Bio-priming Seed Treatment for Biological Control of *Pythium ultimum* Preemergence Damping-off in *sh2* Sweet Corn

NANCY W. CALLAN, Associate Professor, Western Agricultural Research Center, Montana State University, Corvallis 59828; D. E. MATHRE, Professor, Department of Plant Pathology, Montana State University, Bozeman 59717; and JAMES B. MILLER, Research Technician, Western Agricultural Research Center, Montana State University, Corvallis 59828

ABSTRACT

Callan, N. W., Mathre, D. E., and Miller, J. B. 1990. Bio-priming seed treatment for biological control of *Pythium ultimum* preemergence damping-off in *sh2* sweet corn. Plant Dis. 74:368-372.

Sweet corn (*Zea mays*) with the *sh2* gene for enhanced sugar content is highly susceptible to preemergence damping-off, caused primarily by *Pythium ultimum* in Montana's Bitterroot Valley. Rhizosphere bacteria that adhered to hyphae of *P. ultimum* and were antagonistic to the growth of this pathogen were isolated from Bitterroot Valley soils. An isolate of *Pseudomonas fluorescens*, AB254, provided superior seed protection from Pythium damping-off in naturally infested soils. At least 1×10^7 cfu per seed of AB254 was needed to achieve maximum protection. In a process we have termed "bio-priming," dry seed was coated with *P. fluorescens* AB254 and allowed to imbibe water under warm temperatures until a 35–40% moisture content was achieved. During bio-priming, bacterial populations increased from 10 to over 10,000-fold, depending on initial inoculum level. Bio-priming provided protection against damping-off as good as or better than seed treatment with metalaxyl when the seeds were planted in cold soil. This process may be of interest and value to growers who wish to plant sweet corn or other temperature-sensitive crops into cold soils where damping-off is a problem and the use of chemical seed treatments is not desired.

Sweet corn (*Zea mays* L.) carrying the shrunken-2 (*sh2*) gene for elevated sweetness is becoming increasingly popular with consumers. This genotype, however, is characterized by poor stands and low seedling vigor in comparison with standard sweet corn. Stand failure in *sh2* sweet corn has been associated with susceptibility to preemergence damping-off caused by *Pythium* spp., especially when seeds are planted in cold soil (11).

Seed of sweet corn, like that of several other warm-season crops such as snap beans (*Phaseolus vulgaris* L.), lima beans (*P. limensis* Macf.), soybeans (*Glycine max* (L.) Merr.), chickpeas (*Cicer arietinum* L.), and cotton (*Gossypium hirsutum* L.), is subject to imbibitional chilling injury, a physiological disorder that occurs when water uptake begins under low temperature (6,8,14). Membrane damage during low temperature imbibition has been postulated as the mechanism involved (2).

Various substances, including soluble carbohydrates, potassium ions (26), and proteins (29), are released from sweet corn seeds upon imbibition. A high level of electrolyte leakage from sweet corn seed has been found to be an indication of low seed vigor (28,30). The amount of seed exudation during germination was directly related to the incidence of damping-off in beans (23,24), soybeans (17), cucumbers (*Cucumis sativus* L.), and peas (*Pisum sativum* L.) (7). Increased exudation of metabolites is characteristic of seeds germinating in cold soil (13).

In addition to abundant seed exudation, *sh2* sweet corn has structural features that predispose the seed to pathogen attack. The *sh2* seed commonly has large air spaces between the pericarp and the aleurone layer. This allows pericarp breakage (26), which has resulted in increased Pythium damping-off in peas (25). Also, the small seed size of the *sh2* genotype provides less nutrient reserve for the developing embryo (29).

The most practical method of controlling Pythium damping-off is by chemical, physiological, or biological seed treatment. To overcome imbibitional injury of *sh2* corn, hydrating presown seed (4) and osmotic conditioning or osmopriming (5) have been recommended. Osburn and Schroth (21,22) found that osmopriming of sugar beet (*Beta vulgaris* L.) seed protected it from attack by *Pythium* spp. A combination of seed hydration (solid matrix priming) and treatment of cucumber and tomato (*Lycopersicon esculentum* Mill.) seeds with *Enterobacter cloacae* (Jordan) Hormaeche and Edwards or *Trichoderma* spp. reduced Pythium damping-off and enhanced seedling growth (12).

