Biological Control of Pythium Seed Rot and Preemergence Damping-Off of Cotton with *Enterobacter cloacae* and *Erwinia herbicola* Applied as Seed Treatments

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ABSTRACT

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Thirteen strains of *Enterobacter cloacae* and *Erwinia herbicola* were evaluated as biological seed treatments on cotton (Acala SJ-2). All strains reduced the incidence of Pythium seed rot and preemergence damping-off in naturally infested soil. Although three of four strains were as effective as metalaxyl when tested at 25 C, all strains of both bacteria were less effective at 15 C. At 35 C, little disease development was observed in any treatment. Bacterial strains suppressed colonization of germinating seeds by *Pythium* spp. at 15, 25, and 35 C. Control of Pythium seed rot and preemergence damping-off by *E. cloacae* and *E. herbicola* strains was correlated with suppression of seed colonization by *Pythium* spp. during the first 24 hr of seed germination.

Pythium species are major limiting factors to cotton seedling stands, particularly in the northern areas of the cotton belt and California's San Joaquin Valley (2,16,18). Although current controls include the use of seed treatment and in-furrow fungicides, these can be ineffective under conditions of high pathogen inoculum and favorable environment (4).

Recently, the possibilities of biological control of cotton seedling diseases have been investigated (3,7,10–12). Cotton seeds and seedlings can be protected effectively from *Pythium* damage by treating seeds with *Pseudomonas fluorescens* (Trevisan) Migula (12,14,22), *Gliocladium virens* Miller, Gidden, & Foster (10), or the antibiotics they produce (10,12,13,22). Among the bacteria colonizing cotton roots, some genera of enteric bacteria may be effective in reducing Pythium damping-off when applied as seed treatments (7).

The enteric bacterium *Enterobacter cloacae* (Jordan) Hormaeche & Edwards has been identified as an effective biological control agent against seed rots and damping-off caused by *Pythium* spp. (6,25). *E. cloacae* and related strains of *E. herbicola* (Lohnis) Dye are also effective in controlling Fusarium wilt of cucumber (27). The purpose of this study was to evaluate the efficacy of *E. cloacae* and *E. herbicola* as biological seed treatments for control of Pythium seed rot and damping-off of cotton and to determine the influence of temperature on biological control activity.

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MATERIALS AND METHODS

Growth and maintenance of bacterial strains. *Enterobacter cloacae* strains NRRL B-14095 (ATCC 39978) and NRRL B-14096 were isolated from cucumber (*Cucumis sativus* L.) seeds and have been described previously (6). Strains EcH-1 and EcCT-501 were isolated from cotton hypocotyls. Strain EcH-1 was provided by C. R. Howell, National Cotton Pathology Laboratory, USDA-ARS. Strains 0295, 0296, and 0297 were donated by S. V. Beer, Cornell University. *Erwinia herbicola* strain 9S2 was isolated from cucumber seeds. All other *E. herbicola* strains were donated by S. V. Beer, Cornell University.

Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) (50 ml/250-ml flask) was used to grow inoculum of *E. cloacae* and *E. herbicola* used to treat seeds. Cells were grown to mid to late log phase at 30 C and removed from trypticase soy broth by centrifugation (10,000 *g* for 10 min at 4 C). Before use, cells were washed by resuspending them in a saline solution (0.85% NaCl, w/v) and removing them by centrifugation. Cultures were maintained on yeast-dextrose-calcium carbonate agar slants as described previously (25).

Seed treatments and seedling assays. *Enterobacter cloacae* and *Erwinia herbicola* were applied to seeds of cotton (*Gossypium hirsutum* L. 'Acala SJ-2') by procedures described previously (25). The rate applied was 2 ml of suspension (10^9–10^{10} colony-forming units [cfu] per milliliter) per 25 seeds. Populations of *E. cloacae* and *E. herbicola* recovered from air-dried seeds were quite variable and ranged from 10^6 to 10^9 cfu/seed. Metalaxyl (Apron 25WP) was applied to seeds at the rate of 158 μg a.i./g seed in a 1.5% Methocel A4C (Dow Chemical Co., Midland, MI) suspension. Seeds were planted (five seeds per box; five replicates) as described previously (25) in a silt loam soil (pH 6.4) naturally infested with *Pythium* species (primarily *P. ultimum*) (26). Boxes were incubated for 5 days at 15, 25, or 35 C, then placed in a growth chamber at 25 C. Seedling stands from 35 and 25 C treatments were evaluated 5 and 7 days after planting, respectively. Seedling stands from 15 C treatments were evaluated 14 days after planting. Before each experiment, soil populations of *Pythium* spp. were determined with a selective medium (23).

