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ANTAGONISM AND INFECTION INHIBITION OF *PHOMOPSIS PHASEOLI* BY *BACILLUS PUMILUS*

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ABSTRACT--Cells and cell-free culture filtrates of *Bacillus pumilus* inhibited the development of *Phomopsis phaseoli*, the cause of soybean stem canker, in controlled conditions. A permanent zone of inhibition developed between opposing cultures of *B. pumilus* and *P. phaseoli* on cross-culture agar plates. Cell-free filtrates of *B. pumilus* incorporated at different concentrations in agar media significantly reduced mycelium colony growth in diameter, percentage of spore germination, and germ tube length. Reduced symptom development resulted when leaflets of soybean were inoculated simultaneously with the pathogen and bacterial cells.

Stem canker, caused by *Diaporthe phaseolorum* var. *caulivora* Athow and Caldwell, is one of several diseases that appear on soybeans, *Glycine max* (L.) Merr., in Tennessee from mid-season to maturity. Stem canker has occurred in varying degrees of severity in Tennessee since 1981, causing losses approaching 100% in some fields (Hilty, 1981). Plants are inoculated and subsequently infected when spores invade leaf, petiole, or stem tissue (Ploetz and Shokes, 1987). Red-brown cankers may develop, often girdling the stem and causing premature death or severely reducing yields (Sinclair and Backman, 1991).

Control of stem canker has been obtained by using resistant varieties and application of chemical fungicides (Backman et al., 1986). An alternative method not previously explored is biological control, or the use of microorganisms that inhibit growth or virulence of the pathogen on the surface of the soybean plant. Using naturally occurring microorganisms as biocontrol agents is particularly appealing, especially with current concern about the impact of hazardous chemicals on the environment.

Inhibitory activity of several species of bacteria has been demonstrated against fungi pathogenic in the phylloplane (Spurr, 1981). *Bacillus* sp. isolated from either the soil or plant surfaces, are particularly interesting in this regard because they produce a wide range of antimicrobial compounds (Baker et al., 1983). Fravel and Spurr (1977) noted a reduction in *Alternaria* brown spot of tobacco (*Nicotiane tabacum* L.) by application of cells of *Bacillus* sp. in a controlled environment. Rust pustules on snapbean (*Phaseolus vulgaris* L.) leaves were reduced by applications of *Bacillus subtilis* (Ehrenberg) Cohn (Baker et al., 1983). Gayed (1966) noted that lesions caused by *Helminthosporium sativum* Pamm., King, and Bakke were smaller on wheat (*Triticum aestivum* L.) and barley (*Hordeum* L.) when a suspension of cells of *Bacillus pumilus* Gottheil was applied as a protectant. Morgan (1963) reported reduced infection by three rust pathogens of wheat and oats (*Avena sativa* L.) following applications of *B. pumilus*. He also observed germ tube lysis of urediospores on agar containing cell-free culture filtrates of *B. pumilus*.

The bacterium used in the present study was isolated from a soybean seed germinating on agar. Prominent zones of inhibition developed between the bacterial colony and fungal contaminants indicating the possible occurrence of antimicrobial substances. The bacterium was subsequently identified as *B. pumilus* by fatty acid profile determination (L. W. Barnes, pers. comm.). We report the antagonism of *B. pumilus* to the stem canker pathogen and inhibition of infection.

MATERIALS AND METHODS

During the present study, *B. pumilus* was maintained on BBL Microbiology Systems potato dextrose agar slant tubes (PDA). All bacterial cultures were prepared by inoculating nutrient broth with cells from a 3-day culture and incubating in stationary culture for 5 days at 25°C. In some experiments, bacterial culture filtrates were rendered cell-free by passing through a 0.45-μM (pore size) filter. The culture of *D. phaseolorum* var. *caulivora* was isolated from a diseased soybean plant (cv. Essex) in western Tennessee and maintained on PDA. Conidia of the imperfect form, *Phomopsis phaseoli* (Desm.) Sacc., were used as inoculum and were obtained from cultures on sterilized stem segments or leaflets of soybean imbedded in water agar.