Biological seed treatments may not provide adequate seed protection under all conditions. The applied bioprotectant may fail to establish on the seed or in the rhizosphere at sufficient levels for disease control because of unfavorable soil temperature, pH, or moisture. Furthermore, biological control may be difficult to achieve with seeds releasing high levels of exudates during germination (20). A biological seed protection system that addresses problems of seed physiology, such as imbibitional chilling, would appear to have a greater potential for seed protection under adverse germination conditions.

Our objectives were to isolate one or more naturally occurring rhizobacteria that could be applied to *sh2* sweet corn seeds for protection from Pythium preemergence damping-off and to develop a delivery system that would provide consistent control of this disease in cold soil. Using the strategy of Harman and Taylor (12), we have integrated biological and physiological disease control into a system we term "bio-priming." In bio-priming, temperature and moisture conditions during seed imbibition are optimized to eliminate imbibitional chilling injury (4) while a bacterial bioprotectant becomes established on the seed. Bio-priming differs from osmopriming in that water uptake is regulated through adjustments in temperature, duration, and water quantity rather than by the use of an osmotic solution.

MATERIALS AND METHODS

Isolation and characterization of potential seed bioprotectants. An isolation procedure was designed to identify rhizosphere bacteria having an apparent affinity for hyphae of *Pythium* spp. Isolates of *Pythium ultimum* Trow were obtained from infected corn seeds planted at the Western Agricultural Research Center (WARC), Corvallis, Montana. Cultures were maintained on cornmeal agar (CMA).

To obtain a broad spectrum of rhizobacteria with potential as seed protectants, soils were sampled from 10 locations in the Bitterroot Valley in western Montana. Flats of each soil were sown with seeds of *sh2* sweet corn (cv. Crisp'n'Sweet 710) and incubated at 25 C for 96 hr. After removal of the larger

Contribution from the Montana Agricultural Experiment Station, Journal Series 2364.

Accepted for publication 29 November 1989 (submitted for electronic processing).

soil particles and the coleoptile, each seedling with adhering seed remnant was placed in a 5-ml sterile water blank. Plugs of *P. ultimum* (2 mm diam.) were incubated for 48 hr at 25 C in small culture dishes containing 3 ml of cornmeal broth (CMB) (9). The resultant mycelial mats were then transferred individually to the tubes containing seedlings, shaken thoroughly, and incubated for 16 hr at 25 C. Mycelial mats were removed, rinsed in sterile water, and sonicated for 5 min in 100 ml of sterile water to release any attached bacteria. Serial dilutions were plated on CMA, potato-dextrose agar (PDA), or one-tenth-strength tryptic soy agar (TSA) (Difco) and incubated at 25 C for 96 hr, when individual colonies were selected. The selected isolates were streaked, six per plate, around the edge of PDA plates, and a 2-mm plug of *P. ultimum* was placed in the center of each plate. After 36 hr, plates were examined for a zone of inhibition around the bacterial colony or for apparent lysis of hyphae in the area of contact. Selected isolates were grown for 24 hr in 3.5 ml of King's medium B, after which 2 ml of 80% glycerol was added and cultures were stored at −5 C until further testing (D. Sands, *personal communication*).

Inhibition of fungal growth. Liquid cultures containing mycelial mats were used to assess the ability of selected bacteria to inhibit the growth of *P. ultimum* in vitro (10,20). *P. ultimum* was grown from CMA plugs (2 mm diam.) for 16 hr at 18–20 C in individual 12 × 50 mm culture dishes containing 4 ml of CMB. The resultant mycelial mats showing uniform growth (6 mm diam.) were inoculated with 0.1 ml of a 48-hr-old (25 C) nutrient broth (BBL, Cockeysville, MD) culture of selected bacterial isolates (three or four replicates per bacterium). The diameter of the mycelial mat was measured after 24 and 48 hr and compared with cultures without bacteria. Mycelial mats were then removed from the culture dishes, blotted on paper towels, and plated on acidified CMA (1 ml of 25% lactic acid l^{-1}). After 24 hr, mats were checked for mycelial growth. Bacteria that severely limited or prevented growth of *P. ultimum* were tested further as seed protectants.

