Interaction Between *Bacillus subtilis* and Fungi Associated with Soybean Seeds

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ABSTRACT

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A *Bacillus subtilis* isolate from soybean was tested for antagonism against 26 fungi commonly associated with soybean seeds in dual culture on potato-dextrose agar (PDA) and soil-extract agar. The bacteria were fungicidal to *Penicillium* sp. and fungistatic to all others. Autoclaved filtrates of *B. subtilis* cultures inhibited growth and stroma formation of *Phomopsis* sp. A chloroform-soluble component from autoclaved *B. subtilis* culture filtrates was active against seven soybean pathogens on PDA. Suspensions of *B. subtilis* applied as a soybean seed treatment reduced stem infection caused by *Phomopsis* sp., emergence, and plant height in the field but not in greenhouse or growth chamber studies. Suspensions of *B. subtilis* sprayed on soybean plants significantly reduced Phomopsis pod infection in a growth chamber but not in the field.

Additional key words: pod and stem blight

The *Diaporthe* and *Phomopsis* spp. complex of soybeans (*Glycine max* (L.) Merr.), which causes pod and stem blight, stem canker, and seed decay, is endemic in world soybean production areas and causes severe yield losses under warm, moist conditions when harvest is delayed (12,14). Because of latent infection by *Phomopsis* sp. in soybean plants and seeds, media assays are used to detect its presence. In seed assays, *Bacillus subtilis* (Ehrenberg) Cohn occurs frequently and shows antagonistic interactions with certain fungi, particularly *Phomopsis* spp. (15). Several plant-pathogenic fungi are controlled using *B. subtilis* as a seed treatment (3,9,16,17). The antagonistic mechanism of *B. subtilis* is by antibiotic production (5,18), competition (16), or both.

We studied the interaction between a *B. subtilis* isolate from soybean and 26 fungi associated with soybean seeds to determine the potential of the bacterium to serve as a biological control agent of seedborne fungal pathogens on soybean seeds and plants. The bacterium was evaluated as a seed treatment or foliar spray first in the laboratory, then in the greenhouse and growth chamber, and finally in the field.

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MATERIALS AND METHODS

Of 107 *B. subtilis* isolates screened for activity against *Phomopsis* sp. in dual culture, one isolate (BS1) inhibited growth on nutrient media of a *Phomopsis* sp. isolate from a soybean seed. A stock culture of BS1 was maintained by lyophilization or in sterile deionized water at 5 C. BS1 was identified by cell morphology, by noting reactions to the Gram strain, and by using biochemical tests (4): casein hydrolysis, anaerobic growth, catalase production, starch hydrolysis, and acid production from mannitol. The identity was confirmed by L. K. Nakamua, USDA, ARS, Peoria, IL.

Rifampicin-resistant BS1 isolates were selected from sterile nutrient broth amended with 5–1,000 μg/ml of filter-sterilized rifampicin (Sigma Chemical Co., St. Louis). After 48 hr at 28 C, 1-ml samples were plated on rifampicin-amended tryptic soy agar (Difco). Isolates from colonies growing on rifampicin-amended media were transferred to agar media with increasing concentrations of antibiotic until an isolate resistant to 1,000 μg/ml was obtained. The same procedure was used with the rifampicin-resistant isolate to obtain resistance to nalidixic acid. The BS1 isolate used in this study was resistant to 1,000 μg/ml of both rifampicin and nalidixic acid.

Single-cell isolates of BS1 were grown in nutrient broth (Difco) in shake cultures (100 oscillations per minute) for 48 hr at 28 C. Suspensions were diluted with sterile nutrient broth to a concentration of 1×10^8 colony-forming units. Density was checked using a spectrophotometer at a wavelength of 580 nm. Cultures were lyophilized or maintained in sterile distilled water at 25 C. Heat-killed cultures were autoclaved for 30 min at 124.2×10^3 Pa at 121 C.

Culture filtrates from 48-hr-old BS1 cultures grown in either soybean (50 g/L) or nutrient broth were centrifuged at 3,000 *g* for 15 min and the supernatant decanted, filtered through a sterile (Sybron/Nagle) filter (0.1 μm), and the

pH recorded.

