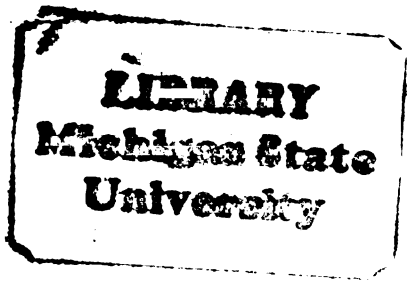

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IN VITRO AND IN VIVO INHIBITION OF BACTERIAL AND FUNGAL PATHOGENS OF BEANS BY BACTERIAL ANTAGONISTS

By

Linda Tully Pontius

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ABSTRACT

IN VITRO AND IN VIVO INHIBITION OF
BACTERIAL AND FUNGAL PATHOGENS OF BEANS
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By

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Internal bacterial contaminants from bean seed were screened *in vitro* and *in vivo* for the ability to inhibit growth of bacterial and fungal plant pathogens. *In vitro* studies were conducted to test the effect of different culture ages on the inhibitory activity of promising antagonists.

Bean plants (*Phaseolus vulgaris* L.) were inoculated with mixtures of bacterial pathogens and antagonists. Several antagonists prevented infection, but only for short periods of time. Longer periods of protection did occur, but not consistently.

In greenhouse studies seed inoculation with several bacterial antagonists protected bean seedlings subsequently inoculated with *Xanthomonas campestris* pv. *phaseoli* which causes common bacterial blight of beans. Spray inoculation of plants with antagonist 143a significantly decreased the severity of bean anthracnose (*Colletotrichum lindemuthianum*) and bean rust (*Uromyces phaseoli*).

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INTRODUCTION AND LITERATURE REVIEW

Research on biological control of plant pathogenic microorganisms is aimed at developing effective, environmentally safe, economical, and technically sound methods of disease control. Such control may be achieved either by introduction of microorganisms antagonistic to plant pathogens, or by manipulation of the environment in such a way as to favor inhibitory activity of natural antagonist populations. In recent years, increased interest has been shown in the control of plant diseases by bacterial antagonists. Much of this interest was directly due to the discovery of a particular bacterium (35) which effectively controlled crown gall disease caused by some strains of Agrobacterium radiobacter var. tumefaciens.

A large number of plant species are attacked by A. radiobacter var. tumefaciens including fruit trees, roses, and grapes (19,32); the pathogen enters these plants through wounds. Only pathogen strains possessing a specific plasmid, the Ti plasmid, cause disease (53). A segment of DNA from the virulence plasmid is passed to and becomes incorporated into host cell DNA (50). Unregulated division of host cells is induced, leading to gall formation (19).

In addition to pathogenic agrobacteria, strains were discovered which did not possess the Ti plasmid and did not cause disease. The ratio of pathogens to nonpathogens was high in soil around diseased plants but low in soil around healthy plants (35). This discovery suggested the possibility of biological control of pathogenic strains through the use of nonpathogenic agrobacteria. Further investigations showed that one strain of nonpathogenic agrobacterium, A. radiobacter var. radiobacter strain 84, protected tomato seedlings from crown gall when the pathogen/nonpathogen ratio was less than one (19).

Strain 84 was shown to produce a specific antibiotic (Agrocin 84) which inhibits most of the pathogenic agrobacteria (16). Currently strain 84 is being used commercially to control crown gall of stone fruit and roses; inoculation of plants with this strain effected virtually 100% control of crown gall under field conditions. This is greater control than that achieved by use of standard bactericides (32). The treatment is also inexpensive and simple, requiring no special equipment; cost of inoculation is only a few cents per plant (32).

Bacterial antagonists may inhibit pathogens in several ways: through antibiosis, by direct parasitism, by altering the environment to make it unfavorable for growth of the pathogen, or by stimulating host plant defenses (3). Antibiosis is the mechanism whereby strain 84 controls crown gall infections caused by Agrocin 84-sensitive strains of

A. radiobacter var. tumefaciens (19). Antibiosis is also implicated as the mode of action in control of Pseudomonas syringae pv. phaseolicola by a strain of P. fluorescens (48). Preinoculation of bean plants by inserting toothpicks coated with P. fluorescens in the stem between the cotyledons, protected leaves above this point from infection by P. syringae pv. phaseolicola. P. fluorescens could not be recovered from the protected leaves, suggesting that an antibiotic substance produced by the bacterium was transported up within the plant (48).

Bacteria may directly parasitize bacterial and fungal plant pathogens by causing lysis. In most cases lysis is believed accomplished through the production of enzymes which attack components of the pathogen cell wall, causing disintegration (3). Bdellovibrio bacteria are known to have the ability to lyse other bacteria (3,38). An Arthrobacter sp. was shown to cause lysis of Pythium debaryanum hyphae in vitro. When tomato seed was dipped in a suspension of the antagonist and planted in soil infected with P. debaryanum, a 37% reduction in disease resulted. The bacterium was thought to produce proteases or glucanases which were primarily responsible for lysis (31). A strain of Bacillus pumilus was isolated which caused lysis of germ tubes from uredospores of Puccinia graminis, P. coronata, and P. graminis f. sp. tritici in vitro (33). When bacterial suspensions were applied to wheat leaves prior to

inoculation with the pathogen, disease was significantly reduced (33).

Several bacteria control plant pathogens by presumably altering the environment in such a way as to make it unfavorable for pathogen growth. Antagonists may accomplish this by altering the pH of the substrate or by depleting the substrate of nutrients required for growth of the pathogen. Erwinia herbicola affects growth of several bacterial pathogens by lowering the pH of the substrate (9,37). Hsieh and Buddenhagen (1974) found E. herbicola inhibited Xanthomonas oryzae in vitro (15). When pH of the media was adjusted to 7.0, no inhibition was observed. They also found that while live cells of E. herbicola provide some protection to rice plants, killed cells, which did not lower the pH, provided no protection (15). Blakeman and Fraser found that germination of Botrytis cinerea conidia on chrysanthemum leaves was inhibited by bacteria present on the leaves (2). Antifungal compounds were not detected in droplets containing the bacteria, and subsequent experiments revealed that antagonistic bacteria removed amino acids from the environment at a rate faster than the fungus. Depletion of nutrients from the environment was proposed as the cause of inhibition in this case (4).

How bacterial antagonists inhibit plant pathogens by stimulation of host plant defenses is not well understood. Several excellent articles dealing with this subject are found in the literature (12,27,36,52). Because antagonists

that inhibit only by this method are not identified using normal screening procedures for bacterial antagonists, this topic will not be discussed extensively. The subject is, however, mentioned later in the discussion of *in vitro* screening procedures.

General procedures to detect bacterial antagonists involve isolation, and *in vitro* screening, followed by *in vivo* testing and/or screening (41). Most researchers have isolated antagonists from the same species of plant which they wish to protect from disease (5,7,10,21-25,31,33,45-48). In some cases, antagonists were isolated from soil (6,11,17, 29,40,49). Soil isolation is often used when the pathogen invades host seed or root tissues. An antagonistic isolate obtained from or near the plant part to be protected is preferable, since such isolates are theoretically better adapted to the particular plant part; therefore, these isolates are more effective in controlling disease, than an antagonist taken from a completely different site (1,6).

Potential bacterial antagonists are screened *in vitro* for ability to inhibit pathogens by several methods. One of the most common methods is to place potential antagonist and pathogen near each other in an agar medium. Inhibition of pathogen growth is evidenced by formation of an inhibition zone. Antagonists found to inhibit pathogens *in vitro* are then subjected to *in vivo* testing. In some cases only antagonists causing formation of especially large inhibition zones were retained (1,21).

Type of media used, incubation temperature, and testing technique all affect the results of in vitro screening tests. Relative to effect of media, Weinhold and Bowman found that a Bacillus sp. produced greater antibiosis on soybean tissue extract agar than on barley extract agar (54). Vasudeva et. al. reported that culture filtrates of a B. subtilis isolate grown in dextrose asparagin phosphate media reduced Fusarium udum colony growth by 61%; whereas culture filtrates from potato dextrose broth cultures of the antagonist reduced colony growth by only 13% (51). Dunleavy found inhibitory compounds produced by B. subtilis more active at pH 4.0 (8). Teliz-Ortiz and Burkholder found that P. fluorescens inhibited P. syringae pv. phaseolicola actively on potato glucose agar but not on nutrient agar (48). Spiers found that although A. radiobacter var. radiobacter strain 84 produced Agrocin 84 on all media tested, inhibition was not. Agar plugs were removed from such plates and placed in holes in plates containing media normally allowing inhibition, sensitive A. radiobacter var. tumefaciens isolates were inhibited (42).

The temperature at which test plates are incubated influences in vitro inhibition of a particular pathogen by an antagonist. In one study, five isolates of Bacillus sp. were tested for inhibition of Rhizoctonia solani at different temperatures (26). Each antagonist grew at temperatures below 12°C but none inhibited the pathogen at temperatures below 12°C (26). Vasudeva et. al. found

maximum growth inhibition of Fusarium udum colonies by B. subtilis at 24-34°C while size of inhibition zones decreased at higher or lower temperatures (51).

Techniques of testing may also affect results. When potential bacterial antagonists were placed on one side of the plate and Typhula idahoensis mycelia on the other, fungal colony growth was inhibited by several bacteria. When the test was repeated placing the bacteria in the middle of the fungal colonies no inhibition of the pathogen resulted (18).

In vitro screening has several other limitations, in addition to problems in interpreting the results, due to the effects of cultural environment and testing technique. Because the testing environment differs greatly from actual in vivo conditions, antagonists successful at inhibiting pathogens in vitro may not necessarily be successful in vivo. These inconsistencies may arise because: (1) media employed may provide a nutrient spectrum different from that found in the host environment, and (2) effects of normal host environment and microflora on the antagonist/pathogen interaction are not encountered in vitro.

Frequently, bacteria which inhibit pathogens in vitro through antibiosis fail to do so in vivo, possibly because nutrients available for antibiotic production in vitro may not be available in/on the host plant (21,24,28,30,45). Even if antibiotics are produced in vivo they may be

inactivated, degraded or adsorbed onto soil particles or plant tissues (3,39).

Failure of successful *in vitro* antagonists to control pathogens *in vivo* may also be due to loss of viability of the antagonist *in vivo*. In one study, a bacterial antagonist inhibitory to *Colletotrichum lagenarium in vitro* gave no control when applied to cucumber plants in the field prior to pathogen inoculation. Further study revealed that 99% of the antagonist cells were no longer viable 24 hours after application. Viability was not significantly improved by adding nutrients to the inoculum (25). Loss of viability may have been due to environmental conditions, to antagonism from other naturally occurring microorganisms, or to competitive weaknesses of the antagonist as compared to normal microflora.

Even when disease control is obtained *in vivo*, it can not be assumed that the mode of inhibition is the same as that *in vitro*. Siminoff and Gottlieb found that *Streptomyces griseus* inhibited *B. subtilis in vitro* by the production of antibiotics. *S. griseus* also inhibited growth of *B. subtilis* in soil. When a mutant *S. griseus*, unable to produce antibiotics, was added to soil, growth of *B. subtilis* was similarly inhibited. Therefore, control originally obtained was not due to antibiosis (39).

It is difficult to predict *in vivo* screening performance of antagonists which do not exhibit activity *in vitro* (5). Despite the limitations of *in vitro* screening, the

procedure remains an inexpensive, rapid way to screen large numbers of potential antagonists; thus it is almost always used as the initial step in the screening procedure. The many successful attempts at biological control using bacterial antagonists originally selected *in vitro* indicate its utility (6,8,11,22,31,33,40,46,48,49).

After *in vitro* screening of potential antagonists, *in vivo* tests are performed to determine whether such isolates will control specific diseases on greenhouse or field grown plants. Many factors affect the outcome of these tests, including mode of antagonist application, nutrients available in the inoculum, application time and populations of the antagonist relative to the pathogen.

In most cases, potential antagonists are applied to the target by only one mode so it is difficult to determine to what extent mode of application affects *in vivo* screening results. In the few cases where several modes of application were attempted, results suggest that the mode is an important factor. *A. radiobacter* var. *radiobacter* strain 84 decreased crown gall on peach trees by 95% when roots of trees were dipped in the inoculant prior to transplanting. When both seeds and roots were so treated, 99% control was achieved. When just seeds were inoculated, only 78% control was obtained (16). Strain 84 was more effective in controlling pathogenic strains insensitive to Agrocin 84 when applied to the seed and roots rather than when applied to aerial plant

parts (32). Swineburne controlled Nectria galligena infection of apple trees when B. subtilis was applied to the leaf scar tissue. Bacteria applied to internodal areas did not persist and did not control disease (46). In contrast, P. fluorescens controlled P. syringae pv. phaseolicola infections of bean equally well whether applied as a spray, by a toothpick method, or as a seed treatment (48).