Greenhouse tests. Three greenhouse trials involving 52 bacterial isolates were used to further delimit potential bioprotectants for field testing. Bacterial isolates were grown on PDA for 48 hr at 25 C. One plate of each isolate was scraped into 4 ml of 1.5% methylcellulose (MC), medium viscosity. Sixty seeds of Crisp'n'Sweet 710 corn (97% germination), previously surface-disinfested for 5 min in 0.25% NaOCl, were coated with the bacterial suspension and allowed to dry for 20 hr (16). Twelve seeds were planted 2 cm deep in one row of a 72-cell flat in a randomized complete block design with four replications. Flats contained field soil from WARC, a Burnt Fork Sandy Loam naturally infested with 1,000 propagules g^{-1} of *Pythium* spp., primarily *P. ultimum*, with 5.6 ppm Fe and a pH of 7.4. Populations of *Pythium* spp. were determined by the method of Ali-Shtayeh et al (1). Each trial included surface-disinfested, nontreated seeds and seeds treated with MC, metalaxyl (0.3 g a.i. kg^{-1} seed), captan (1.0 g a.i. kg^{-1} seed), or diazinon (1.4 g a.i. kg^{-1} seed). Previous testing had determined that a 5-min treatment with 0.25% NaOCl had no effect on seed germination. Untreated seed was also sown into pasteurized soil. Soil temperature averaged 23–27/7 C (max/min).

Field testing of bacterial isolates. The nine most consistently performing isolates in the greenhouse tests were selected for further testing in the field at WARC. Four PDA plates were scraped into 16 ml of MC and applied to 350 *sh2* sweet corn seeds (Crisp'n'Sweet 620, germination 91%). Seed was planted by hand, 3.5 cm deep and 5 cm apart, in a randomized complete block design with six single-row replications of 50 seeds each in the first experiment (26 May 1988) and five replications in the second (22 June). Included were surface-disinfested seed treated with metalaxyl, diazinon, or MC alone and surface-disinfested nontreated seeds. Soil temperature at 5 cm was recorded with a thermograph. Analysis of variance was conducted on data combined over the two field experiments.

Levels of seed bacterization at planting were determined by dilution plating. Three replications of five seeds each were washed in 5 ml of 0.1 M phosphate buffer (pH 6.8) for 30 min with vortex agitation at 10-min intervals (16). Then, 0.10 ml of the appropriate dilution was plated on one-tenth-strength TSA, and bacterial colonies were counted after 48 hr.

Bacterization of dry seed. Crisp'n'Sweet 710 corn seed was treated with isolate AB254 of *Pseudomonas fluorescens* Migula suspended in MC to achieve approximately $\log_{10}$ 3, 5, 7, and 9 cfu $seed^{-1}$. Thirty-six seeds were planted in three rows of a 72-cell flat of naturally infested Burnt Fork Sandy Loam in a randomized complete block design with six replications. Included were surface-disinfested seed treated with metalaxyl, MC, or PCNB and surface-disinfested nontreated seed. Soil temperature averaged 12.7 C during the first 24 hr and was increased to 17.5/12.7 C (max/min) for the remainder of the experiment. Emergence was tabulated daily.

Bio-priming. Bio-priming as described here combines seed bacterization with the hydration technigue of Bennett and Waters (4). Three hundred and fifty Crisp'n'Sweet 710 corn seeds (51 g) were coated with *P. fluorescens* AB254 in MC as described above and either allowed to dry overnight or, after drying 2 hr, placed in 51 g of sterile vermiculite in a self-sealing plastic bag to which 64 ml of sterile water was added. Seeds were incubated for 22 hr at 25 C in the first two experiments and for 20 hr at 22 C in the third experiment. At the end of this period, the seed moisture content was 35–40%. Seed was planted in 72-cell flats of naturally infested Burnt Fork Sandy Loam soil (700–1,000 propagules of *P. ultimum* g^{-1}) as described above. Seed treated with metalaxyl, PCNB, or MC alone, MC-treated and nontreated hydrated seed, and surface-disinfested nontreated seed were also included. This experiment was conducted three times with similar results. Greenhouse soil temperature was adjusted to approximately 11–13 C for the first 20–24 hr, then raised to 17–18/11–13 C (max/min) for the remainder of each experiment.