In Vitro Tests--A modified cross-culture method described by Dhingra and Sinclair (1985) was used to determine inhibitory effects of the bacterial culture on mycelium growth. Agar plugs with mycelium, 12 mm in diameter, were cut from the margins of 10-day cultures of *Phomopsis* and placed on one side of each of 20 Petri plates containing PDA. Sterile filter paper discs, 12 mm in diameter, were dipped into 3-day-old cultures of the bacterium, excess bacteria wiped on the plate edge, and placed opposite the fungus plug at a distance of 4 cm. Controls were sterile filter paper discs. After 5 days at 24°C, zones of inhibition were measured.

The effect of bacterial cells on spore germination and growth was studied. A 6-day broth suspension of bacterial cells was mixed with an equal volume of a spore suspension of *Phomopsis* adjusted to 200,000 spores/ml. A 20-μl drop was placed in each of two wells on two acid-cleaned, sterile depression slides and incubated for 48 h at 25°C in closed Petri dishes with moist filter paper. Spores were stained with acid fuchsin, and 50 spores/well were observed.

Bacterial Filtrates--Presence of a toxin in the filtrate and its effect on mycelial growth in diameter was investigated by incorporating the filtrate in PDA at 5, 10, and 20% by volume and autoclaving. Controls were unamended PDA. Plates were incubated for 7 days at 25°C. The largest colony diameter and the diameter on the perpendicular were determined and averaged for each plate (10 plates/treatment; each plate considered a replication). The experiment was repeated, and data were combined for analysis of variance tests.

The effect of the filtrate on spore germination and germ tube growth was determined by incorporating filtrate in water agar at 50% by volume. Part of the agar-filtrate medium was autoclaved. A 0.5-ml suspension containing approximately 50,000 conidia/ml was spread over the surface of the agar and incubated for 48 h at 24°C. The percentage of germination was determined by counting 100 spores on each of nine plates. Ten germ tubes on each of the nine plates were measured. The experiment was repeated, and data were combined for analysis of variance tests.

In Vivo Biocontrol--Biocontrol of the pathogen was studied by inoculating soybean leaflets in controlled conditions. Individual leaflets were harvested from the seventh node on 60-day-old greenhouse-grown plants, cv. Essex. Leaflets were washed briefly in 70% ethanol and rinsed in sterile distilled water to reduce natural populations of microflora. Inoculum was prepared by mixing equal amounts of unfiltered nutrient broth culture or autoclaved broth culture with a water suspension of conidia for a final spore concentration of approximately 200,000 spores/ml. Leaflets were supported on fiberglass screen above water in covered glass containers, and the abaxial surface was inoculated with a 50-µl drop of the mixture. Controls were inoculated with a water suspension of conidia. Containers were closed to provide 100% relative humidity, and leaflets were incubated at 25°C with 8 h of light. Symptom development was evaluated after 7 to 10 days using the following scale: 1 = no symptoms; 2 = chlorotic spot with few necrotic flecks at point of inoculation; 3 = necrotic spot; 4 = extensive necrosis and cholorisis.

RESULTS

In Vitro Tests--Cells and cell-free culture filtrates of *B. pumilus* inhibited mycelium growth and reduced spore germination and germ tube development of *Phomopsis*. Opposing cultures of *Phomopsis* and *B. pumilus* on cross-culture plates resulted in the formation of a distinct and permanent inhibition zone with an average width of 6.3 mm (Fig. 1). Mycelium covered control plates (9.0 cm diameter) in 5 to 7 days.

Nearly 100% of the spores in the presence of live bacterial cells in depression slides were disintegrated. The few spores observed were swollen and round with no indication of papillae or germ tubes. Germination on control slides was 80%.

Bacterial Filtrates--All concentrations of the cell-free filtrate incorporated in agar significantly reduced growth in diameter of *Phomopsis* mycelium (Fig. 2). Diameters of colonies were significantly different between treatments with the greatest reduction occurring at the 20% concentration and the least at the 5% concentration. The control medium was covered by mycelium in 5 to 7 days.

Spore germination and germ tube growth in length were significantly less on medium containing autoclaved or nonautoclaved cell-free filtrate than those of controls. The percentage of germination was significantly less on the autoclaved than on the nonautoclaved filtrate (Fig. 3). Germ tubes on the autoclaved and nonautoclaved filtrates extended only 131 to 158 µ and were one-third the length of those of controls (Fig. 4). Many spores, germinated or not, on filtrate-amended media were round and nearly double in size. Germ tubes developed abundant vesicles on the hyphae and at hyphal tips. Conidia germinated, and hyphae developed with no malformations on control medium.

