BIOLOGICAL CONTROL OF SEPTORIA TRITICI BLOTCH

By

FERIDOON MEHDIZADEGAN
Bachelor of Science in Animal Science
Pahlavi University
Shiraz, Iran
1978

Master of Science in Teaching
Tarleton State University
Stephenville, Texas
1980

Doctor of Philosophy in Animal Science
University of Arkansas
Fayetteville, Arkansas
1983

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 1990

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Thesis Approved:

Francis J. Gough
Thesis Adviser

Jacqueline Fletcher

Robert M. Hunger

Margaret K Essenberg

Norman N. Durham
Dean of the Graduate College

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Dr. Francis J. Gough, Major Adviser, for his thoughtfulness, constant guidance and support throughout this study. Appreciation is also expressed to the members of the advisory committee, Dr. Hassan A. Melouk, Dr. Robert M. Hunger, Dr. Jacqueline Fletcher, and Dr. Margaret Essenberg, for giving of their expertise during this study.

My special gratitude is expressed to my wife Farzaneh Katkhordeh and my daughter and son, Iden and Abtin, for their understanding and patience throughout this study.

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CHAPTER I

GENERAL INTRODUCTION

This dissertation is arranged into three chapters. Chapter I consists of an introduction and a statement of the general research objectives. Chapter II, entitled "Biological Control of Septoria tritici by Bacterial Antagonists", discusses the methods and results of the research effort. It is presented in the format and style of papers published in Phytopathology, the official journal of the American Phytopathological Society. Chapter III consists of an appendix.

Introduction

Septoria tritici Rob. ex Desm. (perfect state = Mycosphaerella graminicola (Fuckel) Schroeter causes septoria tritici blotch (syn. septoria leaf blotch, speckled leaf blotch) of wheat. Septoria tritici blotch occurs in most major wheat producing areas of the world. The disease attacks wheat in all stages of development and under favorable conditions may cause up to 70% loss of foliage (3,4). Grain yield losses seldom exceed 10-20% (1,2,3), but they are often accompanied by a corresponding decrease in kernel weight (2).

Wheat occupies approximately 20% of the world crop land, provides about 20% of all human food calories (11) and is the staple food for nearly 40% of the world population (10). It has been estimated that increases in world population and changing food habits could cause world wheat consumption to double between 1980 and 2005. A two-fold increase in grain production in 25 years requires an annually compounded increase of about 3.25%. Between 1950-1977, wheat production in the U.S. more than doubled. Yield increases (67%) were more responsible than added crop acres (32%) for the increased productivity (5). From 1961 to 1971 wheat yields increased about 30% (23.9 to 33.9 bu per acre) (5) for an annually compounded rate of about 3.5%. From 1971 through 1982, the U.S. yield increased about 4.8% (33.9 to 36.6 bu per acre) for an annually compounded rate of about 0.5%.

Lowering of the production trend has caused speculation that yields have reached a plateau and can be expected to decline. Thus, development of cost effective methods to prevent or suppress yield reducing diseases could contribute significantly to increasing total wheat production.

Genes in wheat for resistance to septoria tritici blotch do not usually condition high levels of resistance, and the current cash value of the crop does not justify the cost of chemical treatment. Furthermore, most chemicals used to control pathogens and pests are nonspecific in the

variety of organisms they affect. This may alter the microbial balance with usually unknown side effects. The targeted disease organism may be suppressed by these chemicals but at the expense of eliminating harmless and/or beneficial saprophytes with which they are in competition. This may result in dominance of some nontarget phytopathogenic microorganisms. Use of biocontrol agents in conjunction with plant resistance may provide an equivalent level of control with less adverse impact upon the environment than the use of chemicals.

The buffering capacity of the phylloplane microflora against pathogenic microorganisms has been known for years (6), but biological control of pathogens of aerial plant parts has been less studied than control of those in the soil rhizosphere. This is partially due to availability of foliar fungicides, whose effectiveness against diseases of high-cash crops, ease of application, and reliability under environmental extremes discouraged the use of biological agents to control the foliar pathogens. Nevertheless, recent exploration of foliar microflora and their impact on disease incidence has stimulated an interest in managing phylloplane microflora to reduce the need for fungicides. To date, there have been only three short reports on efforts to identify microorganisms inhibitory to infection of wheat by S. tritici (8,9,10).

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CHAPTER II

BACTERIAL ANTAGONISTS TO *SEPTORIA TRITICI*

Abstract

Four bacterial antagonists to *Septoria tritici* were isolated from soil of wheat fields and from wheat leaves. Two bacteria isolated from soil were identified as *Bacillus subtilis* and *B. pumilus* and two isolated from leaves as *B. subtilis* and *Pseudomonas fluorescens*. All four bacteria and their cell-free culture filtrates inhibited the growth of *S. tritici* on V-8 agar medium. Application of these antagonist to wheat seedlings in greenhouse tests significantly reduced infection by *S. tritici*. Populations of *P. fluorescens* declined sharply when applied to wheat seedlings 24 hr before, simultaneously, or 24 hr after inoculation with *S. tritici* in a 6-day study under greenhouse condition. *P. fluorescens* reduced the incidence of septoria tritici blotch in all cases, but was most effective when applied simultaneously with *S. tritici* inoculation. Application of *Bacillus* spp. and *P. fluorescens* in the field reduced natural infection by *S. tritici*. Approximate molecular weights of active compounds were, 11,500, 34,000, and 26,000 for *P. fluorescens*, *B. subtilis* and *B. pumilus*, respectively.

Introduction

Septoria tritici blotch caused by Mycosphaerella graminicola (Fuckel) Schroeter, (anamorph, Septoria tritici Rob. ex Desm.), occurs in most countries where wheat is grown. Losses caused by the disease seldom exceed 10-20% (8,14,24,45) although yield reductions up to 40% (14) and even 70% (20) have been reported. Chemical control of septoria tritici blotch has not been practiced extensively in the major wheat production areas of the United States because the return on investment in most years is only marginally, if at all, profitable. In recent years, breeding for resistance has intensified at many state, federal, and international crop improvement centers, but the levels of resistance have seldom, if ever, been fully successful. Accumulating data indicates that innocuous epiphytic microorganisms on leaf surfaces play important roles in the suppression of several plant diseases (4,43). If microorganisms which suppress development of septoria tritici blotch were sustained on wheat leaves in the field during periods favorable for blotch development, they would enhance the value of partial plant resistance by slowing the rate of infection, and they would serve as a second line of defense if plant resistance were to become ineffective due to virulence shifts in S. tritici.

Objectives of this study were: 1) to identify bacteria inhibitory to in vitro growth and reproduction of S. tritici; 2) to determine if antagonistic bacteria reduce

septoria tritici blotch under greenhouse and field conditions; and 3) to partially characterize the inhibitory compounds produced by the bacteria.

Materials and Methods

The Pathogen. A culture of S. tritici (St83A) was used in tests to determine the antagonistic activity of several bacteria isolated from soil and wheat leaves. Culture St83A characteristically budded copiously for 3-5 days before producing mycelium when inoculated onto agar media with high concentrations of conidia (10^6/ml) in water. This culture produced conidia in "pseudopycnidia" on media when inoculated by point or streak inoculations with a needle or dilute conidial suspension. Culture St83A, isolated in 1983 from wheat grown near Stillwater, Oklahoma, was obtained from stocks maintained at the Plant Science and Water Conservation Laboratory, USDA-ARS, Stillwater, Oklahoma.

Media. A V-8 juice (Campbell Soup Co., Camden, NJ) agar was used as a general nutrient medium, unless otherwise specified, in experiments involving S. tritici. The medium was prepared by mixing 177 ml of V-8 juice and 3.0 g of $CaCO_3$ in 820 ml of distilled water in a graduated cylinder and then placing the mixture in a refrigerator (4 C) until the solid material had settled (12-24 hours). The supernatant was collected, filtered through Whatman No. 1 paper, and 20 g of agar and distilled water were added to

make 1 L. The medium was autoclaved and poured into petri plates. Other commercial media (Difco Laboratories, Detroit, MI) including nutrient agar, potato dextrose agar (PDA), and Pseudomonas isolation agar were prepared as directed.

Isolation of Potential Antagonists from Soil and Leaves. Soil samples from the top 5 cm of fields that had been sown annually with wheat for several years were collected and stored at 5 C for 1-3 days. After being pulverized and thoroughly mixed within the plastic bags, 10 g of each soil sample was placed in 90 ml of sterile water and sonicated (Ultramet II, Buehler, Ltd. Evanston, IL 60204) for 30 seconds. Ten-fold serial dilutions to 10^{-7} of each soil suspension were prepared in water and 0.1 ml of each dilution was transferred to each of four petri plates containing approximately 20 ml of either V-8 juice agar, nutrient agar, PDA, or water agar. Following incubation for 48 hours at 30-32 C, single bacterial colonies were transferred to fresh V-8 agar and nutrient agar and maintained at 25 C until used.