Soil iron content and pH. A greenhouse trial was conducted to examine the ability of *P. fluorescens* AB254 to protect *sh2* corn seeds in two naturally infested soils varying in iron content and pH. The Burnt Fork Sandy Loam soil described above was compared with a Bass Coarse Sandy Loam soil having 35.3 ppm Fe, a pH of 6.6, and a mean of 300 propagules g^{-1} of *P. ultimum*. Seeds of Crisp'n'Sweet 710 were bio-primed as above, and six replications of 36 seeds each were planted in a randomized complete block design. Surface-disinfested seeds treated with metalaxyl, PCNB, or MC alone were also included. Soil temperature averaged 10.8 C for the first 20 hr, increasing to 18.6/10.8 C (max/min) during emergence. Seedling height to the top of the first leaf was measured at 21 days.

RESULTS

Bioprotectant screening. Because *P. ultimum* was isolated consistently from *sh2* sweet corn seed planted in naturally infested field soil and because metalaxyl provided good protection against damping-off under such conditions, we directed our efforts toward rhizobacteria that were antagonistic toward this pathogen. The lack of control provided by the insecticide diazinon or the fungicide PCNB suggested that neither insects nor fungi other than *Pythium* spp. were involved in problems of seedling emergence. Of approximately 1,300 bacterial isolates used to challenge *P. ultimum* on PDA, 247 produced a zone of inhibition and 61 caused a degradation and vacuolation of hyphal cytoplasm. Of these 308 isolates, 96 caused the mycelial mat to cease growth and 18 showed fungicidal activity, i.e., the mycelial mat did not grow when plated on one-tenth-strength CMA. Of the 47 isolates that were involved in all three greenhouse trials, 19 significantly increased emergence over the nontreated control in two of three trials and nine did so in all three. The most effective and consistently per-

forming isolate, AB254, was identified as *P. fluorescens*.

When the nine most effective isolates from the greenhouse trials were applied to field-planted seed, *P. fluorescens* AB254 provided significant seed protection when compared with the surface-disinfested control and the other eight isolates (Table 1). In the May experiment, which was conducted in cold soil, *P. fluorescens* AB254 provided less protection than did the metalaxyl treatment.

Bacterial populations on dry seed. When applied to dry seed, 5.1 $\log_{10}$ cfu seed^{-1} of *P. fluorescens* AB254 increased the emergence of *sh2* corn over that of the controls, but at least 7.4 $\log_{10}$ cfu seed^{-1} were needed for control of damping-off equivalent to that provided by metalaxyl under the conditions of this greenhouse experiment (Fig. 1). Emergence of surface-disinfested nontreated seed was similar to that of MC-treated seed (*data not given*).

Bio-priming. When seed hydration was combined with bacterial seed inoculation, seedling emergence in cold greenhouse soil was greatly increased over that of the controls (Table 2). Application of *P. fluorescens* AB254 to dry seed was considerably less effective than bio-priming with this organism. Low temperature imbibition increased the severity of this test, as indicated by the poor emergence of metalaxyl-treated seed and the increase in emergence after seed hydration alone.

During bio-priming, bacterial populations on seed rose substantially, with the magnitude of increase apparently dependent on initial inoculum level. For example, in various experiments, initial populations of 2.9, 4.9, 6.8, 7.4, and 8.9 $\log_{10}$ cfu seed^{-1} increased to 6.8, 7.0, 7.6, 8.2, and 9.2 $\log_{10}$ cfu seed^{-1}, respectively, after bio-priming. This indicates that relatively low seed treatment bioprotectant levels may be relied on to increase to effective populations during the bio-priming process.

Bio-primed seed emerged at a more rapid rate than did metalaxyl-treated seed (Fig. 2). Seed hydration with no additional treatment improved emergence compared with that of MC-treated or nontreated seed, but to a lesser extent than did bio-priming or metalaxyl. Seed hydrated after treatment with MC emerged at a higher rate than seed hydrated after only surface-disinfestation.