Seed colonization assays. The effects of *Enterobacter cloacae* and *Erwinia herbicola* on colonization of cotton seeds by *Pythium* spp. were determined by planting 10 treated seeds in petri plates (five replicates) filled with the *Pythium*-infested soil described previously. Plates were incubated at 15, 25, or 35 C. Seeds were removed at 3- or 6-hr intervals over a 24- to 48-hr period, rinsed thoroughly with tap water to remove adhering soil particles, and plated on a *Pythium*-selective medium (23). In some experiments, a duplicate sample of seeds was transplanted after various periods of time from *Pythium*-infested soil into sterile quartz sand and incubated at 25 C. Seedling stands were then determined 7 days after transplanting. Untreated and metalaxyl-treated seeds served as controls. Characteristic growth of *Pythium* spp. was evident from seeds after 16–24 hr of incubation on the selective medium at 25 C. In preliminary experiments, colonies developing on the selective medium were grown in pure culture and identified as *P. ultimum*.

All experiments were repeated at least once. Results were analyzed using analysis of variance and means were separated with Duncan's multiple range test and the LSD test.

RESULTS

Suppression of preemergence damping-off. All strains of *Enterobacter cloacae* and *Erwinia herbicola* tested were effective in reducing Pythium seed rot and preemergence damping-off (Table 1). The protection provided by most strains was as good as or better than that provided by metalaxyl.

At 35 C, little or no damping-off was apparent among any of the treatments after 7 days and seedling stands ranged from 84 to 100% (Table 2). At 25 C, however, *E. cloacae* strains ATCC 39978 and EcCT-501 and *E. herbicola* strain 240 were as effective as metalaxyl. Although *E. cloacae* strain EcH-1 was not as effective as metalaxyl, seedling stands from EcH-1-treated seeds were significantly greater than those from the control seeds.

The ability of *E. cloacae* strains and *E. herbicola* strain 240 to protect against *Pythium* was greatly reduced at 15 C. None of the bacterial seed treatments were as effective as metalaxyl. However, *E. cloacae* strain EcCT-501 and *E. herbicola* strain 240 gave rise to significantly better seedling stands at 15 C than untreated or Methocel-treated seeds.

Seed colonization by *Pythium* spp. Colonization of untreated cotton seeds by *Pythium* spp. was dramatically affected by temperature (Fig. 1). One hundred percent of the seeds recovered from *Pythium*-infested soil incubated at 15 and 25 C were colonized by *Pythium* spp. within 24 and 12 hr, respectively, and remained at that level for at least 48 hr. Maximum colonization of cotton seeds incubated at 35 C in *Pythium*-infested soil was observed 12 hr after planting. Percentage of seed colonized declined thereafter to levels not different ($P = 0.05$) from uncolonized seeds.

The level of *Pythium* colonization of cotton seeds occurring within 24 hr was directly related to final seedling stands 7 days after planting. Percentages of seeds colonized by *Pythium* spp. after 24 hr at 15, 25, or 35 C were 92, 40, and 8, respectively. When these seeds were transplanted into sterile quartz sand, 7-day seedling stands were 4, 24, and 88%, respectively. Seeds not colonized by *Pythium* species gave rise to 92–100% seedling stands after 7 days.

Suppression of *Pythium* seed colonization by *Enterobacter cloacae* and *Erwinia herbicola*. Biological control activity of *E. cloacae* and *E. herbicola* as determined by suppression of seed colonization by *Pythium* species was evident within 24 hr of planting (Table 3). Levels of suppression of *Pythium* seed colonization by *E. cloacae* strains and *E. herbicola* strain 240 did not differ from metalaxyl treatments at 15 C. At 25 C, however, only *E. cloacae* strain EcH-1 was effective in suppressing seed colonization to levels equivalent to metalaxyl treatment. At 35 C, no significant ($P = 0.05$) colonization of cotton seeds by *Pythium* was observed among any of the treatments after 24 hr.

DISCUSSION

Enterobacter cloacae and *Erwinia herbicola* were effective biological control agents for Pythium seed rot and preemergence damping-off of cotton under growth chamber conditions. Similar results have been obtained where strains of these bacteria were applied as seed treatments on other plant species (6,25; E. B. Nelson, *unpublished*). For example, *E. cloacae* protects cucumber, table beet, rye, and pea from Pythium damping-off (6,25), whereas certain strains of *E. herbicola* are effective against Pythium damping-off of cucumber (E. B. Nelson, *unpublished*).