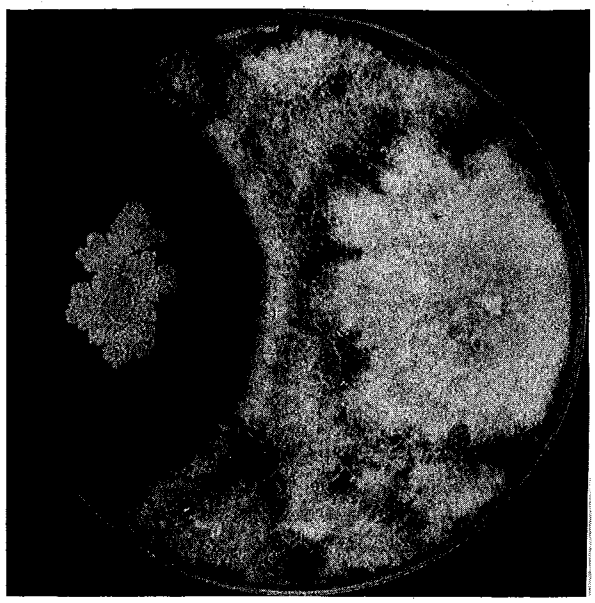

FIG. 1. *Bacillus pumilus* (left) and *Phomopsis phaseoli* (right) on potato dextrose agar with zone of inhibition after 5 days.

In Vivo Biocontrol--Simultaneous application of bacteria and pathogen to the leaflet surface significantly reduced disease development and demonstrated that biocontrol of the pathogen was possible in a controlled environment. Symptom development was not significantly different on leaves treated with live or autoclaved bacterial cells but was

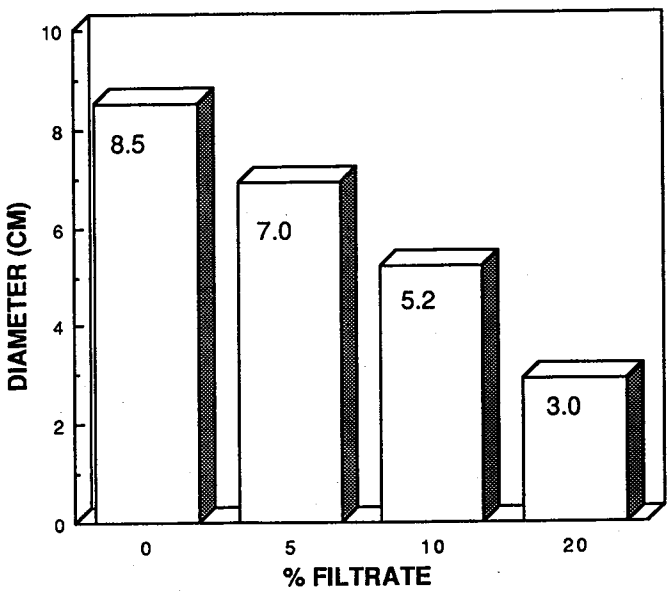

FIG. 2. Growth in diameter of *Phomopsis phaseoli* on media containing different concentrations of culture filtrate of *Bacillus pumilus*. The amount of growth with each concentration of filtrate is significantly different from the amount of growth with all other concentrations (Duncan's new multiple range test; $P \leq 0.05$).

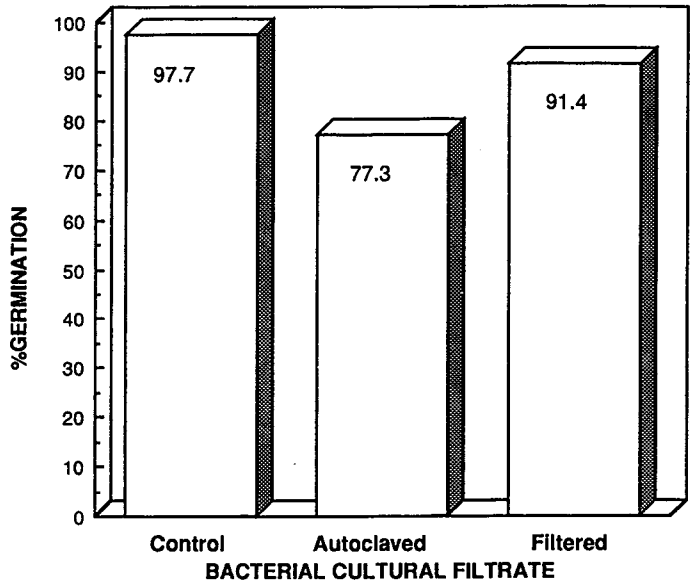

FIG. 3. Effect of culture filtrate of *Bacillus pumilus* on the percentage of germination of conidia of *Phomopsis phaseoli*. The percentage of germination on each type of filtrate is significantly different from the percentage on all other filtrates (Duncan's new multiple range test; $P \leq 0.05$).

