Biocontrol of Tobacco Brown-Spot Disease by Bacillus cereus subsp. mycoides in a Controlled Environment

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ABSTRACT

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Sixteen bacterial isolates, primarily from tobacco leaf surfaces, were screened in vitro for effects on spore germination and germ tube development of three *Alternaria alternata* isolates. Most bacterial isolates suppressed number of germ tubes, and the length and branching of the longest germ tube. Appressorium formation data were inconclusive, but tended toward inhibition. Bacterial isolates which were most inhibitory to growth of *A. alternata* in vitro also were most inhibitory to that fungus on the leaf surface. Using this in vitro system, five bacterial isolates were selected for foliar biocontrol tests. A *Pseudomonas maltophilia* isolate did not alter lesion severity. Two bacterial isolates reduced lesion severity significantly. One isolate, *Bacillus cereus* subsp. *mycoides,* effectively controlled tobacco brown-spot lesion development in a controlled environment. Microscopic observations of the leaf surface showed that conidial germination was 10% in the presence of *B. cereus* subsp. *mycoides,* whereas, 98% germinated in the control and produced brown-spot symptoms.

Biocontrol of foliar fungal pathogens has received less attention than biocontrol of soilborne pathogens (1, 11). Both bacteria and fungi have been applied to foliage as biocontrol agents (1, 2, 4, 8, 10, 12, 13). Several examples of bacterial control of foliar fungal pathogens have been reported. Lesions caused by *Helminthosporium sativum* were less severe on wheat and barley when *Bacillus pumilus* was applied to leaves as a protectant (8). Two *Bacillus* spp., which originally were isolated from lysed germ tubes, inhibited infection by three *Puccinia* spp. on wheat and oats (15). Culture filtrates of these bacteria which were incorporated in water agar lysed uredospore germ tubes.

Application of nonpathogenic fungi to leaf surfaces was employed to reduce severity of diseases caused by *Alternaria* on different hosts (5, 9, 17). Brown-spot control resulted when nonpathogenic *Alternaria* conidia were applied as a protectant to tobacco (*Nicotiana tabacum* L.) leaves prior to inoculation with the pathogen, *A. alternata,* in both laboratory and field experiments (17). Bacteria observed in association with the nonpathogenic fungus at the time of hyphal dissolution on tobacco leaf surface indicated that the bacteria may have caused hyphal disintegration of the protectant organism, thereby altering its protective activity. These bacteria were isolated and found to modify the brown spot lesion-reducing interaction (6, 7). The purpose of this research was to examine the effects of these bacteria on germination and growth of *Alternaria* conidia and to select bacterial isolates as biocontrol agents for brown spot.

MATERIALS AND METHODS

Bacterial and conidial suspensions for all experiments were prepared in the same manner. Bacterial isolates were obtained primarily from water washings of greenhouse- and field-grown tobacco leaves (6). Bacterial suspensions were prepared from 24-hr cultures grown on nutrient agar at room temperature and were standardized to approximately 10^9 cells/ml. Bacterial suspensions then were mixed with equal volumes of aqueous conidial suspensions or water.

Conidial suspensions of *Alternaria* isolates were prepared from 7- to 10-day cultures grown on V-8 agar at 21 C under fluorescent light (16). Conidia were suspended in cold (6 C), distilled water to retard germination, and filtered through cheesecloth to eliminate hyphal fragments. Spore concentration was adjusted to 20,000 conidia/ml and immediately mixed with an equal volume of bacterial suspension or with water, resulting in a final concentration of 10,000 conidia/ml.

In vitro screening.—Acid-cleaned depression slides

were sterilized inside petri dishes containing moistened filter paper. Sixteen bacterial isolates were tested against three *Alternaria* isolates: Isolate A5, the brown-spot pathogen *A. alternata;* F646, a nonpathogenic *A. alternata* isolated from tobacco and used to control brown spot (17); and AT, an unidentified *Alternaria* sp. isolated from a tomato early blight lesion (6). A 0.01 ml drop of each bacterial suspension or sterile water was mixed with an equal volume of each conidial suspension in the well of the depression slide. After 24 hr, conidia and germ tubes were stained with cotton blue in lactophenol. Ten spores were examined in each depression slide. Each conidium was treated as a replicate and the experiment was repeated three times with each bacterial and fungal isolate. This experiment also was performed using five bacterial isolates at dilutions of 0 to 10^{-7}.

