Inhibitory Effect of *Bacillus subtilis* on *Uromyces phaseoli* and on Development of Rust Pustules on Bean Leaves

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ABSTRACT

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Two isolates of *Bacillus subtilis* and one isolate each of *B. cereus* (subsp. *mycoides*), *B. thuringiensis*, and *Erwinia ananas* pv. *uredovora* were effective in controlling rust on beans under certain conditions. The most effective isolate was *B. subtilis* APPL-1. This isolate gave greater than 95% reduction in the subsequent number of rust pustules when it was applied in liquid culture to plants in the greenhouse 2 to 120 hr prior to inoculation with *U. phaseoli* var. *typica* urediospores. The number of pustules was reduced on all tested bean cultivars, and with several different isolates of the fungus. When APPL-1 was applied after inoculation with urediospores, there was no effect on pustule number. Microscopic observations of *B. subtilis*-treated bean leaves showed urediospore germination was greatly reduced and no normal germ tubes were produced. Some urediospores developed abnormal cytoplasmic protrusions. An inhibitory component present in culture filtrates was nondialyzable and heat stable. Dialyzed culture filtrates were subjected to preparative gel filtration. The eluted material had an apparent molecular weight of 5 to 10 Kdaltons, was inhibitory to *U. phaseoli* spore germination, and reduced bean rust severity. Analysis of the eluted fraction showed that it contained approximately 5% carbohydrate and 95% protein.

Additional key words: biological control.

In recent years, bean rust (which is caused by *Uromyces phaseoli* (Reben.) Wint.) has been a major disease problem on snap and dry beans (*Phaseolus vulgaris* L.) in the United States. In 1981, yields of rust susceptible pinto beans were reduced by up to 78% in a severe epidemic in Michigan (8). *U. phaseoli* is a highly variable pathogen, with many races having been described over the past 40 yr (19). Although much resistance is available in the bean genome (16), to maintain and enhance its role in controlling rust requires time, effort, and resources, as well as constant vigilance. Certain fungicides help control bean rust, but they are not always economically practical (6). Furthermore, general concern about possible adverse effects of chemical pesticides makes it desirable to seek additional control methods. One attractive alternative, not previously investigated for bean rust, is the use of parasitic or antagonistic microorganisms (1,15). *Bacillus* spp., isolated from either the soil or leaf surfaces, are particularly interesting in this regard because they produce a wide range of antimicrobial compounds (5,7,9). Several *Bacillus* spp., including *B. subtilis* (Ehrenberg) Cohn, have proven antagonistic to plant pathogenic fungi and bacteria (1,4,5,10,13,18).

In this paper we report the effect of *B. subtilis* on bean rust under greenhouse and cold frame conditions. In addition we report the partial characterization of the active component and its effect on urediospore germination and pustule development. The initial results were reported in an abstract (17).

MATERIALS AND METHODS

Bacterial cultures and cultural conditions. All bacterial cultures were maintained on nutrient agar at 25 C. A culture of *B. subtilis*, originally isolated from soil by Thirumalachar and O'Brien (18), was obtained from M. O'Brien and designated APPL-1. A second isolate of *B. subtilis*, designated APPL-2, was isolated from soil in Delaware. *B. cereus* subsp. *mycoides* isolate B23, which controls lesion production on tobacco by *Alternaria alternata* (4), and *B. thuringiensis* HD-1 were obtained from H. W. Spurr, Jr. (Oxford Research Lab., ARS, USDA, Oxford, NC 27565). *Erwinia ananas* pv. *uredovora* was isolated from a rust pustule on oats in Delaware. An isolate of *Pseudomonas syringae*, avirulent on beans, was obtained from safflower in Montana. Most of the work reported here was done with *B. subtilis* APPL-1.

Each of the isolates was transferred from nutrient agar to 1 L of Eugon broth (Difco Laboratories, Detroit, MI 48232) in a 2.8-L Fernbach flask. The cultures were incubated on a circulatory shaker at 125 rpm, 30 C, and in the dark until the early stationary phase was reached. These same cultural conditions were used for *B. subtilis* (APPL-1) throughout these experiments, except the cultures were usually incubated for only 24–48 hr.

