Effects of Seed Factors on Spermosphere and Rhizosphere Colonization of Cotton by *Bacillus subtilis* GB03

Walter F. Mahaffee and Paul A. Backman

Department of Plant Pathology and Alabama Agricultural Experiment Station, Auburn University, AL 36849.
We gratefully thank Joseph Kloepper, Rodrigo Rodríguez-Kábana, Kira Bowen, Joseph Shaw, and Robert Gudauskas for reviewing the manuscript and further acknowledge the assistance of Kusum Pai for technical support and Ellen Bauske for statistical assistance.
Accepted for publication 30 June 1993.

ABSTRACT

Mahaffee, W. F., and Backman, P. A. 1993. Effects of seed factors on spermosphere and rhizosphere colonization of cotton by *Bacillus subtilis* GB03. Phytopathology 83:1120-1125.

Cotton seed factors, including seed-surface pH, cultivar, and presence of a seed-treatment fungicide, were investigated for their effect on spermosphere and rhizosphere colonization by the commercial biological control agent *Bacillus subtilis* strain GB03 (GB03). Commercial (nonneutralized) cotton seed is traditionally acid-delinted and in these studies was found to have surface pH values ranging from 1.90 to 3.50. Following seed treatment with GB03, spermosphere populations of GB03 were up to 5–10-fold lower on nonneutralized (commercial acid-delinted) cotton seed than on neutralized seed grown in sterile sand or in field soil, respectively; however, rhizosphere populations of GB03 on 2-wk-old cotton root systems did not differ significantly. GB03 populations 24 h after planting on nonneutralized seed did not significantly increase from initial seed populations at the time of planting, and fewer vegetative cells were recovered on nonneutralized seed than on neutralized seed. Spermosphere and radicle populations of GB03 on seed of four cotton cultivars differed significantly, although whether the difference was due to genotype is unclear. Seed treatment with metalaxyl/pentachloronitrobenzene did not affect spermosphere colonization by GB03; however, treatment with these fungicides resulted in an increase of up to 1 log unit in GB03 rhizosphere populations compared to the level for no fungicide treatment. The spermosphere environment can affect the root colonization potential of bacteria applied as seed treatments to cotton seed, thereby possibly affecting the biological control or plant growth-promoting potential of the inoculants.

Additional key words: bacterization, *Gossypium*, PCNB, PGPR, plant growth-promoting rhizobacteria, seed colonization, metalaxyl.

Rhizobacteria (rhizosphere bacteria that exhibit root colonization [13,25]) have been routinely investigated for their biocontrol and plant growth-promoting characteristics in both glasshouse and field trials. While there have been numerous reports of the successes of these inoculants, the disease suppression and/or growth promotion afforded by these treatments is frequently not consistent in the field (6,15,17,19,27,31,34). In many cases, variable performance of introduced rhizobacteria has been attributed to insufficient root colonization (15,17,30), a process whereby the bacteria inoculated onto seed or into soil survive and/or multiply in the spermosphere in response to seed exudates, attach to the root surface, and colonize the developing root system (16). Most investigations of factors affecting root colonization have examined how various bacterial traits (attachment, motility, and antibiotic and siderophore production [31]) affect colonization, and few investigations have focused on effects of plant and abiotic factors, e.g., plant genotype (7,12), edaphic factors (23), and soil moisture (20). Poor root colonization could be caused by unfavorable environmental conditions in the spermosphere and/or rhizosphere.

Since beneficial rhizobacteria are often introduced as seed treatments, they must survive and/or proliferate in the spermosphere before they can begin to colonize the emerging root system. Adaptability of rhizobacteria to the spermosphere environment can also affect root colonization, biological control, and/or plant growth promotion efficacy. The environment of the spermosphere is different from that of the rhizosphere in both nutrient and microflora composition (9). Seed factors, such as cultivar (plant genotype), presence of seed treatment fungicides, and in some instances, seed-surface pH (seed treated with acid, e.g., cotton and tomato), could affect spermosphere and rhizosphere colonization by an introduced rhizobacterium. Pathogens such as *Rhizoctonia* and *Pythium* (causal agents of pre- and postemergence damping-off of cotton and other plants) can attack seed as soon as 6 h after planting (10,22,24,26). Thus, colonization of the spermosphere by beneficial rhizobacteria may be essential if the plant is to be protected against disease.

Bacillus subtilis strain GB03 is a cotton-adapted variant of *B. subtilis* strain A13 (14). A13 was isolated from lysed mycelium of *Sclerotium rolfsii* in 1971 by Broadbent et al (5). Strain A13 has shown biological control and plant growth-promotion activity in field studies on peanuts (28,29), whereas strain GB03 has shown yield enhancement on cotton (D. Kenney, *personal communication*, P. A. Backman, *unpublished data*). GB03 inoculated onto cotton seed was chosen as a model system to examine the effects of seed factors on root colonization. This system was chosen because GB03 is commercially available as the seed inoculant Kodiak (Gustafson, Inc., Dallas, TX) used on cotton, peanut, and common bean.