In vivo biocontrol.—Biocontrol of the pathogen on the leaf surface was studied using five bacterial isolates. A previously described method for producing brown spot on 9-cm diameter leaf disks cut from greenhouse-grown Coker 298 tobacco plants was used (16). Disks were washed with 70% ethanol and rinsed with sterile water to reduce natural populations of microflora (18). Inverted leaf disks were placed on trays over water in plastic boxes and inoculated by placing 12 drops of A5 + bacteria, or A5 + water on the abaxial surface. Inoculation drops were dried for 3 hr and the boxes closed and incubated at 21 C with 8 hr of illumination. Lesion development was evaluated after 7 days. The five bacterial isolates tested were B7, B18, B23, B24, and B31. Microscopic observations of B23 + A5 were made at the time of inoculation, and 1, 2, 3, and 7 days after inoculation.

Bacterial filtrates.—Tests to determine the presence of soluble bacterial metabolites responsible for the observed effects on fungal germination and growth were conducted. Suspensions of B23 were filtered through a 0.22-μm (pore size) filter and the filtrate was combined with an equal volume of A5 conidia in depression slides and incubated as in the in vitro screen test experiment.

TABLE 1. Effects of 16 bacterial isolates on germination of *Alternaria alternata* isolate A5 in vitro after 24 hr of incubation[a]

Isolate	Germ tube length (mm)	Germ tubes/ conidium (no.)	Branches longest germ tube (no.)	Appressoria/ conidium (no.)
Control	0.21	2.43	1.00	0.33
B6	0.11*[b]	1.73	0.57	0.07*
B7	0.11*	1.33*	0.17*	0.00*
B8	0.12*	1.33*	0.10*	0.00*
B9	0.26	2.13	0.73	0.00*
B11	0.16	2.16	0.63	0.23
B12	0.08*	1.40*	0.10*	0.00*
B16	0.15	1.70*	0.07*	0.07*
B18	0.55*	2.50	1.23*	0.16*
B23	0.07*	1.13*	0.23*	0.13*
B24	0.10*	1.77*	0.67	0.03*
B25	0.12*	1.46*	0.47*	0.07*
B26	0.25	1.60*	0.80	0.00*
B28	0.14*	1.86*	0.43*	0.00*
B30	0.09*	1.16*	0.17*	0.03*
B31	0.06*	1.03*	0.13*	0.03*
B32	0.21	2.00*	0.93	0.07*
LSD 0.05	0.06	0.41	0.51	0.14

[a]Figures represent the mean of 30 replicates from three trials.
[b]Asterisks (*) indicate that figure is significantly different from the control ($P = 0.05$) as calculated by the method of least significant difference.

RESULTS

In vitro screening.—Bacterial isolates had different effects on fungal germination and growth, but a bacterial isolate which inhibited one *Alternaria* isolate generally inhibited the other two *Alternaria* isolates being tested (6). Most bacterial isolates inhibited number of germ tubes, germ tube length, and branching of the longest germ tube (Table 1). Data on appressorium formation by F646 and AT were too variable to be definitive, but indicated that most bacterial isolates were inhibitory. When all values were expressed as a percentage of the control, F646 was found to be more susceptible to inhibition by all bacteria than A5 or AT. Generally, number of germ tubes, germ-tube length, branching on longest germ tube, and number of appressoria were simultaneously inhibited. The three *Alternaria* isolates were inhibited most frequently by B7, B23, and B31. Isolates B7 and B23 have been identified as *Pseudomonas maltophilia* and *Bacillus cereus* subsp. *mycoides,* respectively, by the American Type Culture Collection, Rockville, MD 20852. In some instances B18 and B24 stimulated *Alternaria* growth.

