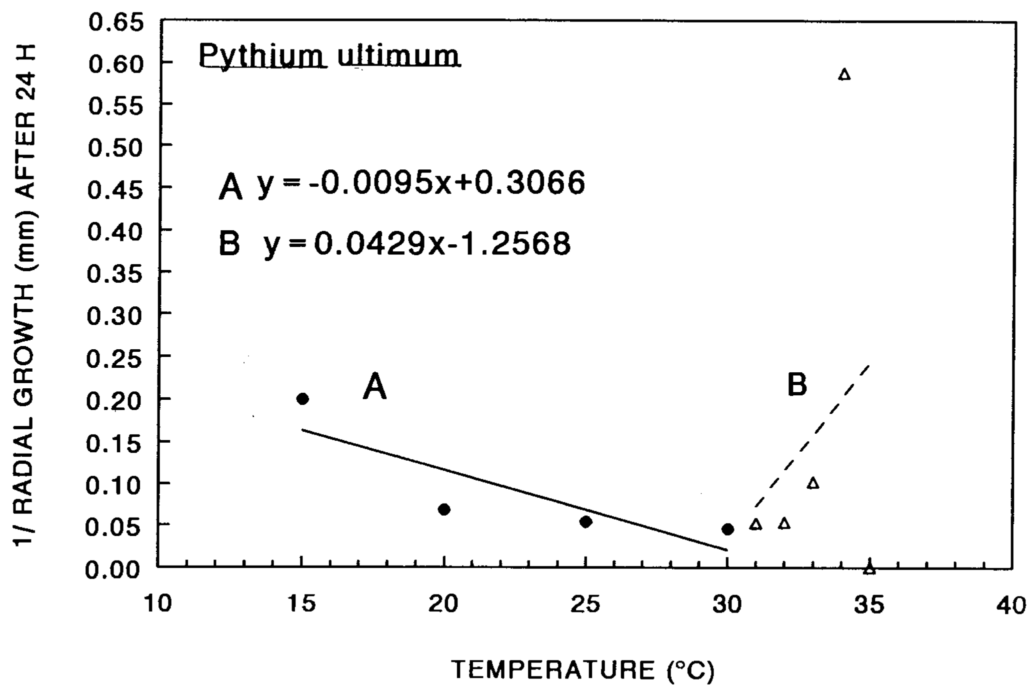

Pythium ultimum-radial growth
RADIAL GROWTH (mm)
TIME (hours)
31°C
32°C
33°C
34°C
35°C
0
10
20
30
40
0
9
18
27
36
45
54
63
72
81
90

Fig. 6. Relationship between radial growth of *Pythium ultimum* and temperature. Regression lines illustrate linear relationship between 15 and 30°C and between 31 and 35°C.


Pythium ultimum
A y = -0.0095x+0.3066
B y = 0.0429x-1.2568
A
B
1/ RADIAL GROWTH (mm) AFTER 24 H
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
10
15
20
25
30
35
40
TEMPERATURE (°C)

between 33 and 34°C after 24 hr (Fig 2). For *P. ultimum*, growth at the five temperatures from 15 to 35°C was significantly different (Fig 4a). Within the range of 31-35°C, no significant differences were observed between 31 and 32°C, nor between 34 and 35°C (Fig 5). This species showed a marked growth response in this temperature range. Growth was maximal at 31 and 32°C, intermediate at 33°C and negligible at 34 and 35°C (Fig 5).

Mycelial Dry Weight

The greatest biomass of *P. aphanidermatum* was produced at 30°C (Fig 1b). However, the greatest mean relative growth rate (MRGR, calculated using the following equation: natural log w_2 - natural log w_1 / T_2 - T_1), was observed at 25°C.

For *P. ultimum*, there was no significant difference (P=0.05) between growth at 25 and 30°C, and these temperatures were optimal for this species (Fig 4b). When MRGR was calculated, the optimum temperature was found to be 25°C.

3.2.2 Bacteria

Pseudomonas fluorescens 63-28

The results from studies on the effects of temperature are illustrated in Figs 7 and 8. The mean square values for all bacteria are listed in Appendix II. *Ps. fluorescens* 63-28 attained a maximum population level after 12 hr at 30°C (Fig 7a). At all other temperatures, maximum growth occurred after 24 hr. There was no significant difference (P=0.05) between growth (cfu/ml) at 20 and 25°C at 24hr. Also, no significant difference was found for growth between 15 and 35°C nor between 25 and 35°C at this time. When the combined effects of time and

temperature were compared for all sampling times (12, 24, 36, and 48 hr), growth was significantly ($P<0.025$) different at all temperatures. When all temperatures were compared to 20°C for the last sampling times (24, 36, and 48 hr), 15 and 20°C, and 20 and 30°C were not significantly different. The optimum temperature was in the range of 20 to 25°C, although this strain grew well at all temperatures tested, except 35°C.

Pseudomonas fluorescens 63-49

This strain did not grow as well at 35°C as it did at all other temperatures (Fig. 7b). The optimum temperature for this strain was 25°C. Growth at this temperature differed significantly ($P<0.0045$) from all other temperatures after 24 hr.

Enterobacter aerogenes B 8

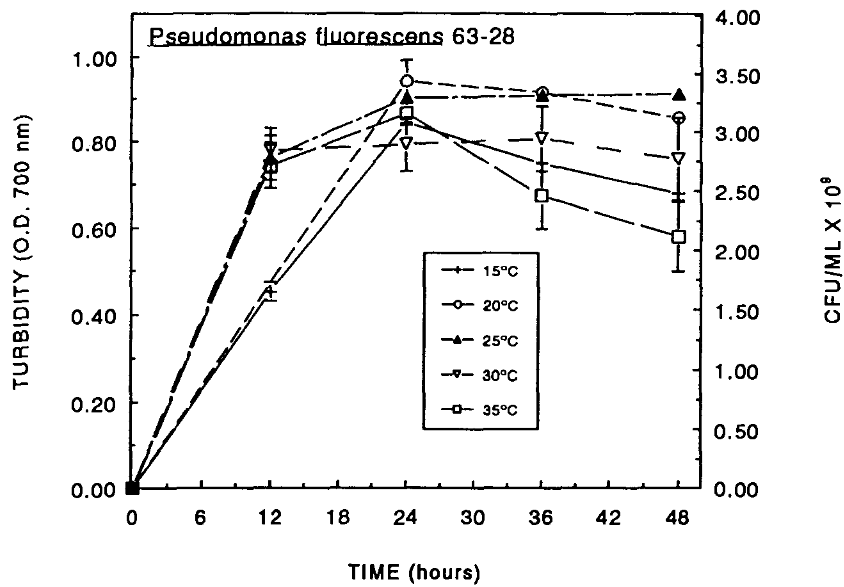
Growth of B 8 was not significantly different ($P=0.05$) at any of the temperatures tested except 15°C, which differed significantly from 25, 30 and 35°C (Fig 8a). Growth was similar at 20, 25, and 35°C.

Bacillus subtilis AB 8

This species grew slowly at 15°C and at 20°C as compared to the other temperatures tested (Fig 8b). Its growth was not significantly different at 25, 30 and 35°C and it grew most rapidly at these temperatures.

Fig. 7a. Growth of *Pseudomonas fluorescens* 63-28 in nutrient broth at 15-35°C.

Fig. 7b. Growth of *Pseudomonas fluorescens* 63-49 in nutrient broth at 15-35°C.


Pseudomonas fluorescens 63-28
TURBIDITY (O.D. 700 nm)
CFU/ML X 10⁹
TIME (hours)
15°C
20°C
25°C
30°C
35°C
0.00
0.20
0.40
0.60
0.80
1.00
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
0
6
12
18
24
30
36
42
48