Ninety-five percent of commercial cotton seed in the United States is acid-delinted with sulfuric acid, and seed often retains high amounts of residual sulfuric acid. The amount of residual acid on the cotton seed coat (seed-surface pH) could be important in survival, germination, and/or proliferation of GB03 endospores in the spermosphere, thereby affecting the colonization process.

Colonization of cotton by GB03 also could be affected by genotype. Seed and root exudates differ qualitatively and quantitatively among cotton cultivars (10,11,27), which could affect spermosphere and rhizosphere colonization by GB03. Rhizosphere colonization by some pseudomonad rhizobacteria is affected by cultivars of potato and wheat (3,4,21).

Fungicides used as seed treatments have been shown to affect growth of rhizobacteria in vitro (27,35) and could also affect spermosphere and rhizosphere colonization whenever an inoculant is applied to seed previously treated with fungicide. Seed treatment fungicides are used almost universally in cotton production; thus, rhizobacteria inoculated onto cotton seed should be compatible with these fungicides. Fungicides used in peanut production did not affect survival of A13 endospores (8); however, those used in cotton are different and could interfere with endospore germination and/or growth of GB03.

The objective of this study was to examine the effects of seed-surface pH, host genotype (cultivar), and seed-treatment fungicides on colonization of the cotton spermosphere and rhizosphere by *B. subtilis* strain GB03. An understanding of these effects would allow for more appropriate formulation of and improved use

recommendations for *B. subtilis* GB03 on cotton and could lead to more consistent field performance with this commercial plant growth-promoting rhizobacteria inoculant.

MATERIALS AND METHODS

Seed. Nonneutralized (commercially acid-delinted) seed of cotton (*Gossypium hirsutum*, L.) cultivars Dixie Experiment Station 119 (DES-119), Delta Pine 50 (DP-50), Gerik and Pustejobski 1005 (GP-1005), German Kleenseed 510 (GC-510), and Lankart Paymaster 571 (LX-571) were used in all experiments. These cultivars were selected because they represent important cotton cultivars grown in the United States; cultivar DES-119 was also included because of its low seed-surface pH (1.90).

Seed-surface pH was determined by stirring 50 g of acid-delinted cotton seed in 100 ml of deionized distilled water for 1 h and then measuring the pH of the solution with a combination pH electrode. Four samples of each cultivar were tested upon receipt of the seed and after storage for 2 and 6 mo at 4 C in paper bags. Seed were neutralized to a pH of 7.00 ± 0.2 by washing 300 g in three changes of 500 ml of 0.02 M potassium phosphate buffer, pH 7.0, for 5 min. Seed were then air dried, overnight, under a laminar flow hood at room temperature. Seed-surface pH of neutralized seed was determined, and any seed not at a pH of 7.0 ± 0.2 was not used in experiments.

Seed treatments. A planter-box formulation of *B. subtilis* strain GB03 (Kodiak HB), which contained a formulation of $>1.0 \times 10^{10}$ endospores per gram, was used as inoculum in experiments at the recommended rate of 7.5 g/kg of seed, resulting in log 6.8 ± 0.2 cfu/seed, unless otherwise noted. Seed and inoculum were placed in a plastic bag and shaken for 2 min to simulate field inoculation. Seed were treated with metalaxyl/pentachloronitrobenzene (PCNB) (1:4) at the recommended rate (3.75 g/kg of seed) (Gustafson, Inc., Dallas,TX) before being inoculated with GB03 for experiments examining the effects of a fungicide seed treatment.

Seed and plants. Seed were planted either in petri dishes (five seed per 150- × 15-mm dish) or in Conetainers (5 × 25 cm, Ray Leach Nursery, Canby, OR, at a depth of 0.7 cm), both filled with coarse, sterile, blasting sand or natural Dothan sand field soil (98% sand). The sterile sand was selected to avoid the confounding effects of soil biotic factors and because it had a texture similar to that of the field soil; the field soil was included to investigate the effects of competitive microflora on GB03 colonization. Water content of the sterile sand and field soil was adjusted to field capacity (0.33 bar or 13% moisture v/v) with 10% sterile Hoagland's solution for the sand or distilled water for the soil. Petri dishes and Conetainers were incubated at 21 C with a 14-h light period in a growth chamber. Conetainers were mist-watered three times with 2.5 cm of water on days 2, 6, and 11 after planting. Petri dishes were not watered after planting.