Dual-culture studies. The activity of BS1 was tested in dual cultures for the following fungi isolated from soybean seeds of several cultivars: *Aspergillus* sp., *Cercospora kikuchii* (T. Matsu & Tomoyaso) Gardner, *C. sojina* Hara, *Chaetomium cupreum* Ames, *Cladosporium* sp., *Colletotrichum truncatum* (Schw.) Andrus & Moore, *Drechslera* sp., *Epicoccum purpurascens* Ehrenb.: Schlecht., *Eurotium* sp., *Fusarium oxysporum* Schlecht. f. sp. *glycines* Armst., *Macrophomina phaseolina* (Tassi) Goid., *Myrothecium roridum* Tode ex Fr., *Nigrospora* sp., *Ostrachoderma* sp., *Paecilomyces* sp., *Penicillium* sp., *Phomopsis* sp., *Phyllosticta sojicola* Massal., *Rhizoctonia solani* Kühn, *Sclerotinia sclerotiorum* (Lib.) de Bary, *Scopulariopsis* sp., *Septoria glycines* Hemmi, *Stachybotrys parvispora* Hughes, *Torula* sp., *Tricothecium roseum* (Pers.) Link: Gray, and *Verticillium albo-atrum* Reinke & Berth. The fungi were identified by the first author; J. L. Crane, Illinois Natural History Survey; J. B. Manandhar, graduate research assistant; and P. N. Thapliyal, visiting professor, on the bases of culture characteristics and conidial ontogeny.

Dual cultures were prepared on PDA or soil-extract agar by streaking a 0.01-ml sample of a BS1 suspension 5 mm from the edge of each of four 9-cm culture plates. After 3 days at 25 C, a 7-mm agar disk of one of the test fungi was placed 70 mm opposite the bacterial streak (10). An agar disk of the same fungus placed on a culture plate without the bacteria served as a control. After 14 days at 25 C, inhibition was recorded if the radial growth of the test fungus was greater on the control than on the bacterium-fungus plate. At that time, a 1-mm^2 piece of mycelium cut from the colony margin was transferred to fresh medium and radial growth was recorded after 14 days at 25 C to determine if inhibition was fungistatic or fungicidal.

Organic solvent studies. Filter-sterilized culture filtrates of BS1 grown in nutrient broth were extracted four times with 50 ml of chloroform, ether, or ethyl acetate. The solvent fraction was flash-evaporated and the residue resuspended to original volume in sterile distilled water. These fractions, the original culture filtrate, and a control of sterile distilled water or nutrient broth were mixed separately with PDA (10% v/v) and autoclaved. Radial growth of *Cercospora kikuchii, C. sojina, Colletotrichum truncatum, F. oxysporum* f. sp. *glycinea, Macrophomina phaseolina, Phomopsis* sp., and *R. solani* on this medium was recorded after 14 days at 25 C. During chloroform extraction, an emulsion layer formed between the two separated layers. Each fraction and emulsion layer was assayed separately as described previously with an additional assay of *Drechslera* sp. and *Nigrospora* sp.

Greenhouse studies. Soybean seeds were treated with a suspension of either live or heat-killed BS1, a sterile culture filtrate of BS1, or nutrient broth (control). Two experiments were conducted using two seed lots of Wells soybean, one with 93% and one with 65% germination, determined by germinating seeds on moist cellulose pads (Kimpac) at nearly 100% RH for 5 days at 25 C. One hundred seeds were dipped into one of the suspensions, then coated with a mixture of screened (16-mesh, 1-mm^2), sterile peat moss (10 g) containing 0.01 g of $CaCO_3$ and 1 g of methyl cellulose (13). Twenty-five seeds per replicate were planted in a 13-cm-diameter clay pot containing either soil infested with oat grains colonized by *Phomopsis* sp. (1:5, v/v) or uninfested, pasteurized field soil. The 32 pots were arranged in a completely randomized design in the greenhouse under ambient conditions. Soil-oat inoculum was prepared by autoclaving 1,500 cm^3 of oat seeds once each day on two successive days. Five 7-day-old, 9-cm-diameter culture plates of *Phomopsis* sp. mycelium were added to oats, incubated for 3 wk at room temperature (28 ± 5 C), and the infested oats mixed in the top 1 cm of soil. Five and 10 days after planting, root length and emergence were recorded. Ten days after planting, plant height and the bacterial population of each of five plants from each replicate were recorded.