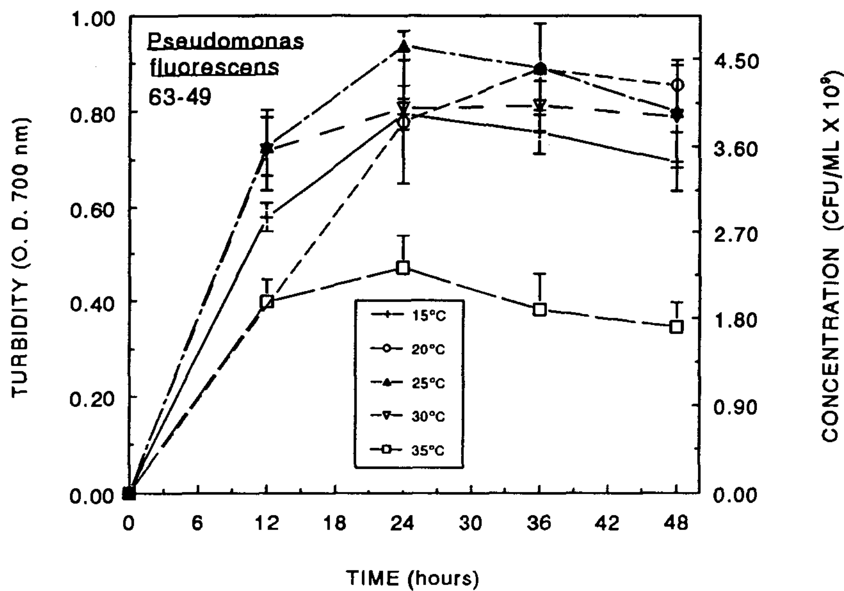
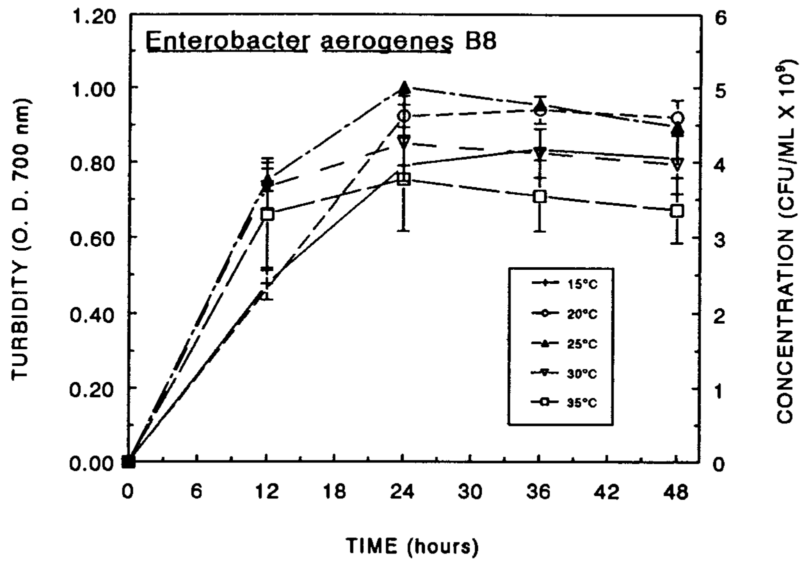
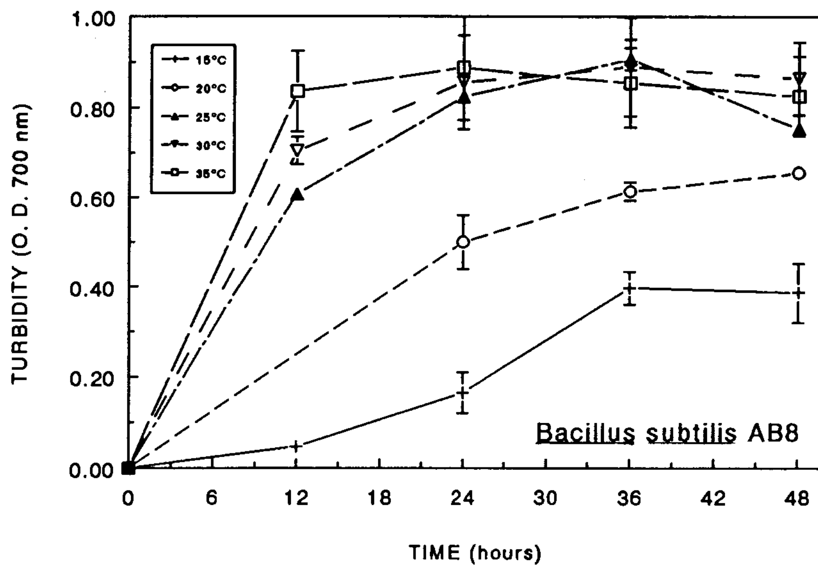

Pseudomonas fluorescens 63-49
TURBIDITY (O. D. 700 nm)
CONCENTRATION (CFU/ML X 10⁹)
TIME (hours)
15°C
20°C
25°C
30°C
35°C
0.00
0.20
0.40
0.60
0.80
1.00
0.00
0.90
1.80
2.70
3.60
4.50
0
6
12
18
24
30
36
42
48

Fig. 8a. Growth of *Enterobacter aerogenes* B 8 in nutrient broth at 15-35°C.

Fig. 8b. Growth of *Bacillus subtilis* AB 8 in potato dextrose broth at 15-35°C.


Enterobacter aerogenes B8
TURBIDITY (O. D. 700 nm)
CONCENTRATION (CFU/ML X 10⁹)
TIME (hours)
15°C
20°C
25°C
30°C
35°C
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
1
2
3
4
5
6
0
6
12
18
24
30
36
42
48


Bacillus subtilis AB8
TURBIDITY (O. D. 700 nm)
TIME (hours)
15°C
20°C
25°C
30°C
35°C
1.00
0.80
0.60
0.40
0.20
0.00
0
6
12
18
24
30
36
42
48

3.3 Effect of Cucumber Age on Infection by *Pythium*

None of the plants inoculated with *P. ultimum* on days 0, 6, and 9 and only 20 % of the plants inoculated on days 3, 12, and 15 survived (Fig 9). Regression was significant, indicating that increase in plant age yielded a measurable increase in survival (Fig 10). In contrast, 60 and 100% of seedlings inoculated on days 18 and 21, respectively, survived.

When the trial was repeated with *P. ultimum*, very few seeds emerged and the experiment was abandoned. When the seeds were later unearthed, many had germinated and subsequently suffocated because they had been unable to emerge through the compact soil.

3.4 Biocontrol of *Pythium* Damping-Off of Cucumbers

The results from the first two trials are shown in Figure 11. Since there was no significant difference between treatments, i.e. between strains or between times of planting, when the two trials were compared, the results were combined. When treatments 2 and 3 (strains 63-28 and 63-49 of *Pseudomonas*) were combined, there was no significant ($P=0.058$) difference between them and the untreated control. Similarly, when treatments 2 and 4 were combined, i.e. *Ps. fluorescens* 63-28 regardless of time, there was no significant ($P=0.119$) difference between the treatment and the untreated control. This was true of *Ps. fluorescens* 63-49 as well. Treatments 1 (control), 2 (*Ps. fluorescens* 63-28 and *P. aphanidermatum*), and 5 (*Ps. fluorescens* 63-49 and *P. aphanidermatum*, delayed planting) were each significantly ($P<0.05$) different from treatment 6 (*Pythium* alone).

The results from the subsequent two trials are illustrated in Figures 12 and 13. In trial 3, none of the treatments differed significantly from the *Pythium*-treated plants ($P=0.057$ for

Fig. 9. Effect of plant age at time of inoculation on mortality of cucumber plants caused by *Pythium aphanidermatum*.

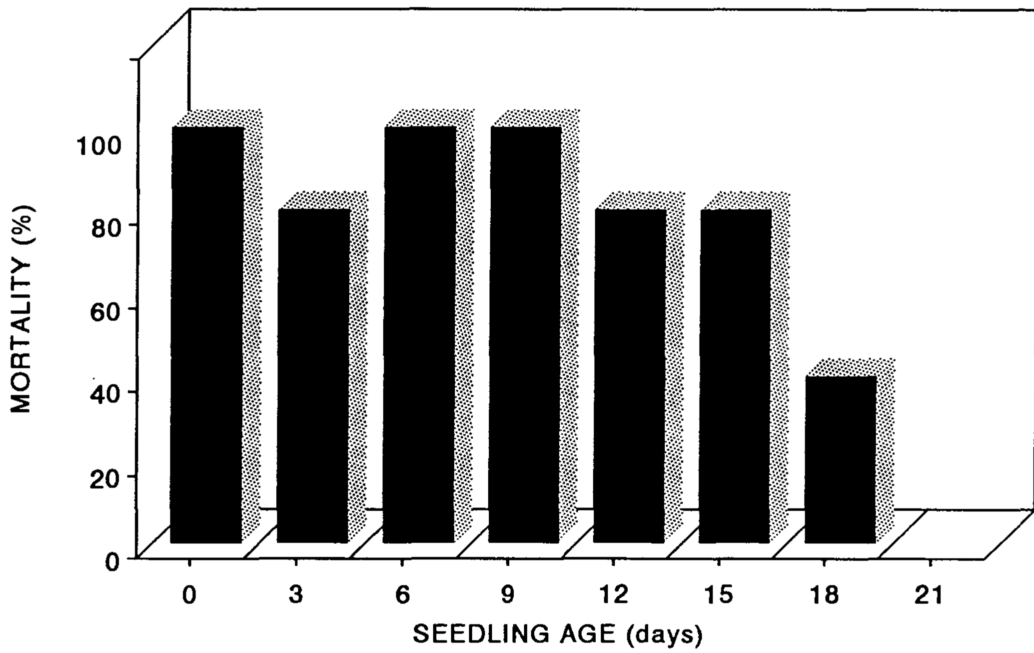

MORTALITY (%)
100
80
60
40
20
0
0
3
6
9
12
15
18
21
SEEDLING AGE (days)

Table 4: Effect of age on survival of cucumber plants transplanted into sandy loam soil infested with *P. aphanidermatum*

Plant age at time of transplant into infested soil	# seedlings emerged		#plants alive on day 39	
	treatment	control	treatment	control
0	0	4	0	4
3	3	4	1	4
6	0	2	0	2
9	3	5	0	5
12	4	3	1	3
15	5	4	1	3
18	3	3	3	3
21	5	4	5	4

Fig. 10. Relationship between arcsin of the proportion survival of cucumber plants inoculated with *Pythium aphanidermatum* and plant age at the time of inoculation.

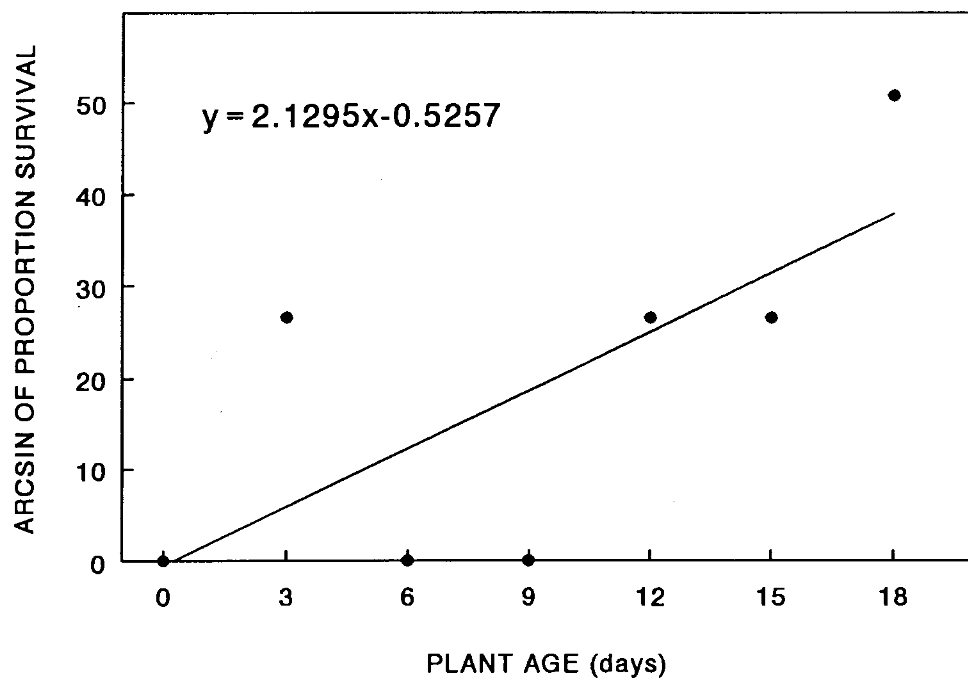

y = 2.1295x-0.5257
ARCSIN OF PROPORTION SURVIVAL
0
10
20
30
40
50
0
3
6
9
12
15
18
PLANT AGE (days)

