POTENTIAL OF *PSEUDOMONAS CEPACIA* AS A BIOLOGICAL CONTROL AGENT FOR SELECTED SOILBORNE PATHOGENS

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Dedicated to my lovely wife

Elaheh Amouzadeh

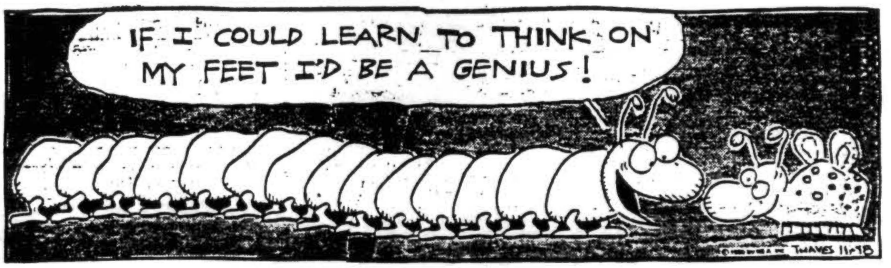
IF I COULD LEARN TO THINK ON MY FEET I'D BE A GENIUS!

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TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION	1
II.	LITERATURE REVIEW	3
	Morphology	3
	Major Synonyms	4
	Importance of *P. cepacia* as Human Pathogen	5
	Importance of *P. cepacia* as Plant Pathogen	6
	Importance of *P. cepacia* as an Industrial Bacterium	7
	Potential of *P. cepacia* as Biological Control Agent	8
	P. cepacia As Biocontrol Agent of Foliar Diseases	8
	P. cepacia as Biocontrol Agent of Soilborne Diseases	10
	Possible Mechanisms of *P. cepacia*	10
	Root Colonization	11
	Concept of Rhizosphere Competence	11
	Definitions: "Rhizosphere Competence (RC)"	11
	Problems Involved with Selection of *P. cepacia* as Biocontrol Agent	13
	Production of Antibiotics by *P. cepacia*	16
	Pyrrolnitrin	16
	Pyoluteorin	17
	Pyrrole and the Derivatives	17
	Altercidins	17
	Xylocandin	18
	Production of Bacteriocin	18
	Production of Siderophores by *P. cepacia*	18
	Cepabactin	19
	Pyochelin	20
	Possible Production of Volatile(s) by *P. cepacia*	20
	Genetic Investigation on Phytopathogenic *P. cepacia*	23
	P. cepacia as a Promoter of Plant Growth	24
	Distinction between Phytopathogenic and Clinical Strains of *P. cepacia*	25
	Production of Enzymes by *P. cepacia*	25
	Formulation of *P. cepacia*	27
	Selective Media for Isolation of *P. cepacia*	30
	Influence of Environmental Factors on *P. cepacia*	30
	Statement of Thesis Problem	32
	References	33

Chapter Page

III. CHARACTERIZATION OF *PSEUDOMONAS CEPACIA* STRAINS AND EVALUATION OF RHIZOSPHERE COLONIZATION ON ROOTS OF VINCA (*CATHANTHUS ROSEUS* L.) 41

Abstract 41
Introduction 42
Materials and Methods 44
Description of *P. cepacia* Strains 44
Preparation of Bacterial Inoculum 44
Pathogenicity Test of Strains of *P. cepacia* on Onion 44
Petri Dish Assay for Fungal Inhibition 45
Production of Siderophore(s) by *P. cepacia* and *P. aeruginosa* 45
Production of Pectolytic Enzymes by *P. cepacia* and *P. aeruginosa* 46
Inoculation of Seed by Antagonistic Bacteria 46
Directed Bioassay of *P. cepacia* on Seed Germination 47
Effect of *P. cepacia* Concentration on Seed Germination 47
Determination of Root Colinization Ability of *P. cepacia* 48
Determination of Doubling Time of *P. cepacia* OK-2 48
Antagonistic Effect of *P. cepacia* against *R. solani* OK-330 (AG-4) 49
Drenching Method Using Different Concentrations of *P. cepacia* OK-2 50
Results 50
Pathogenicity Test of Strains of *P. cepacia* on Onion 50
Petri Dish Assay for Fungal Inhibition by Strains of *P. cepacia* 51
Production of Siderophore(s) by *P. cepacia* and *P. aeruginosa* 51
Production of Pectotytic Enzymes by *P. cepacia* and *P. aeruginosa* 51
Direct Bioassay of *P. cepacia* on Seed Germination 51
Determination of Root Colinization Ability of *P. cepacia* 52
Determination of Doubling Time of *P. cepacia* OK-2 52
Antagonistic Effect of *P. cepacia* against *R. solani* 330 (AG-4) 53
Drenching Method Using Different Concentrations of *P. cepacia* OK-2 53
Discussion 54
References 64

Chapter Page

IV. PRODUCTION OF AMMONIA BY *PSEUDOMONAS CEPACIA* AND *PSEUDOMONAS AERUGINOSA*: QUANTIFICATION AND EFFECT ON HOST AND PATHOGEN 67

Abstract 67
Introduction 68
Materials and Methods 70
Characteristics of *P. cepacia* Strains 70
Indirect Effect of *P. cepacia* on Seed Germination 70
Detection and Identification of Volatile Compound(s) 70
Effect of Volatile Compound(s) on Seed Germination and Root Elongation of Vinca Seeds and Growth of Selected Fungi 72
Suppressive Effect of Volatile Compound(s) on Selected Plant Pathogenic Fungi 73
Development of a Bioassay for Ammonia Production 73
Results 74
Indirect Effect of *P. cepacia* on Seed Germination 74
Detection and Identification of Volatile Compound(s) 75
Effect of Volatile Compound(s) on Seed Germination and Root Elongation of Vinca Seeds and Growth of Selected Fungi 76
Suppressive Effect of Volatile Compound(s) on Selected Plant Pathogenic Fungi 77
Development of a Bioassay for Ammonia Production 78
Discussion 79
References 97

V. SUMMARY AND CONCLUSIONS 101

Pseudomonas cepacia, Biocontrol Agent or Deleterious Bacterium? 101
Advantages of *P. cepacia* 101
Disadvantages of *P. cepacia* 102
References 106

LIST OF TABLES

Table Page

CHAPTER III

1. Comparison of Inhibition Caused by Antibiotic Production by Strains of *Pseudomonas cepacia* OK-1 and OK-5, and *P. aeruginosa* B-4 against Selected Fungi on KMB^{x} and PDA^{Y} Media 58

2. Effect of Inoculum Density of Strains of *Pseudomonas cepacia* on Percent Germination of Vinca Seeds over an Eleven Day Period 59

3. Average Root Length of Vinca Seedlings Treated by *P. cepacia* OK-2 Using Four Different Methods of Inoculation with and without the Addition of *Rhizoctonia solani* (Ag-4) 330 to the Soil System 61

4. Mean Stand Radish Seedling Srenched [a] with Four Concentrations (Log 2, 4, 6, and 8 cfu/ml) of *Pseudomonas cepacia* (OK-2) in the Presence or Absence of *Rhizoctonia solani* Absence of *Rhizoctonia solani* 62

CHAPTER III

1. Production of Ammonia by *Pseudomonas cepacia* Strains Detected by EM Quanta Ammonia Test Kit 87

2. Production of Ammonia (ppm) by *Pseudomonas cepacia* Detected by Lachet Instrument 88

3. Detection and Identification of Volatile Ammonia by Strains of *Pseudomanas cepacia* (OK-1 to OK-5) and *P. aeruginosa* (B-4) Using Mass Spectra Analysis 89

4. Effects of Volatiles Produced by Strains of *Pseudomonas cepacia* OK-1 to OK-5) Grown on Two Different Media, after One Week, on Germination and Root Length of Vinca 91

Table Page

5. Quantification of Ammonia Production by Strains of *Pseudomonas cepacia,Pseudomonas aeruginosa* by Comparing Inhibition of Growth of Soilborne Fungi in the Presence of the Bacteria to Inhibition Caused by Ammonia Released from Solutions of NH_4OH 94

CHAPTER V

1. List of Diseases Controlled by Application of *P. cepacia* 105

LIST OF FIGURES

Figure Page

CHAPTER III

1-3. Rhizosphere Colonization of *Pseudomonas cepacia* OK-2 on Three Sections of Vinca Root for a 20 Day Period 60

4. Comparison of Three Application Methods of *Pseudomonas cepacia* (OK-2) on Percent Stand of Vinca Seedling LSD (0.05)=8.0 63

CHAPTER IV

1-4. Detection of Ammonia Produced by Two Strains of *Pseudomonas cepacia* (OK-2 and OK-3) Using Mass Spectra Analysis 90

5-10. Calibration and Detection of Ammonia from Hydroxide Compared to Pure Ethanol and Three Strains of *Pseudomonas* 91

11-14. Suppression of Fungal Pathogens Affected by Volatile Compound(s) Produced by Strains of *P. cepacia* OK-2, *P. aeruginosa* (B-2, B-4), and *Bacillus subtilis* (B-6) 93

15. Bioassay System Used to Compare the Inhibition of *Trichoderma harzianum* by Ammonium to Inhibition Caused by Strains of *Pseudomonas* spp. 95

16. Bioassay System Used to Compare the Inhibition of *Macrophomina phaseolina* by Ammonium to Inhibition Caused by Strains of *Pseudomonas* spp. 95

17. Bioassay System Used to Compare the Inhibition of *Fusarium oxysporum* by Ammonium to Inhibition Caused by Strains of *Pseudomonas* spp. 96

18. Bioassay System Used to Compare the Inhibition of *Scterotium rolfii* by Ammonium to Inhibition Caused by Strains of *Pseudomonas* spp. 96

CHAPTER I

INTRODUCTION

At the beginning of the twenty-first century, biological control will be an alternative way to assist the development of agriculture. Biotechnology has been improving during the present decade; specifically, a great deal of information on molecular biology and interaction between host and pathogen has been acquired. Recently, some bacterial biocontrol agents have been commercially introduced, but this field of investigation cannot compete with the development of numerous pesticides because the performance for biocontrol agents is still inadequate for substitution of chemical control. Therefore, it will be a remarkable achievement if biocontrol agents can be substituted for hazardous, synthetic chemicals which damage the natural environment. For this reason, the attempts to introduce a new biocontrol agent will be beneficial to agriculture as well as the environment.

The purpose of this research is to investigate the potential of *Pseudomonas cepacia* as a biocontrol agent, to study the *P. cepacia* / host / pathogen interaction, and to investigate factors involved in the enhancement of biocontrol to protect crops from soilborne diseases. The literature review looks at the previous and present investigations conducted on *P. cepacia* as biocontrol agent. Moreover, the review surveys similar aspects of closely related species of *Pseudomonas*. The focus of discussion is the phytopathogenic strains; however, some aspects of clinical and industrial strains of *P. cepacia* are mentioned in order to develop key points about the possible hazard to human

health and the environment.

Since the detection, identification, and pathogenicity of ammonia produced by *P. cepacia* will not be discussed in the literature chapter, additional information comparing this bacterium to other microorganisms producing ammonia during pathogenic interactions will be discussed.

It is intended that this effort find a convincing answer to the main question of whether or not *P. cepacia* is a beneficial or deleterious organism.

CHAPTER II

LITERATURE REVIEW

Pseudomonas cepacia Burkholder (1950) Palleroni and Holmes 1981 has been described in Bergey's Manual of Determinative Bacteriology as a member of the family Pseudomonadaceae in the kingdom of prokaryotae (Palleroni, 1984). The species name, "*cepacia*", from the latin word *caepa* or *cepa*, means onion and *cepacia*, signifies "of or like an onion". Many strains of these obligate aerobic bacteria have been isolated from onion; others are either soil inhabitants or human pathogens (Palleroni, 1984).

Morphology

This species is characterized as a gram-negative, non-spore forming bacterium, having straight rods with rounded ends. It is motile with polar, multitrichous flagella and peritrichous fimbriae which occur singly or in pairs. The size of the cells varies from 1 um to 2.8 μm by 1.9 to 3.2 μm. Sometimes, they may contain two granules (Palleroni, 1984). *P. cepacia* Burkholder (1950) has nonfluorescent, round colonies (5 mm in diameter) on chemically defined media with yellow or greenish pigmentation (Fuerst and Hayward, 1969). The pathogenic strains that affect onion commonly produce two types of colonies: first, a smooth, white, rapidly growing round colony, which turns into a pin-point form when cultured on beef extract-peptone agar; second, a rough yellow colony.

P. cepacia can grow on many substrates and has been isolated from soil,

water, and clinical specimens. The mechanism of survival is not known (Starr, 1981). The range of temperature for growth is from 9 C to 41 C; it does not grow below 4 C. Optimum temperature for growth is 30 C (Burkholder, 1950, Ballard, et al., 1970).

Major Synonyms

1. *P. cepacia* Burkholder,(1950): There are 35 strains of *P. cepacia* but many other isolates were finally included under *P. cepacia* Burk., (1950) due to nomenclatural priority. This group is comprised of plant pathogens causing onion rot (Snell, et al., 1972; Burkholder, 1950). No strain of *P. cepacia* produces purple pigment which is unique among *P. multivorans* strains (Sneath, et al., 1981; Strainer, et al, 1966).

2. *P. multivorans* Stainer, Palleroni, and Doudoroff (1966): These bacteria are isolated from soil (Sinsabaugh and Howard, 1975; Snell et al., 1966). Two strains of *P. multivorans* are known to be human opportunistic pathogens. At the present time, there is no phenotypic distinction between *P. multivorans* and *P. cepacia*. Purple pigmentation is unique among *P. multivorans* strains (Sneath, et al., 1981; Stainer, et al., 1966). Stainer, et al., (1966) named *P. multivorans* based on nutritional, cytological, and biochemical similarities. These strains are nutritionally versatile in type and number of organic substances used as carbon sources and they are able to accumulate poly-beta-hydroxybutyric acid (PHB). Numerical taxonomy of pseudomonads, based on the utilization of substrates, indicated that there were some differences between *P. multivorans* and *P. cepacia* (Sneath, et al., 1981). Isolates of *P. cepacia* from soil, water, and clinical material cannot be distinguished based on phenotypical characteristics. Two strains of *P. cepacia*

and *P. multivorans* share a level of genetic homology. Experimental inoculation indicated that *P. multivorans* was also capable of producing onion rot (Ballard, et al. 1970).

3. *P. kingii* Jonsson, 1970: Three strains of this group were isolated from clinical specimens (Jonsson, 1970). Snell et al. (1972) proposed that *P. kingii* was synonymous with *P. cepacia*. *P. kingii* EO-1 was designated as Eugonic Oxidizer due to their great utilization of many carbohydrates. These obligate aerobes are pleomorphic with wavy polar flagella and may have a lateral one. Pigmentation occurs on iron containing media and colonies are nonfluorescent (Sinsabaugh and Howard, 1975). *P. multivorans* and *P. kingii* were described as synonyms for *P. cepacia*. Based on 52 biochemical, physiological, and morphological characteristics, *P. kingii* and *P. multivorans* showed the following similarities: (a) Both show lysis (blood cell lysin) but not hemolysin destruction or decoloration of blood agar. Only *P. multivorans* has a slightly greenish area around the edge of "lysis" zones (Sinbaugh and Howard, 1975). (b) Both are so similar that additional tests such as determination of flagellar number and/or arrangement, and antibiotic susceptibility are required, however, their ability to utilize numerous carbon compounds can be used to separate these organisms (Sinsabaugh and Howard, 1975; Snell, et al., 1975).

4. *P. alliicola* Burkholder, 1942: Is the causal agent of onion bulb rot.

5. *P. marginata* Mc Culloch, 1921: Causes the rot of gladiolus. In vitro DNA hybridization of *P. alliicola* and *P. marginata* showed a high level of genetic homology (Ballard, et al., 1970).

Importance of *P. cepacia* as Human Pathogen

Although *P. cepacia* causes stem and bulb rot of onion, this organism

has special significance due to the continuous isolation of this bacterium in clinical specimens such as blood cultures, wounds, urine, etc.; and miscellaneous sources such as disinfectants (e.g. Cetavlon, Savlon), respirators, drip-lines, and even crystal violet (Snell, et al., 1972). Thus, some strains become potentially dangerous human pathogens and cause serious diseases to compromised human patients if an opportunity to enter into the blood stream becomes possible (Starr, et al., 1981; Snell, et al., 1972).

Importance of *P. cepacia* as Plant Pathogen

Soft rot bacteria are associated with onion decay. Approximately half of the bacteria which are isolated from decaying onions or organic soils cropped with onion belong to the genus *Pseudomonas* (Kawamoto and Lorbeer, 1967; 1974; 1976). For selective isolation of soft rot bacteria of onion, non-contaminated, fresh slices of onion were either streaked with tissue of decayed onion or with 0.05 to 0.2 g of soil. Infected onion slices were incubated on moist filter paper in a petri dish at 30 C for 1-4 days (Kawamoto and Lorbeer, 1964, 1972, a,b). Two bacterial diseases have caused considerable losses in stored onion. Burkholder (1950) identified these phytopathogenic bacteria from stored onions as *P. alliicola, P. cepacia* and some other isolates which were similar if not identical to *P. marginalis* (Kawamoto and Lorbeer, 1967). *P. alliicola* frequently causes disease of onion in Hungary, Australia and *P. cepacia* occurs in the USA. The susceptibility of onion cultivars and several *Allium* sp. to these bacterial isolates, methods for the inoculation of bulbs and foliage, and the evaluation of symptoms were investigated extensively by Burkholder (Darvas, et al., 1985; Cother aand Dowling, 1985). In 1942, *Pseudomonas allicola* was identified as the causal agent of bacterial scale rot of onion. Also, another rot of

onion was described by A.G. Newell (1942) as "sour skin and slippery skin". The infection caused by *P. alliicola* does not have a watery or glossy appearance but is slimy and yellow colored under the epidermis of the scale (Burholder, 1950).

Importance of *P. cepacia* as an Industrial Bacterium

Many synthetic chemicals are produced for agricultural products or are developed by manufacturing processes. These substances are major sources of pollution to the environment because they may be highly toxic, carcinogenic, or persistent in the nature (Kilbane, et al., 1983). Most microorganisms have limited ability to degrade a large variety of synthetic chemicals. Some microbial populations are able to degrade these compounds in a process called cometabolism (Kilbane, 1983). Cometabolism was defined by Reineke and Knachmuss (1988), as the widespread ability of microorganisms to catalyze partial transformation to products that do not support growth". For example, *P. cepacia* AC1100 has ability to utilize 2,4,5-trichlorophenoxyacetic acid as carbon source. The removal activity in contaminated soil is more than 99% at 1 mg/g of soil within one week. The benefit of this process is to support the growth of sensitive plants when the concentration is low. No serious ecological disturbance occurs after application of this strain in the contaminated area (Kilbane, et al., 1983). In addition, halogenated aromatic compounds such as pesticides, herbicides, lubricants, insulators, hydraulic fluid and many toxic wastes (e.g. nonvolatile residue of nylon manufacturing) can be metabolized partially or completely by *P. cepacia* (Ramsay, 1986; Karns, et al., 1983). The ability of this bacterium to degradate substances is dependent on the enzyme systems (Karns, et al., 1983). The industrial characteristics of *P. cepacia* are an

additional advantage for this organism to be more beneficial to the polluted environment as well as removal agent of excessive chemical residues in agriculture.

Potential of *P. cepacia* as Biological Control Agent

P. cepacia As Biocontrol Agent of Foliar Diseases

P. cepacia has been considered as a useful bacterium to control foliar diseases. This organism is able to control southern corn leaf blight in greenhouse trials. In addition, it has an inhibitory effect on conidial germination of *Alternaria alternata* causing tobacco Alternaria leaf spot (Spurr and Sasser, 1982). Cercospora leaf spot of peanut and Alternaria leaf spot of tobacco have been successfully controlled by spraying *P. cepacia* obtained from corn leaves infected by *Bipolaris maydis* (Mu kerjii and Garg, 1988). Higher concentrations of 10^8 cells/ml of this organism are capable of inhibiting entire conidial populations of *A. alternata* (Spurr and Sasser, 1982).

Xanthomonas translucens and *P. cepacia* combined with *Septoria nodorum* were applied to flag leaves of wheat at the 50% heading stage. Although apparent photosynthetic rate and transpiration rate of flag leaves did not change statistically, *P. cepacia* showed a very effective antagonism against *S. nodorum* (Jones, et al., 1981). Spore germination of *S. nodorum* was completely inhibited 96 hrs after inoculation of wheat with *P. cepacia.* Both *X. translucens* and *P. cepacia* reduced the germ tube development of *S. nodorum.*

Although *Bacillus subtilis* was considered as a successful biocontrol agent for postharvest diseases, a new bacterial isolate was identified by Janisiewicz (1987, a, b) as *Pseudomonas* strain (L-22-64). Later it was identified as *P. cepacia.* This strain was tested against *Penicillium expansum*

causing blue mold of pome fruit, and *Botrytis cinerea*, the causal agent of gray mold. In vitro tests on apples and pears demonstrated this strain of *Pseudomonas* was most effective when applied at $5x10^7$ cells/ml on 3 mm deep wounded fruits, compared with the untreated fruits (Janisiewicz, 1987, a). Fortunately, this isolate was effective in vitro as well as in vivo where lesion development was totally inhibited. The period of time apples were submerged in the bacterial suspensions played an important role. In fact, there was a direct relationship between the effectiveness of the biocontrol agent and the fungal spore concentrations. Higher spore concentrations of pathogen were controlled by higher concentration of biocontrol agent. This protection was effective even after subsequent reinoculation of wounds by the pathogen. Persistence of *P. cepacia* L-22-64 on fruit stored at 1 C lasted for a period of two months. Thus, this bacterium can control postharvest diseases of stored fruits at low temperature (Janisiewicz, 1987, b).

The antagonistic effects of *P. cepacia* were demonstrated in vitro on nutrient yeast dextrose agar (NYDA) against *B. cinerea* and *Mucor* sp.. Due to an antifungal compound, *P. cepacia* significantly reduced Mucor rot lesions on wounded apples (Janisiewicz and Roitman, 1987). When different combinations of antagonistic *P. cepacia* ($2.0x10^5$ cfu/ml) were tested on fruit against mixture of *B. cinerea* and *Penicillium expansum* spores (10^4 spores/ml), fruits were protected in excellent conditions. The development of lesions on apples was dependent on the quantitative relationship between the bacterial population and the pathogen spore concentration (Janiciewicz, 1988).

Recently, new strains of *P. cepacia* have been isolated from the caryopses of the grass *Tripsacum dactyloides* with antagonistic activity against important corn fungal pathogens. These strains produce an antifungal compound with antimicrobial activity on cornmeal agar and potato dextrose

agar toward *Trichoderma viride* (Jayaswal, et al., 1990). Successful application of *P. cepacia* as a foliar biocontrol agent is due to the production of effective antimicrobial compounds against a wide range of foliar fungal pathogens. It is important to know whether or not *P. cepacia* can perform efficiently against soilborne pathogens with similar mechanism.

P. cepacia as Biocontrol Agent of Soilborne Diseases

Onion seeds were infested using a suspension of 10^7 cells/ml of *P. cepacia* to determine whether the bacterium could inhibit *Fusarium oxysporum* f.sp. *cepae* (Kawamoto and Lorbeer, 1972, a, b). *P. cepacia* colonized the root tips, the root-stem zone, and the seed coat of onion. The number of damped-off seedlings was reduced compared to uninfested seed (Kawamoto and Lorbeer, 1972, a, b). Although protection of seedlings by treating seed with antagonistic microorganisms against soilborne fungi has been reported many times, selection of *P. cepacia,* an onion pathogen, for the protection of onion as a host was an unusual situation. *P. cepacia* was never recommended for commercial planting in spite of the potential to colonize the rhizosphere of many plants (Kawamoto and Lorbeer, 1972, a, b). Among four selected biocontrol agents for soilborne microorganisms, *P. cepacia* significantly increased plant fresh weight of china aster in the field and reduced the incidence of Fusarium wilt (Cavileer and Peterson, 1985).

Possible Mechanisms of *P. cepacia*

In spite of many investigations during the past ten years, few biological control agents are commercially available. To achieve the goal of a more efficient biocontrol agent, it is essential to know the mechanisms involved in

biological control (Baker, 1986). Enhancement of biocontrol efficiencies, plant growth, and increased yield are dependent on rhizosphere colonization. Therefore, extensive research is required to determine the mechanisms involved in the increased efficiency of rhizosphere-competent organism (Ahmad and Baker, 1987, b).

Root Colonization

Biocontrol agents should have ability to colonize along the root (internally or on the surface), to become distributed in natural soil near the root (rhizosphere), and to survive for several weeks in the presence of competitive indigenous microflora of the root (Weller, 1988).

Concept of Rhizosphere Competence

Definitions: "Rhizosphere Competence (RC)"

According to Ahmad and Baker (1987,a), RC is "the ability of a biocontrol agent to grow and function in the rhizosphere". Different bacteria have various RC; rhizosphere incompetent microorganisms lack this ability. The term "Rhizosphere" refers to "the zone of activity of microbial growth in the immediate vicinity of a plant root" (Goldberg, et al., 1989).

Ahmad and Baker (1987, b) developed a rhizosphere competence assay to determine the population density of *Trichoderma harzianum* along the root of cucumber plants. This method provided a quantitative measurement of rhizosphere competent fungi at the root tip. Ahmad and Baker (1987, d) found that mutants of *T. harzianum* tolerant to benomyl (10 µg/g of soil) became rhizosphere competent. The mutants affected *Pythium* spp., increased seed germination, enhanced plant growth, and reduced pre-emergence damping-off

compared to the non-rhizosphere competent parent strains. Population densities of two RC mutants of *T. harzianum* were higher than wild-type strains.