Since antibiotic production can be enhanced in vitro by nutrient modifications, attempts have been made to increase inhibitory activity of antagonists in vivo by including nutrients in the inoculum. B. subtilis controlled R. solani more effectively in greenhouse tests when a nutrient solution high in nitrogen was added to soil containing the antagonist (8). A bacterial antagonist used to control cucumber anthracnose was more effective when applied in nutrient broth, soybean meal broth or glucose (23,24). B. subtilis significantly reduced rust infections (Uromyces phaseoli) on greenhouse grown beans only when Eugonbroth was applied with the bacterium; Eugonbroth alone provided no protection from infection (44).

The time at which the antagonist is applied in relationship to the time of inoculation or infection by the pathogen also affects disease control. P. fluorescens protected bean plants from infection by P. syringae pv. phaseolicola only when applied prior to the pathogen (48). B. subtilis controlled Uromyces phaseoli infections on

greenhouse grown bean plants only when applied 0-3 days prior to the pathogen. Weekly applications to plants in the field, however, did not significantly control bean rust (44). Strain 84 of A. radiobacter var. radiobacter was more effective in reducing crown gall on Mazzard cherry trees when applied after the pathogen than when applied prior to the pathogen (32). P. cepacia sprayed weekly on field grown peanuts reduced Cercospora leaf spot by 38%; weekly applications were necessary because antagonist populations declined rapidly after application (43).

Populations of antagonists relative to those of the pathogen may also affect efficiency of the antagonist. Strain 84 was only found effective in reducing crown gall on tomato when the pathogen/nonpathogen population ratio was less than 1 (19). E. herbicola was more effective in controlling X. oryzae infections of rice when the pathogen/nonpathogen population ratio was less than 1 (15).

In summary, a large number of factors may influence the results of in vivo testing. Antagonists successful in controlling disease in the protected environment of a mist chamber or greenhouse are often ineffective in the field (7,20,25,26,43). In one study, B. subtilis protected corn from corn root infections in the field during a wet season but did not give protection during two dry growing seasons. A fungal antagonist showed the ability to protect corn during the two dry seasons but was less

effective during the wet season. It was suggested that a mixture of antagonists might have protected the seedlings under both extremes of field conditions (20). The fact that mixtures of bacterial antagonists have not been tested to control disease is probably a shortcoming of the screening procedures used to identify potential antagonists (1).

Successful control of bean rust and halo bacterial blight with bacterial antagonists suggests that biological control methods may be useful in controlling some other bean diseases (44,48). Bean halo blight and common bacterial blights are generally managed through preventive measures such as use of clean seed and tolerant varieties (14). Other bean diseases, such as white mold can be partially controlled by cultural practices and chemical applications (13,34). In each case however, the diseases can be difficult and expensive to control once pathogen inoculum is present in the field. Development of biological control agents for control of bean diseases could provide safe and inexpensive methods to supplement current control methods.

The purposes of this study were to: (1) screen internal bacterial contaminants of bean seed *in vitro* for the ability to inhibit various bean pathogens, (2) to determine factors affecting *in vitro* antagonism, (3) to conduct preliminary screening experiments *in vivo* to select promising bacterial antagonists and, (4) to study factors

affecting the ability of selected antagonists to control bean diseases *in vivo*.

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MATERIALS AND METHODS

Isolation and In Vitro Screening of Antagonists

A total of 214 bacterial isolates were screened for ability to inhibit Xanthomonas campestris pv. phaseoli in vitro. In addition, 131 of the isolates were screened for the ability to inhibit Pseudomonas syringae pv. phaseolicola, and 58 were screened for the ability to inhibit Pseudomonas syringae pv. syringae in vitro.

Most (140) of the isolates were obtained by means of the lacuna method (8), as follows. Bean seeds were surface sterilized for 2 minutes in 2.5% sodium hypochlorite (2.5% Cl^-), and rinsed twice for 1 minute each in sterile distilled water (SDW). The seeds were allowed to air dry, and ground to a fine powder in a mill (screen size 40μ). Samples of ground seed (8.5 g) from each seed lot were then suspended in 50 ml of Kings Medium B Broth (20 g peptone, 2.5 g $K_2HPO_4 \cdot 3H_2O$, 6 g $MgSO_4 \cdot 7H_2O$, 15 ml glycerol in 1000 ml distilled water) (4) or in 50 ml BYE (10 g yeast extract in 1000 ml 0.01M PO_4 buffer pH 7.2) and the mixtures incubated on a rotary shaker at room temperature (22 $\pm$ 2°C).

After 48 hours, serial dilutions of the cultures were made and 0.1 ml of the 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}

dilutions were mixed with 0.1 ml of a pathogen suspension (adjusted to O. D. 0.3 at 600 nm) plus 4.0 ml of soft agar (10 g agar in 1000 ml distilled water). Absorbance was determined using a Bausch and Lomb Spectronic 20 Spectrophotometer. Suspensions were poured over a hard agar (15 g agar in 1000 ml distilled water) base layer. Soft BYE over a hard BYE base layer was used to test antagonists from cultures incubated in BYE, while soft KMB was used over a hard KMB base layer to test antagonists from cultures incubated in liquid KMB.

Antagonistic bacteria selected for further study were those giving distinct areas of inhibition in the pathogen lawn. These colonies were picked out and maintained on modified YCA (10 g yeast extract, 2.5 g calcium carbonate, and 15 g agar in 1000 ml distilled water) or KMB plates for further testing. If more than one isolate with identical colony characteristics was obtained from a given seed lot, only one was retained. Seed lots used, seed type and antagonistic isolates obtained are given in Appendix A.

Numerous (58) isolates of internal bean seed contaminants were obtained from Mintarsih Adimihardja (presently at Kampus University, Lampung, Indonesia), and 6 isolates were obtained from Maureen Mulligan (graduate student, Department of Botany and Plant Pathology, Michigan State University, East Lansing MI). In addition 4 isolates were obtained from bean stem sections after grinding the stem sections in buffered saline and streaking the liquid onto

KMB and YCA plates. The source of each of these isolates is given in Appendix A.

All isolates were further tested for inhibitory activity by spotting or streaking the bacteria on top of a soft agar layer containing the pathogen of interest. Most frequently used was a 10 ml base layer of Nutrient Agar (Difco) with a NBGA soft layer consisting of 8 g Nutrient Broth (Difco), 5 g glucose, and 10 g agar in 1000 ml distilled water. Occasionally NBGYE (consisting of 8 g Nutrient Broth, 5 g glucose, 5 g yeast extract and 10 g agar in 1000 ml distilled water) was used for the soft agar layer.

Certain of the antagonists were also tested on Bean Pod Agar (Difco). Single colonies from 2-3 day old antagonist cultures were streaked on top of a soft BPA layer (15 g Difco Bean Pod Agar in 1000 ml distilled water) containing the pathogen of interest; BPA was also used for the base layer in these experiments. This was done to test the effect of different cultural media on inhibitory activity.

Some antagonists were tested for the ability to inhibit growth of plant pathogenic fungi in culture. In general, tests were performed on PDA (Difco Potato Dextrose Agar), while tests for inhibition of Colletotrichum lindemuthianum were performed on BPA, and tests for the inhibition of Phytophthora megasperma were conducted on LBA (Difco Lima Bean Agar). The source of all fungal isolates used throughout this study are given in Appendix B.

A 5 mm diameter plug of mycelium was removed from the advancing edge of a 4-8 day old colony of each fungal isolate using a cork borer. Each mycelial plug was placed on a fresh plate of PDA, and a 5 μl droplet of a turbid suspension of the antagonist in SDW was spotted onto the plate 2 cm from the mycelial plug. Control plates were those in which only SDW was spotted beside the mycelial plug. Studies included 3 replications and 1 control for each antagonist/pathogen combination. Plates were observed after 4-7 days incubation in the dark at 25^{o}C.

The Effect of Temperature on the Ability of Antagonists to Inhibit Bacterial Pathogens of Beans

Experiments were performed to determine possible temperature effects on the inhibitory activity of antagonists. In these studies, NBGA was used for both soft and base layers of media. Pathogens were grown on a shaker for 36 hours in liquid BYE, adjusted to an absorbance of 0.25 at 600 nm, and 0.1 ml of the pathogen suspension mixed into 4.0 ml of soft agar. The mixture was poured over the 10 ml base layer. Single colonies were then taken from 48 hour old YCA or KMB cultures of each antagonist using sterile Q-tips and streaked on the soft agar layer. Control plates for each pathogen were streaked with sterile Q-tips rubbed on the surface of uninoculated YCA or KMB plates. Up to 5 or 6 replications were made for each antagonist/pathogen combination at each temperature. Plates were incubated

in the dark at 15, 20, 30 and 36°C. After 48 hours, inhibition zones were measured and recorded.

The Effect of Culture Age on In Vitro Inhibition by Antagonists of Bacterial and Fungal Pathogens

Turbid suspensions of selected antagonists were prepared in SDW and 5 µl droplets of the suspensions or of SDW were placed in the middle of plates containing 10 ml BPA. The isolates were allowed to grow for 1 to 9 days before being killed by 30 minutes exposure to chloroform vapor. Control plates were similarly exposed to chloroform vapor.

A 4.0 ml soft layer of BPA containing the bacterial pathogen of interest was then poured over each plate. Pathogens included Xanthomonas campestris pv. phaseoli (Xp), Pseudomonas syringae pv. phaseolicola (Pp), and Pseudomonas syringae pv. syringae (Ps). The sources of all bacterial pathogens used throughout this study are given in Appendix C. In each case, 4 replications were made for each antagonist (of a particular culture age)/pathogen combination. Plates were observed after 1, 2 and 3 days, and inhibition zones recorded.

Isolate 143a, an endospore forming bacterium, could not be killed with chloroform, therefore a number of experiments were performed to detect activity of inhibitory compounds in the culture filtrates of different aged cultures. In the first experiment, inhibition of bacterial pathogens was tested by first mixing 0.1 ml of a suspension of Xp

isolate 520 or Xpf R10 with 4.0 ml of soft NBYEA. This suspension was poured over a NA base layer and allowed to harden. Fungal inhibition was tested by first spreading 0.2 ml of a mycelial suspension of Sclerotinia sclerotiorum or Phoma betae on plates containing 10 ml each of PDA.

Isolate 143a was grown in NBGCC (8 g NB, 5 g glucose, and 5 g calcium carbonate in 1000 ml distilled water) for 24 hours. The culture was passed through a 0.2 μ pore size filter (Nalgene presterilized disposable filter unit) using a vacuum of approximately 1200 mm Hg. Sterile filter paper discs 1 cm in diameter were dipped in the culture filtrate and two such discs were placed in plates containing the test bacterial or fungal pathogen. Control plates contained the pathogen and two filter discs dipped in filter sterilized NBGCC. Seven replications were prepared for each pathogen and plates were incubated in the dark at 25^{o}C. Plates containing bacterial pathogens were examined after 48 hours while those containing fungal pathogens were examined after 4 days. The presence of any inhibition zones around or beneath the filter discs was recorded.

In the second experiment, antagonist 143a was grown in Eugonbroth (BBL) for 4 or 35 days on a shaker at room temperature. The 4 day old culture was filter sterilized using a 0.45 μ pore size filter while the 35 day old culture was filter sterilized using a 0.2 μ pore size filter. Droplets (5 μl) of each culture filtrate were placed on plates

containing 10 ml of BPA, allowed to dry for 1 hour, and then 4.0 ml of soft BPA containing 0.1 ml of a suspension of Xp 11 was poured over this base layer. Plates were incubated for 24 hours in the dark at 25°C then observed for the presence of inhibition zones.

In a third experiment, 143a was grown in Eugonbroth for 1, 2, 4, 6 or 9 days, and culture filtrates obtained by passing each through a filter of 0.45 μpore size. A 10 μl droplet of one of the culture filtrates was spotted onto each of 4 plates containing 10 ml of PDA, and a mycelial plug of S. sclerotiorum (5 mm in diameter) placed 1 cm from each droplet. Control plates were spotted with uninoculated filter sterilized Eugonbroth. Radial growth of the fungal colonies in the direction of the culture filtrate was measured after 3 days.

