

Seed Treatment with a Fungal or a Bacterial Antagonist for Reducing Corn Damping-off Caused by Species of *Pythium* and *Fusarium*

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ABSTRACT

Mao, W., Lewis, J. A., Hebbar, P. K., and Lumsden, R. D. 1997. Seed treatment with a fungal or a bacterial antagonist for reducing corn damping-off caused by species of *Pythium* and *Fusarium*. Plant Dis. 81:450-454.

Bioassays were conducted under greenhouse conditions to test the efficacy of antagonists applied to corn (*Zea mays*) seed for protection against seed rot and seedling damping-off at 18 and 25°C in a field soil artificially infested with a combination of *Pythium ultimum*, *P. arrhenomanes*, and *Fusarium graminearum*. Biomass of *Gliocladium virens* isolates Gl-3 or Gl-21, *Trichoderma viride* isolate Tv-1, or peat-based slurry of *Burkholderia cepacia* isolates Bc-B, Bc-T, or Bc-1 was coated individually onto corn seeds in one test, and Gl-3 or Bc-B at four inoculum levels was used in another test. Seed treatments with most of the biocontrol agents, as well as with the fungicide captan, significantly ($P \leq 0.05$) increased seedling stand, plant height and fresh weight, and decreased root rot severity compared with untreated seeds in pathogen-infested soil. Coating seeds with the biocontrol fungus *G. virens* isolate Gl-3 was the most effective treatment, resulting in greater ($P \leq 0.05$) seedling stand, plant height, and fresh weight, and lower ($P \leq 0.05$) severity of root rot than those parameters from seeds treated with captan or other antagonists at both temperatures. The results from the seeds treated with Gl-3 were similar to those of untreated seeds in noninfested soil. In treatments with Bc-1, Bc-T, Bc-B, or Tv-1, incubation temperature affected plant emergence, root rot severity, plant height, and fresh weight ($P \leq 0.01$). Conversely, in seeds coated with Gl-3 or Gl-21, these parameters were similar at both temperatures. The minimum number of propagules needed per corn seed to obtain plant emergence comparable to that from captan-treated seeds was between 10^4 and 10^5 CFU for Gl-3 and $>10^8$ for Bc-B. When propagules of Gl-3 were applied at a rate $>10^6$ CFU per seed, seedling emergence was greater ($P \leq 0.05$) than that from captan-treated seeds.

Currently, most commercial seeds of corn hybrids sold in the United States are treated with pesticides. One of the pesticides, the fungicide captan, has been used to enhance seed germination and seedling stand by reducing the severity of seed rot and root rot caused by various soilborne pathogens, including *Pythium* spp. and *Fusarium* spp. (1,17,25,26). Even with captan-treated seeds, up to 35% of corn seedlings may be affected in the midwestern areas of the United States (5,18).

Biological control using antagonistic microbes alone, or as supplements to minimize the use of chemical pesticides in a system of integrated plant disease management, has become more important in recent years (8,20). Beneficial microbes, including antagonistic bacteria and fungi, applied as seed treatments provide unique opportunities and benefits for crop protec-

tion, especially for protection against soilborne fungal pathogens (8). Considerable research has been done to investigate antagonistic microbes for use in seed treatments (1,5,11,16,17). Many methods of large-scale production of biocontrol agents (13,15,16,19) and delivery systems for applying these antagonists to seeds have been developed (7,14,16). For example, *Burkholderia cepacia* (syn. *Pseudomonas cepacia*) (27) and *Pseudomonas fluorescens* were applied directly to pea seeds to reduce damping-off and root rot caused by *Pythium* spp. and *Aphanomyces* spp., respectively (2,22). *Gliocladium virens* J. H. Miller, J. E. Giddens, & A. A. Foster was coated onto cotton seeds for protection against *Rhizoctonia solani* (12). *Pseudomonas fluorescens* was applied to corn seeds by a bio-priming method for reducing *Pythium* damping-off (5). A peat-based inoculum of *B. cepacia* was coated onto corn seeds for suppressing soilborne diseases of corn (10,11). Because these microbial strains have the ability to grow and proliferate on germinating seeds, and may eventually colonize the whole root system or rhizosphere, they are referred to as "rhizosphere competent" (8).