Soil iron content and pH. Bio-priming with *P. fluorescens* AB254 and metalaxyl treatment were equally effective in controlling Pythium preemergence damping-off in soil of high or low iron content and of slightly different pH level (Table 3). Emergence was low in either soil when

Table 1. Biocontrol of preemergence damping-off, caused by *Pythium ultimum*, of *sh2* sweet corn by treatment of dry seed with nine bacterial isolates under natural field conditions[x]

Treatment	Emergence (%)[y]		
	26 May 1988	22 June 1988	Average
Metalaxyl[z]	77.7	86.0	81.5 a
AB254	28.0	76.4	52.8 b
AB288	10.3	69.6	40.1 c
AB166	7.7	71.6	40.0 c
AB223	4.0	71.2	37.7 cd
AB302	4.3	70.8	37.5 cd
AB142	3.0	70.0	36.6 cd
AB267	3.7	66.0	35.3 cd
Carrier alone	2.8	63.2	33.0 cd
AB268	3.3	58.8	30.8 de
AB281	4.0	56.0	30.0 de
Disinfested control	1.7	48.8	25.3 e

[x] Planted in Burnt Fork Sandy Loam naturally infested with *P. ultimum* (700–1,000 propagules g^{-1}). Soil temperature at 5 cm averaged 23.4/8.6 C (max/min) during the 10 days following the 26 May planting and 30/14 C following the 22 June planting. Seed was coated with bacteria (6.9–8.8 $\log_{10}$ cfu seed $^{-1}$) in 1.5% methylcellulose and dried overnight.

[y] Values are the means of six (26 May) or five (22 June) replications of 50 seeds each. Means within a column followed by different letters are significantly different ($P \leq 0.05$) according to Student-Newman-Keuls multiple range test. Factorial ANOVA conducted on the arcsin of the square root of the proportion.

[z] Applied at 0.3 g a.i. kg^{-1} seed.

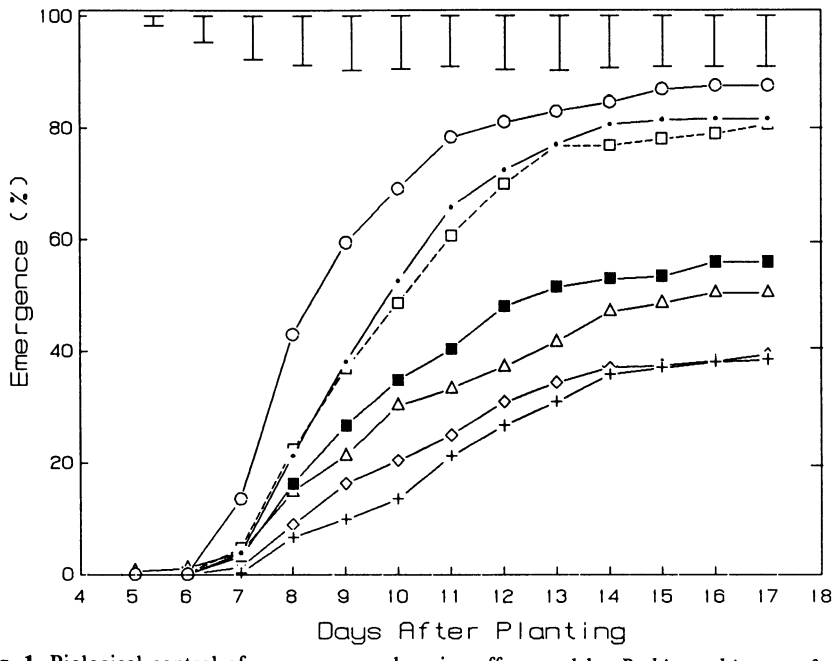

Fig. 1. Biological control of preemergence damping-off, caused by *Pythium ultimum*, of *sh2* corn by application of *Pseudomonas fluorescens* AB254 to dry seed. Seed coated with a methylcellulose suspension of 8.9 $\log_{10}$ (●), 7.4 $\log_{10}$ (□), 5.1 $\log_{10}$ (■), 3.7 $\log_{10}$ (△), metalaxyl (○), PCNB (+), methylcellulose (◇). Bars = protected LSD ($P = 0.05$).