In the final experiment with 143a, flasks containing 75 ml of Eugonbroth were inoculated with 143a at various times (65-0 days) prior to filter sterilization. Cultures were grown at room temperature on a shaker. Control flasks containing Eugonbroth alone were also placed on the shaker for various time intervals prior to filter sterilization. All cultures for a given experiment were filter sterilized the same day using Nalgene presterilized disposable filter units with a pore size of 0.45 μ. Culture filtrates were tested immediately, after autoclaving 20 minutes, after 30 days aging, and after 30 days aging plus autoclaving.

Pathogens used in these experiments included S. sclerotiorum, P. betae, Rhizoctonia solani, Fusarium solani f. sp. phaseoli, C. lindemuthianum, as well as Xp 11 and Xp a.

Plates of bacterial pathogens were prepared by adding 3.0 ml of a suspension (O. D. 0.3) of the bacteria in 0.01M PO_4 buffer pH 7.2 per 100 ml warm (45°C), liquid NA. After mixing, 10 ml of the solution was added to each petri dish and allowed to harden.

To test S. sclerotiorum, R. solani, and P. betae, mycelium of each was scraped off 4 day old PDA cultures and placed in SDW for 20 minutes and shaken frequently. Inoculum of F. solani f. sp. phaseoli was prepared by scraping spores from 7 day old PDA cultures and suspending in SDW. A 0.2 ml aliquot of each mycelial or spore suspension was spread evenly over plates containing 10 ml of PDA.

Plates containing C. lindemuthianum were prepared by first scraping conidia from a 6-9 day old BPAB (22.5 g BPA and the extract from 20 g of navy bean seeds in a total of 1000 ml distilled water) culture of the pathogen. The conidia were suspended in SDW for 20 minutes with frequent shaking. A 0.2 ml aliquot of the spore suspension was spread evenly over plates containing 10 ml each of BPA.

Four or 5 wells of 5 mm diameter were then cut with a cork borer in the agar of the seeded plates. Wells were

spaced equidistant from one another and sides of the petri dishes. A 30 µl aliquot of each test culture filtrate was placed in each well; one well in each plate contained filter sterilized Eugonbroth as a control. At least 3 replications were prepared for each experiment for each pathogen/culture filtrate combination, and all plates were incubated in the dark at 25°C. Plates containing bacterial pathogens were observed after 48 hours, while those with fungal pathogens were observed after 4 days. In each case size of inhibition zones was recorded.

To further study 143a inhibition of fungi in culture, 5 µl of 143a suspension was spotted onto Eugonagar plates. At 10 days after inoculation, 5 mm diameter plugs were removed from areas 2 mm from the bacterial colony. The plugs were transferred to fresh plates of PDA (20 ml/plate). Plugs of S. sclerotiorum mycelium were taken from 4 day old cultures of the fungus and placed on the PDA plates 20 mm from the Eugonagar plugs. After 6 days incubation at 25°C, S. sclerotiorum hyphae were removed from edges of inhibition zones, examined microscopically and transferred to fresh PDA plates. Plates were retained for 20 days to determine whether hyphae were viable.

To determine whether in vitro inhibition of C. lindemuthianum by 143a was due to pH effects, 5 mm diameter plugs of pathogen strains alpha, beta and gamma were placed 5 cm from a colony of 143a. Medium used in these experiments was BPA prepared in 1000 ml 0.01M PO_4 buffer pH 7.2.

Plates were incubated in the dark at 25°C for 11 days at which time inhibition zone size was recorded. Two plates were used for each pathogen strain and the experiment repeated twice.

Physiological Tests

Attempts were made to identify several of the antagonists. Tests included: fluorescence on KMB (1), catalase (3), growth in Thioglycollate Broth (9), gram stain (16), arginine dihydrolase (15), reduction of nitrate (11), Voges-Proskauer Reaction (3), hydrolysis of starch (3), oxidase activity (6), and hypersensitivity in tobacco (5).

Pathogenicity Tests

All antagonists used for *in vitro* studies were first tested for pathogenicity to beans by the seedling injection technique (10). Manitou kidney bean seedlings were injected at 10-12 days with turbid suspensions of each isolate; a minimum of 9 seedlings were used for each isolate. Isolates causing browning of stems, wilting, or any other abnormality in plant growth were discarded.

Screening of Antagonists In Vivo

Use of Mixed Inoculation Techniques to Screen Antagonists for Ability to Protect Bean Plants from Fuscous, Common, and Halo Blight of Beans

Various antagonists were tested for ability to control halo, fuscous and common bacterial blight *in vivo* in two different experiments. In the first experiment, flasks containing 25 ml of BYE were inoculated with 0.1 ml suspension (0.3 O. D.) of the antagonist and 0.1 ml suspension (0.3 O. D.) of the pathogen. After 2 days incubation on a shaker at room temperature, the mixed cultures were injected into 10-day-old Manitou seedlings (10). Control seedlings were injected with BYE alone or the pathogen alone. After 17 days plants were observed for disease symptoms. As there was little or no variation in disease severity between plants in each pot, disease ratings were on a pot basis rather than individual plant basis. The experiment was repeated 3 times.

In the second mixed inoculation experiment, equal suspension volumes of antagonist and pathogen were mixed and immediately injected into the primary leaf nodes of 10-12-day-old Manitou seedlings. Plants were observed 5, 10, 20, and 34 days after injection and rated for disease severity.

In each mixed inoculation experiment, disease ratings were made using the following rating system: (-) healthy, (+) plants with stem browning, (++) plants with stem browning, slight wilting and, (+++) severely wilted plants.

The Effect of Application Time on the Ability of Antagonists to Protect Greenhouse Grown Bean Plants from Bacterial and Fungal Pathogens

Experiments were performed to determine whether time of antagonist application affected ability of the antagonists to control common blight. Manitou bean plants were grown in the greenhouse (1 plant/pot) at temperatures of 22-30°C. Antagonists were grown in BYE at room temperature for 2 days on a shaker, and the cells sedimented by centrifugation for 15 minutes at 10,000g. Bacteria were suspended in 0.01M PO_4 buffer, and adjusted to an O. D. of 0.4. Plants were sprayed with the bacterial suspension to run off at various time intervals (9-1 days) prior to inoculation with the pathogen. Control plants were either unsprayed or sprayed with PO_4 buffer alone. A minimum of 8 pots of plants were sprayed for each antagonist at each time interval.

The common blight pathogen, isolate Xp 11, was prepared in the same manner as antagonists except that the inoculum suspension was adjusted to O. D. 0.25. Plants were sprayed lightly to run off with the pathogen after all plants possessed fully expanded 3rd trifoliolate leaves. Plants were observed after 3 weeks and percentage of the 2nd trifoliolate covered with lesions recorded. Duncan's multiple range test was used in each experiment to compare disease severity of treated plants with that of the controls (13).

The effect of application time on ability of 143a to protect bean seedlings from infection by C. lindemuthianum was also tested. Montcalm kidney bean seedlings were pretreated with the antagonist at various times prior to inoculation with the pathogen. Plants were pretreated by spray inoculation with a suspension of 143a (approximately 2.5×10^9 cfu/ml) in SDW or with SDW alone. Approximately 3.5 ml of the bacterial inoculum or SDW was used per plant.

The plants were then placed in a mist chamber with relative humidity above 95% until inoculation with strain beta of C. lindemuthianum. The seedlings were no more than 18 days old before inoculation with the pathogen. Inoculum was prepared by flooding 6-10 day old BPAB cultures of C. lindemuthianum with SDW, scraping the agar surface and pouring the resulting spore suspension through two layers of cheesecloth into SDW. One plate of C. lindemuthianum was used for each 150 ml of SDW (approximately 1×10^7 spores/ml); 0.1% v/v Tween 80 was added to the inoculum.

Plants were removed from the mist chamber, spray inoculated with the pathogen and immediately returned to the mist chamber. Special attention was given to inoculating the undersides of leaves, the growing point and the stems. After an additional 8 days in the mist chamber, plants were removed to greenhouse benches for 2 days before disease ratings were made.

Plants were rated subjectively and quantitatively to determine disease severity. Subjective data was taken using the following rating system:

0 = healthy
1 = a few lesions on the stem or first trifoliolate leaf
2 = a moderate number of lesions on the stem and first trifoliolate leaf
3 = both first trifoliolate leaf and stem with numerous lesions
4 = severe water-soaking and chlorosis of the first trifoliolate leaf
5 = collapse of the first trifoliolate leaf, lesions on the leaf, stem, and petioles

Quantitative data was obtained by counting numbers of lesions on the stem from soil level to the point of attachment of the first trifoliolate leaf. Both sets of data were analyzed using Duncan's multiple range test.

The Effect of Mode of Application on the Ability of Antagonists to Protect Greenhouse Grown Bean Plants from Bacterial and Fungal Pathogens

Seed treatments with a variety of antagonists were studied to determine whether mode of inoculation affected degree of control of common blight of beans. Manitou bean seeds were surface sterilized by soaking in a solution of 2.5% sodium hypochlorite (2.5% Cl^-) for 2 minutes. The seeds were then rinsed twice for 1 minute each with SDW and allowed to dry.

Cultures of each antagonist were grown for 48 hours in BYE on a shaker. Five Manitou seeds were then placed in each mixture of 10 ml soft BPA with 1.0 ml of an antagonist culture, or in 11 ml of soft BPA alone. Seeds were

incubated in the inoculum for 16 hours in the dark at 25°C then planted in the greenhouse in vermiculite with 1 seed per pot. One month after planting plants were sprayed lightly to run off with Xp 11.

Pathogen inoculum was prepared by centrifuging 48 hour old liquid BYE cultures of Xp 11 for 15 minutes at 10,000g. Sterile distilled water was added to adjust inocula to an O. D. of 0.2. Twenty days after spray inoculation of plants with Xp 11 number of lesions on the first trifoliolate leaf of each plant was recorded.

The experiment was designed with 5 blocks containing 1 replication (1 plant) for each treatment. Plants were randomized within each block and were separated by sufficient space to prevent physical contact between plants. After square root transformation of the data, to obtain homogeneous variance, seed treatments with different antagonists were compared to control treatments using Duncan's multiple range test.

Seed treatments were also evaluated in two experiments as a control for anthracnose. In the first experiment, seeds of the Michigan Improved Cranberry (MIC) variety naturally infected with C. lindemuthianum strain beta were obtained from field plants inoculated with the pathogen during the 1981 growing season. To determine whether seed treatment with 143a would reduce disease severity, seeds were dipped in SDW, soft BPA, or soft BPA containing 143a (approximately 2.5×10^9 cfu/ml). Because of differences

in seed germination the number of plants used for each treatment varied from 14 in one experiment to 22 and 24 in the other experiments.

Seeds were planted immediately after treatment in vermiculite or a mixture of vermiculite and sterile greenhouse soil. Upon germination, plants were transferred to a mist chamber for 7-10 days. Seven days after removal from the mist chamber, number of lesions on the portion of the stem from soil line to the primary leaf node was recorded.

In the second experiment, uninfected seeds of the MIC variety were surface sterilized then dipped into SDW, soft BPA, or soft BPA containing 143a (approximately 2.5 x 10^9 cfu/ml), and planted. When all plants possessed fully expanded 1^{st} trifoliolate leaves they were spray inoculated with C. lindemuthianum strain beta (approximately 1 x 10^7 spores/ml) and placed in a mist chamber for 8 days. Two days after removal from the mist chamber the number of lesions on the stem from soil line to the primary leaf node was recorded. The experiment was repeated twice: 27 plants were used for each treatment in each experiment.

Log transformations were used to achieve homogeneous variance of the data from both experiments and Duncan's multiple range test was then used to compare lesion numbers found in the different treatments within each experiment.

The Effect of Media on the Ability of Antagonist 143a to Control Uromyces phaseoli Infection on Greenhouse Grown Bean Plants

The effect of different suspending solutions on the ability of 143a to control bean rust was tested. An isolate of B. subtilis (obtained from Dr. J. R. Stavely, USDA, Beltsville, MD) reported to protect bean seedlings from bean rust (12) was compared with 143a. Fourteen day old UI-114 pinto bean seedlings were spray inoculated with an average of 3.0 ml/plant of the following solutions: (1) SDW, (2) no spray, (3) sterile Eugonbroth, (4) 143a from a 2 day old Eugonagar culture suspended in SDW, (5) 143a in Eugonbroth (a two day old culture) or (6) B. subtilis in Eugonbroth (a two day old culture).