Living and dead wheat leaves were collected during the spring of 1984 and 1985 when plants were in growth stages 7 through 10.1 (Feekes' scale adapted by Large) (29). Ten grams of live leaf tissue and 1 g of dead leaf tissue were sonicated for 30 sec in 90 and 9 ml of sterile water, respectively. Procedures for making serial dilutions and isolating colonies were similar to those used for soil

samples, except that in 1985 Pseudomonas isolation agar was used in addition to the V-8 and nutrient agar media used in 1984.

Selection of Bacterial Antagonists to S. tritici. More than 600 bacterial isolates from soil and leaf tissue were evaluated for their ability to inhibit growth of culture St83A. A 100 µl suspension of each isolate (approximately 10^7 cfu/ml) was placed at the center of V-8 juice agar in each of five petri plates and incubated at 25 C for 24 hr. Conidia of St83A, collected from actively budding flooded-plate cultures were suspended in sterile distilled water (10^5 conidia/ml) and sprayed aseptically onto each plate until the surface was wetted to near-running when the plates were tilted. The suspension was applied with a sterile atomizer connected to a pressure/vacuum pump (manufactured by General Electric, distributed by Fisher Scientific Co.). Air from the pump was forced (3.5 kPa) through a sterile cotton filter in a side-arm flask and thence through sterile tubing to the atomizer. After 7-8 days of incubation at 25 C, the plates were examined for inhibited fungal growth around the bacterial inoculation sites. Occurrence and width of inhibition zones were considered indicative of either the presence and relative effectiveness of antimycotic products generated by the bacteria, or as a measure of relative abilities by the bacteria to deplete the medium of metabolites essential for growth of St83A.

From among 12 cultures classed as antagonistic, two isolated from soil and one from leaf tissue in 1984 and one isolated from leaf tissue in 1985 were selected for this study.

Identification of Potential Antagonists. The four bacterial cultures were classified on the basis of cultural characteristics and physiological and biochemical tests described by Gibson and Gordon in Bergy's Manual (17). Two cultures that produced inhibition zones of similar size were tentatively identified as Bacillus subtilis and designated BS1 and BS2 (BS1 and BS2 were isolated from a leaf and soil, respectively). The remaining two cultures were tentatively identified as Bacillus pumilus (isolated from soil) and Pseudomonas fluorescens (isolated from leaf tissue) and were designated BP and PF. The cultures were sent to an independent laboratory (American Type Culture Collection, Rockville, MD) for confirmation of their taxonomic placement.

After the bacteria were identified, they were processed and placed in long and short-term storage. For long-term storage, 0.5 ml of each bacterium suspended in nutrient broth or liquid King's medium B (KMB) were added to 0.5 ml of non-fat milk, lyophilized, and stored at 0 C. For short-term storage, the cultures were placed on nutrient agar and KMB agar slants, as appropriate, and stored at 5 C.

Fungicidal vs. Fungistatic Activity. To determine if growth inhibiting products generated by each bacterium were fungicidic or fungistatic, three plugs of agar (6 mm diameter) were removed from the inhibition zone surrounding the bacterial colony and transferred to V-8 agar in a petri plate. Also, three similar plugs of sterile V-8 agar were transferred to the same plate. A 25 µl suspension of conidia from a 7-day old culture of St83A was deposited on the top of each plug. After 7 days of incubation at room temperature, presence of growth of St83A on the plugs from the inhibition zones was compared to that on the sterile agar plugs within the same petri plate. The plugs were then inverted to place their inoculated surfaces against the medium in the plate, incubated another 7 days, and then examined for growth of St83A around each plug.

Nutrient Base and Growth Inhibition. The inhibitory activity of each selected bacterium was compared in each of four petri dishes on V-8 agar, nutrient agar, potato dextrose agar, and on each medium diluted to one-half strength but with the agar content kept constant. The method of inoculating the media with St83A and the bacteria was the same as described above. Similarly, width of inhibition zones was the basis of evaluating nutrient effects.

Effect of Cell-Free Filtrates on Growth of St83A. The four bacterial antagonists were grown at 25 C in 50 ml of

either nutrient broth or liquid KMB (depending upon the culture) on a rotary shaker (100 rpm). After 72 hours, the cultures were centrifuged for 15 minutes at 10,000 g to pellet the bacterial cells. The near-cell free supernatant medium was filtered through 0.22 μm pores in nitrocellulose membranes and then lyophilized. To assure that the antagonistic integrity of each lyophilized filtrate had been maintained, a sample of each concentrate was dissolved in 2.0 ml of 0.05 potassium phosphate buffer (pH 7.0) and 200 μl was placed in 1 cm diameter holes cut in one-quarter strength V-8 agar in each of four petri plates. The agar was then sprayed with a conidial suspension (10^5 conidia/ml) of St83A, incubated for 7 days at 25 C and checked for the presence of inhibition zones. Once antimycotic activity of lyophilized filtrates was established, percentage dilutions from 0 to 100% in 10 unit increments were made for each filtrate. From each dilution, 20 μl were transferred to 3 mm diameter holes cut in one-quarter strength V-8 agar in petri plates. The agar was sprayed with a conidial suspension of St83A. The diameter of growth inhibition zones around the holes after 7 days of incubation was used as the measure of antimycotic activity in each filtrate dilution.

Infection of Greenhouse Grown Wheat by St83A in the Presence of Antagonistic Bacillus cultures. The effects of bacterial cultures BS1 and BP on infection of juvenile wheat by St83A in the greenhouse were studied in 1984 and

1985, and similarly the effect of culture PF was studied in 1985 and 1986. Since Bacillus cultures designated BS1 and BS2 were similar in suppressing growth of St83A in vitro, BS1 was chosen for this test because it was isolated from leaf tissue (although it presumably represented a transient presence on the leaf sample rather than an inhabitory one).

Winter wheat (cv. TAM W-101) susceptible to Septoria tritici was grown in pasteurized soil in pots (10-12 plants/7.5 cm pot) in the greenhouse. When plants reached the second to third leaf stage, they were separated into four lots of five pots each, and each lot was subjected to one of the following treatments:

a) Sprayed with culture BS1 and then inoculated with culture St83A;

b) Sprayed with culture BP and then inoculated with culture St83A;

c) Sprayed with sterile V-8 liquid medium in volume equivalent to that of the bacterial suspensions and then inoculated with culture St83A; and

d) Inoculated with culture St83A.

Three and 7 days prior to applying the treatments, fresh cultures of the bacteria (cultures BS1 and BP) and the pathogen (St83A), respectively, were initiated by transferring 1 ml of cell (conidia in the case of St83A) suspension of each to each of 10 flasks containing 50 ml of liquid V-8 medium. The cultures were incubated on a shaker at room temperature. Serial dilution plating of the

bacteria and hemocytometer counts of St83A conidia, made just prior to inoculating, indicated that cell concentrations used in the 1984 and 1985 tests, respectively, were 8.7×10^7 and 7.6×10^7 cfu/ml for culture BS1, 6.9×10^7 and 6.1×10^7 cfu/ml for culture BP, and about 10^6 conidia/ml each year for St83A.

The bacterial cultures and St83A were applied to the wheat leaves with an electric sprayer. Following inoculation, the plants were placed in a plastic chamber equipped with humidifiers which maintained free water on the plant leaves for 96 hours. The plants were then incubated on greenhouse benches for 21 days at 20-28 C. Following incubation, second and third leaves were harvested from plants in each pot and pooled. From each pooled sample, lesions were counted in five randomly selected leaves which were then dried for 24 hrs at 95 C and weighed.

Pseudomonas culture PF. Three tests were conducted regarding the effect of culture PF on infection by culture St83A. Firstly, because strains of P. syringae are foliar pathogens of wheat which may produce antimycotic compounds (1, 42), attempts were made to isolate Pseudomonas spp. from leaves of wheat in the greenhouse prior to testing the effect of culture PF on infection by S. tritici. Only negative results were obtained. Secondly, the effect of culture PF and its cell-free culture filtrate on infection was determined. Thirdly, the effectiveness of liquid

culture cell suspension and cell-free culture filtrate when applied before, after, and simultaneously with St83A was evaluated using a strain of PF selected for antibiotic resistance. In this test, the population density of the P. fluorescens strain was monitored.

In 1985, plants of wheat TAM W-101 were grown in pots as described for tests with the Bacillus cultures. The pots of plants were separated into lots of five and treated as follows:

a) Sprayed with culture PF,

b) Inoculated with culture St83A,

c) Sprayed with culture PF and then inoculated with culture St83A,

d) Sprayed with cell-free medium filtrate of culture PF and then inoculated with culture St83A,

e) Sprayed with cell-free medium filtrate of culture PF; and,

f) Sprayed with sterile liquid V-8 medium in volume equivalent to that used for St83A in treatment c.

In 1986, the experiment was repeated and KMB was applied as an additional check.