Effect of seed-surface pH and cultivar on spermosphere and rhizosphere colonization. Both the spermosphere and rhizosphere experiments were a randomized complete block design with treatments (seed-surface pH and cultivar) arranged in a 2 × 4 factorial design. Originally, experiments involving seed-surface pH and cultivars were performed using 10 replications; however, the number of replications for subsequent experiments was reduced, based on low variances of the experiments, to four replications for petri dishes (one petri dish per replication) and six replications for Conetainers (one Conetainer per replication). Nonneutralized (acidic) and neutralized seed of cultivars DP-50, GC-510, GP-1005, and LX-571 were inoculated with GB03 (7.5 g of formulation per kilogram of seed) and planted either in petri dishes, for spermosphere samples, or Conetainers, for rhizosphere samples, containing sterile sand. Petri dishes were incubated for 60 h and Conetainers for 14 days. Seed (radicles <0.5 cm long) from each petri dish or up to three root systems from each Conetainer were placed in Tekmar sterile lab bags (Tekmar, Co., Cincinnati, OH) with 10 ml of potassium phosphate buffer. Number of seed and fresh weights for the root systems were recorded. Samples were triturated for 1 min with a Tekmar Stomacher Lab Blender model 80 (Tekmar, Co.), serially diluted, and plated with a spiral plater (Spiral Systems Inc., Bethesda, MD) on salt V-8 agar (25) amended with polymyxin B (100 units/ml) and 200 μg of cycloheximide. Polymyxin B was added to the medium to prevent growth of Gram-negative bacteria. Plates were incubated at 30 C for 48 h, and distinctive colonies of GB03 were visually identified and counted. Experiments were conducted three times with similar results.

Population dynamics in the spermosphere and on radicles in relation to seed-surface pH. The experiment was a randomized complete block design with treatments (seed-surface pH, cultivar, and time) arranged in a 2 × 4 × 4 factorial design. Four replications were used. Neutralized and nonneutralized seed of four cultivars, LX-571, GP-1005, GC-510 and DP-50 (Table 1) were inoculated with 4.0 g of formulation per kilogram of seed and planted in petri dishes filled with sterile sand. Plates were incubated at 21 C and seed were sampled at 0, 24, 48, and 72 h. Samples were processed as above, except for the 72-h sample, in which the radicles were separated from the seed coat and processed separately as above. In addition to total viable cells, spore populations also were determined for each sample by incubating samples in a water bath (80 C), with shaking (100 rpm) for 10 min to kill vegetative cells, and plated as above. Percent vegetative cells for each treatment was determined by subtracting the spore population (counts from heat-treated dilutions plated on salt V-8 agar) of GB03 from the total GB03 population (counts from nonheat-treated dilutions plated on salt V-8 agar) then dividing by total GB03 population and multiplying by 100. The experiment was conducted twice with similar results.

Effect of seed-surface pH and a seed treatment fungicide on spermosphere and rhizosphere colonization. Both spermosphere and rhizosphere experiments had randomized complete block designs with treatments (seed-surface pH and fungicide) arranged in a 2 × 2 factorial design. Four replications were used for spermosphere colonization and 10 replications for rhizosphere colonization. Fungicide-treated neutralized and nonneutralized cotton seed of DES-119 were first treated with metalaxyl/PCNB and either inoculated with GB03 (7.5 g of formulation per kilogram of seed) or not inoculated. Treated seed were planted in petri dishes and Conetainers (to examine spermosphere and rhizosphere colonization, respectively) filled with either sterile sand or field soil. Petri dishes were incubated for 60 h and Conetainers for 14 days before sampling. Experiments were conducted twice in sterile sand and four times in field soil with similar results.

Statistical methods. All colonization data were transformed into $\log_{10}$ cfu/unit (gram, seed, or radicle) to make treatment variances homogeneous and were analyzed as a two-way analysis of variance in PC-SAS (SAS Institute, Cary, NC). Fisher's protected least significant difference ($P \leq 0.05$) was used to separate means. To determine whether a joint relationship exists

TABLE 1. Seed-surface pH of cotton cultivars before and after neutralization

Cultivar	Seed-surface pH[w]	
	Nonneutralized seed[x]	Neutralized seed[x,y]
LX-571	3.50*[z]	7.00
GP-1005	3.12*	7.00
GC-510	2.83*	6.96
DP-50	2.15*	6.94
DES-119	1.90*	6.93

[w] Seed-surface pH was determined by stirring 50 g of acid-delinted cotton seed in 100 ml deionized distilled water for 1 h and then measuring the pH of the solution with a combination pH electrode.

[x] Each value is the mean of four samples.

[y] Seed-surface neutralized by successive washings with 0.2 M potassium phosphate buffer.

[z] Asterisk indicates significant difference from neutralized counterpart ($P = 0.01$).