All bacterial inocula were adjusted to an absorbance of 0.4 at 600 nm. Special care was exercised to completely cover both sides of each primary leaf with the appropriate inoculum. Plants were placed in a mist chamber at relative humidity greater than 95%. After two days plants were spray inoculated with an average of 2.0 ml/plant of a suspension of Uromyces phaseoli uredospores prepared by adding 5 mg of uredospores per 10 ml of SDW containing 0.1% v/v Tween 20.

The experiment was designed to include 2 blocks with 1 replication of each treatment (consisting of 12 samples) randomly arranged within each block. Each treatment was separated from others in the mist chamber by a row of pots containing UI-114 plants.

After 5 days in the mist chamber, plants were rated for number of lesions on each primary leaf. Data were transformed using a square root transformation to achieve homogeneous variance. Duncan's multiple range test was used to compare each treatment with the control treatment of interest. Results should be viewed with caution since the experiment was performed only once.

Viability of Isolate 143a on Spray Inoculated Bean Leaves

In order to determine whether antagonist 143a remains viable on spray inoculated bean leaves, leaf prints were obtained. Seven different varieties of beans were used including Manitou, Montcalm, Michigan Improved Cranberry, UI-114, GN Tara, Sanilac, and Black Turtle Soup beans. Plants possessing fully expanded 2^{nd} trifoliolate leaves were spray inoculated with 143a (approximately 2.5×10^9 cfu/ml) in SDW. Control plants of each variety were spray inoculated with SDW alone.

Inoculum of 143a was prepared by centrifugation of 2 day old Eugonbroth cultures at 10,000g for 15 minutes. The pellet was resuspended in SDW and absorbance adjusted to 0.3 at 600 nm. One, three and eleven days after inoculation, one leaflet of the 2^{nd} trifoliolate was removed, and the upper surface pressed flat onto Eugonagar plates containing cycloheximide (50 ppm) for 1 minute (2). Plates were incubated at $25^{o}C$ in the dark for 2 days. Percentage of leaflet area colonized by bacteria with

colony characteristics identical to isolate 143a was recorded for both treated and control plants.

Control of Damping-Off of Sugar Beet Seedlings with 143a

Sugar beet seeds suspected of being internally infected with P. betae were surface sterilized by soaking for 5 minutes in 0.5% sodium hypochlorite (0.5% Cl^-). Seeds were allowed to dry and placed in petri dishes containing water agar (18 g agar in 1000 ml distilled water). After 3, 5 and 10 days, seeds were observed microscopically in order to determine number from which mycelium characterisitic of P. betae grew out; P. betae hyphae are septate with vesicles. A total of 360 randomly selected seeds from the seed lot were observed, and percentage of seed infected was determined.

To determine whether isolate 143a could control damping-off of sugar beet seedlings, seeds were treated for one and a half hours with 143a suspended in 0.5% w/v cellulose methyl ether (CME) (approximately 1×10^8 cfu/seed) or CME (0.5% w/v) alone (14). Seeds were allowed to dry for 24 hours at $25^{\circ}C$.

Treated seeds were then planted in sand (with a water content of 4.0%), 180 seeds per wooden flat and 2 flats per treatment. Each flat was covered with a plastic top and left at room temperature ($23 \pm 3^{\circ}C$). After 8 and 16 days the number of seeds which did not germinate and the number of infected seedlings were recorded. Germination

and infection data for 143a treated seeds and for control seeds were compared using Duncan's multiple range test after an arcsin square root transformation.

Field Experiments Conducted in 1981

During the summer of 1981, various experiments were performed in the field to test the ability of several potential antagonists to control bean pathogens. All experiments were conducted at the Michigan State University Botany Farm located in East Lansing, Michigan.

Use of Antagonists to Control Anthracnose and White Mold of Beans Under Field Conditions

An experiment was performed to determine whether spray inoculation with 143a would protect plants from infection with C. lindemuthianum strain beta. Seeds of the MIC variety were planted (5 seeds/ft) in 4 blocks with each block containing one 20 foot row of beans for each treatment. Treatments included:

1) Spray inoculation with approximately 1 x 10^8 cfu/ml SDW of 143a, 7 and no days prior to inoculation with the pathogen
2) Inoculation with 143a, at the time of inoculation with the pathogen and 7 days later
3) Inoculation with 143a, 7 and 19 days after inoculation with the pathogen
4) Control (not spray inoculated)

Pathogen inoculum was prepared as previously described for experiments in the greenhouse involving C. lindemuthianum. The pathogen was applied 39 days after planting when plants possessed fully expanded 2nd trifoliolate leaves.

At maturity, 70 pods were selected at random from each row and the number of sporulating lesions on each was recorded. A log transformation was used to obtain homogeneous variance of the data. Duncan's multiple range test was used to compare treatments 1, 2 and 3 to control treatment #4.

Experiments were also conducted to determine whether sprays of 143a to plants of Tuscola and MIC varieties at various times during the growing season would protect them from infection by S. sclerotiorum. Bacterial inoculum was prepared by suspending 2 day old PDA cultures in SDW. Approximately 1 x 10^8 cfu/ml were applied at a rate of 10 ml per foot of row at each inoculation time.

The experiment with Tuscola plants was designed with 6 blocks, each block containing one 22 ft row per treatment. Treatments included:

1) Plants spray inoculated with 143a, 24 and 32 days after planting
2) Plants spray inoculated with 143a, 32, 38 and 47 days after planting
3) Plants spray inoculated with 143a, 38 and 47 days after planting
4) Unsprayed control plants

The same basic experiment was performed using MIC plants. In this case the experimental plot contained 4 blocks with one 18 ft row for each treatment in each block. Treatments included:

1) Plants spray inoculated with 143a, 23 and 47 days after planting
2) Plants spray inoculated with 143a, 47 and 59 days after planting

3) Plants spray inoculated with 143a, 59 days after planting
4) Unsprayed control plants

Plants in both experimental plots became naturally infected with S. sclerotiorum. At harvest 40 plants were chosen at random from each row in both experiments. The percentage of plants out of 40 showing characteristic white mold symptoms (bleached appearance and/or sclerotia) was recorded. Duncan's multiple range test was used in each case to compare treatments 1, 2, and 3 to the control treatment.

Treatment of Bean Seeds with Antagonists for Protection of Plants from Bacterial and Fungal Pathogens Under Field Conditions

Seeds of the MIC, Ouray pinto, and Seafarer navy bean varieties were treated with isolate 143a prior to planting to see whether this antagonist would provide protection from naturally occurring disease pathogens.

Seeds were surface sterilized in 2.5% sodium hypochlorite (2.5% Cl^-) for 2 minutes, rinsed twice for 1 minute each in SDW, and allowed to dry for 24 hours at room temperature. Seeds were then placed in a side arm flask containing BYE alone or 48 hour old cultures of 143a in BYE (containing approximately 2.5×10^9 cfu/ml). Seeds were vacuum infiltrated with the liquids for 3 minutes then rinsed 2 times for 1 minute each with SDW. Seeds were allowed to dry for 3 days at room temperature before planting.

The experiment contained 6 blocks with 1 replication (consisting of 50 seeds planted 5/ft) of each treatment per block. Since no natural infections developed in the beans in the experiment, treatments were only compared for their effect on emergence and final stand. Duncan's multiple range test was used to make these comparisons.

In a similar experiment, seeds of MIC, Olathe pinto bean and Sacramento light red kidney bean varieties were surface sterilized as before, then vacuum infiltrated with BYE or 48 hour old BYE cultures of 143a. The seeds were planted using the same experimental design just described. However these plants were inoculated 26 days after planting with C. lindemuthianum strain beta. Inoculum of the pathogen was prepared as previously described for greenhouse experiments.

Plants were sprayed with the inoculum on a cool humid evening at the rate of 10 ml inoculum/ft of row. To determine whether seed treatment with 143a provided protection from anthracnose, 70 mature pods were harvested at random from each row and number of sporulating lesions per pod recorded. Duncan's multiple range test was used after a square root transformation of the data, to compare number of sporulating lesions on pods from seeds treated with 143a and with BYE for each bean variety tested.

Bean seeds were vacuum infiltrated with different antagonists to determine whether antagonists would provide effective protection of the plants from halo blight. Seeds

of Seafarer, Charlevoix and Manitou bean varieties were surface sterilized, rinsed and allowed to dry as described previously. Seeds of each variety were then vacuum infiltrated with BYE alone, 2 day old BYE cultures of antagonist 169, or 2 day old BYE cultures of 139b; absorbance of bacterial cultures used for inoculum was adjusted to 0.3. Three blocks were used with 2 replications (each consisting of one 10 ft row) of each treatment in each block.

Seedlings were spray inoculated with a 0.3 absorbance suspension of Pp R13 36 days after planting. Unfortunately, no disease symptoms were observed on any of the plants during the summer. Data is therefore only analyzed for any possible effects of isolate 169 or 139b on emergence or final stand count. Dunnett's two tailed test was used to make these comparisons (13).

Protection of Bean Plants from Common Blight and Halo Blight Using Spray Applications of Various Antagonists

Experiments were conducted to determine whether spray inoculation of Charlevoix bean plants with antagonists would protect them from common and halo bacterial blights under field conditions. The experimental design was that of a completely randomized block with 3 replications (each consisting of one 18 ft row) for each treatment. Inoculum of each antagonist was prepared by suspending the bacteria from 2 day old YCA cultures in SDW and adjusting absorbance to 0.3. Plants were sprayed lightly to run off with the suspension at a rate of 10 ml/ft using a knapsack sprayer.

The following treatments were used:

1) Control plants (unsprayed)
2) Plants spray inoculated with isolate 139b, 23 and 36 days after planting
3) Plants spray inoculated with isolate 139b, 36 days after planting
4) Plants spray inoculated with isolate 169, 23 and 36 days after planting
5) Plants spray inoculated with isolate 169, 36 days after planting

At 36 days after planting all plants were spray inoculated with a 0.3 absorbance suspension of isolate a of Xp in SDW.

A similar experiment was conducted to determine if antagonist 195 would protect Charlevoix plants from halo blight. Inoculum for both antagonist and pathogen were prepared as described above, except that 195 was taken from KMB plates. Plants were spray inoculated with one of the following solutions:

1) Control plants (unsprayed)
2) Plants spray inoculated with isolate 195, 23 days after planting
3) Plants spray inoculated with isolate 195, 23 and 36 days after planting

Plants were inoculated with Pp Bruder 53, 36 days after planting.

In both experiments plants were observed throughout the summer for common blight or halo blight symptoms; unfortunately no disease developed.

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RESULTS

In Vitro Studies with Antagonistic Bacteria

Initial screening of bacterial contaminants from bean seeds yielded over two hundred isolates which showed the ability to inhibit Xp, Pp, and Ps in vitro. Several of these isolates also inhibited one or more fungal plant pathogens in vitro (Table 1). Further testing of antagonist 143a showed it to be inhibitory to a wide range of fungal plant pathogens (Table 2).

Type of culture medium used was found to affect the ability of some antagonists to inhibit bacterial pathogens in vitro (Table 3). Isolate 107 did not inhibit Xpf R10 or Pp B53 when tested using a NBGA soft agar layer over a NA base layer, but inhibited both pathogens when tests were conducted using BPA soft and base layers. None of the antagonists inhibited Ps on NA based media while several (107, 169 and 195) successfully inhibited the pathogen on BPA.

Incubation temperature also affected the inhibitory activity of some antagonists in vitro (Table 4). Antagonist 143a inhibited Xpf, Pp and Ps isolates only over narrow temperature ranges with each genera of bacterial

pathogen inhibited over a different temperature range. In contrast antagonist 169 showed the same inhibitory activity against Pp BB2 at all temperatures tested.

Antagonists differed in inhibitory activity in vitro depending on culture age (Table 5). Isolate 139b showed activity against Xp 11 after 1 day of growth in culture, while isolate 161 showed activity only after 6 days of growth in culture. The culture age at which inhibitory activity was detected also differed for specific antagonists depending on the test pathogen. For example, isolate 139b inhibited Xp 11 at all culture ages, but inhibited Ps only after 6 days growth in culture.

Preliminary experiments with antagonist 143a designed to detect activity of inhibitory compounds in culture filtrates of different ages showed no inhibition of Xpf 520, Xpf R10, S. sclerotiorum or P. betae by 24 hour culture filtrates filter sterilized with a 0.2 μ pore size filter. However, inhibitory activity against Xp 11 was detected in filtrate from 4 and 35 day old Eugonbroth cultures of 143a.