Further investigation by Ahmad and Baker (1987, c) indicated that competitive saprophytic ability (CSA) of two RC mutants of *T. harzianum* (T-95 and T-12B) was higher than wild type *Trichoderma* spp. Besides benomyl tolerance, mutants of *T. harzianum* were more efficiently able to utilize substrates found on the rhizoplane or root surface and produced higher biomass in the presence of certain single or complex sugars compared to wild types (Ahmad and Baker, 1987, d). These studies on *Trichoderma* spp. support the hypothesis that RC of mutants can be explained by their ability to utilize cellulose substrate on or near the rhizoplane (Ahmad and Baker, 1988).

Based on the theoretical speculations, many mechanisms might be involved in RC: 1) A diffusible growth factor might be induced to increase the rate of plant emergence. This factor could be absorbed by the rhizosphere competent organisms at the root tip where population densities of these organisms were growing (Ahmad and Baker, 1987, b). 2) Rhizosphere competent mutants may act as biocontrol agents against minor pathogens and higher emergence of seeds treated with mutants may occur (Ahmad and Baker, 1987,d). 3) Higher biomass of the biocontrol agent has ecological importance due to the improvement of RC (Ahmad and Baker, 1988). 4) Mutations increase enzyme activity which results in higher CSA, compared to fungal pathogens (such as *Pythium*), for possession of cellulose substrate near the rhizoplane. Therefore, higher efficiency of cellulose degradation acts as a key factor increasing rhizosphere competent microorganisms. Greater enzyme production is directly related to CSA and efficient utilization of cellulose substrate in the rhizosphere or the rhizoplane (Ahmad and Baker, 1987, d).

Problems Involved with Selection of *P. cepacia* as Biocontrol Agent.

Only *Bacillus subtillis* (Quantum 4000 Gustafson, Inc.) has been commercialized as a seed inoculant for field crops to produce healthy plants, to increase yield, and to control many diseases. Many rhizobacteria did not have consistency during field tests (Knudsen, et al., 1987). Genes regulating toxin, antibiotic, enzyme, and plant growth hormone production have been identified from soil bacteria but genes responsible for RC have not been cloned or identified in non-rhizobacteria. Therefore, extensive investigations on bacterial recombinant DNA technology should be performed to identify rhizosphere competent bacteria, to insert or amplify certain genes into these organisms so they may be developed into seed inoculants. This approach was used when the insecticidal delta-toxin gene of *Bacillus turingiensis* sub sp. *kurstaki* was transferred into *Pseudomonas fluorescens* strains which colonized corn roots (Knudsen, et al., 1987).

One of the major problems in the development of biocontrol agents is the lack of correlation between laboratory and field results. There are several reasons for this. The initial selection of candidate isolates is based on bacterial antagonism toward plant pests in vitro. The best way to screen bacterial isolates is based on their ability to inhibit other common rhizosphere microorganisms and their ability as root colonizers. Therefore, screening methods should be designed and developed in a way that both inhibition and RC characteristics can be evaluated (Juhnke, et al., 1987). Establishment of bacteria coated onto seeds (spermosphere competence) should be studied. When abundant amounts of carbohydrates and amino acids are released during the seed germination, bacteria will be attracted and a zone

(spermosphere) of increased microbial growth will be formed. After incorporation of bacteria at a relatively low population, any successful spermosphere colonizer should compete with indigenous soil microorganisms and colonize the root. Studies on spermosphere colonization by fluorescent pseudomonads shows spermosphere competence is strain specific and can be used to select for superior spermosphere-colonizing bacteria in field soil (Kloepper, et al., 1985). Another major problem for introducing biocontrol agents is that it is very difficult to induce a non-rhizosphere organism to become a part of the rhizosphere population through seed infestation (Kawamoto and Lorbeer, 1972, a). Selection of candidate biocontrol agents is difficult. Only 10% of the total rhizosphere bacteria have the potential to be effective. In fact, there is no general relationship between the ability of a selected bacterium to inhibit a pathogen in vitro in a laboratory experiment and suppress the disease caused by that pathogen in vivo in the field. Good performance of one strain on media by producing a large inhibition zone does not necessarily mean the best biocontrol agent was chosen; therefore, the relationships between in vitro and in vivo tests should be investigated (Weller, 1988).

Formulation of a bacterial biocontrol material is a critical factor which will be discussed later. The threshold population of bacteria on planting material or soil is important. Any reduction in the establishment and maintenance of these bacteria inhibit biological control. In addition, several soil edaphic factors such as temperature, soil moisture, pH, and clay content interfere with their survival and interaction with the pathogen (Weller, 1988).

Pseudomonads are one of the major groups of root-colonizing bacteria and are dominant in the rhizosphere or rhizoplane. Recently, *Pseudomonas* spp. have shown potential as biocontrol agents. *P. fluorescens* and *P. putida* were applied to seeds in order to improve yield of potato, sugar beet, radish,

and other crops. These bacteria were named Plant Growth Promoting Rhizobacteria (PGPR) due to their ability to colonize roots and suppress a majority of plant pathogens (Weller, 1988). *Pseudomonas* spp. may perform inconsistently because they lose their ability to compete and survive naturally (ecological competence) in the rhizosphere. After the first isolation, bacteria are surrounded by a capsular exopolysaccharide (EPS), but EPS-deficient mutants may lose survival ability because they are predominant and fast-multiplier. In the case of biocontrol agents, such as fluorescent pseudomonads, repeated culturing can cause loss of field efficacy due to changes in cell and colony morphology, loss of cell surface structures or reduction in antibiotic and siderophore production (Weller, 1988). All these factors must be evaluated for *P. cepacia*. Researchers do not recommend onion seed infestation by P. cepacia to improve seedling stand. At this point, there is more speculation than investigation about the mechanism by which this bacterium protects the seedling (Kawamoto and Lorbeer, 1976). *P. cepacia* is able to colonize adventitious root meristem. Root tips, root-stem zone and germinating seeds are three areas where the greatest root exudation and microbial activity occurs. *P. cepacia* may interfere with fungal pathogens either by the fungistatic effect on a plant pathogenic fungus such as *Fusarium oxysporum* f.sp. *cepae* or by antagonism. Therefore, the bacterium can have an indirect or direct action upon the fungus (Kawamoto and Lorbeer, 1976). Although the mechanisms of antibiotic production and competition of *P. cepacia* were reported, there is no evidence to prove whether or not this bacterium is capable of producing a phytotoxin. Phytotoxin producing biocontrol agents can not be used even though they may be able to produce strong antibiotics, or are highly competitive or are hyperparasites.

Production of Antibiotics by *P. cepacia*

Pyrrolnitrin

Fluorescent pseudomonads are commonly isolated from plant rhizospheres. Howell and Stipanovic (1979) determined that the antagonistic activity of a strain of fluorescent *Pseudomonas* was due to production of pyrrolnitin. Pyrrolnitrin is a product of tryptophane metabolism. Phenylpyrroles are also produced by this metabolic pathway (Mahoney, et al., 1990). This antibiotic acts as a chemical seed treatment to prevent damping-off of cotton seedlings by *Rhizoctonia solani* and to inhibit the growth of several other fungi (e.g. *Pythium ultimum*) causing disease complexes (Howell and Stipanovic, 1980). One of the advantages of this antibiotic is that it is an effective inhibitor without phytotoxicity, and displaces deleterious minor pathogens (Elad and Chet, 1987).

Investigations in France showed that when 47 maize seedling plants from two varieties were initially colonized by a small number of rhizobacteria including *P. cepacia*, population densities reached up to 10^8 cfu/g of root. Among rhizobacteria, 32 isolates demonstrated antifungal activity against major maize pathogens. Four of these strains were *P. cepacia* with ability to produce pyrrolnitrin as well as another unknown antifungal compound (Lambert, et al., 1987). Another strain of *P. cepacia* from apple leaves, capable of producing these antibiotic was reported by Janisiewicz and Roitman (1988). During an agar diffusion test, 1 mg/L of pyrrolnitrin was able to inhibit conidial germination of blue mold and gray mold of pears and apples. Production of the powerful and broad spectrum antifungal compound pyrrolnitrin, by *P. cepacia*, enhances the possible commercial aspects of this biocontrol agent against postharvest

diseases of various fruits.

Pyoluteorin

Further studies indicated the existence of another antifungal compound, pyoluteorin, which was effective against *P. ultimum* but not *R. solani.* Pyoluteorin is also an effective seed protectant that may be responsible for loss of mobility and activity of fungi by a chelating reaction between pyoluteorin and soil metals. Loss of antibiotic production by fluorescent *Pseudomonas* spp. is correlated to the loss of their effectiveness as plant growth promoters (Howell and Stipanovic, 1980).

Pyrrole and the Derivatives

Recently, several antibiotics derivatives produced by *P. cepacia* were isolated and characterized: pyrrole, aminopyrrolnitrin, isopyrrolnitrin, monodechloropyrrolnitrin, oxyprrolnitrin, 2-chloropyrrolnitrin, 3-(2-amino-3-chlorophenyl)pyrrole, 2,3-dichloro-4-(2-amino 3-chlorophenyl)pyrrole. These compounds have lower antifungal activities than pyrrolnitrin. Separation and quantitative analysis of all phenylpyrroles were done by high-performance liquid chromatographic (HPLC) (Mahoney, et al., 1990).

Altercidins

Cepacin A (2-(2-heptanyl)-3-methyl-4-quinolinol) and cepacin B (2-(2-nonenly)-3-methyl-4-quinolinol): were also produced by *P. cepacia* strains (Homma, et al., 1989). Altercidins antibiotics are considered to play an important role in the suppression or inhibition of many soilborne microorganisms (Homma and Suzui, 1989).

Xylocandin

Xylocandin is an antifungal cyclic peptide containing glycine, serine, asparagine, and an unusual amino acid, beta-hydroxytyrosine which has been isolated from *P. cepacia* strain ATCC 39277 (Bisacchi, et al., 1987). Xylocandin displayed anticandidal and antidermatophytic activities in vitro (Meyers, et al., 1987).

Antibiotic production by *P. cepacia* is a great advantage for this microorganism. The variety of antibiotics produced gives a unique characteristic to this bacterium to be strongly antagonistic against a majority of plant pathogens. In addition to antibiotics, the effect of bacteriocin, siderophores and the possible production of plant growth regulators should be considered as the most important attributes of *P. cepacia* (Homma and Suzui, 1989).

Production of Bacteriocin

Among 34 strains of *P. cepacia* isolated from clinical sources and the rhizosphere of plants, 6 strains were highly active bacteriocin (*cepaciacin*) producers and 20 strains had the capacity to synthesize cepaciacins of new types (Dodatko, et al., 1989). If *P. cepacia* produces cepaciacin in the rhizosphere of plants, in addition to other antimicrobial compounds will be more effective against a larger number of pathogens (Smirrov, et al., 1982).

Production of Siderophores by *P. cepacia*

A group of bacterial strains called *Pseudomonas fluorescens-putida* are able to produce iron-chelating siderophores which play important roles in biological control. Essentially, the siderophores are capable of complexing

Fe^{+3} and making iron unavailable for competing pathogens. When iron chelating agents are present in the growth media, only microorganisms that can compete for the available iron are able to grow. The majority of these isolates are *Pseudomonas* spp., which produce a yellowish fluorescent pigment in the presence of high concentration of these chelators (Park, et al., 1988; Simeoni, et al, 1987). The siderophore-producing pseudomonads are able to colonize roots in high populations. This is one of the fundamental characteristics important for an effective biological control bacterium. Root colonization can be divided into two phases: the "early attraction and interaction" of bacteria with the roots; then, the "persistent phase", when they can utilize root exudates, multiply , and survive. Thus, the siderophore-producing bacteria are supported on the root surface because they utilize nutrients and excrete siderophores (Park, et al., 1988). The mechanism of biological control for the suppression of Fusarium wilt is due to the activity of a siderophore-producing *P. putida*. Iron competition is affected by soil minerals, pH, iron levels, siderophere production, and the plant root. Therefore, knowledge of these interactions and the Fe^{+3} activity is very necessary for optimal biological control. By adjusting the proper iron level during selection of potential biocontrol agents, prediction of biological suppression will be possible if all other factors are known. (Park, et al., 1988).

Cepabactin

Siderophore producing *P. cepacia* ATCC 25416 produces a siderophere in iron-deficiency conditions. Cepabactin is a new siderophere with low molecular mass which is a heterocyclic analogue of catechol (Meyer, et al., 1989).

Pyochelin

Pyrochelin is another siderophere produced by *P. cepacia* and clinical isolates which is unrelated to pyoverdine. *P. fluorescens* produces pyoverdine; therefore, it can not be produced by the siderophore system of non-fluorescent *P. cepacia* (Meyer, et al., 1989).

Recently, Smirov, et al. (1990) studied 46 strains of *P. cepacia* against phytopathogenic fungi. The antifungal effect of the bacteria was dependent on the presence of Fe^{+3} and crude yellow and violet pigments. Addition of $FeCl_3$ (100 mg/ml) to the medium decreased the biosynthesis of these violet and yellow pigments. Therefore, pigments of *P. cepacia* have a participating role in iron transportation. In addition, *P. cepacia* strains showed a resistance to the synthetic iron chelating agents such as hydroxyethylene diphosphonic and diethylene diamino penta acetic acids. The conclusion was that a high Fe^{+3}-binding constant was involved in *P. cepacia* siderophore production. The existence of antibiotics, siderophore and other antimicrobial compounds found in *P. cepacia* increase the potential of this organism as a biocontrol agent.

Possible Production of Volatile(s) by *P. cepacia*

According to Schippers, et al., (1982) volatiles are only secondary products to the main mechanism of soil mycostasis. Any manipulation of these volatiles has potential for restricting the activity and survival of many pathogenic soilborne fungi (Schippers, et al., 1982). Volatiles play an important role in spermosphere ecology. These compounds are stimulants for the germination of spores, sclerotia, and the growth of bacteria and fungi as well as attractants of germ tubes or hyphae (Nelson, 1987). The production of volatile metabolites by *Pseudomonas* strains including *P. aeruginosa, P. maltophilia* and *P. cepacia*

was investigated by using an automated headspace concentrator incorporating a gas chromatography technique. The results showed that *P. aeruginosa* and related species produce characteristic profiles of headspace metabolites when the growth and analytical conditions remained constant. Automated volatile analysis provides a rapid detection for the identification of pseudomonads since conventional bacteriological tests require at least 24 hours or more for reliable result (Zechman and Labows, 1985).

The function of volatile exudates in the initial host-pathogen interactions have been largely overlooked. Many researchers reported the stimulation of fungal spore germination and hyphal growth by these volatiles. Among varieties of volatile compounds (ethane, acetaldehyde, methanol, ethanol,and acetone) collected from the head space of germinating cotton seeds, only two major volatiles, acetaldehyde and ethanol stimulate the germination of *Pythium ultimum* sporangia. Ethanol is very important in establishing rapid infection of seeds and seedlings by *Pythium* spp. (Nelson, 1987). When volatile compounds are released either from plant tissues or from germinating seeds, they stimulate germination of propagules or increase growth of fungi. They induce two mechanisms: chemotactic or chemotrophic, which can be either stimulatory or inhibitory of fungi. Many volatiles are stimulatory at low concentration (nanogram/milliliter), moderate, or inhibitory at high concentrations (Punja, et al., 1984).

There is an evidence that *P. cepacia* is capable of producing volatile compound. Various volatile sulfur compounds stimulate the oviposition of onion maggot *Hylemya antique*. Onion flies can lay eggs in the presence of sulfur odor. When *P. cepacia* (Burkholder, 1940) colonizes either untreated or sterilized onion seedling, it does not increase the attractiveness as oviposition sites. Therefore, *P. cepacia* reduces damage by the onion maggot producing

an inhibitory stimulant and has the potential to be a biocontrol agent of onion maggot and a related species, *H. planura* (Hough, et al., 1981).

The identity of the volatile can be speculated to be ethylene, cyanide, or ammonia. "Ethylene" is one of the most important volatile mycostatic agent in soil even though it is not directly responsible for the inhibition of conidial germination. Ethylene can induce the microbial production of allyl alcohol which is a mycostatic compound (Schippers, et al., 1982).

Some Pseudomonads, known as the deleterious group, produce "cyanide". These bacteria are usually nonfluorescent and are found in the soil with continuous cultivation of potatoes (Campbell, 1989). About 50% of *Pseudomonas* sp. isolates are deleterious due to cyanide production, which requires iron. Plant growth promoting rhizobacteria (PGPR) compete with the cyanide producers to reduce or inhibit toxicity in the root (Campbell, 1989). Hydrogen cyanide can inhibit cytochrome oxidase respiration in potato roots at least 40%. PGPR such as *P. fluorescens* spp. do not produce cyanide (Bakker, et al., 1987). *P. fluorescens* NCIB 11764 can catalyze the conversion of cyanate to ammonia by using the enzyme,"cyanate aminohydrolase". This organism utilizes cyanate (OCN) as a sole source of nitrogen for growth. Therefore, any cyanide induced by deleterious *Pseudomonas* spp. can be reduced in soil by *P. fluorescens* (Kunz and Nagappan, 1989).

One of the static volatiles in the soil atmosphere is "ammonia". This compound is derived as a byproduct of microbial metabolism of bacteria and is responsible for the inhibition of germinating spores and the regulation of fungal activities in soil (Schippers, et al., 1982). Recently, Howell, et al. (1988), indicated a volatile compound was involved in the biological control of Pythium pre-emergence damping-off by *Enterobacter cloacae*. The presence of antibiotic production or hyperparasitic activity by this bacterium was not

detected. In fact, after inhibition, there was no physical contact between bacteria and fungi (*Pythium ultimum* and *Rhizoctonia solani*). The inhibitory volatile was identified as ammonia, an antifungal byproduct, resulting from ammonification of amino acids and acid amines in soil. Ammonia was very toxic to *P. ultimum* at low concentrations. *E. cloacae* produced ammonia in the spermosphere as a part of inhibitory mechanism against *Pythium* (Howell, et al., 1988).

Several volatiles such as methane, isopropanethiols, methyl ketones and secondary alcohols were also produced by Pseudomonads (e.i. *P. fragi* and *P. fluorescens* biotype 1 at 6 C) (Campbell, 1989). Although the production of volatile can not compete with those of antimicrobial compounds, the penetration of a volatile may reach to areas where antibiotic(s) or siderophore(s) can not perform efficiently. Therefore, identification and determination of the role of volatiles in biological should always be considered.

Genetic Investigation on Phytopathogenic *P. cepacia*

Based on the prediction of a computer stimulation model, the dynamics of survival and conjugation of *P. cepacia* carrying the transmissible recombinant plasmid R388:tn 1721 was tested with a non-recombinant recipient strain in a simple rhizosphere and phyllosphere microcosm. Donor and recipient populations (10^6-10^8 cfu/g) on plant or in soil were applied on radish and bean leaves in petri dishes or used in a test tubes with a peat-vermiculite solution (Knudsen, et al.,1988). After one day, transconjugant populations of 10^1 to 10^4 were observed. Also, the initial numbers of transconjugants increased rapidly and declined subsequently in the rhizosphere and on the leaf. The computer model predicted all aspects of these transmissions correctly (Knudsen, et al.,

1988).

P. cepacia has the unusual ability to use penicillin as a source of carbon and energy. Lysine auxotrophic mutant derivatives of several strains have lost their multiple functions including utilization of penicillin (lys- strains). Alteration of plasmid WB1 indicated that the same region of plasmid was affected (Beckman, et al., 1982). By a gene replacement technique, the metabolite hydrogen cyanide, was produced by *P. fluorescens* to suppress black root rot of tobacco caused by *Thielaviopsis basicola* (Voisard, et al., 1989). Similar methods can be applied if *P. cepacia* produces certain volatiles. It is necessary to evaluate the ability of introduced microorganisms to transfer genetic materials when releases into the environment.

Recent research was performed to detect conjugal DNA plasmid transfer under various environmental conditions. Donor *P. cepacia* containing pR388::Tn1721 and *P. cepacia* recipients were coincubated in soil slurries containing autoclaved or natural soil. Highest numbers of transconjugants, $1.5x10^7$ cfu/ml soil slurry, were observed when enriched nutrient suppliment was added to the soil whereas these numbers were low, 10^3 cfu/ml soil slurry, under low nutrient or pH stress. This system of investigation estimates the effects of changing environmental factors on plasmid transfer rates and on the survival of recombinant microorganisms (Walter, et al., 1989).

P. cepacia as a Promoter of Plant Growth

Four strains of *P. cepacia* promoted nodulation of the actinorhizal plants *Alnus rubra* when co-inoculated with several other bacteria including infective *Frankia* spp. Under nonsterile conditions, non-endophytic bacteria were not detected within nodule tissue. There was no evidence to suggest these "helper"

bacteria in rhizosphere enter the host tissue but the effect of the "helper" was at the surface of the root hair (Knowlton, et al., 1980).

Distinction between Phytopathogenic and Clinical Strains of *P. cepacia*

Despite taxonomic changes, the question remained whether isolates of *P. cepacia* originating from plants were equivalent to the bacterial strains isolated from clinical sources. Gonzalez and Vidaver, (1979) tested 22 strains of *P. cepacia* from both plant and clinical sources using conventional biochemical tests and antibiotic sensitivity patterns. They concluded that *P. cepacia*, *P. kingii,* and *P. mutivorans* are synonymous. Differentiation of plant pathogenic and clinical strains are based on the following tests: a) biochemical Tests, b) onion maceration test, (Gonzalez and Vidaver, 1979), c) hydrolysis of low pH pectate agar (Gonzalez and Vidaver, 1979, Urlich, 1975), d) pattern of bacteriocin, e) LD_{50} In mice, f) minimal inhibitory concentration of antibiotics, g) plasmid analysis of strains (Gonzalez and Vidaver, 1979).

Production of Enzymes by *P. cepacia*

Production of **pectolytic enzymes** by pathogens are known to be important in plant diseases. Virulence of many plant pathogenic strains is associated with the ability to secrete pectinases. These enzymes include: (a) Polygalacturonase (PG) (Exo- and Endo-). The optimum activity of these enzymes are from pH 4.4 to 4.6. The difference between these two enzymes is the way each one splits the pectin molecules in the middle and viscosity reduction. The pectin molecules were isolated and purified from the macerated tissues of onion. (PG) penetrates into the middle lamella and cell wall (Ulrich,

1975). (b) Pectin Methylestrase (PME), PME of *P. cepacia* is different from those of *P. marginalis* (Ulrich, 1975). (c) Polygalacturonate trans-eliminase (PGTE): The enzyme isolated from diseased onion is similar to those reported for *P. marginalis* (Ullrich, 1975). Clinical strains of *P. cepcia* have the ability to produce extracellular products and 70% of selected strains had the ability to produce lipase, protease, and lecithinase but hemolysin was produced by 4% of opportunistic pathogens of *P. cepacia* (Nakazawa, et al., 1987).

An unexplained phenomenon has been observed in some of Pseudomonads. These bacteria can survive or grow better in buffered or chlorinated water distribution system. Eventhough iodine (I_2) and hyiodic acid (HOI) are germicidally active, *P. cepacia* was isolated from water system of the NASA shuttle orbitors. The rate of increase in iodinated *P. cepacia* was greater than comparable cultures of *P. aeruginosa*. There are several possibilities that explain this rate of increase. Disinfection with iodine may cause a physiological change in cell surviving iodination that recover from injury better than untreated cells. This may be due to the proteins produced as the result of starvation or oxidative agents. Also, this process makes them more readily available as a nutrient; therefore, disinfection of water may stimulate the growth of surviving Pseudomonads in weak phosphate water and permit these bacteria to multiply during the storage and distribution after disinfection. If these bacteria are pathogenic, there would be significance health risk (Pyle and Mc Feters, 1990).

Both plant pathogens and clinical strains of *P. cepacia* have already been exposed to the environment, causing different diseases but the potential of transferring genes from one cell to another one is not always a rare possibility. EPA has always stressed biohazard agents and has emphasized "worst case scenarios" especially about gene manipulated biocontrol agent. Although differences among phytopathogenic and clinical strains are more than

their similarities, our strain of *P. cepacia* should be investigated to assure that this bacterium does not damage the environment or does not harm human health. Certainly, differentiation of plant or soil isolates from clinical strains should be a part of extensive investigation on a candidate biocontrol agent such as *P. cepacia.*

Formulation of *P. cepacia*

Formulation of *Pseudomonas* spp. as biocontrol agents has many problems due to the fact that these gram negative organisms are very sensitive to drying and heat. A granular peat formulation of *P. fluorescens* (Dagger G, Ecogen Inc.) has shown satisfactory control of cotton seedling pathogens. Also, a dried formulation of PGPR strains mixed with Xanthin gum and talc was introduced for potato (Weller, 1988). Formulation of a product has potential to compensate for certain natural "deficiencies" of a biocontrol agent. Addition of a nutrient source into pellets or micro-capsules may allow the biocontrol agent to compete more effectively against indigenous microorganisms (Mukerji and Gary, 1988). Survival of biocontrol agents in hostile environments is important. An appropriate formulation of a bacterial product using techniques such as pelletization, microencapsulation, desiccation, and ultraviolet radiation can improve the shelf life of these products for several months (Knudsen and Spurr, 1985).