Fig. 11. Influence of bacterial treatments on survival of cucumber plants growing in sandy loam soil and inoculated with *Pythium aphanidermatum* (Py). Results of Trials 1 and 2. (*= treatments differing significantly from treatment with *Pythium aphanidermatum* alone)

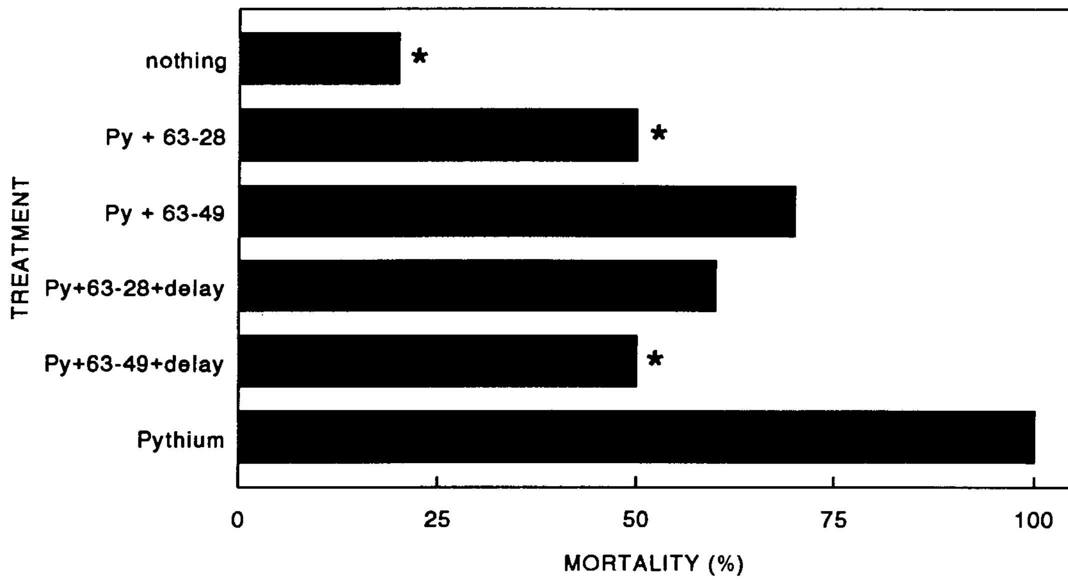

TREATMENT
nothing
Py + 63-28
Py + 63-49
Py+63-28+delay
Py+63-49+delay
Pythium
*
*
*
0
25
50
75
100
MORTALITY (%)

Fig. 12. Influence of bacterial treatments on survival of cucumber plants growing in sand and vermiculite and inoculated with *Pythium aphanidermatum*. Results of Trial 3.

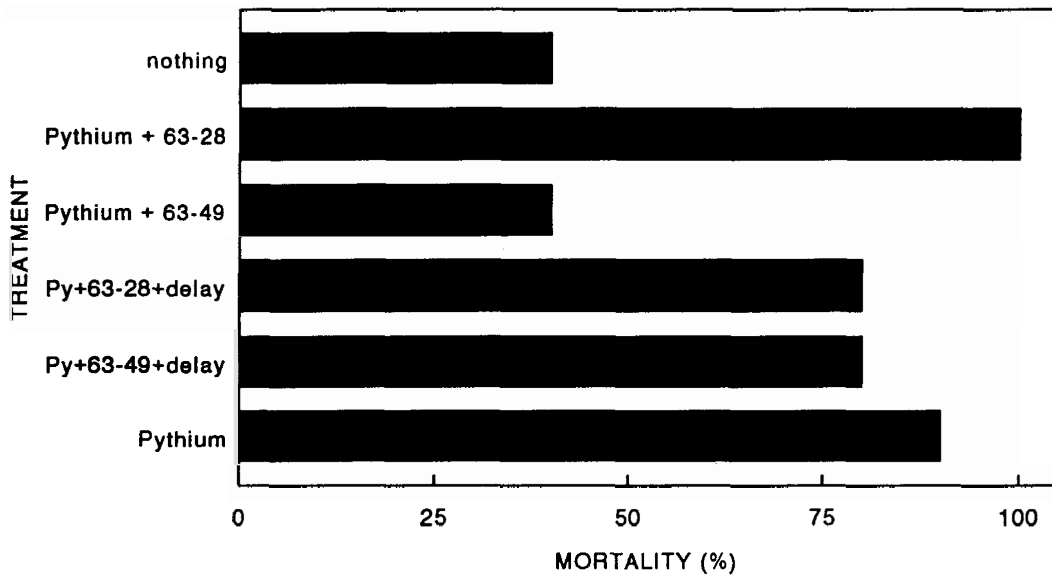
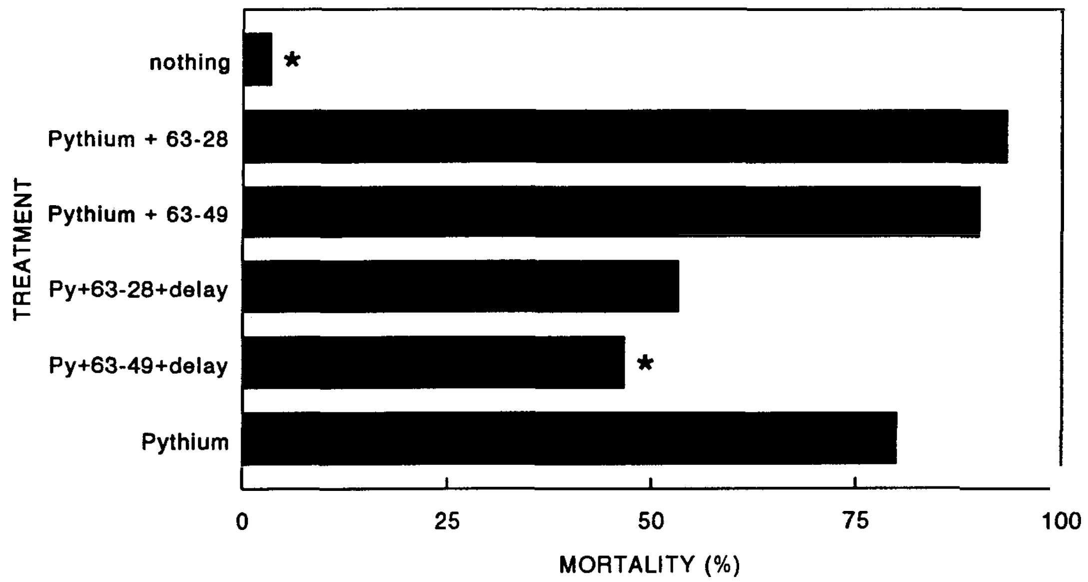

nothing
Pythium + 63-28
Pythium + 63-49
Py+63-28+delay
Py+63-49+delay
Pythium
TREATMENT
0
25
50
75
100
MORTALITY (%)

Fig. 13. Influence of bacterial treatments on survival of cucumber plants growing in sand and vermiculite and inoculated with *Pythium aphanidermatum*. Results of Trial 4. (*=treatments differing significantly from treatment with *Pythium aphanidermatum* alone)


nothing
Pythium + 63-28
Pythium + 63-49
Py+63-28+delay
Py+63-49+delay
Pythium
TREATMENT
*
*
0
25
50
75
100
MORTALITY (%)

treatment 3, 63-49 + *Pythium*). There was a high percentage of mortality in the untreated plants, however, which makes the results uninformative. In trial 4 (Fig 13), treatment 1 (untreated control) and 5 (*Pythium* combined with 63-49 and delayed planting) were significantly different from treatment 6 (*Pythium* alone) ($P=0.001$ and $P=0.015$, respectively).

3.5 Biocontrol of Crown Rot of Cucumbers in Hydroponic Culture

Both *Pseudomonas* strains showed marked growth promoting ability in the first few days after seeding. The treated seeds germinated more rapidly, and reached the first true leaf stage 2 days before the positive and negative controls (see Plate 1). By day 29, 11 days after the first bacterial drench, the negative effects of inoculation with *P. aphanidermatum* were evident (see Table 6). Average fresh weight of mature plants treated with *Pythium* alone was 17.3 % lower than that of the untreated control, and 16 % lower than plants treated with *Pythium* and 63-49 (Table 6). With regard to the immature fruits remaining on the plants at the time of harvest, the *Pythium*-treated plants weighed 22.6 % less than the untreated plants and 13.6 % less than plants treated with 63-49 and *Pythium*. None of these differences was significant at the 0.05 level. *Pythium*-treated plants produced considerably more commercial-size fruit than did the untreated control. When these data are considered, total biomass of the untreated control plants is only 8.7 % greater than that of the *Pythium*-treated plants. However, fruit production by *Pythium*-treated plants had decreased greatly by the time the plants were harvested. Average dry weights of untreated plants and plants treated with 63-49 and *Pythium* were significantly higher ($P=0.05$) than that of the *Pythium*-treated plants ($P=0.05$).