TABLE 2. Effects of seed-surface pH and cultivar on populations of *Bacillus subtilis* strain GB03 on spermosphere and rhizosphere of cotton planted in sterile sand

Cultivar	Spermosphere population of GB03[t,u] (log cfu/seed)			Rhizosphere population of GB03[t,v] (log cfu/g fresh weight root)		
	Nonneutralized seed	Neutralized seed[w]	Cultivar mean[x,y]	Nonneutralized seed	Neutralized seed[w]	Cultivar mean[x,y]
LX-571	6.88	7.19	7.03 A	5.46	5.47	5.46 D
GP-1005	6.59	6.81	6.70 B	5.45	5.49	5.48 D
GC-510	5.69	6.69	6.19 C	4.93	5.40	5.21 D
DP-50	5.92	6.36	6.14 C	5.12	5.29	5.20 D
Neutralization mean[z]	6.27	6.76		5.29	5.37	

[t] Randomized complete block design with a 2 × 4 factorial (seed-surface pH × cultivar).
[u] 60 h after planting. Each value is the mean of four replications.
[v] 14 days after planting. Each value is the mean of six replications.
[w] Seed-surface neutralized by successive washings with 0.2 M potassium phosphate buffer.
[x] Means in a column followed by the same letter are not significantly different at $P = 0.05$.
[y] LSD (0.05) among spermosphere cultivar means = 0.29; among rhizosphere cultivar means = not significant.
[z] LSD (0.05) between spermosphere neutralization means = 0.21; between rhizosphere neutralization means = not significant.

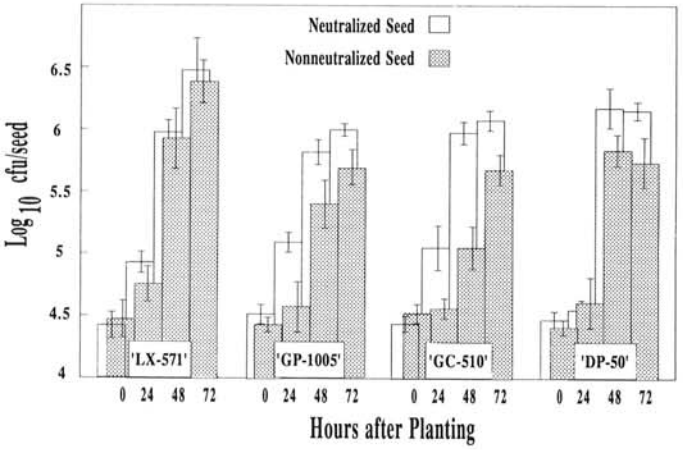

Fig. 1. Cotton spermosphere population dynamics of *Bacillus subtilis* strain GB03 in sterile sand as effected by cultivar and seed-surface pH. Nonneutralized (acidic) and neutralized seed were treated with Kodiak HB (4.5 g/kg seed), placed in uncovered petri dishes containing sterile sand, and incubated at 21 C. Error bars indicate the standard error for each treatment. Columns without overlapping error bars are significantly different ($P \leq 0.05$). Populations were determined as described in Materials and Methods.

between seed-surface pH and spermosphere colonization and between seed-surface pH and percent vegetative cells, Pearson correlation coefficients were calculated. To determine whether colonization varied with these characteristics in a curvilinear fashion, second-order regression models were fit with each factor as the independent variable and colonization as the dependent variable. Data presented is from a single trial that was representative of the results from each experiment.

RESULTS

Seed-surface pH. Seed-surface pH of cultivars ranged from 1.90 to 3.50 (Table 1). DES-119 had the lowest seed-surface pH (1.90), and LX-571 had the highest (3.50). Seed surface pH remained approximately the same (± 0.1 pH units) for all sampling times. Typical seed-surface pH values of neutralized and nonneutralized seed are presented in Table 1.

Effect of seed-surface pH and cultivar on spermosphere and rhizosphere colonization. Spermosphere populations of GB03 were significantly different among cultivars ($P > 0.0001$) and between neutralized and nonneutralized seed ($P > 0.0001$). Rhizosphere populations of GB03, at 14 days after planting, were not significantly different between nonneutralized and neutralized seed ($P = 0.44$); however, there was a trend to significance ($P = 0.06$) among cultivars (Table 2). GB03 rhizosphere populations on cultivar GC-510 were erratic and generally smaller than on other cultivars. Rhizosphere populations on neutralized seed were also lower in direct relationship to their original seed-surface pH before neutralization. No significant interactions were detected between seed-surface pH and cultivar for spermosphere ($P = 0.43$) and rhizosphere ($P = 0.11$) colonization.

TABLE 3. Effects of seed-surface pH and cultivar on *Bacillus subtilis* strain GB03 spermosphere and radicle colonization of cotton planted in sterile sand[v,w]

Cultivar	Radicle population of GB03 (log cfu/radicle)		
	Nonneutralized seed	Neutralized seed[x]	Cultivar mean[y]
LX-571	3.57	3.78	3.67
GP-1005	3.38	3.71	3.52
GC-510	3.17	3.76	3.47
DP-50	3.17	3.56	3.36
Neutralization mean[z]	3.32	3.70	

[v] Randomized complete block design with a 2 × 4 factorial (seed-surface pH × cultivar).
[w] Populations on radicles detached from seed 72 h after planting in sterile sand. Each value is the mean of four replications.
[x] Seed-surface neutralized by successive washings with 0.2 M potassium phosphate buffer.
[y] LSD (0.05) among cultivar means not significant.
[z] LSD (0.05) between neutralization means = 0.28.