P. cepacia can be stored as a wettable powder of lyophilized cells of bacteria at room temperature and can be applied easily to peanut foliage using spray equipment for control of Cercospora leaf spot disease (Knudsen and Spurr, 1988). The persistence and efficacy of five lyophilized bacteria against Cercospora leaf spot of peanut were tested in the field. Bacteria were applied

at biweekly intervals as aqueous suspension of wettable powders or as a dust, to peanut cultivars. Population density of each bacterial strain recovered on leaflets declined from 10^4 to 10^3 cfu/leaflet over most two week intervals. Survival of *Bacillus* spp. formulated as wettable powder was less variable than *P. cepacia* with the same formulation, even though *P. cepacia* was a nonspore-former. Log populations of the *P. cepacia* were higher on the leaflet and controlled the disease more effectively than *Bacillus* spp. (Knudsen and Spurr, 1987).

Population dynamics of *P. cepacia* (L-22-64) were determined against *Botrytis cinerea* and *Penicillium expansum* on wounded apples over a 30 days period. Application of a bacterial suspension was either in water or mixed with Ortho-X 77 as a surfactant. During the first ten days, a rapid increase (100-1000 fold) in population was followed by a small decline after which the bacteria populations became stable. Even application of the antioxidant diphenylamine at concentrations of 2000 ppm had little reduction on viable numbers of *P. cepacia* (Janiseiwicz, 1987, b).

An expert systems model was designed that couples a computer stimulation of peanut Cercospora leaf spot development with population models for applied antagonistic bacteria such as *P. cepacia* and *Bacillus thuringensis*. This model assumed the mortality of *P. cepacia* vegetative cells was very high (95%) on the day they were applied as an aqueous suspension of lyophilized vegetative cells. The results indicated that under high relative humidity (>95%) a slower exponential increase in the rate of growth would occur. The model for *Bacillus thuringensis* assumed a simple exponential decline over time. This model was reasonably accurate for field observations of *P. cepacia* because it provides an evaluation method to improve field performance even though the field results are unpredictable. This system helped investigators understand the

survival of biocontrol agents and their interactions with pathogens in a dynamic environment, to optimize spray timing or dosage, and predicted the field results. Disease progress could be predicted and correlated to functions of weather, pathogen characteristics, plant growth and biocontrol agent (Knudsen and Spurr, 1985).

The antagonistic effect of *P. cepacia* against *Pythium aphanidermatum* was tested in vitro. Populations of bacteria in soil were assayed with a selective medium. One week after the addition of nutrient solution to a sandy loam soil, the population increased from 0.7 to 55.4×10^4 cfu/g soil but then declined. *P. cepacia* performed better at pH 5.6 than 6.6, at 20 C than 30 C, and at -0.3 bars (high moisture) than at 5.0 bars (low moisture). Populations of *P. cepacia* increased rapidly in nonsterile soil with dried organic matter from 0.23 to 3.78×10^7 cfu/g soil followed by addition of corn, cotton, and bean tissues. Populations were high up to one month on alfalfa and bean tissue. After addition of corn, bean, and cotton into soil, populations increased from nondetectable to 4.5×10^4 indicating a dynamic behavior for *P. cepacia* survival in soil (Lumsden, 1982).

Proliferation of biocontrol agents can be enhanced by substitution of food additives which are non-toxic to non-target microorganisms (Fravel, et al., 1985). The use of $CaCl_2$ as the gelling agent for pellet formation with *P. cepacia* was not successful and only 0.9% of the cells survived in Ca-gluconate. Bacteria were viable for two weeks in pellets gelled in calcium gluconate (Fravel, et al., 1985).

Any attempt to improve formulation or delivery systems for biocontrol agents will enhance their potential as commercial products. To summarize this discussion, gelling agents are used to maintain the initial population as well as to improve the survival of biocontrol agents under environmental condition.

Best performance of *P. cepacia* will depend on appropriate formulations. Wettable powders of lyophilized cells were applied to foliar parts of plants but it is not well known whether this kind of preparation can improve the survival of the bacteria in soil under low humidity or pH changes.

Selective Media for Isolation of *P. cepacia*

A selective medium was used to identify and count *P. cepacia* in mixed populations from soil and mature tobacco roots. Water extracts were prepared and aliquots were spread on agar media. After incubation, the total pure bacteria extracted from soil was 10^3-10^5 cfu, 0.4-2.6% of the population and the concentration of bacteria isolated from tobacco roots was 10^5-10^7 cfu/cm^2 of root surface area. The density of *P. cepacia* was 10^2-10^4 cfu or 0.1-1% of the population (Spurr and Sasser, 1982). Another selective medium for *P. cepacia* (PCAT) performed successfully for plant and soil strains of *P. cepacia* (Burbage and Sasser, 1982). A highly selective medium consisting of glucose as the sole source of carbon and asparagine as the sole of nitrogen was formulated. A combination of Trypan blue (TB) and tetracycline (T) was added to this medium (TB-T) at pH 5.5. Crystal violet, nystatin or both were used as inhibitors of molds. The defined formation of this medium allow the recovery of *P. cepacia* strains (76 to 86%) from low soil dilution (10^1 to 10^3) (Hagedorn, et al., 1987).

Influence of Environmental Factors on *P. cepacia*

One of the majors problem for plant growth promoting rhizobacteria is their inconsistency as the result of environmental factors. Poor colonization of these bacteria along the length of roots will result in poor control of soilborne plant pathogens. Plant regulating substances and optimum physical conditions

are possible factors involved in bacterization to improve plant growth but the microbial interaction in the rhizosphere and on the rhizoplane play a major role in colonization (Davis and Whitebread, 1989). Any manipulation in either the biotic or abiotic environments can interfere with the populations along the root system. Factors such as soil moisture, water movement, soil microflora, temperature, and soil types are required to enhance plant growth (Davis and Whitebread, 1989). It is not really known how to optimize conditions for colonizing bacterial populations of *P. cepacia*. Therefore, part of this research includes the complex interaction of environmental factors affecting this biocontrol agent as well as methods of application.

Recently, Conway, et al., (1989) isolated a new strain of *P. cepacia* from soil in Washington, Oklahoma. This isolate inhibited many important fungal pathogens on potato dextrose agar (PDA) including: *Macrophomina phaseolina, Rhizoctonia solani, Fusarium oxysporum, Pythium irregulare,* and the biocontrol agent *Laetisaria arvalis.* On King's Medium B (KMB), this bacterial strain exhibited strong inhibition toward *Sclerotium rolfsii* but not on PDA medium indicating the possible involvement of a siderophore in addition to antibiotic production.

Finally, seeds of cotton cv Del Cerro were submerged for 1 hour into suspensions of log 4, 6, and 8 cfu/ml of *P. cepacia*. Populations of *P. cepacia*, on the cotton root system, increased logarthmically during the first seven days indicating that this isolate was rhizosphere competent (Delgado and Conway, 1989). Unfortunately, seed germination was reduced at higher concentrations especially at 2.1×10^7 cfu/ seed. It was speculated that the strain produced a toxic substance causing reduction of germination particularly during the early stages of germination. After this period, higher bacterial populations did not affect root elongation. In some cases, the growth of cotton root was enhanced

compared to untreated cotton plants (Delgado and Conway, 1989).

Statement of Thesis Problem

My research involved the continuation of studies on this strain of *P. cepacia* isolated from soil at Washington, OK. and two additional strains of *P. cepacia* isolated from soil planted with squash at Plant Pathology farm, OSU, Stillwater, OK.

The purpose of the first part of this study is **to determine whether *Pseudomonas cepacia* is rhizosphere competent on selected hosts**. This research is only focused on the RC ability of the biocontrol candidate(s). Another objective is **to determine whether *Pseudomonas cepacia* can be effective against selected pathogens**. Thus, the ability of the biocontrol candidates to produce antimicrobial compounds such as antibiotics, siderophore and other substances has been investigated. The effects of *P. cepacia* on host and/or pathogen are not well understood; therefore, the last objective of this study is **to evaluate the mechanism(s) involved in *Pseudomonas cepacia* / host / pathogen interaction**.

REFERENCES

Ahmad, J.S., and Baker, R. 1987, a. Rhizosphere competence of *Trichoderma harzianum*. Phytopathology 77:182-189.

Ahmad, J.S., and Baker, R. 1987, b. Implications of rhizosphere competence of *Trichoderma harzianum*. Can. J. Microbiol. 34:299-234.

Ahmad, J.S., and Baker, R. 1987, c. Competitive saprophytic ability and cellulytic activity of rhizosphere competent mutants of *Trichoderma harzianum.* Phytopathology 77:358-362.

Ahmad, J.S., and Baker, R. 1987, d. Growth of rhizosphere-competent mutants of *Trichoderma harzianum* on carbon substrates. Can. J. Microbiol. 34:807-814.

Ahmad, J.S., and Baker, R. 1988. Rhizosphere competence of benomyle-tolerant mutants of *Trichoderma* spp. Can. J. Micribiol. 34:694-696.

Asanuma, S., Tanaka, H. and Yatazawa, M. 1980. *Pseudomonas cepacia*- A characteristic rhizoplane microorganisms in rice plant. Soil Sci. Pl. Nutr. 26(1):71-78.

Baker, R. 1986. Biological control: an overview. Can. J. Pl. Pathol. 8:218-221.

Bakker, A.W., Schippers, B. 1987. Microbial cyanide production in relation to potato yield in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp-mediated plant growth-stimulation. Soil Biol. Biochem. 19(4):541-457.

Ballard, R.W., Palleroni, N.J., Doudoroff, M., Stanier,R.Y., and Mandel, M. 1970. Taxonomy of the aerobic Pseudomonads: *Ps. cepacia, Ps. marginata, Ps. alliicola,* and *Ps. caryophylli*. J. Gen. Microbiol. 60:199-214.

Baligh, M., Conway, K.E., and Delgado, M.A. 1990. Production of ammonia by *Pseudomonas cepacia* interferes with seed germination and root elongation. Phytopathology 80(10):1048.

Baligh, M., Conway, K.E., and Delgado, M.A. 1991. Development of a bioassay system to compare quantities of ammonia produced by strains of *Pseudomonas* spp., potential biocontrol agents against soilborne fungi Phytopathology 81(10):1228 (abstract)

Beckman, W., Gaffney, T., and Lessie, T.G. 1982. Correlation between auxotrophy and plasmid alteration in mutant strains of *Pseudomonas cepacia.* J. Bacteriol. 49(3):1154-1158.

Bisacchi, G.S., Hockstein, D.R., Koster, W.H., Parker, W.L. Rathnum, M., Unger, S.E. 1987. Xylocandin: A new complex of antifungal peptides. J. Antibiot. 40(11):1520-1529.

Bowen, G.D. 1991. Microbial dynamic in the rhizosphere: possible strategies in managing rhizosphere populations. in "The Rhizosphere and Plant Growth" by Keister D.L. and Cregan, P.B. (Eds.)25-32.

Burbage, D.A., Sasser, M. 1982. A medium selective for *Pseudomonas cepacia.* Phytopathology 72:706 (Abstract).

Burkholder, W.H. 1950. Sour Skin, Bacterial rot of onion bulbs. Phytopathology 40:115-117.

Conway, K. E., Foor, C. J., Malvick, D., and Bender, C. 1989. Inhibition of *Pseudomonas cepacia,* a potential biocontrol agent of selected soilborne pathogens. Phytopathology 79:1159 (Abstract).

Campbell, R. 1989. Biological control of microbial plant pathogens Cambridge University Press New York, Port Chester, Melbourne, Sydney pp.218.

Cavileer, T.D. ,and Peterson, J.L. 1985. Biological activities of soilborne microorganisms against Fusarium wilt of China aster Phytopathology 75(11):1344 (Abstract).

Chet, I., Ordentlich, A., Shapira, R., and Oppenheim, A. 1991. Mechanisms of Biocontrol of soilborne plant pathogens by rhizobacteria. p.229-236. in The rhizosphere and plant growth Kiester, D.L. and Creagan, P.B. (eds.) Kluwer Academic Publishers pp.386.

Cother, E.J., and Dowling, V. 1985. Association of *Pseudomonas cepacia* with internal breakdown- a new record for Australia. Aust. Pl. Pathol. 14(1):10-12.

Darvas, B., Szentesi, A., and Viranyi, F. (Eds.) 1985. Reactions of onion varieties to bacterial infection and the inheritance of resistance. Proc. Int. Con. Integ. Pl. Prot. Vol.3 Integrated Plant Production of Vegetables, Ornamental and Medicinal Plants. p. 106- 110.

Delgado, M., and Conway, K.E. 1989. Population dynamics of three potential biocontrol *Pseudomonas* strains on seeds and roots of cotton. Phytopathology 80:434 (Abstract).

Davis, K.G. and Whitebread, R. 1989. Factors affecting the colonization of a root system by fluorescent Pseudomonads: the effects of water temperature,

and soil microflora. Plant Soil 116:247-256.

Dodatko, T.A., Kiprianova, E.A., and Smirrov, V.V. 1989. Bacteriocin typing of *Pseudomonas cepacia* strains isolated from clinical sources and the rhizosphere of plants. Zh. Microbiol. Epidemiol. Immunobiol. 1:21-26.

Durrands, P.K. and Cooper, R.M. 1988. Development and analysis of pectic screening media for use in the detection. Appl. Microbiol. Biotechnol. 28:463-467.

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

Fravel, D.R., Marios, J.J., Lumsden, R.D., and Connick, W.J. Jr.. 1985. Encapsulation of potential biocontrol agents in an alginate-clay matrix. Phytopathology 75:774-777(Abstract).

Fuerst, J.A.,and A.C. Hayward 1969. Surface appendages similar to fimbriae (pili) on *Pseudomonas* species. J. Gen. Microbiol. 58:227-229.

Goldberg, N.P., Hawes, M.C.,and Stranghellini, M.E. 1989. Specific attraction to an infection of cotton root cap cells by zoospores of *Pythium dissotocum.* Can. J. Bot. 67:1760-1767.

Gonzalez, C.F. and Vidaver, A.K. 1979. Bacteriocin, plasmid, and pectolytic diversity of *Pseudomonas cepacia* of clinical and plant strains. J. Gen. Microbiol. 110:161-170.

Hagedorn, C., Gould, W.D., Bardinelli, T.R., and Gustavson, D.R. 1987. A selective medium for enumeration and recovery of *Pseudomonas cepacia* biotypes from soil. Appli. Environ. Microbiol. 53(9):2256-2268.

Hasegawa, S., Fujio, K., Mutsuyasu, N., and Haruyoshi, M. 1990. Isolation of *Pseudomonas* spp. and antifungal agents against phytopathogenic fungi. Bull. Coll. Agric. Vet. Med. Nihon. Univ. 0(47):224-232.

Homma, Y., Sato, Z., Hirayama, F., Konno, K. Shrahama, H.,and Suzui, T. 1989. Production of antibiotics by *Pseudomonas cepacia* as an agent for biological control of soilborne plant pathogens. Soil Biol. Biochem. 21(5):723-728.

Homma, Y. and Suzui, T. 1989. Role of antibiotic production in suppression of radish damping-off by seed bacterization with *Pseudomonas cepacia.* Ann. Phytopathol. Soc. Japan 55:643-652.

Hough, J.A., Harman, G.E., Eckenrode, C.J. 1981. Microbial stimulation of onion maggot oviposition. Envir. Entomol. 10(2):206-210.

Howell, R.C., 1976. Use of enzyme-deficient mutant of *Verticillium dahliae* to assess the importance of pectolytic enzymes in symptom expression of Verticillium wilt of cotton. Physiol. Pl. Pathol. 9:279-283.

Howell, C.R., and Stipanovic, R.D. 1979. Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology 69:480-482.

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

Howell, C.R., Beier, R.C., and Stipanovic, R.D. 1988. Production of ammonia by *Enterobacter cloacae* and its possible role in the biological control of Pythium pre-emergence damping off by the bacterium. Phytopathology 78:1075-1078.

Janisiewicz, W.J. 1985. Biological control of postharvest diseases of pome fruits. Phytopathology 75:1301 (Abstract).

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

Janisiewicz, W.J. 1987, b. Population dynamic of two biological control agents on apple and their compatibility with postharvest treatment. Phytopathology 77:1777 (Abstract).

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

Janisiewicz, W.J. and Roitman, J. 1987. Postharvest Mucor rot control on apples with *Pseudomonas cepacia*. Phytopathology 77:177 (Abstract).

Janisiewicz, W.J. and Roitman, J. 1988. Biological control ofblue mold and gray on apple and pear with *Pseudomonas cepacia*. Phytopathology 78:1697-1700.

Janisiewicz, W.J. and Yourman, L., Roitman, J. and Mahony, L. 1991. Postharvest control of blue mold and gray mold of apples and pears by dip treatment with pyrrolnitrin, a metabolite of *Pseudomonas cepacia*. Pl. Dis. 75:490-494.

Jones, J.B., Roane, C.W., and Wolf, D.D. 1981. The effect of *Septoria nodorum* and *Xanthomonas translucens* f.sp. *undulosa* on photosynthesis and transpiration of wheat flag leaves. Phytopathology 71(11):1173-1177.

Jonsson, V. 1970. Proposal of a new species, *Pseudomonas kingii*. Int. J. Sys. Bacteriol. 20:255-257.

Juhnke, M.E., Mathre,D.E., and Sands, D.C. 1987. Identification and characterization of rhizosphere-competent bacteria of wheat. Appli. Envir. Microbiol. 53(12):2793-2799.

Kawamoto, S.O. and Lorbeer, J.W. 1964. Selective isolation of soft-rot bacteria of onion. Phytopathology 54:897 (Abstract).

Kawamoto, S.O. and Lorbeer, J.W. 1967. Soft rot bacteria associated with onion decay. Phytopathology 57:341 (Abstract).

Kawamoto, S.O. and Lorbeer, J.W. 1972, a. Multiplication of *Pseudomonas cepacia* in onion leaves Phytopathology 62:1263-1265.

Kawamoto, S.O., and Lorbeer, J.W. 1972, b. Histology of onion leaves infected with *Pseudomonas cepacia.* Phytopathology 62:1266-1271.

Kawamoto, S.O., and Lorbeer, J.W. 1974. Infection of onion leaves by *P. cepacia.* Phytopathology 64:1440-1445.

Kawamoto, S.O., and Lorbeer, J.W. 1976. Protection of onion seedling from *Fusarium oxysporum* f.sp. *cepae* by seed and soil infestation with *Pseudomonas cepacia.* Pl. Dis. Rep. 60(3):189-191.

Karns, J.S., Kilbane, J.J., Duttagupta, S., and Chakrabarty, M. 1983. Metabolism of halophenols by 2,4,5-trichlorophenoxyacetic acid-degrading *Pseudomonas cepacia* Appli. Envir. Microbiol. 46(5):1176-1181.

Keister, D.L. and Cregan, P.B. (Eds.) 1991. The rhizosphere and plant growth. Beltsville Symposia in Agricultural Research Vol.14 Kluwer Academic Publisher pp.386.

Kilbane, J.J., Chatterjee, D.K., and Chakrabarty, A.M. 1983. Detoxification of 2,4,5 trichlorophenoxyacetic acid from contaminated soil by *Pseudomonas cepacia.* Appli. Environ. Microbiol. 45(5):1697-1700.

Kirinuki, T., Iwanuma, K., Suzuki, N., Fukami, H., and Ueno, T. 1977. Altercidins, a complex of polypeptide antibiotics, produced by *Pseudomonas cepacia* and their effect for the control of black spot of pear caused by *Alternaria kikuchiana tanaka,* Sci. Rep. Faculty Ag., Kobe Univ. 12:223-230.

Kloepper, J.W., Scher, F.M., Laliberte', M., and Zaleska, I. 1985. Measuring the spermosphere colonizing capacity (spermosphere competence) of bacterial inoculants Can. J. Microbiol. 31:926-929.

Knowlton, S.A., Berry, T., and Torrey, J.G. 1980. Evidence that associated soil bacteria may influence root hair infection of actinorrhizal plant by *Frankia* Can. J. Microbiol. 26:971-977.

Krieg, N.R. and Holt, J.G. (eds.) 1984. Bergey's Manual of Systematic Bacteriology vol.1 Williams and Wilkins publication pp.964.

Knudsen, G.R. and Spurr, H.W. Jr. 1985. Computer stimulation as a tool to evaluate foliar biocontrol strategies. Phytopathology 74:1343.

Knudsen, G.R. and Spurr, H.W. 1985. A computer model for evaluating foliar biocontrol agents. Phytopathology 75(11):1343 (Abstract).

Knudsen, G.R. and Spurr, H.W.Jr. 1987. Field persistence and efficacy of five bacterial preparations for control of peanut leaf spot. Pl. Dis. 71(5):442-445.

Knudsen, G.R., Walter, M.V., Porteous, A., Prince, V.J. Armstrong, J.L., and Seidler, R.J. 1988. Predictive model of conjugative plasmid transfer. Appl. Environ. Microbiol. 54(2):343-347.

Kunz, D.A. and Nagappan, O. 1989. Cyanase-mediate utilization of cyanate in *pseudomonas fluorescens* NCIB 11764. Appl. Envir. Microbiol. 55(1):256-258.

Lambert, B., Leyns, F., Van-Rooyen, L., Gossele, F., Papon, Y., and Swings, J. 1987. Rhizobacteria of maize and their antifungal activities. Appl. Environ. Microbiol. 53(8):1866-1871.

Lumsden, R.D. 1982. Ecological study of *Pseudomonas cepacia* in soil using a new selective medium. Phytopathology 72:709 (Abstract).

Mahoney, N.E.,and Roitman, J.N. 1990. High performance liquid chromatographic analysis of phenlpyrrols produced by *Pseudomonas cepacia*. J. Chromatography 508:247-251.

Meyer, J.M., Hohnadel, and D. Halle', F. 1989. Cepabactin from *Pseudomonas cepacia*, a new type of siderophore. J. Gen. Microbiol. 135:1479-1487.

Meyers, E. Bisacchi, G.S., Dean, L. Liu. W.C., Minassian, B. Slusarchyk. D. S., Sykes, R.B., Tanaka, S.K., and Trewjo, W. 1987. Xylocandin: A new complex of antifungal peptides. I. taxonomy, isolation, and biological activity. J. Antibiotics 40(1):1515-1519.

Mukerji, K.G. and Garg, K.L. (eds.) 1988. Biocontrol of plant disease. Vol. I and II CRC Press, Inc. Boca Raton, Florida Vol.I pp.211, Vol.II pp.198.

Nelson, E.B. 1987. Rapid germination of sporangia of *Pythium* species in response to volatiles from germinating seeds. Phytopathology 77:1108-1112.

Nawazawa, T., Yamada, Y., and Ishibashi, M. 1987. Characterization of hemolysin in extracellular products of *Pseudomonas cepacia* J. Clin.

Microbiol. 25(2):195-198.

Noel, G.R. 1990. Inability of a seed treatment with *Pseudomonas cepacia* to control *Herterodera glycines* on soybean. Suppliment to J. Nematol. 22(45):792-794.

Palleroni, N.J., and Holmes, B., 1981. *Pseudomonas cepacia* sp.nov.,nom. rev.. Int. J. Syst. Bacteriol. 31(4):479-481.

Palleroni, N.J., 1984. Family I. Pseudomonadaceae. Winslow, Broadhurst, Buchanan, Krumiede, Rogers, and Smith 1917. 555 Al, p.141-219 in Krieg, N.R. and Holt, J.G. (eds), Bergey's Manual of Systematic Bacteriology, Vol. I. The Williams and Wilkin Co., Baltimore.

Park, C.S., Paultiz, N., and Baker, R. 1988. Biocontrol of Fusarium wilt of cucumber resulting from interactions between *Pseudomonas putida* and nonpathogenic isolates of *Fusarium oxysporum*. Phytopathology 78:190-194.

Punja, Z K., Jenkins, S.F. and Grogan, R.G. (1984). Effect of volatile compounds, nutrients, and sources of sclerotia on eruptive sclerotial germination of (*Sclerotium rolfsii*). *Phytopathology* **74**, 1290-1295.

Pyle, B.H., McFeters, G.A. 1990. Population dynamics of Pseudomonads after iodination. Can. J. Microbiol. 36:801-803.

Ramsay, B.A., Znoj., G.M., and Cooper, D.G. 1986. Use of a nylon manufacturing waste as an industrial fermentation substrate. Appli. Envir. Microbiol. 52(1):152-156.

Reineke, W. and Knachmuss, H.J. 1988. Microbial degradation of haloaromatics. Ann. Rev. Microbiol. 42:263-287.

Schippers, B., Meijer, J.W., and Liien, J.I., 1982. Effect of ammonia and other soil volatiles on germination and growth of soil fungi. Trans. Br. Mycol. Soc. 72(2):253-259.

Sinsabaugh, H.A., and Howard Jr. G.W. 1975. Enumeration of the description of *Pseudomonas cepacia* Bukholder (synonyms: *P. multivorans* Stainer et. al. *P. kingii* Jonsson; EO-1 Group. Int. J. Syst. Bacteriol. 25(2):187-201.

Simeoni, L.A., Lindsay, W.L. and Baker, R. 1987. Critical iron level associated with biological control of Fusarium wilt. Phytopathology 77:1057-1061.

Smirrov, V.V., Garagulya, A.D., Kipranova, E.A., 1982. Antibiotic properties of *Pseudomonas cepacia*. Antibiotiki. 27(8):577-580.

Smirrov, V.V., Kiprianova, E.V., Garagulia, A.D., Dodatko, T.A. 1990. Antibiotic

activity and siderophores of *Pseudomonas cepacia*. Priki. Biokhim. Microbiol. 26(1):75-80.

Sneath, P.H.A., Stevens, M., and Sackin, M.J. 1981. Numerical taxonomy of *Pseudomonas* based on published record of substrate utilization. Antoni Van Leewean Hoeck 48:423-448.

Snell, J. J. S., Hill, L.R., Lapage, S. P., and Curtis M. A. 1972. Identification of *Pseudomonas cepacia* Burholder and its synonymy with *Pseudomonas kingii* Jonsson. Int. J. Syst. Bacteriol. 22:127-138.