Culture filtrates from 6 and 9 day old cultures passed through 0.45 μpore size filters significantly inhibited growth of S. sclerotiorum when spotted onto plates 2 cm from mycelial plugs of the fungus. Filtrates from 6 day old cultures of 143a inhibited radial growth of the fungal colony to an average 18 mm in the direction of the filtrate while in control plates growth averaged 36 mm. No

TABLE 1. Inhibition of plant pathogenic fungi *in vitro* by various antagonists.[1]

Pathogen	Inhibition[2] by Antagonist								
	112	114	139b	143a	152	153	158	196	196b
S. sclerotiorum	-	±	-	+	-	±	+	-	-
C. lindemuthianum	+	-	-	+	NT	-	-	-	±
Macrophomina sp.	-	-	-	+	-	-	NT	-	-

[1]A plug of mycelium was cut from the advancing edge of a 4-8 day old colony of each fungus using a cork borer (5 mm diameter). Each plug was placed on a fresh plate of medium. Droplets (5 μl) of turbid suspensions of each antagonist were placed 2 cm from the mycelial plug. Tests with *C. lindemuthianum* were conducted on BPA, and tests with *S. sclerotiorum* and *Macrophomina* sp. were performed on PDA. Plates were incubated for 7 days in the dark at 25°C.

[2](-) no distinct inhibition zone, (+) inhibition zone present, (±) inhibition was detected in at least one but not all replicates, (NT) not tested.

TABLE 2. Inhibition of plant pathogenic fungi *in vitro* by antagonist 143a.[1]

Pathogen	Inhibition[2]
Alternaria sp.	+
Aphanomyces cochlioides	+
Botrytis cinerea	+
Cephalosporium sp.	+
Colletotrichum lindemuthianum	+
Fusarium solani	+
Fusarium solani f. sp. *phaseoli*	+
Macrophomina sp.	+
Phoma betae	+
Phytophthora megasperma fi	+
Phytophthora megasperma r3	+
Phytophthora megasperma r1-m	+
Pyrenochaeta terrestris	+
Pythium aphanidermatum	+
Pythium ultimum	±
Sclerotinia cepivorum	+
Sclerotinia rolfsii	+
Sclerotinia sclerotiorum	+
Thielaviopsis sp.	+

[1]A 5 μl droplet of a turbid suspension of 143a in SDW was spotted onto fresh media in plates. All experiments were conducted on PDA except those involving C. *lindemuthianum* (BPA) and P. *megasperma* (LBA). A 5 mm diameter mycelial plug from a 4-8 day old fungal culture was placed 2 cm from each bacterial droplet. Plates were observed after 4-7 days incubation in the dark at 25°C.

[2](+) inhibition zones present, (-) no inhibition of the fungus, (±) inhibition was detected in at least one but not all replicate plates.

TABLE 3. The effect of different culture media on inhibitory activity of antagonists against *Xanthomonas phaseoli* var. *fuscans* (Xpf), *Pseudomonas syringae* pv. *phaseolicola* (Pp), and *Pseudomonas syringae* pv. *syringae* (Ps) *in vitro*.[1]

	Inhibition[2]					
	Tested Using NBGA Soft Agar and NA Base Layer			Tested Using BPA Soft and Base Layers		
	Pathogen			Pathogen		
Antagonist	Xpf R10	Pp B53	Ps	Xpf R10	Pp B53	Ps
105	-	-	NT	-	-	-
107	-	-	NT	+	+	+
112	-	-	-	-	-	-
138	-	-	-	+	-	-
143a	-	-	-	+	-	-
153	NT	-	-	-	-	-
158	-	-	-	-	-	-
169	+	±	-	+	-	+
195	-	±	-	-	-	+
315	+	±	-	+	+	-

[1] A suspension (0.1 ml) of each pathogen was mixed with 4.0 ml of NBGA or BPA soft agar. The suspension was poured over 10 ml thick base layers of NA or BPA. Single colonies from 2 or 3 day old antagonist cultures were streaked on top of the soft agar layer. Four replicate plates were prepared to test each antagonist/pathogen combination on each media. Observations were made after 48 hours incubation in the dark at 25°C.

[2] (-) no inhibition of the pathogen, (+) inhibition of the pathogen did occur, (±) inhibition of the pathogen occurred in at least 1 but not all replicate plates, (NT) not tested.

TABLE 4. The effect of temperature on the ability of antagonists to inhibit bean bacterial pathogens *in vitro*.[a]

Pathogen	Antagonist	Size of Inhibition Zone (in mm)[b]			
		15°C	20°C	30°C	36°C
P. phaseolicola (Pp)					
Pp BB2	169	12.5	12.2	11.0	NT[c]
Pp BB2	195	9.3	10.3	3.0	NT
Pp BB2	143a	0.3	0	22.7	NT
Pp BB4	143a	0	0	7.2	0
X. phaseoli var. *fuscans* (Xpf)					
Xpf R10	143a	0	7.5	20.0	NT
Xpf wv	143a	0	10.0	0	0
P. syringae	143a	0	0	0	14.3

[a]Pathogens were grown on a shaker for 36 hours in BYE. The absorbance of each was adjusted to 0.25. Suspensions (0.1 ml) were mixed with 4.0 ml of soft NBGA, poured over a 10 ml base layer of NBGA. Single colonies of each antagonist were streaked on plates containing the pathogen. All plates were incubated in the dark for 48 hours at the temperature given ± 2°C.

[b]Values are the average of 6 replicates.

[c](NT) indicates the test was not made.

TABLE 5 Effect of culture age on *in vitro* inhibition by antagonists of *X. campestris* pv. *phaseoli* (*Xp*), *P. syringae* pv. *phaseolicola* (*Pp*), and *P. syringae* pv. *syringae* (*Ps*).

Age of Antagonist Culture Prior to Chloroform Treatment[1]	Inhibition[2]																
	Pathogen																
	Xp 11										*Pp* B53				*Ps*		
	112	114	138	139b	152	153	158	161	169	199	139b	196	196b	299	107	139b	169
1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	+	+	-	-	+	-	+	-	+	-	-	+	-	-	+
6	-	-	±	+	-	-	+	+	+	-	+	-	-	+	-	+	X[3]
9	X	-	-	+	-	-	X	X	X	X	X	X	X	X	X	X	X

[1]Droplets (5 µl) of a turbid suspension of an antagonist in SDW was spotted onto plates containing 10 ml of BPA. Isolates were allowed to grow for 1-9 days before being killed by a 30 minute exposure to chloroform vapor. Control plates (lacking antagonists) were exposed to chloroform vapor at corresponding times. Samples of 0.1 ml from 2 day old cultures of each pathogen were mixed with 4.0 ml of soft BPA and poured over each plate containing antagonists previously killed with chloroform vapor. Four replicate plates were prepared for each antagonist (of a particular culture age)/pathogen combination. Plates were observed after 1, 2 and 3 days.

[2](+) inhibition zones present, (-) no inhibition of the pathogen, (±) inhibition zones were found in at least one but not all replicate plates. All control plates were (-).

[3](X) antagonists were still viable after exposure to chloroform vapor.

inhibitory activity was detected in plates containing culture filtrate from 1, 2 or 4 day old cultures of 143a.

Further testing revealed a wide range of plant pathogenic fungi to be inhibited by culture filtrates of 143a of different ages (Figure 1). Of those tested P. betae appeared to be most sensitive to inhibitory substances in the filtrates while F. solani f. sp. phaseoli was found least sensitive. In general inhibitory activity reached a peak in 8-12 day old culture filtrates. Inhibitory activity remained stable or gradually decreased in filtrates from older cultures.

Culture filtrates of 143a were found to inhibit S. sclerotiorum even after autoclaving, after aging for 30 days, or aging for 30 days then autoclaving prior to use (Figure 2). Both aging and autoclaving decreased inhibitory activity.

Xp a was also inhibited by 143a culture filtrates (Figure 3). Autoclaving reduced inhibitory activity of culture filtrates. In general inhibitory activity against bacterial pathogens Xp 11 and Xp a, was highest in filtrates from 7-12 day old cultures. Culture filtrates from older cultures (50-65 days) showed no activity against either pathogen.

In an experiment to determine how 143a inhibits S. sclerotiorum, plugs were taken 2 mm from a 10 day old colony of 143a. These plugs were placed 2 cm from a mycelial plug of the fungus on a fresh plate of PDA.

Figure 1. Inhibition of various plant pathogenic fungi by filtrates from different aged cultures of antagonist 143a. Turbid spore suspensions of C. lindemuthianum strain beta or Fusarium solani f. sp. phaseoli, or turbid mycelial suspensions of R. solani or P. betae were spread over plates containing 10 ml of PDA. Five wells were cut in each plate using a 5 mm diameter cork borer. Antagonist 143a was grown in Eugonbroth on a shaker for various times (0-65 days) prior to filter sterilization. Culture filtrates were used immediately after filtration. A 30 μl aliquot of each test culture filtrate was placed in each well. Plates were incubated in the dark at 25°C for 4 days and inhibition zone size recorded. Values are the average of 5 replicates.

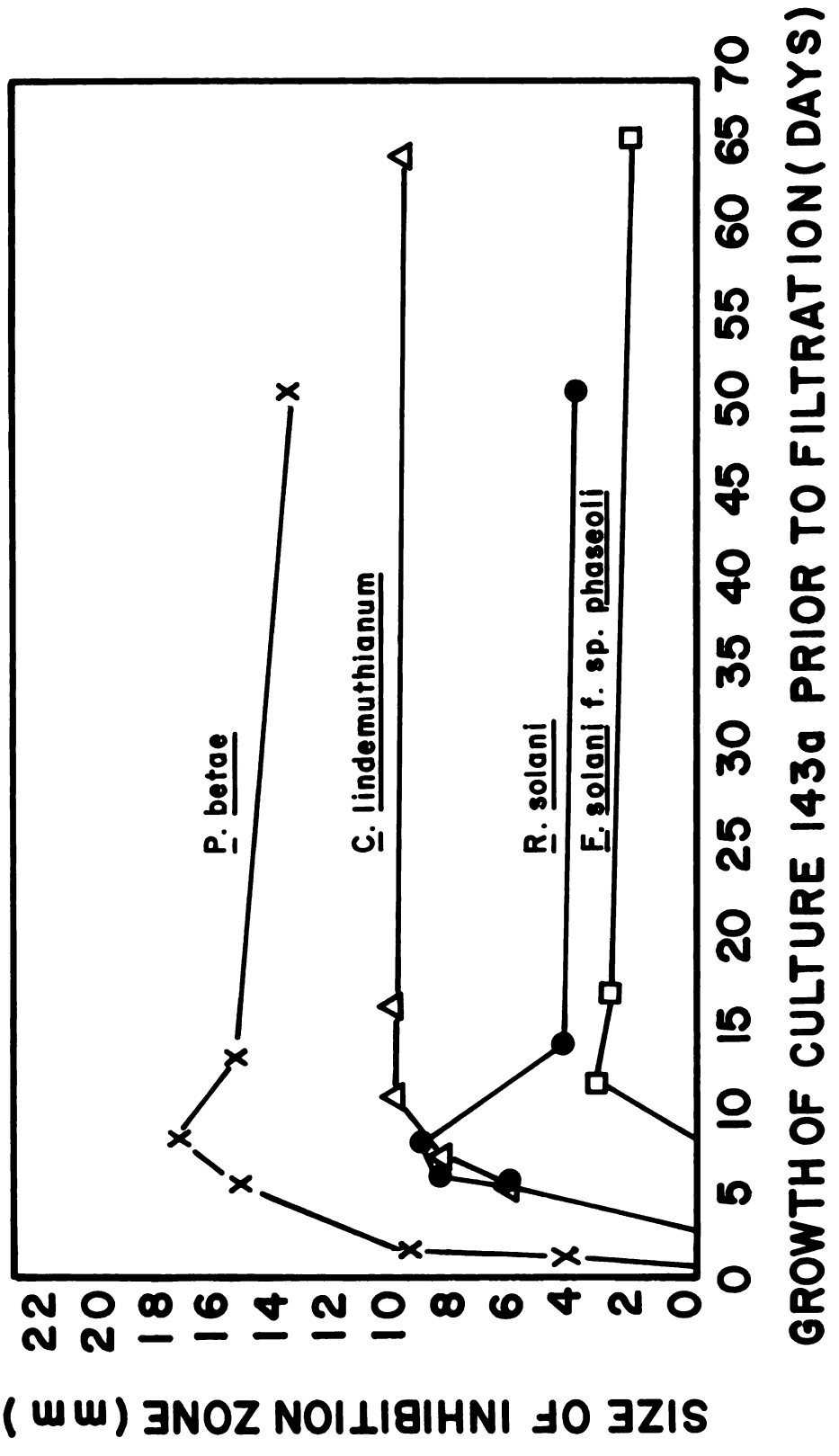
SIZE OF INHIBITION ZONE (mm)
0
2
4
6
8
10
12
14
16
18
20
22
GROWTH OF CULTURE 143a PRIOR TO FILTRATION (DAYS)
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
P. betae
C. lindemuthianum
R. solani
F. solani f. sp. phaseoli

Figure 1.

