To the Graduate Council:

I am submitting herewith a thesis written by Patrice Nicole Smith entitled "Biological Control of *Rhizoctonia solani* on Tomato." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

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BIOLOGICAL CONTROL OF *RHIZOCTONIA SOLANI* ON TOMATO

A Thesis

Presented for the

Master of Science Degree

The University of Tennessee, Knoxville

Patrice Smith

December 1997

DEDICATION

This thesis is dedicated to my parents

Mrs. Annie Bell Smith

and

Mr. Mark Smith Jr.

who have always supported my educational endeavors.

ACKNOWLEDGMENTS

I would like to thank Dr. Ownley for her research guidance. I would also like to thank Dr. Ownley for offering me a research assistant position while attending graduate school.

I would like to thank Dr. Ownley, Dr. Starman, Dr. Bernard, and Dr. Faust for serving on my committee.

I would like to thank Dr. Carroll Southards for willingly accepting me into the Department of Entomology and Plant Pathology.

I would like to thank Mary Dee, Robert Reeder, Joseph McMahan, Bendik Clark, and Jay Follis for making my experience as a graduate student unforgettable.

I would like to thank the faculty, staff, and students in the Department of Entomology and Plant Pathology.

I would like to thank my family for their continual and unconditional love and support.

ABSTRACT

Rhizoctonia solani causes damping-off and root rot of tomato plants. Post-emergence damping-off begins as a canker on the stem located above the soil line. Growers traditionally have managed damping-off and root rot of tomato with chemical pesticides. Due to the increasing awareness of the harmful effects that chemical pesticides impose on the environment and humans, alternative control options are needed. The focus of this study was to assess bacterial isolates for biological control of *R. solani* on tomato.

Three collections of *Bacillus* and *Pseudomonas* isolates were evaluated for biocontrol activity. The collections were from a tobacco field soil, rhizosphere of tomato and within tomato root tissue (endophytes). Of the 204 bacterial isolates screened, 13 decreased disease severity of *R. solani*, 10 increased shoot height, and 11 decreased plant mortality. Of 94 bacterial isolates collected from tobacco field soils, five reduced disease severity of *R. solani* BA19, BA77, BA101, BA23, and BA106. Tobacco field soil isolates decreased disease severity from 13 % to 64 %, and decreased plant mortality by 25 % to 92 %. Eight of the 60 endophytic isolates decreased disease severity, ranging from 31 % to 60 %. The endophytes also decreased plant mortality (31 to 76 %) and increased shoot height of tomato seedlings from a range of 25 to 76 %. Isolates collected from the tomato rhizosphere did not affect disease caused by *R. solani.*

One tobacco field soil isolate inhibited growth of *R. solani* in culture on Min 3C agar. Many of the bacterial endophytes suppressed growth of *R. solani* on Min 3C. Isolates exhibiting the greatest zones of inhibition were BA101, E69,

E723, E721, E722, E729, and E730. While antibiosis was not the sole mechanism of action involved, it does appear to be a contributing factor, particularly with the endophytic isolates.

Characterization and identification of the 13 bacterial isolates effective against *R. solani* was relevant for determining the possible mechanism of suppression of *R. solani*. Bacterial isolate BA106 was Gram-negative, and the remaining isolates were Gram-positive. Tobacco field soil isolates BA77, BA19, BA23, BA101, and all of the endophytic isolates were catalase positive. Bacterial isolates E726, BA77, BA19, E61, and E65 multiplied anaerobically. Starch hydrolysis was negative for isolate BA106, and positive for isolates BA77, BA19, BA101, BA23 and the endophytic isolates. Bacterial isolates BA101, E61, and BA106 utilized citrate and the remaining isolates did not. Both the endophytic isolates and the tobacco field soil isolates were tolerant of 7% NaCl.

Acid production on mannitol medium was positive for bacterial isolates BA101, BA23, and BA106. Acid production on arabinose medium was positive for BA23 and BA106. Acid production on xylose was positive for bacterial isolates BA77, BA19, and BA23. Tobacco field soil isolates and the endophytic isolates digested protein and decomposed chitin, characteristics that might be important for inhibition of *R. solani*.

According to the MicroLog identification system, Gram-positive bacterial isolates were identified as bacilli, and the Gram-negative bacterium was identified as a pseudomonad. The bacterial isolates were identified as *B. thuringiensis/cereus* BA77, *B. megaterium* BA101, *P. corrugata* BA106, *B.*

pasteurii BA19, *B. azotoformans* BA23, *B. subtilis* E726, *B. pasteurii* E66, *B. megaterium* E61, *B. coagulans* E69, *B. megaterium* E65, and *B. brevis* E21. Two isolates of *Bacillus*, E727 and E723, were not identified to species.

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CHAPTER I: LITERATURE REVIEW

INTRODUCTION

Tomato is an important economic crop worldwide. Tennessee ranks fifth in the nation in fresh market tomato production (Duffy Barr et al., 1996). Farmers spend millions of dollars on chemicals used to control diseases of tomato (Duffy Barr et al., 1996). Due to the increasing attention given to the problems that chemical pesticides cause to the environment and to humans, many chemicals are being prohibited.

The Bhopal gas tragedy on December 2, 1984, in India is an example of the magnitude of danger involved in chemical pesticides (Upadhyay and Rahe, 1988). Methyl isocyanate leaked from a Union Carbide factory that was engaged in manufacture and commercial production of pesticides. More than 2,000 people were killed and over 50,000 were treated for injuries. This incident is considered the worst industrial accident in history. Scientists are now realizing the importance of developing alternative control measures such as biological control.

Biological control of plant pathogens is the use of one or more biological processes to lower inoculum density of the pathogen or reduce its disease-producing activity (Baker, 1987). Beneficial microorganisms, such as *Bacillus* and *Pseudomonas* spp., introduced in soil to control soilborne pathogens, has been studied for over 65 years, but during most of that time commercial use of these microorganisms was not considered feasible. Today many companies have programs to develop biocontrol agents as commercial products. Biological

control offers many advantages to growers and to society in general and it must be pursued. The ultimate pay-off will be a more stable, sustainable, and safer food supply that is produced often at lower cost.

The focus of this research project is selection of potential bacterial biocontrol agents applied as seed treatments to control *Rhizoctonia* diseases of tomato.

TOMATO PRODUCTION

The word tomato (*Lycopersicon esculentum* Miller) was derived from the Aztec word *xitomate* or *zitotomate* (Tracy, 1923). The crop was grown by Native Americans long before Christopher Columbus took plants to Europe. As recently as the nineteenth century, tomatoes were thought to be poisonous and were grown only as ornamentals (Tracy, 1923). We now know that tomatoes are a rich source of vitamins A and C, and some B (Cox, 1940). Tomato is one of the most popular vegetables in the United States, especially for home gardeners. In the Southeastern United States, Tennessee ranks fifth in the nation in fresh market tomato production behind Florida, California, Georgia, and South Carolina (Barr et al., 1996). Tomato is also commercially important worldwide.

Tomato plants are usually planted in the field during the spring season, and in either late spring or late fall in the greenhouse. Tomato grows best at day temperatures between 21 to 26°C and night temperatures between 14 to 15 °C (Schultheis, 1993). The preferred soil should be rich, loamy, well drained, and high in organic matter with a sub soil (Schultheis, 1993). High levels of phosphorus and potash with a soil pH between 6.5 to 6.8 are recommended.

RHIZOCTONIA SOLANI

Rhizoctonia spp. are an ecologically diverse group of fungi. *Rhizoctonia* spp. may be pathogenic on many hosts, live as saprophytes in soil, or in mycorrhizal associations with orchidaceous plants (Adams and Butler, 1983). *Rhizoctonia solani* (Kühn) is the asexual stage or anamorph of the sexual stage, *Thanatephorus cucumeris* (A. B. Frank) Donk. *Thanatephorus cucumeris* produces basidiospores, which are hyaline, smooth, oblong to ellipsoid and average 9.0 x 5.5 μm. The spores are formed more often during extended periods of moderate temperature (20 to 30 °C). In addition, spores can be induced with increased levels of nitrogen and glucose in growth medium as well as by drying the sporulation medium (Adams and Butler, 1983).

Rhizoctonia solani exists as sterile mycelium. Characteristics of *R. solani* include branching near the distal septum of cells in young vegetative hyphae, formation of a septum in the branch near the point of origin, constriction of the branch, dolipore septa, no clamp connections, no conidia except monilioid cells, sclerotia not differentiated into rind and medulla, and no rhizomorphs. Right angle branching and large hyphae with three or more nuclei are characteristics of *R. solani.* When the mycelium is young it is colorless, but with age it becomes yellow-brown. *R. solani* overwinters as mycelium. When the hyphae comes into contact with a root or stem it branches profusely to form an infection cushion (Ogoshi, 1987).

Different strains of *R. solani* are separated on the basis of anastomosis

grouping (AG). An anastomosis is the fusion of hyphae, which represents vegetative compatibility. Anastomosis grouping is also host specific. For example, AG2 causes cankers on root crops and root diseases of crucifers (Ogoshi, 1987; Sneh et al., 1991). AG groups can be either pathogenic or nonpathogenic. The AG groups of *R. solani* pathogenic on tomato include AG1, AG3, and AG4. These AG groups cause damping-off, leaf blight, and fruit rot respectively. The group AG4 represents the type described as *Corticium practicola* by Kotilas (Ogoshi, 1987), which can be subdivided into two groups, HGI and HGII. These groups are based on differences in DNA homology and form of sclerotia (Ogoshi, 1987). Isolates grouped as AG4 HGI have dark brown sclerotia on potato dextrose agar (PDA), and AG4 HGII isolates have gray or whitish-brown sclerotia on PDA.

DAMPING-OFF AND ROOT ROT

Damping-off and root rot of tomato is caused by *R. solani*. Damping-off appears first as a canker on the stem above the soil line. As the disease progresses the fungus girdles the stem. Eventually the stem becomes constricted and the plant damps off or falls over. Brown discoloration of roots is a symptom of root rot. Diseases caused by *R. solani* develop in a wide range of humid, heavy soil and in lighter, drier soils, at temperatures between 15 and 26°C. The infected debris of previous crops often serves as the primary source of inoculum. The fungus attacks during propagation or after planting.

CHEMICAL CONTROL

Since the discovery of Bordeaux mixture in 1885, fungicides have been essential for crop management. With public interest and increasing knowledge about hazards such as mammalian toxicity and environmental pollution, we are confronted with the dilemma involved with continual use of fungicides. The public is asking for strict regulation of fungicides and other chemicals used in crop management. Methyl bromide has been used as a soil fumigant on a wide range of crops and ornamentals. Recently, methyl bromide has been identified as an ozone-depleting chemical; its use will be phased out in the United States by the year 2001 (United States Environmental Protection Agency, 1995).

Mancozeb is applied to tomato seeds to control damping-off. It is the active ingredient in the commercial product Dithane. Mancozeb is toxic to fish and other aquatic organisms if not applied suitably. There are several chemicals on the prohibition acts list enforced by the United States Environmental Protection Agency in the 1994 settlement that places restraints on harmful chemicals that may induce cancer (US EPA, 1995).

BIOLOGICAL CONTROL

With increasing awareness of the possible deleterious effects of fungicides on the ecosystem, growing interest in pesticide-free agricultural products, and time-consuming breeding programs, biological control of plant pathogenic fungi has received considerable attention. Biological control is the use of natural or

modified organisms, genes, or gene products to reduce the effects of undesirable organisms and to favor desirable organisms (Baker, 1987; Campbell, 1989; Nigam and Mukerj, 1988). Reducing the effects of undesirable organisms includes affecting such qualities as the growth, infectivity, aggressiveness, virulence, and reproduction of pathogens. The "natural or modified organisms" include avirulent or hypovirulent pathogens, plants with genetic resistance, or any microorganisms that interfere with the survival or disease-producing activities of the pathogen. Biological control of soilborne pathogens may be accomplished through destruction of existing inoculum, exclusion from the host, or the suppression or displacement of the pathogen after infection.