The PF cultures used in the treatments were obtained by transferring a 1 ml cell suspension from 5 and 6 day old cultures to each of 20 flasks containing 50 ml of KMB 4 days prior to their use. Incubation conditions and the method of applying the cells to the plants were essentially the same as those described for the Bacillus cultures.

Concentrations of the suspensions were determined to be about 7.9×10^7 cfu/ml in 1985 and 8.7×10^7 cfu/ml in 1986. The medium from 10 flasks of the 4 day old cultures was centrifuged at 10,000 g for 10 minutes and the supernatant passed through membrane filters with 0.22 μm diameter pores.

Seven days prior to application of the treatments, 10 flasks containing 50 ml of liquid V-8 medium were each inoculated with five 1 cm agar plugs from 7 day old cultures of St83A and incubated on a shaker at room temperature. After 7 days of incubation the conidia containing medium was filtered through a single layer of muslin cloth to remove small mats of mycelium that would interfere with inoculation of the plants.

The PF cultures, PF medium filtrate, St83A inoculum, and sterile V-8 and KB medium were applied to the plants with an electric powered sprayer. Following inoculation, the plants were placed in a humidity chamber and kept moist for 96 hours with time-clock controlled humidifiers. The plants were then placed on a greenhouse bench. After 21 days of incubation, random samples of second and third leaves were collected and transferred to the laboratory where fresh weight, lesion counts, relative water content, and dry weight measurements were obtained.

The leaves were cut into 2-3 cm lengths and soaked in 20 ml of distilled water in petri plates for 24 hours. The leaf segments then were blot-dried and weighed, placed in a

drying oven (95 C) for 24 hours and weighed again. The relative water content (RWC), determined by the following formula, was then used to estimate leaf damage due to infection.

$$RWC = \frac{\text{fresh weight - dry weight}}{\text{saturated weight - dry weight}} \times 100$$

To test survival of P. fluorescens on wheat leaves, a strain resistant to streptomycin sulfate and nalidixic acid was developed from culture PF. First, a 100 µl suspension of PF and its 10-fold dilutions to 10^7 cfu/ml were spread on plates of KMB agar containing 0, 10, 20, 40, 60, 80, and 100 ppm of streptomycin-sulfate and 100 ppm of cyclohexamide. The cultures were incubated for three days at room temperature. The number of cfu/ml on plates of each streptomycin concentration was determined and the frequency of resistant colonies at each concentration was calculated based on the cfu/ml of the original suspension. A single colony strain (PFCS) resistant to 100 ppm of streptomycin was isolated and used to select for nalidixic acid resistance. The same procedure was followed in selecting a strain resistant to nalidixic acid as was used for selecting strain PFCS, except that KMB amended with both streptomycin (100 ppm) and cycloheximide (100 ppm) served as the basal medium. A strain (PFCNS) resistant to both streptomycin (100 ppm) and nalidixic acid (75 ppm) was obtained.

Stability of strain PFCNS was tested on KMB broth without antibiotics for five consecutive transfers, and

then on the same medium containing agar and either antibiotics or no antibiotics. Colony forming units/ml were compared across the media used.

Before testing survival of PFCNS on wheat plants, a preliminary test was conducted to determine the background population of antibiotic resistant pseudomonads in the greenhouse and to compare the efficacy of sonication and shaking for harvesting the bacterium from leaves of greenhouse cultured wheat.

The procedure was as follows: 4 days prior to applying PFCNS, 20 ml of a fresh PFCNS culture was prepared by placing a 1 ml suspension of PFCNS in each of four test tubes containing 5 ml of KMB, supplemented with 100 ppm of both cyclohexamide and streptomycin, and 75 ppm of nalidixic acid. The cultures were incubated on a rotary shaker (125 rpm) for 4 days at room temperature. After adding 200 µl of Tween-20 (polyoxyethylene sorbitan monolaurate) to the medium, the culture of PFCNS was applied with an electrically powered atomizer to 10-12 seedlings of cv. Danne (in the third leaf stage) in each of five pots. Twenty ml of sterile uninoculated medium (broth) was applied to plants in an additional five pots to serve as a control.

Prior to applying PFCNS to the plants, 1 ml of the bacterial suspension was used to determine cfu/ml using the dilution plating method.

Populations of the bacterium on leaves were measured by sampling approximately 0.2 g of third leaf tissue from plants in each pot on the 0 (immediately following application of the double-marker strain), first, third, sixth, ninth, and twelfth day after application. Each sample was placed in a moistened polyethylene bag and transferred to the laboratory.

To compare the effect of shaking and sonication as methods for estimating numbers of bacteria harvested/sample, approximately 0.1 g sample of leaf tissue from each pot was placed in 9.9 ml of sterile tap water in a 20 ml test tube. The test tubes were randomly divided into two groups. One group was placed in a sonicator (Ultramet II, Buehler Ltd. Evanston, IL 60204) for 30 seconds and one was placed on a reciprocal shaker (125) rpm for 30 min. Immediately following each treatment, each sample was dilution plated on KMB containing cyclohexamide and the two antibiotics. Plates were then incubated for three days at room temperature, and cfu/g were determined for each sample.

After the background population of PFCNS in the greenhouse was established and a method of harvesting the bacterium was selected, the following tests were used to determine the effects of time of application of PFCNS (relative to the time of inoculation with S. tritici), on infection levels of S. tritici, and to monitor survival of PFCNS on leaves over time.

A. Application of PFCNS 24 hours prior to inoculation of plants with S. tritici.

Thirty pots of 10-12 seedlings of Danne were grown to third leaf stage. Five pots of seedlings were randomly assigned to each of the following treatments.

1) Application of strain PFCNS by spraying as described earlier.
2) Application of strain PFCNS and inoculation with S. tritici.
3) Inoculation with S. tritici.
4) Application of sterile KMB (broth) and inoculation with S. tritici.
5) Application of cell-free filtrate of PFCNS.
6) Application of cell-free filtrate of PFCNS and inoculation with S. tritici.

B. Application of PFCNS at the time of plant inoculation with S. tritici.

Forty pots of 10-12 seedlings of Danne were grown as in test A and subjected to eight treatments. Six treatments were similar to those in test A, except that S. tritici inoculation immediately (within 15 min) followed application of PFCNS. Two additional treatments were added;

7) Application of PFCNS and S. tritici conidia mixed together immediately before applying,

8) Application of cell-free culture filtrate of PFCNS and S. tritici conidia mixed together immediately before applying.

C. Application of PFCNS 24 hours after inoculating seedlings with S. tritici. Treatments in this test were similar to those in test A except for time of application.

Prior to application of PFCNS in test A, 1.0 ml of pooled suspension of PFCNS was used to measure the cfu/ml, by serial dilution plating. The optical density of the bacterial suspensions was determined at 590 nm using a spectrophotometer (Spectronic 20, Coleman Instrument, CA). The concentration of the bacterial suspensions used in test B and C were adjusted to the optical density values of the suspension used in test A.

One liter of PFCNS suspension was prepared for each test, using the procedure described for the test for background levels of antibiotic resistant P. fluorescens. For each test, 500 ml of PFCNS suspension was used to prepare a cell-free culture filtrate by first centrifuging the suspension at 10,000 g for 10 min and then passing the liquid phase through a 0.22 μm membrane. In each test, sterile KMB containing the antibiotics and cyclohexamide was used as a control.

In each test, 0.5 ml of Tween-20 was added to each aliquot of cell-free filtrate and control before their

application to the seedlings. The application procedure was similar to that used in preliminary tests.

The population of PFCNS was determined from approximately 0.1 g of leaf tissue collected from each PFCNS treated pot at 0, 1, 3, and 6 days after application. The samples were individually weighed and placed in 9.9 ml of sterile tap water on a shaker (125 rpm) for 30 minutes. Following the incubation period serial dilutions of each sample were prepared and 0.1 ml aliquants of each dilution were spread on KMB agar plates containing cyclohexamide and both antibiotics. Plates were incubated for three days and cfu/gm of wheat leaf was determined for each sample.

Seven days prior to inoculation of plants with S. tritici, one liter of conidial suspension of S. tritici was prepared for each test by placing a 5 ml suspension of S. tritici in each of twenty 250 ml flasks containing 50 ml of V-8 medium. The flasks were incubated on a rotary shaker (125 rpm) at room temperature. Prior to inoculation of plants with S. tritici, the number of conidia in the suspension was adjusted to about 10^7/ml by addition of sterile water or concentrated conidial suspension. Following the application of treatments, the plants were placed in a humidity chamber for 6 days, and then moved to a bench in a greenhouse for 21 days. On the 28th day after inoculation, a third leaf from each pot in each treatment was randomly sampled, placed in a moist polyethylene bag, and transferred to the laboratory for total pycnidia

counts. Actual numbers of pycnidia on each leaf were determined using a dissecting microscope. The samples were then placed in individual envelopes, dried to constant weight in a drying oven, and weighed. Pycnidia/gm dry of leaf was the criterion for comparing the impact of the treatment on disease development.