Spurr, H.W. 1981. Experiment on foliar disease control using bacteria antagonists 370-381. "Microbial Ecology of the Phyloplane" Academic Press edited by J. P. Blackeman London, New York, Toronto, Sydney, San Francisco pp.502.

Spurr, H.W. and Sasser, M. 1982. Distribution of *Pseudomonas cepacia,* a broad-spectrum antagonist to plant pathogens Phytopathology 72:710 (Abstract).

Starr, M.P., Balows, A., and Schlegel (ed.) 1981. Selection from the prokaryotes, a handbook on habits, isolation, and identification of bacteria "Phytopathogenic Bacteria" Spinger-Verlog New York, Berlin, Heidelberg, Tokyo pp.2284.

Stainer, R.Y., Palleroni, N.J., and Doudoroff, M. 1966. The Aerobic Pseudomonads: A taxonomy study. J. Gen. Microbiol. 43:159-271.

Ulrich, J. M. 1975. Pectic enzymes of *Pseudomonas cepacia* and penetration of polygalacturonase into cells. Physiol. Pl. Pathol. 5:37-44.

Ullrich, W. R., Novacky, A., and Kunz, G. 1984. The role of ammonium and the effect on the electrical membrane potential in cotton and tobacco. in "Membrane Transport in Plant" proc. Symp. held in Prague, Czechoslovakia Aug.15-21, 1983 , Crani, W. J., et al. (eds), A Wiley and Sons-Interscience Publication pp.592.

Voisard, C., Keel, C., Haas, D., and Defago, G. 1989. Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnobiotic conditions. EMBO J. 8(2):351-358.

Walter, M. V., Porteous, L. A., and Seidler, R. J. 1989. Evaluation of a method to measure conjugal transfer of recombinant DNA in soil slurries. Curr. Microbiol. 19(6):365-370.

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

Zechman, J. M., Labows, J. N., Jr., 1985. Volatiles of *Pseudomonas aeroginosa* and related species by automated head-space concentration-gas chromatography. Can. J. Microbiol. 31(3):232-237.

CHAPTER III

CHARACTERIZATION OF *PSEUDOMONAS CEPACIA* STRAINS AND EVALUATION OF RHIZOSPHERE COLONIZATION ON ROOTS OF VINCA (*Catharanthus roseus* L.)

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ABSTRACT

Three strains of *Pseudomonas cepacia,* isolated from soil in Oklahoma, were not pathogens on onion bulbs compared to the pathogenic strain. These isolates showed moderate amounts of pectolytic enzyme production and the levels of siderophore production varied among strains. Production of antimicrobial compounds was tested against selected pathogenic fungi and strains of bacteria. Selected isolates of *P. cepacia*, strains OK-1, OK-2, and OK-5, were identified as soil inhabitants. Strain OK-2 was inhibitory to germination of peanut seed, whereas the germination of cowpea was delayed. Strain OK-2 colonized the rhizosphere of vinca when applied to seed. Population densities were near log 7 colony forming unit (cfu/cm) of the root. Doubling time for bacterial populations was from 4.0 to 4.9 h when the initial density was log 2 cfu/ml of bacterial suspension. This strain of *P. cepacia* was tested against

Rhizoctonia solani OK-330 (AG-4), causing damping-off of radish, using three methods of application including; drenching soil with a suspension of log 6 cfu/ml, soaking seeds in bacterial suspension (log 6 cfu/ml) and sticking bacteria onto the seed with carboxymethyl cellulose. Only the drenching method was slightly inhibitory to the fungal pathogen.

INTRODUCTION

P. cepacia has been used as a successful biocontrol agent against foliar and post-harvest diseases (Janiseiwicz, 1988; Janiseiwicz and Roitman, 1988; Janiseiwicz, Yourman, Roitman, and Mahony, 1991; Wilson and Chalutz, 1989). Mechanisms of action were determined as production of antibiotics such as pyrrolnitrin, pyoluteolin (Janiseiwicz and Roitman, 1988, Janisieiwicz, et al., 1991) and siderophores such as cepabactin. Certain strains of *P. cepacia,* isolated from the caryopsis of grass plants had the ability to produce antifungal compounds suppressive to many phytopathogens (Jawaswal, Fernandez, and Scherder, 1990). *P. cepacia* has also been reported to be an antagonist of soilborne pathogens as well as having deleterious effects on host plants (Conway, Foor, Malvick, and Bender, 1989).

In some cases, the beneficial affects of biocontrol agents have failed due to poor root colonization and lack of optimum conditions for physico-chemical and biological factors (Parke, 1991). In spite of numerous organisms evaluated each year as potential biocontrol agents, formulation is the key for their successful use. These organisms must be handled carefully to insure maintenance of viability throughout processing, storage and application (Connick, 1989). One of the major problems associated with biological control agents is the field variability that limits their agronomic application. This

variability is due to any factor interfering with either the population size of a biocontrol agent or its expression of activity. The key characteristics contributing successs as a biocontrol organism as well as physico-chemical factors which determine success are not well known (Loper, 1988).

Another strain of *P. cepacia* isolated from rhizosphere of rice, reduced the weight and the length of seedlings. These effects were due to the accumulation of nitrite by *P. cepacia* (Asanuma, Tanaka, and Yatazawa, 1980). Treatment of pea seeds with a low initial concentration of *P. cepacia* successfully suppressed the preemergence damping-off caused by *Pythium ultimum* and *P. sylvaticum*. The severity of Aphanomyces root rot was reduced when pea seeds were treated with *P. cepacia* and yield of pea was also increased in a natural infested soil containing *Aphanomyces euteiches* and *Pythium* spp. In addition, doubling time of *P. cepacia* was shortest (3.1 h) when the initial concentration on the seed was low whereas high population densities applied on pea seeds greatly increased doubling times (Parke, 1990).

Information about factors involved in rhizosphere competence (RC) is limited. Certain bacteria contribute specific characteristics for successful root colonization and the prediction of RC of bacteria along the root is important (Hozore and Alexander, 1991). In order to further evaluate these strains of *P. cepacia* as potential biocontrol agents, we characterized *P. cepacia* strains based on their pathogenicity, production of antimicrobial compounds, enzyme activity and investigated the root colonization of isolates, methods of application of the bacterium to seeds and soil, and production of several compounds that may determine success as a biological control agent.

MATERIALS AND METHODS

Description of *P. cepacia* Strains. Five isolates of *P. cepacia*: OK-1 (Wild type), isolated from soil at the State Forest Nursery in Washington, OK.; OK-2, an antibiotic selection from OK-1 with antibiotic markers 0.12 mg/ml nalidixic acid and 0.8 mg/ml chloramphenicol; Pathogenic strain of *P. cepacia* 945 or OK-3 from the National Collection of Plant Pathogenic Bacteria, Harpenden, England, with several antibiotic markers (carbenicillin 1 µg/ml, streptomycin 200 µg/ml, tetracycline 50 µg/ml, neomycin 200 µg/ml, and penicillin 200 µg/ml); OK-4 isolated from fruit by W.J. Janisiewicz; and OK-5 isolated from soil in Stillwater, OK.

Preparation of Bacterial Inoculum. Isolates of *P. cepacia* were streaked on King's Medium B (KMB) and incubated for 48 hrs at 28-30 C. Bacterial suspensions were prepared by removing the colonies from the media using a sterile solution of 0.85% sodium chloride. The concentration of bacterial suspensions was standardized to approximately log 8 colony forming units (cfu)/ml using a Spectronic 20 at an absorbance value of 0.1 at 660 nm. Other concentrations (log 2 to log 6 cfu/ml) were prepared by appropriate dilutions with sterile 0.85% sodium chloride.

Pathogenicity Test of Strains of *P. cepacia* on Onion. Suspensions of log 8 cfu/ml were prepared from the fresh cultures of five strains of *P. cepacia* (OK-1 to OK-5) in 250 ml sterile flasks. Several cottage cheese containers and lids were soaked in 95% ethanol and 0.525% clorox solution (50%/50% v/v) for 1 h and washed with sterile water. Clean tissue papers were placed inside each container and were moistened with 5 ml sterile distilled water. For each strain of *P. cepacia*, two fresh onions were selected. Scales were removed and the

surface of each onion was cleaned with 95% ethanol. Two inoculation techniques were used for each isolate. In first method, 0.5 ml of log 8 cfu/ml suspension of each bacterial strain was injected into the onion bulb at three locations at different depths. A covering scale was placed back at the original position to protect the injected area from drying and contaminating organisms. For the control, onions were injected with 0.5 ml distilled water. The second method involved the removal of small triangular pieces of the onion flesh. Cells of *P. cepacia* from colonies were transferred in mass into the depth of each triangle cut. Control onions were cut in a similar manner but no bacteria were applied. Onions were kept in closed containers and incubated at 27 C for one week. The experiment was repeated once.

Petri Dish Assay for Fungal Inhibition. Agar blocks (2mm diam.) from fresh cultures of *Fusarium oxysporum, Macrophomina phaseolina, Sclerotium rolfsii, Rhizoctonia solani,* and *Pythium ultimum* were separately tested in a dual culture on PDA and KMB media against *P. cepacia* (OK-2 and OK-5) and *P. aeruginosa* (B-4) for inhibition of growth. All cultures were incubated at 27 C for 72 h. The experiment was repeated once.

Production of Siderophore(s) by *P. cepacia* and *P. aeruginosa*. Dilutions (control, from 0.1 to 1.0, 100, 500, and 1000 μM) of ferric chloride ($FeCl_3$) were prepared by addition into KMB. Dual cultures were prepared by using fresh cultures of *S. rolfsii, P. cepacia* (OK-1 to OK-5) and *P. aeruginosa* (B-4). A block (3mm diam.) of *S. rolfsii* was placed on the agar 1 cm from the margin of the petri dish and a loopful of a bacterial suspension was streaked opposite on the medium. All cultures were incubated for 72 h at 27 C. All treatments were replicated three times.

Production of Pectolytic Enzymes by *P. cepacia* and *P. aeruginosa*. For detection of polygalacturonase (PG) and pectate lyase (PL), a highly modified medium was prepared at two pH levels: pH 5 for PG and at pH 7 for PL (Hankin and Anagonstakis, 1975; Gonzalez and Vidaver, 1979). Fresh cultures of *P. cepacia* and *P. aeruginosa* (positive control) were used with different inoculation methods such as zigzag, streak, and 0.2 ml drops to observe the halo zone of enzyme activity after addition of the reagent. All cultures were incubated for 5 days at 27 C. After five days of incubation, a fresh solution of 1% aqueous hexadecyclotrimethyl ammonium bromide (HMAB) was prepared and gently poured on the entire surface of media to cover all colonies (Hankin and Anagnotakis, 1975,; Durrands and Cooper, 1988). The experiment was repeated three times.

Inoculation of Seed by Antagonistic Bacteria. Vinca seeds were disinfected with 0.525% $NaClO_3$ and 95% ethanol (50%/50% v/v) for 1 min. and immediately rinsed several times with distilled water. Seeds were dried in a forced air horizontal hood for 12 h. Seeds were submerged for 1 h in four concentrations of bacterial suspensions (log 2, 4, 6, and 8 cfu/ml) were prepared to determine the number of bacteria per seed. Initial densities of bacteria on seeds were determined by placing 10 vinca seeds into 5 ml of 0.85% NaCl solution for 1 hr. Seeds were shaken for 30 sec every 10 min during this period to facilitate release of the bacteria from the seed coat. Dilutions of the suspensions were plated in 1% potato dextrose agar (PDA) and incubated at 27 C for 72 h. Bacterial colonies were counted, using a Gallenkamp colony counter, to determine cfu/seed. Twenty seeds of vinca, peanut and cowpea were immersed in suspensions (log 2, 4, 6, and 8 cfu/ml) of *P. cepacia*. Twenty untreated, clean seeds were used as control. Seeds were

placed on sterile moisten filter paper and seed germination was recorded daily for a 7 day period. Each treatment was replicated five times.

Direct Bioassay of *P. cepacia* on Seed Germination. Five groups of vinca seed, each containing 20 seeds, were disinfected and rinsed with distilled water. Four different suspensions (log 2, 4, 6, and 8 cfu/ml) of *P. cepacia* strains OK-1 to OK-5, were adjusted using a spectrophotometer. Seeds were submerged into a bacterial suspension for 1 h, removed and dried under aseptic condition for 2 h. The last group of seeds was soaked in distilled water as control for each concentration and five replications were prepared. Sterile filter paper was placed in petri dishes and moistened daily with distilled water. Control seeds and treated seeds were set on the surface of the paper equidistance (2.5 cm) from each other. Numbers of germinated seeds were recorded daily from all petri dishes up to eleven days.

Effect of *P. cepacia* Concentration on Seed Germination. Eighty peanut seeds were disinfected using a mixture of clorox and 95% ethanol solution (50%/50% v/v) and washed several times with distilled water. Seeds were separated into eight groups of 10 seeds each. Fifty ml of bacterial suspensions of log 2, 4, 6, and 8 cfu/ml of *P. cepacia* (OK-2) were prepared in 250 ml sterile flasks. The first group of seeds was submerged into 50 ml distilled water for 30 min, removed, dried, and used as a control. Other groups were submerged into each bacterial suspension for 30 min and dried under aseptic condition. Ten seeds of each concentration were placed equidistant in sand contained in pots and 50 ml distilled water was poured gradually on the sand surface. All containers were placed under fluorescent light for 10 days at room temperature. Similar procedures were also conducted with cowpea seeds.

Determination of Root Colonization Ability of *P. cepacia*. Plastic centrifuge tubes (50 ml) were cut longitudinally into two halves, washed carefully in clorox and soap for 1 h, dried, microwaved for 90 sec. and wrapped with parafilm. One hundred tubes were filled with 25 g of Redi-Earth (RE) (W.R. Grace, Inc.) were moistened to 15% water holding capacity. Germinated vinca seeds, with radicles of 1 mm in length, were placed 1 cm below the surface of the RE and 0.1 ml of polysurf C gel (modified hydroxyethyl cellulose, Hercules, Inc.) was placed on top of the seed. Seeds were covered by RE. Sets of germinated seeds were submerged into either log 2, 4 or 6 cfu/ml suspension of *P. cepacia* OK-2 for 30 min and dried for 1 h before placing them into the RE and covering with gel in each tube. Tubes were incubated in a growth chamber adjusted to 27 C day/30 C night, 12/12 h cycle. No additional water was added during the experiment.

Shoots emerged after 3 days, and 3 tubes per concentration level were split and soil was gently removed. Roots and rhizosphere soil were weighed, and sectioned into 3 equal parts. A 1 cm length from each section was removed and separately ground in 1 ml distilled water with a sterilized mortar and pestle. This suspension was used to prepare a 10-fold dilution series with 1% PDA and plated. Cultures were incubated at 27 C for 48 h. The number of colonies were counted and densities were determined from each root segment. Agar medium for dilutions was later modified due to contaminating microorganisms by amending *Pseudomonas* F agar (Difco) with 0.12 g nalidixic acid and 0.8 g chloramphenicol (dissolved in 5 ml ethanol).

Determination of Doubling Time of *P. cepacia* OK-2. Doubling time (T_d) for *P. cepacia* during the first 48 h after planting was determined for the three initial densities on vinca seed according to the formula:

$$(\log N_t - \log N_o) / 0.301\ t = 1 / T_d$$

where N_o = Original cfu/seed, N_t = final cfu/seed and roots, t = time in h (Parke, 1990).

Antagonistic Effect of *P. cepacia* OK-2 against R. solani OK-330 (AG-4). Inoculum of *R. solani* was prepared in a sand-cornmeal-medium, dried, and stored in a paper bag at room temperature. *R. solani* was added to RE at a predetermined rate equivalent to LD_{50} value for vinca. Sixty cottage cheese containers were soaked in 10% clorox, washed with soap, rinsed with water, and dried. RE was microwaved for 90 sec to kill contaminating fungi (Ferriss, 1984) and *R. solani* was added to RE as 0.1 g of inoculum /100 g RE and mixed in a twin-shell blender (Bison Gear and Engineering Corp.) for 15 min. Approximately 50 g of non-infested RE was added to each container and an additional 50 g of infested RE was layered on top. Similar series of containers were prepared with non-infested RE. Suspensions of log6 cfu/ml from the fresh cultures of *P. cepacia* OK-2 were prepared and vinca seeds were soaked in the suspensions for 30 min and dried. Ten seeds were placed into the top layer of RE just below the surface using a small template and an additional covering layer of non-infested RE was added. Distilled water was sprayed on each container every day when containers were removed from the incubator (27 C) for observation. All containers were randomly arranged and there were 10 replicants per treatment. Treatments included containers with/without *R. solani* and combinations with/without *P. cepacia* OK-2, and controls contained no bacterial or fungal inocula. Germination and incidence of damping-off were recorded daily.

A second set of experiments investigated if the method of application of *P. cepacia* would have an effect on control of *R. solani.* Ten vinca seeds were

placed on the surface of RE, and 8 g of additional RE with or without *R. solani* (0.01 g/100 g RE) was added to cover seeds. *P. cepacia* OK-2 was applied using three different methods: soak, seed treatment with carboxmethylcellulose (CMC) and drenching. For the **soak treatment**, disinfected vinca seeds were soaked in a bacterial suspension of log 6 cfu/ml for 1 h and dried at room temperature. For the **CMC seed treatment**, vinca seeds were covered with a suspension of *P. cepacia* OK-2 (log 6 cfu/ml) in sterile 1% CMC and dried. The **drench treatment** utilized *P. cepacia* OK-2 (log 6 cfu/ml) at a rate of 118 ml/232 cm^2, an equal amount of distilled water was applied to controls. The fourth treatment was used as a **control** without addition of bacteria. There were 10 seeds per container and each treatment was replicated 8 times. All containers were randomly placed inside an incubator at (27 C) with 12 h dark/12 h light.

Drenching Method Using Different Concentrations of *P. cepacia* OK-2. Similar to the previous procedure, 10 radish seeds were grown in plastic containers and bacteria were applied using the soil drench method. Suspensions of log 2, 4 and 6 cfu/ml of *P. cepacia* OK-2 were prepared and applied to each container at a rate of 118 ml/232 cm^2. An equal amount of distilled water was poured on the control seeds. Treatments were replicated four times. Treatments were arranged in a random order in an incubator set at 27 C, 12 h/12 h light cycle. Percent germination of radish was recorded for 1 week.

RESULTS

Pathogenicity Test of Strains of *P. cepacia* on Onion. Strain OK-3 produced the largest macerated area on onions, whereas strains OK-5

exhibited the least damage. Isolates from soil (OK-1, OK-2 and OK-5) caused less damage that the plant isolate OK-3.

Petri Dish Assay for Fungal Inhibition by Strains of *P. cepacia*. Inhibition zones were observed against selected fungi on KMB and PDA (Table 1). *P. cepacia* OK-1 inhibited all fungi except *S. rolfsii* when grown on PDA medium but the growth of this fungus was inhibited on KMB. Also, *P. cepacia* OK-5 inhibited the growth of *Macrophomina phaseolina* and *Pythium ultimum* on KMB but not on PDA. In addition, *P. aeruginosa* B-4 inhibited only the growth of *P. ultimum* when on KMB and PDA. *F. oxysporum* was only inhibited by *P. aeruginosa* on PDA medium.

Production of Siderophore(s) by *P. cepacia* and *P. aeruginosa*. Strains of *P. cepacia* could inhibit the growth of *S. rolfsii* in concentrations with lesser amounts of $FeCl_3$ in the medium. Lower amount of $FeCl_3$ in the medium had a definite effect on the size of the zones of inhibition indicating the production of siderophore under iron deficiency conditions. Each strain varied in production of siderophores with OK-1, OK-2, and OK-3 showing activity below 0.1 to 0.4 µM/ml and OK-5 showing no activity (data not presented).

Production of Pectotytic Enzymes by *P. cepacia* and *P. aeruginosa*. Halo zones appeared around all colonies in some media and indicated that strains OK-4 and OK-5 showed weak PG and PL activities. Other strains of *P. cepacia* (OK-1 and OK-2) and *P. aeruginosa* (positive control) exhibited moderate PG and PL activities (data not presented).

Direct Bioassay of *P. cepacia* on Seed Germination. There was a delay ($p=0.05$) in seed germination directly related to seed treatment with *P. cepacia*

(OK-1 to OK-5) compared to the control (Table 2). Greatest delay was observed during the early days (1-2) of germination. However, by day 7 and 11, germination in treated and control seeds was similar. After two days of incubation, strains OK-1, OK-2 and OK-3 reduced germination of vinca seeds greater ($p=0.05$) than other isolates and the control. In a few cases, high initial concentrations of *P. cepacia* on the seed reduced germination compared to lower concentration but there was no significant effect due to the increased concentration for all strains. On the other hand, peanut seeds were very sensitive to seed treatment with isolates of *P. cepacia* since they did not germinate at any concentrations (data not presented). All control seeds germinated and produced healthy shoots. In the case of cowpea, there was a delay in germination (data not shown) but all seed treatments had similar growth rates compared to non-treated seeds.

Determination of Root Colonization Ability of *P. cepacia*. Population dynamics of *P. cepacia* on roots of vinca over a 17 day period are presented in Figures 1-3. Regardless of the initial concentrations of *P. cepacia* on vinca seeds, the population densities usually increased rapidly for the first 3 days and were maintained near log 6 cfu/cm for the 17 day period. High initial concentrations of bacteria on seeds declined for the first 3 days at the top and tip of the root, but increased to log 8 cfu/cm by the fifth day.

Determination of Doubling Time of *P. cepacia* OK-2. Doubling time for *P. cepacia* during the first 48 h was shorter for the lower initial concentrations. Doubling time for log 2 cfu/ml ranged from 4.0 to 4.9 h for all three sections of roots. Times increased for log 4 cfu/ml to 6.3 to 8.0 h. Bacterial densities on roots of seeds treated with log 6 cfu/ml decreased at the tip and upper root

segments but had a doubling time of 6.25 h for the middle sections.

Antagonistic Effect of *P. cepacia* against *R. solani* 330 (AG-4). There was a reduction in germination rate of vinca seeds in all treatments with *P. cepacia* and *R. solani* compared to the non-infested control (Table 3). Root length was reduced (P=0.05) when seeds were soaked in suspensions of *P. cepacia* and planted into RE amended with *R. solani* compared to planting into non-infested RE and the non-infested control. Seed treatment with bacteria generally reduced the germination rate but had no effect on root length in non-*R. solani*-infested RE. Only when *P. cepacia* was used as a drench was length and precent germination greater, but not significantly, in the presence of the pathogen compared to non-infested RE. In a second experiment under more disease pressure, there was a reductino in the number of vinca seedlings when *P. capacia* was applied by any method in the absence of *R. solani* compared to the control (P=0.05) (Figure 4). Applying bacteria to seeds with CMC resulted in a greater stand (P=0.05) than soaking seeds in a bacterial suspension. When these seeds were placed into Rhizoctonia-infested soil, there was a greater stand (LSD=0.05) in the CMC treatment compared to the control.

Drenching Method Using Different Concentrations of *P. cepacia* OK-2. There was no difference in mean stand of radish seedlings due to *P. cepacia* compared to the control in the absence of *R. solani.* However, stand of radish seedlings was reduced (P=0.05) by *P. capacia* in Rhizoctonia amended soil when drenched at concentrations of log 2 and 4 cfu/ml compared to drenching with log 8 cfu/ml but was not different than stand in the control. Stand was reduced (P=0.05) in Rhizoctonia amended soil in drench treatments of log 2 and log 6 cfu/ml and in the control compared to stand in the same treatment of

non-amended soil (Table 4).

DISCUSSION

Only *P. cepacia* OK-3 was identified as a plant pathogenic strain using the onion puncture test. The limited maceration of onion suggested that the other strains were not pathogenic. *P. cepacia* could not be reisolated from the macerated tissue. In previous investigations (Omidiji and Ehimidu, 1990), inoculation of purple onion bulbs with *P. cepacia* resulted in the death of 80% of bacterial cells 3 days after the inoculation of the tissue and an increase in phenolic concentration up to 5 days after inoculation.

P. cepacia and *P. aeruginosa* produce antibiotic(s) and siderophore(s), although these products were not identified. However, others have indicated that compounds such as pyrrolnitrin and pyoluteolin are commonly produced by *P. cepacia* (Lievens, Rijsbergen, Leyns, Lambert, Tenning, Swing, and Joos, 1989; Meyer, Hohnadel, and Halle', 1989). According to Ulrich (1975), endo-polygalacturonase produced by *P. cepacia* and associated with a severe stem and bulb rot of onion, influenced the virulence of this disease. The optimum pH for the activity of PG produced by *P. cepacia* was between 4.4 and 4.6 which is lower than those reported for *Erwinia* spp.

Seeds differ in their sensitivity to *P. cepacia,* in the case of peanut and onion seeds, germination was almost completely inhibited. Other seeds may exhibit a delay in germination or may have a reduction in root elongation. There is evidence that some strains of *P. cepacia* enhance root elongation of wheat (de Freitas and Germida, 1990; Homma, and Suzui, 1989). Scanning Electron Microscopy (SEM) of inoculated wheat roots revealed that *P. cepacia* had significant enhancing effect on lateral root and root hair elongation. There

is no evidence to indicate that our isolate of *P. cepacia* had any enhancing effect since microscopic observation showed the lateral vinca root were reduced and root hairs were poorly grown.