The advantages of biological control include low economic cost and safety to man and the environment. A successful biological control agent must grow fast with limited nutrients and environmental requirements, be affective at colonizing organic matter, be adapted to disturbed cultivated environments, and produce some type of survival structure (Cook, 1993; Fravel and Keinath, 1991; Nigam and Mukerj, 1988).

There are a few microbial biocontrol agents registered in the United States for use against soilborne plant pathogens. These include bacteria, (*Agrobacterium radiobacter* K-84, *Pseudomonas fluorescens*, *Bacillus subtilis*), and fungi (*Gliocladium virens* Miller, Giddens, & Foster, *Trichoderma harzianum* Rifai, and *T. harzinum/polysporum* (Link) Rifai (Cook, 1993).

The mechanism by which a biocontrol agent reduces disease severity is an important factor when screening potential biocontrol agents. Antibiosis,

mycoparasitism and lysis, competition, cross protection, and induced resistance are the most likely modes of action of biocontrol agents. Some agents may utilize more than one of these mechanisms.

MODES OF ACTION

ANTIBIOSIS

Antibiosis is the inhibition or destruction of one organism by a metabolic product of another (Fravel, 1988). Antibiotics are low molecular weight organic compounds produced by microorganisms (Campbell, 1989). Soil-inhabiting microorganisms mostly produce antibiotics. To inhibit plant pathogens antibiotics must be produced in extremely small quantities, and secreted at the site where it is most effective (Campbell, 1989). For many years purified antibiotics were used to control foliar plant pathogens (Baker, 1987). However, the efficacy of antibiotics against soilborne pathogens was questioned. The antibiotic phenazine produced by *P. fluorescens* to control the take-all fungus, *Gaeumannomyces graminis* (Sacc.) Von Arx & Olivier var. *tritici* Walker, relieved doubt that antibiosis could be the primary mechanism for control of soilborne plant pathogens (Thomashow and Weller, 1991). *Pseudomonas flurorescens* 2-79 and *P. aureofaciens* 30-84 produce the antibiotic phenazine-1-carboxylic acid (PCA). To determine whether the antibiotic was produced *in situ*, wheat seeds were treated with strain 2-79, strain 30-84, or non-phenazine non-producing mutants, then sown in natural or steamed soil in the field or growth chamber (Thomashow et al., 1990). Roots from which the antibiotic was extracted had

significantly less disease than roots with no antibiotic, indicating that suppression of take-all was related directly to the presence of the antibiotic in the rhizosphere. This study provided the first evidence that antibiotics were able to survive in soil without established infection by the pathogen. PCA was recovered from roots in natural soils whether or not *G. graminis* var. *tritici* was introduced, and the amounts were sufficient to provide significant disease control in the presence of the pathogen.

Research with antibiotic-deficient mutants has provided evidence both for and against the role of antibiosis as a mechanism to control plant pathogens. In a recent study (Liu et al., 1995), UV-induced mutants of *Gliocladium virens* that were deficient for production of the antibiotic gliotoxin, and gliotoxin-producing parent strains were compared for their biocontrol efficacy against *R. solani* AG4 in cotton seedlings. There were no significant differences between antibiotic-deficient and parent strains for control of *R. solani*. This indicated that antibiosis was not the primary mechanism in disease control for this interaction.

Providing evidence for antibiosis as a mechanism of action against a soilborne pathogen has been challenging. *In vitro* assays are primarily used to demonstrate antibiotic production. O'Brien and coworkers (1984) evaluated 433 actinomycete isolates obtained from phloem and xylem of American elm for antagonism *in vitro* against *Ceratocystis ulmi* (Buism.) C. Mor., the causal agent of Dutch elm disease. One isolate each of *Streptomyces albovinaceus* and *S. griseus* inhibited various isolates of *C. ulmi* by 64 to 90% *in vitro*, but failed to prevent symptoms of Dutch elm disease when inoculated into elm samplings one

week before challenge with *C. ulmi*. Many studies show no correlation between antibiosis and biocontrol in the field or greenhouse.

In contrast, there are studies with a positive correlation between antibioitc production *in vitro* and *in vivo*. Twenty-one isolates of the bacterium *Bacillus subtilis* were tested on cornmeal agar for antagonism to six isolates of *Phytophthora cactorum* (Lebert & Cohn) J. Schrot., the causal agent of apple crown rot (Utkhede, 1984). The antagonists were further evaluated in a greenhouse trial for their ability to control infection of crown rot on apple seedlings. No correlation was observed between width of inhibition zone *in vitro* and protection from *P. cactorum* infection in the apple seedlings. In field tests, severity of bean rust, caused by *Uromyces appendiculatus* (Pers.:Pers.) Unger, was reduced at least 75% with three applications per week of *Bacillus subtilis* (Papavizas et al., 1982). One of the isolates had an inhibitory effect on yield and a stimulatory effect on plant growth. In some treatments *B. subtilis* was more effective than the weekly application of the fungicide mancozeb.

Antibiosis is usually tested first as a possible mode of action when screening potential biocontrol agents, but due to reasons such as genes for antibiotic production not turned on, low production, or microbial degradation, antibiosis is not always the primary mode of action.

MYCOPARASITISM OR HYPERPARSITISM

Mycoparasitism is a phenomenon that involves fungi parasitizing plant pathogenic fungi (Nigam and Mukerj, 1988). Hyperparasites are parasites of parasites, such as bacteria parasitizing plant-parasitic fungi or nematodes

(Campbell, 1989). The best known mycoparasite is the fungus *Trichoderma harzianum*, which has been shown to reduce the inoculum density of many soilborne pathogens. *Trichoderma harzianum* is one of the few agents that are commercially available mycoparasites (Cook, 1993). The hyphae of *T. harzianum* penetrate resting structures or parasitize growing hyphae. The fungus coil around the parasitizing hyphae and cause lysis. *Pythium nunn* Lifshitz, Stanghellini and Baker is also a mycoparasite of several different soilborne pathogens (Campbell, 1989). When *P. nunn* attacks *P. ultimum* Trow, the hyphae of *P. nunn* coils around the hyphae of *P. ultimum* and causes lysis (Campbell, 1989). However, when *P. nun* attacks *Phytophthora* spp., it forms an infection peg which penetrates the pathogen (Campbell, 1989; Papavizas, 1992). *Gliocladium virens*, also acts as a mycoparasite by coiling around and penetrating hyphae of *R. solani* (Papavizas, 1992).

COMPETITION

Competition results when two or more organisms attempt to gain an advantage for use of a substrate supply that is not sufficient for all (Campbell, 1989; Paulitz, 1990). Parasitic organisms can compete for nutrients and space while in a dormant state, saprophytic stage, or during the time the pathogen is actively living on the surface of living plants or in a lesion. Competition for nutrients may result in competition for space by exclusion of the pathogen from the host rhizosphere. Organisms usually compete for space, carbon, nitrogen, oxygen, iron (siderophores), and growth factors. For example, in nonsterile soil, oxygen is a limiting factor for wood-inhabiting pathogens, and competition for

oxygen between beneficial microorganisms and plant pathogens may destroy the pathogen (Baker, 1987).

The plant pathogens, *P. ultimum*, *Erwinia caratovora*, and *Fusarium oxysporum* Schlecht.:Fr. are suppressed by various species of siderophore-producing Pseudomonads. The mechanism in the suppression of *Fusarium* wilt of radish by *Pseudomonas putida* strain WCS358 involved siderophore-mediated competition for iron (III) (Raaijmakers et al., 1995). *Pseudomonas* spp. sequester iron causing it to become the limiting factor, hence *Fusarium* starves for iron (Fravel, 1988; Thomashow and Weller, 1991). This biocontrol mechanism is effective only in high pH soils where iron is limited. In addition, iron is known to regulate the synthesis of several secondary metabolites such as antibiotics (Fravel, 1988).

CROSS PROTECTION

Cross protection is a mechanism that is rarely mentioned because it is sometimes confused with the mechanism of induced resistance. Cross protection is the infection of the host by one organism that greatly reduce subsequent infection by a second organism (Campbell, 1987). For example, tomato can be protected from *F. oxysporum* Schlecht.:Fr. f. sp. *lycopersici* (Sacc.) W. C. Snyder and H. N. Hans. by dipping the roots in a suspension of *F. oxysporum* Schlecht.:Fr. f. sp. *dianthi* (Prill. & Delacr.) W. C. Snyder & H. N. Hans. days before exposure (Wymore and Baker, 1982).

INDUCED RESISTANCE

Induced resistance involves challenge of the host with an organism or

chemical, which induces an increase in host resistance or activates host defense mechanisms (Campbell, 1989). Later when the host is challenged by a second organism it is resistant to it. If the resistance is only in the vicinity of the first challenge organism it is considered local acquired resistance. If, however, resistance is seen in parts of the plants that are at a distance from where the first organism was applied, it is systemic acquired resistance.

Tomatoes which were inoculated first with *Cethalosporium* sp. (foliar pathogen) showed systemic resistance to *F. oxysporum* f. sp. *lycopersici* (soilborne pathogen) (Upadhyay and Rai, 1988). *Pseudomonas putida* 89B-27 and *Serratia marcescens* 90-166 induced systemic resistance in cucumber against cucumber anthracnose caused by *Collectotrichum obriculare* (Wei et al., 1996) and bacterial angular leaf spot caused by *Pseudomonas syringae* pv. *lachrymans* (Liu et al., 1995). *Pseudomonas fluorescens* strain WCS417 induced systemic resistance against the virulent bacterial pathogen *P. syringae* pv. *tomato*, the fungal leaf pathogen *Alternaria brassicola* (Schwein.) Wiltshire, and the xylem pathogen *F. oxysporum* on tomato (Hoffland et al., 1996).

Synthesis of several low molecular weight pathogenesis-related proteins is commonly associated with induced acquired resistance (Lewis et al., 1990). These proteins include chitinases and ß-1,3 glucanases. It has been suggested that the signal from the first challenge organism is salicyclic acid (SA) (Klessig and Malamy, 1994). However, SA inhibits catalase (converts hydrogen peroxide (H_2O_2) to H_2O and O_2), which in turn increases H_2O_2 concentration. This suggests that the signal may be H_2O_2 (Klessig and Malamy, 1994).

MULTIPLE MODES OF ACTION

Most biocontrol agents use a combination of mechanisms to suppress pathogens. For example, *Pseudomonas* spp. produce antibiotics such as pyoluteorin and pyrrolnitrin, which are effective against damping-off of cotton, caused by *R. solani* (Howell and Stipanovic, 1979). *Pseudomonas* spp. also produces siderophores, which limit growth of pathogens by chelating the available iron in soil (Schroth and Hancock, 1982; Thomashow and Weller, 1991). *Trichoderma* spp. are antagonistic against *Botrytis cineria* Pers.:Fr. through different mechanisms such as competition, antibiosis, or direct parasitic action (Chet, 1987). *Gliocladium virens* has been shown to use both parasitism and antibiosis as modes of action against plant pathogens (Papavizas, 1992). Although fungi clearly have the potential to control soilborne pathogens, the remainder of this review will focus on two genera of potential bacterial biocontrol agents, *Bacillus* and *Pseudomonas*.

BACTERIAL BIOCONTROL AGENTS

BACILLUS SPP.