Field Evaluation of Antagonistic Bacteria as Biological Control Agents of Septoria Tritici Blotch. Bacillus cultures (BS1 and BP) and the Pseudomonas culture (PF) were tested in the field for their ability to suppress natural development of septoria tritici blotch. Cultures BS1 and BP were tested in 1984 and 1985. Culture PF and its cell-free culture filtrate were tested in 1987.

In 1984, three 1 m^2 subplots were established side-by-side, approximately 6 m from both the western and southern edges, in each of eight (replications) large plots (15x30 m) of the winter wheat TAM W-101. The subplots were separated from each other and from surrounding plants by a 30-45 cm alley created by removing plants along the perimeter of the subplots. Each subplot was randomly assigned to one of the following treatments: a) sprayed with 250 ml of a 3 to 4 day old culture of BS1 in liquid V-8 medium, b) sprayed with a similar culture of BP and c) sprayed with water as a check. Beginning on April 1, the bacterial suspensions were applied seven times at 7 to 10 day intervals using a "Sure Shot" sprayer (1 L capacity, Milwaukee Sprayer, Milwaukee, WI 53218) pressurized with a

CO_2 cartridge. Prior to applying the cultures, the cfu/ml of each was determined by serial dilution plating. The concentration of bacterial cells in each spray was 7.7 x 10^8, 8.7 x 10^8, 1.2 x 10^9, 7.7 x 10^8, 1.1 x 10^9, 1.01 x 10^9, and 8.43 x 10^8, for culture BS1; and 9.15 x 10^8, 7.9 x 10^8, 9.3 x 10^8, 9.0 x 10^8, 8.9 x 10^8, 8.02 x 10^8, and 6.9 x 10^8 for culture BP. The sprays were applied on April 13, 19, 26, and May 3, 8, 15, and 23.

One week after the last spray was applied, a sample of ten flag leaves was randomly collected from near the center of each plot (an attempt was made to restrict the collection area to a 50 x 50 cm square but this was not always possible due to row spacing and missing plants). The total number of lesions and the dry weight of each sample were determined, and the percentage of variation from the control was calculated.

The experiment was repeated in 1985, with approximately the same concentrations of bacteria. Each plot was divided into four subplots, with the fourth one sprayed with liquid V-8 medium. Sampling subplots, determining lesions per gram of dry leaf weight, and calculations of percentage of variation from control treatments were conducted in the same manner as in 1984.

Inhibition of septoria tritici blotch by culture PF and its cell-free culture filtrate were tested in the field in the spring of 1987. Eight plots (replications) approximately 2 x 2 m, located about 6 m from both the

western and southern borders of large (15 x 30 m) plots of winter wheat cv. TAM W-101 were divided into 1 m^2 quadrants (subplots) separated from each other and from surrounding plants by 30-45 cm alleys. Each subplot within a replication was assigned to one of the following treatments: a) sprayed with PF in liquid KMB, b) sprayed with cell-free filtrate from PF cultures, c) sprayed with sterile KMB, and d) left unsprayed.

Cultures of PF were prepared for application in the field by inoculating liquid KMB in 250 ml flasks with 10 ml of cell suspension from a 4 day old stock culture, then incubating the fresh cultures for 4 days on a shaker at room temperature. Just prior to the first application, the number of cfu/ml of suspension was determined to be 5.9 x 10^7 by serial dilution plating, and optical density was measured at 590 nm with a spectrophotometer (Spectronic 20). The bacterial suspensions for subsequent applications were adjusted to the same optical density.

Before each application, the total bacterial cell suspension volume (after adjusting for optical density) was divided into two equal parts. One part, stored at 4 C in a refrigerator until all treatments were prepared, was sprayed directly on the plots (treatment a); and one part was used as the source of cell-free filtrate. The cell-free filtrate was prepared by centrifuging the cell suspension at 10,000 g for 15 min and then filtering the supernatant through membrane filters with a pore size of

0.22 νm (Gelman Instrument Co. Ann Arbor, MI). The control treatment of sterile KMB was prepared as for culturing PF. A few drops of Tween-20 were added to each liquid treatment before application to enhance spreading.

Two-hundred-fifty ml of PF cell suspension, culture filtrate and sterile KMB were sprayed (as described for the Bacillus culture) on the assigned subplots five times during the season (beginning April 20) at approximately weekly intervals. One week after the fifth application of treatments, lesions/g dry weight were determined from 10 flag leaves collected randomly from near the center of each subplot and percent variation from the control was calculated.

Heat Stability of Antimycotic Products in Culture Filtrates. Cell-free culture filtrates were prepared from 3 day old cultures of BS1 and BP, and from 4 day old cultures of PF grown in 100 ml of V-8 broth and KMB broth, respectively. The filtrates were prepared by centrifugation and filtration as described previously. A 10-ml aliquant of lyophilized culture filtrate of each bacterium was rehydrated in 0.05 M phosphate buffer (pH 7) (50 mg/ml) and divided into five 2-ml aliquots. Each aliquot was subjected to one of the following treatments: 1) autoclaved at 121 C for 15 min, 2) maintained at 60 C for 1 hr, 3) maintained at 5 C for 24 hr, and 4) maintained at 0 C for 24 hr. Two hundred μl of each sample was placed in each of ten 1-cm wells cut in 1/4 strength V-8 agar in

five petri plates. A near equal volume of a S. tritici conidial suspension was sprayed on each plate and incubated at room temperature for 5 to 7 days. The width of inhibition zone around the wells was the criterion for measuring activity of each sample. The average value for each pair of wells in a petri plate was treated as a replication when the data were analyzed.

Partial Purification and Characterization of Antimycotic Compounds Produced by Bacterial Cultures. Cultures BS1 and BP were grown in V-8 broth for 3 days and culture PF was grown in KMB broth for 4 days. The number of cfu/culture was determined by dilution plating. Immediately after preparing the dilution plate series, a 100-ml cell-free filtrate of each culture was prepared by centrifuging the culture at 10,000 g for 15 min, and then passing the supernatant through membrane filters with pore diameters of 0.22 μm. The filtered supernatants were lyophilized and stored at 0 C until partial characterization was initiated. Prior to storage, a subsample (50 mg) of each lyophilized sample was dissolved in 1.0 ml of 0.05 M phosphate buffer (pH 7.0) and 200 μl of each was placed in 1-cm diameter wells cut in 1/4 strength V-8 agar in petri plates. The agar was sprayed with a conidial suspension of S. tritici, incubated for 7 days, and checked for presence of inhibition zones.

Approximate molecular weights (MW) of the active compounds were determined first by dissolving the

lyophilized crystals in 10 ml of potassium phosphate buffer (0.05 M, pH 7.0), dividing the solution into two lots, and then passing one lot through an ultrafiltration system (Amicon Corp., Danvers, MA 01923) and the other by dialyzing against the buffer. In the ultrafiltration procedure, 1.0 ml samples of rehydrated culture filtrate was passed through membranes having MW cut-offs of 8,000, 10,000, 30,000, and 50,000. In the dialysis procedure, 1 ml of each sample was placed in dialysis tubing with MW cut-offs of 1,000, 5,000, 10,000, 30,000 and dialyzed at 4 C against 1 L of the phosphate buffer for 48 hr, with a change of buffer after 24 hr. In each case, samples were collected successively at each MW cut-off. The samples were lyophilized as collected and stored at 0 C until tested for inhibitory activity against conidial suspensions S. tritici as described above.

After a MW range for each active compound was determined, 2.0 ml of concentrated cell-free culture filtrate of each antagonist was placed on appropriate Sephadex gel (G 50-80, Sigma Chemical Co., St. Louis, MO) columns (2.5 x 100 cm) and eluted with phosphate buffer (0.05 M, 7.0 pH) with a hydrostatic pressure of 35 cm of buffer solution. Fractions were collected at the rate of 0.5 ml/min at room temperature. Appropriate MW markers (Sigma Chemical Co., St. Louis, MO) were run before and after each filtrate sample under the same conditions as a check for any change in flow rate and gel condition. The

filtrate was not mixed with the standard markers to avoid the possibility of the active compound binding with the markers. Each fraction was measured for absorbance at 280 nm with a Gilford spectrophotometer and lyophilized. To test the activity of fractions, each was dissolved in 200 µl of eluting buffer and bioassayed as previously described.

The column fractionation tests were repeated under the same conditions. For each antagonist, adjacent fractions that showed activity against S. tritici were pooled, lyophilized, and stored for later use.

Results

Four bacterial strains, isolated from soil and leaf tissue, that exhibited the most antagonistic activity to growth in vitro of S. tritici were identified as B. subtilis (two cultures) B. pumulis (one culture) and P. fluorescens (one culture). Tests conducted at the American Type Culture Collection Laboratory confirmed the identification of the four cultures and further classified P. fluorescens as biovar I.