Population dynamics in the spermosphere and on radicles in relation to seed-surface pH. As expected, there was a significant interaction ($P = 0.03$) between seed-surface pH and sampling time, indicating that population growth of GB03 was differentially affected by seed-surface pH. After 24 h, spermosphere populations of GB03 on nonneutralized seed of all cultivars, grown in sterile sand, were not significantly different from those of the time-0 sample, whereas populations on neutralized seed, except on DP-50, were significantly greater ($P \leq 0.05$) than at time 0 for all cultivars (Fig. 1). Spermosphere populations of GB03 on nonneutralized seed, 24 h after planting, were significantly correlated with seed-surface pH ($r = 0.83$, $P = 0.001$). At 48 and 72 h, spermosphere populations of GB03 were significantly greater ($P \leq 0.05$) on neutralized seed than on nonneutralized seed for all cultivars except LX-571. No significant interactions were detected between seed-surface pH and cultivar ($P = 0.17$) or among seed-surface pH, cultivar, and time ($P = 0.34$).

Radicle populations of GB03 from nonneutralized seed, 72 h after planting, were significantly ($P = 0.01$) lower than populations from their neutralized counterparts (Table 3), but no significant ($P = 0.41$) cultivar effect was found. There was a significant correlation ($r = 0.087$, $P = 0.001$) between spermosphere populations and radicle populations at 72 h.

Percent vegetative cells of GB03 on seed of the four cultivars 24 h after planting were significantly greater ($P = 0.03$) on neutralized seed than on nonneutralized (Table 4) but were not significantly different at 48 h ($P = 0.17$) and 72 h ($P = 0.25$) after planting (*data not shown*). Percent vegetative cells of GB03 decreased with lower seed-surface pH of the cultivars and were significantly correlated ($r = 0.94$, $P = 0.0001$) with seed-surface pH.

Effect of seed-surface pH and seed treatment fungicides on spermosphere and rhizosphere colonization. Spermosphere populations of GB03 were significantly different between neutralized and nonneutralized seed ($P = 0.0015$), although there was no significant difference between fungicide-treated and nontreated seed ($P = 0.22$) (Table 5). A significant interaction ($P = 0.021$) was found between seed-surface pH and fungicide treatment for rhizosphere populations of GB03. Treatments differed by only log 0.08 cfu/g between neutralized and nonneutralized seed treated with fungicide, while there was a difference of log 0.50 cfu/g between neutralized and nonneutralized seed not treated with fungicide. Supporting these data, neither spermosphere nor rhizosphere colonization was affected ($P = 0.78$ and $P = 0.65$, respectively) by the presence of metalaxyl/PCNB on seed planted in sterile sand (*data not shown*). Spermosphere and rhizosphere populations of GB03 on nonneutralized seed planted in field soil were significantly lower than the populations of their neutralized counterparts (Table 5), confirming the results for spermosphere and rhizosphere colonization in sterile sand (Table 2).

TABLE 4. Effects of seed-surface pH and cultivar on percent vegetative cells of *Bacillus subtilis* strain GB03 in the spermosphere of cotton, 24 h after planting[u]

	Percent vegetative cells of GB03 in the spermosphere[v]		
Cultivar	Nonneutralized	Neutralized[w]	Cultivar mean[x,y]
LX-571	67.6	73.1	70.5 A
GP-1005	56.6	61.1	58.3 AB
GC-510	32.0	57.3	46.3 BC
DP-50	14.9	41.9	29.2 C
Neutralization mean[z]	42.2	69.5	

[u] Randominzed complete block design with a 2 × 4 factorial (seed-surface pH × cultivar).
[v] Determined as described in text.
[w] Seed-surface neutralized by successive washings with 0.2 M potassium phosphate buffer.
[x] Means followed by the same letter are not significantly different at $P = 0.05$.
[y] LSD (0.05) among cultivar means = 17.8.
[z] LSD (0.05) between neutralization means = 12.6.

DISCUSSION

Spermosphere colonization of cotton by GB03 appeared to be greatly affected by the seed-surface pH of cotton seed. Populations were as much as 90% (1 log unit) lower on nonneutralized seed than on neutralized seed. Whether the increase was due to neutralization of residual acid or to removal of seed leachates or other compounds that may have inhibited spermosphere colonization of GB03 could not be determined conclusively. However, there is strong evidence to indicate that the overriding factor affecting spermosphere colonization is the residual acid on the seed coat (i.e., seed-surface pH). Total populations (Fig. 1) and percent vegetative cells (Table 3) of GB03 on nonneutralized seed 24 h after planting were significantly correlated with seed-surface pH.