Bacillus spp. are Gram-positive, rod-shaped, endospore-producing bacteria. Production of endospores gives this group of bacteria great potential for biocontrol. Endospores are resistant to heat and other harmful agents such as drying, radiation, acids, and chemical disinfectants (Brock and Madigan, 1991). Endospore-producing bacteria are most commonly found in soil, and can be isolated by exposing the sample to heat (75 to 85 °C) for 10 min. This

treatment completely destroys vegetative cells while the spores remain viable. Bacilli usually grow well on synthetic media containing sugars, organic acids, alcohol, etc., as the sole carbon source and ammonium as the sole nitrogen source. Many *Bacillus* spp. produce extracellular hydrolytic enzymes that break down polysaccharides, nucleic acids, and lipids, permitting the organisms to use these products as carbon sources. Secondary metabolites such as bacitracin, alboleutin, tyrocidin, iturin, fengycin, gramiddin, and circulin can be produced from a wide variety of *Bacillus* spp. (Sonenshein et al., 1990). Some of these antibiotics aid in suppression of plant diseases (Sonenshein et al., 1990). In most cases antibiotics are released in culture at the beginning of the stationary phase of growth. Antibiotic excretion is often concurrent with sporulation (Sonenshein et al., 1990).

Bacillus spp. have been studied extensively as biocontrol agents. For example, *B. subtilis* was highly effective as an inoculant against avocado postharvest diseases caused by *Collectotrichum gloeosporioides* (Penz.) Penz. & Sacc. on avocado. *Bacillus* spp. comprised a major component of the microflora of avocado plants and several species of *Bacillus* were screened, with an isolate of *B. subtilis* proving to be most effective. In an artificial inoculation study, increasing the concentration of *B. subtilis* cells was effective against increasing populations of the pathogens *C. gloeosporioides*, *Fusarium solani* (Mart.) Sacc., and *Thyronectria pseudotrichia* (Berk. & M. A. Curtis) Seeler. Furthermore, *B. subtilis* has demonstrated effective control against established infections (Zuber et al., 1993).

In another study, *B. subtilis* was used as a biocontrol agent against the soilborne tomato wilt pathogen, *Fusarium oxysporum* f. sp. *dianthi*, (Upadhaya and Rai, 1988). In pot trials with tomato plants, treatment of the soil with *B. subtilis* resulted in improved root development and increased yield of fruit. Ashby (1995) compared the ability of several chemicals and biologicals control of target spot on tobacco in a greenhouse float bed system. Control of target spot on tobacco was significantly greater on seedlings treated with *Bacillus cereus* BA-55, fluazinam, iprodione, and mancozeb than the control.

***PSEUDOMONAS* SPP.**

Pseudomonas spp. are Gram-negative rods or coccus shaped bacteria. Strains of *P. fluorescens* have been studied widely as biocontrol agents. Several strains are known to reduce the severity of take-all of wheat. Mechanisms of biocontrol associated with *Pseudomonas* spp. include competition, induced resistance, and antibiosis. Fluorescent pseudomonads isolated from a soil naturally suppressive to black root rot of tobacco caused by *Thielaviopsis basicola* (Berk. and Broome) Ferraris, seem to be the major group of organisms responsible for suppression of this disease (Shew and Lucas, 1991). *Pseudomonas fluorescens* strain CHA0 suppresses both *T. basicola* and *G. graminis* var. *tritici*. Strain CHA0 produces 2,4-diacetylphoroglucinol which is a metabolite with antifungal, antibacterial, and phytotoxic properties (Keel et al., 1989). In greenhouse trials this strain also significantly suppressed *T. basicola* (Stutz et al., 1982).

Pseudomonas putida plays a role in suppressing Fusarium wilt of flax,

radish, and cucumber (Scher and Baker, 1982). *Pseudomonas* spp. suppress parasites on potato (Escande and Echandi, 1991), peanut (Ganesan and Granamanickam, 1987), cotton (Lewis et al., 1990), wheat (Weller and Cook, 1986), carnation (Yuen et al., 1985), and poinsettia (Upadhaya and Rai, 1988) by production of antibiotics. The antibiotics, pyoleutorin and pyrrolnitrin, are involved in suppression of *R. solani* on cotton (Lewis et al., 1990). *Bacillus* spp. and *Psuedomonas* spp. have demonstrated enormous potential as biocontrol agents and should be further studied.

OBJECTIVES

This research project was focused on assessment of several bacterial isolates in the genera *Pseudomonas* and *Bacillus* for biological control activity against *R. solani* on tomato. The specific objectives of this research were:

1. to screen a collection of *Bacillus* and *Pseudomonas* isolates from tobacco field soils (TFS) for inhibition of *R. solani* in culture medium as evidence of antibiosis by the bacteria;
2. to screen the TFS bacterial collection applied as seed treatments for biocontrol of *R. solani* on tomato in the greenhouse;
3. to develop a collection of bacterial endophytes and rhizosphere colonists from tomato seedlings and screen the isolates for inhibition of *R. solani in vitro* and for biocontrol of damping-off of tomato in the greenhouse; and
4. to characterize and identify effective isolates.

CHAPTER II

SCREENING BACTERIA FOR BIOCONTROL OF *RHIZOCTONIA SOLANI* ON TOMATO

Root-associated bacteria may provide the front line of defense for roots attacked by pathogens. Pathogens encounter antagonism from rhizosphere microorganisms before and during primary infection, and during secondary spread on the roots. Therefore, microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents. Studies show that rhizosphere bacteria with the ability to provide biological control appear to comprise less than 10% of the total population of bacteria in the rhizosphere (Schroth and Hancock, 1981; Weller, 1988).

Because of the time and expense required for field testing, better methods are needed to select potential field-effective strains (Weller, 1988; Schroth and Hancock, 1982). Selection of effective strains can be improved by isolating bacteria from the same environment in which the organism will be used, for example, selecting endophytes from tomato root tissue if the target pathogen causes a root disease of tomato.

Some studies show no correlation between a biocontrol agent inhibiting a pathogen *in vitro* and suppressing disease caused by that pathogen *in vivo*. *Trichoderma viride* and other fungi in the rhizosphere did not exhibit antagonism *in vitro* to *Verticillium*, however, in gnotobiotic culture with *Trichoderma viride,* the total number of tomato plants infected with Verticillium was half the number inoculated with *Verticillium* alone (Schippers et al., 1985). In another study

(Weller et al., 1985), fluorescent pseudomonads isolated from a soil suppressive to *Fusarium* wilt significantly reduced take-all in wheat and *Ophiobolus* patch (*G. graminis* var. *avenae* (E. M. Turner) Dennis) in *Agrostis* turfgrass. There were significantly fewer diseased wheat roots in the treatments with the bacteria and pathogen than in those with the pathogen alone. However, there was no correlation between *in vitro* antibiosis on agar plates and suppression of disease in pot experiments. Therefore, *in vitro* antibiosis was considered a poor criterion for selecting bacterial isolates effective against take-all.

Selecting field-effective strains can be further facilitated by the use of greenhouse assays. Greenhouse tests are more useful when screening biological control agents because the host is present. Inoculum potential of the pathogen, environmental conditions, temperature and moisture content of the soil, and the amount of the candidate bacterium are important variables of greenhouse assays. Greenhouse assays have been developed to screen antagonists against *Pythium* and *Gaeummannomyces* on wheat (Weller and Cook, 1986). Bacteria selected to treat seed sown in a field naturally infested with *Pythium* spp. eliminated symptoms typical of *Pythium* similar to the effect of chemical treatment (Weller and Cook, 1986).

The way in which bacteria are applied to protect plants against pathogens should also be considered when screening potential biocontrol agents. The application of biocontrol agents by broadcast spreaders, or by direct application in the seed furrow at the time of sowing, has the greatest potential for use in commercial greenhouse operations or small scale field experiments (Cook and

Baker, 1983). For biological control of root pathogens in the field, introducing antagonists as seed treatments has greater potential (Mukerj and Garg, 1988). This method gives the antagonist an opportunity to colonize the roots before the pathogen. This treatment is more useful for biological protection of the plant, protection of germinating seeds, roots, or emerging shoots. Seed treatments also have certain advantages over chemicals. Seed treated in excess of that needed for planting can be fed to poultry or livestock with less risk than if it were chemically treated. Also, in countries where the chemicals are not available, or cannot be purchased, a microorganism could be produced locally and used in place of the chemical.

Seed inoculated with antagonistic microorganisms offer potential for the control of root diseases, and there are many reports of success. Corn seed inoculated with *Chaetomium globosum* Kunze:Fr. controlled *Fusarium roseum* Link:Fr. f. sp. *cerealis* (Chang and Kommedahl, 1968; Mukerj and Garg, 1988). Rhizosphere bacteria were collected from a variety of fields in southern Alberta, and applied as seed treatments for biocontrol of damping-off of safflower caused by *Pythium ultimum*. Isolates of *Erwinia carotovora*, *E. rhapontici*, *Pseudomonas putida*, *P. fluorescens*, *Bacillus polymyxa*, *B. subtilis*, and *E. herbicola* were identified that significantly reduced pre-emergence damping-off caused by *P. ultimum* and increased seedling emergence in safflower. Application of *Gliocladium virens* to seed potatoes in storage suppressed *Rhizoctonia solani* by the production of a highly potent antibiotic, gliotoxin (Aluko and Hering, 1970). *T. harzianum* applied as a seed treatment controlled seed rot of pea or radish in soil

infested with *Pythium* spp. or *R. solani*, respectively, if soil temperatures were between 17 and 34 °C and seeds had been treated with 100 ml of conidial suspension (Harman et al., 1981). In a field test, isolates of *Penicillium* and *Aspergillus* reduced the incidence of galling on cherry seedlings (Cooksey and Moore, 1980).

The goal of this research was to isolate bacteria that were effective as biological controls of *R. solani* on tomato. The specific objectives were to 1) screen a collection of *Bacillus* and *Pseudomonas* isolates from tobacco field soils (TFS) for inhibition of *R. solani* in culture medium as evidence of antibiosis by the bacteria; 2) screen the TFS bacterial collection applied as seed treatments for biocontrol of *R. solani* on tomato in the greenhouse; 3) develop a collection of bacterial endophytes and rhizosphere colonists from tomato seedlings; 4) screen the endophytic and rhizosphere isolates for inhibition of *R. solani in vitro* and for biocontrol of damping-off of tomato in the greenhouse.

MATERIALS AND METHODS

Development of bacterial collections. Three collections of *Bacillus* and *Pseudomonas* isolates were evaluated. The first collection consisted of 94 isolates from tobacco field soils in Greeneville, TN (Ashby, 1995). These isolates were designated 'BA.' The second and third groups included 60 endophytes (designated 'E') and 50 rhizosphere colonists (designated 'R') from tomato root tissue.

Tomato seeds ('Better Boy') were sown in a 1:1 w/w mix of Etowah silt

loam (collected from the Knoxville Experiment Station, TAES) and Fafard mix (Conrad Fafard, Inc., Agawam, MA). After about 4 weeks, the seedlings were carefully removed from the soil mix, excess soil was removed and the root system was detached. Serial dilutions of the adhering rhizosphere soil were made in phosphate buffered saline solution (PBS; [8 g NaCl, 0.34 g KH_2PO_4, and 1.21 g K_2HPO_4 in 1L deionized water]) and plated on *Pseudomonas* isolation agar (PIA [Simon et al., 1973]) and tryptic soy agar (TSA; [3 g tryptic soy broth, Difco Laboratories, Detroit, MI, and 15 g agar, Sigma Chemical Co., St. Louis, MO, and 1L deionized water]). For isolation of *Bacillus*, dilutions were heated to 80°C for 30 min, then plated on Aerobic Spore Formers Medium (ASFM [Wollum, 1982]). For isolation of endophytes, the remaining soil was removed by washing in tap water, and the roots were surface sterilized in 30% hydrogen peroxide for 30 min. The disinfested root tissue was macerated with a sterile mortar and pestle. Serial dilutions were prepared in PBS, and 0.1-ml aliquots were plated on PIA, TSA, and ASFM (after heating).