Volatile ammonia produced by *P. cepacia* has been implicated as one of the factors affecting seed germination and root elongation (Baligh, Conway, and Delgado, 1990, 1991). SEM observations of *P. cepacia* in the rhizosphere of radish revealed bacteria along the junction of epidermal cells of young root immediately after seed emergence. These rod-shaped bacteria adhered to the surface of root cells with fine threads or were embedded in mucilaginous materials. Thus, *P. cepacia* multiplied in radish seed spermosphere and colonized along the surface of young root emerged from coated seed (Homma and Suzui, 1989, Homma, Sato, Hirayama, Konno, Shrahama, and Suzui, 1989). Among various bacteria from different crops, *Pseudomonas* spp. were very aggressive colonizers of the rhizosphere of several plants and proved to have a broad-spectrum biological activity against soilborne phytopathogenic fungi. In fact, these rhizobacteria are not different from other soilborne bacteria in the production of antifungal compounds but they do differ in their ability to colonize many different crops compared to other soil bacteria (Lievens, et al., 1989).

Recently, specific characteristics of some root colonizing bacteria have been evaluated (Horzore and Alexander, 1991). Six traits including: growth rate, extent of growth on root exudates, chemotaxis to root exudates and tolerance of low osmotic potentials are essential for a successful root colonization. *P. cepacia* OK-2 strain inoculated on vinca seed increased population densities and colonized roots. Comparison of bacterial populations distributed among three sections of root (top, middle, and tip) indicated that there are stable numbers of bacteria along all parts. Eventually populations

stabilize and were maintained near log 6 cfu/cm of root for a 17 day period. Investigations conducted by Delgado and Conway (1990) indicated that *P. cepacia* was able to maintain population densities on cotton roots up to log 10 cfu/g.

The doubling time of *P. cepacia* OK-2 was shortest when the initial concentration was low on vinca seed. Populations of indigenous bacteria associated with the seeds in natural soil may be reduced due to antibiotic production of *P. cepacia.* In spite of strong antibiotic and siderophore production, drenching suspensions of *P. cepacia* OK-2 at concentrations ranging from log 2 to log 6 cfu/ml failed to protect host plants against infection by *R. solani* compared to the infested control. However, the drench treatment of *P. cepacia* at log 8 cfu/ml reduced the loss of radish seedlings in Rhizoctonia-infected soil vs. non-amended soil compared to other drench treatment and control treatment. Even though doubling times are shorter for the lower concentrations, they may not reach population densities needed to effectively control *R. solani.* Population densities greater than log 8 cfu/ml in drenches may provide for greater control of *R. solani*, but also may adversely affect stand. Concentrations of *P. cepacia* (log 6 cfu/ml) used as a drench method were deleterious to vinca root elongation.

According to Homma and Suzui (1989), bacterization of the planting materials such as seeds or roots of the host plants with *P. cepacia* successfully restricted soilborne pathogens. When radish seeds were submerged into three different concentrations of *P. cepacia*, root diseases were controlled by rapid colonization of the bacteria in the rhizosphere of the host plant. Colonization of the rhizosphere changed the quantity and quality of the rhizosphere microflora, and suppressed the pathogen in the infection court on seed surface or young root by producing antibiotics or siderophores.

In our experiments, *P. capacia* reduced the number of vinco seedlings compared to the control in non-amended soil, however, under severe disease pressure (>90% reduction in the control) the stand of vinca was increased when *P. cepacia* was applied to the seeds using a CMC sticker. Improved formulations and methods of applying this bacterium to seeds in soil should greatly improve its efficacy as a biological control for Rhizoctonia damping-off diseases. Another example is the formulation of a mixture of *Trichoderma harzianum* and *P. cepacia* into granule and/or powder using sodium alginate, zeolite or diatomaceous earth. Both organisms are antagonistic to *Phytophthora capsaci,* causing blight of red pepper. An amendment of rice in the alginate formation increased the viability of *P. cepacia*. It is interesting to know that suppression of the disease on red pepper was significant when *P. cepacia* was applied to soil as a pellet compared to direct drenching (Park, Jang, Kim, and Lee, 1989).

In addition, lyophilized formulations of *P. cepacia* were also successful for control of foliar diseases (Knudsen and Spurr, 1987). A commercialized preparation of *P. cepacia* has been introduced in 1989 by Blue Circle Inoculant, Stine Seed Farm, Adel, IA. This strain failed to control *Heterodera glycines* and diseases of maize and soybean (Noel, 1990). Perhaps a combination of genetic manipulation and formulation technology will allow the potential of this bacterium to be exploited as a biocontrol agent on a reasonable scale (Jutsum, 1988).

TABLE 1

COMPARISON OF INHIBITION CAUSED BY ANTIBIOTIC PRODUCTION BY STRAINS OF *PSEUDOMONAS CEPACIA* OK-1 AND OK-5, AND *P. AERUGINOSA* B-4 AGAINST SELECTED FUNGI ON KMB[x] AND PDA[y] MEDIA

BACTERIAL STRAIN	K M B	P D A	FUNGAL STRAIN
B-4	-	+	*Fusarium*
OK-1	+	+	*oxysporum*
B-4	-	-	*Macrophomina*
OK-1	+	+	*phaseolina*
OK-5	+	-	
B-4	-	-	*Sclerotium*
OK-1	+	-	*rolfsii*
B-4	-	-	*Rhizoctonia*
OK-1	+	+	*solani*
B-4	+	+	*Pythium*
OK-1	+	+	*ultimum*
OK-5	+	-	

[x] King's Medium B [y] Potato Dextrose Agar

\+ Inhibition zone was observed around *P. cepacia* colonies after 72 h incubated at 27 C.

\- No inhibition zone was observed around *P. cepacia* colonies after 72 h incubated at 27 C.

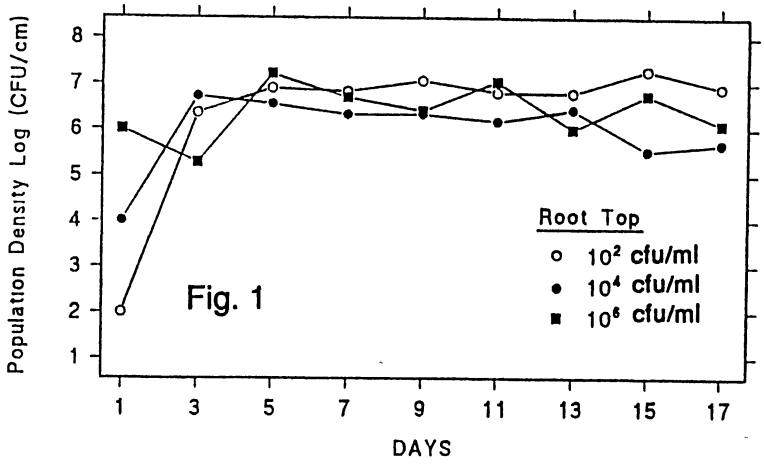
TABLE 2

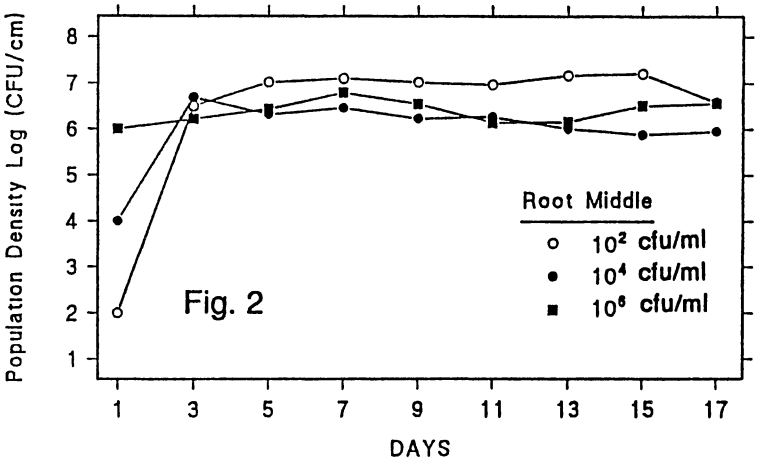
EFFECT OF INOCULUM DENSITY OF STRAINS OF *PSEUDOMONAS CEPACIA* ON PERCENT GERMINATION OF VINCA SEEDS OVER AN ELEVEN DAY PERIOD

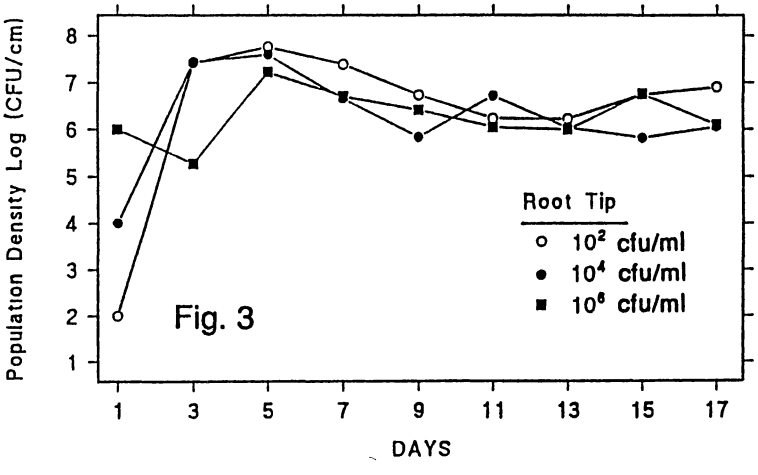
STRAIN	INITIAL DENSITY Log cfu/ml	PERCENT GERMINATION DAYS OF INCUBATION [2] 2	7	11
OK-1	2.0 [1]	9	66	66
	4.0	10	66	67
	6.0	17	68	68
	8.0	11	59	60
OK-2	2.0	18	57	58
	4.0	8	66	66
	6.0	6	62	63
	8.0	12	66	66
OK-3	2.0	24	76	77
	4.0	13	62	63
	6.0	13	67	67
	8.0	12	62	62
OK-4	2.0	25	68	69
	4.0	22	73	75
	6.0	24	78	81
	8.0	20	75	75
OK-5	2.0	27	76	77
	4.0	16	74	74
	6.0	22	66	67
	8.0	20	73	74
CONTROL		50	71	74
LSD (0.05)		11.27	16.60	15.85
S E		5.67	8.35	7.97

[1] Solution was prepared from a 48 hrs culture of each strain by diluting the suspension (log 8 cfu/ml) adjusted at 0.1 on spectrophotometer at 660 nm.

[2] Incubation temperature was adjusted at 27 C. Number of seeds were 20 per petri dish, replicated 4 times.







Figures 1-3 : Rhizosphere Colonization of *Pseudomonas cepacia* OK-2 on Three Sections of Vinca Root for a 20 Days Period. Fig. 1 Top Third of Root. Fig. 2 Middle Third of Root. Fig. 3 Bottom Third of root. Initial Inoculum on Seed Was Log 2, 4, and 6 cfu/seed.

TABLE 3

AVERAGE ROOT LENGTH OF VINCA SEEDLINGS TREATED BY *P. CEPACIA* OK-2 USING FOUR DIFFERENT METHODS OF INOCULATION WITH AND WITHOUT THE ADDITION OF *RHIZOCTONIA SOLANI* (AG-4) 330 TO THE SOIL SYSTEM

TREATMENT [6]	ROOT LENGTH (cm) [1]	PERCENT GERMINATION [2]
Control with *R. solani*	5.0 ab	73.3
Control without *R. solani*	6.3 a	90
Soaking[3] with *R. solani*	4.4 b	65.5
Soaking without *R. solani*	6.6 a	71.1
Drenching[4] with *R. solani*	5.6 ab	77.7
Drenching without *R. solani*	5.0 ab	63.3
CMC[5] with *R. solani*	4.6 ab	57.7
CMC without *R. solani*	5.9 ab	74.4

1 Root length and percent germination were calculated from 10 seeds/container.

2 Letters are significantly different (P=0.05), One way ANOVA randomized complete design.

3 Soaking in bacterial suspension of log 6 cfu/ml for 60 min. of log 6 cfu/ml.

4 Drenching with 118 ml/232 cm^2 from bacterial suspension of log 6 cfu/ml.

5 Carboxymethylcellulose 1% (CMC) used as a sticker and mixed with log 8 cfu/ml of bacterial suspension.

6 Treatements were replicated 9 times.

TABLE 4

MEAN STAND RADISH SEEDLING DRENCHED [a] WITH FOUR CONCENTRATIONS (LOG 2, 4, 6, AND 8 CFU/ML) OF *PSEUDOMONAS CEPACIA* (OK-2) IN THE PRESENCE OR ABSENCE OF *RHIZOCTONIA SOLANI* OK-330 (AG-4).

CONCENTRATION (log cfu/ml)	MEAN STAND [b] *Rhizoctonia solani* (-)	(+)
2	6.75 a*[c]	3.50 b
4	5.00 a	3.25 b
6	8.25 a*[d]	5.00 ab
8	7.00 a	6.50 a
CONTROL	8.25 a*	5.25 ab

[a] Data were collected 8 days after drench treatment (118 ml/ 232 cm^2).

[b] Number of seeds per container were 10 with four replications. Experiment was conducted in a completely randomized design.

[c] Mean followed by different letters are significantly different. Student-Newman-Keuls Test (p=0.05).

[d] * - indicates significant differences within treatments between *Rhizotonia* amended (+) and non-amended (-) treatments.

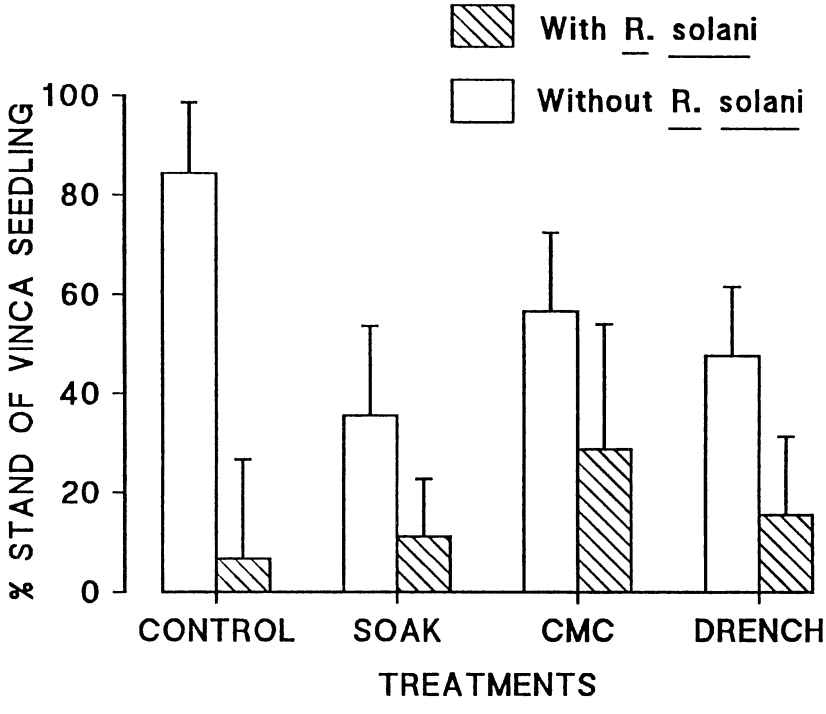


Figure 4. Comparison of Three Application Methods of *Pseudomonas cepacia* (OK-2) on Percent Stand of Vinca Seedling LSD(0.05)=8.0.

REFERENCES

ASANUMA, S., TANAKA, H. AND YATAZAWA, M. (1980). *Pseudomonas cepacia*- A characteristic rhizoplane microorganisms in rice plant. *Soil Science Plant Nutrition* **26**(1), 71-78.

BALIGH, M., CONWAY, K.E. AND DELGADO, M.A. (1990). Production of ammonia by *Pseudomonas cepacia* interferes with seed germination and root elongation. *Phytopathology* **80**(10), 1048 (Abstract).

BALIGH, M., CONWAY, K.E. AND DELGADO, M.A. (1991). Development of a bioassay system to compare quantities of ammonia produced by strains of *Pseudomonas* spp., potential biocontrol agents against soilborne fungi. *Phytopathology* **81**(10), 1228 (Abstract).

CONWAY, K.E., FOOR, C.J., MALVICK, D. AND BENDER, C. (1989). Inhibition of *Pseudomonas cepacia,* a potential biocontrol agent of selected soilborne pathogens. *Phytopathology* **79**, 1159 (Abstract).

CONNICK, W.J. JR. (1989). Formulation of biocontrol agents for use in plant pathology. *Journal of Cellular Biology* Suppl. **13** A Abstract 18th annual meetings.

DELGADO, M. AND CONWAY, K.E. (1989). Population dynamics of three potential biocontrol *Pseudomonas* strains on seeds and roots of cotton. *Phytopathology* **80**, 434 (Abstract).

DE FREITAS, J.R. AND GERMIDA, J.J. (1990). A root tissue culture system to study winter wheat-rhizobacteria interactions. *Applied Microbiology and Biotechnology* **33**, 589-595.

DURRANDS, P.K. AND COOPER, R.M. (1988). Development and analysis of pectic screening media for use in the detection. *Applied Microbiology and Biotechnology* **28**, 463-467.

FERRIS, R.S. (1984). Effects of microwave oven treatment on microorganisms in soil. *Phytopathology* **74**, 121-126.

GONZALEZ, C.F. AND VIDAVER, A.K. (1979). Bacteriocin, plasmid, and pectolytic diversity of *Pseudomonas cepacia* of clinical and plant strains. J. Gen. Microbiol. 110:161-170.

HANKIN, L. AND ANAGNOSTAKIS, S. (1975). The use of solid media for

detection of solid media for detection of enzyme production by fungi. *Mycologica* **67**(3), 597-607.

HOMMA, Y., SATO, Z., HIRAYAMA, F., KONNO, K. SHRAHAMA, H. AND SUZUI, T. (1989). Production of antibiotics by *Pseudomonas cepacia* as an agent for biological control of soilborne plant pathogens. *Soil Biology and Biochemistry* **21**(5), 723-728.

HOMMA, Y. AND SUZUI, T. (1989). Role of antibiotic production in suppression of radish damping-off by seed bacterization with *Pseudomonas cepacia*. *Annual Phytopathological Society of Japan* **55**, 643-652.

HORZORE, E. AND ALEXANDER, M. (1991). Bacterial characteristics important to rhizosphere competence. in *The Rhizosphere and Plant Growth* (ed. by D.L. Keiser and P.B. Cregan) Kluwer Academic Publishers. p. 108.

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

JANISIEWICZ, W. J. AND ROITMAN, J. (1988). Biological control of blue mold and gray on apple and pear with *Pseudomonas cepacia*. *Phytopathology* **78**, 1697-1700.

JANISIEWICZ, W.J. AND YOURMAN, L., ROITMAN, J. AND MAHONY, L. (1991). Postharvest control of blue mold and gray mold of apples and pears by dip treatment with pyrrolnitrin, a metabolite of *Pseudomonas cepacia*. *Plant Disease* **75**, 490-494.

JAWASWAL, R.K., FERNANDEZ, M.A. AND SCHREDER III, R.G. (1990). Isolation and characterization of a *Pseudomonas* strain that restricts growth of various phytopathogenic fungi. *Applied Environmental Microbiology* **56**(4), 1053-1058.

JUTSUM, A.R. (1988). Commercial application of biological control: status and prospects, Philosophical Transactions of the Royal Society of London **B318**, 357-373.

KNUDSEN, G.R. AND SPURR, H.W. (1987). Field persistence and efficiency of five bacterial preparations for control of peanut leaf spot. *Plant Disease* **71**, 442-445.

LIEVENS, K.H., RIJSBERGEN, V.R., LEYNS, F.R., LAMBERT, B.J., TENNING, P., SWINGS, J. AND JOOS, H.J.P. (1989). Dominant rhizosphere bacteria as a source of antifungal agents. *PesticideScience* **27**(2), 141-154.

LOPER, J.E. (1988). Role of fluorescent siderophore production in biological control of (*Pythium ultimum*) by *Pseudomonas fluorescens* strain. *Phytopathology* **78**, 166-172.

MEYER, J.M., HOHNADEL AND D. HALLE', F. (1989). Cepabactin from *Pseudomonas cepacia*, a new type of siderophore. *Journal of General Microbiology* **135**, 1479-1487.

NOEL, G.R. (1990). Inability of a seed treatment with *Pseudomonas cepacia* to control *Herterodera glycines* on soybean. Supplement to *Journal of Nematology* **22**(45), 792-794.

OMIDIJI, O. AND EHIMIDU, J. (1990). Changes in the content of antibacterial isorhamnetin 3-glucoside and quercetin 3'-glucoside following inoculation of onion (*Allium cepa* L. cv. Red Creole) with *Pseudomonas cepacia*. *Physiological Molecular Plant Pathology* **37**, 281-292.

PARK, K.S., JANG, S.W., KIM, C.H. AND LEE, E.J. (1989). Studies on biological control of Phytophthora blight of red pepper: III Formulations of *Trichoderma harzianum* and *Pseudomonas cepacia* antagonistic to *Phytophthora capsici* and their preservation. *Korean Journal of Plant Pathology* **5**(2), 131-138.

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

PARKE, J.L. (1991). Root colonization by indigenous and introduced microorganisms. in *The Rhizosphere and Plant Growth* (ed. by D.L. Keister and P.B. Cregan) Kluwer Academic Publishers pp.33-42.

ULRICH, J.M. (1975). Pectic enzymes of *Pseudomonas cepacia* and penetration of polygalactronase into cells. Physiological Plant Pathology **5**, 37-44.

WILSON, C.L. AND CHALUZ, E. (1989). Postharvest biological control of *Penicillium* rots of citrus with antagonistic yeasts and bacteria. *Scientia Horticulturae* **40**, 105-112.

CHAPTER IV

PRODUCTION OF AMMONIA BY *PSEUDOMONAS CEPACIA* AND *PSEUDOMONAS AERUGINOSA*: QUANTIFICATION AND EFFECT ON HOST AND PATHOGEN

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ABSTRACT

Different concentrations of *P. cepacia* were applied to vinca and other seeds as a biological seed treatment to control soilborne diseases. Direct and indirect contact of bacteria with seeds delayed seed germination and reduced root elongation especially at high inoculum concentrations. When *P. cepacia* and seeds were incubated in separate sections of a split-half petri dish, germination was delayed suggesting the existence of a volatile compound(s). This was confirmed when pH indicator papers suspended over bacterial cultures showed a pH change from 7 to 8. The volatile was collected in water traps connected to cultures grown in Czapek's broth with and without 20 g/l of peptone. The volatile was identified as ammonia using an EM Quanta Ammonia Kit, a calorimetric processes using a Lachet instrument and by mass spectral analysis. Production of ammonia varied among strains of *P. cepacia* and *P. aeruginosa* and depended on the amount of peptone or amino acids

added to the media. Production of ammonia by each bacterial strain was quantified by comparison to known concentrations of ammonia (NH_4OH) in a bioassay system and resultant inhibition of selected fungi. Among fungi, *Sclerotium rolfsii* was the most sensitive being completely inhibited by <3 µg/ml, while *Trichoderma harzianum* was stimulated at this concentration. Production of ammonia varied among bacterial strains but was equivalent to NH_4OH concentrations ranging from 6 to 18 µg/ml. Volatiles produced by rhizobacteria should be considered as additional factors involved in the inhibition of pathogens in the rhizosphere and/or spermosphere of host plants.

INTRODUCTION

Previous investigations concerning *P. cepacia* indicated that this biocontrol candidate interfered with seed germination and root elongation of host plants (Baligh, Conway, and Delgado, 1990; Delgado and Conway, 1989). It was not clear whether this retardation was due to the direct phytotoxicity of *P. cepacia* or due to the release of a volatile compound produced by this bacterium in the spermosphere and/or the rhizosphere of host plants. In addition, a preliminary experiment indicated that ammonia might be involved in this process and the volatile component should be investigated.

The roles of antibiotics and siderophores produced by *P. cepacia* have been extensively investigated (Homma and Suzui, 1989; Janiseiwicz and Roitman, 1988; Janiseiwicz, Yourman, Roitman, and Mahony, 1991). The existence of these antimicrobial compounds were detected and the ability to colonize the rhizosphere was confirmed on vinca and other small seeds (Baligh, et al., 1990). In some investigations the involvement of volatile compounds in the host, pathogen, and biocontrol agent interaction has been

noted (Punja, Jenkins, and Grogan, 1984). Sources of volatiles in the spermosphere and rhizosphere can be either from plant tissues or exudates from germinating seeds and roots. The response of soilborne fungi to volatiles at low concentrations is stimulatory but almost all are inhibitory at high concentrations. Many volatiles which are released from plant tissues and germinated seeds stimulate germination of fungal propagules. Compounds such as methanol, ethanol, and butanol induce chemotactic or chemotropic responses of fungi but can be inhibitory at high concentrations. Sources of organic volatiles in soil include "primary" compounds derived from plants or organisms, or "secondary" materials resulting from microbial transformation (Stotzky and Schenick, 1975).

Recently, an additional mechanism of *P. putida* strain NIR against *Pythium ultimum* was investigated by Paulitz, (1991). Although this bacterium did produce antibiotics and was deficient in siderophore production, it was still able to control pre-emergence and post-emergence damping-off and root rot of vegetable crops. In this process, *P. putida* rapidly metabolizes volatile seed exudates and suppressed the stimulation of sporangia of *P. ultimum* and reduced daming-off of soybean and pea seeds. Volatile exudates, such as ethanol and acetaldehyde, are an early germination signal and nutrient source that are released from seeds prior to the release of water soluable exudates. It is essential to know how a volatile affects seeds or roots of a host plant as well as to understand the reaction of a pathogen, especially when a volatile is released by a biocontrol candidate. In addition, production of phenylalanine ammonia lyase (PAL) by *P. cepacia* was shown to suppress spore germination and inhibit fungal pathogens (Huang, Deverall, and Morris, 1991). The present study focuses on the detection and identification of a volatile compound(s) which may inhibit seed germination. The positive and negative effects of this

volatile on host plants and pathogens were evaluated. Preliminary reports of this work have appeared elsewhere (Baligh, Conway, and Delgado, 1991).