Nelson et al (25) observed differences in performance of *E. cloacae* as a seed treatment when applied to seeds of different plant species. A strain of *E. cloacae* was not effective in protecting plants such as snap bean, soybean, lima bean, and corn, whose seeds rapidly release carbohydrates during germination. However, it remained highly effective on plant species such as cucumber and rye, whose seeds release very low levels of carbohydrates during germination. The absence of biological control activity in association with high-carbohydrate-exudation seeds has been attributed to the presence of sugars in the spermosphere that interfere with the ability of *E. cloacae* to attach to hyphae of *P. ultimum* and inhibit its growth before seed infection occurs. Under conditions where attachment to hyphae is eliminated, biocontrol activity is greatly reduced or eliminated.

At 25 C, cotton seeds release low levels of carbohydrates during the first 24 hr of germination (24; E. B. Nelson, *unpublished*). Consequently, exudate

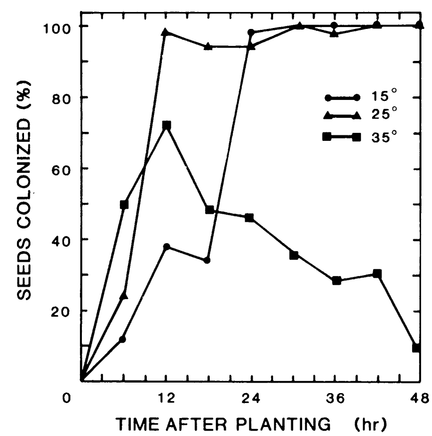

Fig. 1. Influence of temperature on the colonization of untreated cotton (Acala SJ-2) seeds by *Pythium* spp. Soil populations of *Pythium* spp. at time of planting were 323 cfu/g dry wt of soil. LSD (0.05) at 48 hr = 13.8.

Table 1. Effects of cotton seed treatments with *Enterobacter cloacae* and *Erwinia herbicola* on seedling stands

Seed treatment	Seedling stand[x] (%)
Untreated	11 d
Methocel adhesive	40 cd
Metalaxyl[y]	72 abc
E. cloacae strains[z]	
0295	88 ab
0296	84 ab
EcH-1	80 ab
EcCT-501	76 ab
ATCC 39978	72 abc
NRRL B-14096	64 abc
E. herbicola strains[z]	
240	96 a
112Y	80 ab
282	76 ab
242	72 abc
159	68 abc
181	60 abc
9S2	56 bc

[x]Determined 7 days after planting at 25 C. Numbers followed by the same letter are not significantly ($P = 0.05$) different according to Duncan's multiple range test.
[y]Applied at 158 μg a.i./g seed.
[z]Applied to seeds in a 1.5% suspension of Methocel A4C; recoverable populations of bacteria from treated seed were 10^8–10^9 cfu/seed.

Table 2. Effects of temperature on biological control of Pythium seed rot and preemergence damping-off by strains of *Enterobacter cloacae* and *Erwinia herbicola*

Seed treatment	Seedling stand[x] (%) 15 C	25 C	35 C
Untreated	18 d	8 d	84 b
Methocel adhesive	20 d	24 cd	88 b
Metalaxyl[y]	92 a	92 a	100 a
E. cloacae strains[z]			
Ech-1	30 cd	56 bc	96 ab
EcCT-501	42 bc	76 ab	92 ab
ATCC 39978	34 bcd	72 ab	100 a
E. herbicola strain[z]			
240	60 b	76 ab	100 a

[x]Determined 5, 7, and 14 days after planting at 35, 25, and 15 C, respectively. Numbers in each column followed by the same letter are not significantly ($P = 0.05$) different according to Duncan's multiple range test.
[y]Applied at 158 μg a.i./g seed.
[z]Recoverable populations of bacteria from treated seeds were 10^7–10^8 cfu/seed.