The lower populations on nonneutralized seed were probably not caused by a reduction in populations per se, but rather by a delay in spore germination, as indicated by the percent vegetative cell data (Table 3). Inhibition of spore germination would result in lower bacterial populations on the nonneutralized seed by increasing the length of the lag phase and thereby the length of time before bacteria reached the exponential growth phase. These data agree with the work of Akiba et al (1,2), in which spore germination of *B. thuringiensis* was dependent on soil pH. Spore germination was greatest at pH 7.0 and decreased until it stopped at pH 5.0. In addition, West et al (32,33) demonstrated that cellular growth of *B. thuringiensis* and *B. cereus* was similarly dependent on soil pH. The lag phase could have been extended because bacterial spores, inoculated onto nonneutralized seed, may remain dormant in the acidic environment of the spermosphere until the environment becomes favorable for germination. This delay in germination appears to be a major reason for population differences in the spermosphere because of the following: 1) percent vegetative cells were as much as 30% lower on nonneutralized seed than on neutralized seed 24 h after planting, and spermosphere populations 24 h after planting were not significantly different from the initial populations on nonneutralized seed; 2) spermosphere populations on neutralized seed were significantly greater than initial seed populations 24 h after planting; and 3) bacteria appeared to grow at the same rate (i.e., have the same doubling time) on both neutralized and nonneutralized seed.

Seed surface pH appears to cause a delay in spore germination that later results in reduced spermosphere and radicle populations. Reductions of radicle populations may translate into lower rhizosphere populations. A combination of decreased spermosphere and radicle populations along with plant genotype effects may account for the lower rhizosphere populations found on cultivar GC-510.

Seed-surface pH ≥3.50 does not appear to affect spermosphere colonization by GB03. The apparent tolerance of GB03 to seed-

TABLE 5. Effects of seed-surface pH and a seed treatment fungicide on *Bacillus subtilis* strain GB03 spermosphere and rhizosphere colonization of cotton[q] planted in field soil[r]

	Spermosphere population of GB03[s] (log cfu/seed)			Rhizosphere population of GB03[t-v] (log cfu/g fresh weight root)	
Treatment	Nonneutralized seed	Neutralized seed[w]	Fungicide mean[x]	Nonneutralized seed	Neutralized seed[w]
GB03	6.02	6.41	6.23	4.53 A	5.03 B
GB03 and fungicide[y]	6.01	6.44	6.21	5.48 C	5.56 C
Neutralization mean[z]	6.01	6.42			

[q] Cultivar DES-119 (seed-surface pH, 1.90).
[r] Randomized complete block design with a 2 × 2 factorial (seed-surface pH × fungicide).
[s] 60 h after planting. Each value is the mean of four replications.
[t] 14 days after planting. Each value is the mean of six replications.
[u] Means followed by the same letter are not significantly different at $P = 0.05$.
[v] LSD (0.05) among rhizosphere fungicide × pH means = 0.42.
[w] Seed-surface neutralized by successive washings with 0.2 M potassium phosphate buffer.
[x] LSD (0.05) between fungicide means = not significant.
[y] Metylaxyl/pentachloronitrobenzene (3.5 g/kg seed).
[z] LSD (0.05) between neutralization means = 0.30.

surface pH of ~ 3.50 could be used in developing formulations for seed treatments using GB03. Calcium carbonate and/or other buffers could be added to the formulation to neutralize the residual sulfuric acid, thereby raising seed-surface pH above 3.50.

Rhizosphere populations in field soil were significantly increased 5–10-fold by the presence of seed-treatment fungicides. This increase could be the result of inhibition of competitive fungi and bacteria in the rhizosphere. Inhibition of indigenous microflora, coupled with tolerance to metalaxyl/PCNB and other seed treatment fungicides (8), would give GB03 an ecological advantage, thus allowing greater colonization. Seed-surface pH also affected rhizosphere colonization in field soil; however, the negative effect of the residual acid on nonneutralized seed was overridden by the positive effects of the metalaxyl/PCNB seed treatment. Protection of the developing root system afforded by metalaxyl/PCNB on nonneutralized seed could have given GB03 the opportunity to colonize niches that would otherwise have been occupied by indigenous microflora, thereby increasing its effectiveness as a biological control agent. The protection theory is supported by two observations: 1) rhizosphere populations of GB03 were not affected by the presence of metalaxyl/PCNB when the seed were grown in sterile sand; and 2) rhizosphere populations on nonneutralized seed were lower than those on neutralized seed in the absence of seed treatment fungicides.