Rust inhibition bioassay. Five seeds of *P. vulgaris* 'Lake Shasta' were planted per 10-cm-diameter plastic pot, germinated in a greenhouse at 24–30 C, and thinned to three uniform plants per pot 5–7 days after seeding. Culture supernatant and other culture fractions tested for rust inhibition activity were applied to young plants with primary leaves one-fifth to one-third expanded. Bacterial suspensions or test solutions were sprayed onto plants with a Sprā-tool (Crown Industrial Products Co., Hebron, IL 60034) from a distance of approximately 30–40 cm to give thorough coverage of all aboveground plant surfaces. The plants were then allowed to dry.

Urediospores of *U. phaseoli* were added to a solution of distilled water containing 0.01% Tween-20 and stirred on a magnetic stirrer for ~5 min. Urediospore concentration was determined with a hemacytometer and adjusted to about 20,000 spores per milliliter. This suspension was lightly sprayed onto the upper surface of leaves of plants that had been either pretreated or nontreated with *B. subtilis*. After the film dried, the plants were placed overnight in a dew chamber (Percival Mfg. Co., Boone, IA 50036) set to regulate

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a wall temperature of 13 C and a water temperature of 23 C. The plants were moved to the greenhouse the next morning. After 8–14 days, uredial pustules had developed and were counted. In some experiments conducted in May and June, plants were moved from the dew chamber to an uncovered, outdoor cold frame. All of these in vivo treatments were applied to at least 10 replicate plants and the experiments were repeated at least once.

Cultures of several unique pathotypes of *U. phaseoli* were tested against *B. subtilis* APPL-1. Collection 73-16, in which the major component resembles race 32 (20), was included in all experiments. This pathotype is the principal one on snap beans in the eastern United States (16) and produces large pustules on plants of cultivar Lake Shasta, which was used in much of this study. However, to determine if APPL-1 was broadly effective, plants of five bean cultivars were sprayed with the bacterium, then inoculated with five different pathotypes of *U. phaseoli*. These pathotypes are readily distinguishable on differential bean cultivars, but have not been assigned race numbers. The cultivars tested included BBL 47, Lake Shasta, Mountaineer White Half Runner, Mount Hood, and Pinto 111. The last three cultivars are highly resistant or immune to collection 73-16, but all five cultivars are susceptible to collection 75-22, another one of the pathotypes that were used. Another collection of *U. phaseoli*, 73-23, produces small necrotic spots, but no urediospores, on the leaves of cultivar Lake Shasta. This combination was used to determine the effect of APPL-1 on the necrotic reactions. Finally, urediospores were harvested from several individual pustules that occasionally occur on an APPL-1 pretreated Lake Shasta plant inoculated with collection 16. These spores were increased on a nontreated Lake Shasta plant in a rust free greenhouse, and then used to test for the presence of a pathotype of *U. phaseoli* resistant to *B. subtilis*.

TABLE 1. Effect of pretreatment with certain bacteria on the number of uredial pustules produced by *Uromyces phaseoli* on bean leaves[y]

Pretreatment	Uredial pustules per leaf	
	Number (mean)	Control (%)[z]
Bacillus subtilis (APPL-1)	0.5 ± 0.2	99 a
B. subtilis (APPL-2)	6.9 ± 1.0	84 b
B. cereus subsp. *mycoides*	9.2 ± 1.5	80 b
B. thuringiensis	6.9 ± 1.6	85 b
Erwinia ananas pv. *uredovora*	6.4 ± 1.0	86 b
Pseudomonas syringae	47.2 ± 7.1	0 c
Untreated control	45.3 ± 6.5	0 c
Culture medium control	45.7 ± 6.2	0 c

[y] Bean cultivar Lake Shasta and *U. phaseoli* collection 73-16 were used in this experiment.

[z] Percent control or reduction in number of pustules as compared to the number on untreated plants. Any two figures followed by the same letter are not significantly different according to Duncan's multiple range test, $P = 0.05$.

Spore germination bioassay. Approximately 1 mg of *U. phaseoli* urediospores, collection 16, were placed in a 2.5-cm-diameter petri dish that contained approximately 5 ml of tap water and 0.01% Tween-20. Germination of urediospores was best in hot tap water initially cooled to room temperature before the urediospores were applied. Apparently this water supplies inorganic nutrients that improve germination. After 10 min, a Pasteur pipette was used to remove the solution from beneath the floating spores. This washing procedure was repeated four times to remove the germination self-inhibitor from within the spores. Washed spores were then transferred to wells (400 μl) of a microtiter plate that contained the suspensions or fractions derived from *B. subtilis*. The transfer was accomplished by allowing spores to adhere to the top of a 2-mm-diameter glass rod. Each solution to be tested was serially diluted. The microtiter plates were placed on a moist paper towel in a covered baking dish and incubated overnight at 16 C. The following morning the dishes were observed under a microscope at ×100 to determine the percentage of spores that had germinated.