Plate 1. Growth promoting effects of *Pseudomonas fluorescens* strains 63-28 (*P. fluorescens* 1) and 63-49 (*P. fluorescens* 2) on 34-day old cucumber seedlings growing in rockwool and inoculated with *Pythium aphanidermatum*

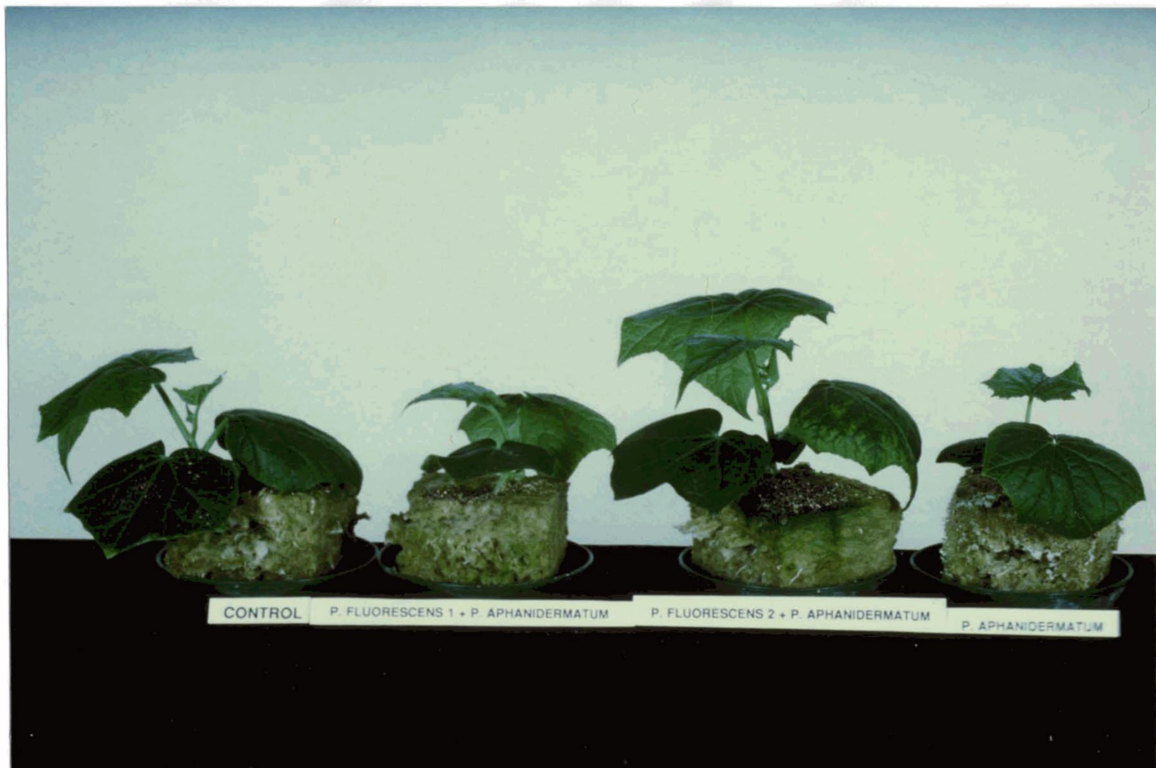

CONTROL
P. FLUORESCENS 1 + P. APHANIDERMATUM
P. FLUORESCENS 2 + P. APHANIDERMATUM
P. APHANIDERMATUM

Table 5. Height of hydroponically grown cucumber plants seed-treated and inoculated with *Pseudomonas fluorescens*, strains 63-28 and 63-49 on days 9 and 35 and inoculated with *Pythium aphanidermatum* on days 18 and 35 (n = 9).

Treatment	Average plant height (cm)		
	Day 29	Day 42	Day 54
None	8.38	99.3	201.7
63-28+*Pythium*	9.5	83	203.1
63-49+*Pythium*	10.7	82.2	204.8
Pythium	6.75	75.25	187.5

Table 6: Fresh weight, fruit weight, and dry weight of hydroponically grown cucumber plants seed-treated and inoculated with *Pseudomonas fluorescens* strains 63-28 and 63-49 on days 9 and 35 and inoculated with *Pythium aphanidermatum* on days 18 and 35.

	Treatment			
	None	Strain 63-28	Strain 63-49	*Pythium*
Average fresh weight (g)	1030.5	940.6	1015	853
% of untreated control (n=9)		91.3	98.5	82.7
Average immature fruit weight (g)	442.3	335	303.9	286.4
% of untreated control (n=9)		75.7	68.7	64.7
Average fresh weight + immature fruit weight (g)	1472.8	1275.5	1318.9	1139.4
% of untreated control (n=9)		86.6	89.5	77.4
Average commercial fruit weight (g)	586.5	864.5	693.4	739.8
% of untreated control (n=9)		147.4	118.2	126.1
Average total biomass (g)	2059.3	2140	2012.8	1879.2
% of untreated control (n=9)		103.9	97.7	91.3
Average dry weight (g)	89.2*	80.1	84.3*	69.6
% of untreated control (n=9)		89.7	94.5	78

* Significantly different from plants treated with *Pythium* alone

Chapter 4

Discussion

Antagonism on agar media *in vitro* is often used as a criterion for the selection of organisms for use as biocontrol agents in the field. The validity of this criterion is still being debated. Schroth and Hancock (1981) argued that data of this nature cannot be correlated with biocontrol activity in natural soil. Weller (1988) stated "Since no general relationship exists between the ability of a bacterium to inhibit a pathogen *in vitro* and suppress disease caused by that pathogen *in vivo*, strains producing the largest zones of inhibition on agar media do not always make the best biocontrol agents". Utkhede and Gaunce (1983) observed no correlation between growth of *Phytophthora cactorum* in dual culture with *Enterobacter aerogenes* B 8 and protection of McIntosh apple seedlings from infection under greenhouse conditions.

In contrast, other researchers have found antagonism *in vitro* to be a good indication of biocontrol performance in the field. Alconero (1980) tested *Agrobacterium radiobacter* K 84 against 10 strains of *Agrobacterium tumefaciens* and found that those strains of the pathogen that were least susceptible in petri dish assays caused the greatest gall development on peach and tomato in the greenhouse, and *in vitro* inhibition was correlated with protection *in planta*. In a trial conducted by Howell and Stipanovic (1980), *Pseudomonas fluorescens* Pf-5 inhibited *Pythium ultimum* on King's B medium. When *Ps. fluorescens* or its antibiotic, pyoluteorin, were applied as a seed treatment at the time of planting in *Pythium*-infested soil, they increased seedling survival significantly. Weller and Cook (1983) found that 40% of the fluorescent

pseudomonads that showed *in vitro* inhibition of *Gaeumannomyces graminis* (Sacc.) Oliver and Von Arx, the causal agent of take-all of wheat, protected wheat seedlings from take-all in greenhouse pot tests where they were applied as seed and soil treatments. They used *in vitro* performance for initial selection of candidate bacteria.

In the present research, *in vitro* antagonism on agar was not correlated with *in planta* protection. The only bacterium which produced an inhibition zone in trials against both *Pythium* species (*E. aerogenes* B 8), did not protect cucumbers from infection by *Pythium aphanidermatum*. On the other hand, both strains of *Pseudomonas* showed potential as biocontrol agents *in planta* yet neither showed any antagonism *in vitro*. This does not imply that strain B 8 would not have inhibitory capabilities under different experimental conditions. Many factors come into play in the root zone that can affect the ability of a bacterium to become established and to be an effective antagonist. Similarly, the two strains of *Ps. fluorescens* were tested on only one agar medium (CMA) and could show some antagonism *in vitro* on a different medium, such as PAF or PDA. *Bacillus subtilis* AB 8 did not exhibit any antagonism *in vitro* nor *in planta*.

In the evaluation of the effect of cucumber age on infection by *Pythium*, the results appeared to indicate that the plants were most susceptible to damping-off during the first 15 days after seeding. After this critical age, cucumber tissues may have become resistant. Mellano et al. (1970) determined the critical age for infection of snapdragon by *Pythium* to be 25 days. Red clover becomes resistant after 5 days, and alfalfa and sweet clover after 3 days (Endo and Colt,1974). The explanation for this age-induced resistance is that the cell walls thicken and become lignified as the plant grows. Pectin is converted to calcium pectate in the cell wall, and

cross-linkages are formed among the cell wall polymers. The formation of a suberized layer of cells in resistant geraniums and strawberries was also reported to stop the spread of *Pythium* in these plants (Endo and Colt, 1974). However, while invasion of the pathogen was checked in mature tissues, root tips and feeder roots remained susceptible. In cucumbers, infection of mature plants manifests itself as crown rot and results in a decrease in yield and overall vigour of the plant. When the plant is stressed by other factors, it may wilt, in some cases irreversibly. The purpose of this experiment was therefore to determine at which point a plant could escape damping-off in a soil infested with a high population of *Pythium* propagules. Of those plants exposed to the pathogen on day 15, only one survived compared to 60% and 100% of those plants exposed on days 18 and 21, respectively. Although plant roots were not examined for the presence of *Pythium*, at the high inoculum level used (2500 colony forming units/g soil), it can be assumed that the pathogen was present in the root zone. The maturing cucumber tissue could have limited infection to root tips and feeder roots and allowed plants to escape death due to damping-off. The poor germination of the seeds in the control in this experiment caused by the density of the unamended sandy loam from the Summerland Research Station complicates the validity of the results, however. Normally, the germination rate of Fidelio cucumber seeds is approximately 99%. When this trial was conducted using soil infested with *P. ultimum*, seedling emergence was so poor that the trial was not completed. It is necessary, therefore, to consider emergence as well as mortality, since the emergence was low in the control, but mortality was insignificant (one plant) when compared to the *Pythium* treatment (see Table 4). The data presented here for *P. aphanidermatum* will only be useful when the experiment is repeated using a different growth medium with a larger number of replicates.

Studies on radial growth of *P. aphanidermatum* indicated that hyphal elongation was most rapid at 35°C. Growth rate decreased with decreasing temperature, and at 15°C, this species grew very slowly. On the other hand, measurements made of mycelial dry weight seemed to indicate that 30°C was the optimum temperature. Differences in optimum temperatures between radial growth on agar and mycelial growth in broth seem quite typical. Bateman and Dimock (1959) found that the optimum temperature range for three fungi, including *P. ultimum,* was greater when growth was measured as an increase in colony diameter compared to increase in dry weight and that hyphal growth on agar was rapid at temperatures considered to be below optimum.