Fungicidal vs. Fungistatic Activity. Culture ST83A failed to grow on the tops of the 6 mm diameter plugs cut from clean inhibition zones surrounding the bacterial isolates and placed on sterile V-8 agar in petri plates; whereas, pink masses of budding cells developed on the tops of plugs cut from sterile V-8 agar. When the plugs were

inverted, pink masses of cells developed around the edges of all plugs where they contacted the sterile V-8 agar in the plates. From these results, it was concluded that the antimycotic activity of substances produced by the four bacterial cultures was fungistatic but not fungicidal.

Nutrient Base and Growth Inhibition. The antimycotic activity against ST83A by individual cultures did not differ significantly (P=0.05) on three commonly used general media (V-8, nutrient, and potato dextrose agars) supplied at full and one-half strength. Also, no significant differences occurred among cultures on a single medium, or among replications (Table I). These data, while not singly conclusive, indicate that the inhibition zones were not a result of competition for nutrients in the medium.

TABLE I

GROWTH INHIBITION OF SEPTORIA TRITICI (ST83A) BY FOUR BACTERIAL CULTURES ON V-8, NUTRIENT (NA), AND POTATO DEXTROSE (PDA) AGAR MEDIA AT FULL AND ONE-HALF STRENGTH

	Width of Inhibition Zone on Indicated Medium (mm)[a] [b]					
Culture	V-8	1/2V-8	NA	1/2NA	PDA	1/2PDA
BS1	13	12	12	12	11	11
BS2	14	11	13	11	13	11
BP	12	12	12	11	12	10
PF	14	12	15	14	13	11

[a] Values are the mean of five replications

[b] An ANOVA indicated that differences within and between treatments (media), cultures, replications, and interactions were not significant at P=0.05.

Effect of Cell-Free Filtrates on Growth of ST83A.

Effects of dilution of the bacterial culture filtrates on growth of S. tritici are shown in Figure 1. The inhibitory activities of all culture filtrates were reduced as the percent dilution of filtrates increased. The inhibitory activities of the culture filtrate obtained from P. fluorescens was higher (P=0.05) at some dilution levels (60, 70, and 80%) than those from the other bacteria at the same levels. Filtrates from Bacillus spp. had similar activities on all dilution levels. It seems that the

inhibitory activities of culture filtrate obtained from P. fluorescens was higher for most dilution levels. Filtrates from B. subtilus had the lowest level of activity among the four bacterial antagonists.

Infection of Greenhouse Grown Wheat by ST83A in the Presence of Antagonistic Bacteria. Bacillus: Cultures BS1 and BP. Bacillus cultures BS1 and BP, applied singly to wheat leaves just prior to inoculation with ST83A (followed by a 96-hour wet-leaf period), significantly (P=0.05) reduced the number of septoria blotch lesions that developed per gram of dry leaf tissue in the 1984 and 1985 tests (Table II). Compared to control plants sprayed with V-8 liquid medium before inoculation with ST83A, the percentages of lesion reduction attributed to the two bacteria in 1984 and 1985, respectively, were 23.3 and 33.7% for culture BS1, and 31.5 and 41.4% for culture BP.

TABLE II

EFFECT OF BACILLUS SUBTILIS (BS1) AND B. PUMULIS (BP) ON INFECTION OF WHEAT CV. TAM W-101 BY SEPTORIA TRITICI (ST83A) IN GREENHOUSE TESTS

	Lesions of Septoria Tritici Blotch			
	Number of lesions per g of Dry Leaf Tissue[b]		% of Control	
Treatment[a]	1984	1985	1984	1985
BS1 + ST83A	40.7[y]	29.3[x]	23.3	33.7
BP + ST83A	36.3[x]	25.9[x]	31.5	41.4
V-8 + ST83A	53.0[y]	44.2[y]	-	-
H_2O + ST83A	56.7[y]	48.5[y]	-	-

[a] Bacterial cultures (BS1 and BP), sterile liquid V-8 medium (V-8), and water (H_2O) were applied in equal volumes to the leaves immediately prior to inoculation with ST83A.

[b] Lesion numbers are the mean for five randomly selected leaves. Numbers followed by the same letter within the same column are not significantly (P=0.05) different according to Duncan's multiple range test.

Pseudomonas: Culture PF. Culture PF of P. fluorescens and the cell-free filtrate of its medium applied to wheat leaves significantly (P=0.05) reduced the lesion numbers and amount of damaged tissue caused by inoculation with culture ST83A (Table III). Expressed as lesions per gram of dry leaf tissue, application of PF cell suspensions reduced infection by an average of 67.5% in the

1985 and 1986 tests. In the same tests, determination of relative water content indicated an average reduction of 50.7% in leaf damage for the two years. Corresponding mean values for reduced leaf tissue damage attributed to application of cell-free filtrate was 51.9% in lesion numbers and 44.8% in RWC.

TABLE III

THE EFFECT OF PSEUDOMONAS FLUORESCENS (PF) AND CELL-FREE MEDIUM OF CULTURE PF ON INFECTION OF WHEAT CV. TAM W-101 BY SEPTORIA TRITICI (ST83A) IN TWO GREENHOUSE TESTS

	Septoria Tritici Blotch Infection[b]					
	Relative Water Content(%)		Lesions/g Dry Weight		% Inhibition[c]	
Treatment[a]	1985	1986	1985	1986	1985	1986
V-8	83.6^{x}	-	-	-	-	-
PF	78.8^{x}	82.9^{x}	-	-	-	-
PF Filtrate	84.3^{x}	83.8^{x}	-	-	-	-
PF+ST83A	82.9^{x}	79.9^{x}	12.1^{x}	6.8^{x}	61.6	73.4
PF Filtrate +ST83A	73.2^{y}	72.3^{y}	16.9^{y}	11.0^{y}	46.9	57.03
KMB+ST83A	-	44.1^{z}	-	26.8^{z}	-	-
H_2O+ST83A	40.0^{z}	40.2^{z}	31.8^{z}	25.6^{z}	-	-

[a] The volume of sterile V-8, medium, culture PF filtrate, suspension of culture PF, KMB and water were equivalent.

[b] Lesion numbers and relative water content values are means obtained from five or more randomly selected leaves. Within columns, numbers followed by the same letter are not significantly (P=0.05) different according to Duncan's multiple range test.

[c] The percentage of inhibition was calculated using lesion per gram of dry leaf tissue and the water + ST83A treatment.

A strain of PF (designated PFCNS) resistant to streptomycin (100 ppm) and nalidixic acid (75 ppm) was selected to evaluate the survival of P. fluorescens on leaves and to determine the effect of time of application relative to time of inoculation with culture ST83A on disease development. The double-marker strain was stable after five serial transfers on KMB lacking the antibiotics. A few other fluorescent pseudomonads and non-fluorescent bacteria resistant to both antibiotics were also isolated from leaf samples of uninoculated plants used as checks, but their numbers (mean = 0.03/petri plate) were considered negligible. The source of these bacteria is unknown; some may have originated as spontaneous mutants and some may have been PFCNS that had been transferred inadvertently to the plants before or during sampling.

The total number of cfu/ml of PFCNS harvested from wheat leaves by sonication was higher at each sampling than those harvested by shaking (Table IV), but the difference in numbers was not significant three days after application. Shaking was selected as the method of choice in further studies because small differences in agitation time were not as critical with the shaker as for sonication. In further population studies in the greenhouse, the marker strain was harvested from wheat leaves through the sixth day following its application to the wheat seedlings.

TABLE IV

COMPARISON BETWEEN SONICATION AND SHAKING FOR HARVESTING *P. FLUORESCENS* OVER A 12-DAY PERIOD

Sampling days after application and statistics	Cfu x 10^4 of culture PFCNS/g of fresh wheat leaf[a]	
	Sonication	Shaking
0	310.00	209.00
1	16.70	8.70
3	0.61	0.40
6	0.59	0.31
9	0.58	0.53
12	0.42	0.38
CV[b]	43.7%	

[a] Initial cell population in the inoculum suspension was 3.6×10^7 cfu/ml.

[b] CV = coefficient of variation.

Patterns of bacterial population on wheat leaves over time are shown in Figure 2. Regardless of the harvesting method the population of PFCNS declined sharply until the third day following its application to the leaves, then stabilized at relatively low population densities for the duration of the experiment (12 days after application). Therefore, in further population studies in the greenhouse, the marker strain was harvested from wheat leaves through the sixth day following its application to the wheat seedlings.

The effects of time of application of P. fluorescens on infection by S. tritici are shown in Table V. In all tests, application of sterile KMB (broth) did not affect (P=0.05) the infection of wheat seedlings by S. tritici. This confirms results of previous greenhouse tests (33). Regardless of relative time of application, PFCNS significantly reduced leaf blotch expressed as number of pycnidia/g dry leaf tissue, but most did so effective by (P=0.05) when applied simultaneously with S. tritici. There was no difference (P=0.05) in the amount of infection between the postmix and premix treatment.

The filtrate of the antagonist was effective (P=0.05) in reducing the amount of infection only when applied simultaneously with, or 24 hours before, applying inoculum of S. tritici, but was most effective when applied simultaneously with S. tritici.