Histological procedures. Leaves were removed from treated and control bean plants immediately after the plants were removed

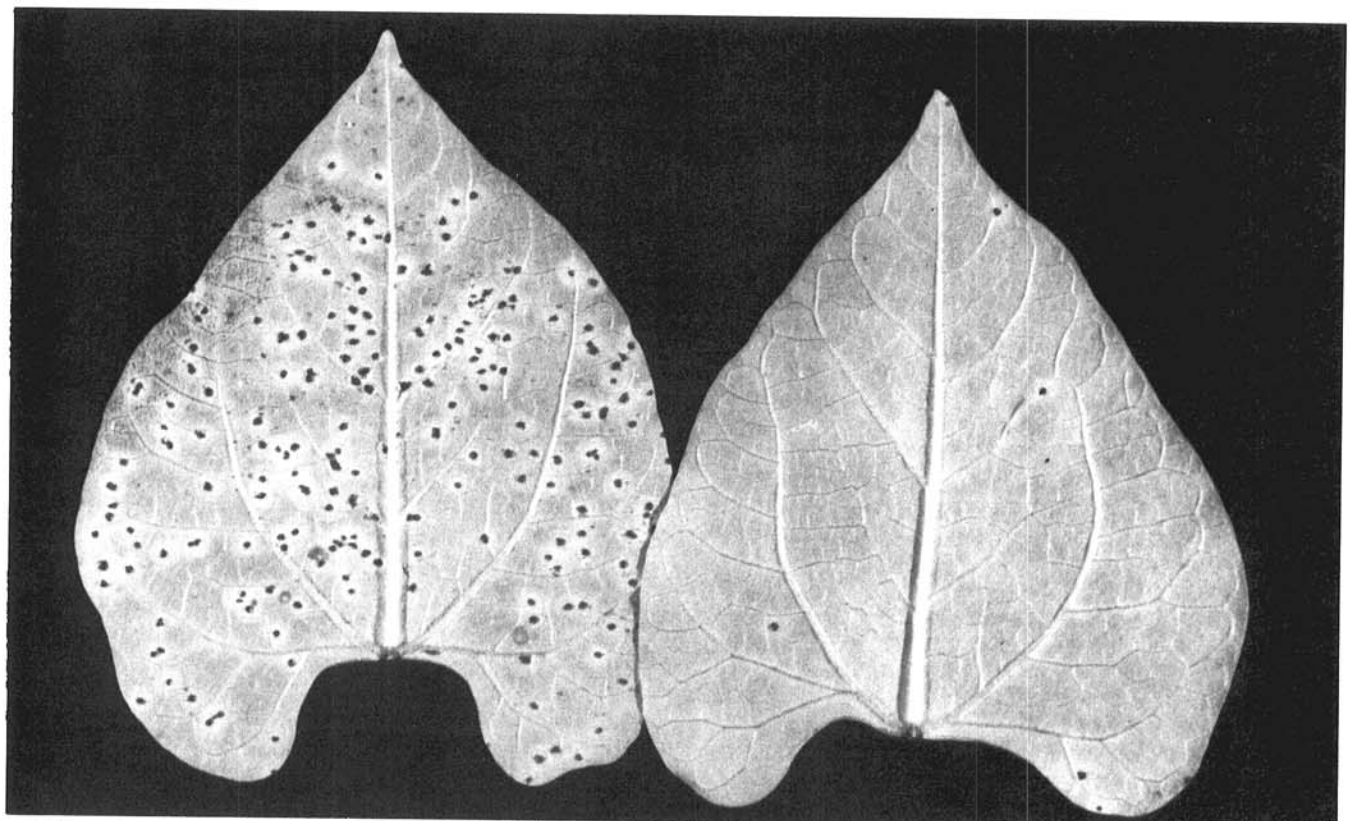

Fig. 1. Effect of *Bacillus subtilis* on *Uromyces phaseoli* and bean rust. Leaves from bean cultivar Lake Shasta, treated with *B. subtilis* 1 day prior to inoculation with *U. phaseoli* (right), and leaf from control plant not pretreated with *B. subtilis* (left). Both plants were inoculated at the same time and incubated under identical conditions.