Figure 2. Inhibition of S. sclerotiorum by filtrates from different aged cultures of antagonist 143a. Mycelial suspensions (0.2 ml) of S. sclerotiorum were spread evenly over plates containing 10 ml of PDA. Five wells of 5 mm diameter were then cut with a cork borer in the agar of the seeded plates. Antagonist 143a was grown in Eugonbroth shaker cultures for various times (0-20 days) prior to filter sterilization. Filtrate was used immediately, after autoclaving, after aging for 30 days, or after aging for 30 days and autoclaving. A 30 µl aliquot of each test culture filtrate was placed in each well. Plates were incubated in the dark at 25°C for 4 days prior to observation. Values are the average of at least 3 replicates.

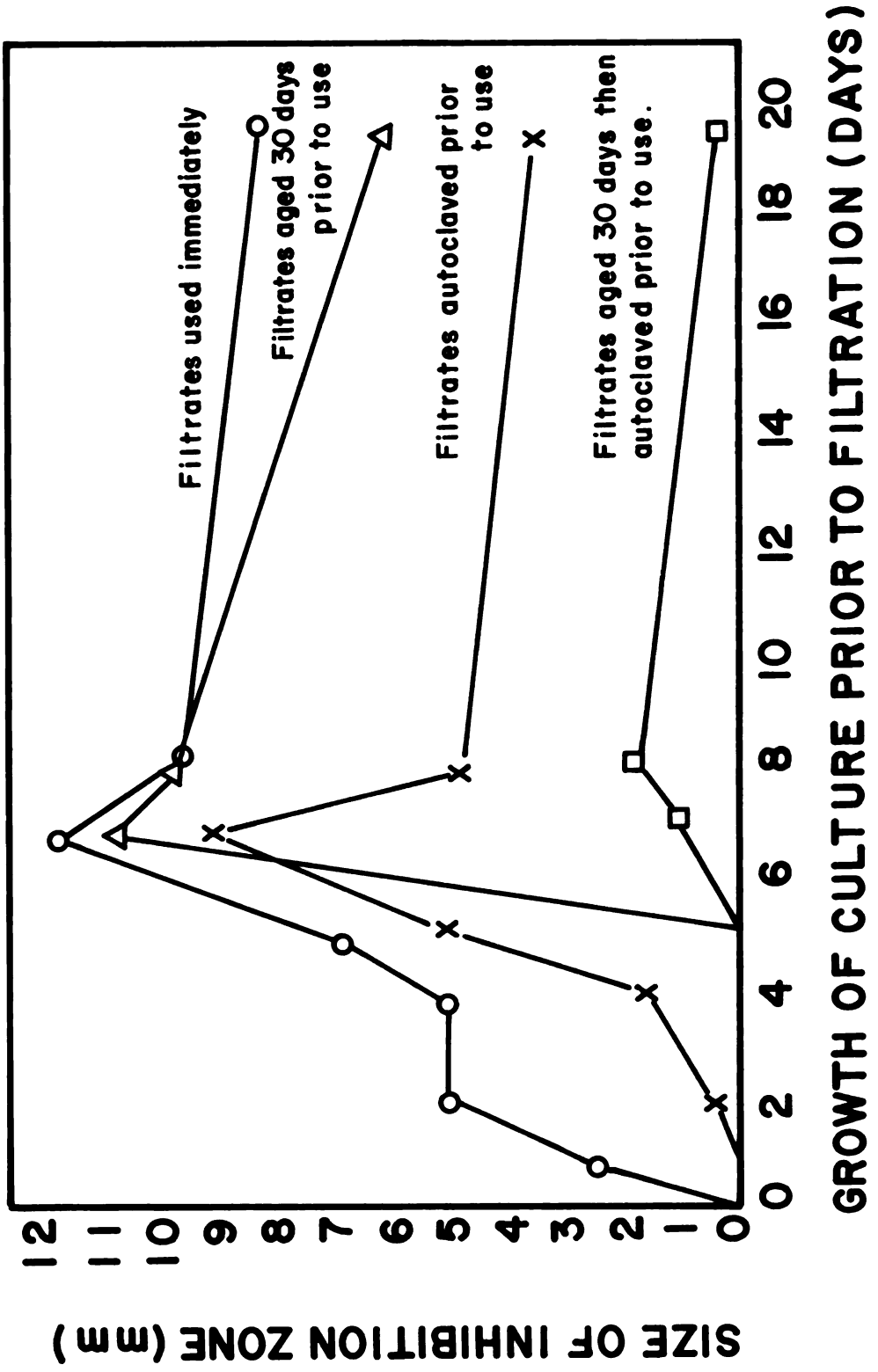
SIZE OF INHIBITION ZONE (mm)
0
1
2
3
4
5
6
7
8
9
10
11
12
0
2
4
6
8
10
12
14
16
18
20
GROWTH OF CULTURE PRIOR TO FILTRATION (DAYS)
Filtrates used immediately
Filtrates aged 30 days prior to use
Filtrates autoclaved prior to use
Filtrates aged 30 days then autoclaved prior to use.

Figure 2.

Figure 3. Inhibition of isolate a of Xanthomonas campestris pv. phaseoli by culture filtrates from different aged cultures of antagonist 143a. Suspensions (3.0 ml) of Xp a (O. D. 0.3) were added per 100 ml of liquid NA (warmed to 45°C), and 10 ml of the solution was added to each petri dish and allowed to harden. Four or five wells 5 mm diameter were then cut with a cork borer in each plate. Antagonist 143a was grown in Eugonbroth for various times (0-65 days) prior to filter sterilization. Filtrate was used immediately or autoclaved prior to use. A 30 µl aliquot of each culture filtrate was placed in each well, and plates were incubated for 48 hours in the dark at 25°C. Inhibition zones are reported as the average of at least 3 replicates.

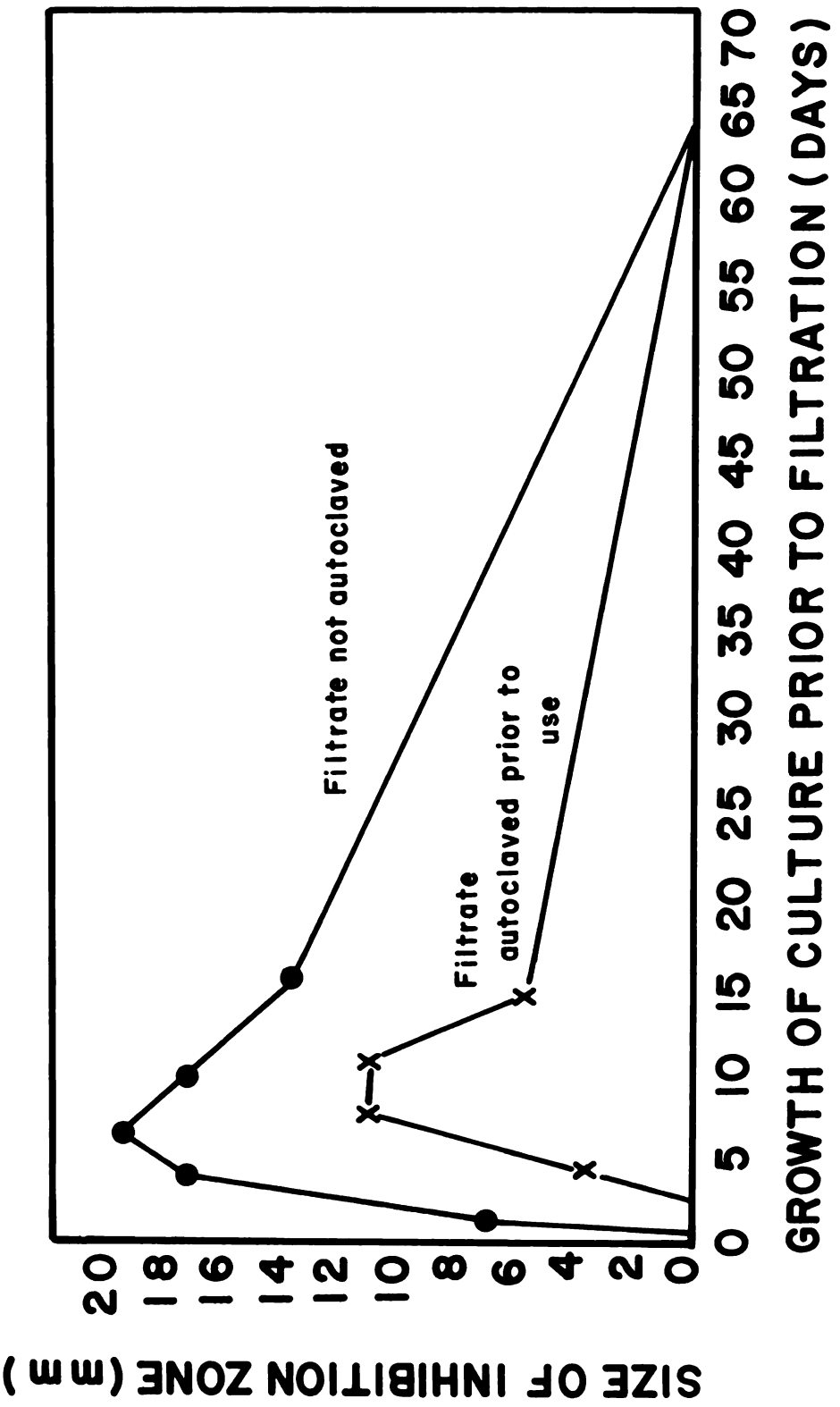
SIZE OF INHIBITION ZONE (mm)
0
2
4
6
8
10
12
14
16
18
20
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
GROWTH OF CULTURE PRIOR TO FILTRATION (DAYS)
Filtrate not autoclaved
Filtrate autoclaved prior to use

Figure 3.

After inhibition zones formed hyphae were taken from the edges of the inhibition zones and placed on fresh PDA plates. None of these hyphae showed signs of growth after 20 days incubation.

Experiments were conducted to determine whether inhibition of C. lindemuthianum by antagonist 143a in vitro was due to pH effects. Inhibition of all pathogen strains tested was found to be the same on BPA prepared with PO_4 buffer (pH 7.2) as previously found in tests conducted on BPA prepared in distilled water.

Physiological and Pathogenicity Tests

Isolate 143a, tentatively identified as Bacillus subtilis, was found to be gram positive, aceotin positive, oxidase positive, positive for hydrolysis of starch, catalase positive, and strictly aerobic. Microscopic examination showed that endospores are produced mostly centrally with no distension of the sporangium. Both tobacco hypersensitivity and bean pathogenicity tests were negative.

Isolates 195 and 196 were presumed saprophytic pseudomonads. Both isolates were fluorescent on KMB, gram negative, oxidase positive and arginine dihydrolase positive. Each gave no response in the tobacco hypersensitivity test and bean pathogenicity test.

Isolate 139b was a gram negative, oxidase positive, facultative anaerobe capable of reducing nitrate. It

gave no hypersensitive response in tobacco and was not pathogenic to beans. Further study will be needed to identify this isolate.

In Vivo Studies with Antagonistic Bacteria

Screening of Antagonists In Vivo Using Mixed Inoculation Techniques for the Ability to Control Fuscous, Common and Halo Blight of Beans

When antagonists and pathogens were grown together in culture, and the cultures injected into Manitou variety seedlings, inconsistent results were obtained. In one experiment (Table 6) isolates 169 and 108 completely protected seedlings from fuscous blight infection, and other antagonists provided partial protection. In two repetitions of the experiment, no disease control was obtained with these isolates.