Preparation of biological seed treatments. Bacterial isolates were grown in Minimal 3C broth (Min 3C [Handelsman et al., 1990]) for three days at room temperature (25 to 30 °C) with shaking (150 rpm). The bacterial cultures were then centrifuged at 3500 rpm for 15 min. The supernatant from each culture was discarded, 10 ml of freshly prepared 1 % methyl cellulose (Sigma Chemical Co., St. Louis, MO) solution was added to each pellet, and the mixture was vortexed until bubble formation. 'Better boy' tomato seeds were surface-sterilized in 30% hydrogen peroxide for 30 min, then air-dried under a laminar

flow hood. Approximately 0.2 g of the surface-disinfested tomato seeds was added to the bacterial solution and vortexed. The bacterized seeds were then air-dried under a laminar flow hood.

Production of pathogen inoculum. *Rhizoctonia solani* was cultured on potato dextrose agar (PDA; [24 g potato dextrose broth, Difco Laboratories, Detroit, MI, and 15 g agar, Sigma Chemical Co., St. Louis, MO, in 1 L deionized water]) and incubated at 25 °C for a maximum of three days. Agar plugs of the fungus were then transferred to twice autoclaved rice grain medium (25 g rice to 8 ml deionized H_2O) and incubated at room temperature for 7 to 14 days.

Greenhouse assay for assessment of disease severity rating, shoot height, and percent plant mortality. For each replicate of each treatment ten treated seeds (five seeds in two rows) were planted in one tray containing ProMix (Premier Horticulture Inc., Red Hill, PA). After 2 to 4 weeks, 3.5 g of 7 to 14-day-old rice inoculum of *R. solani* was added in a row down the center of the tray. After 2 to 4 additional weeks, plants were removed from the trays and the roots were washed free of Pro-Mix. The roots were rated for development of lesions on a scale of 0 to 7. The scale was defined with 0 = healthy seedling, 1 = lesion < 2.5 mm long, 2 = lesion 2.5 to 5.0 mm long, 3 = lesion > 5.0 mm long, 4 = lesion girdling the stem, 5 = lesion girdling and constricting the stem (< 75%), 6 = lesion girdling and constricting the stem (> 75%), and 7 = dead seedling. The percent mortality (death rate) of seedlings was calculated and the shoot height of living plants was determined. Shoot height was measured in centimeter from the soil line to the tallest leaf.

Antibiosis assay. All isolates were tested for antibiosis activity against *R. solani in vitro*. Isolates were grown in Min 3C broth and Nutrient Broth Yeast Extract (NBY [Vidaver, 1967]) for 3 days at 25 to 30 °C. Ten-µl aliquots of the broth culture of each isolate were inoculated down the center of Min 3C agar and NBY agar plates, then incubated for six days at room temperature. Six days after inoculation of the plates, 5 mm-plugs of *R. solani* on PDA were placed on both sides of the bacterial streak. Plates were incubated for an additional five days, then the zones of inhibition were measured on both sides of the bacterial streak (Figure 2.1).

Experimental design and statistical analysis. For the greenhouse assay, 31 separate experiments were conducted. Each experiment was arranged in a randomized complete block design with four replicates per treatment and ten plants per replicate. The number of treatments per experiment varied. For the antibiosis assay, there were three plates (replicates) per bacterial isolate with 2 observations per plate, arranged in a randomized complete block design. Data was analyzed by the GLM procedure of PC-SAS. Significant effects were further analyzed with Fisher's protected least significant difference (LSD) test. Mortality data were transformed (arcsin) before analysis. For each experiment in which the effect of seed treatment was significant for disease severity rating, shoot height, or percent plant mortality, individual treatments were then compared with the untreated control. The percent change in the measured variables was calculated for those treatments that differed from the untreated control.

RESULTS

Development of endophytic and rhizosphere colonist collections. The majority of the endophytic bacteria (30 isolates) were selected on ASFM. Twenty endophytic isolates were selected on PIA and ten isolates were from TSA. The majority of rhizosphere isolates (25) were taken from ASFM.

Greenhouse assay. Bacterial seed treatments of tomato reduced disease severity and percent mortality caused by *R. solani*, in six of 31 greenhouse experiments (Tables 2.1 to 2.6). In two experiments, bacterial seed treatments resulted in greater shoot height (Tables 2.5 and 2.6). Seed treatments were separately compared to the controls. Of 204 bacterial isolates screened, only 13 decreased disease severity (Table 2.7), ten increased shoot height (Table 2.8), and 11 reduced plant mortality (Table 2.9).

Of 94 bacterial isolates collected from tobacco field soils, only five (5.3%) reduced disease severity of *R. solani* (Figure 2.2) BA19, BA77, BA101, BA23, and BA106. Tobacco field soil isolates decreased disease severity from 13 to 64% (Figure 2.2). Eight (13%) of the 60 endophytic isolates decreased disease severity E21, E61, E65, E66, E69, E723, E726, and E727. Endophytic isolates decreased disease severity 30 to 60% (Figure 2.3). The rhizosphere isolates did not reduce disease severity.

Shoot height was calculated to determine the effect of seed treatments on plant growth (Table 2.8). Tobacco field soil isolates and endophytic isolates increased shoot height significantly. However, some of the treatments that increased shoot height did not decrease disease severity or percent mortality of

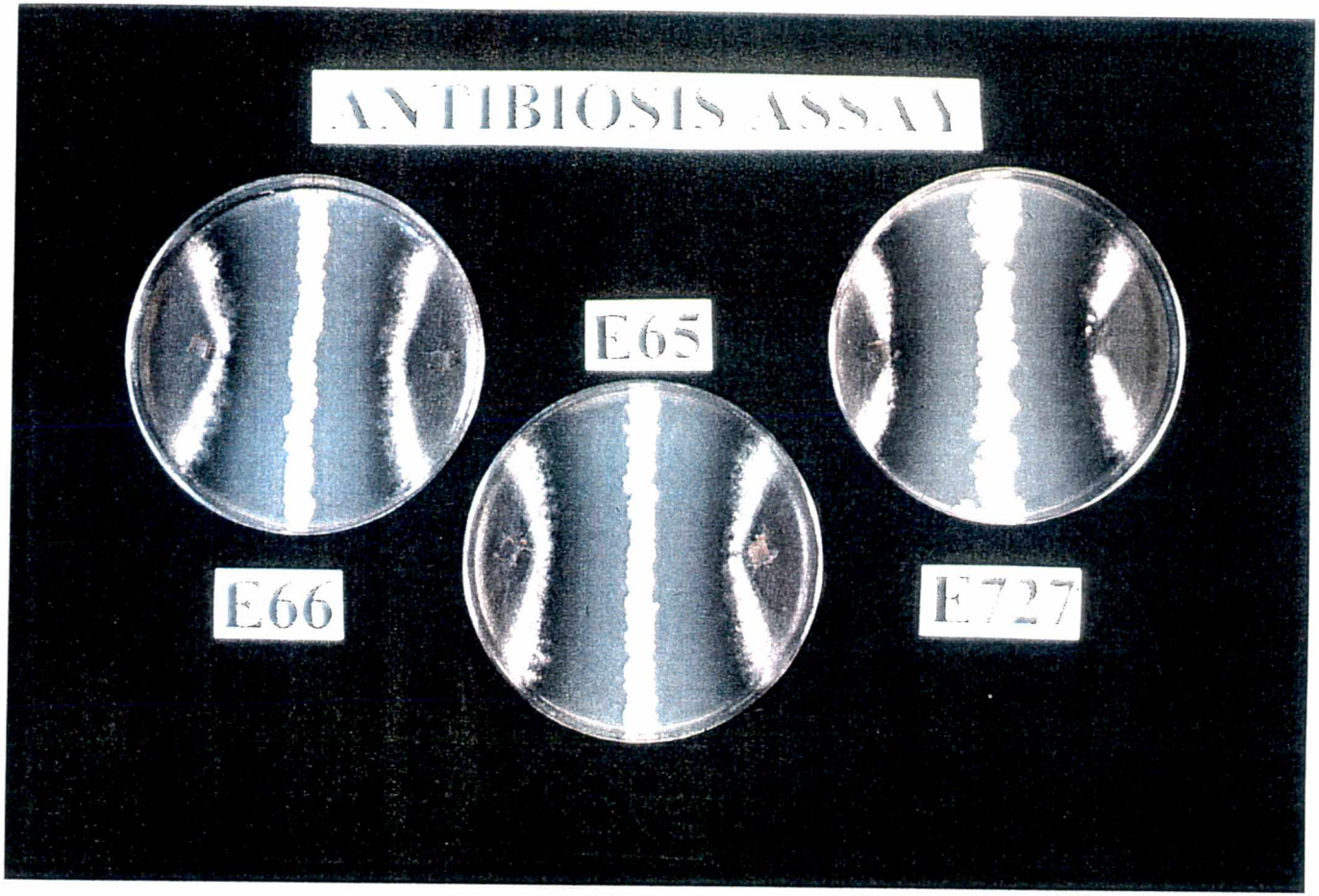

Figure 2.1. The antibiosis assay plates containing Min 3C were inoculated with bacterial isolates (center of plate) which inhibited growth of *R. solani* (on either side of bacterial streak).

Table 2.1. Analysis of variance for the effect of seed treatment on disease rating and percent mortality of tomatoes inoculated with *R. solani* in Experiment 4.

Source	DF	Sum of Squares	Mean Squares	F Value	Pr>F
Replicate	3	6.283	2.094	27.02	0.0007
Disease rating					
Treatment[y]	2	34.102	17.051	220.01	0.0001
Model	5	40.384	8.078	104.22	0.0001
Error	6	0.465	0.078		
Corrected Total	11	40.849			
Percent mortality[z]					
Replicate	3	0.411	0.137	4.55	0.0548
Treatment[y]	2	2.583	1.291	42.82	0.0003
Model	5	2.994	0.599	19.85	0.0011
Error	6	0.181	0.030		
Corrected Total	11	3.175			

[y]Experiment 4 included BA77, BA80, and a methyl cellulose control.
[z]Data for percent mortality were transformed (arcsin) before statistical analysis.

Table 2.2. Analysis of variance for the effect of seed treatment on disease rating and percent mortality of tomatoes inoculated with *R. solani* in Experiment 11.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Disease rating					
Replicate	3	0.942	0.314	1.02	0.4006
Treatment[y]	9	10.716	1.191	3.86	0.0031
Model	12	11.658	0.972	3.15	0.0065
Error	27	8.338	0.309		
Corrected Total	39	19.996			
Percent Mortality[z]					
Replicate	3	0.130	0.043	0.71	0.5551
Treatment[y]	9	1.474	0.164	2.68	0.0231
Model	12	1.604	0.134	2.18	0.0449
Error	27	1.653	0.061		
Corrected Total	39	3.256			

[y]Experiment 11 included BA19, BA21, BA22, BA23, BA24, BA25, BA26, BA27, BA28, and a methyl cellulose control.
[z]Data for percent mortality were transformed (arcsin) before statistical analysis.

Table 2.3. Analysis of variance for the effect of seed treatment on disease rating and percent mortality of tomatoes inoculated with *R. solani* in Experiment 19.

Source	DF	Sum of Squares	Mean Squares	F Value	Pr>F
Disease rating					
Replicate	3	0.538	0.179	0.71	0.5580
Treatment[y]	7	4.825	0.689	2.72	0.0357
Model	10	5.363	0.536	2.12	0.0712
Error	21	5.324	0.254		
Corrected Total	31	10.687			
Percent mortality[z]					
Replicate	3	0.064	0.021	0.58	0.6329
Treatment[y]	7	0.770	0.110	3.01	0.0237
Model	10	0.834	0.083	2.28	0.0537
Error	21	0.769	0.037		
Corrected Total	31	1.603			

[y]Experiment 19 included BA101, BA106, BA108, BA110, BA105, and a methyl cellulose control.
[z]Data for percent mortality were transformed (arcsin) before statistical analysis.