A dosage-response relationship was determined for B7 and B23 with respect to inhibition of germ-tube length, but was not observed with B18, B24, or B31. At four concentrations ranging from 5.0×10^8 through 5.0×10^7 of B23, a dosage-response was noted for the number of germ tubes. With this exception, germ-tube number, branch number, and appressorium formation did not show a dosage-response. The ED_{50}, calculated for germ-tube length, of B7 and B23 were 1.1×10^9 and 2.5×10^7 bacteria/ml, respectively.

In vivo biocontrol.—Application of bacteria and the pathogen to the leaf surface confirmed that bacteria which inhibited germination and growth of the pathogen in vitro did the same on the leaf surface and that biocontrol of tobacco brown-spot lesions by bacteria was possible in a controlled environment. Bacterial isolates

TABLE 2. Biocontrol of brown-spot disease on tobacco leaf disks by five bacterial isolates in a controlled environment

Treatment[a]	Disease index[b]
Control	4.08 A[c]
A5 + B7	3.93 AB
A5 + B18	4.02 A
A5 + B23	1.75 C
A5 + B24	3.71 B
A5 + B31	3.87 AB

[a]The pathogenic *Alternaria alternata* isolate employed was A5. Bacterial isolates used were B7, B18, B23, B24, and B31.
[b]Maximum lesion development was rated 5 and no visible symptom was rated 1. Figures represent the mean of 36 replicates from three trials.
[c]Averages of three replicates, any two numbers followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

varied in degree of lesion suppression. Isolate B23, the most inhibitory to fungal growth in vitro, gave the most disease control (Table 2). Those that were less inhibitory (B7, B31) consistently reduced lesion development, but not significantly. Bacterial isolates B18 and B24, which stimulated *Alternaria* germination and growth in some instances, differed from each other in vivo. Lesion severity was reduced by B24, but B18 was ineffective. Microscopic observations of B23 + A5 showed that fewer conidia germinated on the leaf surface than in vitro. Less than 10% germination was observed on the leaf surface in the presence of B23 whereas, there was an average of 60% germination in vitro.

Bacterial filtrates.—When bacterial filtrates rather than cells were used in depression slides, no differences from the check were observed for either B7 or B23.

DISCUSSION

Biocontrol of brown spot resulted when B23 was inoculated simultaneously with the pathogen in a controlled environment. Selection of this bacterial isolate as a biocontrol agent was facilitated by the use of the in vitro screen test. *Bacillus cereus* subsp. *mycoides* has also been used to control needle rust, *Melampsora medusae* Thum., on *Pseudotsuga menziesii* (Mirb.) Franco. Previous reports of bacterial-fungal interactions indicated lysis as a probable cause of fungal inhibition (1, 2, 4, 5, 15). In this study, bacterial suppression of germ-tube growth was observed rather than lysis. Hence, different mechanisms are involved and the present mechanism could involve a toxic substance(s) rather than a lysing agent. Contrary to previous reports (14, 15), bacterial filtrates did not affect germination, growth or infection by the pathogen. Still it seems probable that B23 produces an inhibitory metabolite because conidia failed to germinate in its presence. Competition for a germination factor(s) could also induce this response, but this mechanism seems unlikely because A5 conidia germinate readily in distilled water. Further work will be necessary to determine the mode-of-action of these bacteria.

Naturally occurring leaf surface microflora may interfere with biocontrol experiments. The treatment of leaf surfaces with ethanol reduced this interference and resulted in more disease following inoculation with pathogenic spores (18). This facilitated the in vivo assessment of biocontrol microorganisms. Agricultural chemicals may have similar effects (1, 3) and they should be studied for integration with biocontrol. Also, foliar protection may result from applications of selected microorganisms to seeds as recently described for soybeans (12).

Successful biocontrol in a regulated environment encourages research into field applications. Practical field control will depend on having adequate populations of the control agent present at appropriate times. *Bacillus cereus* subsp. *mycoides* was effective at concentrations 100-fold less than those of *P. maltophilia* in vitro; thus it was easier to reach an effective concentration of B23. Also, fewer bacteria (B23) were necessary to retard germination and growth of the pathogen on the leaf surface than in vitro. Hence, the ED_{50} for disease control may be less than for in vitro inhibition. The relationships between the application of protective microorganisms and their growth and survival in effective numbers on leaf surfaces under field conditions remains a primary challenge to be resolved.

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