When antagonist and pathogen were mixed immediately prior to seedling injection inconsistent results were again obtained. In one experiment (Table 7) isolate 195 protected seedlings from halo blight infection by Pp BB2. Such protection occurred once again in a repetition of the experiment, but no control was obtained in a third experiment. Isolates 139b and 169 delayed development of common blight (Xp a) in one experiment but not in two subsequent experiments. Isolate 195 delayed development of halo blight caused by Pp R13. These results were repeated in two subsequent experiments.

TABLE 6. Screening of antagonists *in vivo* for ability to control X. *campestris* pv. *phaseoli* var. *fuscans* (Xpf) in beans.

Pathogen	Antagonist[1]	Disease Rating[2]
Xpf 520	None	+++
None[3]	None	-
Xpf 520	99	++
"	106	++
"	108	-
"	114	++
"	139a	+
"	139b	+
"	143a	+
"	158	+
"	166	++
"	169	-

[1]Flasks containing 25 ml BYE (10 g yeast extract in 1000 ml 0.01M PO_4 buffer pH 7.2) were inoculated with 0.1 ml of a suspension (0.3 O. D.) of an antagonist followed by 0.1 ml of a suspension (0.3 O. D.) of the pathogen. After incubation for two days on a shaker, the mixed cultures were injected into the primary leaf nodes of 10-day-old Manitou kidney bean seedlings. After 17 days plants were rated for disease severity on a pot basis.

[2](-) indicates plants were healthy, (+) stem browning was observed, (++) stem browning and slight wilting, (+++) severely wilted plants.

[3]Plants were injected with BYE alone.

TABLE 7. Screening of antagonists *in vivo* for the ability to control *X. campestris* pv. *phaseoli* (*Xp*) and *P. syringae* pv. *phaseolicola* (*Pp*) in beans.

Pathogen[1]	Antagonist	Disease Rating[2] Observation Time (Days after Inoculation) 5	10	20	34
Pp BB2	None	-	+	+	+
Pp BB2	174a	-	+	+	+
Pp BB2	139b	-	+	+	+
Pp BB2	169	-	+	+	+
Pp BB2	195	-	-	-	-
Pp R13	None	+	++	+++	+++
Pp R13	169	+	++	+++	+++
Pp R13	195	-	+	+++	+++
Xp a	None	-	+	++	++
Xp a	139b	-	-	-	±
Xp a	169	-	-	-	±

[1]Equal amounts of antagonist and pathogen suspensions were mixed and immediately injected into the primary leaf nodes of 10-12-day-old Manitou kidney bean seedlings. Four pots containing 4-5 plants/pot were injected with each antagonist/pathogen mixture.

[2]Plants were rated on a pot basis using the following disease rating system: (-) healthy plants, (+) plants showing stem browning, (++) plants showing stem browning and slight wilting, (+++) severely wilted plants, (±) some pots healthy, some with plants showing stem browning.

The Effect of Time of Application on the Ability of Antagonists to Control Bacterial and Fungal Bean Pathogens on Greenhouse Grown Bean Plants

Greenhouse grown Manitou bean seedlings were spray inoculated with 139b in PO_4 buffer or buffer alone 2, 7 or 9 days prior to inoculation with Xp 11 (Table 8). None of the 139b sprayed plants showed a significantly higher or lower percentage of lesions than control plants. In a second experiment inoculation of Manitou plants at various times after spray application of antagonists showed that time of application did affect the ability of one antagonist to protect seedlings from common blight (Table 9). Antagonist 161 significantly reduced average percentage of 2^{nd} trifoliolate leaf area showing typical common blight lesions, (necrotic areas surrounded by chlorotic tissue), only when applied 7 days prior to the pathogen. Protection was not statistically significant when 161 was applied 6 days prior to the pathogen. No other isolate gave significant disease control.

Pretreatment of Montcalm seedlings with 143a, 7 and 2 days or 2 days prior to inoculation with C. lindemuthianum strain beta, decreased anthracnose severity 86% and 53% in terms of average number of stem lesions, respectively (Table 10). When 143a was applied 9 and 2 days prior to the pathogen, no significant control resulted. Subjective data from these experiments gave more conservative results. Analysis of the data showed significant control of anthracnose only when 143a was applied 7 and 2 days prior to the

TABLE 8. The effect of application time on ability of antagonist 139b to control X. campestris pv. phaseoli (Xp) in beans.[1]

	Average Percentage of 2nd Trifoliolate Leaf with Lesions.[2]		
	Time of Treatment (in days prior to inoculation with Xp 11)		
Treatment	2	7	9
139b	27.8 a[3]	29.6 a	47.9 a
PO_4 buffer	34.5 a	27.6 a	43.2 a

[1]Manitou kidney bean plants were sprayed lightly to run off with a suspension of 139b in PO_4 buffer (pH 7.2, O. D. 0.4) or PO_4 buffer alone at various times prior to inoculation with Xp 11 (O. D. 0.25). Plants were then sprayed lightly to run off with a suspension of Xp 11 in PO_4 buffer. Plants were observed after 3 weeks.

[2]Values are the average of 10 replicates. Averages were obtained after an arcsin square root transformation of the data.

[3]Means in the same column with the same letter do not differ significantly at $\alpha = 0.1$ according to Duncan's multiple range test.

TABLE 9. Control of Xanthomonas campestris pv. phaseoli (Xp) in beans by preinoculation at various times with antagonists.

Antagonist[1]	Application Time (in days prior to inoculation with Xp 11)	Average Percentage of the 2nd Trifoliolate Leaf with Lesions[2]	
None		16.3	bc[3]
138	2	11.3	ab
139b	2	20.0	cd
139b	6	22.5	d
161	6	11.9	ab
161	7	10.0	a
169	1	13.8	ab

[1]Antagonists were prepared by centrifuging 2 day old BYE shaker cultures. The pellets were resuspended in PO4 buffer (pH 7.2) and absorbance was adjusted to 0.4 at 600 nm. Plants were sprayed lightly to run off with the appropriate solution. One to seven days after application of the antagonists plants were spray inoculated with Xp 11. Inoculum of the pathogen was prepared in the same manner except that absorbance was adjusted to 0.25. Observations were made 3 weeks after inoculation with the pathogen.

[2]Values are the average of 8 replicates.

[3]Values in the same column which have the same letter do not differ significantly at $\alpha = .05$ using Duncan's multiple range test.

TABLE 10. The effect of preinoculation of Montcalm bean plants with antagonist 143a on severity of anthracnose (C. lindemuthianum).

Treatment[1]	Average Number of Stem Lesions for each Time of Treatment (in days prior to inoculation with C. lindemuthianum)		
	7 and 2 days[2]	2 days[3]	9 and 2 days[4]
SDW	3.103 a[5]	2.182 a	0.629 a
None	NT	2.284 a	NT
143a in SDW	1.114 b	1.426 b	0.425 a

[1]Montcalm variety seedlings were spray inoculated with SDW alone, antagonist 143a suspended in SDW (approximately 2.5 x 10^9 cfu/ml) or not sprayed. Two days after the last treatment plants were spray inoculated with C. lindemuthianum strain beta (10^7 spores/ml). Plants were placed in a mist chamber for 8 days then a greenhouse bench for 2 days prior to observation. The number of lesions on each stem, from soil level to the node of the first trifoliolate leaf was counted.

[2]Values are the average of 54 replicates after a square root transformation of the data.

[3]Values are the average of 54 replicates after a log transformation of the data.

[4]Values are the average of 162 replicates.

[5]Means in the same column with the same letter do not differ significantly at $\alpha = .05$ according to Duncan's multiple range test. (NT) indicates the test was not made.

pathogen (with a 61% decrease in the average disease rating). Unfortunately disease symptom development in experiments in which 143a was applied 7 days prior to the pathogen or at the time of inoculation with the pathogen was inconsistent, so no analysis was possible.

The Effect of Mode of Application on the Ability of Antagonists to Control Bacterial and Fungal Pathogens of Beans

Several antagonists significantly reduced severity of common blight disease on greenhouse grown Manitou plants when applied as a seed treatment (Table 11). Isolate 138 was the most effective antagonist in this regard. Isolates 105, 112, 143a, 152, and 161 were also effective in controlling disease.

Treatment of MIC variety bean seeds naturally infected with C. lindemuthianum with antagonist 143a in BPA did not protect seedlings from anthracnose. In two other experiments, healthy MIC variety seeds were treated with 143a in BPA and plants were later inoculated with C. lindemuthianum, and no decrease in average number of stem lesions was detected.

The Effect of Media on the Ability of Antagonist 143a to Control Uromyces phaseoli Infections on Greenhouse Grown Bean Plants

Media affected the ability of 143a to control bean rust (Table 12). For example, 143a suspended in SDW did not significantly control bean rust while 143a in

TABLE 11. The effect of seed treatment with various antagonists on disease severity in Manitou variety plants subsequently inoculated with Xanthomonas campestris pv. phaseoli (Xp).[1]

Antagonist	Average Number of Lesions on the 1st Trifoliolate Leaf[2]	
105	1.7	ab[3]
112	1.6	ab
138	1.4	a
143a	1.7	ab
152	1.7	ab
153	2.5	c
158	2.4	c
161	1.6	ab
169	2.0	abc
None	2.8	c

[1]Surface sterilized Manitou bean seeds were placed in 10 ml of soft BPA with 1.0 ml of a 48 hour old BYE culture of each antagonist or in 11 ml of soft BPA alone. Seeds were incubated in the inoculum for 16 hours at 25°C before planting. One month after planting a 0.2 O. D. suspension of Xp 11 was sprayed lightly to run off on each plant. Twenty days after inoculation number of lesions on the first trifoliolate leaf of each plant was recorded.

[2]Values are the averages of 5 replicates after a square root transformation of the data.

[3]Means in this column with the same letter are not significantly different at α = .01 level according to Duncan's multiple range test.

TABLE 12. The effect of culture medium on ability of antagonist 143a to control bean rust (Uromyces phaseoli) on greenhouse grown bean plants.

Treatment[1]	Average Number of Lesions[2]	
None	5.04	a[3]
Sterile Distilled Water	5.04	a
Sterile Eugonbroth	5.01	a
143a in SDW	4.57	a
143a in Eugonbroth	0.26	c
B. subtilis in Eugonbroth	1.54	b

[1]Fourteen day old Pinto (UI-114) variety bean seedlings were preinoculated with antagonists (O. D. 0.4) or control solutions. Two days later plants were spray inoculated with U. phaseoli uredospores (5 mg spores/10 ml SDW with 0.1% v/v Tween 20) at a rate of 2 ml per plant and were placed in a mist chamber. The number of lesions on each primary leaf was counted 5 days later.

[2]Values are the average of 2 replications with 12 samples each, after a log transformation of the data.

[3]Means in the same column with the same letter are not significantly different at $\alpha = .01$ level according to Duncan's multiple range test.

Eugonbroth resulted in a 99% reduction in rust lesions on primary leaves. While treatment with B. subtilis reduced the average number of rust lesions, antagonist 143a was significantly more effective. Plants pretreated with nothing, with SDW or Eugonbroth did not differ significantly in average number of rust lesions.

Viability of Isolate 143a on Spray Inoculated Bean Leaves

Circumstantial evidence was found to indicate that isolate 143a remains viable on bean leaves for at least 11 days after inoculation. Eleven days following inoculation of treated plants with 143a in SDW and control plants with SDW alone, prints of leaves from 143a inoculated plants revealed 15-60% more of the 2nd trifoliolate leaf area showing bacterial growth characteristics identical to 143a than control leaves. Highest populations of 143a-like bacteria were found on leaves of GN Tara variety (60% greater than control), while the lowest populations were found on leaves of Manitou variety plants (15%). Leaves of Montcalm, MIC, and Black Turtle Soup bean plants all had 50% higher populations of 143a-like bacteria after 11 days than control plants. While populations of 143a-like bacteria on UI-114 pinto bean plants remained 50% higher than control plants at 3 days after inoculation, populations fell to only 20% greater than control plants by 11 days.

Control of Damping-Off of Sugar Beet Seedlings with Antagonist 143a

Sugar beet seed (USH20) suspected of being infected with P. betae were tested in vitro and 20.8% were found to carry the fungus. When samples of this seed lot were treated with CME or 143a in CME prior to planting, percentage of infected seedlings at 16 days after planting was significantly less in those pretreated with 143a in CME than in CME alone (Table 13). Emergence was not affected by treatment with 143a.