In the present study, *P. aphanidermatum* grew very slowly at 15°C and growth ceased on some of the replicate plates, which explains the very high standard deviation after 24 hr. Gold and Stanghellini (1985) found that this species caused significant yield reductions on hydroponically grown spinach at 27 and 21°C, but not at 17°C. *P. aphanidermatum* is classified as a high temperature fungus which, like *Pythium arrhenomanes* and *Pythium carolinianum*, is more damaging at elevated temperatures (Hendrix and Campbell, 1973). Luna and Hine (1964) found that mycelial growth of *P. aphanidermatum* from an agar disc in soil was optimum at temperatures between 28 and 31°C. Ayers and Lumsden (1975) observed satisfactory oospore production by *P. aphanidermatum* at temperatures ranging from 15 to 35°C.

In contrast, *P. ultimum*, like *P. irregulare* and *P. spinosum,* is considered to be a low-temperature species. In the present study, *P. ultimum* exhibited the most rapid radial growth at 30°C and produced the greatest mycelial dry weight at 25 and 30°C. It did not grow at all on agar at 35°C and barely grew at 34°C and also grew very poorly in PDB at 35°C. Other

workers have reported the optimum temperature for growth of *P. ultimum* to be between 28 and 32°C (Bateman and Dimock,1959). If these temperatures seem rather high for a low temperature species, it should be noted that temperature optima for growth may be different from those for infection. Kraft and Roberts (1969) found that disease caused by *P. ultimum* on pea was most severe at 18 or 24°C when the soil water tension was between - 1/3 and - 1 bar. Bateman and Dimock (1959) observed that root rot of poinsettia caused by this species developed best at 17°C, and plants were not seriously damaged at 26°C or above. They pointed out that disease was most severe at temperatures unfavourable for growth of the host plant regardless of temperatures favouring growth of the fungus. However, if a fungus shows no growth at certain temperatures *in vitro*, it can be assumed that it would be nonpathogenic at these temperatures in nature.

Under conditions found in British Columbia, temperatures of the nutrient solution in the greenhouse typically range from 16 to 30°C, providing the appropriate temperature range for growth of the two pathogens used in this study. In areas where *P. ultimum* is a problem, temperatures at night as well as daytime temperatures during the cooler months would be conducive to development of this pathogen. In areas where *P. aphanidermatum* is the dominant pathogen, as it is in B.C., the hotter summer temperatures not only favour the fungus but put the plant at a disadvantage, particularly when it is stressed by other factors, such as fruit production.

According to Bergey's Manual (Krieg and Holt, 1984), the optimum temperature for growth of *Ps. fluorescens* is 25 - 30°C. The two strains tested here showed optimum growth in this general range. Strain 63-49 was somewhat more sensitive to high temperature than 63-28

since it did not grow as well at 35°C. The significance of these findings is that these two strains, which are soil inhabiting aerobes, are favoured by the same temperatures as the two species of *Pythium*. The competitive ability of these bacteria may therefore be maintained under environmental conditions found in the rhizosphere of greenhouse cucumbers. Under appropriate conditions, *Pseudomonas* species are aggressive colonizers and vigorous competitors (Kloepper et al., 1980), and may place *Pythium* spp., which are weak competitors, at a disadvantage.

E. aerogenes B 8 was not affected by temperature and grew well at 20 to 35°C. *B. subtilis* AB 8 grew well at high temperatures, with an optimum at 30 - 35°C. It grew poorly at 15 and 20°C. In trials conducted by Gupta and Utkhede (1986), *E. aerogenes* B 8 produced the greatest amount of anti-fungal compounds at 14 - 21°C and showed maximum population growth in sterilized soil at 18°C. In the same study, *B. subtilis* produced the most antifungal compounds between 21 and 28°C, and the optimum temperature for maximum population growth in sterilized soil was 25°C. These two organisms are known antagonists of *Phytophthora cactorum*, the causal agent of crown rot of apple trees, which has an optimum temperature between 18 and 25°C (Gupta and Utkhede, 1986).

The results from the first biocontrol trial indicated that none of the four species of bacteria alone had an adverse effect on the emergence and survival of cucumber seedlings. Since strains B 8 and AB 8 did not appear to provide protection against *Pythium*, they were not included in subsequent trials. The two species of *Pseudomonas* provided some protection when applied at planting and were thus tested again in subsequent trials. When data from trials 1 and 2 were combined, there were no differences between any of the treatments in which both *Pythium* and a bacterium were applied. In treatments with strains 63-28 and 63-49, 50 and 30

% of plants survived, respectively. In treatments with strains 63-28 and 63-49 applied 48 hr prior to *P. aphanidermatum*, 40 and 50 % of plants survived, respectively.

In trial 3, there was poor germination in the control, making the results uninformative. In trial 4, the medium used was sand and vermiculite and a higher rate of emergence was obtained in the control compared to the sandy loam used in other trials. In this trial, there was a significant difference between the two times of planting but no difference between the two strains of *Ps. fluorescens*.

The inconsistent results obtained in these trials reflect the inherent problem associated with much of the work being conducted on biological control of plant diseases. Capper and Campbell (1986) obtained 0 to 114% increase in yield of wheat using *Bacillus pumilus* as an antagonist against *Gaeumannomyces graminis*. Weller and Cook (1983) observed a 6 to 27% increase in wheat using *Ps. fluorescens* against the same pathogen. A -3 to 136% yield increase was observed when *Ps. fluorescens* E 6 was inoculated against minor pathogens on zinnia (Yuen and Schroth, 1986). Weller (1988) attributed inconsistent performance to a multitude of factors, including loss of ecological competence on the part of the bacterium, the absence of the target pathogen or interference by nontarget pathogens, and variable root colonization by bacteria. Loss of ecological competence can occur when a bacterium is cultured repeatedly in the lab. For fluorescent pseudomonads, this may result in changes in colony morphology, loss of cell surface structures, or reduction in antifungal substances. Interference of a nontarget pathogen can occur if suppression of the target pathogen occurs to the advantage of another pathogen. Finally, if a bacterium cannot become established on the plant root or if after establishment the population declines greatly over time, its competence as an antagonist will be diminished.

The sources of variability in the biocontrol experiments in this study have not been determined. It is unlikely that the bacteria had undergone mutation since they were stored at - 70°C and were frequently transferred. The target pathogen was not absent and there was no interference from nontarget pathogens since sterilized medium was used. However, populations of *Pythium* did vary from 10,000 cfu/ml medium in trials 1 and 2 to 2800 cfu/ml medium in trials 3 and 4. However, low *Pythium* population was not correlated with higher protection. While the same methods of bacterial inoculation were used in all trials, it is quite possible that variability existed in the extent of root colonization between trials and even between replicates within a treatment. Variability could also be due, in part, to differences that may have existed in environmental conditions between trials. Temperature, for instance, may fluctuate a few degrees as the growth chamber adjusts to changes in outside temperature. Bacterization at lower temperatures may be influenced by increased amounts of carbohydrates released from seeds as temperatures decrease (Hayman, 1969), making the seeds more susceptible to infection by *Pythium* (Keeling, 1974). Irrespective of whether seeds were sown at the same time that bacteria were applied or 48 hr after bacteria had been introduced to the medium, there was no effect in trials 1, 2, or 3. In trial 4, a difference was observed between the two times. This may indicate that the medium used (sand and vermiculite) was more conducive to establishment of the antagonist than the soil used in previous trials.

The mechanisms by which the two species of *Pseudomonas* provided protection against *Pythium* infection was not explored here, since it was beyond the scope of this project. However, as discussed in the introduction, there are reports that antibiosis may be involved. Cotton seed survival was increased by inoculation with antibiotics isolated from *Ps. fluorescens*

strain Pf-5 (Howell and Stipanovic, 1980). Biological control of *P. ultimum* on cotton was due, in part, to the antibiotic oomycin A produced by *Ps. fluorescens* strain Hv37a, since mutants which did not produce the antibiotic were less efficient (Gutterson et al., 1986; Howie et al., 1989). *Ps. fluorescens* 2-79 is inhibitory to *G. graminis* var. *tritici*, the causal agent of take-all of wheat. Gurusiddaiah et al. (1986) isolated the antibiotic phenazine-1-carboxylic acid (PCA) from this strain, tested it against several fungi, and found excellent inhibitory activity. Thomashow et al. (1990) treated wheat seeds with PCA-producing and PCA-nonproducing strains of *Ps. fluorescens*. The antibiotic was later isolated only from the roots of seedlings which had been seed-treated with the wild-type bacterium. In both steamed and natural soils, these roots had significantly less disease than roots from which the antibiotic had not been isolated.

Disease suppression by pseudomonads has also been ascribed to induced resistance of plants to pathogens (Anderson and Guerra, 1985; Zhou et al., 1992), to the production of siderophores (Becker and Cook, 1988; Loper. 1988), and to competition for carbon substrates (Paulitz, 1991). Strain 63-28 has been tested for ability to increase average root length of cucumber, tomato, and canola (M.S. Reddy, personal communication)[2] and was found to increase cucumber root length by 21% over the untreated control. However, root elongation activity was not correlated with biocontrol activity, suggesting that other mechanisms are involved.

The results from this project demonstrate the potential of two strains of *Pseudomonas* as biocontrol agents for protection of cucumber plants against damping-off caused by *P.*

---

[2]Research Scientist, Esso Ag. Biologicals, Saskatoon, Saskatchewan.

aphanidermatum. However, much work remains to be done to determine the sources of variability between trials and the conditions required to achieve the greatest biocontrol activity. This might involve testing a variety of environmental conditions such as moisture level, temperature, fertilizer and light regimes, as well as other parameters such as bacterial populations, *Pythium* inoculum levels, and inoculation techniques. The antagonists themselves should be studied thoroughly to elucidate conditions which would enhance root colonization and survival. This could involve studies to determine temperature requirements not just for growth but for colonization. Osmotolerance, nutrient requirements, and other factors should also be examined. An understanding of the mechanisms involved in this system is necessary if we are to utilize these organisms to their full potential. Molecular techniques appear to be useful in this regard. Should antibiotics or lytic enzymes prove to be the mechanisms by which such bacteria function, conditions affecting the production of these substances must be elucidated so that the rhizosphere can be manipulated to enhance such production.