Growth chamber studies. Cultivar Wells soybean seeds planted in pasteurized soil were thinned to five plants per pot after 2 wk. All plants were grown under fluorescent and incandescent light (2,500 lux) at nearly 100% RH with 14-hr days at 27 C and nights at 22 C. At growth stage R6 (6), pods were wounded with a multineedle inoculator and sprayed to runoff with a live or heat-killed suspension of BS1, a culture filtrate of BS1, or nutrient broth control prepared as described previously. All plants were sprayed after 30 min with a 1×10^5 spore suspension of *Phomopsis* sp. Five replicates of each treatment were arranged in a completely randomized design.

Bacterial leaf populations were determined 1, 2, and 5 days after inoculation from three trifoliolate leaves per plant treated with a live BS1 suspension by shaking leaf tissue separately in 10 ml 0.01 M $MgSO_4$ for 15 min. Each suspension was diluted serially and plated on tryptic soybean agar amended after autoclaving with sterile solutions of dichloran (Botran 75W, 100 μg/ml), cycloheximide (150 μg/ml), and nalidixic acid (100 μg/ml) and incubated for 48 hr at 28 C (13).

For fungal assay at 30 days, pods, stems, and roots were cut into 1-cm sections, washed for 3–4 hr in tap water, surface-disinfested in 0.05% NaOCl (Clorox) for 3 min, rinsed in 1,000 μg/ml sodium thiosulfate, double-rinsed in sterile distilled water, and plated on acidified PDA (pH 4.5). Occurrence of fungi was recorded after 7 days at 25 C.

Field studies. Field studies were conducted in 1982 and 1983 at the Plant Pathology Research Farm, Urbana, IL. Plots were arranged in a randomized complete block design, with eight treatments replicated four times. Four 5-m rows on 76-cm center plots were used. Data were collected from the two center rows. In 1982, cultivar Beeson 80 soybeans were planted on 19 May in a plot that had been in continuous soybeans for 8 yr and on 2 June 1983 in a plot that had been in continuous soybeans for 14 yr.

In 1982, seeds of four experimental plots were sprayed with a suspension of live or heat-killed BS1 as described previously; four experimental units were sprayed on 1 June (growth stage V1), 12 July (growth stages R2 to R3), and 6 August (growth stage R6) with either of the two BS1 suspensions using a Hudson 6622 Duralite hand sprayer. In 1983, the plot design was identical, except one plot was an unsprayed control. Beginning at 3 wk, five plants were harvested biweekly for a total of six times. Stem sections were taken 8 and 16 cm above the soil line from plants less than and more than 25 cm tall, respectively (7). Ten stem, pod, and root sections were assayed for fungi as described previously.

Field data were analyzed as a split plot in time (with treatments as the main factor and time as the split factor) with

Table 1. Inhibition of growth of two fungi grown on potato-dextrose agar amended with organic soluble components of a *Bacillus subtilis* culture filtrate (CF) (10%, v/v) after 14 days at 25 C

Treatment[a]	Mean radial growth (mm)[b]	
	Colletotrichum truncatum	*Phomopsis* sp.
Ether	85.5	87.5*
CF-ether	70.3*[c]	89.0
Ethyl acetate	85.3	90.0
CF-ethyl acetate	63.3*	87.3*
Original CF	61.8*	88.0*
Control (NB)	86.0	90.0
Control (SDW)	86.0	90.0
Chloroform	65.8*	86.0*
CF-chloroform	85.8	90.0
LSD (P= 0.05)	5.05	1.62
C.V. (%)	4.54	1.26

[a] Bacterial filtrates were obtained from a *B. subtilis* culture grown in nutrient broth (NB) for 48 hr at 28 C. The filtrate and organic solvent were mixed, extracted four times, flash-evaporated at 60 C, adjusted to the original CF volume with sterile distilled water (SDW), amended to agar, and autoclaved.

[b] Means based on four replicates.

[c] * = Significant difference from controls at P= 0.05.

multiple mean comparisons with the heat-killed suspension of BS1 at each treatment stage. All analyses of variance and mean calculations were conducted with the Statistical Analysis Program (SAS) (11). Significant differences were at $P = 0.05$ unless otherwise indicated.

RESULTS

Dual-culture studies. BS1 was a gram-positive, spore-forming rod and was positive for casein hydrolysis, catalase production, starch hydrolysis, and acid production from mannitol. BS1 was negative for anaerobic growth. BS1 significantly inhibited radial growth of all test fungi after 14 days except for *Septoria glycines*. After reisolation from inhibited colonies on fresh PDA, all fungi produced normal growth except *Penicillium* sp., which produced no growth. BS1 apparently produced a compound fungicidal to *Penicillium*.