Table 2.4. Analysis of variance for the effect of seed treatment on disease rating and percent mortality of tomatoes inoculated with *R. solani* in Experiment 26.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Disease rating					
Replicate	3	2.586	0.862	1.02	0.3957
Treatment[y]	10	42.757	4.276	5.08	0.0002
Model	13	45.343	3.488	4.15	0.0006
Error	30	25.241	0.841		
Corrected Total	43	70.584			
Percent mortality[z]					
Replicate	3	0.222	0.074	0.71	0.5528
Treatment[y]	10	4.223	0.422	4.06	0.0014
Model	13	4.445	0.342	3.29	0.0035
Error	30	3.122	0.104		
Corrected Total	43	7.566			

[y]Experiment 26 included E21, E22, E23, E24, E25, E26, E27, E28, E29, E210 and a methylcellulose control.
[z]Data for percent mortality were transformed (arcsin) before statistical analysis.

Table 2.5. Analysis of variance for the effect of seed treatment on disease rating, percent mortality, and shoot height of tomatoes inoculated with *R. solani* in Experiment 23.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Disease rating					
Replicate	3	3.663	1.221	1.04	0.3893
Treatment[y]	10	31.965	3.197	2.72	0.0165
Model	13	35.628	2.741	2.33	0.0273
Error	30	35.235	1.175		
Corrected Total	43	70.863			
Percent Mortality[z]					
Replicate	3	0.238	0.079	0.77	0.5223
Treatment[y]	10	2.448	0.245	2.37	0.0334
Model	13	2.686	0.207	2.00	0.0581
Error	30	3.106	0.104		
Corrected Total	43	5.792			
Shoot height					
Replicate	3	9.839	3.280	1.15	0.3465
Treatment[y]	10	1296.476	129.648	45.43	0.0001
Model	13	1306.950	100.535	35.23	0.0001
Error	28	79.899	2.854		
Corrected Total	41	1386.849			

[y]Experiment 23 included E61, E62, E63, E64, E65, E66, E67, E68, E69, E610, and a methyl cellulose control.
[z]Data for percent mortality were transformed (arcsin) before statistical analysis.

Table 2.6. Analysis of variance for the effect of seed treatment on disease rating, percent mortality, and shoot height of tomatoes inoculated with *R. solani* in Experiment 25.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Disease rating					
Replicate	3	1.448	0.483	0.66	0.5848
Treatment[y]	10	67.905	6.791	9.25	0.0001
Model	13	69.353	5.335	7.26	0.0001
Error	30	22.035	0.734		
Corrected Total	43	91.388			
Percent Mortality[z]					
Replicate	3	0.024	0.008	0.13	0.9394
Treatment[y]	10	5.419	0.542	8.99	0.0001
Model	13	5.443	0.419	6.95	0.0001
Error	30	1.808	0.060		
Corrected Total	43	7.251			
Shoot height					
Replicate	3	11.732	3.911	1.03	0.3962
Treatment[y]	10	1375.976	137.598	36.09	0.0001
Model	13	106.759	107.234	28.12	0.0001
Error	28	106.759	3.812		
Corrected Total	41	1500.807			

[y]Experiment 25 include E721, E722, E723, E724, E725, E726, E727, E728, E729, E730, and a methyl cellulose control.
[z]Data for percent mortality were transformed (arcsin) before statistical analysis.

Table 2.7. Effect of individual bacterial seed treatments on disease rating of tomato seedlings inoculated with *R. solani*.

Experiment	Bacterial seed treatment[x]	Disease rating of treatment[y]	Disease rating of control[yz]	Significance level (Pr>F)
4	BA77	1.98	5.50	0.0010
11	BA19	5.58	6.95	0.0314
11	BA23	5.78	6.95	0.0327
19	BA101	6.08	7.00	0.0365
19	BA106	5.95	7.00	0.0204
23	E61	2.50	5.83	0.0180
23	E65	3.68	5.83	0.0422
23	E66	4.05	5.83	0.0389
23	E69	3.60	5.83	0.0110
25	E723	2.68	5.80	0.0047
25	E727	2.88	5.80	0.0034
25	E726	2.30	5.80	0.0003
26	E21	2.93	5.80	0.0161

[x]Bacteria with an 'E' designation were isolated as endophytes from tomato root tissue, and those designated 'BA' were isolated from tobacco field soils.
[y]Each value is the mean of four replicates with 10 plants per replicate.
[z]Control was treated with 1 % methyl cellulose only.

Table 2.8. Effect of individual bacterial seed treatments on shoot height of tomato seedlings inoculated with *R. solani*.

Experiment	Bacterial seed treatment[x]	Shoot height of treatment[y]	Shoot height of control[yz]	Significance level (Pr>F)
25	E721	26.8	7.4	0.0002
25	E722	24.1	7.4	0.0004
25	E724	21.4	7.4	0.0066
25	E727	21.3	7.4	0.0010
25	E728	12.0	7.4	0.0063
25	E729	13.5	7.4	0.0122
25	E730	14.4	7.4	0.0063
25	E723	20.5	7.4	0.0040
25	E726	14.2	7.4	0.0053
23	E68	19.2	15.4	0.0395

[x]Bacteria with an 'E' designation were isolated as endophytes from tomato root tissue.
[y]Each value is the mean of 4 replicates with ten plants per replicate.
[z]Control was treated with 1 % methyl cellulose only.

Table 2.9. Effect of individual bacterial seed treatments on percent mortality of tomato seedlings inoculated with *R. solani*.

Experiment	Bacterial seed treatment[x]	% mortality of treatment[y]	% mortality of control[yz]	Significance level (Pr>F)
4	BA77	5.0	65.0	0.0115
11	BA23	67.5	97.5	0.0240
11	BA19	67.5	97.5	0.0052
19	BA106	75.0	100.0	0.0032
19	BA101	75.0	100.0	0.0154
25	E723	17.5	72.5	0.0034
25	E726	17.5	72.5	0.0003
25	E722	45.0	72.5	0.0486
25	E727	22.5	72.5	0.0012
25	E729	50.0	72.5	0.0374
26	E21	20.0	72.5	0.0179

[x]Bacteria with an 'E' designation were isolated as endophytes from tomato root tissue, and those designated 'BA' were isolated from tobacco field soils.
[y]Each value is the mean of 4 replicates with ten plants per replicate.
[z]Control was treated with 1 % methyl cellulose only.

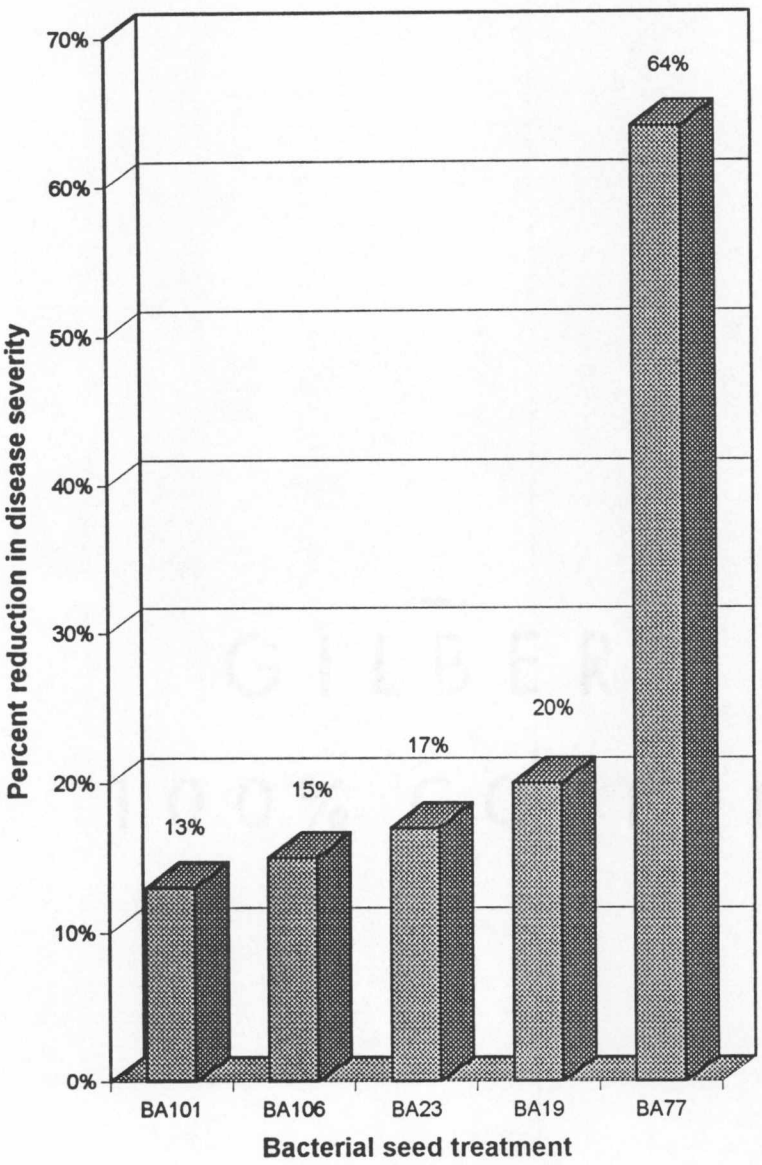

Figure 2.2. Percent reduction in disease severity caused by *Rhizoctonia solani* on tomato seedlings treated with bacteria collected from tobacco field soil.

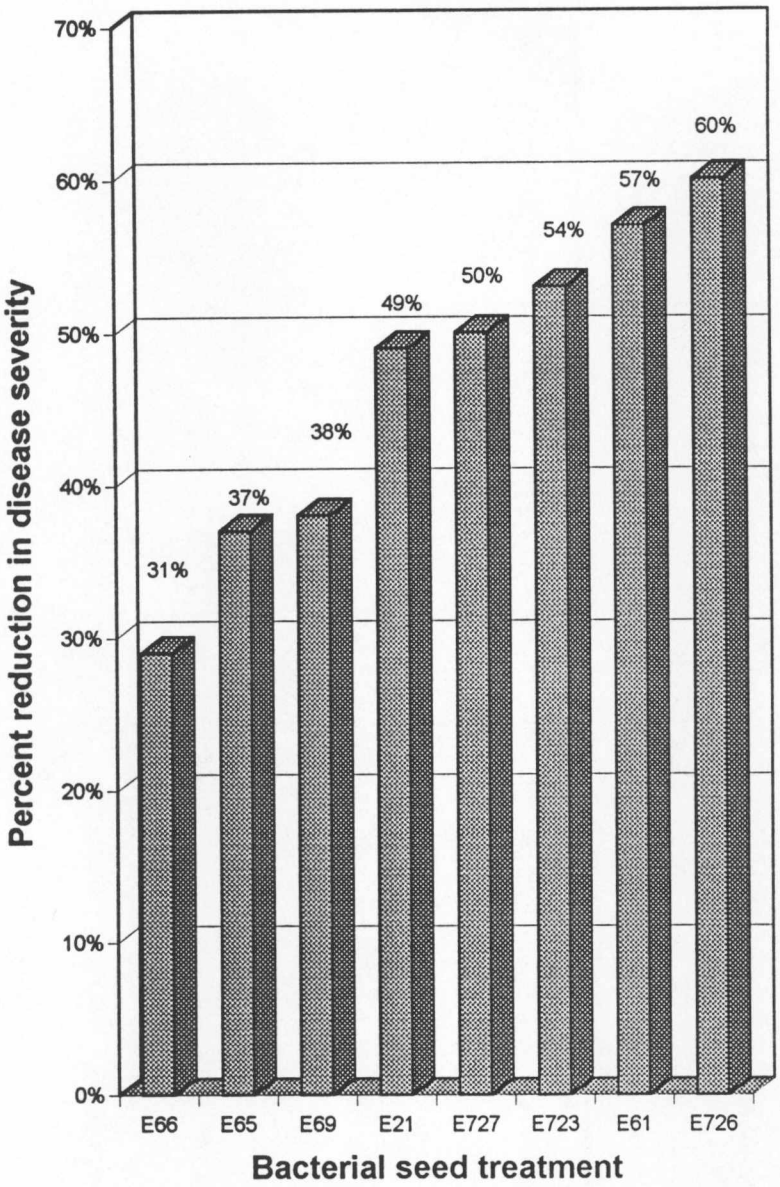

Figure 2.3. Percent reduction in disease severity caused by *Rhizoctonia solani* on tomato seedlings treated with endophytic bacteria isolated from tomato root tissue.

seedlings ranged from 25 to 262 % (Figure 2.4).