Table 2. Bio-priming of *sh2* sweet corn for control of damping-off, caused by *Pythium ultimum*, in naturally infested soil under cold greenhouse conditions[t]

Treatment	Emergence (%)[u]
AB254 bio-primed[v]	39.4 a
Metalaxyl[w]	30.1 b
Hydrated only[x]	19.8 c
AB254 seed bacterization[y]	8.3 d
PCNB[z]	1.4 d
Disinfested control	1.0 d
Methylcellulose	1.0 d

[t] Planted in Burnt Fork Sandy Loam. Soil temperature during the first 24 hr averaged 10.8 C and during emergence, 17.5/13.0 C (max/min).

[u] Values are the untransformed means of six replications of 36 seeds each. ANOVA conducted on the arcsin of the square root of the proportion. Means within a column followed by different letters are significantly different ($P \leq 0.05$) according to Student-Newman-Keuls multiple range test.

[v] *Pseudomonas fluorescens* isolate AB254 applied in 1.5% methylcellulose; 350 seeds incubated with 51 g of vermiculite and 64 ml of water for 22 hr at 25 C. AB254 population was 9.2 $\log_{10}$ cfu seed^{-1} at planting.

[w] Applied at 0.3 g a.i. kg^{-1} seed.

[x] Seeds incubated in moist vermiculite as above.

[y] AB254 applied in 1.5% methylcellulose and allowed to air-dry. AB254 population was 8.5 $\log_{10}$ cfu seed^{-1} at planting.

[z] Applied at 1.875 g a.i. kg^{-1} seed.

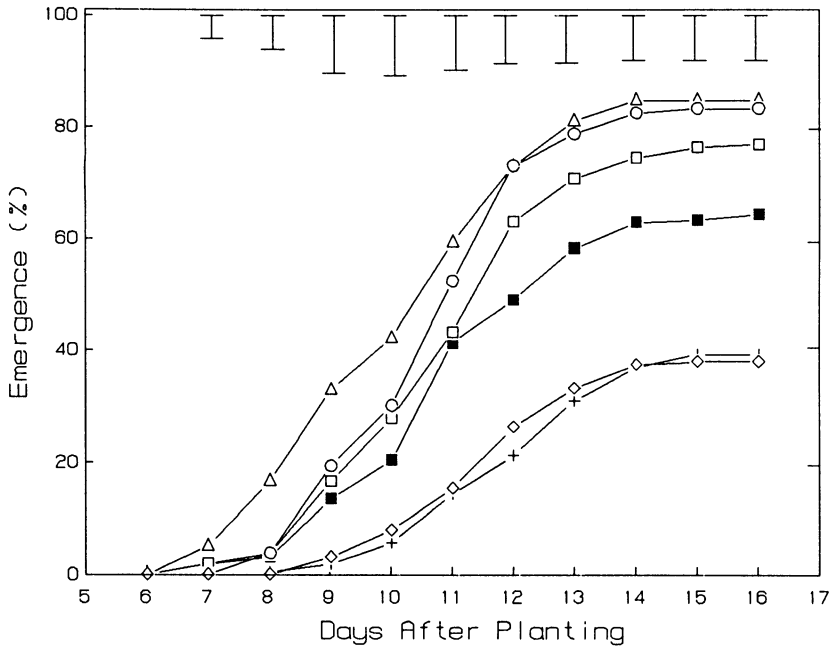

Fig. 2. Control of preemergence damping-off, caused by *Pythium ultimum*, of *sh2* corn by bio-priming with *Pseudomonas fluorescens* AB254. Bio-primed (△), metalaxyl (○), methylcellulose-coat hydrated (□), nontreated hydrated (■), PCNB (+), methylcellulose (◇). Bars = protected LSD ($P = 0.05$).

seeds were untreated or treated with PCNB. Seedling growth in infested soil was increased by either bio-priming or metalaxyl treatment. There was no interaction ($P \leq 0.05$) between seed treatment and soil type.

DISCUSSION

Various isolates of *P. fluorescens* have been found to be effective seed bioprotectants against *Pythium* spp. in a number of crop species (15,18,27,31). The Bitterroot Valley isolate of *P. fluorescens*, AB254, was effective in protecting *sh2* sweet corn against Pythium preemergence damping-off under a range of soil conditions and temperatures, as evidenced by the control it provided during various times of the year. Bacterization with *P. fluorescens* AB254 had no effect on final emergence of seed planted in pasteurized soil compared with nontreated seed (*unpublished*), indicating that this organism per se is neither germination promotive nor phytotoxic.