If biocontrol of *Pythium* on hydroponic cucumbers is to have any practical use, the antagonists must be convenient and simple to use. This requires the development of a formulation which allows for the absorption and survival of the antagonist, has good storage life, is not harmful to the plant, and is easy to deliver. A formulation which would also provide the antagonists with a food base would be ideal. Campbell et al. (1992) tested Promix G, Allegro, perlite and vermiculite as formulation carriers of strain 63-28 and found vermiculite to be the most promising, although populations declined by 2 log units after 4 weeks. Further studies of this nature are required.

Research is also required for nutrient film technique (NFT) systems, which are becoming

increasingly popular given the build-up of used rockwool, the waste of fertilizer and the subsequent pollution associated with the present methods of hydroponic cultivation.

When many of these questions have been addressed, commercialization of PGPR's and fungal antagonists will be feasible, and it will be possible to offer greenhouse growers new products which will increase yield and quality of their crops.

REFERENCES

Agrios, G.N. 1988. Plant Pathology. Academic Press Inc., San Diego, pp. 293-299.

Allen, M.C., and Haenseler, C.M. 1935. Antagonistic action of *Trichoderma* on *Rhizoctonia* and other soil fungi. Phytopathology 25:244-252.

Alconero, R. 1980. Crown gall of peaches from Maryland, South Carolina and Tennessee and problems with biological control. Plant Dis. 64:835-838.

Anderson, A.J., and Guerra, D. 1985. Response of bean to root colonization with *Pseudomonas putida* in a hydroponic system. Phytopathology 75:992-1005.

Anonymous, 1988. Nursery, greenhouse, vegetable and ornamental production guide for commercial growers. Province of British Columbia, Ministry of Agriculture and Food, Victoria, B.C. 24pp.

Ayers, W.Y., and Lumsden, R.D. 1975. Factors affecting production and germination of oospores of three *Pythium* species. Phytopathology 65:1094-1100.

Barton, R. 1957. Germination of oospores of *Pythium mamillatum* in response to exudates from living seedlings. Nature 180:613-614.

Bateman, D.F., and Dimock, A.W. 1959. The influence of temperature on root rots of poinsettia caused by *Thielaviopsis basicola, Rhizoctonia solani* and *Pythium ultimum*. Phytopathology 49:641-647.

Becker J.O., and Cook, R.J. 1988. Role of siderophores in suppression of *Pythium* species and production of increased growth response of wheat by fluorescent pseudomonads. Phytopathology 78:778-782.

Campbell, S.E., Reddy, M.S., and Brown, G.L. 1992. Delivery, persistence and efficacy of *Pseudomonas fluorescens* 63-28 as a commercial biocontrol agent for greenhouse grown cucumbers. Can.J.Plant Path. 14:239 (Abstr).

Capper, A.L., and Campbell, R. 1986. The effect of artificially inoculated antagonistic bacteria on the prevalence of take-all disease of wheat in field experiments. J. Appl. Bacteriol. 60:155-160.

Carrier, A. 1989. Concombres de serre; nos plants viellisent mal. Producteur Horticole. 5:11-12.

Chen, W., Hoitink, H.A., and Madden, L.V. 1988. Microbial activity and biomass in container media for predicting suppressiveness to damping-off caused by *Pythium ultimum*. Phytopathology 78:1447-1450.

Chen, W., Hoitink, H.A., and Schmitthenner, A.F., and Tuovinen, O.H. 1988. The role of microbial activity in suppression of damping-off caused by *Pythium ultimum*. Phytopathology 78:314-322.

Elad, Y., and Chet, I. 1987. Possible role of competion for nutrients in biocontrol of *Pythium* damping-off by bacteria. Phytopathology 77:190-195.

Endo, R.M., and Colt, W.M. 1974. Anatomy, cytology and physiology of infection by *Pythium*. Amer Phytopath. Soc. Proc. 1:215-223.

Favrin, R. 1987. Crown rot of cucumbers in coastal British Columbia greenhouses. Master of Pest Management Professional Paper. Simon Fraser University, Burnaby, B.C.

Favrin, R., and Rahe, J.E., and Mauza, B. 1988. *Pythium* spp. associated with crown rot of cucumbers in British Columbia greenhouses. Plant Dis. 72:683-687.

Gold, S.E., and Stanghellini, M.E. 1985. Effects of temperature on *Pythium* root rot of spinach grown under hydroponic conditions. Phytopathology 75:333-337.

Gupta, V.K., and Utkhede, R.S. 1986. Factors affecting the production of antifungal compounds by *Enterobacter aerogenes* and *Bacillus subtilis*, antagonists of *Phytophthora cactorum*. J. Phytopathology 117:9-16.

Gurusiddaiah, S., Weller, D.M., Sarkar, A., and Cook, R.J. 1986. Characterization of an antibiotic produced by a strain of *Pseudomonas fluorescens* inhibitory to *Gaeumannomyces graminis* var. *tritici* and *Pythium* spp. Antimicrob. Agents Chemother. 29:488-495.

Gutterson, N.I., Layton, T.J., Ziegle, J.S., and Warren, G.J. 1986. Molecular cloning of genetic determinants for inhibition of fungal growth by a fluorescent pseudomonad. J. Bacteriol. 165:696-703.

Hadar, Y., Harman, G.E., Taylor, A.G., and Norton, N.M. 1983. Effects of pregermination of pea and cucumber seeds and of seed treatment with *Enterobacter cloacae* on rots caused by *Pythium* spp. Phytopathlogy 73:1322-1325.

Harman, G.E., Chet, I., and Baker, R. 1980. *Trichoderma hamatum* effects on and seedling disease induced in radish and pea by *Pythium* spp. or *Rhizoctonia solani*. Phytopathology 70:1167-1172.

Harman, G.E., Eckenrode, C.J. and Webb, D.R. 1978. Alteration of spermosphere ecosystems affecting oviposition by the bean seed fly and attack by soilborne fungi on germinating seeds. Ann. Appl. Biol. 90:1-6.

Harman, G.E., and Hadar, Y. 1983. Biological control of *Pythium* species. Seed Sci. and Technol. 11:893-906.

Hayman, D.S. 1969. The influence of temperature on the exudation of nutrients from cotton seeds and on pre-emergence damping-off by *Rhizoctonia solani*. Can. J. Bot. 47:1663-1668.

Hendrix, F.F., Jr., and Campbell, W.A. 1973. Pythiums as plant pathogens. Ann. Rev. Phytopathology 73:77-98.

Howell, C.R., and Stipanovic, R.D. 1980. Suppression of *Pythium ultimum* induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic pyoluteorin. Phytopathology 70:712-715.

Howell, C.R., and Stipanovic, R.D. 1983. Gliovirin, a new antibiotic from *Gliocladium virens* and its role in the biological control of *Pythium ultimum*. Can. J. Microbiol. 29:321-324.

Howie, W., Matsubara, D., Gutterson, N., and Suslow, T. 1989. Directed enhancement of biocontrol in *Pseudomonas* by constitutive antibiotic synthesis. Phytopathology 79:1160 (Abstr.).

Johnson, L.F. 1952. Control of root rot of corn under greenhouse conditions by microorganisms antagonistic to *Pythium arrhenomanes*. Phytopathology 73:1284-1289.

Kao, C.W., and Ko, W.H. 1986. The role of calcium and microorganisms in suppression of cucumber damping-off caused by *Pythium* splendens in a Hawaiian soil. Phytopathology 76:221-225.

Keeling, B.L. 1974. Soybean seed rot and the relation of seed exudate to host susceptibility. Phytopathology 64:1445-1447.

Kloepper, J.W., Schroth, M.N., and Miller, T.D. 1980. Effects of rhizosphere colonization by plant growth promoting rhizobacteria on potato plant development and yield. Phytopathology 70: 1078-1082.

Ko, W., and Lockwood, J.L. 1967. Soil fungistasis: relation to fungal spore nutrition. Phytopathology 57:894-901.

Kraft, J.M., and Roberts, D.D. 1969. Influence of soil water and temperature on the pea root rot complex caused by *Pythium ultimum* and *Fusarium solani* f. sp. pisi. Phytopahtology 59:149-152.

Kraus, J., and Loper, J.E. 1992. Lack of evidence for a role of antifungal metabolite production by *Pseudomonas fluorescens* Pf-5 in biological control of *Pythium* damping-off of cucumber. Phytopthology 82:264-271.

Krieg, N.R., and Holt, J.G. 1984. Bergey's manual of systematic bacteriology. Vol 1. Williams and Wilkins, Baltimore, pp.142-165.

Liu, S., and Vaughan, E.K. 1965. Control of *Pythium* infection in table beet seedlings by antagonistic microorganisms. Phytopathology 55:986-989.

Loper, J.E. 1988. Role of fluorescent siderphore production in biological control of *Pythium ultimum* by a *Pseudomonas fluorescens* strain. Phytopathology 78:166-172.

Luna, L., and Hine, R.B. 1964. Factors affecting saprophytic growth of *Pythium aphanidermatum* in soil Phytopathology 54:955-959.

Martin, S.B., Hoch, H.C., and Abawi, G.S. 1983. Population dynamics of *Laetisaria arvalis* and low-temperature *Pythium* spp. in untreated and pasteurized beet field soils. Phytopathology 73:1445-1449.

Mellano, H.M., Munnecke, D.E., and Endo, R.M. 1970. Relationship of seedling age to development of *Pythium ultimum* on roots of *Antirrhinum majus*. Phytopathology 60:935-942.

Mircetich, S.M., and Kraft, J.M. 1973. Efficiency of various media in determining *Pythium* populations in soil. Mycopathol. Mycol. Appl. 50:151-161.

Mitchell, R., and Hurwitz, E. 1965. Suppression of *Pythium debaryanum* by lytic rhizosphere bacteria. Phytopathology 55:156-158.