Many of the seed treatments that reduced disease severity also reduced plant mortality (Table 2.9). The bacterial isolate BA77 decreased mortality by 92% (Figure 2.5). The remaining seed treatments decreased percent plant mortality from 25 to 76%. As expected, there was a correlation between disease severity and percent plant mortality, as disease severity decreased, percent mortality decreased.

Antibiosis assay. Only one of the tobacco field soil isolates inhibited *R. solani* in culture on Min 3C agar. The bacterial endophytes decreased disease severity and inhibited *R. solani* on Min 3C (Table 2.10). Isolates exhibiting the greatest zones of inhibition were BA101, E69, E65, E21, E723, E721, E722, E729, and E730.

DISCUSSION

Approximately 5% of the bacterial isolates from tobacco field soils, 13% of the endophytic isolates, and none of the rhizosphere isolates from tomato root tissue controlled damping-off and stem rot of tomato seedlings caused by *R. solani*. Typically, 10 % of potential isolates screened for biocontrol activity are effective (Fravel, 1988). However, compared to the isolates from tobacco field soil and rhizosphere colonists, a higher percentage of endophytic isolates decreased disease severity. Since endophytic isolates have a greater impact on disease severity, for antagonists against *R. solani* should focus on root endophytes

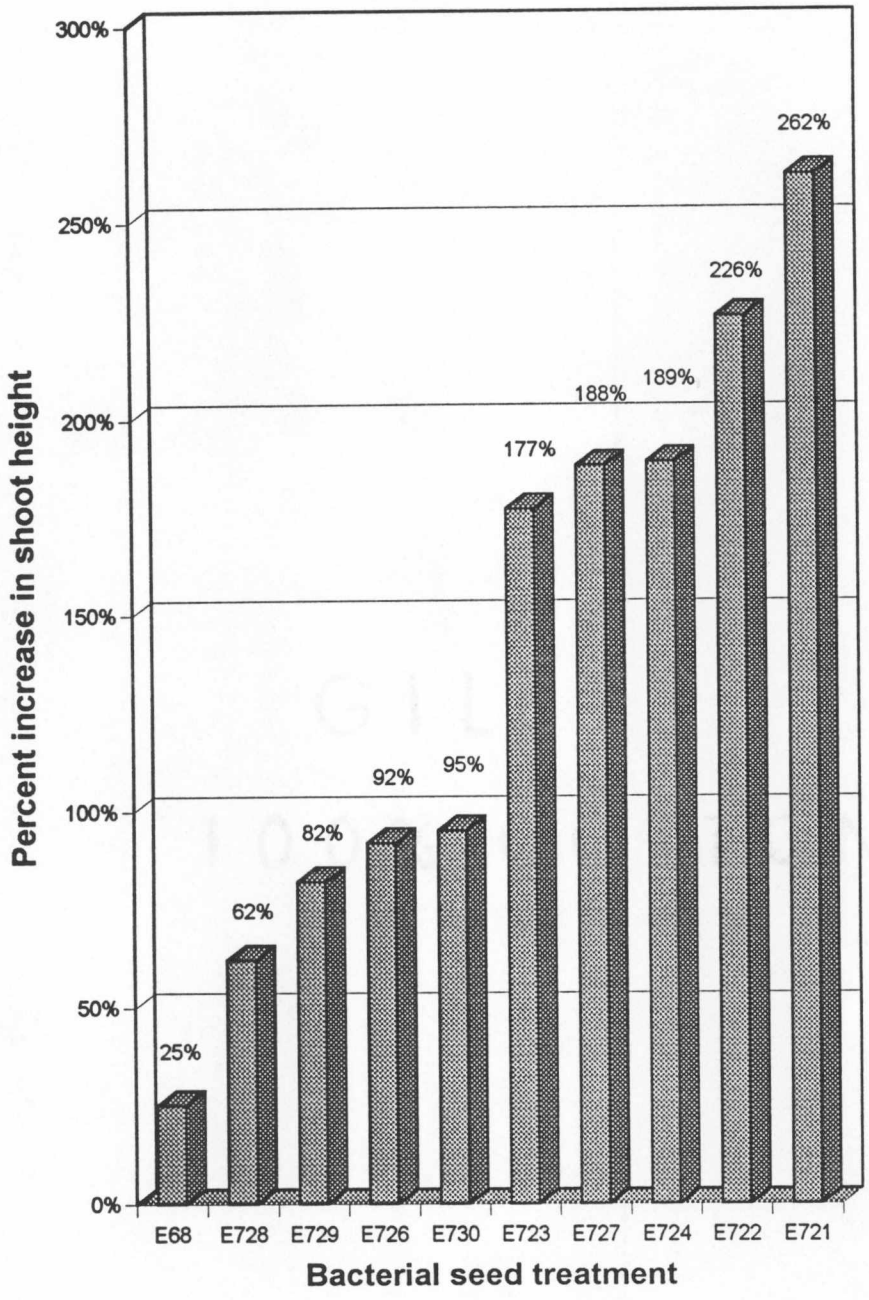

Figure 2.4. Percent increase in shoot height as a result of tomato seed treated with endophytic bacteria.

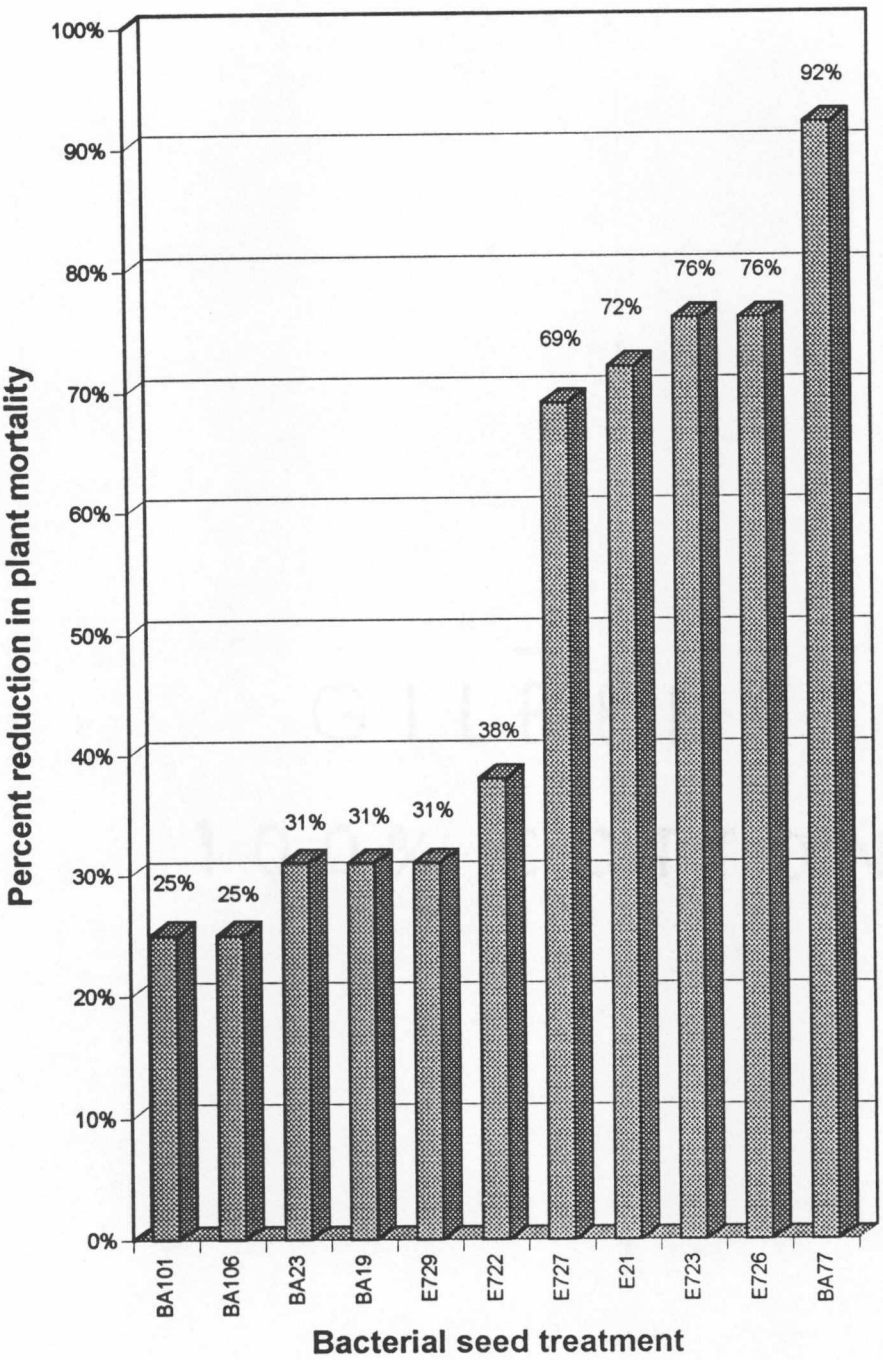

Figure 2.5. Percent reduction in plant mortality of tomato seedlings from bacteria-treated seeds.

Table 2.10. Antibiosis activity of tobacco field soil, endophytic and rhizosphere isolates on Minimal 3C agar against *R. solani*.

Bacterial Isolates[y]	Minimal 3C agar[z]
BA23	-
BA19	-
BA106	-
BA101	++
BA77	-
E66	+
E69	++
E65	++
E61	+
E21	Nd
E727	+
E723	++
E726	+
E726	+
E722	+
E724	++
E728	+
E729	++
E730	++
E68	+

[y]Bacteria with a 'BA' designation were isolated from tobacco field in Greenville, TN and those with 'E' designations were isolated as endophytes from tomato root tissue.

[z]The (-) represents no inhibition, (+) represents a mean inhibition zone less than 2 cm, and (++) represents a mean inhibition zone greater than or equal to 2 cm, and Nd represents not determined.

It is possible that a greater number of isolates with biocontrol potential would have been detected if other factors, such as temperature, inoculum potential, seed germination, potting mix, moisture content, and method of application had been altered. For example, seed germination decreased as the temperatures fluctuated in the greenhouse from January to March. Variability in soil moisture also may have affected seed germination. In many previous studies, sterilized soil or potting mixes were utilized to lessen competition between the potential biocontrol organism and indigenous microbial populations (Fravel, 1988). The potting mix was not sterilized in this study.

The age and amount of pathogen inoculum are also critical factors, because a pathogen must be viable to incite disease. In this study, inoculum of *R. solani* was not utilized if more than two weeks old. The amount of inoculum utilized in the assays was assumed to be greater than the level of *R. solani* found in natural soils; therefore, disease severity might be reduced even more if the isolates were applied to seeds and transplanted to field soil.

In some cases, disease control was accompanied by a significant increase in shoot height of tomato. Bacterial biocontrol agents may improve growth by suppressing either major or minor pathogens (Fravel, 1988). Major pathogens produce the well-known root or vascular diseases with obvious symptoms (Salt, 1979; Fravel, 1988). Minor pathogens are parasites or saprophytes that damage mainly juvenile tissue such as root hairs and tips, and cortical cells (Salt, 1979; Fravel, 1988), and the disease symptoms are not obvious. For this reason,

beneficial root-associated bacteria with an increase shoot height are referred to as plant growth promoting rhizobacteria (PGPR) (Fravel, 1988).

PGPR are thought to improve plant growth by colonizing the root system and preempting the establishment of, or suppressing, deleterious rhizosphere microorganisms (DRMO) on the roots (Schroth and Hancock, 1981; Schroth et. al., 1982; Suslow and Schroth, 1982; Fravel, 1988). Studies in the Netherlands suggest that PGPR promote potato growth primarily by suppressing cyanide-producing DRMO (Fravel, 1988). Other studies suggest that PGPR promote growth by stimulating plant hormones which in turn, increase shoot height (Kloepper et al., 1991). The biocontrol candidates in this study that increased shoot height should be further studied for production of plant hormones.