Organic solvent studies. Culture filtrates, ethyl extracts of culture filtrate, and chloroform-soluble fractions significantly reduced the growth of *Colletotrichum truncatum* and *Phomopsis* sp. Ether extract of culture filtrates reduced growth of *C. truncatum* (Table 1). Inhibition of *Phomopsis* sp. by aqueous fractions of the culture filtrate was significantly greater than by nonpolar organic solvent extract. Chloroform-soluble fractions were significantly more inhibitory to *Phomopsis* sp. than all others except the ether-soluble fraction. Solvent systems were not significantly different for any of the other fungi tested.

In the chloroform extraction studies, the suspension from the emulsion layer significantly inhibited the growth of five of the fungi tested (Table 2).

Greenhouse and growth chamber studies. Seedling emergence and root length data from greenhouse and growth chamber studies, respectively, were significantly lower than the controls in *Phomopsis*-infested soil (Table 3). Seedling emergence and plant height data from growth chamber and greenhouse studies, respectively, were reduced in *Phomopsis*-infested soil but were not significantly different from the control (Table 3). In the growth chamber, the BS1 treatment had significantly less *Phomopsis* sp. (18%) in pods than the control (35%). Heat-killed culture filtrate broths and the control were not significantly different. Recovery from stems was not significantly different for any treatment (Table 4). Leaf populations of BS1 ranged from 1.4×10^5 to 3.6×10^2 after 24 and 48 hr, respectively. After 5 days, no bacteria were recovered from the trifoliolates.

Field studies. Soybean seeds treated with the suspension of BS1 had significantly less *Phomopsis* sp. in the stems than the heat-killed control (Table 5). The percentage of root infection was lowest on plants from seeds treated with BS1 but was not significantly different from the heat-killed treatment. There were no significant differences between treatments in 1983.

DISCUSSION

Many pathogens are sensitive to *B. subtilis* or its culture filtrate, which contains antibiotics (1,2,5,18). Using nutrient media assays for antagonism of a microorganism in culture primarily selects for the ability of that microorganism to produce antibiotics (10). Activity of BS1 against pathogens of other hosts (9,13,16) suggests that the bacterium can be an effective biological

Table 2. Radial growth (mm) of various fungi grown on potato-dextrose agar amended with chloroform-soluble components of a *Bacillus subtilis* culture filtrate (CF) (10%, v/v) after 14 days at 25 C

Treatment[a]	*Cercospora kikuchii*	*Colletotrichum truncatum*	*Drechslera* sp.	*Nigrospora* sp.	*Phomopsis* sp.
Extracted CF	70.5[b]	74.0	67.0*	76.3*	78.5*
CF-chloroform 1	69.5	74.5	70.3	84.0	81.5*
CF-chloroform 2	50.5*[c]	46.3*	45.5*	61.8*	75.3*
Control (NB)	69.3	75.5	86.8	87.3	87.8
Control (SDW)	71.0	57.3	88.8	88.3	87.7
LSD ($P = 0.05$)	2.78	2.35	6.34	4.72	2.89
C.V. (%)	3.21	2.91	2.95	3.84	1.99

[a] Bacterial filtrates were obtained from a *B. subtilis* culture grown in nutrient broth (NB) for 48 hr at 28 C. The filtrate and chloroform were mixed, extracted four times, flash-evaporated at 60 C, adjusted to the original CF volume with sterile distilled water (SDW), amended to agar, and autoclaved.
[b] Means based on four replicates.
[c] * = Significant difference from the controls at $P = 0.05$.

Table 3. Percentage emergence and plant height in the greenhouse and percentage emergence and root length of cultivar Wells soybean seedlings from seeds untreated or treated with a live or heat-killed (HK) isolate or a culture filtrate (CF) of *Bacillus subtilis* in pasteurized soil uninfested or infested with *Phomopsis* sp. in a growth chamber

	Greenhouse		Growth chamber	
Treatment[a]	Emergence[b] (%)	Plant height[c] (mm)	Emergence[b] (%)	Root length (cm) at 5 days[d]
B. subtilis				
Treated seed in infested soil	34.0	75.2	59.0	29.9
Treated seed in uninfested soil	49.0	92.6	93.8	37.5
B. subtilis (HK)				
Treated seed in infested soil	40.0	89.2	67.0	32.3
Treated seed in uninfested soil	39.0	92.4	77.0	35.6
B. subtilis (CF)				
Treated seed in infested soil	38.0	85.3	64.0	35.8
Treated seed in uninfested soil	59.0	85.8	89.0	39.3
Control				
Untreated seed in infested soil	54.0	86.4	64.0	40.7
Untreated seed in uninfested soil	69.0	98.6	93.5	41.8
LSD ($P = 0.05$)	14.6	11.2	11.6	7.9
C.V. (%)	29.8	23.6	14.9	15.7