It is not clear from these data whether cultivar differences affect colonization, even though spermosphere and radicle populations of GB03 significantly differed among cultivars. Spermosphere colonization and percent vegetative GB03 cells of both neutralized and nonneutralized seed were correlated with the initial seed-surface pH. Not only were the populations of GB03 decreased as seed-surface pH decreased on nonneutralized seed, but the populations on neutralized seed also decreased with decreasing seed-surface pH. The latter trend could be caused by four factors: 1) sulfate salts were formed when the seed were neutralized and insufficiently rinsed, which resulted in a suppression of spore germination and/or growth rate of GB03; 2) acid was absorbed by the seed coat and was not neutralized but was later released during imbibition; 3) the amount of acid retained on the seed following the delinting process was due to a cultivar trait that affects colonization; and/or 4) compounds inhibitory to GB03 could have been released by or present on the seed.

Growth-promoting effects of root-colonizing *Bacillus* spp. and pseudomonads have previously been demonstrated to be cultivar specific on wheat (7,18) and potato (12,19) and may be due to differences in root colonization of the cultivar by the introduced rhizobacterium; thus, the indications of cultivar specificity for GB03 presented in this article need to be investigated further. To determine whether cultivar factors affect GB03 colonization potential, population dynamics of GB03 need to be examined on many cultivars representing various parentage groups. Some cultivars of interest would be those with broad resistance to pathogens, e.g., cultivars similar to GC-510, a multiadversity resistant cultivar. Mechanically delinted seed may be more useful than acid-delinted seed for examining cultivar factors on colonization because the confounding effects of seed-surface pH would be eliminated; however, spermosphere colonization of the seed could be altered by the lint remaining on the seed.

Variability of root colonization by GB03 is in part the result of seed factors that influence both spermosphere and rhizosphere colonization when the bacterium is applied as a seed treatment. The extent to which these factors affect root colonization may depend on the physical and chemical environment of a soil, environmental conditions, and the indigenous microflora present. The effect of these factors must be further examined in order to better formulate GB03 inoculants so that field performance is less variable.

LITERATURE CITED

1. Akiba, Y., Sekijima, Y., Aizawa, K., and Fujiyoshi, N. 1977. Microbial ecological studies of *Bacillus thuringiensis*. III. Effect of pH on growth of *Bacillus thuringiensis* in soil. Jpn. J. Appl. Entomol. Zool. 23:220-223.
2. Akiba, Y., Sekijima, Y., Aizawa, K., and Fujiyoshi, N. 1978. Microbial ecological studies of *Bacillus thuringiensis*. IV. The growth of *Bacillus thuringiensis* in soils of mulberry plantations. Jpn. J. Appl. Entomol. Zool. 24:13-17.
3. Atkinson, T. G., Neal, J. L., Jr., and Larson, R. I. 1975. Genetic control of the rhizosphere microflora of wheat. Pages 116-22 in: Biology and Control of Soil-Borne Plant Pathogens. G. W. Bruehl, ed. American Phytopathological Society, St. Paul, MN.
4. Azad, H. R., Davis, J. R., Schnathorst, W. C., and Kado, C. I. 1985. Relationship between rhizoplane and rhizosphere bacteria and Verticillium wilt resistance in potato. Arch. Microbiol. 140:347-351.
5. Broadbent, P., Baker, K. F., Waterworth, Y. 1971. Bacteria and actinomycetes antagonistic to fungal root pathogens in Australian soils. Aust. J. Biol. Sci. 24:925-944.
6. Buyer, J. S., and Leong, J. 1986. Iron transport-mediated antagonism between plant growth-promoting and plant-deleterious *Pseudomonas* strains. J. Biol. Chem. 261:791-794.
7. Chanway, C. P., Nelson, L. M. and Holl, F. B. 1988. Cultivar-specific growth promotion of spring wheat (*Triticum aestivum* L.) by coexistent *Bacillus* species. Can. J. Microbiol. 34:925-929.
8. Clay, R. P. 1986. Evaluation of *Bacillus subtilis* as a biological seed treatment for the 'Florunner' peanut plant. M.S. thesis. Auburn University, Auburn, AL. 91 pp.
9. Curl, E. A., and Truelove, B. 1986. The Rhizosphere. Springer-Verlag, Berlin. 288 pp.
10. Hayman, D. S. 1970. The influence of cotton seed exudate on seedling infection by *Rhizoctonia solani*. Pages 99-102 in: Root Diseases and Soilborne Pathogens. T. A. Toussoun, R. V. Bega, and P. E. Nelson, eds. Univ. Calif. Press, Berkeley.
11. Hayman, P. A. 1969. Influence of temperature on the exudation of nutrients from cotton seeds and on pre-emergence damping-off by *Rhizoctonia solani*. Can. J. Bot. 47:1663-1669.
12. Howie, W. J., and Echandi, E. 1983. Rhizobacteria: Influence of cultivar and soil type on plant growth and yield of potato. Soil. Biol. Biochem. 15:127-132.
13. Kloepper, J. W. 1983. Effect of seed piece inoculation with plant growth-promoting rhizobacteria on populations of *Erwinia carotovora* on potato roots and daughter tubers. Phytopathology 73:217-219.
14. Kloepper, J. W., Mahaffee, W. F., McInroy, J. A., and Backman, P. A. 1991. Comparative analysis of five methods for recovering rhizobacteria from cotton roots. Can. J. Microbiol. 37:953-957.
15. Kloepper, J. W., and Schroth, M. N. 1978. Plant growth-promoting rhizobacteria on radishes. Pages 879-882 in: Proc. Int. Conf. Plant Pathog. Bact., 4th.
16. Kloepper, J. W., and Schroth, M. N. 1981. Plant growth-promoting rhizobacteria and plant growth under gnotobiotic conditions. Phytopathology 71:642-644.
17. Kloepper, J. W., Zablotowicz, R. M., Tipping, E. M., and Lifshitz, R. 1991. Plant growth promotion mediated by bacterial rhizosphere colonizers. Pages 315-326 in: The Rhizosphere and Plant Growth. D. L. Keister and P. B. Cregan, eds. Kluwer Academic Publishers, The Netherlands.
18. Kucey, R. M. N. 1988. Plant growth-altering effects of *Azospirillum brasilense* and *Bacillus* C-11-25 on two wheat cultivars. J. Appl. Bacteriol. 64:187-196.
19. Leben, S. D., Wadi, J. A., and Easton, G. D. 1987. Effects of *Pseudomonas fluorescens* on potato plant growth and control of *Verticillium dahliae*. Phytopathology 77:1592-1595.
20. Liddell, C. M., and Parke, J. L. 1989. Enhanced colonization of pea taproots by a fluorescent pseudomonad biocontrol agent by water infiltration into soil. Phytopathology 79:1327-1332.
21. Neal, J. L., Jr., Atkinson, T. G., and Larson, R. I. 1970. Changes in the rhizosphere microflora of spring wheat induced by disomic substitution of a chromosome. Can. J. Microbiol. 16:153-158.
22. Nelson, E. B., and Craft, C. M. 1989. Comparative germination of culture-produced and plant-produced sporangia of *Pythium ultimum* in response to soluble seed exudates and exudate components. Phytopathology 79:1009-1013.
23. Ownley, B. H., Weller, D. M. and Alldredge, J. R. 1990. Relation of soil chemical and physical factors with suppression of take-all by *Pseudomonas fluorescens* 2-79. In: Plant Growth-Promoting Rhizobacteria—Progress and Prospect. C. Keel, B. Koller, and G. Défago, eds. IOBC/WPRS Bull. XIV/8:299-301.
24. Schlub, R. L., and Schmitthenner, A. F. 1978. Effects of soybean seed coat cracks on seed exudation and seedling quality in soil infested with *Pythium ultimum*. Phytopathology 68:1186-1191.
25. Schroth, M. N., and Hancock, J. G. 1982. Disease-suppressive soil and root-colonizing bacteria. Science 216:1376-1381.