Table 3. Influence of temperature on suppression of *Pythium* colonization of cotton seeds by strains of *Enterobacter cloacae* and *Erwinia herbicola*

Seed treatment	Seeds colonized[x] (%) 15 C	25 C
None	94 b	100 a
Methocel adhesive	100 a	100 a
E. cloacae strains[y]		
EcH-1	4 c	4 c
EcCT-501	0 c	38 b
ATCC 39978	4 c	40 b
E. herbicola strain[y]		
240	4 c	34 b
Metalaxyl[z]	0 c	2 c

[x]Determined 24 hr after planting seeds in *Pythium*-infested soil. Soil populations of *Pythium* spp. were 200 cfu/g dry wt. Numbers in each column followed by the same letter are not significantly ($P = 0.05$) different according to Duncan's multiple range test.
[y]Recoverable populations of bacteria from treated seeds were 10^8–10^9 cfu/seed.
[z]Applied at 158 μg a.i./g seed.

sugars may not be present at levels that would interfere with attachment to hyphae and inhibition of growth of *P. ultimum* at that temperature. However, seeds germinating at lower temperatures can release increased amounts of carbohydrates (9). For example, cotton seeds germinating at 12 C release four times more glucose equivalents per seed within 48 hr than at 24 C (9). The ability of *E. cloacae* and *E. herbicola* to suppress seed colonization by *Pythium* spp. and suppress seed rot at 15 C is evidence that these strains are active despite increased levels of carbohydrate exudation. Therefore, either sugars are not released at a time or level sufficient to interfere with attachment of bacterial strains to hyphae or other mechanisms of suppression are operative in the expression of biocontrol activity. For example, *E. cloacae* can reduce the levels of ethanol released from germinating seeds (5). Ethanol is an effective stimulant of sporangium germination in the apparent absence of other stimuli (24) and reductions in ethanol production may reduce or delay sporangium germination of *Pythium* spp. and thus delay seed colonization.

Pythium spp. respond rapidly to germinating cotton seeds. Sporangia are capable of germinating 1–2 hr after exposure to an imbibing seed (24). Therefore, bacteria must be active as soon after planting as possible to prevent or reduce seed infection. The ability of *E. cloacae* to protect seeds and seedlings against Pythium damping-off has been related to its ability to suppress colonization of seeds by *Pythium* spp. within the first 24 hr of germination (25). Suppression of *Pythium* colonization of germinating cucumber seeds by *E. cloacae* within 12 hr of planting is sufficient to effectively protect seedlings from Pythium damping-off at 25 C. Both *E. cloacae* and *E. herbicola* were effective in suppressing colonization of cotton seeds by *Pythium* spp. at 15 and 25 C, and this was related to increases in seedling stands. *E. cloacae* strain EcCT-501 was capable of suppressing seed colonization by *Pythium* spp. as early as 6 hr after planting at 25 C and held colonization to levels similar to those observed with untreated seeds germinating at 35 C. Therefore, activity of *E. cloacae* or *E. herbicola* resulting in reductions in seed colonization by *Pythium* spp. during initial stages of germination are apparently most important in the control of Pythium seed rot and preemergence damping-off.

Temperature has rarely been considered as a factor affecting the performance of biological seed treatments. Harman et al (8) observed that *Trichoderma hamatum* applied to pea seeds was not effective in controlling Pythium damping-off at temperatures below 17 or above 34 C. Likewise, strains of *T. harzianum* and *T. koningii* were not as effective at 19 as at 26 C (21). Control of Pythium damping-off of cotton is particularly important at low temperatures (1,15,19), because cotton, a high-temperature crop, is more susceptible to low-temperature damage than cool-temperature crops like pea (20). This is reflected in the lower disease incidence at 35 than at 15 and 25 C. Although *E. cloacae* and *E. herbicola* suppressed *Pythium* damage at temperatures as low as 15 C, the level was not as great as at 25 C. Reduced biological control activity at 15 C may simply result from reduced growth and survival in the spermosphere or the inability of *E. cloacae* and *E. herbicola* to colonize emerging radicals and developing hypocotyls as quickly or as efficiently as *Pythium* spp. Perhaps, the development of strains more adapted to cooler temperatures may provide control as effective as metalaxyl at low temperatures when applied as seed treatments. Attempts to isolate cold-tolerant strains of biocontrol agents from cotton soils have been reported (17).

In this study, considerable variation was observed in the performance of *E. cloacae* and *E. herbicola* as seed treatments on cotton. Whereas recoverable seed populations varied from 10^6 to 10^9 cfu/seed, these appear to be within the range adequate for suppression of *Pythium*. In previous studies, populations of *E. cloacae* as low as 10^6–10^7 cfu/seed were effective in suppressing Pythium seed rot of cucumber (6,25). Although soil populations of *Pythium* spp. were fairly uniform from experiment to experiment, variations in populations may account for some of the observed variability. In addition, factors influencing both the inoculum efficiency of *Pythium* spp. and the biological control efficiency of antagonist strains may be responsible for this variation in performance.

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