[a] Bacterial filtrates were obtained from isolate BS1 of *B. subtilis* culture grown in nutrient broth at 28 C. Cell concentrations of *B. subtilis* were adjusted to 1×10^8 colony-forming units per milliliter, and heat-killed suspensions were prepared by autoclaving for 30 min. Soybean seeds had a predetermined germination of 65% for greenhouse and 93% for growth chamber studies.
[b] Means based on four replicates of 25 seeds each.
[c] Values represent combined means of two experiments with four replicates of five plants each, recorded 10 days after planting by measuring length between cotyledons and leaf primordia.
[d] Values represent the means of four replicates of either 25 seeds or five roots measured from the soil line to the root tip.

Table 4. Percentage recovery of *Phomopsis* sp. from soybean pods on plants sprayed first with *Bacillus subtilis*, then with a suspension of the test fungus in a growth chamber

Treatment[a]	Mean recovery[b] (%)
B. subtilis (live)	18.0
B. subtilis (HK)	41.0
B. subtilis (CF)	25.0
Control (NB)	35.0
LSD (0.05)	13.2
C.V. (%)	25.4

[a] Cell concentrations of *B. subtilis* were adjusted to 1×10^8 colony-forming units per milliliter, and heat-killed (HK) suspensions were prepared by autoclaving for 30 min. The culture filtrate (CF) was obtained from a *B. subtilis* culture grown in nutrient broth (NB) for 48 hr at 28 C. Treatments were applied to soybeans at growth stage R6 30 min before inoculation with a 1×10^5 conidial suspension of *Phomopsis* sp.
[b] Values represent the combined means of two experiments with five replicates each of 10 1-cm pod sections assayed on potato-dextrose agar (pH 4.5).

Table 5. Recovery of fungi from cultivar Beeson 80 soybean plant stems or treated seeds, or from plants sprayed with *Bacillus subtilis*

Treatment[a]	Mean recovery (%)[b]		
	Alternaria sp.	*Fusarium* sp.	*Phomopsis* sp.
B. subtilis (live) seed treatment	1.2	2.2	5.8
B. subtilis (HK) seed treatment	1.7	1.2	6.8
B. subtilis (live) spray at V1	1.6	1.5	6.6
B. subtilis (HK) spray at V1	1.2	1.5	6.7
B. subtilis (live) spray at R2–R3	2.2	1.8	6.7
B. subtilis (HK) spray at R2–R3	1.4	2.2	6.9
B. subtilis (live) spray at R6	1.5	2.0	6.4
B. subtilis (HK) spray at R6	1.5	2.0	7.1
LSD ($P = 0.05$)	0.80	0.80	0.70
C.V. (%)	68.04	78.54	18.09

[a] Cell concentrations of *B. subtilis* were adjusted to 1×10^8 colony-forming units per milliliter and heat-killed suspensions prepared by autoclaving for 30 min. All bacterial sprays were applied to runoff at dusk at the respective growth stage (6) using an atomizer.

[b] Means based on four replicates of ten 1-cm stem sections assayed on potato-dextrose agar (pH 4.5) at six sampling dates.

control agent. Our greenhouse and growth chamber data provide evidence for *Phomopsis* sp. control, but it was not shown to occur under our field conditions. Soybean seeds and plants are sensitive to BS1 or its antibiotics. The reduction of *Phomopsis* sp. in the field could be caused in part by the decrease in plant stand that would in turn alter the canopy and microclimate, thus affecting the occurrence of the pathogen in soybean stems. The reduced emergence associated with seed treatment may have been influenced by the age and concentration of BS1 inoculum, the method of application, and environmental factors, which may act in consort (8). Additional work is being conducted to examine these parameters and to identify the heat-stable, diffusable substances produced by BS1. Selection of *B. subtilis* isolates or its by-products that are not phytotoxic to soybean seeds or plants may prove useful in controlling other seedborne soybean pathogens and should be evaluated with several years of replicated field trials.

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