26. Schroth, M. N., Weinhold, A. R., and Hayman, D. S. 1966. The effect of temperature on quantitative differences in exudates from germinating seeds of bean, pea and cotton. Can. J. Bot. 44:1429-1432.
27. Suslow, T., and Schroth, M. N. 1982. Rhizobacteria of sugar beets: Effects of seed application and root colonization on yield. Phytopathology 72:199-206.
28. Turner, J. T., and Backman, P. A. 1989. Severity, distribution and losses from tap root cankers caused by *Rhizoctonia solani* in peanuts. Peanut Sci. 15:73-75.
29. Turner, J. T., and Backman, P. A. 1991. Factors relating to peanut yield increases after seed treatment with *Bacillus subtilis*. Plant Dis. 75:347-352.
30. Weller, D. M. 1983. Colonization of wheat roots by a fluorescent pseudomonad suppressive to take-all. Phytopathology 73:1548-1553.
31. Weller, D. M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. 26:379-407.
32. West, A. W., Burges, H. D., Dixon, T. J., and Wyborn, C. H. 1985. Survival of *Bacillus thuringiensis* and *Bacillus cereus* spore inocula in soil: Effects of pH, moisture, nutrient availability and indigenous microorganisms. Soil Biol. Biochem. 17:657-665.
33. West, A. W., Burges, H. D., and Wyborn, C. H. 1984. Effect of incubation in natural and autoclaved soil upon potency and viability of *Bacillus thuringiensis*. J. Invertebr. Pathol. 44:121-127.
34. Xu, G.-W., and Gross, D. C. 1986. Field evaluations of the interactions among fluorescent pseudomonads, *Erwinia carotovora*, and potato yields. Phytopathology 76:423-430.
35. Zablotowicz, R. M., Press, C. M., Lyng, N., Brown, G. L., and Kloepper, J. W. 1992. Compatibility of plant growth promoting rhizobacterial strains with agrichemical applied to seed. Can. J. Microbiol. 38:45-50.