MATERIALS AND METHODS

Characteristics of *P. cepacia* Strains. There were five strains of *P. cepacia* used in this study: OK-1 (the wild type), isolated from soil at the State Forest Nursery in Washington, OK.; OK-2, an antibiotic selection from strain OK-1 with two antibiotic markers, 120 µg/ml nalidixic acid and 80 µg/ml chloramphenicol; *P. cepacia* 945 or OK-3 from the National Collection of Plant Pathogenic Bacteria, Harpenden, England, isolated from onion tissue and with five antibiotic markers, carbenicillin 1 µg/ml, streptomycin 200 µg/ml, tetracycline 50 µg/ml, neomycin 200 µg/ml, and penicillin 200 µg/ml from the National Collection of Plant Pathogenic Bacteria; OK-4 ,isolated from fruit by W.J. Janiseiwicz, and OK-5, isolated from soil in Stillwater, OK. Two isolates of *P. aeruginosa,* B-2 and B-4 from Peru were also used in this study.

Indirect Effect of *P. cepacia* on Seed Germination. *P. cepacia* OK-1 or OK-2 were inoculated into split-half petri dishes with potato dextrose agar (PDA) on one side. The other half of the petri dish was lined with moist sterile filter paper and 20 surface sterilized vinca (*Catharanthus roseus* L.), sage (*Salvia officialis* L.) or onion (*Alluim cepa* L.) seeds were separately placed on the filter paper. All seeds were incubated for 48 h at 28 C. This experiment was repeated twice.

Detection and Identification of Volatile Compound(s). The following broths were prepared and adjusted to pH 7.0: Nutrient (NB), potato dextrose (PDB), King's B (KBB) and Czapek's (CZB). Each broth (100 ml) was poured

into each of five-250 ml flasks. Four sterile strips of pH paper (10 cm length) with different pH ranges were hung from the neck of each flask. A 0.5 ml aliquot from suspensions (log 8 cfu/ml) of five strains of *P. cepacia* (OK-1 to OK-5) was each inoculated into one of the broth cultures except the control. All flasks were incubated on a shaker (84 rpm, Eberbach 115 volt, 60 cycle) at room temprature so that strips of the pH papers did not touch the media.

Further tests were conducted using CZB amended with 20 g/l peptone (CZPB). Another series of 250 ml flasks containing either CZB or CZPB were inoculated with strains (OK-1, OK-2, OK-3, OK-4, OK-5) of *P. cepacia* and incubated on a rotary shaker at room temperature. Sterile filtered air was passed over cultures of *P. cepacia* and the controls in the flasks and was trapped in a water blank. The initial bacterial suspension (0.5 ml of log 8 cfu/ml) was added after adjusting the media to pH 7.0. After 72 h of incubation, water samples were tested using an EM Quanta Ammonia Test Kit (EM Science Co., Gibbtown, N.J.). In addition, similar samples were prepared and tested at the Oklahoma State University, Soil and Water Quality Testing Laboratory on a Lachet instrument, which uses a calorimetric process to measure the quantity of ammonia in water. Samples were also prepared for analysis by mass spectrometry (MS).

Volatile compound(s) were similarly collected using 20 ml absolute ethanol instead of water in the traps. Ethanol traps were surrounded by ice inside an ice chest. Valves between the bacterial cultures and the water traps were opened 36 h after incubation of *P. cepacia* strains and *P. aeruginosa* (B-4) in CZPB or CZB (300 ml). Ethanol traps were disconnected and plugged after 72 h for analysis with a VG-11-250 integrated mass spectrometer. The mass range was initially monitored over a mass-to-charge ratio (M/Z) of 16 to 20 and then expanded from 17 to 18 to improve accuracy. Software peak matching

was used to determine the accuracy of the mass of the suspected peak (Howell, et al., 1988). All tests were conducted three times.

Effect of Volatile Compound(s) on Seed Germination, and Root Elongation of Vinca Seeds and Growth of Selected Fungi. Ten grams of moist sterilized sand were added to each of two opposite sections of quadrad portion petri dishes. Five surface sterilized vinca seeds were placed on each of these sections. The other two sections of the petri dishes were filled with 5 ml of either Czapek's Agar (CZA) or CZA amended with peptone (20 g/l) (CZPA). After placing vinca seeds on the surface of moist sand, 0.5 ml of log 8 cfu/ml suspension of *P. cepacia* strains (OK-1 to OK-5) were added to the surface of the agar media. All petri dishes were sealed with parafilm and masking tape and incubated at 28 C. All treatments were replicated five times. Numbers of germinated seeds and the length of roots were recorded after a week.

In another experiment, two opposite sections of quadrad petri dishes were filled with 5 ml of CZPA. A suspension of strain OK-2 was spread on the agar surface in the two quarters of the petri dishes. The other agar sections was filled with 5 ml of trypticase soy agar (TSA) medium and were inoculated with a small block of a selected pathogenic fungus. Tested fungi included *Macrophomina phaseolina* (M-9, OK-98, and OK-342), *Rhizoctonia solani* (OK-337), *Fusarium oxysporum* (OK-209, OK-97), *Pythium ultimum* (OK-288), *P. aphanidermatum* (OK-317), *Phytophthora cactorum* (OK-316), and *Sclerotium rolfsii* (OK-315). All petri dishes were sealed with parafilm and masking tape and incubated at 28 C.

Another variation of the experiment evaluated the effect of peptone on volatile production by comparing CZPA and CZA. *P. cepacia* strains (OK-1 to OK-5) were streaked on the surface of quadrad of either CZPA or CZA. The

radial growth of fungal colonies was measured after 18, 24, and/or 48 h, depending on the growth rate of each fungal pathogen. A similar experiment was conducted using non-sterilized seeds. Numbers of germinated seeds and the length of the root were measured after one week.

Suppressive Effect of Volatile Compound(s) on Selected Plant Pathogenic Fungi. Fresh cultures of eight pathogenic fungi including *Pythium aphanidermatum, Fusarium oxysporum* (OK-209 and OK-97), *Macrophomina phaseolina* (M-9 and OK-98), *Phytophthora cactorum, Rhizoctonia solani* (OK-330), and *Sclerotium rolfsii* (OK-64) were prepared. Three drops of log 8 cfu/ml suspensions from a 24 h culture of either *P. cepacia* (OK-2), *P. aeruginosa* (B-2, B-4), or *Bacillus subtilis* (B-6) were separately added to the surface of two opposite sections of quadrad portion petri dishes containing CZPA medium. After 24 h incubation, two small blocks of each selected fungus were placed on the surface of the other two quarters containing TSA medium. All petri dishes were sealed with parafilm and masking tape and incubated at 28 C. Treatments were replicated five times. The radial growth of two opposite fungal colonies were measured from each petri dish, depending on their growth rate, after a 18, 24, or 48 h period.

Development of a Bioassay for Ammonia Production. Five ml of CZPA was added to each one of two opposite sections of quadrad petri dishes. The other two sections were filled with 5 ml TSA, which has a nitrogen source but not in the form of an ammonium salt for culturing the selected fungi. One half ml of each bacterial suspension (log 8 cfu/ml) was added to the entire area of two sections of CZPA except in the control petri dishes. Twenty four hours after the incubation of the bacteria at 27 C, a 2 mm diameter block of a fungus was

placed at the center of each one of two opposite sections of TSA. All petri dishes were sealed with parafilm and masking tapes and placed inside an incubator at 27 C.

In a second part of the experiment, various amounts of ammonia from 3 µl to 54 µl in 1 µl increments were prepared by addition of ammonium hydroxide (NH_4OH) to 1 ml aliquot of sterile water. These dilutions, 0.5 ml/section, were placed into the empty opposite sections of quad-petri dishes. A block of fungus was placed on each one of the two TSA media sections. All petri dishes were sealed immediately with parafilm and masking tape and incubated at 27 C. Depending on the rapidity of fungal growth, the incubation period ranged from 18 to 72 h. The radial growth of fungi was measured and compared with the untreated control and to fungal growth in the presence of strains of *P. cepacia* (OK-1 to OK-5) and *P. aeruginosa* (B-2 and B-4). Data on germination was recorded at 18, 24, and 48 h depending on growth rate of the fungus. This experiment was repeated once.

RESULTS

Indirect Effect of *P. cepacia* on seed germination. Previous studies indicated that when vinca seeds were soaked in suspensions (log 2, 4, 6, and 8 cfu/ml) of *P. cepacia* strains (OK-1 to OK-5), there was a delay in germination of seeds compared to the non-treated control seeds (Baligh, et al., 1990). This delay mostly occurred during the early stage of germination due to direct contact with *P. cepacia.* However, after 11 days, germination rates of seeds were similar to the control. The indirect bioassay indicated that germination of sage, vinca and onion was also reduced by the volatile produced by *P. cepacia* OK-1 and OK-2. Among selected seeds, onion seemed to be more sensitive to

the volatile than the other seeds (Data not presented). Both strains of *P. cepacia* produced similar effects on germinating seeds.

Detection and Identification of Volatile Compound(s). Color changes of pH papers suspended above cultures of *P. cepacia* were compared to the color standards for each paper. Maximum pH detected was 8.2 above NB and KMB, but only a slight change in pH was noted for cultures grown in PDB. No color changes were observed in control flasks. Greatest changes of pH occurred when *P. cepacia* was grown in CZB amended with peptone. The volatile compound was detected and identified as ammonia using the EM Quanta Ammonia test kit and the Lachet calorimetric apparatus. The greatest amount of ammonia was detected from strain OK-3 which consistently had the highest values for ammonia production in three different tests (Table 1). The OSU Soil and Water Quality Laboratory also detected ammonia using calorimetric changes with the Lachet apparatus but indicated much smaller quantities were produced by each strain (Table 2).

According to the EM Quanta test kit, the range of ammonia produced was 5 to 60 ppm. On the other hand, the Lachet instrument indicated that the range was 0.05 to 8.93 ppm in the presence of peptone. When these tests were repeated, both methods showed variable quantities of ammonia were produced by each strain. According to the EM Quanta method higher quantities of ammonia were produced by strain OK-3, whereas the Lachet test indicated that strain OK-5 and OK-3 produced more ammonia compared to the other strains of *P. cepacia*.

Volatile ammonia was not detected in ethanol traps connected to the control. Mass spectral analysis (Table 3 and Figures 1-4 and Figures 5-10) indicated that the only major difference in the spectra occurred near the mass to

charge ratio of 17.0265. This compared favorably to the calculated amount for NH_3:17.0266 and to a standard calibration for ammonia. The ammonia peak from the bacterial cultures only occurred in ethanol samples connected to CZPB samples but not from CZB. Comparison of the M/Z 17.0265 peak areas of strains of *P. cepacia* and *P. aeruginosa* indicated strain *P. aeruginosa* B-4 produced the greatest amount of ammonia. The soil strain *P. cepacia* OK-3 had the smallest peak compared to OK-1, OK-2, and OK-4. No ammonia was detected from strain OK-5 using MS technique. No other peak(s) was detected between the ammonia and the OH peaks except an additional unidentified peak from strain OK-4.

Effect of Volatile Compound(s) on Seed Germination and Root Elongation of Vinca Seeds and Growth of Selected Fungi. Results of three experiments were analyzed to determine whether or not the addition of peptone to CZA had any effect on the mean root length and/or percent germination of vinca seeds. In general, none of the *P. cepacia* strains, except strain OK-4, had an effect on the root length compared to the control (Table 4). In two out of the three tests, only strain OK-4 significantly reduced mean root length compared to the control when grown on CZA. Strain OK-4 produced an unknown volatile without addition of peptone into CZA medium. Therefore, none of CZA media had any effect on ammonia production by other strains of *P. cepacia* (OK-1, OK-2, OK-3, OK-5) to reduce mean root length compared to the control.

Addition of peptone to CZA media activated most strains of *P. cepacia* to release volatile ammonia. In general volatilization of ammonia by strain OK-3, known as the pathogenic strain, significantly reduced mean of root length compared to the control in all three tests. The effect of volatile ammonia produced by strains OK-1, OK-2, and OK-5 on mean root length of vinca varied

but differed from the control. The mean root length of vinca varied when germinated in the presence of *P. cepacia* (OK-1 to OK-5) strains growing on CZA medium, but did not differ from the control. Root length of vinca was reduced (LSD=0.05) due to the volatile produced when *P. cepacia* (OK-1, OK-2, OK-3 and OK-5, excluding OK-4 during test 2 was grown on CZPA compared to CZA. Strain OK-3 produced the greatest reduction among all *P. cepacia* strains. During the experiments, strain OK-4 grown on CZA reduced mean of root length compared to CZPA (Tables 4). There was a significant reduction (LSD=0.05) of root length by strain OK-4 on CZPA.

On the other hand, percent germination of vinca seeds in the presence of bacterial strains varied among isolates and media. Only strain OK-3 reduced percent germination when grown on CZPA medium. The results also showed that percent germination of seeds varied when volatile ammonia was released by the other strains grown on either CZA or CZPA.

Among selected pathogenic fungi, the radial growth of *S. rolfsii* (OK-315), *F. oxysporum* (OK-209), and *P. cactorum* (OK-316) were reduced (p=0.05) whereas this suppression varied among other selected pathogenic fungi. Some of the selected fungi such as *Trichoderma harzianum, Fusarium oxysporum* tolerated or were slightly stimulated by the volatile.

Suppressive Effect of Volatile Compound(s) on Selected Plant Pathogenic Fungi. Bacterial isolates growing on CZPA differed in their effects on the growth of various pathogenic fungi (Figures 5-8). During the first 18 h period, the radial growth of *Pythium aphanidermatum* was suppressed (P=0.05) due to the volatile produced by *P. aeruginosa* (B-2 and B-4) compared to the control. The radial growth of *R. solani* colonies, measured after 24 h incubation, was suppressed (P=0.05) by the volatile(s) released by *P. aeruginosa* (B-2)

compared to the control. None of the bacterial strains were able to reduce the growth of either isolate of *M. phaseolina* during this period of time. *P. aeruginosa* (B-2, B-4), and *B. subtilis* suppressed the radial growth of *Phytophthora cactorum* (p=0.05), compared to the control when measured after 48 h incubation. Among selected fungi, *Sclerotium rolfsii* was the most sensitive fungus and was suppressed by volatile(s) produced by all bacteria except *B. subtilis*.

Development of Bioassay for Ammonia Production. The sensitivity of selected fungi to produce volatile ammonia varied (Table 5). The most sensitive fungus, *Sclerotium rolfsii,* was inhibited by <3.0 µl/ml. Among bacterial isolates, *P. aeruginosa* (B-2 and B-4) produced higher amounts of ammonia compared to strains of *P. cepacia.* Values indicating total inhibition (LC_{100}) of each fungus as well as 50% (LC_{50}) and 90% inhibitions (LC_{90}) of selected fungi were determined. The ability of each bacterial strain to produce ammonia was compared to these values. Among selected fungi, *Fusarium oxysporum, Pythium myriotylum* and *R. solani* had the highest tolerance to volatile ammonia produced by *Pseudomonas* spp.. Among bacterial strains, only *P. aeruginosa* B-4 produced sufficient ammonia (equivalent or above LC_{50} values) to suppress *M. phaseolina, F. oxysporum, P. myriotylum, P. aphanidermatum,* and *S. rolfsii.* None of the bacterial strains produced sufficient amounts of volatile, equvalent to LC_{50} values, to inhibit *R. solani, P. cactorum*, and *T. harzianum.* In addition, ammonia equivalent values varied among *P. cepacia* strains OK-1 to OK-5. Figures 15-18 (photos) demonstrate the bioassay for *Trichoderma harzianum, Macrophomina phaseolina, Fusarium oxysporum*and *Sclerotium rolfsii.*

DISCUSSION

Information about the effect of volatile compound(s) produced by microorganisms against soilborne pathogens is limited. The efficacy of a biocontrol agent can be enhanced by the production of volatiles on the spermosphere, rhizosphere, or rhizoplane of a host plant where the antagonistic biocontrol agent encounters the pathogen. Previous studies indicated that direct contact of *P. cepacia* with the seed or seedling interfered with germination and root elongation (Baligh, et al., 1990). Both direct and indirect contact of *P. cepacia* with seeds had similar effects on the delay in germination and reductions in root elongation. These observations suggested that volatile compounds produced by *P. cepacia* have phytotoxic effects on the metabolism of host plants. Some strains of *P. cepacia* enhanced root elongation (De Freitas and Germida, 1990). Our strains reduce root elongation but they are successful root colonizers on selected host plants (Delgado and Conway, 1989).

Although the use of sterile pH paper was not an accurate detection method, the change of pH produced above NB, PDB, and KBB containing *P. cepacia* indicated that a volatile compound was released from these media and the degree of alkalinity was directly correlated to the source of nutrients added to the broth.

During this study several techniques, such as the EM Quanta ammonia kit and the Lachet analytical system with a Quical II calibration, were used to identify and quantify the volatile ammonia. Even though these methods identified ammonia, there were large discrepancies between the two methods. The EM kit recorded a range of 5 to 60 µg/ml and indicated that the amounts varied among the water samples used to trap volatiles, while the Lachet system

recorded a range of 1.4 to 8.93 µg/ml when peptone was present in the culture media. Both methods indicated that the addition of peptone to CZB increased the amount of ammonia released compared to when bacteria were grown in CZB alone. MS analysis could detect and quantify ammonia but the results varied among samples from each bacterial strain. In the first attempt to use MS analysis, strain OK-3 produced the lowest quantity (3600 Abs.Ht.) of ammonia among all *Pseudomonas* strains (Table 3). However, during subsequent analyses, strain OK-3 had higher ammonia peak than those of OK-2 (Figures 1-4 and Figures 5-10).

Many other methods have been introduced for ammonia determination including formation of blue color indophenol compound with sodium salicylate (Yerdouw, Van Echteld, and Dekkers, 1988), the modified calorimetric method of Kjeldahl digestion based on the sensitivity color reaction between NH_4^+ and a weakly alkaline mixture of sodium salicylate and dichloroisocyanurate (Cooke and Simpson, 1971), Nessler's reagent, and an ammonia gas sensitive electrode (Underhill, 1990). Indeed, all above methods have some difficulties. Modification of the water trapping system for collecting the volatile allowed the use of an EM Quanta Ammonia test kit to determine the amount of ammonia. The Lachet analytical system quantified and identified the volatile as ammonia but none of these techniques could quantify ammonia directly. Results of these tests for the same strain of *P. cepacia* were variable. In order to better quantify the amount of ammonia produced by bacterial isolates, a bioassay system was developed that relied on comparisons to the volatilization of ammonia from a series of solutions of ammonium hydroxide.

The bioassay technique determined that all strains of *P. cepacia* were able to produce volatile ammonia to suppress individual fungal pathogens. The amount of ammonia produced by strains of *Pseudomonas* varied. Comparison

of *P. cepacia* (OK-1 to OK-5), *P. aeruginosa* (B-2 and B-4) and *Bacillus subtilis* (B-6) for production of volatile compound(s) indicated that the amounts of ammonia produced by each strain was generally less than LC_{50} values. Comparison of ammonia equivalent values (AEV) of each bacterial strain to LC_{50}, LC_{90}, and LC_{100} values of the same fungus indicated how much inhibition of mycelial growth was possible, under ideal conditions, by volatile ammonia. Values of AEV varied among all strains of *P. cepacia* and *P. aeruginosa.* Among selected pathogenic fungi mycelial growth of *S. rolfsii* was the most sensitive and *Pythium aphanidermatum* the most tolerant to ammonia. Tolerance of fungi to ammonia varied and indicated that production of ammonia, by bacteria will not completely control pathogens. However, suppression of fungal pathogens by ammonia can be effective when mycelial growth toward germinating seeds is retarded allowing for additional growth of the seedling.

According to Chun, Filonow, and Lockwood, (1984), low concentrations of ammonia were not toxic to sclerotia of *M. phaseolina*. Other studies (Tabak and Bridge Cook, 1968) indicated that NH_3, produced as byproduct of organisms, reduces or inhibits the sporulation of *Fusarium* spp. in closed dishes or on media containing nitrogenous compounds that releases ammonia. However, Loffler, van Dongen, and Schippers, (1986) have shown that low concentration of NH_3 (15 µg/ml) stimulated the formation of chlamydospore by *Fusarium* spp. in both germinated and ungerminated conidia, but higher concentrations of NH_3 (150 µg/ml) had inhibitory effects. Also, low concentrations of NH_3 (10 µg/ml) had stimulatory effects on conidial germination whereas higher concentration (>150 µg/ml) had inhibitory effects (Palvica, Hora, Bradshaw, Skogerboe, and Baker, 1978). In our experiment, mycelial growth of all fungi tested was completely inhibited by 30 µg/ml of

ammonia. Variability in reactions of pathogens to NH_3 may limit the potential of NH_3 on a field scale basis (Smiley, Cook, and Papendick, 1972). Therefore, it is important to know at what stage of the life cycle, a pathogen is most sensitive to the volatile produced by a biocontrol agent. It is expected that the tolerance of various propagules (e.g. zoospore, chlamydospore, mycelia) are different (Zakaria, Lockwood, and Filonow, 1980, Loffler, et al., 1986, Leach and Davey, 1935). Our bioassay can be modified to evaluated such effects.

All selected strains of *Pseudomonas* demonstrated total inhibition of mycelial growth of *Sclerotium rolfsii* since more than 3 µl/ml of volatile ammonia were produced by all strains. According to Leach and Davey (1935), aqueous solutions of ammonia of less than 50 ppm, were lethal to the mycelium of *S. rolfsii* after a 24 h exposure and at 150 ppm within 2 h. Minimum concentrations needed to kill resistant sclerotia within 24-72 h was 250 ppm (Fang and Lui, 1988).

According to Howell, Deverall, and Morris, (1988) low concentrations of ammonia produced by *Enterobacter cloacae* in the spermosphere were highly toxic to *Pythium ultimum* causing pre-emergence damping-off of cotton. Our strains of *Pythium* were highly tolerant to ammonia up to 27 µl/ml and at low concentrations, ammonia was somewhat stimulatory. Other researchers reported that low concentrations of ammonia had fungistatic effects and populations of *P. ultimum* and *M. phaseolina* slowly declined (Chun, et al., 1984). All strains *P. cepacia* and *P. aeruginosa* suppressed the growth of *P. aphanidermatum* similar to the LC_{50} level (13.4 µg/ml) but were not as inhibitory of mycelial growth of *Phytophthora cactorum.* Zoospore germination of *P. cinnamoni* was completely inhibited by 17 µg/ml ammonia in buffer solution after 24 h (Lockwood and Filonow, 1981, Gilbert, Hanelsman, and Parke, 1989). Thus, the effect of ammonia produced by *P. cepacia* may have

similar activity against zoospore of *Pythium* spp. and *Phytophthora* spp.

Stimulatory effects of volatile ammonia produced by *P. cepacia* at low concentrations were observed for *Trichoderma harzianum,* although *P. aeruginosa* (B-2 and B-4) strains had inhibitory effects on this fungus. The efficacy of *Trichoderma* spp. as biocontrol agent may be limited by the concentration of ammonia in soil (Linderman and Gilbert, 1969). The bioassay system indicated this fungus was completely inhibited at 15 µl/ml. Information about factors involved in inhibition and/or stimulation are important since it is essential to understand or interpret the behavior of pathogen at the site of infection.

There is evidence that ammonia is involved in the pathogenicity by bacteria. For example, *Pseudomonas tomato,* causing bacterial speck of tomato, produces ammonia that increases tissue necrosis. In addition to chlorosis and necrosis, electrolyte leakage occurred which coincided with ammonia accumulation (Bashan, Okan, and Henis, 1980). Similar observations were reported in diseased plants infected by *P. coronafaciens* (Trabulsi, Whitebread, and Duckett, 1978). *Pseudomonas fluorescens, P. aeruginosa* and several other Pseudomonads produce ammonia when they are grown on yeast peptone (Bashan, et al., 1980). Ammonium is not toxic to the host in concentrations of approximately 100 µg/ml whereas ammonia has a necrotic effect at this concentration. *P. tabaci* produces tabtoxin, which is responsible for water soaking and subsequently dark-brown necrosis due to an increase in pH and the formation of ammonia gas in the infected tissues (Lovrekovich, Lovrekovich, and Goodman, 1969). According to Fazzolari (1990), formation of ammonia is during the late log phase of the growth cycle and accounts for 50% of the initial nitrate N content at the end of incubation. Accumulation of ammonia results in the consequent loss of glutamine synthetase activity.

Therefore, the metabolic activity of bacteria grown in the inoculated tissue contributes to the accumulation of ammonia and the increase in pH of the lesion tissue (Turner, 1981). Also, ammonia is involved in the tissue necrosis in the wildfire disease caused by *Erwinia herbicola* and the stem tissue of twig blight of apple caused by *E. amylovora* (Sasser, Stall, and Cook, 1968).

Saxena and Karan (1985) studied the effect of ammonia solutions on 14 different fungi that were isolated from 20 samples of pea seeds, species of *Alternaria, Aspergillus, Cephalosporium, Penicillium, Mucor,* and *Fusarium* were among these fungi. Ammonia solutions had a strong effect on the mycofloral growth but the germination of pea seeds was retarded. Similar effects were observed by strains of *P. cepacia* when non-disinfected vinca seeds were tested during the indirect bioassay. Volatile ammonia had inhibitory effects on most seed contaminating fungi such as *Mucor* sp., *Aspergillus* sp. and *Rhizopus* sp. growing on seeds (personal observation).