TABLE V

PYCNIDIA PER GRAM DRY WEIGHT OF WHEAT LEAVES INOCULATED WITH *SEPTORIA TRITICI* BEFORE, AFTER, AND SIMULTANEOUSLY WITH APPLICATION OF *PSEUDOMONAS FLUORESCENS* (PFCNS) AND ITS CELL-FREE CULTURE FILTRATE

	Numbers of pycnidia (x1000)			
	Inoculation time relative to PFCNS/filtrate application			
	24 hr before	24 hr after	Simultaneously[a]	
Treatment			postmix	premix
S. tritici + PFCNS	7.0[xb]	6.5[x]	3.1[y]	2.7[y]
S. tritici + PFCNS filtrate	7.4[x]	11.7[w]	5.7[z]	4.3[z]
S. tritici	11.8[w]	12.5[w]	11.4[w]	-
S. tritici + sterile KMB	11.6[w]	11.9[w]	12.2[w]	-

[a] Postmix = antagonist (culture and cell-free filtrate) applied to leaves immediately after inoculation with *S. tritici*; premix = antagonist (culture and cell-free filtrate) mixed with *S. tritici* inoculum and applied to leaves in a single operation.

[b] Values followed by the same letter are not significantly different according to Duncan's multiple range test (P = 0.05).

Regardless of relative time of application, populations of PFCNS on wheat declined sharply but stabilized after the third day following application to wheat seedlings in both P. fluorescens and P. fluorescens - S. tritici treated plants (Figure 2). Except for the third day, in all tests, population densities of PFCNS were lower in the presence of S. tritici than in populations of PFCNS alone (control). However, greater densities were evident when PFCNS and S. tritici were applied simultaneously (Test B) than when PFCNS was applied 24 hours before (Test A) or 24 hours (Test C) after inoculation of S. tritici to wheat seedlings (Table VI). Mixing of PFCNS with the conidia suspension of S. tritici prior to inoculation of wheat seedling resulted in higher population density of PFCNS as compared to those values in other treatments (Table VI).

TABLE VI

SURVIVAL OF *PSEUDOMONAS FLUORESCENS* (PFCNS) ON WHEAT LEAVES WHEN APPLIED BEFORE, AFTER, AND SIMULTANEOUSLY WITH INOCULUM OF *SEPTORIA TRITICI*

Sampling time (days after application)	Sampling cfu x 10^4 of culture PFCNS/g of fresh wheat leaf[a]						
	(Test A) 24 hr before		(Test B) simultaneously			(Test C) 24 hr after	
	PF	PF/S	PF	PF/S POSTMIX	PF/S PREMIX	PF	PF/S
0	448.0	428.0	716.0	792.0	588.0	540.0	556.0
1	27.8	8.3	66.6	26.6	44.2	44.0	7.4
3	0.8	0.8	0.6	0.3	0.5	0.5	0.3
6	0.6	0.2	0.5	0.2	0.5	0.4	0.2

A Initial cell population in the suspension applied 24 hr before inoculation with *S. tritici* was 6.8 CFU x 10^6. Cell populations in the two succeeding tests were adjusted to the same spectrophotometric value as in first test.

B PF and PF/S indicate application of PFCNS only and application of both PFCNS and *S. tritici* at indicated times, respectively.

C Postmix = antagonist PFCNS in culture filtrate applied to leaves immediately after inoculation with *S. tritici*, premix = antagonist PFCNS in culture filtrate mixed with *S. tritici* inoculum and applied to leaves in a single operation.

Although the initial population of PFCNS in tests B and C were spectrophotometrically adjusted to the same sample values as the cell suspension used in test A, variations in population densities of PFCNS were indicated on leaves at day 0 (immediately after application of the antagonist) in each of the three tests (Table VI). Thus, the density differences observed could be partially due to differences in the initial number of cells applied to the leaves.

This study indicated that under greenhouse conditions, PFCNS or its culture filtrate is effective in reducing the amount of infection when applied simultaneously or 24 hours before applying inoculum of S. tritici.

Field Evaluation of Antagonistic Bacteria as Biological Control Agents of Septoria Tritici Blotch. The application of Bacillus cultures BS1 and BP, to wheat leaves in the field caused significant reduction in the numbers of septoria tritici blotch lesion caused by natural infection in both 1984 and 1985 (Table VII). Application of sterile V-8 liquid medium did not significantly affect the numbers of septoria tritici blotch lesions. These results indicate that the reduction in lesion numbers was caused by antagonistic activity by the cultures against Septoria tritici. These results are in apparent agreement with those described earlier for these cultures on young plants in the greenhouse.

TABLE VII

EFFECTS OF *BACILLUS SUBTILIS* (BS1) AND *B. PUMULIS* (BP) APPLIED TO WHEAT LEAVES ON NATURAL INFECTION BY *SEPTORIA TRITICI*

Treatment and statistics[ab]	1984[c]		1985	
	lesions/ gdw	%inhibition[d]	lesions/ gdw	%inhibition[d]
BS1	58.6x	27.5	25.1x	36.8
BP	49.6y	37.1	22.8x	42.6
Check, H_2O	78.9y	--	39.7y	--
Check, V-8 med.	--	--	37.2y	6.3
CV	27.2%	17.5%		

[a] Mean of seven application of *B. subtilis* and *B. pumulis* was 9.4×10^8 and 7.3×10^8, respectively, in 1984; and 8.5×10^8 and 8.1×10^8, respectively, in 1985.

[b] Bacterial cultures were applied in liquid V-8 medium. CV = coefficient of variation.

[c] Lesions per gram of dry leaf weight (gdw) is based on 10 randomly selected flag leaves from each of eight replications. Values within columns not followed by the same letter are significantly different (P=0.05) according to Duncan's multiple range test.

[d] Percent inhibition compared to water check.

The application of *P. fluorescens* in culture medium significantly reduced natural infection by *S. tritici*, whereas, its cell-free culture filtrate did not (Table VIII). Suppression of infection by the combination of

living cells and culture medium agrees with the results obtained earlier in greenhouse tests. However, failure of the cell-free filtrate to inhibit infection does not agree with the greenhouse tests.

TABLE VIII

THE EFFECT OF PSEUDOMONAS FLUORESCENS (PF) IN KINGS MEDIUM B AND THE CELL-FREE CULTURE FILTRATE APPLIED TO WHEAT LEAVES ON NATURAL INFECTION BY SEPTORIA TRITICI IN 1987

Treatment and statistics[ab]	lesion/gdw[c]	% inhibition[d]
PF	18.85x	42.9
Cell-free filtrate	31.70y	4.1
KMB	34.23y	0.0
Unsprayed, check	33.04	--.-
CV	23.2%	

[a] Approximately 5.9 x 10^7 cfu/ml of P. fluorescens was applied five times.

[b] KMB = sterile King's medium B; cell-free filtrate and sterile KMB were applied in a volume equivalent to that of the culture suspension; CV = coefficient of variation.

[c] Lesion per gram of dry leaf weight (gdw) is based on 10 randomly selected flag leaves from each of eight applications. Values not followed by the same letter are significantly different (P=0.05) according to Duncan's multiple range test.

[d] Percent inhibition based on unsprayed check.

Heat Stability of Antimycotic Products in Culture Filtrates. Cell-free culture filtrates of the two Bacillus spp. remained almost fully antagonistic (14% and 17% loss of activity by filtrates of B. subtilis and B. pumulis; respectively) to growth of S. tritici in vitro after being autoclaved for 15 min at 121 C; whereas, the cell-free culture filtrate of P. fluorescens was rendered ineffective by the same treatment. A sample cell-free filtrate of P. fluorescens maintained at 60 C for 1 hr was 21% less effective as a growth inhibitor than one held at 25 C for 24 hr (Table IX). The differences in heat sensitivity may result from differences in molecular weight of the active products of the two genera. When active compounds produced by the antagonist were lyophilized and redissolved in phosphate buffer and tested for activity against S. tritici, no loss in activity was observed, indicating that active compounds are water soluble.

TABLE IX

EFFECT OF TEMPERATURE ON THE EFFICACY OF CELL-FREE CULTURE FILTRATES FROM BACILLUS SUBTILIS AND PSEUDOMONAS FLUORESCENS TO INHIHIBIT GROWTH OF SEPTORIA TRITICI IN VITRO

Treatment	Width of inhibition zone (mm)[a]		
	BS1 F[b]	BP2 F[b]	PF F[b]
121 C (15 min)	12	10	0
60 C (1 hr)	12	11	11
25 C (24 hr)	13	12	14
5 C (24 hr)	12	12	13
0 C (24 hr)	14	12	14

[a] Based on five replications per treatment. Each replication represented the mean value of filtrate in two 1-cm diameter wells cut into V-8 agar in a petri plate. Coefficient of variation = 11.4%

[b] BS1 F, BP2 F, and PF F designates 200 µl of cell-free filtrate (derived from 500 mg of lyophilized filtrate rehydrated in 10 ml of potassium phosphate buffer pH 7.0) of B. subtilis, B. pumulis and P. fluorescens, respectively.