Despite these findings, seed treatment with antagonistic microbes, especially with fungal antagonists, to reduce damping-off

of field corn has been limited. In this paper, we report the potential of seed treatments with the biocontrol fungi *G. virens* isolates Gl-3 or Gl-21, and *Trichoderma viride* Rifai isolate Tv-1, and the biocontrol bacteria *B. cepacia* isolates Bc-B, Bc-T, or Bc-1 to reduce corn seed rot and seedling damping-off caused by a combination of the pathogens *Pythium ultimum* Trow, *Pythium arrhenomanes* Drechs., and *Fusarium graminearum* Schwabe. Emphasis of this research was placed on (i) the effectiveness of the antagonists in reducing disease severity, (ii) the minimum number of propagules needed to coat a corn seed to have an equal or superior efficacy to the fungicide captan, and (iii) the effect of temperature on antagonists, pathogens, and corn plants.

MATERIALS AND METHODS

Microbial cultures and soil. The biocontrol fungi used in these studies were *G. virens* isolates Gl-3 and Gl-21, and *T. viride* isolate Tv-1 from the collection of the Biocontrol of Plant Diseases Laboratory. The bacteria used were *B. cepacia* isolates Bc-B (provided by Stine Seed Company, Adel, IA), Bc-T (provided by T. Heulin, Centre National de la Recherche Scientifique, Nancy, France), and Bc-1 (isolated from a soybean root in Beltsville, MD). The bacteria were grown on nutrient agar (Difco, Detroit, MI) at 24°C and maintained at -80°C in glycerol. The pathogens *F. graminearum* and *Pythium arrhenomanes* were isolated from corn field soil in Beltsville, and the isolate of *Pythium ultimum* was provided by D. E. Mathre, Montana State University. These pathogen isolates were grown on potato dextrose agar (PDA) (Difco) at 24°C and maintained at -80°C in glycerol. The soil used was a loamy sand, pH 6.0 to 6.5, collected from a corn field in Beltsville.

Preparation of inocula. The three fungal antagonists were grown on molasses yeast medium (19) in liquid fermentation for 10 days. The biomass was filtered on muslin, air-dried overnight in a transfer hood, milled to pass a 425- μ m screen, and stored at 4°C. Biomass of the three antagonists consisted mostly of chlamydospores, and propagule counts were 10^6 CFU/g as determined by a semiselective medium (21). Bacterial cultures were grown in 250-ml Erlenmeyer flasks containing 100 ml of sterilized tryptic soy broth (TSB) (Difco). The flasks were placed on a rotary shaker

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for 48 h at $23 \pm 2^\circ\text{C}$. The cultures were then formulated with Nitrugin Sterile (NS) peat (LiphaTech, Milwaukee, WI) at a ratio of 1:1 (wt/vol) and maintained as a slurry at 4°C for no more than 7 days before use. The bacterial slurry contained 10^9 to 10^{10} CFU/g as determined on the selective medium *Pseudomonas cepacia* azelaic acid tryptamine (PCAT) (3).

Pythium spp. were grown on a sterilized semisolid medium consisting of 600 ml of Redi-Earth 3 CP (Grace-Sierra, Milpitas, CA), 330 ml of 60% V8 juice, 10 g of potato dextrose broth powder (Difco), and 0.6 g of CaCO_3 in foil-covered polypropylene flats ($12 \times 23 \times 45$ cm). The flats were autoclaved for 1 h on each of two successive days, inoculated with PDA plugs of the isolates, and incubated for 2 weeks at $23 \pm 2^\circ\text{C}$. The inocula contained approximately 10^5 CFU/g as determined by the most probable number (MPN) assay (9). *F. graminearum* was cultured on a semisolid wheat bran and water (1:1 wt/wt) medium for 10 days at $23 \pm 2^\circ\text{C}$. After incubation, the inoculum was air-dried for 3 days and milled in a blender to pass a 3.36-mm screen. The inoculum contained 10^4 to 10^5 CFU/g as determined by serial dilution on a peptone pentachloronitrobenzene (PCNB) medium (4).