Nelson, E.B., and Craft, C.M. 1992. A miniaturized and rapid bioassay for the selection of soil bacteria suppressive to Pythium blight of turfgrasses. Phytopathology 82: 206-210.

Parke, J.L. 1990. Population dynamics of *Pseudomonas cepacia* in the pea spermosphere in relation to biocontrol of *Pythium*. Phytopathology 80:1307-1311.

Paulitz, T.C. 1991. Effect of *Pseudomonas putida* on the stimulation of *Pythium ultimum* by seed volatiles of pea and soybean. Phytopathology 81:1282-1287.

Paulitz, T.C., and Baker, R. 1987. Biological control of *Pythium* damping-off of cucumbers with *Pythium nunn*: influence of soil environment and organic amendments. Phytopathology 77:341-346.

Paulitz, T.C., Zhou, T., and Rankin, L. 1992. Selection of rhizosphere bacteria for biological control of *Pythium aphanidermatum* on hydroponically grown cucumbers. Biological Control 2:226-237.

Schroth, M.N., and Hancock, J.G. 1981. Selected topics in biological control. Ann. Rev. Microbiol. 35:453-476.

Thomashow, L.S., Weller, D.M., Bonsall, R.F., and Pierson, L.S. 1990. Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. Appl. Environ. Microbiol. 56:908-912.

Utkhede, R.S., and Gaunce, A.P. 1983. Inhibition of *Phytophthora cactorum* by a bacterial antagonist. Can. J. Bot. 61:3343-3348.

Weller, D.M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Ann. Rev. Phytopathology 26:379-407.

Weller, D.M., and Cook, R.J. 1983. Suppression of take-all of wheat by seed treatments with fluorescent pseudomonads. Phytopathology 73:463-469.

Yuen, G.Y., and Schroth, M.N. 1986. Interactions of *Pseudomonas fluorescens* strain E6 with ornamental plants and its effect on the composition of root-colonizing microflora. Phytopathology 76:176-180.

Zhou,T., Rankin,L., and Paulitz, T.C. 1992. Induced resistance in the biological control of *Pythium aphanidermatum* by *Pseudomonas* spp. on European cucumber. Phytopathology 83:872-876.

Appendix I

Optical Density Curves to Measure Bacterial Populations

Enterobacter aerogenes B 8 and *Pseudomonas fluorescens* strains 63-28 and 63-49 were grown overnight at 28°C, 150 rpm, in nutrient broth. *Bacillus subtilis* AB 8 was grown in PDB. One ml of each suspension was pipetted onto each of five plates containing the appropriate medium (NA for *E. aerogenes*, PAF for *Ps. fluorescens* 63-28 and 63-49 and PDB for *B. subtilis*). After 24 hr the growth on all five plates was scraped into 1 L SDW. Ten dilutions were made by adding .5 ml SDW to a bacterial suspension (i.e. 5 : 0 to 0.5 : 4.5, bacterial suspension : DW). The optical densities of the 10 dilutions were measured at 700 nm using a Bausch and Lomb spectrophotometer with water as a blank.

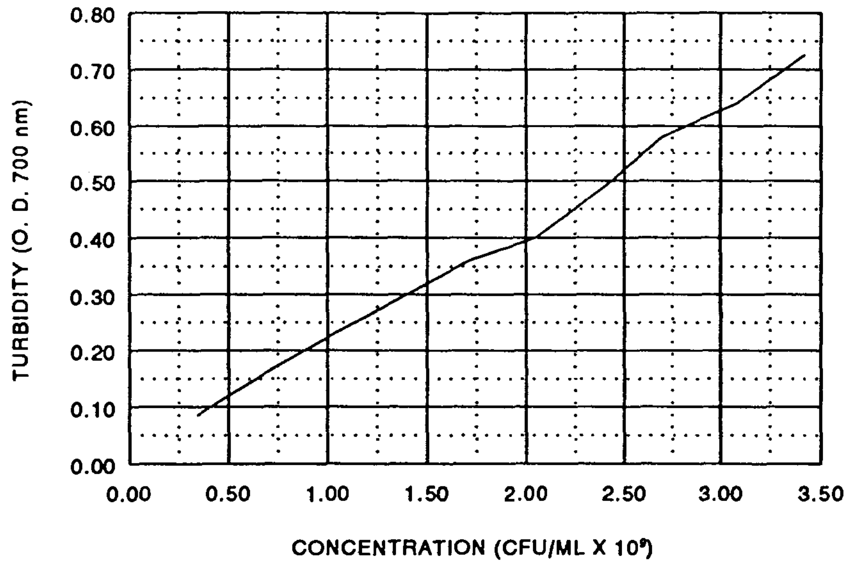
To measure the population of the original solution, 10 ml were pipetted into a 90 ml SW blank and shaken. Ten ml samples were then transferred immediately through successive 90 ml sterile water blanks until a 1:100,000,000 dilution was reached. Each of the 8 dilutions were then spiral plated using a Spiral Plater Model D (Spiral Systems Inc.) onto five plates of the appropriate medium and incubated at 28°C for 24 hr. Dilutions yielding reasonable numbers of colonies were counted and an average of the five plates was calculated. This number was multiplied by the dilution factor to obtain the number of CFU per ml of solution. An average of all countable dilutions was taken to be the population of the original solution. This population was then calculated for the 10 optical densities obtained yielding the following graphs (figures 1, 3, 5, and 7).

To measure the population of a bacterium in broth for the purpose of the temperature-growth studies, the same procedure was followed except the plates were scraped into the broth

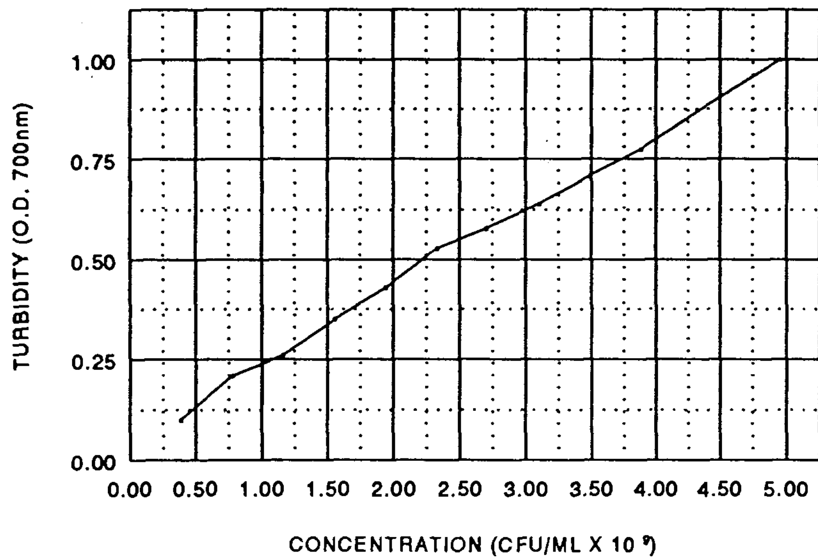
and pure broth was used as a blank in the spectrophotometer (see figures 2, 4, and 6). This procedure was not successful for AB 8 so although growth (Section 2.4.2) of the bacterium was measured using optical density, this optical density is not correlated with population.

Fig. 1. Curve relating optical density of *Pseudomonas fluorescens* 63-28 to population levels using water as a blank.

Fig. 2. Curve relating optical density of *Pseudomonas fluorescens* 63-28 to population levels using nutrient broth as a blank.



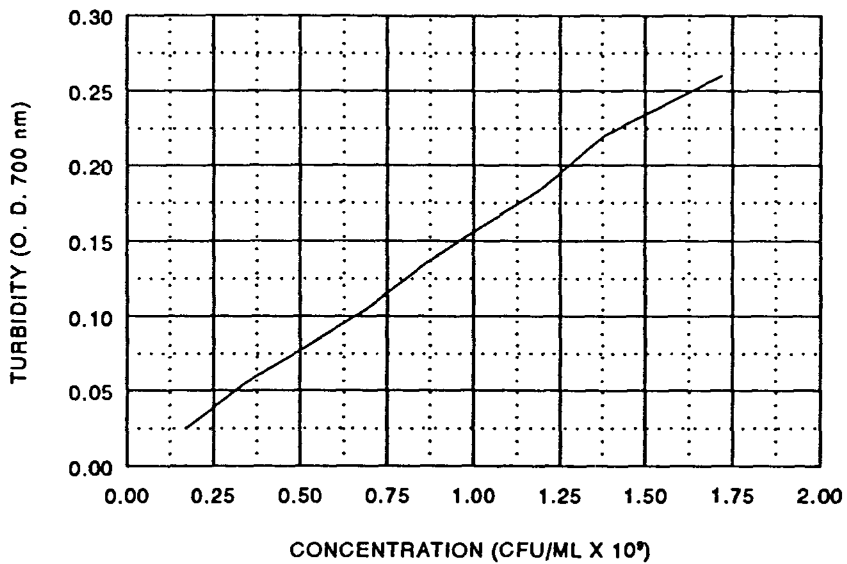
TURBIDITY (O. D. 700 nm)
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.00 0.50 1.00 1.50 2.00 2.50 3.00 3.50
CONCENTRATION (CFU/ML X 10⁹)



TURBIDITY (O.D. 700nm)
1.00
0.75
0.50
0.25
0.00
0.00 0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50 5.00
CONCENTRATION (CFU/ML X 10⁹)

Fig. 3. Curve relating optical density of *Pseudomonas fluorescens* 63-49 to poluation levels using water as a blank.

Fig. 4. Curve relating optical density of *Pseudomonas fluorescens* 63-49 to population levels using nutrient broth as a blank.