Molecular Weight Estimates of Compounds Produced by B. subtilis (culture BS1), B. pumulis (culture BP), and P. fluorescens (culture PF) that Inhibit Growth of S. tritici. Dialysis and ultrafiltration produced similar estimates of molecular weight range (upper and lower limits) for the antimycotic compound in the filtrate of each bacterial

culture (Table X). In each case, the molecular weight indicated by gel filtration fell within the range estimated by dialysis and ultrafiltration (Table X). According to the gel filtration procedure, molecular weights of the active compounds produced by P. fluorescens (culture PF), B. subtilis (culture BS1), and B. pumulis (culture BP) were 11,500, 34,000, and 26,000, respectively. When absorbance spectra of fractions eluted from the Sephadex column were scanned, only the active material produced by P. fluorescens exhibited an absorbance maximum at 280 nm. This may indicate that the active materials of P. fluorescens are proteinaceous in nature.

TABLE X

MOLECULAR WEIGHT ESTIMATES FOR THREE COMPOUNDS ANTAGONISTIC TO GROWTH OF *SEPTORIA TRITICI* AND SINGLY PRODUCED BY *PSEUDOMONAS FLUORESCENS* (PF), *BACILLUS SUBTILIS* (BS1), AND *B. PUMULIS* (BP).

Filtrates from cultures	Molecular weight estimated by indicated method		
	Dialysis	Ultra-Filtration	Gel Filtration
PF	>5,000[a] <15,000	>10,000 <30,000	11,500
BS1	>30,000 <50,000	>30,000 <50,000	34,000
BP	>10,000 <30,000	>10,000 <30,000	26,000

[a] The symbols > and < signify greater than and less than, respectively.

Discussion

The results establish *B. subtilis* (culture BS1) *B. pumulis* (culture BP), and *P. fluorescens* (culture PF) as antagonistic to growth of *S. tritici in vitro*, and as inhibitors to infection by *S. tritici* of wheat seedlings in the greenhouse and adult wheat plants in the field. Although not demonstrated on leaves, the antagonism of each bacterial culture was assumed to result from fungistasis conditioned by the production of an antimycotic compound.

The fact that culture BS1 was isolated from wheat leaf tissue and culture BP was isolated from soil had no apparent effect on their ability to suppress septoria tritici blotch. B. subtilis has been shown to be antagonistic to many soil-borne pathogens (5, 6, 10, 12, 27, 32) but there also are reports of its effect on foliar pathogens (3, 32, 43). The culture filtrate of B. pumulis was reported to lyse germ-tubes of cereal rusts (35) both before and after autoclaving. B. licheniformis controlled tan spot of wheat (16) and net blotch of barley (37) in greenhouse and field tests, respectively. However, none of the Bacillus spp. have been adopted as biological control agents of foliar diseases.

It is unlikely that the Bacillus spp. used in the present study will be used directly as control agents of septoria tritici blotch because they are normally inhabitants of soil rather than the phylloplane; and even prepared in encapsulated form the cost of application would be prohibitive. The most promising strategy for use of the cultures would be to insert segments of their DNA that regulate production of antimycotic substances into species of microflora that commonly inhabit the wheat phylloplane.

Pseudomonas fluorescens is indigenous to both above- and below- ground plant parts. It occurs on the leaves of wheat, at least when moisture is present, for sustained periods of time, and is suspected of playing a role in

natural suppression of septoria tritici blotch (Appendix I).

Pseudomonas spp., like those of Bacillus, have been used successfully to control pathogens residing in the rhizosphere (7, 15, 19, 21, 22, 28) but not those infecting the foliar parts of plants. However, it is well established that colonization of leaf surfaces with Pseudomonas spp. does reduce infection by fungal pathogens, including those causing southern maize leaf blight (40), cecospora leaf spot of peanut (2), scleroderris canker of conifers (28), and septoria tritici blotch and leaf rust of wheat (30, 31).

In this study, survival of P. fluorescens on wheat seedlings in the greenhouse was examined relative to the time of application and inoculation with S. tritici. The population of P. fluorescens applied to leaves in a relatively high density (10^7/ml), declined rapidly to a low but stable density of a few hundred cfu/g dry leaf (Fig. 2). This observed occurrence was similar to those observed by other workers. Knudsen (28) reported a rapid decrease in population of P. fluorescens following application to red pine seedlings. A rapid decline in population also occurred when other bacteria were added to soil (9, 13). Agrobacterium tumefaciens decreased sharply when added to soil (13). Casida (9) reported that Micrococcus luteus rapidly declined, with less than 10% of the initial population surviving two weeks after addition to soil.

The application of P. fluorescens (culture PF) in culture medium, but not of its cell-free culture filtrate, significantly reduced natural infection by S. tritici (Table VIII). Suppression of infection by the combination of living cells and culture medium in the field agreed with results obtained earlier in the greenhouse tests (Tables II and III). However, failure of the cell-free filtrate to inhibit infection did not agree with the greenhouse tests in which the filtrate inhibited infection if applied simultaneously with, or 24 hr before, inoculation with conidia, but not when applied 24 hr after inoculation with conidia (Table III).

The reasons for failure of the culture filtrate to suppress infection by S. tritici in the field (Table VIII) are unknown. It is probable that the active compound was quickly degraded either by nonsensitive microorganisms on leaves, or by a physical factor such as sunlight. Regardless of the cause, these data indicate that the active compound, per se, may be of little or no value as a protectant against S. tritici. Consequently, a strategy to utilize P. fluorescens as a biocontrol agent of septoria tritici blotch should include a method for maintaining high density populations of living antagonistic bacterial cells on the leaves, or for the transfer of the gene(s) regulating production of the compounds to well adapted inhabitants of the phylloplane.

Three products of P. fluorescens strains have been identified that have antimycotic properties. A product of P. fluorescens strain 2-79 which is inhibitory to the wheat take-all fungus, Gaeumannomyces graminis var. tritici and Pythium spp. was identified as a dimer of phenazine carboxylic acid (19). A strain of P. fluorescens (PF-5) isolated from rhizosphere soil from cotton produces an antibiotic identified as pyrrolnitrin which is active against Rhizoctonia solani, weakly active against Fusarium spp., and inactive against Pythium ultimum (21). Later it was shown that strain PF-5 produced a second antibiotic, pyoluteorin, that was highly inhibitory to P. ultimum but not to R. solani, Alternaria sp., Fusarium sp., Thielaviopsis basicola and Verticillum dahliae (22).

Activity of the active compound was lost when Howell's (21, 22) methods (used for purification of pyrrolnitrin and pyoluteorin) were used to purify the active compound produced by my culture (PF) of P. fluorescens. This loss of activity and the fact that the active compound was ineffective against both R. solani and P. ultimum (Mehdizadegan and Gough, unpublished) indicates that it is unlikely that the partially defined compound isolated in this study from culture PF is one of the above antibiotics. A fourth compound produced by an unidentified species of a fluorescent Pseudomonas and identified as 1-hydroxyphenazine was inhibitory to S. tritici and Puccinia recondita (31). If the active chemical partially

characterized in this study is a phenazine related substance, it is probably associated with a proteinaceous compound, since it possesses a high molecular weight (11,500), tracks a sigmoid activity curve when diluted (characteristic of proteins having more than one subunit), shows a light absorbance peak at 280 nm, and becomes inactive when heated.