significantly less than that of controls (Fig. 5). Although flecking and some chlorosis occurred at the point of inoculation on leaflets receiving bacteria, no mycelium developed nor did symptoms advance. Profuse aerial mycelium occurred on control leaflets 4 days after inoculation, and entire leaflets became chlorotic.

DISCUSSION

Bacillus pumilus inhibited *Phomopsis* in culture and suppressed symptom development in vivo. Interference with pathogen development in the absence of bacterial cells supports the proposal of Morgan (1963) of an inhibitory soluble toxin. Activity after autoclaving suggests a heat-stable substance. Christensen and Davis (1940) in studies of a toxin produced by *B. mesentericus* = *B. pumilus* (cf., Berey, 1948) noted that it retained activity against *Helminthosporium sativum* for at least 4 months, even after repeated autoclaving.

In the present study, numerous vesicles developed on germ tubes and at hyphal tips of spores on filtrate-amended media. In some instances, the vesicles lysed, and elongation ceased. These structures may be similar to those previously described as forming only at the hyphal tip of germinating urediospores (Morgan, 1963). Gayed (1966) and Christensen and Davis (1940) reported morphological changes in mycelium of *H. sativum* by *B. pumilus* and *B. mesentericus*, respectively.

A reduction in infection by *Phomopsis* resulted when soybean leaflets were inoculated simultaneously with the pathogen and *B. pumilus*. Previous reports of bacterial-fungal interactions indicate lysis as a probable cause of fungal inhibition, thereby reducing infection (Morgan, 1963). Fravel and Spurr (1977) observed germ tube suppression rather than lysis, suggesting that different mechanisms may function. Suppression of germination and germ tube development was observed in vitro in the present study, but the behavior of spores on the leaf surface was not observed. Thus, it is assumed that the rich suspension of *B. pumilus* cells or the toxin caused lysis or inhibition of spores and mycelium. Although the activity of the culture filtrate on the plant surface was not tested in the present study, it effectively reduced rust infections in small grains (Morgan, 1963). Characterization of the toxin of *B. pumilus* is necessary to further understand its inhibitory activity and mode of action. The reduction of infection by *B. pumilus* in a controlled environment encourages further research to define the potential for biological control in field applications.

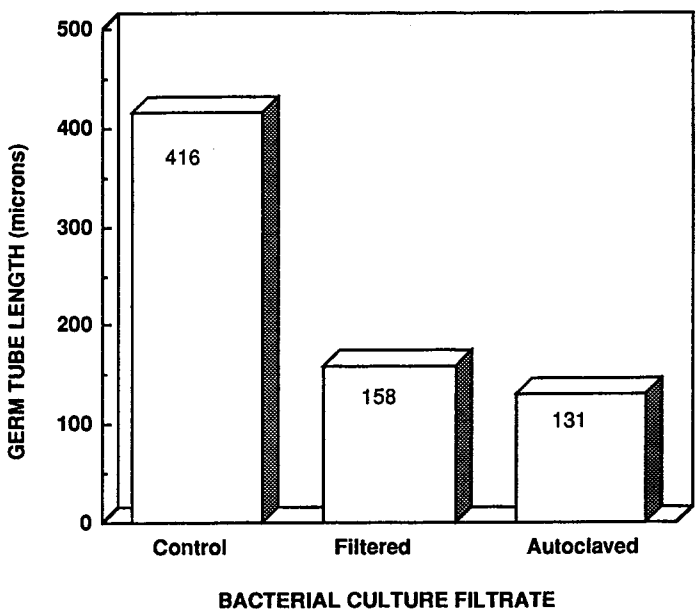

FIG. 4. Effect of culture filtrate of *Bacillus pumilus* on germ tube length of conidia of *Phomopsis phaseoli*. Lengths on filtered and autoclaved filtrates were not significantly different from each other (Duncan's new multiple range test; $P > 0.05$) but were significantly different from lengths on the control filtrate ($P \leq 0.05$).