Volatiles produced by strain OK-3 on CZPA media greatly reduced root length of vinca. The existence of an unknown volatile compound for OK-4 was confirmed by mass spectral analysis when an additional peak was detected between the ammonia and -OH- group peaks. This suggested an additional mechanism other than ammonia may be involved in inhibition of germination and/or root length. Mass spectra analysis was the most accurate and reliable technique to detect and identify ammonia but problems exist with quantification.

The application of a buffer substance or other microorganisms to the rhizoplane of the host plant may prevent the harmful effect of ammonia production while the volatile itself may protect the root from invasive soilborne pathogens around the rhizosphere. For instance, the weight and length of seedlings of rice cultured, used as padding treatment, was depressed by *P. cepacia* (2.6×10^6 cfu/ml) but not when *Bacillus* spp. (3.7×10^6 cfu/ml) were

applied. Plants treated with *P. cepacia* had shorter root length in padding soil suspension (PSS) but many lateral roots were formed in the vicinity of the root apices. It was also found that *P. cepacia* can form nitrite (NO_2^-). Rice seedlings are sensitive to nitrite produced by *P. cepacia* but not *Bacillus* sp. Therefore, retardation of rice roots might be caused by nitrite accumulation (Krieg, Walker, Senaratna, and Mc Kersiae, 1984).

According to Mac Millan (1956), ammonia readily penetrates the cells of many organisms by passive diffusion of its undissociated molecules. Under suitable conditions, ammonia equilibrates rapidly and accumulates in the cells at a concentration greater than that of the external medium. Ammonia has a disruptive action causing membrane deterioration. The toxicity of NH_3 is different than NH_4^+. Ammonium ion toxicity is the result of the initial reaction of the gas when it enters the cell regardless of the final ionic form of the molecule (Rush and Lyda, 1982). If the ratio of NH_4^+-N/NO_3^--N can be manipulated when *P. cepacia* starts to establish its population on the rhizoplane or in the rhizosphere, the toxicity of volatile ammonia may be eliminated. In this regard, the use of certain compounds or microorganisms to convert harmful NH_4^+-N into NO_3^--N would be helpful. The form of nitrogen can be influenced by the pH of the rhizosphere soil. This is significant if toxic NH_3 produced by *P. cepacia* can be converted to nontoxic NH_4^+ in rhizosphere. Perhaps this conversion would suppress damage caused by NH_3 and allow the bacteria to continue to produce antibiotics and siderophores.

Ammonia production by bacterial biocontrol agents has two important functions. Suppression or inhibition of the mycelial growth of soilborne pathogens is a positive attribute. On the other hand, interference with the seed germination and root elongation constitutes a negative aspect. Therefore, it is necessary to evaluate ammonia production by *P. cepacia* and to determine how

quantities of ammonia can be manipulated to allow for control of pathogenic fungi but yet cause no harm to the developing seedling. There are many factors involved in the complex environment of soil that interfere with the level of volatile produced by a biocontrol agent. In fact, the rhizopshere of a plant is not a closed system and volatiles may penetrate beyond the active area of antibiotics or siderophores and suppress or inhibit certain indigenous, minor and/or pathogenic soilborne microorganisms. The release of volatile ammonia is one of the factors in suppression or inhibition of pathogens.

TABLE 1

PRODUCTION OF AMMONIA BY *PSEUDOMONAS CEPACIA* STRAINS DETECTED BY EM QUANTA AMMONIA TEST KIT [1]

STRAIN AND SOURCE	AMMONIA (ppm) Test 1	Test 2	Test 3
OK-1 Soil, Washington, OK.	10	10	12
OK-2 Selection from OK-1	5	15	10
OK-3 Onion	20	55	60
OK-4 Apple	10	30	40
OK-5 Soil, Stillwater,OK.	5	5	8

[1] Amounts of ammonia present in water blanks was determined by comparing a color change on a test strip to a standard color chart.

TABLE 2

PRODUCTION OF AMMONIA (PPM) BY *PSEUDOMONAS CEPACIA* DETECTED BY LACHET INSTRUMENT [1].

Strains	Test 1 CZB[2]	Test 1 CZPB[3]	Test 2 CZB_2	Test 2 CZPB[3]
OK-1	----	----	----	7.35
OK-2	0.10	1.87	0.10	1.04
OK-3	0.17	5.82	2.16	8.89
OK-4	0.05	8.06	----	4.24
OK-5	----	----	----	8.93
Control	0.024	1.40	----	2.06

1 Lachet Instrument operated by OSU Soil and Water Laboratory.
2 CZB : Czapek-Dox Broth
3 CZPB: Czapek-Dox Broth + 20 g/L Peptone

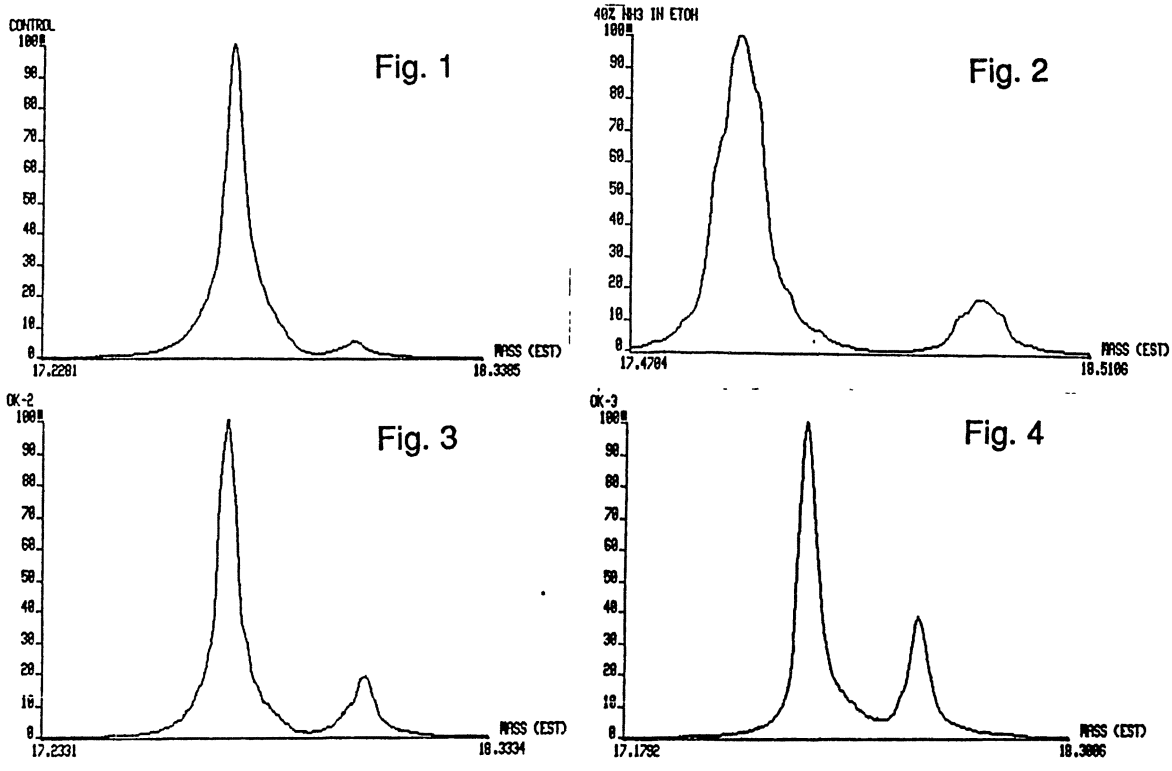
TABLE 3

DETECTION AND IDENTIFICATION OF VOLATILE AMMONIA BY STRAINS OF *PSEUDOMANAS CEPACIA* (OK-1 TO OK-5) AND *P. AERUGINOSA* (B-4) USING MASS SPECTRA ANALYSIS [A]

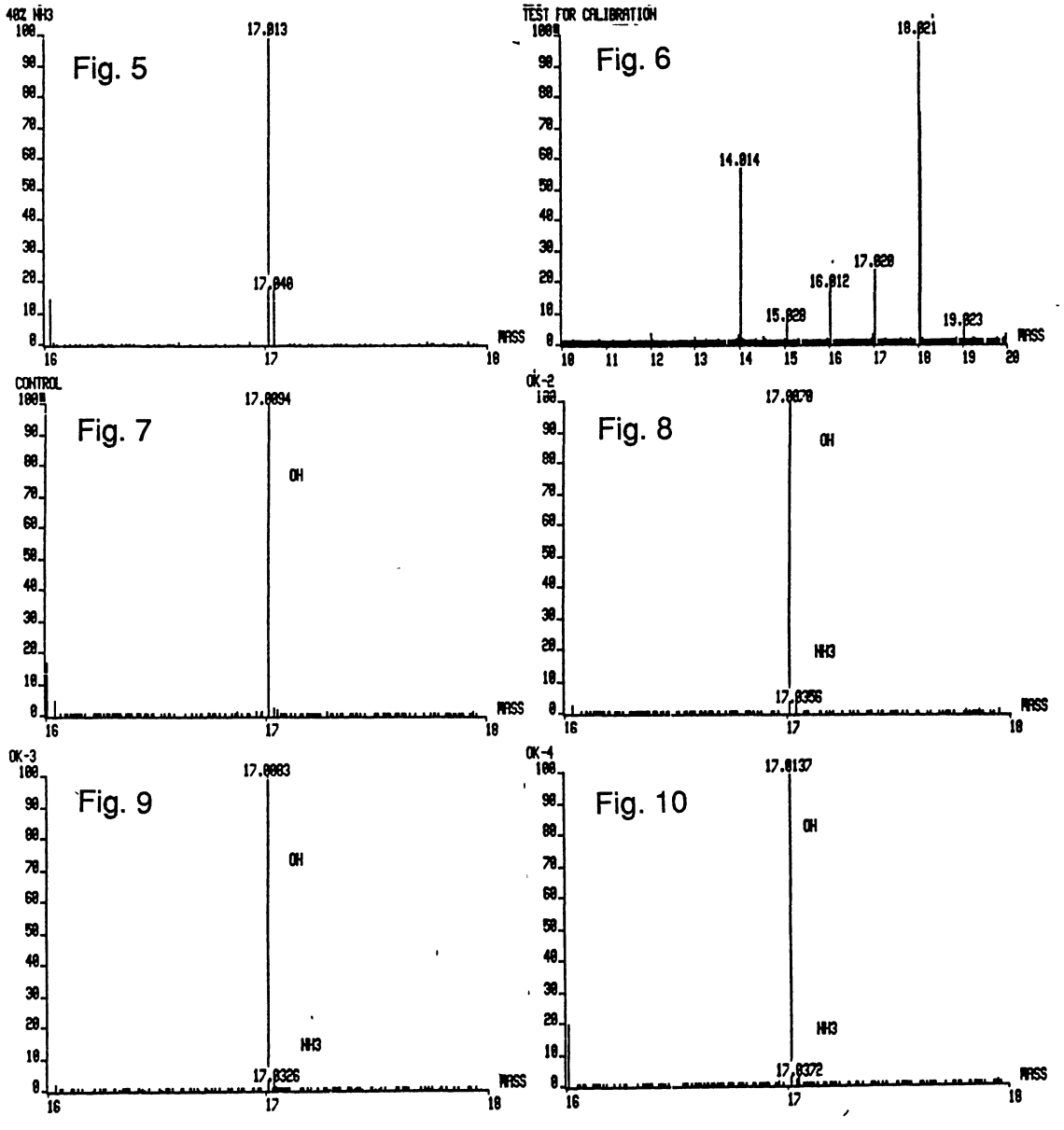
STRAIN	MASS OH	MASS NH_3	ABS. HT. OH	ABS. HT. NH_3	HT. NH_3/ HT. OH	% Base
OK-1	17.007	17.045	131000	9200	**0.070**	0.25
OK-2	17.007	17.035	340000	9600	**0.028**	0.23
OK-3	17.008	17.032	552400	3600	**0.0065**	0.08
OK-4	17.009	17.038	680100	6400	**0.0094**	0.16
OK-5	[b]	----	----	----	----	----
B-4	17.002	17.032	821000	10000	**0.1218**	0.30
CONTROL	17.009	----	303700	----	**----**	9.85

[a] Samples were prepared by collecting air above bacterial cultures in ethanol traps. 0.5 µl of each sample was injected into the apparatus.

[b] No ammonia was detected from strain OK-5 during this experiment.



Figures 1-4. Detection of Ammonia Produced by Two Strains of *Pseudomonas cepacia* (OK-2 and OK-3) Using Mass Spectra Analysis: The large peak represents hydroxyl (OH) group of either ethanol or water. Small peak represents ammonia production. Fig. 1. Control. Note: Small peak on the control sample is the transfer of volatilized trace of ammonia due to the presence of peptone in CZPB; Fig. 2 Peak of ammonia from 40% ammonium hydroxyde; Fig. 3. *P. cepacia* Strain OK-2; Fig. 4 *P. cepacia* strain in the presence of peptone in CZPB.



Figures 5-10. Calibration and Detection of Ammonia from Hydroxide Compared to Pure Ethanol and Three Strains of *Pseudomonas.* Note: The amounts of ammonia production varied among three strains. Fig. 5. Peak of ammonia from 40% ammonium hydroxide. Fig. 6. Test for calibration indicating ammonia peak between 17 to 18. Fig. 7. Control. Fig. 8. *P. cepacia* strain OK-2. Fig. 9. *P. cepacia* strain OK-3. Fig. 10. *P. cepacia* strain OK-4.

TABLE 4

EFFECTS OF VOLATILES PRODUCED BY STRAINS OF *PSEUDOMONAS CEPACIA* OK-1 TO OK-5) GROWN ON TWO DIFFERENT MEDIA[1], AFTER ONEWEEK, ON GERMINATION AND ROOT LENGTH OF VINCA

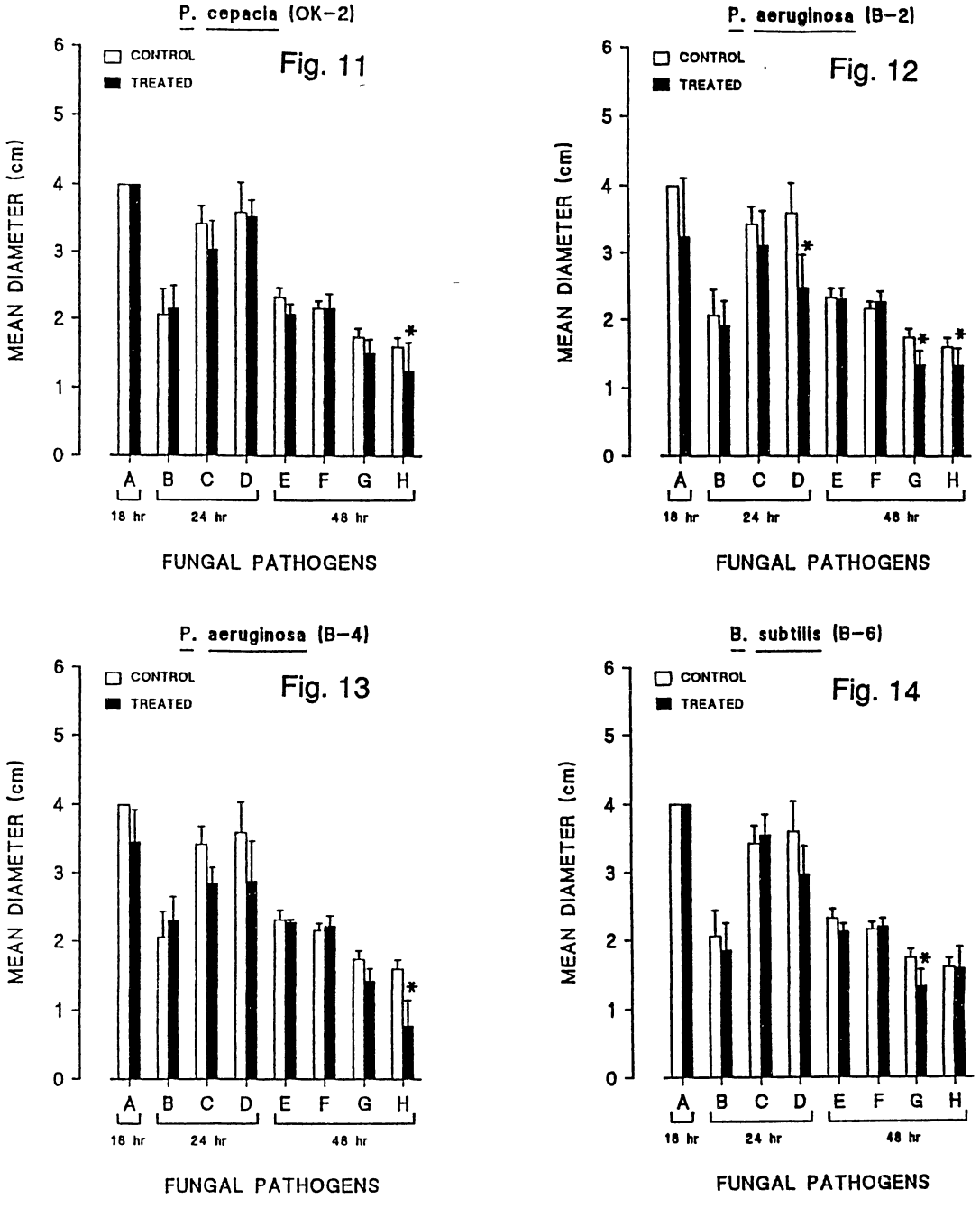
STRAIN	MEAN ROOT LENGTH[2]		LSD[3]	% GERMINATION	
	CZA	CZPA		CZA	CZPA
OK-1	1.661	0.985	0.365	78	54
OK-2	1.477	1.047	0.342	70	76
OK-3	1.445	0.141	0.392	66	48
OK-4	0.445	1.164	0.467	30	56
OK-5	1.451	0.818	0.368	62	64
CONTROL	1.358	1.266	0.344	72	72
LSD[4]	0.808	0.816			

1 CZA- Czapek's Agar, CZPA - 20 g/l peptone added.

2 Means are from 50 observations and number of replications were 5.

3 LSD (0.05) values were calculated among the number of germinated seeds.

4 LSD (0.05) values were calculated between two groups of CZA and CZPA media.



Figures 11-14. Suppression of Fungal Pathogens Affected by Volatile Compound(s) Produced by Strains of *P. cepacia* OK-2 (11), *P. aeruginosa* (B-2, B-4) (12, 13), and *Bacillus subtilis* (B-6) (14). Alphabets represent selected fungi at different time periods: A. *Pythium aphanidermatum* OK-317 (18 h, LSD (0.05)= 0.3990), B. *Macrophomina phaseolina* M-9, C. *M. phaseolina* OK-98, D. *Rhizoctonia solani* 330 (AG-4) (24 h, LSD (0.05)= 0.8905), E. *Fusarium oxysporum* OK-209, F. *F. oxysporum* OK-97, G. *Phytophthora cacturom* OK-316, H. *Sclerotium rolfsii* OK-64 (48 h (0.05)= 0.1558). * Represents significant effect.

TABLE 5

QUANTIFICATION OF AMMONIA PRODUCTION BY STRAINS OF *PSEUDOMONAS CEPACIA,PSEUDOMONAS AERUGINOSA* BY COMPARING INHIBITION OF GROWTH OF SOILBORNE FUNGI[X] IN THE PRESENCE OF THE BACTERIA TO INHIBITION CAUSED BY AMMONIARELEASED FROM SOLUTIONS OF NH_4OH

SELECTED FUNGI	AMMONIA EQUIVALENT VALUES (µG/ML) FOR INHIBITION[Y]									
				P. cepacia					*P. aeruginosa*	
	LC50	LC90	LC100	OK-1	OK-2	OK-3	OK-4	OK-5	B-2	B-4
Macrophomia phaseolina	10.6	17.2	18.0	8.3	8.7	9.0	9.3	8.9	9.3	10.6
Fusarium oxysporum	18.7	28.7	30.0	13.8	16.3	16.3	16.3	17.3	17.5 1	9.2
Sclerotium rolfsii	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
Rhizoctonia solani	17.2	20.2	21.0	7.2	15.0	16.1	17.8	7.6	N.A.[Z]	17.1
Pythium myriotylum	12.4	22.8	27.0	13.0	12.8	12.6	12.9	12.8	<3.0	13.3
Pythium aphanidermatum	13.4	16.6	18.0	13.9	13.7	13.4	13.2	13.6	14.0	13.9
Phytothora cactorum	12.8	14.6	5.0	7.2	8.2	7.8	8.2	6.4	7.2	9.0
Trichoderma harzianum	11.1	14.1	15.0	6.3	6.3	8.0	3.0	3.8	9.1	9.1

[X] Fungi were grown on trypticase soy agar (TSA).

[Y] Ammonia equivalents determined by comparing growth of fungi in the presence of volatile produced by *Pseudomonas* spp. to growth of fungi exposed to solutions of NH4OH.

[Z] Data not available.

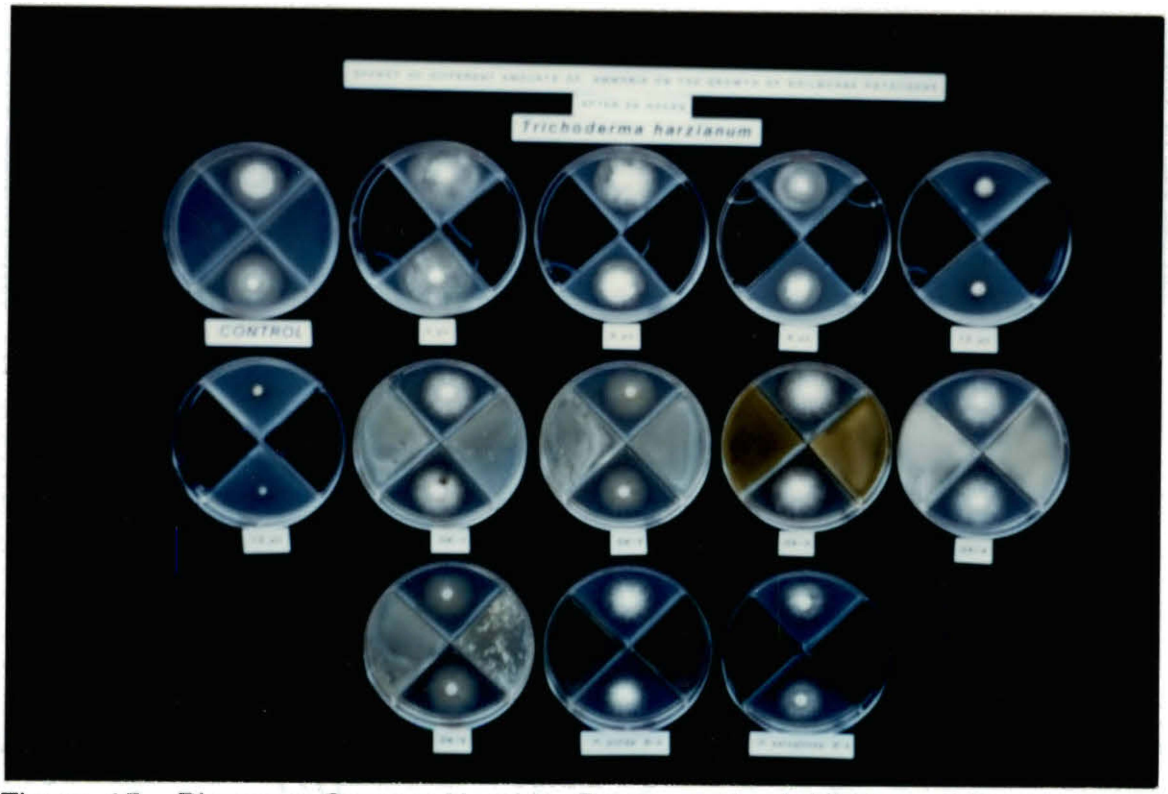


Figure 15. Bioassay System Used to Compare the Inhibition of *Trichoderma harzianum* by Ammonium to Inhibition Caused by Strains of *Pseudomonas* spp.