TABLE 2. Effect of varying the time interval between treatment with *Bacillus subtilis* APPL-1 and inoculation with *Uromyces phaseoli* collection 73-16 on the subsequent number of uredial rust pustules formed on leaves of bean cultivar Lake Shasta in the greenhouse and in an outdoor cold frame

Time between *B. subtilis* application and rust inoculation (hr)	Uredial pustules per leaf					
	Cold frame			Greenhouse		
	Treated (mean)	Untreated[y] (mean)	Control[z] (%)	Treated (mean)	Untreated[y] (mean)	Control[z] (%)
−24	...	...	...	29.9 ± 5.49	23.9 ± 9.63	0
2	0.2 ± 0.07	22.8 ± 3.56	99 a	1.4 ± 0.46	18.1 ± 4.69	92 a
24	0.1 ± 0.11	3.0 ± 0.65	97 a	4.7 ± 1.47	46.8 ± 8.07	90 a
48	0.3 ± 0.06	52.8 ± 10.68	99 a	1.2 ± 0.32	26.0 ± 6.56	95 a
72	...	...	...	8.4 ± 4.15	210.4 ± 25.34	96 a
96	18.6 ± 7.41	193.5 ± 21.52	90 ab	14.0 ± 2.94	279.8 ± 28.78	95 a
120	24.1 ± 6.33	125.9 ± 21.14	81 b	3.6 ± 1.25	72.8 ± 5.52	95 a

[y]The number of pustules on untreated leaves tends to increase as the leaf size increases at time of inoculation.

[z]Percent control or reduction in number of pustules as compared to the number on untreated plants. Any two figures followed by the same letter are not significantly different according to Duncan's multiple range test, $P = 0.05$.

from the dew chamber. The leaves were sectioned with a scalpel into approximately 1-cm-square pieces. The sections were immersed for 10 sec in alcoholic lactophenol cotton blue (14) modified to contain 0.1 g aniline blue per 40 ml. The sections were then rinsed briefly with 50% glycerin and mounted on a glass slide under a coverslip for observation with an Olympus model BH-2 light microscope.

Partial characterization. Cultures of *B. subtilis* APPL-1 were grown in Eugon broth that had been passed through Spectrapor dialysis tubing (Spectrum Medical Ind., Los Angeles, CA 90054) of molecular weight limitation of 10,000, to remove nondialyzable material from the medium. Bacterial cells were removed from 24- to 48-hr-old cultures by centrifugation or were inactivated by autoclaving. Following centrifugation, the supernatant was dialyzed with the same type of Spectrapor tubing. The portion of the culture filtrate (100 ml) retained by the dialysis tubing was lyophilized and dissolved in 50 ml of distilled water. This reconstituted aqueous filtrate (10 ml) was further purified by gel filtration on a Bio-gel P-10 column (2.5-cm diameter × 90 cm) equilibrated with 0.1 M tris, pH 7.0. Fractions (5 ml) were assayed for protein (12) and carbohydrate (3). In vitro, and in some cases, in vivo bioassays were conducted at appropriate steps in this procedure.

RESULTS

Effective bacteria. Cultivar Lake Shasta bean plants pretreated with cultures of *B. subtilis, B. cereus* subsp. *mycoides, B. thuringiensis*, or *E. ananas* pv. *uredovora* had 80–99% fewer uredial pustules than those pretreated with either avirulent *P. syringae* or sterile Eugon broth or given no pretreatment (Table 1 and Fig. 1). The bacterial isolate most inhibitory to rust development was *B. subtilis* APPL-1, which consistently reduced the number of pustules by more than 90% compared to the nontreated control. This was a significantly greater reduction than given by APPL-2 or the other effective bacteria. There was no significant difference among the percent reduction in pustule numbers caused by *B. subtilis* APPL-2, *B. cereus* subsp. *mycoides, B. thuringiensis*, or *E. ananas* pv. *uredovora*.

In vivo studies. Comparisons of various time intervals and sequences between application of bacterial culture and urediospores showed that *B. subtilis* APPL-1 had to be applied to bean plants prior to inoculation with urediospores in order to inhibit the subsequent number of pustules (Table 2). When *B. subtilis*-pretreated plants inoculated with *U. phaseoli* were placed outdoors in a cold frame following the postinoculation dew period, the effect on pustule development was similar to that occurring when they were placed in a greenhouse. Once applied to the bean leaves, *B. subtilis* was effective for at least 5 days in reducing the number of subsequent rust pustules.