Seed treatment. In the first series of tests, 10 g of corn seeds (N 6330, Northrup King Corporation, Golden Valley, MN) were mixed with 4.0 ml of an aqueous (12%) Pelgel (LiphaTech) solution as a sticker and 2.1 g of a slurry of each bacterial isolate. Ten grams of seeds were mixed with 6 ml of sticker and 1.2 g of biomass powder for each fungal isolate. The seeds, sticker, and biomass were mixed thoroughly; then the seeds were covered with sufficient dry NS peat to form an even, dry coating and stored at 4°C for no more than 5 days before planting. Seeds contained $>10^6$ CFU/seed for each fungal antagonist or $>10^8$ CFU/seed for each bacterium.

In the second test, seeds were coated the same way except that NS peat was used to dilute GI-3 biomass to obtain 10^5 , 10^4 , or 10^3 CFU/seed. For biomass of Bc-B, a slurry of NS peat and sterile TSB medium was used to dilute the original slurry to obtain 10^8 , 10^7 , or 10^6 CFU/seed. Seeds of N 6330 commercially pretreated with captan were provided by Northrup King.

Bioassays. Pathogen inocula were applied to soil at a rate of 3 g of *F. graminearum* and 5 g of each *Pythium* sp. per 150 g of soil. The infested soil and noninfested soil (control) were placed in 10-cm-diameter plastic pots and immediately planted with 3 seeds per pot of treated or nontreated corn seeds. Treatments for the first series of tests are shown in Table 1 and those for the second series in Figure 1. Both tests were repeated, and the experimental design was a randomized complete block with 10 replicate pots per treatment. The first series of tests was con-

ducted in greenhouse cubicles maintained at 18 or 25°C , and the second series at 18°C only. Pots were watered daily for 18 days; then seedlings were harvested to determine plant stand and root rot severity. A root rot severity index was used in which 1 = $<2\%$ (healthy plant), 2 = 3 to 30% (slight disease), 3 = 31 to 60% (moderate disease), 4 = 61 to 90% (severe disease), and 5 = $>91\%$ (dead plant). Plant height and fresh weight were evaluated (based on all planted seeds) only for the first series of tests. Values of 0 cm and 0.3 g were given for plant height and fresh weight, respectively, when the seeds did not germinate.

Statistical analyses. Statistical analyses were conducted using SAS Version 6.08 ed. (SAS Inc., Cary, NC) for all parameters in both tests, and for coating treatments

across temperatures and the interaction between temperature and coatings in the first series of tests only. Mean separation was accomplished using Duncan's Multiple Range Test, and all tests of significance were conducted at $P \leq 0.05$. Since the results from repeated experiments were similar, data from only one experiment are presented.

RESULTS

In general, the biocontrol agents had a beneficial effect when applied to corn seeds. Only 26 and 40% of nontreated seeds germinated at the low and high temperatures, respectively, in pathogen-infested soil compared with 87 and 90%, respectively, for that of untreated seeds in noninfested soil (noninfested control) (Fig.

Table 1. Effect of corn seed treatments on plant height and fresh weight of seedlings in soil infested with a combination of *Pythium ultimum*, *P. arrhenomanes*, and *Fusarium graminearum* at 18 and 25°C ^y

Treatments	Plant height (cm)		Fresh weight (g)	
	18°C	25°C	18°C	25°C
Untreated seed in pathogen-infested soil	2.9 d ^z	3.5 e	1.3 e	1.4 e
Untreated seed in noninfested soil	12.8 b	16.6 a	3.7 b	5.1 a
Captan	7.5 c	9.7 cd	2.7 d	3.1 cd
<i>Gliocladium virens</i> isolate GI-3	15.1 a	17.9 a	4.5 a	5.0 a
<i>G. virens</i> isolate GI-21	11.3 b	12.1 bc	3.2 c	3.6 c
<i>Trichoderma viride</i> isolate Tv-1	8.4 c	10.9 cd	2.7 c	3.5 c
<i>Burkholderia cepacia</i> isolate Bc-B	11.4 ab	15.0 a	2.9 c	4.2 bc
<i>B. cepacia</i> isolate Bc-T	6.1 c	8.9 d	1.5 e	2.7 d
<i>B. cepacia</i> isolate Bc-1	5.7 c	11.4 cd	1.9 de	3.6 de

^y Evaluations were made at harvest (18 days after planting).