While antibiosis may not be the sole mechanism of action involved, it does appear to be a contributing factor, particularly with the endophytic isolates. Antibiosis was tested on both a nutrient depleted (Min 3C) agar and a nutrient rich (NBY) agar. The endophytic isolates inhibited *R. solani* on Min 3C, a nutrient depleted agar. Nutrient-limiting conditions stimulate antibiotic production.

Additional mechanisms such as competition for nutrients and induced resistance should be considered for future studies. Understanding the mode of action of biocontrol candidates might enable us to improve the inconsistencies in performance sometimes demonstrated by biocontrol agents.

CHAPTER III

CHARACTERIZATION OF BACTERIAL BIOCONTROL CANDIDATES

Bacteria shown or thought to have potential for biocontrol activity occur in many genera, including *Agrobacterium*, *Erwinia*, *Pseudomonas*, and *Bacillus*. Bacilli are screened on a wide variety of plant species for the ability to control disease. They are appealing candidates for biocontrol because they produce endospores that are tolerant to heat and desiccation and can survive inside plants.

Bacillus subtilis is a well-known biocontrol agent that can control green mold, sour rot, stem end rot of citrus (Sholberg et al., 1995), damping-off of sugar beet (Utkhede, 1984), and apple crown rot (Sholberg et al., 1995). The antagonist, *B. subtilis*, improves plant growth by suppressing major and minor pathogens and possibly by directly stimulating plant growth (Sholberg et al., 1995). *B. subtilis* is a common endophytic bacterium.

Utkhede and Rahe (1983) studied *B. subtilis* and its effect on *Sclerotium cepivorum* Berk. which causes onion white rot. The authors found that *B. subtilis* significantly reduced symptoms of onion rot and increased yield of the susceptible cultivar, Autumn Spice. Kommedahl and Ocamb (1994) investigated the effect of *B. subtilis* on kernels of corn. They inoculated seeds with *B. subtilis* and observed reduced disease severity caused by *Collectotricum globosum* for one year.

Additional species of *Bacillus* have been studied for biocontrol activity. Broadbent (1971) isolated potential biocontrol candidates from different soils throughout the United States, and found several biocontrol candidates in the genus Bacillus including *B. subtilis, B. megaterium, B. cereus, B. pumilis, B. polymyxa*, and *B. badius*. Some strains of *B. cereus* grow rapidly on rice, spices, meat, egg, and dairy products, but other strains are biocontrol agents. *B. cereus* UW85 suppressed damping-off caused by *Phytophthora megasperma* f. sp. *medicaginis* of alfalfa seedlings (Handelsman et al., 1990). Furthermore, *B. cereus* UW85 decreased severity of damping-off of tobacco and alfalfa (Handelsman et al., 1990). The population biology of *B. cereus* UW85 was investigated in the rhizosphere of soybeans grown in the field. The spores germinated on seeds, colonized roots, and persisted until harvest (Handelsman et al., 1993).

Bacillus spp. are Gram-positive, rod-shaped (0.5-2.5 x 1.2-10 μm), in length), and often are arranged in pairs or chains, with rounded or squared ends. The cells are motile by peritrichous flagella and form oval-shaped endospores, one per cell. Sporulation is not suppressed by exposure to air. Catalase production is the distinguishing biochemical reaction, which differentiates the genus *Bacillus* from the other endospore-forming genera (Schaad, 1988).

There are many commonly used methods for identifying unknown bacterial isolates. The first step is to determine morphological characteristics. Bacteria are differentiated on the basis of cell wall structure, which is revealed by the Gram-stain reaction. An insoluble crystal violet-iodine complex of the Gram-

stain reaction is formed inside cells and this complex is extracted by alcohol from Gram-negative but not from Gram-positive bacteria (Brock and Madigan, 1990). The alcohol dehydrates Gram-positive bacteria, which have thick cell walls consisting of several layers of peptidoglycan. Thus, the pores in the cell wall close preventing the crystal violet-iodine complex from permeating the cell wall. The alcohol dissolves freely in Gram-negative bacteria, which have a thin peptidoglycan layer, and penetrates the outer layer of the cell wall. As a result, the Gram-negative does not retain the crystal-violet iodine complex. A second stain, safranin is applied to adhere to the cell wall of Gram-negative bacteria. Biochemical tests aid in the identification and characterization of unknown microorganisms and may aid in deterimining the mechanism of suppression by a biocontrol strain. For example, digestion of chitin by a biocontrol candidate indicates that chitinase production is a potential biocontrol mechanism.

In addition to individual biochemical tests, commercial identification systems, such as the Biolog Identification System, are available. The Biolog Identification System is based on the ability of a microorganism to utilize or oxidize a pre-selected panel of different carbon sources. The Biolog plates yield a characteristic pattern of purple wells, which constitutes a "metabolic fingerprint" of the inoculated organism. The objective of this study was to identify and characterize the effective biocontrol candidates with biological stains, biochemical tests, and the Biolog system.

MATERIAL AND METHODS

Standard Biological Stains. Gram, spore, and flagella stains were used to identify Gram-positive and Gram-negative bacterial cells, spore positions, and positions of flagella (polar, bipolar, or peritrichous flagella). Gram-stains were prepared by spreading a thin layer of bacterial cells across glass microscope slides to air dry. The air-dried bacterial cells were heat-fixed to the slides by lightly flaming the underside of each slide. The slides were covered with crystal violet solution for 1 min, then rinsed with tap water. Iodine solution was applied to slides for 1 minute then rinsed with tap water. Gram's decolorizer solution was applied to the slides for 10-15 sec. After rinsing with water, the slides were flooded with safranin solution. Then slides were blotted dry, observed with an oil immersion lens at 1000x.

The protocol to stain spores of bacterial cells required 5% malachite green and 0.5% aqueous safranin. A loopful of bacterial cells was suspended in a drop of water on a glass microscope slide. After air-drying the slides were flooded with malachite green for 10 min and rinsed with tap water. The slides were flooded with safranin solution for 15 sec and rinsed with tap water. Lastly the slides were blotted dry and observed under oil immersion 1000x magnification.

The procedure to stain the flagella of bacterial cells required tannic acid solution [(5 g tannic acid, 1.5 ferric chloride, 2 ml formalin (15%), 2 ml sodium hydroxide (1%), in 90.5 ml of distilled water) (Schaad, 1988)] and ammoniated silver nitrate solution [(100 ml of silver nitrate (2%) (Schaad, 1988)]. Bacterial

cells were suspended in a drop of water on microscope slides and air-dried. The cells were covered with tannic acid solution for 2 to 4 min and rinsed with distilled water. Ammoniated silver nitrate solution was applied to the slides for 30 sec and immediately rinsed with distilled water. Slides were blotted dry, and observed under 1000x oil immersion magnification.

Standard Biochemical Assays. Identifying and characterizing unknown isolates can be accomplished through a series of biochemical reactions. In this study the following biochemical tests were chosen to differentiate genera and species: anaerobic/aerobic growth, catalase production, utilization of mannitol, arabinose, xylose, and citrate, protein digestion, chitin utilization, starch hydrolysis, production of hydrogen cyanide, and salt tolerance. Unless otherwise stated, chemical reagents were purchased from Sigma Chemical Co., St. Louis, MO, and incubations were performed at room temperature (25 C).

Anaerobic growth was determined by inoculating the bacterial isolates in glucose broth. The broth was covered with sterile mineral oil. Turbidity in the broth layer signified a positive reaction for anaerobic growth (Schaad, 1988).

Detection of catalase production separates *Bacillus* spp. from other endospore-forming bacteria. Catalase is the decomposition of H_2O_2 to H_2O and O_2 gas. The catalase reaction prevents the toxin, H_2O_2, from destroying bacterial cells by utilizing the H_2O produced from the reaction. Catalase production was determined by incubating bacteria on nutrient agar slants [8 g nutrient broth (Difco Laboratories, Detroit, MI) and 15 g agar in 1000 ml of deionized H_2O (Schaad, 1988)] until growth was visible. Then drops of 3 % H_2O_2 were placed

on the bacterial colony. If the H_2O_2 effervesced (O_2 generation) the reaction was positive for catalase production.

To determine if the unknown bacterial isolates yielded acid from arabinose, xylose, and mannitol, a basal medium [1.0 g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 12 g purified agar, 1 ml bromthymol blue, and 15 agar in 900 ml deionized water (Schaad, 1988)] was prepared for each carbon source. Each carbohydrate solution was transferred to the basal medium and the mixtures were poured into petri dishes. The unknown bacterial isolates were inoculated on each medium and observed for color change. Media color change was an indication of acid production.

Protein hydrolysis was investigated on yeast nutrient agar (YNA) and skim milk. The medium contained three solutions: 4 g nutrient broth, 4 g agar in 500 ml deionized water; 5 g yeast, 7.5 g agar in 900 ml of deionized water; 10 g skim milk in 100 ml of deionized water (Schaad, 1988). The skim milk solution was added to the yeast and agar mixture. A thin layer of the nutrient agar was added to the petri dishes and allowed to solidify. Then, a layer of the YNA + skim milk mixture was added to the petri dishes. The bacterial isolates were spot inoculated and observed for a clear zone around the colonies after 3, 5, and 7 days.

Citrate utilization was determined on sodium citrate agar [5 g NaCl, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 1 g $NH_4H_2PO_4$, 1 g K_2HPO_4, 2 g anhydrous sodium citrate, 20 g agar, 15 ml of soluble (0.2 g + 20 ml ethanol) bromythymol blue in 1000 ml deionized water (Schaad, 1988)]. The pH of the medium was adjusted to 6.8

before autoclaving. The agar medium was slanted in test tubes to solidify. The bacterial isolates were inoculated in the tubes and incubated for approximately 4 days. A positive reaction for citrate utilization was evident by color change of the agar slants from green to blue.

To determine the amount of salt the bacterial isolates could tolerate, growth on 7% Nutrient Broth Salt Agar [8 g nutrient broth, 7 g NaCl, 15 g agar in 1000 ml deionized water (Lelliot and Stead, 1987)] was observed. Bacterial cells were inoculated on the medium and incubated for 3 days. Growth on the medium indicated a positive reaction.

To test for the ability of the bacterial isolates to hydrolyze starch, bacterial cells were streaked on starch hydrolysis medium [13 g nutrient broth, 2 g soluble starch, 15 g agar in 1000 ml of deionized water (Schaad, 1988)]. The plates were incubated for approximately 7 days or until bacterial growth. The plates were flooded with potassium iodide. Starch hydrolysis was evident by clear zones in the black-stained medium around the colonies.

The cell walls of many fungi are partially composed of chitin. Therefore, the ability to hydrolyze chitin is an important feature of potential biocontrol agents. Chitin medium [10 g ball-milled chitin, 1 g $MgSO_4 \cdot 7\ H_2O$, 2 g K_2HPO_4 (Schaad, 1988)] was prepared to test the ability of bacterial isolates to hydrolyze chitin. The bacterial isolates were spot inoculated on the medium, and incubated for approximately 3 days. Clear zones around the colonies indicated a positive reaction.

Hydrogen cyanide (HCN) is an anti-fungal metabolite produced by microorganisms. To determine if the bacterial isolates produced HCN, filter paper strips were immersed in (13 %) picric acid for 48 h and dried under a laminar flow hood. The picric acid impregnated filter strips were attached with Velcro to the cover of petri dishes inoculated with the bacterial isolates growing on NBY (Vidaver, 1967) agar. After 7 days of incubation at 28 °C, the filter paper strips were observed for color change. A color change of the filter paper strips from yellow to orange indicated a positive reaction.