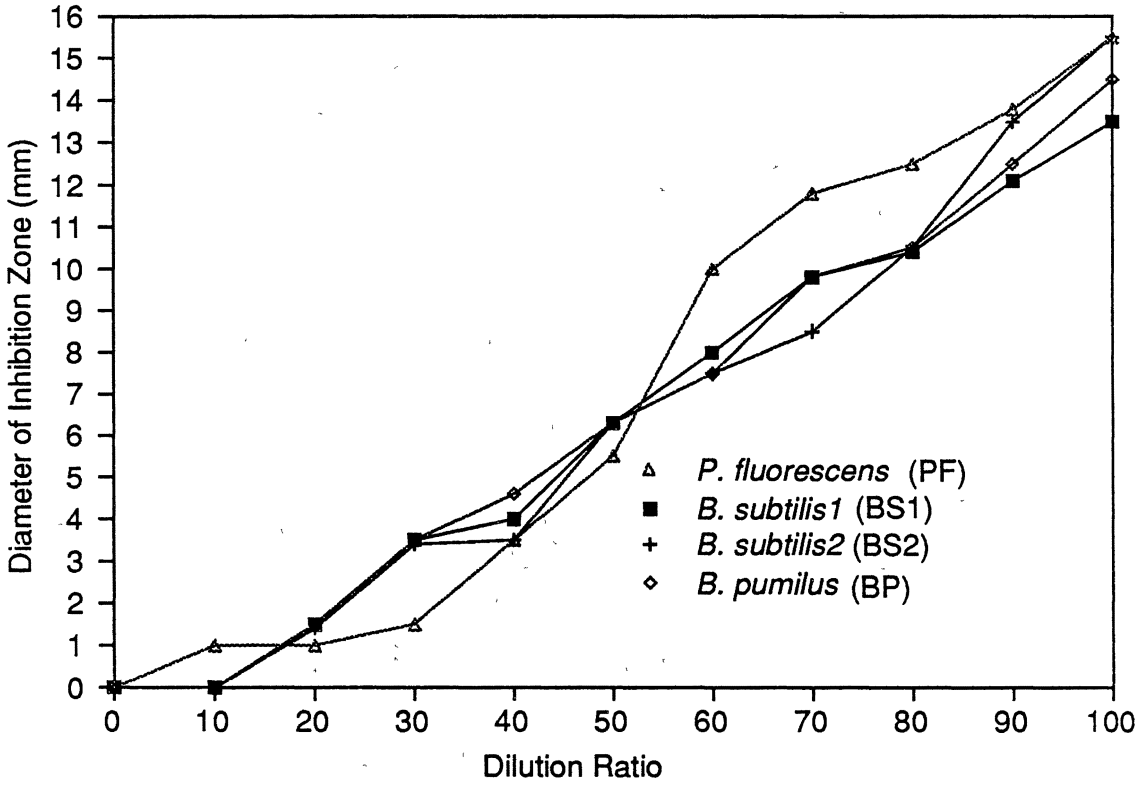

Figure 1. Effects of dilution of cell-free culture filtrates of bacterial antagonists on growth of S. tritici, measured as diameter of inhibition zone in mm.

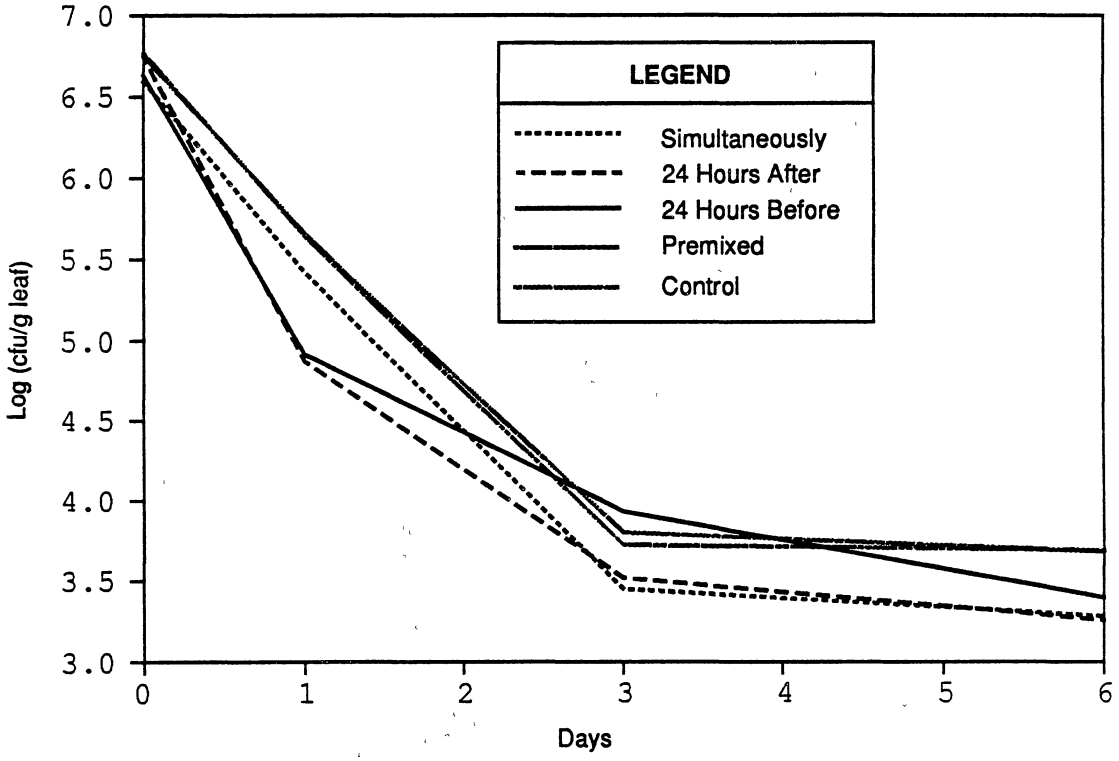

Figure 2. Population levels of PFCNS over six days following application to wheat leaves 24 hr before; 24 hr after; simultaneously with; and Premixed in Suspension with conidial inoculum of S. tritici and when applied singly as a control.

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CHAPTER III

APPENDIX I

Effect of Streptomycin on Development of Septoria Tritici Blotch

The effect of streptomycin on development of septoria tritici blotch in flag leaves of winter wheat cv. TAM W-101 was tested in the field in the spring of 1984 and again in 1985. In 1984, plots were established within large (30 m x 15 m) no-till plots (having a full complement of residue from the previous wheat crop) used in a crop residue management study. In 1985, the plots were established within both no-till and clean-till plots of a crop residue study. A randomized complete block design with four replications was used in all tests. The treated and untreated plots were each 1 m^2, and so arranged that they occupied the centers of diagonally opposed quadrants of 2 m x 2 m squares. This arrangement permitted maximum access to the plots, minimized spray drift, and maintained close proximity of the treated and check plots.

Streptomycin sulfate (125 ppm) in distilled water containing a drop of Tween 20/L was applied to runoff with a hand-held CO_2 pressurized mist sprayer. In 1984,

applications were made on May 8, 14, 21, and 31, when the plants were at growth stages (Feekes' scale) 9.0, 10.0, 10.3, and 10.5, respectively. In 1985, streptomycin was applied on April 13 and 20 and on May 1 and 11; plant growth stages were not recorded.

Ten flag leaves were collected randomly from each plot on June 6, 1984, and on May 20, 1985. The number of septoria tritici blotch lesions/g of dry leaf tissue was determined. Differences (P=0.05) between treatment means were determined and compared by analysis of variance (Table XI) and the test for least significant differences between means.

Attempts were made each year, during the time the streptomycin was being applied, to isolate bacteria antagonistic to growth of S. tritici from leaves collected from plants adjacent to the plots. Nutrient agar and V-8 agar were used as the isolation medium in 1984, and nutrient agar and Pseudomonas isolation medium were used in 1985.

The application of streptomycin resulted in a 52.6% increase in number of lesions/g of dry leaf tissue in 1984, and in a 14.2% and 22.1% increase, respectively, in similar tissue from plants in the no-till and clean-till plots in 1985 (Table XI). These data strongly indicate that naturally occurring bacteria inhibit infection by S. tritici. Attempts to isolate bacteria antagonistic to S. tritici failed in 1984. However, two bacteria, identified

as Bacillus subtilis and Pseudomonas fluorescens biovar I, which inhibited growth of S. tritici were isolated in 1985.

TABLE XI

EFFECT OF STREPTOMYCIN ON THE DEVELOPMENT OF SEPTORIA LEAF BLOTCH LESIONS IN FLAG LEAVES OF WINTER WHEAT CV. TAM W-101

Crop year	Tillage[a]	Lesions/g of dry leaf		% Increase	LSD
		Streptomycin	Check		
1984	No-till	11.9	7.8	52.6	1.08
1985	No-till	43.4	38.0	14.2	0.84
1985	Clean-till	45.8	37.5	22.1	1.25

[a] No-till = all residue from prior wheat crop left on soil surface; clean-till = residue buried with a moldboard plow.

VITA

Feridoon Mehdizadegan

Candidate for the Degree of

Doctor of Philosophy

Thesis: BIOLOGICAL CONTROL OF SEPTORIA TRITICI BLOTCH

Major Field: Plant Pathology

Biographical:

Personal Data: Born in Ahwaz, Iran, 22 June 1955, the son of Mr. and Mrs. Mohamad Mehdizadegan. Married to Farzaneh Katkhordeh; father of a son and a daughter, Iden and Abtin.

Education: Graduated from Pahlavi University High School, Shiraz, Iran in 1972; received Bachelor of Science Degree in Botany from Pahlavi University, Shiraz, Iran in 1978; received Master of Science Degree from Tarleton State University, Stephenville, Texas in 1980; completed requirements for the Doctor of Philosophy degree in Animal Sciences at the University of Arkansas, Fayetteville, Arkansas in 1983; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in 1990.

Professional Experience: Research Assistant, Agricultural Department, Tarleton State University August 1979, to December, 1980; Teaching and Research Assistant, Department of Animal Sciences, University of Arkansas, January, 1980, to December 1983; Teaching and Research Assistant, Department of Plant Pathology, Oklahoma State University, August 1984 to December 1988.

Professional Affiliations: Member of the American Phytopathological Society, Phi Kappa Phi, and Mycological Society of America.