^z Numbers in each column followed by the same letter are not significantly different from each other according to Duncan's Multiple Range Test ($P < 0.05$).

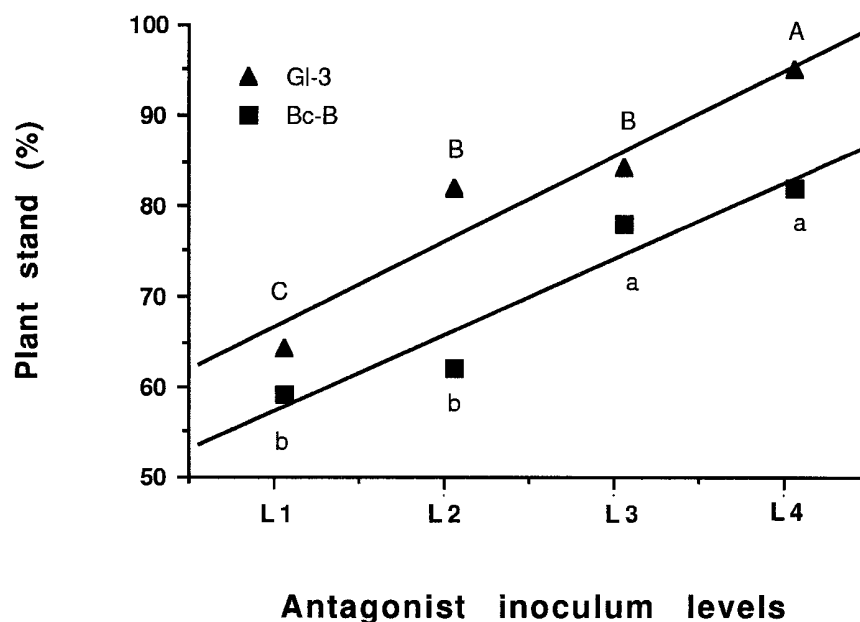


Fig. 1. Effect of inoculum level of corn seed treatment with isolates of *Gliocladium virens* or *Burkholderia cepacia* on plant stand in soil infested with a combination of *Pythium ultimum*, *P. arrhenomanes*, and *Fusarium graminearum* at 18°C . Inoculum levels from L1 to L4 for GI-3 were 10^3 , 10^4 , 10^5 , and 10^6 , and for Bc-B were 10^6 , 10^7 , 10^8 , and 10^9 . Y values were $\text{GI-3} = 54.0 + 10.5X$, $r^2 = 0.967$, and $\text{Bc-B} = 47.5 + 8.9X$, $r^2 = 0.956$. Evaluations were made at harvest (18 days after planting). Means with the same letter are not significantly different from each other according to Duncan's Multiple Range Test ($P < 0.05$).

2). At the low temperature, all seed treatments with antagonists, except for Bc-1, increased seedling stand from 15 to 65% ($P \leq 0.05$) (Fig. 2). At the high temperature, all treatments increased seedling stand from 15 to 50% ($P \leq 0.05$). Coating seeds with Gl-3 resulted in seedling stand comparable to the noninfested control at the low temperature. Coating seeds with Gl-3 or Bc-B resulted in plant stands similar to the noninfested control at the high temperature.

All treatments containing biocontrol agents increased plant height ($P \leq 0.05$) compared with that of untreated seeds in pathogen-infested soil (Table 1). At the low temperature, plant height from Gl-3-coated seeds was greater ($P \leq 0.05$) than from the noninfested control, whereas treatments with Gl-21 or Bc-B resulted in plant height similar to the noninfested control. At the high temperature, treatment with Gl-3 or Bc-B resulted in plant height comparable to that of the noninfested control. Similarly, all seed treatments except Bc-T and Bc-1 increased ($P \leq 0.05$) fresh weight compared with that of untreated seeds in pathogen-infested soil (Table 1). Coating seeds with Gl-3 was the only treatment to have fresh weight similar to, or greater ($P \leq 0.05$) than, the noninfested control at the high or low temperature, respectively.