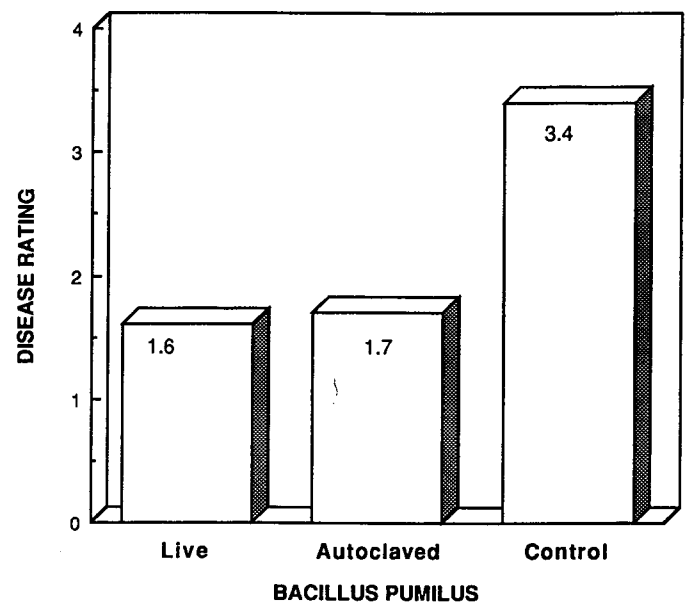

FIG. 5. Biocontrol of *Phomopsis phaseoli* by *Bacillus pumilus* on soybean leaflets. Absence of symptoms was rated 1, and maximum disease was rated 4. Disease ratings with live and autoclaved *B. pumilus* were not significantly different from each other (Duncan's new multiple range test; $P > 0.05$) but were significantly different from disease ratings with the control ($P \leq 0.05$).

LITERATURE CITED

BACKMAN, P. A., M. A. CRAWFORD, AND E. SMITH. 1986. Management of stem canker with fungicides. Proc. Southern Soybean Dis. Workers, Baton Rouge, Louisiana.

BAKER, C. J., J. R. STAVELY, C. A. THOMAS, M. SASSER, AND J. S. MCFALL. 1983. Inhibitory effect of *Bacillus subtilus* on *Uromyces phaseoli* and on development of rust pustuies on bean leaves. Phytopathology, 73:1148.

BERGEY, D. H. 1948. Manual of determinative bacteriology. Waverly Press, Inc., Baltimore, Maryland.

CHRISTENSEN, J. J., AND F. R. DAVIS. 1940. Variation in *Helminthosporium sativum* induced by a toxic substance produced by *Bacillus mesentericus*. Phytopathology, 30:1017-1033.

DHINGRA, O. D., AND J. B. SINCLAIR. 1985. Basic plant pathology methods. CRC Press, Inc., Boca Raton, Florida.

FRAVEL, D., AND H. W. SPURR, JR. 1977. Biocontrol of tobacco brownspot disease by *Bacillus cereus* subsp. *mycoides* in a controlled environment. Phytopathology, 67:930.

GAYED, S. K. 1966. *Bacillus pumilus* and its mycolytic action against *Helminthosporium sativum*. Plant and Soil, 24:178-180.

HILTY, J. W. 1981. Soybean stem canker, a major disease in 1981. Tennessee Farm and Home Sci., 120:16-17.

MORGAN, F. L. 1963. Infection inhibition and germ tube lysis of three cereal rusts by *Bacillus pumilus*. Phytopathology, 53:1346.

PLOETZ, R. C., AND F. M. SHOKES. 1987. Infection of different plant parts of soybean seedlings by southern *Diaporthe phaseolorum* and its role in the development of stem canker symptoms. Canadian J. Bot., 65:2104-2108.

SINCLAIR, J. B., AND P. A. BACKMAN, EDS. 1991. Compendium of soybean diseases. Amer. Phytopathological Soc., St. Paul, Minnesota.

SPURR, H. W. 1981. Experiments on foliar disease control using bacterial antagonists. Pp. 369-381 *in* Microbial ecology of the phylloplane (J. P. Blakeman, ed.). Academic Press, London.