0.30
0.25
0.20
0.15
0.10
0.05
0.00
TURBIDITY (O. D. 700 nm)
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
CONCENTRATION (CFU/ML X 10⁹)

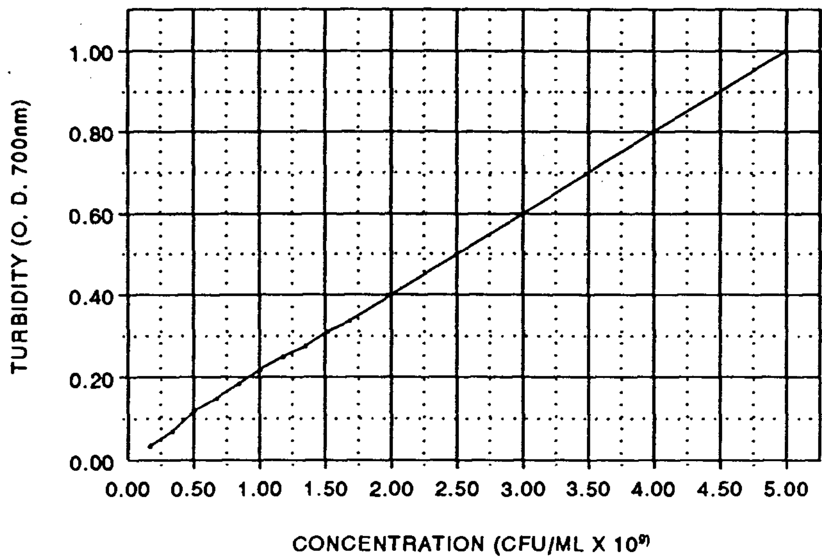
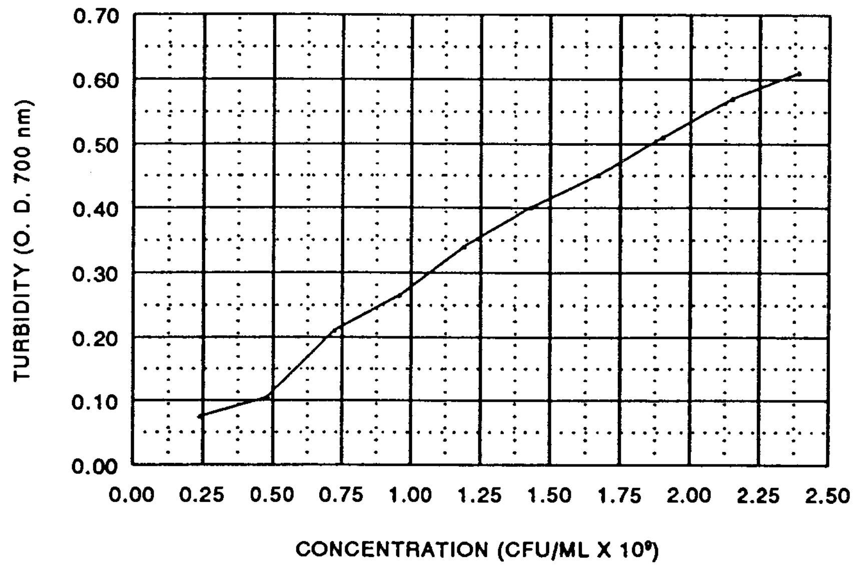
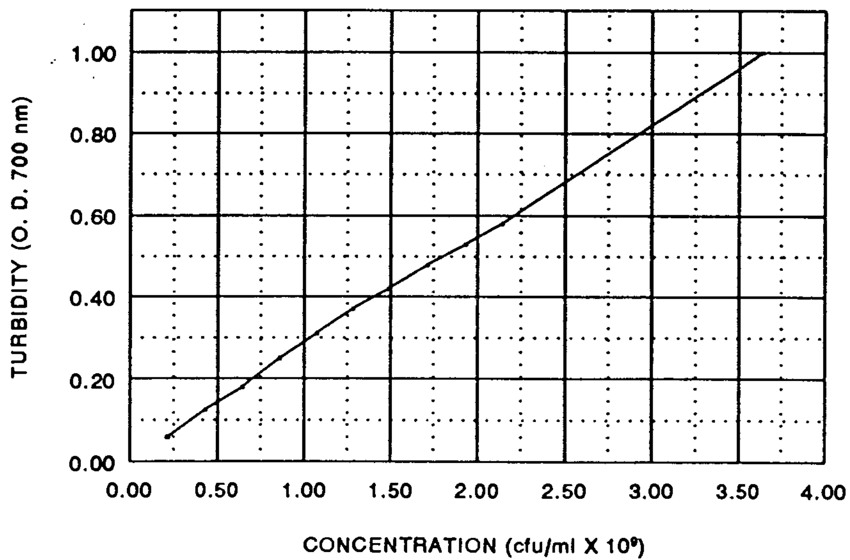

1.00
0.80
0.60
0.40
0.20
0.00
TURBIDITY (O. D. 700nm)
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
CONCENTRATION (CFU/ML X 10⁹)

Fig. 5. Curve relating optical density of *Enterobacter aerogenes* B 8 to population levels using water as a blank.

Fig. 6. Curve relating optical density of *Enterobacter aerogenes* B 8 to population levels using nutrient broth as a blank.

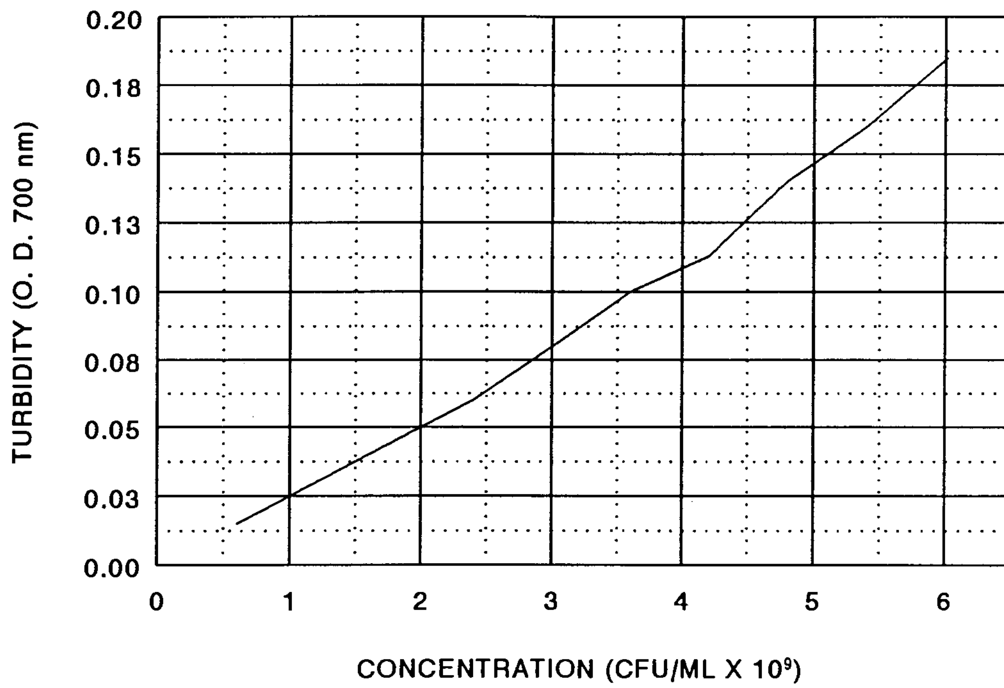


TURBIDITY (O. D. 700 nm)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.00 0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00 2.25 2.50
CONCENTRATION (CFU/ML X 10⁹)



TURBIDITY (O. D. 700 nm)
1.00
0.80
0.60
0.40
0.20
0.00
0.00 0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00
CONCENTRATION (cfu/ml X 10⁹)

Fig. 7. Curve relating optical density of *Bacillus subtilis* AB 8 to population levels using water as a blank.


0.20
0.18
0.15
0.13
0.10
0.08
0.05
0.03
0.00
TURBIDITY (O. D. 700 nm)
0
1
2
3
4
5
6
CONCENTRATION (CFU/ML X 10⁹)

Appendix II

M.S. values comparing growth of four bacterial antagonists at various temperatures generated by an ANOVA with repeated measures.

Pseudomonas fluorescens 63-28

Temperature	12h	24h	36h	48h
15-20		0.021	0.06	0.07
15-25	0.213	0.008	0.056	0.12
15-30	0.237	0.0055	0.007	0.014
15-35	0.19	0.001	0.0125	0.022
20-25		0.004	0.0001	0.008
20-30		0.055	0.03	0.023
20-35		0.014	0.1452	0.19
25-30	0.0007	0.03	0.026	0.058
25-35	0.0008	0.0034	0.137	0.276
30-35	0.003	0.013	0.044	0.081

Pseudomonas fluorescens 63-49

Temperature	12h	24h	36h	48h
15-20		0.0006	0.043	0.06
15-25	0.056	0.05	0.044	0.027
15-30	0.048	0.0003	0.0066	0.019
15-35	0.069	0.0126	0.017	0.034
20-25		0.062	0.00001	0.0073
20-30		0.002	0.013	0.0094
20-35		0.239	0.643	0.648
25-30	0.00002	0.037	0.0137	0.0002
25-35	0.0006	0.0126	0.117	0.1221
30-35	0.0008	0.007	0.042	0.098

Enterobacter aerogenes B 8

Temperature	12h	24h	36h	48h
15-20		0.044	0.027	0.03
15-25	0.19	0.11	0.035	0.017
15-30	0.17	0.009	0.0003	0.001
15-35	0.085	0.003	0.039	0.05
20-25		0.015	0.0005	0.0014
20-30		0.013	0.034	0.0397
20-35		0.07	0.132	0.154
25-30	0.0008	0.056	0.042	0.026
25-35	0.02	0.151	0.149	0.125
30-35	0.0137	0.023	0.032	0.037

Bacillus subtilis AB 8

Temperature	12h	24h	36h	48h
15-20		0.28	0.117	0.18
15-25	0.79	1.089	0.65	0.34
15-30	1.08	1.197	0.6	0.57
15-35	1.56	1.31	0.52	0.48
20-25		0.262	0.215	0.025
20-30		0.317	0.19	0.108
20-35		0.376	0.144	0.072
25-30	0.02	0.0026	0.0007	0.03
25-35	0.13	0.0102	0.007	0.013
30-35	0.04	0.0026	0.003	0.0036