Treatments with antagonists or captan reduced severity of root rot ($P \leq 0.05$) compared with the plants from untreated seeds in pathogen-infested soil (Fig. 3). Only the treatment with Gl-3 at both temperatures and Bc-B at the high temperature reduced severity of root rot to values similar to those of the noninfested control. Root rot severity in treatments with other antagonists was comparable to the plants from captan-treated seeds, except with Bc-1 at the low temperature and with Bc-T at the high temperature.

In general, there were greater plant stands, plant height, fresh weight, and lower severity of root rot at the higher than at the lower temperature. However, there were significant interactions between temperature and seed treatments on plant stand ($P \leq 0.05$), plant height ($P \leq 0.01$), and fresh weight ($P \leq 0.01$), but not on severity of root rot. Plants from untreated seeds in non-infested soil, or from seeds treated with Bc-B, Bc-T, Bc-1, or Tv-1, had less plant height and fresh weight at the lower than at the higher temperature. In addition, plant stand was decreased from 8 to 20% in treatments with Bc-B, Bc-T, Bc-1, Tv-1, or with untreated seeds in pathogen-infested soil at the lower temperature (Fig. 2, Table 1).

In the second series of tests, the ability of the two isolates Gl-3 and Bc-B to in-

crease plant stand depended upon the amount of inoculum applied to each corn seed. There was a significant linear response for the percentage of plant stand with the amount of inoculum of both Gl-3 ($Y = 54.0 + 10.5X$, $r^2 = 0.967$) and Bc-B ($Y = 47.5 + 8.9X$, $r^2 = 0.956$) (Fig. 1). Coating seeds with 10^6 CFU of Gl-3 per seed resulted in greater ($P \leq 0.01$) plant stand than for the seeds treated with lower rates of inoculum. However, even 10^4 CFU of Gl-3 per seed resulted in a plant stand comparable to the noninfested control. Similarly, seed treatment with the bacterium Bc-B at rates of 10^8 and 10^9 CFU/seed resulted in a plant stand similar to that of the noninfested control. However, stands from Bc-B-treated seeds were less ($P \leq 0.01$) than those from seeds treated with the same amount of propagules of Gl-3.

DISCUSSION

The application of biocontrol agents to corn seeds led to effective control of damping-off of corn caused by a combination of *Pythium* spp. and *F. graminearum* under controlled environmental conditions. In this study, seed treatments with almost all of the antagonists significantly decreased severity of root rot and increased seedling stands, as well as increasing plant height and fresh weight compared with those from untreated seeds in pathogen-infested soil. Although there is little information on the biocontrol of corn diseases (1,5,10,11,17), these data indicate that some fungal or bacterial antagonists have disease control potential comparable to or superior to the fungicide captan for corn. In this study, isolate Gl-3 of *G. vires* was the most effective of the antagonists. Not only did it have the best efficacy in suppressing disease, which was significantly superior in effectiveness to that of captan and other antagonists, but also its performance was more consistent at both high and low temperatures compared with *T. viride* isolate Tv-1 and the bacterial strains. This suggests that isolate Gl-3 has considerable potential for commercial application for protection against seed rot and root rot of field corn, especially under high disease pressure. In other studies, Gl-3 significantly reduced damping-off diseases of eggplant, pepper, and zinnia caused by *R. solani* and snap bean caused by *Sclerotium rolfsii* (15, 20).

Temperature has been cited as one of the most important environmental factors affecting the pathogenicity of *Pythium* spp. on corn, as well as the disease suppression abilities of antagonistic microbes on other crops (5,17,24). In the first series of tests, the ability of Tv-1 and three bacterial strains to reduce severity of root rot was significantly decreased when they were placed at 18°C compared with at 25°C. The interaction between temperature and seed treatment suggested that the efficacy of some biocontrol agents was significantly