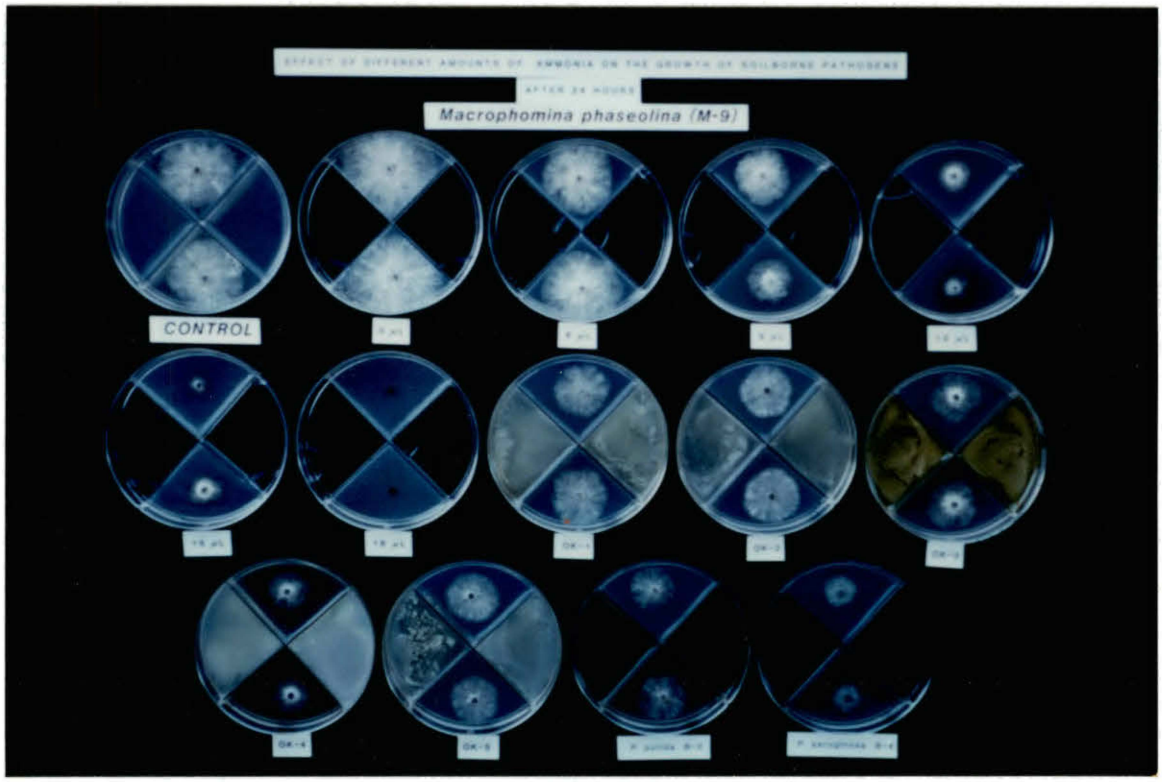


Figure 16. Bioassay System Used to Compare the Inhibition of *Macrophomina phaseolina* by Ammonium to Inhibition Caused by Strains of *Pseudomonas* spp.

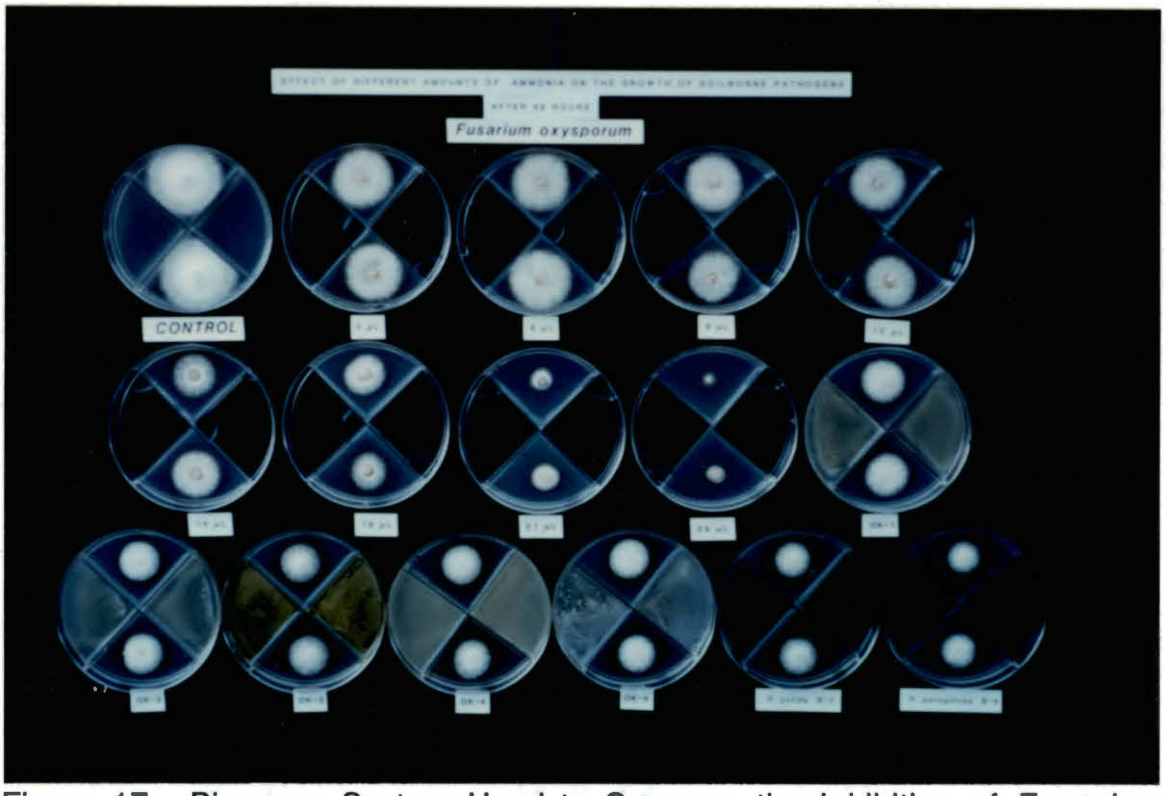


Figure 17. Bioassay System Used to Compare the Inhibition of *Fusarium oxysporum* by Ammonium to Inhibition Caused by Strains of *Pseudomonas* spp.

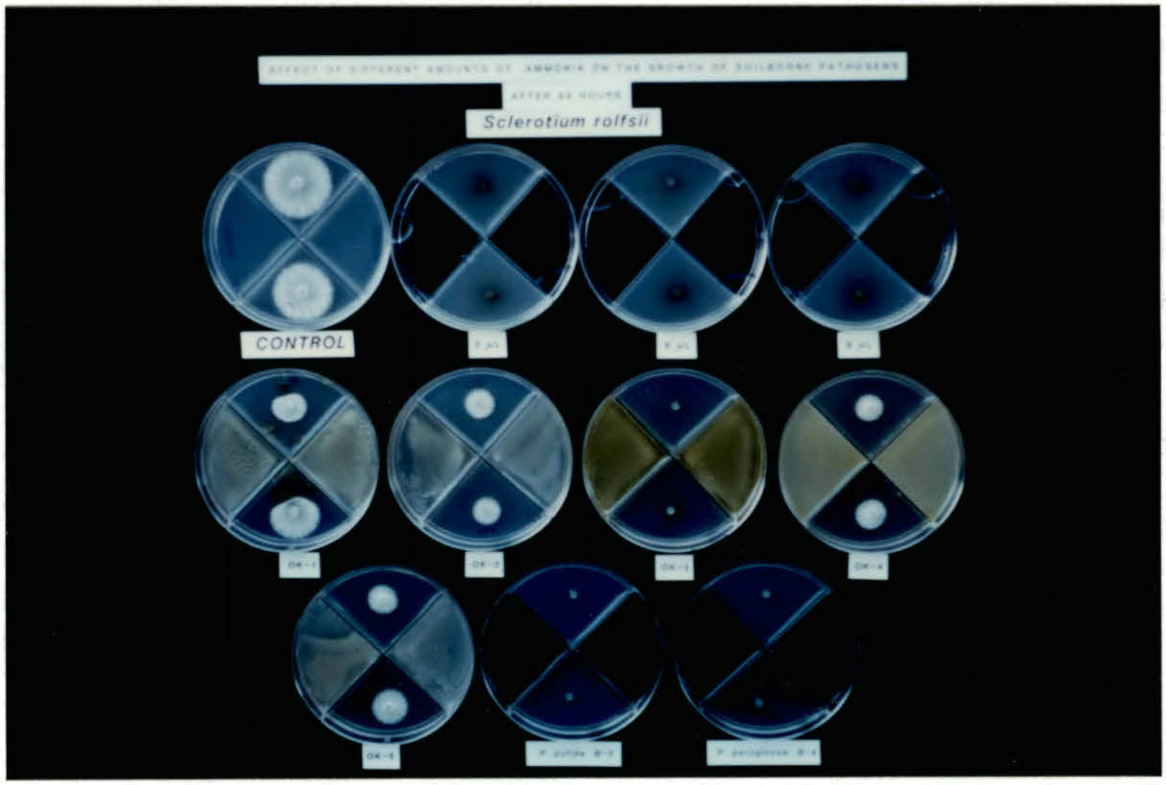


Figure 18. Bioassay System Used to Compare the Inhibition of *Scterotium rolfii* by Ammonium to Inhibition Caused by Strains of *Pseudomonas* spp.

REFERENCES

ASANUMA, S., TANAKA, H. AND YATAZAWA, M. (1980). *Pseudomonas cepacia*- A characteristic rhizoplane microorganisms in rice plant. *Soil Science Plant Nutrition* **26**(1), 71-78.

BALIGH, M., CONWAY, K.E. AND DELGADO, M.A. (1990). Production of ammonia by *Pseudomonas cepacia* interferes with seed germination and root elongation. *Phytopathology* **80**(10), 1048 (Abstract).

BALIGH, M., CONWAY, K.E. AND DELGADO, M.A. (1991). Development of a bioassay system to compare quantities of ammonia produced by strains of (*Pseudomonas*) spp., potential biocontrol agents against soilborne fungi. *Phytopathology* **81**(10), 1228 (Abstract).

BASHAN, Y., OKAN, Y. AND HENIS, Y. (1980). Ammonia causes necrosis in tomato leaves infected with *Pseudomonas tomato* (Okabe) Alstatt. *Physiological Plant Pathology* **17**, 111-119.

CROOKE, W.M. AND SIMPSON, W.E. (1971). Determination of ammonium in Kjeldahl digests of crops by an automated procedure. *Journal of Science Food Agriculture* **22**(1), 9-10.

CONWAY, K.E., FOOR, C.J., MALVICK, D. AND BENDER, C. (1989). Inhibition of *Pseudomonas cepacia,* a potential biocontrol agent of selected soilborne pathogens. *Phytopathology* **79**, 1159 (Abstract).

CHUN, D.A., FILONOW, A.B. AND LOCKWOOD, J.L. (1984). Interactive effect of ammonia and pH on viability and C-labelled exudation from fungal propagules. *Canadian Journal of Botany* **62**, 1220-1226.

DE FREITAS, J.R. AND GERMIDA, J.J. (1990). A root tissue culture system to study winter wheat-rhizobacteria interactions. *Applied Microbiology and Biotechnology* **33**, 589-595.

DE PASQUALE, D.A. AND MONTVILLE, T.J. (1990). Mechanism by which ammonium bicarbonate and ammonium sulphate inhibit mycotoxigenic fungi. *Applied Environmental Microbiology* **56**(12), 3711-3717.

DELGADO, M.A. AND CONWAY, K.E. (1989). Population dynamics of three potential biocontrol *(Pseudomonas)* strains on seeds and roots of cotton. *Phytopathology* **80**, 434 (Abstract).

FANG, H.C. AND LUI, T. M.E. (1988). Effect of urea and nitrogenous compounds on the sclerotial germination and viability of *Sclerotium rolfsii*in soil and its relationship with soil microorganism. *Plant Protein Bulletin* **30**, 235-244.

FAZZOLARI, E. (1990). Nitrate reduction to ammonia: a dissimilary process in *Enterobacter amnigenus. Canadian Journal of Microbiology* **36**, 779-785.

GILBERT, G.S., HANELSMAN, J. AND PARKE, J.L. (1990). Role of ammonia and calcium in lysis of zoospores of *Phytophthora cactorum* by *Bacillus cereus* strain UW85. *Experimental Mycology* **14**, 1-8.

HOMMA, Y. AND SUZUI, T. (1989). Role of antibiotic production in suppression of radish damping-off by seed bacterization with *Pseudomonas cepacia. Annual Phytopathological Society of Japan* **55**, 643-652.

HOWELL, C.R., BEIER, R.C. AND STIPANOVIC, R.D. (1988). Production of ammonia by *Enterobacter cloacae* and its possible role in the biological control of Pythium pre-emergence damping off by the bacterium. *Phytopathology* **78**, 1075-1078.

HUANG, Y., DEVERALL, B.J. AND MORRIS, S.C. (1991). Promotion of infection of orange by *Penicillium digitatum* with a strain of *Pseudomonas cepacia, Phytopathology* **81**, 615-618.

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

JANISIEWICZ, W.J. AND ROITMAN, J. (1988, b). Biological control of blue mold and gray on apple and pear with *Pseudomonas cepacia. Phytopathology* **78**, 1697-1700.

JANISIEWICZ, W.J., YOURMAN, L., ROITMAN, J. AND MAHONY, L. (1991). Postharvest control of blue mold and gray mold of apples and pears by dip treatment with pyrrolnitrin, a metabolite of *Pseudomonas cepacia, Plant Disease* **75**, 490-494.

KRIEG, L.C., WALKER, M.A., SENARATNA, T. AND MC KERSIAE, B.D. (1990). Growth, ammonia accumulation and glutamine synthetase activity in alfalfa (*Medicago sativa* L.) shoots and cell cultures treated with phosphinothricin. *Plant Cell Report* **9**, 80-83.

LEACH, L.D. AND DAVEY, A.E. (1935). Toxicity of low concentrations of ammonia to mycelium and sclerotia of *Sclerotium rolfsii. Phytopathology* **25**, 957-965.

LINDERMAN, R.G. AND GILBERT, R.G. (1969). Stimulation of *Sclerotium rolfsii*

in soil by volatile components of alfalfa hay. *Phytopathology* **59**, 1366-1372.

LOCKWOOD, J.L. AND FILONOW, A.B. (1981). Responses of fungi to nutrient-limiting conditions and inhibitory substances in natural habitats. in *Advances in Microbial Ecology* Vol 5 (ed. M. Alexsander) Plenum Publishing Corporation p.1-61.

LOFFLER, H.J.M. VAN DONGEN, M., AND SCHIPPERS, B. (1986). Effect of NH_3 on chlamydospore formation on *Fusarium oxysporum* f.sp. *dianthi* in a NH_3-flow system. *Journal of Phytopathology* **117**, 43-48.

LOVREKOVICH, L., LOVREKOVICK, H. AND GOODMAN, R.N. (1969). The role of ammonia in wildfire disease of tobacco caused by *Pseudomonas tabaci*. *Phytopathology* **59**, 1713-1716.

MAC MILLAN, A. 1956. The entry of ammonia into fungal cells. *Journal of Experimental Botany* **7**(19), 113-126.

PAULITZ, T.C. (1991). Effect of (*Pseudomonas putida*) on the stimulation of *Pythium ultimum* by seed volatiles of pea and soybean. *Phytopathology* **81**(10), 1282-1287.

PUNJA, Z K., JENKINS, S.F. AND GROGAN, R.G. (1984). Effect of volatile compounds, nutrients, and sources of sclerotia on eruptive sclerotial germination of *Sclerotium rolfsii*. *Phytopathology* **74**, 1290-1295.

RUSH, C.M. AND LYDA, S.D. 1982. The effects of anhydrous ammonia on membrane stability of *Phymatotrichum omnivorum*, *Mycopathologia* **79**, 147-152.

SASSER, J.M., STALL, R.E. AND COOK, A.A. (1968). Membrane disruption in pepper cells induced by *Xanthomonas vesicatoria* and by volatile produced by the bacterium. *Phytopathology* **58**, 1066 (Abst.).

SAXENA, N. AND KARAN, D. (1985). Effect of ammonia Solution and acetic acid on the mycoflora of pea seeds and also their effect on germination. *Indian Journal of Botany* **8**(10), 35-38.

SCHIPPERS, B., BOERWINKEL, D.J. AND KONINGS, H. (1978). Ethylene not responsible for inhibition of conidium germination by soil volatiles. *Netherlands Journal Plant Pathology* **84**, 101-107.

SCHIPPERS, B., MEIJER, J.W. AND LIIEN, J.I. (1982). Effect of ammonia and other soil volatiles on germination and growth of soil fungi. *Transactions of the British Mycology Society* **72**(2), 253-259.

SMILEY, R.W., COOK, R.J. AND PAPENDICK, R.I. (1972). Fusarium foot rot of wheat and peas as influenced by soil applications of anhydrous

ammonia and ammonia--potasium azide solutions. *Phytopathology* **62**, 86-91.

STOTZSKY, G. AND SCHENICK, S. (1975). Volatile organic compounds and microorganisms. *CRC in Microbiology* **4**, 333-382.

TABAK, H. H. AND BRIDGE COOKE, W.M. (1968). The effects of gaseous environments on the growth and metabolism of fungi. *Bot.anical Review* **34**, 126-252.

TRABULSI, L.Y., WHITEBREAD, R. AND DUCKETT, J.G. (1978). Temporal relationship between changes in ultrastructure and ammonia levels induced in oats by tabtoxin, *Proceedings of the 4th International Conference on Plant Pathogenic Bacteria* pp. 687-698, Angers.

TURNER, J.G. (1981). Tabtoxin, produced by *Pseudomonas tabaci*, decreases *Nicotiana tabcum* glutamine synthetase in vitro and causes accumulation of ammonia. *Physiological Plant Pathology* **19**, 57-67.

UNDERHILL, S.E. (1990). Techniques for studying the microbial ecology of nitrification in *Methods in Microbiology* vol.22 (ed. by R. Grigorova and J.R. Norris), Academic press. pp.

YERDOUW, H.C., VAN ECHTELD, J.A. AND DEKKERS, E.M.J. (1977). Ammonia determination based on indophenol formation with sodium salicylate. *Water Research* **12**, 399-402.

ZAKARIA, M.A., LOCKWOOD, J.L. AND FILONOW, A.B. (1980). Reduction of *Fusarium* population density in soil by volatile degradation products of oil seed meal amendment. *Phytopathology* **70**, 495-499.

CHAPTER V

SUMMARY AND CONCLUSIONS

Pseudomonas cepacia, Biocontrol Agent or Deleterious Bacterium?

During the past few years, *P. cepacia* has been recognized as a successful biocontrol agent for the control of foliar diseases of plants. However, the use of this bacterium for the control of soilborne pathogens has not progressed as rapidly. Soilborne diseases are more difficult to control with biocontrol agents rather than chemicals because of the complexity of the soil environment. Certain fungicides are very effective but are being withdrawn from use because of their risks to the environment and to human health. The exclusive use of certain chemicals may lead to the development of resistant pathogens (Parke, J.L., 1990). Therefore, in some cases, the alternative choice would be new biocontrol candidates such as *P. cepacia*. To answer whether or not *P. cepacia* is a beneficial organism, several characteristics of this bacterium should be evaluated and many aspects about the application of this organism should be considered.

Advantages of *P. cepacia*

P. cepacia is a gram negative, non-sporing rod which is nutritionally versatile and it is stimulated by root exudates comparing to gram positive rods and coccoids. Strains of *P. cepacia* are rhizosphere competent on the root

systems of certain host plants, especially small seeds. *P. cepacia* has several mechanisms that may control pathogens such as the production of antibiotics, siderophores, enzymes, and volatile ammonia. Therefore, it may be usefully applied against many diseases due to its diverse metabolic productions which may overcome a majority of soilborne pathogens. This investigation indicated that *P. cepacia* OK-1, OK-2, and two strains of OK-5 are colonizing the rhizosphere of vinca and they produce antimicrobial compounds. These are the most important characteristics required for *P. cepacia* to be a biocontrol agent. In addition, the production of ammonia by this biocontrol candidate was demonstrated against selected fungi and the its significance was evaluated by the bioassay technique. Indeed, volatile production against fungi is not a rare phenomenon. Production of volatile ammonia by *P. cepacia* is a mediated mechanism of antibiosis. This additional mechanism suppresses or inhibits many seed-contaminating and pathogenic fungi. Volatile ammonia may act beyond the territory of direct action of antimicrobial compounds in the rhizosphere. If *P. cepacia* is characterized as a pathogen, host range is limited to onion and *Allium* species (Hayward, 1988). Strains of *P. cepacia* are mildly pathogenic on host plants and cannot be transferred to a non-host plants. *P. cepacia* strains can suppress or inhibit numerous pathogens (Table 1) indicating that *P. cepacia* is a biocontrol agent or beneficial bacterium.

Disadvantages of *P. cepacia*

Strains of *P. cepacia* cause delay in seed germination and interfere with root elongation, specially when the initial density of the bacteria is high. Volatile ammonia, produced by *P. cepacia* is harmful to the root of host plant, especially during the early stages of seed germination and root elongation. If harmful

effects of the volatile produced by *P. cepacia* in the rhizosphere or rhizoplane cannot be prevented by manipulation of genes involved in ammonia production, there are chances that special formulations can convert the toxic NH_3 into NH_4^+-N or NO_2^--N forms by the use of biotic or abiotic agents. Therefore, simply because ammonia reduces potential of *P. cepacia* as a biocontrol agent, it does not mean that this unique organism should be ignored.

Knowledge of the antagonistic mechanism is necessary for EPA to register a new biocontrol agent. When an organism produces non-target toxic metabolites, EPA will never approve it. Our strain does not have such products and it is a well known industrial bacterium which degrades many wasteful compounds in the environment. *P. cepacia* as an opportunistic human pathogen which only infects patients with immune deficiency and it has already been exposed to the environment. This means an optimistic view for further investigation as long as EPA does not restrict any progress in the area of biotechnology. This policy may would improve the opportunities required for a biocontrol agent to compete with chemicals (Jutsun, A.R., 1988).

Unfortunately, this research did not investigate the effect of environmental factors in the performance of *P. cepacia*. The point is that if *P. cepacia* does not perform properly in petri dishes or growth chambers, it will not be a wise decision to go to the next step of formulation or commercialization. Application of *P. cepacia* should not have any legal problem as long as it is not genetically manipulated. The efficacy of *P. cepacia* is one of the major problems since moisture, temperature, sunlight and pH for optimum growth of this biocontrol is not known. There are many questions about application methods which needed to be answered. It is not clear whether or not lyophilized forms, mixture with diamaceous earth or powdered of glass beads (Homma and Suzui, 1989) can applied to our strains. Mixture with *Trichoderma*

harzianum or nitrogen fixing bacteria are other choices.

Future plan should emphasize three aspects of *P. cepacia* studies: 1) determination of factors that can prevent the deleterious effects of ammonia production, 2) manipulation of genes involved in the production of antimicrobial compounds, using biotechnology to enhance the stability of the biocontrol candidate, 3) development of new formulations and application methods.

TABLE 1

LIST OF THE DISEASES CONTROLLED BY APPLICATION OF *P. CEPACIA*

DISEASE	PATHOGEN	REFERENCE
Tobacco Alternaria-Leaf Spot	*Alternaria sp.*	Janiseiwicz 1988
Southern Leaf Blight	*Bipolaris maydis*	Janiseiwicz 1988
Gray Mold of Apple	*Botrytis cinerea*	Janiseiwicz 1988
Peanut Leaf Spot	*Cercospora arachidicola*	Knudsen 1987
Fusarium-Wilt of Onion	*Fusarium oxysporum f.sp. cepae*	Kawamoto and Lorbeer 1988
Fusarium-Wilt of China Aster	*Fusarium Oxysporum f.sp. callestephi*	Cavillar 1984
Fusarium-Wilt of Tomato	*Fusarium oxysporum f.sp. lycopersici*	Homma and Suzui 1989
Onion Maggot	*Hylemy antiqua & H. planta*	Hough et al., 1981
Brown Rot of Nectarines	*Monilia fructicola*	Smilanick, et at., 1989
Blue Mold of Apple	*Penicillium expansum*	Janiseiwicz 1988
Rots on Citrus Fruit	*Penicillium italicum & P. digitatum*	Lievens, et al., 1989
Damping-off of Bean,Pepper, Melon, Tomato, and Cotton	*Pythium aphanidermatum*	Elad and Chet, 1987
Damping-off of Cucumber	*Pythium ultimum*	"
Damping-Off of Radish	*Rhizoctonia solani*	Homma and Suzui, 1989
Glume Blotch of Wheat	*Septoria nodorum*	Jones, et al., 1981
Verticillium-Wilt of Egg Plant	*Verticillium dahliae*	Homma and Suzui 1989

REFERENCES

Cavileer, T.D. ,and Peterson, J.L. 1985. Biological activities of soilborne microorganisms against Fusarium wilt of China aster Phytopathology 75(11):1344 (Abstract).

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

Hayward, A.C. 1988. *Plant Bacterial Diseases, A Diagnostic Guide* (ed. P.C. Fahy and G.J. Persley) Academic press, Australia, pp. 393.

Homma, Y. and Suzui, T. 1989. Role of antibiotic production in suppression of radish damping-off by seed bacterization with *Pseudomonas cepacia.* Ann. Phytopathol. Soc. Japan 55:643-652.

Hough, J.A., Harman, G.E., Eckenrode, C.J. 1981. Microbial stimulation of onion maggot oviposition. Envir. Entomol. 10(2):206-210.

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

Janisiewicz, W.J. and Roitman, J. 1988. Biological control ofblue mold and gray on apple and pear with *Pseudomonas cepacia.* Phytopathology 78:1697-1700.

Jones, J.B., Roane, C.W., and Wolf, D.D. 1981. The effect of *Septoria nodorum* and *Xanthomonas translucens* f.sp. *undulosa* on photosynthesis and transpiration of wheat flag leaves. Phytopathology 71(11):1173-1177.

Kawamoto, S.O., and Lorbeer, J.W. 1976. Protection of onion seedling from *Fusarium oxysporum* f.sp. *cepae* by seed and soil infestation with *Pseudomonas cepacia.* Pl. Dis. Rep. 60(3):189-191.

Knudsen, G.R. and Spurr, H.W.Jr. 1987. Field persistence and efficacy of five bacterial preparations for control of peanut leaf spot. Pl. Dis. 71(5):442-445.

Jutsum, A.R. (1988). Commercial application of biological control: status and prospects, Philosophical Transactions of the Royal Society of London B318:357-373.

Lievens, K.H., Rijsbergen, V.R., Leyns, F.R., Lambert, B.J., Tenning, P., Swings, J. and Joos, H.J.P. (1989). Dominant rhizosphere bacteria as a source of antifungal agents. PesticideScience 27(2):141-154.

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

Smilanick, J.L., Ho, M.Y., Henson, D. and Anderson, P. 1989. Novel chemical and biological methods to control postharvest brown rot of nectarines. Phytopathology 79:1177 (Abstract).

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