The high levels of nutrient exudation by *sh2* corn seeds during imbibition provide a challenge to biocontrol of seed-rotting pathogens. Nelson et al (19,20) found that seeds of species that produced very little exudate during imbibition were more effectively protected by *E. cloacae* than those that released abundant exudate. *P. fluorescens* AB254 may be able to defend the *sh2* sweet corn spermoplane against attack by *Pythium* spp. by monopolizing this abundant nutrient source (10), by competing for iron through siderophore release (3,18), or by antibiotic production (15). *P. fluorescens* AB254 protected seeds equally well in soil with high or low iron content, which may indicate that iron-mediated siderophore content is not an essential component of seed protection. However, further investigation of the mode of action of *P. fluorescens* AB254 in seed protection is needed.

Bio-priming with *P. fluorescens* AB254 enhanced the ability of this biocontrol agent to protect *sh2* corn seeds from attack by *Pythium* spp. in cold to warm soils. The effects of bio-priming on both seed physiology to relieve chilling injury and bacterial growth to provide pathogen control may be involved in this response.

Seed hydration improved seedling emergence over that with nontreated seed but did not provide protection equal to either bio-priming or treatment with metalaxyl. This is in contrast to results presented by Osburn and Schroth (22), who found that osmopriming protected sugar beet seeds from Pythium damping-off as well as did treatment with metalaxyl. The relative effectiveness of hydration alone in seed protection may depend on crop species, soil temperature, and the level of disease pressure.

Because of the rapidity of seed attack by *Pythium* spp. (24), establishment of the bioprotectant on the seed before planting, i.e., during bio-priming, was undoubtedly an important aspect of protection. We found that under field conditions, colonization of nontreated *sh2* corn by *P. ultimum* was extensive 24 hr after planting and nearly total by 48–72 hr, depending on soil temperature (*unpublished*). The value of bio-priming over that of seed bacterization alone was clearly seen when the seed was planted in cold soil.

Bio-priming can provide a high level of protection against preemergence damping-off of *sh2* sweet corn seed caused by *P. ultimum*. This seed treatment appears to be especially effective when seed is planted in cold soil. Protection by bio-priming was generally equal or superior to the control provided by metalaxyl. As such, it may be of interest and value to growers who want to grow the supersweet corns in areas where planting conditions involve cold soils and to growers who wish to avoid chemical treatment of their seed.

Table 3. Influence of soil iron content on control of preemergence damping-off, caused by *Pythium ultimum*, and growth of *sh2* corn by bio-priming with *Pseudomonas fluorescens* AB254[u]

Treatment	Emergence (%)	Height[v] (cm)
Soil iron content		
35.3 ppm	64.7 a[w]	3.9 a
5.6 ppm	61.3 a	3.1 b
Seed treatment		
Metalaxyl[x]	85.0 a	4.0 a
AB254 bio-primed[y]	84.7 a	4.3 a
PCNB[z]	42.1 b	3.0 b
Methylcellulose	40.3 b	2.9 b

[u] Planted in Burnt Fork Sandy Loam naturally infested with *P. ultimum*, pH 7.4 (5.6 ppm Fe), or in Bass Coarse Sandy Loam, pH 6.6 (35.3 ppm Fe). Soil temperature averaged 10.8 C during the first 20 hr, then 18.6/10.8 C (max/min). Values are the untransformed means of six replications of 36 seeds each and are averaged across the four seed treatments or both iron contents. Factorial ANOVA conducted on the arcsin of the square root of the final germination proportion. The interactions were nonsignificant.

[v] Seedling height to the top of the first leaf at 21 days.

[w] Column means (within treatments) followed by the same letter are not significantly different ($P \leq 0.05$) according to Student-Newman-Keuls multiple range test.

[x] Applied at 0.3 g a.i. kg^{-1} seed.

[y] AB254 applied in 1.5% methylcellulose; 9.2 $\log_{10}$ cfu $seed^{-1}$ after bio-priming.

[z] Applied at 1.875 g a.i. kg^{-1} seed.

ACKNOWLEDGMENTS

We thank D. C. Sands and Alice Pilgeram for identifying *Pseudomonas fluorescens* AB254 and Crookham Co., Caldwell, Idaho, for donating seed.

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