Biolog Bacterial Identification System. The Biolog MicroPlates™ contain carbon sources that are pre-filled and dried into 96-well plates. Tetrazolium violet is used as a redox dye to colorimetrically indicate utilization of the carbon sources. After characterizing the bacterial isolates with the preliminary biochemical reactions, the Biolog system provides a system for species identification.

Gram-positive isolates were grown on a Biolog Universal Growth Medium (BUGM + 1 % glucose) from 12 to 18 h at 28 °C (Biolog, Inc., 1994), and the Gram-negative organism was grown on Tryptic Soy Medium (TSA). Isolates were incubated from 12 to 18 h at 28 °C. A uniform suspension of inoculum of each isolate was prepared by removing the cells from the agar plates with a sterile cotton swab and dipping the swab into sterile saline solution. Bacterial cells were suspended in the saline solution until a concentration of approximately 3×10^8 cells/ml for Gram-negative isolates and 4.5×10^8 cells/ml for Gram-positive

isolates was established. The MicroPlates™ were inoculated with an 8-Channel Repeating Pipette and incubated at 28 °C for 4 to 24 h.

The results were interpreted using the MicroLog computer software. Purple wells were interpreted as positive reactions and wells visually resembling the A-1 well were interpreted as negative reactions. Wells with extremely faint color, or with small purple flecks or clumps, were scored as borderline.

RESULTS

Standard Biochemical Stains and Assays (Tables 3.1 and 3.2). The tobacco field soil isolate BA106 was a Gram-negative bacterium. Tobacco field soil isolates BA77, BA19, BA23, and BA101 were Gram-positive bacteria. Endophytic bacterial isolates were Gram-positive. With the exclusion of BA106 (polar flagellum), the tobacco field soil isolates had peritrichous flagella. Isolate BA106 did not contain spores, but the remaining tobacco field soil and endophytic isolates produced centrally located spores.

Catalase production was observed on the agar slants of tobacco field soil isolates BA77, BA19, BA23, and BA101, but not BA106. The endophytic isolates were catalase positive. Bacterial isolates E726, BA77, BA19, E61, and E65 multiplied anaerobically. Starch hydrolysis was negative for isolate BA106, and positive for isolates BA77, BA19, BA101, BA23, and the endophytic isolates. Bacterial isolates BA101, E61, BA106, E69, and E66 utilized citrate on sodium citrate agar. Both the endophytic isolates and the tobacco field soil isolates were tolerant of 7% NaCl.

Table 3.1. Characterization of tobacco field soil isolates.

Tobacco field soil isolates	BA77	BA19	BA23	BA106	BA101
Gram stain	+[z]	+	+	-	+
Anaerobic growth	+	+	-	-	-
Flagella stain					
Peritrichous	+	+	+	-	+
Polar	-	-	-	+	-
Bipolar	-	-	-	-	-
Spore stain					
Central spore	+	+	+	-	+
Acid production					
Mannitol	-	-	+	+	+
Arabinose	-	-	+	+	-
Xylose	-	-	-	+	+
Salt tolerance (7 % NaCl)	+	+	+	+	+
Citrate utilization	-	-	-	+	+
Starch hydrolysis	+	+	+	-	+
Catalase production	+	+	+	-	+
Protein digestion	+	+	+	+	+
Chitin hydrolysis	+	+	+	+	+
HCN production	-	-	-	-	-

[z](+) designates a positive reaction and (-) designates a negative reaction

Table 3.2. Characterization of endophytic isolates.

Endophytic isolates	**E726**	**E727**	**E723**	**E69**	**E61**	**E66**	**E65**	**E21**
Gram stain	+[z]	+	+	+	+	+	+	+
Anaerobic growth	+	-	-	-	+	-	+	-
Flagella stain								
Peritrichous	+	+	+	+	+	+	+	+
Polar	-	-	-	-	-	-	-	-
Bipolar	-	-	-	-	-	-	-	-
Spore stain								
Central spore	+	+	+	+	+	+	+	+
Acid production								
Mannitol	+	+	+	+	+	+	+	+
Arabinose	-	-	-	-	-	-	-	-
Xylose	+	+	+	+	+	+	+	+
Salt tolerance (7 % NaCl)	+	+	+	+	+	+	+	+
Citrate Utilization	-	-	-	+	+	+	-	-
Starch hydrolysis	+	+	+	+	+	+	+	+
Catalase production	+	+	+	+	+	+	+	+
Chitin hydrolysis	+	+	+	+	+	+	+	+
Protein digestion	+	+	+	+	+	+	+	+
HCN production	-	-	-	-	-	-	-	-

[z] (+) designates a positive reaction and (-) designates a negative reaction.

Acid production was observed on mannitol medium with the tobacco field soil isolates BA101, BA23, BA106, and all of the endophytic isolates. Bacterial isolates BA19 and BA77 did not produce acid on mannitol medium. Acid production was detected on arabinose medium with the tobacco field soil isolates BA23 and BA106, but the remaining endophytic and tobacco field soil isolates did not produce acid on arabinose medium. Acid production on xylose medium was detected with the endophytic and tobacco field soil isolates, with the exceptions of BA77, BA19, and BA23.

Digestion of protein was observed on YNA + skim milk with both the tobacco field soil and the endophytic isolates. Tobacco field soil and endophytic isolates were positive for chitin hydrolysis. Neither the tobacco field soil isolates nor the endophytic isolates produced hydrogen cyanide.

Bacterial Biolog Identification System (Table 3.3). According to the Biolog Identification System, Gram-positive bacterial isolates were identified as *Bacillus*, and the Gram-negative bacterium was identified as *Pseudomonas corrugata* (similarity index = 0.586). The similarity index of isolates BA77, BA101, BA19, BA23, E726, E66, E69, E65, E723, and E21 ranged from 0.500 to 0.812, and included *B. thuringiensis/cereus*, *B. megaterium*, *B. pasteurii*, *B. azotoformans*, *B. subtilis* var. *globigii*, *B. coagulans*, and *B. brevis*. Two isolates, E727 and E61, had similarity indices < 0.500 and were not identified.

DISCUSSION

Characterization and identification of the potential biocontrol isolates is

Table 3.3. Identification of tobacco field soil and endophytic isolates with the Biolog System.

Bacterial Isolates	Similarity Index	Identified Isolates
BA77	0.631	*Bacillus thuringiensis/cereus*
BA101	0.793	*Bacillus megaterium*
BA106	0.586	*Pseudomonas corrugata*
BA19	0.518	*Bacillus pasteurii*
BA23	0.611	*Bacillus azotoformans*
E726	0.505	*Bacillus subtilis* var. *globigii*
E727	0.388[y]	*Bacillus coagulans*[z]
E66	0.500	*Bacillus pasteurii*
E61	0.483[y]	*Bacillus megaterium*[z]
E69	0.662	*Bacillus coagulans*
E65	0.668	*Bacillus megaterium*
E723	0.812	*Bacillus brevis*
E21	0.706	*Bacillus brevis*

[y]Similarity index <0.5 is not an accurate identification of species
[z]Closest match but not considered a valid identification.

crucial information to determine the possible mechanism by which bacterial isolates inhibit *R. solani* and for future studies. The biological assays identified morphological features, and the biochemical reactions characterized possible biocontrol activity and physiology of the bacterial isolates. The Biolog Identification system identified the genus and species of the bacterial isolates.

Isolate BA106 was identified as *Pseudomonas corrugata*. This is consistent with its Gram-negative stain reaction, polar flagella, lack of endospore production, and inability to grow anaerobically. *P. corrugata* strain BA106 produced acid on mannitol, arabinose, and xylose, tolerated 7% NaCl, utilized citrate, digested protein and decomposed chitin. According to Schaad (1988), *P. corrugata* is normally negative for acid production on arabinose. Isolate BA106 was also negative for starch hydrolysis, catalase production, and hydrogen cyanide production. Catalase production is characteristic of strictly aerobic bacteria and should have been detected in BA106.

Isolates of *P. corrugata* can cause tomato pith necrosis. However, some strains have been studied as biocontrol agents. For example, *P. corrugata* biologically controlled brown rot of nectarines and peaches caused by *Monilinia fructicola* (G. Wint.) Honey (Smilanick et al., 1993), green mold of lemons (Smilanick and Denis-Arrue, 1992), and *Fusarium* dry rot (Schisler et al., 1997).

Bacterial isolates BA77, BA101, BA19, BA23, E726, E66, E69, E65, E723, and E21 were identified as *Bacillus thuringiensis/cereus*, *B. megaterium*, *B. pasteurii*, *B. azotoformans*, *B. subtilis* var. *globigii*, *B. pasteurii*, *B. coagulans*, *B.*

megaterium, *B. brevis*, and *B. brevis*. There are reports on *B. azotoformans, B. megaterium*, *B. cereus*, *B. brevis*, and *B. subtilis* as biocontrol agents. *B. azotoformans* biologically controlled root rot of avocado seedlings (Duvenhage and Kotze, 1993). *B. megaterium* decreased disease severity of root rot caused by *Phytophthora cinnamomi* Rands (Duvenhage and Kotze, 1993). Many reports have focused on *B. subtilis* as a biocontrol agent such as, biocontrol of *R. solani* on cotton (Fiddaman and Rossall, 1993), and biocontrol of *R. solani* on wheat (Wilhelm, 1973).

Ordinarily, strains of *Bacillus brevis*, *B. pasteurii*, and *B. azotoformans* are strict aerobes (Schaad, 1988; Sonenshein and Losick, 1993). Strains of *B. thuringiensis/cereus*, *B. megaterium*, and *B. subtilis* are able to grow at least weakly in the absence of oxygen (Sonenshein and Losick, 1993). *B. coagulans* grows anaerobically (Sonenshein and Losick, 1993). As expected, *B. pasteurii* E66, *B. azotoformans* BA23, *B. brevis* E723, and *B. brevis* E21 did not replicate anaerobically; however, *B. pasteuri* BA19 replicated in glucose broth anaerobically; suggesting that BA19 is a facultative anaerobe. *B. coagulans* E727 and E69, *B. thuringiensis/cereus* BA77, *B. subtilis* E726, *B. megaterium* E61, and E65 also multiplied in glucose broth anaerobically, while *B. megaterium* BA101 did not.

Generally, some strains of *B. pasteurii* have limited ability to produce acid from sugars (Sonenshein et al., 1993). *B. azotoformans* and *B. brevis* do not produce acid from any sugars (Sonenshein et al., 1993), and *B. cereus* does not produce acid from mannitol (Schaad, 1988). Concurring, *B. thuringiensis/cereus*

BA77 and *B. pasteuri* BA19 did not produce acid from mannitol. In contrast to additional reports, *B. brevis* E723 and E21 produced acid from mannitol and xylose, and *B. azotoformans* BA23 produced acid from mannitol and arabinose. Leary and Chun (1988) discovered that *B. brevis* produced acid from mannitol and xylose variably. Acid production from xylose was observed with each species except *B. thuringiensis/cereus* BA77 and *B. pasteurii* BA19.

Leary and Chan (1988) found that *B. coagulans* and *B. brevis* could not tolerate 7% NaCl. In contrast, in this study *B. coagulans* E69 and *B. brevis* E723 and E21 tolerated 7% NaCl. As expected, *B. megaterium* BA101, *B. coagulans* E69, *B. megaterium* E61, and *B. pasteurii* E66 utilized citrate. All of the Gram-positive isolates produced catalase.

To determine how the bacterial isolates suppressed disease caused by *R. solani*, it is important to know if the bacterial species digest protein, hydrolyze chitin, and/or produce hydrogen cyanide. All of the biocontrol isolates were able to digest protein and break down chitin of fungi. However, neither the *Pseudomonas* sp. nor the *Bacillus* spp. produced the anti-fungal metabolite hydrogen cyanide.

Isolates E727 and E61 had similarity indexes less than 0.5, therefore further study is needed to identify these isolates. Compared to previous studies minor differences in biochemical reactions were observed.

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