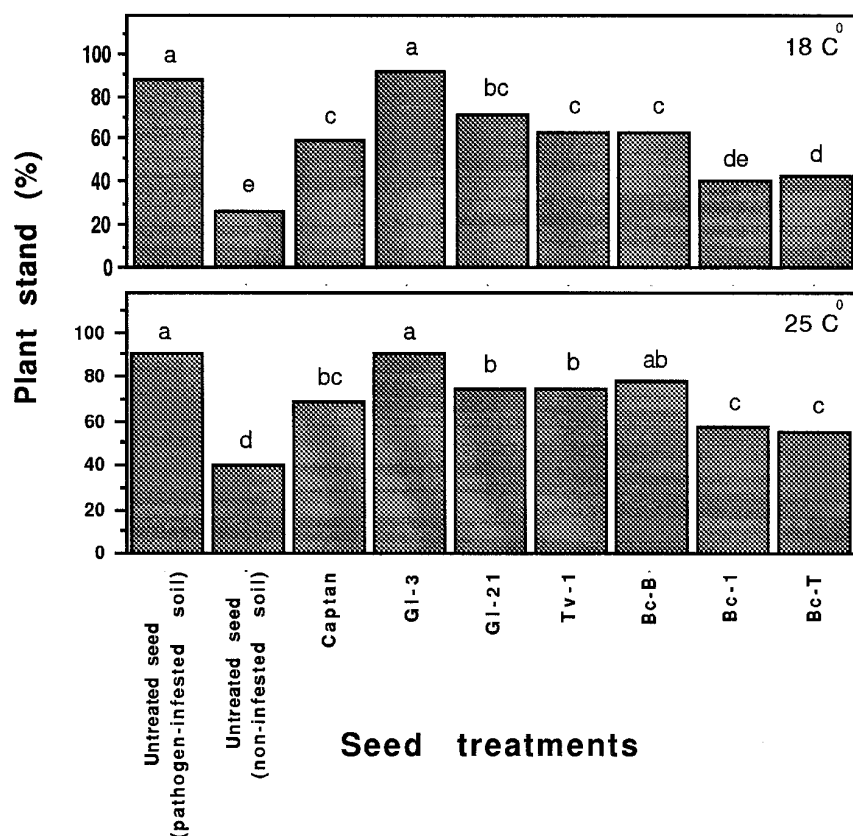


Fig. 2. Effect of corn seed treatments on plant stand in soil infested with a combination of *Pythium ultimum*, *P. arrhenomanes*, and *Fusarium graminearum* at 18 and 25°C. Evaluations were made at harvest (18 days after planting). Means with the same letter are not significantly different from each other according to Duncan's Multiple Range Test ($P \leq 0.05$).

influenced by temperature, indicating that some of the antagonists may be less able to compete with and effectively protect against the pathogens at lower temperature. Upadhyay et al. (24) reported that the antifungal ability of *B. cepacia* was greatly influenced by temperature. They showed that only a small amount of the antifungal compound, pyrrolnitrin, was produced at 18°C, with maximum production at 37°C. However, in this study, the disease suppression ability of *G. virens* isolates GI-3 and GI-21 were relatively consistent at both temperatures. This indicated that these isolates might have a broad temperature range for their antifungal activity. In the noninfested control treatment, plant height and fresh weight were increased by high temperature compared with low temperature, indicating that high temperatures can increase the growth rate of corn. This increased growth might enhance the host's physiological defenses against these pathogens (5,17).

The methods of applying biocontrol agents to a target area are critical in the development of biocontrol strategies for protection against different diseases. Callan et al. (5) reported that colonization of corn seeds by *Pythium ultimum* could be

extensive within 24 h after planting and nearly total by 48 to 72 h, depending on soil temperature and moisture. Application of antagonists by seed treatment appears to represent the most suitable method for biocontrol in the spermosphere and rhizosphere (14). Results of this study suggest that seed treatment with an antagonistic microbe is an effective delivery system to provide a conducive environment for the biocontrol agents. In addition, a large population of an introduced antagonist in the immediate proximity of a germinating corn seed might provide a competitive advantage over resident soil microorganisms (6,10,11).

In this study, to achieve activity similar to that of captan in increasing seedling stand, the minimum population needed was between 10^4 and 10^5 CFU/seed for GI-3 and $>10^8$ for Bc-B. However, when the population of GI-3 was $>10^6$ CFU/seed, the effectiveness in increasing plant stand was superior to that from captan. These data suggest that an adequate population of biocontrol agent needs to be applied to corn seeds to achieve comparable seedling stands and plant growth rate to those from captan-treated seeds. In some cases, the applied biocontrol agents may fail to es-

tablish on the seeds or in the rhizosphere at sufficient levels for disease suppression because of unfavorable soil temperature, pH, or moisture (5). In order to achieve a uniform colonization of sugar beet roots, Suslow and Schroth (23) indicated that the inoculum of a rhizobacterium needed to contain at least 10^5 propagules per seed. Hebbar et al. (10,11) reported that at least 10^7 CFU of *B. cepacia* per seed were needed to suppress soilborne diseases of corn.