Field Experiments

MIC bean plants spray inoculated with 143a at different times during the growing season then inoculated with C. lindemuthianum showed similar numbers of pod lesions as uninoculated control plants. Spray inoculation with 143a at various times provided no control of white mold in Tuscola and MIC variety plants. Vacuum infiltration of Seafarer, Ouray and MIC variety bean seeds with antagonist 143a did not significantly affect emergence or final stand count.

Vacuum infiltration of Sacramento seeds with 143a significantly reduced the number of sporulating lesions found on pods after plants were artificially inoculated with C. lindemuthianum. A 47% decrease in the average number of lesions was obtained. Similar treatment of Olathe variety and MIC variety seeds with 143a, however did not result in significant disease control.

TABLE 13. Control of damping-off of sugar beet seedlings with antagonist 143a.[1]

Treatment	8 Days After Planting		16 Days After Planting	
	Average Percent[2] Not Emerging	Average Percent Infected	Average Percent Not Emerging	Average Percent Infected
Cellulose Methyl Ether (CME)	31.43 a[3]	10.06 a	26.91 a	83.08 a
143a in CME	27.22 a	2.28 a	21.99 a	49.74 b

[1]USH20 sugar beet seeds were treated with 143a suspended in 0.5% w/v CME (approximately 1 x 10^8 cfu/seed) or CME alone. Seeds were allowed to dry for 24 hours and then planted in flats containing sand which were covered with plastic tops.

[2]Values are the average of 2 replicates with 180 plants in each replication. An arcsin square root transformation of the data was used to achieve homogeneous variance.

[3]Means in the same column with the same letter do not differ significantly at $\alpha = .05$ according to Duncan's multiple range test.

Vacuum infiltration of Seafarer and Manitou bean seeds with antagonists 139b and 169, did not significantly affect emergence or stand. Vacuum infiltration of Charlevoix variety seeds with antagonist 139b did result in an emergence and stand increase of 15% over seeds vacuum infiltrated with BYE alone. Vacuum infiltration of Charlevoix seeds with antagonist 169 did not affect emergence or stand.

DISCUSSION

Successful inhibition of numerous plant pathogens was achieved *in vitro* and *in vivo* with bacterial antagonists isolated from bean seed. Media, incubation temperature, and testing techniques were found to influence inhibitory activity of some antagonists *in vitro*. Isolates exhibiting inhibitory activity *in vitro* were tested *in vivo* for ability to control several plant pathogenic bacteria and fungi. Selected bacterial antagonists were used successfully to control *C. lindemuthianum*, *U. phaseoli*, and *X. campestris* pv. *phaseoli* in beans as well as *P. betae* in sugar beets. Success of *in vivo* disease control depended on mode of application and composition of suspending solutions used.

Results of *in vitro* tests on the effect of media and incubation temperature on inhibitory activity support the findings of several previous studies (4,5,12,15,16,17). In some cases inhibitory activity of an antagonist towards a particular pathogen was only detected on a specific type of culture media, or over a very narrow temperature range. No correlation was found between success in *in vivo* disease control and ability to exhibit inhibitory activity over wide ranges of media or incubation temperatures. This research suggests that in order to detect

all potential antagonists in vitro, screening tests should be conducted on a variety of media at several different temperatures. In addition, it was found that size of inhibition zone, used in the past as an indicator of the potential worth of an antagonist (8) could be misleading, since zones differed markedly for some antagonist/pathogen combinations on different media and/or at different temperatures.

This study also shows that testing technique can influence the results of in vitro studies. When isolates were grown in culture, then killed by exposure to chloroform vapor prior to introduction of the pathogen, many exhibited no inhibitory activity; even though each isolate had previously been found to inhibit the same pathogen in tests using the lacuna method or when streaked over media seeded with the pathogen. Huber and Watson demonstrated the same principal when testing potential bacterial antagonists against Typhula idahoensis (7). Using two different testing techniques they obtained different results. No in vivo tests were performed, however, to determine which screening procedure was most effective in identifying potential antagonists (7). In this study in vivo tests were conducted with antagonists which did not exhibit inhibitory activity using the chloroform method. Two of these isolates, 112 and 152, protected Manitou kidney beans from the common blight bacterium (X. campestris pv. phaseoli) when the antagonists were applied to

seeds prior to planting. If the chloroform test had been used for initial screening these isolates would have been missed.

In vivo studies to determine the effect of application time on disease control were inconclusive. In experiments where plants were pretreated by spray inoculation with antagonists at various times prior to inoculation with the common blight bacterium, the ability of isolate 161 to reduce disease was affected by application time. Success in control of common blight by the spray application method was so limited however, that no generalizations on the effect of application time can be made. Different degrees of control were obtained when Montcalm variety seedlings were inoculated at various times with antagonist 143a prior to inoculation with C. lindemuthianum. Examination of the data, however, reveals that 143a provided relatively greater control in experiments where disease was more severe. More controlled experiments will be needed to determine whether application time does affect the ability of 143a to control anthracnose.

Mode of application was found to influence the ability of several isolates to control disease in vivo. Isolate 138 did not protect Manitou seedlings from X. campestris pv. phaseoli when applied as a spray inoculant prior to the pathogen. When applied as a seed treatment, however, isolate 138 was very effective in controlling the same pathogen. This work differs from that of

Teliz-Ortiz and Burkholder who found an isolate of Pseudomonas fluorescens which controlled P. phaseolicola regardless of the plant part to which it was applied (15). In greenhouse tests, antagonist 143a significantly reduced disease severity of anthracnose on Montcalm seedlings when spray inoculated onto the plants prior to the pathogen, but did not protect seedlings when used as a seed treatment. These results are similar to those of Htay and Kerr (1974) who found that Agrobacterium radiobacter var. radiobacter strain 84 was far more effective in controlling crown gall on peach when applied to roots than seeds (6). My results indicate that in vivo screening tests should include use of different modes of application in order to detect successful antagonists and to determine the most efficient method of application.

Suspending media used markedly affected the ability of 143a to control bean rust. When the antagonist in Eugonbroth was applied to plants prior to inoculation with the pathogen significant reductions in disease severity resulted. When 143a suspended in sterile distilled water was used no control was achieved. These results are much the same as those of Stavely et. al. (14) who found B. subtilis provided protection to bean plants from bean rust only when suspended in Eugonbroth.

Unfortunately, knowledge of the importance of suspending media for disease control with antagonist 143a was not available when field studies were conducted. Failure

of antagonist 143a to control white mold and anthracnose on field grown bean plants may have been due to improper suspending solutions. Addition of nutrients in the inoculum have been shown to increase inhibitory activity of antagonists in several studies (4,9,10,14) and have been postulated to stimulate antibiotic production *in vivo* by providing constituents necessary for their production (1,2). Addition of nutrient solutions might also act to prolong the life cycle stage during which antibiotics are produced. It has been shown that antibiotic production is often characteristic of a single stage in the life cycle of a bacterium (3). In this study it was found that many antagonists inhibited specific pathogens only at certain culture ages. Culture filtrate of antagonist 143a was found to be more effective in inhibiting most pathogenic bacteria and fungi tested when taken from cultures incubated at least 8-10 days prior to filtration. At the present time the *in vivo* mode of action of 143a against *C. lindemuthianum* and *S. sclerotiorum* is not known so it is difficult to determine how nutrients might affect inhibitory activity.

Further knowledge of *in vivo* modes of pathogen inhibition are needed to improve selection procedures and to provide more consistent and efficient *in vivo* pathogen inhibition. At the present time little is known of the fate of the antagonists themselves, or inhibitory compounds they produce on or within plants. Antibiotic

resistance markers have been used successfully in the past (13,18) to study population dynamics of plant pathogenic bacteria. Resistance markers might be useful to determine the ability of antagonists to survive under different conditions *in vivo*. Information of this type could be used to find compatible mixtures of antagonists which actively inhibit under different conditions, leading to more consistent disease control. Use of resistance markers would also aide in determining proper mode of application, suspending media and antagonist/pathogen population ratios for disease control.

Further knowledge on the fate of inhibitory compounds would lead to development of more efficient screening procedures to identify potentially successful antagonists. Knowledge of this type might be obtained by more comprehensive studies of successful antagonists such as *Agrobacterium radiobacter* var. *radiobacter* strain 84 (12). If certain types of antibiotics or lytic enzymes are found to be particularly effective *in vivo*, *in vitro* screening tests could be adjusted to select specifically for microorganisms producing them.

In the course of this study several bacterial antagonists were isolated which successfully inhibited plant pathogens *in vivo*. Successful use of antagonists as seed treatments to control common blight of beans and damping-off of sugar beet seedlings was very encouraging since seed treatment is simple and far less expensive than

treatment requiring application in the field. Experiments conducted on mode of application and suspending media show the importance of these factors in optimizing disease control by bacterial antagonists. Preliminary studies conducted to control bean diseases with bacterial antagonists have shown that substantial disease control can be achieved. Because of the need for safe and inexpensive disease control methods further research in this area is warranted.

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APPENDICES

APPENDIX A

Sources of Bacterial Antagonists

TABLE 14. Sources of bacterial antagonists.

Bean Type	Seed Lot Number and/or Variety Name	Antagonists
Small black beans	73327	1, 2, 3, 114
	74049	100, 100a, 104
	79596	170, 173, 174, 174a, 175a, 175b, 175c, 175d, 176, 176a, 177, 177a, 178, 178b, 192, 193, 194, 195
	80160	218, 218a, 219, 225, 227, 228, 229, 230, 231, 233, 248, 250, 251
Pinto beans	73382	238, 239, 240, 241, 260, 267
	74022	105, 106
Kidney beans	73977/Red Kloud	149, 150, 151, 152, 155, 157
	74052	153, 154, 156
	74054	102, 103, 112
	74055	133, 134, 135
Cranberry beans	72369	158, 160, 161, 162

TABLE 14. (cont'd)

Bean Type	Laboratory Seed Lot Identification Number and/or Variety Name	Antagonists
White beans	72368	181, 186, 190, 191, 197, 198, 199
	73300/Kentwood	108, 113
	73302/Sanilac	107, 109, 110, 111, 111b
	73336	299, 300, 301, 302, 303, 304, 305, 306
	73370/Fleetwood	224, 249, 253, 265, 266, 268
	73397/Tuscola	221, 223, 226, 261
	73498/Fleetwood	4, 5, 6, 12, 13
	73715	99, 122, 122a, 122b, 123, 124, 125, 126, 127, 128
	73993	129a, 129b, 130, 131, 132
	74016/Tuscola	115a, 115b, 116, 117, 120, 121
	74041	163
	80040	179, 180, 182, 183, 184, 185, 187, 188, 189, 196, 196a, 196b, 196c, 200, 201

Antagonists obtained from Mintarsih Adimihardja (Kampus University, Lampung, Indonesia):

136, 138, 139a, 139b, 139c, 140, 141, 142, 143a, 143b, 143c, 144, 145, 146, 148, 164, 165, 166, 167, 168, 169, 171, 172, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 216, 217, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 284, 295, 296, 298, 308, 309, 310, 311, 313, 314

Antagonists obtained from Maureen Mulligan (Michigan State University, East Lansing, MI):

288, 289, 290, 291, 293, 294

Antagonists obtained from bean stem sections:

213, 214, 280, 281

APPENDIX B

Sources of Plant Pathogenic Fungi

The following isolate was obtained from Dr. D. Fulbright (Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI): *Cephalosporium* sp.

Dr. J. L. Lockwood's laboratory (Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI) provided: *Fusarium solani*, *Macrophomina* sp., *Phytophthora megasperma* (isolates fi, r3, and rl-m), *Sclerotinia cepivorum*, *S. rolfsii*, *S. sclerotiorum*, and *Thielaviopsis* sp.

Dr. A. W. Saettler (Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI) provided: *Colletotrichum lindemuthianum*, and *Uromyces phaseoli*.

Dr. G. R. Safir's laboratory (Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI) provided: *Alternaria* sp., *Fusarium solani* f. sp. *phaseoli*, and *Pyrenochaeta terrestris*.

Dr. Schneider (Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI) provided: Aphanomyces cochlioides, Botrytis cinerea, Phoma betae, Pythium aphanidermatum, Pythium ultimum, and Rhizoctonia solani.

APPENDIX C

Sources of Plant Pathogenic Bacteria

All plant pathogenic bacterial isolates used in this study were provided by Dr. A. W. Saettler (Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI). Pathogenic bacteria were isolated from infected bean tissue collected in Michigan. References dealing with isolation of rifampin resistant mutants, Xp a (2) and Pp R13 (1) are given below.

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