The effect of *B. subtilis* APPL-1 on rust was the same on all tested cultivars of *P. vulgaris* and with all tested pathotypes of *U. phaseoli*. A 96 to 98% reduction occurred in the number of pustules

TABLE 3. Effect of *Bacillus subtilis* APPL-1 on rust development on five bean cultivars[a]

Bean cultivar	Uredial pustules/leaf		
	B. subtilis-treated (mean)	Nontreated (mean)	Control[b] (%)
Lake Shasta	9 ± 2.9	231 ± 24.3	96
Mount Hood	12 ± 4.4	317 ± 29.4	96
Pinto 111	8 ± 2.9	346 ± 19.9	98
Mountaineer White Half Runner	8 ± 2.1	322 ± 27.6	98
BBL 47	3 ± 0.6	159 ± 27.9	98

[a]The pustules on primary leaves of nine plants were counted and averaged for each treatment and control. Rust collection 75-22, compatible with all cultivars tested, was used for these inoculations.

[b]Percent control or reduction in number of pustules as compared to the number on untreated plants. An *F*-test indicates no difference in the percentages of inhibition ($P = 0.05$ or 0.01).

TABLE 4. Effect of *Bacillus subtilis* APPL-1 on symptom development on bean cultivar Lake Shasta inoculated with five pathogenically unique collections of *Uromyces phaseoli*

Collection	Average uredia per leaf		
	Treated (mean)	Control (mean)	Control[b] (%)
79-4	1.6 ± 0.5	39.1 ± 14.5	96
73-23[a]	9.7 ± 0.37	14.5 ± 1.1	95
73-16	0.5 ± 0.2	14.7 ± 4.0	97
75-22	0.6 ± 0.19	26.8 ± 5.5	98
73-32	0.4 ± 0.21	12.0 ± 2.7	97

[a]Collection 73-23 causes necrotic spots without sporulation on cultivar Lake Shasta; therefore, necrotic spots were counted rather than uredia.

[b]Percent control or reduction in the number of pustules as compared to the number on untreated plants. An *F*-test indicates no significant difference between these figures either at $P = 0.05$ or 0.01.

produced by collection 75-22 on all five tested cultivars (Table 3). There was no statistical difference in the effect among cultivars or among five pathotypes of *U. phaseoli* (Table 4) analyzed according to a one-way analysis of variance. Pretreatment of Lake Shasta prior to inoculation with collection 73-23 reduced the number of necrotic spots as much as it reduced the number of pustules produced by collections 79-4, 73-16, 75-22, and 73-32. Ten additional collections of *U. phaseoli* that contained pathotypes different from those listed in Table 4 were also tested. The *B. subtilis* pretreatment had an identical inhibitory effect on development of either the necrotic or pustule reaction, whichever was characteristic of the appropriate host-pathotype combination. *B. subtilis* pretreatment was also effective against isolates of *U. phaseoli* obtained from the occasional pustules that develop on the

pretreated plants.

Histological observations. Leaves from treated and untreated bean plants inoculated 18 hr earlier were examined with a light microscope after being stained briefly in alcoholic lactophenol cotton blue. Leaf sections treated with *B. subtilis* had many nongerminated urediospores floating in the mounting solution. Cytoplasm had ballooned out of about 30% of the spores that remained on the leaf surface. No normal germ tubes were observed on leaves treated with *B. subtilis*. More than twice as many spores remained on the surface of the nontreated leaves as on the treated leaves. Nearly 100% of the spores on the nontreated leaves had developed germ tubes 5–20 spore diameters in length during the overnight incubation.

In vitro studies and partial characterization of inhibitor. Bacterial growth on the culture medium made from the dialyzable fraction of Eugon broth was similar to that of unmodified Eugon broth. Cultures, 24- to 48-hr-old, were highly inhibitory to spore germination. When cultures were subjected to centrifugation to remove bacterial cells, the rust-inhibiting activity remained in the supernatant and could withstand autoclaving for 10 min. When subjected to dialysis, the inhibitor of both rust and spore germination was retained by the dialysis tubing. This second dialysis removed the remainder of the components in the original Eugon broth. Dialyzed culture filtrate retained inhibitory activity after lyophilization and being dissolved in distilled water. Fractions (5 ml) for gel filtration of this reconstituted aqueous phase that were assayed for protein and carbohydrate gave the elution profile shown in Fig. 2. Inhibitory activity was strongest in fraction numbers 50 to 60. When analyzed, these fractions, which had an apparent molecular weight of 5–10 Kdaltons, contained 95% protein and 5% carbohydrate.