This study demonstrated that (i) seed treatment with sufficient propagules of a fungal or a bacterial antagonist was an effective delivery system for suppressing seed rot and root rot of corn under greenhouse conditions, (ii) temperature influenced the ability of an agent to suppress disease, and (iii) *G. virens* was superior to other biocontrol agents tested and has great potential to be utilized for commercial application.

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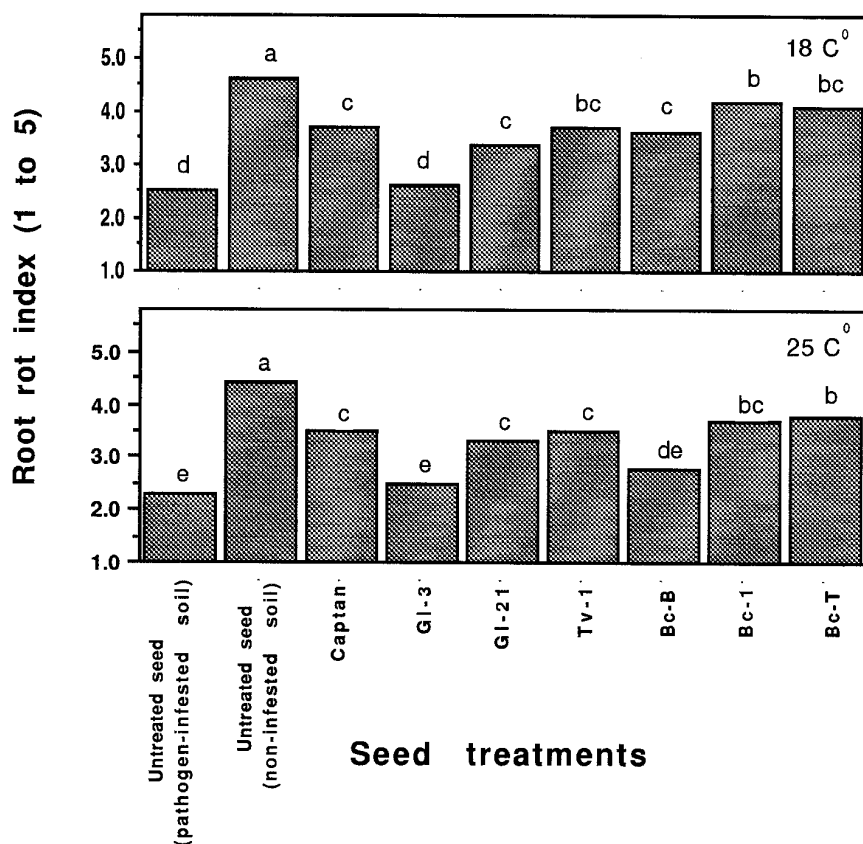


Fig. 3. Effect of corn seed treatments on severity of root rot in soil infested with a combination of *Pythium ultimum*, *P. arrhenomanes*, and *Fusarium graminearum* at 18 and 25°C. Root rot severity index: 1 = <2% (healthy plant), 2 = 3 to 30% (slight disease), 3 = 31 to 60% (moderate disease), 4 = 61 to 90% (severe disease), and 5 = >91% (dead plant). Evaluations were made at harvest (18 days after planting). Means with the same letter are not significantly different from each other according to Duncan's Multiple Range Test ($P < 0.05$).

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ERRATUM / Volume 81, Number 5, 1997

In the article "Seed treatment with a fungal or a bacterial antagonist for reducing corn damping-off caused by species of *Pythium* and *Fusarium*" by W. Mao, J. A. Lewis, P. K. Hebbar, and R. D. Lumsden on pages 450 to 454, in Figures 2 and 3, the label for the first bar should read "Untreated seed (noninfested soil)" and for the second bar should read "Untreated seed (pathogen-infested soil)."