DISCUSSION

The results from this study demonstrate that a product (and possibly more than one product) from *B. subtilis* inhibits *U. phaseoli* from causing rust on beans. The inhibitory effect of *B. subtilis* on rust development appeared to be due to its effect on urediospore germination. The in vivo histological observations, in vitro spore germination studies, and inability of *B. subtilis* to inhibit rust development after the urediospores had germinated all support this conclusion. The inhibitory effect persists on bean leaves for at least 4–5 days under greenhouse and cold frame conditions. Results from purification studies indicate that the bacterial component responsible for these results is heat stable with an apparent molecular weight of ~5–10 Kdaltons. The crude fractions containing the component are composed of about 95% protein and 5% carbohydrate.

There are numerous studies of the use of antagonistic microorganisms to control plant disease (1). Most of these studies deal with microbial interactions in the soil. Few workers have attempted to manipulate populations of microorganisms on the leaf surface (2,11). Successful antagonists and/or their antagonistic or inhibitory metabolites introduced into the leaf environment must be able to withstand the rapidly changing conditions on the leaf surface, including daily weather fluctuations, other microflora, and plant metabolites. In this regard, *B. subtilis* would seem to be a good candidate as a successful antagonist. *B. subtilis* is a spore-forming bacterium that seems to survive well, at least in the soil (10), and it is well documented for its production of numerous antibiotics (7,9).

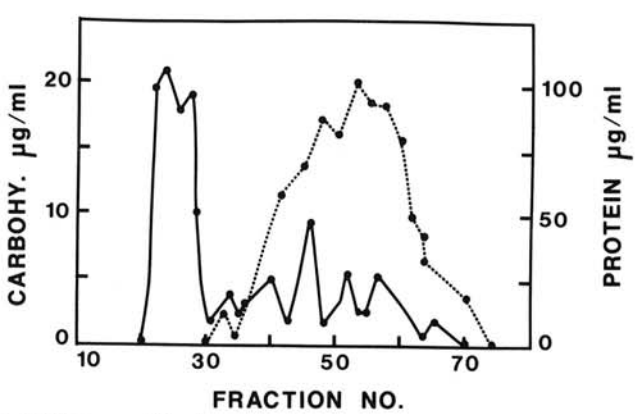

Fig. 2. Elution profile of a culture filtrate of *Bacillus subtilis* from a Bio-gel P-10 column (2.5 × 90 cm) equilibrated with 0.1 M tris buffer, pH 7.0. Fractions were collected and assayed for carbohydrate (•——•) and protein (•----•).

There have been several studies of leaf diseases that relate to the one reported here. Fravel and Spurr (4) found that *B. cereus* subsp. *mycoides* controlled brown spot, caused by *Alternaria alternata*, on tobacco leaves. Although the mode of action was not completely determined, it was suggested that there was involvement of an inhibitory metabolite or a toxic substance that suppressed conidial germination. Morgan (13) showed that cereal rust could be controlled by spray treatment of wheat and oats with *B. pumilus*. The mechanism of inhibition was not determined, but germ tube lysis was shown not to be involved. The active factor from *B. pumilus* was heat labile, but the inhibitor from *B. subtilis* reported here is not heat labile. Another method of biological rust control was reported by Spencer (15). Production of uredial pustules by *U. dianthi* on carnations was reduced by 40–60% when plants were sprayed with a conidial suspension of mycoparasitic *Verticillium lecanii*, which had no effect on spore germination and caused no lysis of germ tubes.

The success of *B. subtilis*, or any other antibiotic-producing antagonist, as a biological control agent depends on three criteria. First, the antagonist must both survive well and produce the antibiotic under the given conditions. Next, the antagonist must produce sufficient concentrations of inhibitor to affect the pathogen. This requires that the rate of production must overpower any inactivation or degradation of the organism and the antibiotic by the plant, other microflora, or the environment. Finally, the pathogen must remain susceptible to the effects of the antibiotic under given conditions. The last criterion is by far the most significant, for, although the first two requirements can be either modified or overridden by direct application of the antibiotic, the entire system becomes ineffective when the latter criterion is not met. The above criteria are apparently met in the greenhouse and cold frame, and preliminary results suggest that they are also met under field conditions. The further purification of the *B. subtilis* (isolate APPL-1) inhibitor will be necessary before we can determine the importance of each of these criteria.

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