Field Persistence and Efficacy of Five Bacterial Preparations for Control of Peanut Leaf Spot

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ABSTRACT

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Five lyophilized bacterial preparations were evaluated in field trials for persistence and efficacy against Cercospora leaf spot of peanut (causal agent *Cercospora arachidicola*). Bacteria were applied as aqueous suspensions of wettable powders or as dusts, at biweekly intervals, to peanut cultivar Florigiant foliage at three locations in North Carolina and Virginia. Leaflets were sampled periodically to determine survival of applied bacteria and disease progression. Numbers of each bacterial strain recovered declined from about 10^3–10^4 cfu/leaflet (3×10^3–3×10^4 cfu/g fresh weight) to 10–10^3 cfu/leaflet (30–3×10^3 cfu/g) over most 2-wk intervals. Background populations in untreated plots averaged about 10^4 cfu/leaflet (3×10^4 cfu/g) and mostly consisted of gram-negative rod-shaped bacteria. Survival of gram-positive spore-forming bacteria (*Bacillus* spp.) formulated as wettable powder was less variable than survival of gram-negative, non-spore-forming *Pseudomonas cepacia* formulated as wettable powder or dust, and mean log populations were higher for *Bacillus* spp. However, *P. cepacia* controlled disease more effectively. End-of-season disease severity and area under the disease progress curve were significantly lower with several bacterial formulations than in control plots, but bacteria were less effective than the chemical fungicide chlorothalonil.

Epiphytic bacteria have potential to control foliar plant diseases through antagonistic activity against pathogens (1,4–6,9,10,13,14); however, the level of control observed in field trials has been variable and usually lower than that obtained with the best available fungicides (13,14). Inadequate disease control after application of antagonistic bacteria may result from poor survival of the biological control (biocontrol) agents in the field (5,6,9), but few researchers have attempted to monitor survival of potential biocontrol agents on plant surfaces during field trials. Field application of foliar antagonists is in some ways analogous to application of chemical fungicides (14), and quantitative assessment of residue dynamics is necessary to predict their performance in the field. This information is also necessary to increase biocontrol efficacy through selection of superior agents, improved formulation techniques, or more appropriate application schedules. In this study, bacterial strains with potential as biocontrol agents were formulated as wettable powders and dusts and applied to peanut foliage on a biweekly schedule at three locations. Their survival and efficacy in controlling peanut leaf spot (causal agent: *Cercospora arachidicola* Hori) were monitored in the field over a 12-wk period, and disease control was compared with that obtained using chlorothalonil fungicide.

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MATERIALS AND METHODS

Biocontrol agents. Four strains of bacteria recognized as potential biocontrol agents for peanut leaf spot (4,13) were selected for this study: *Bacillus thuringiensis* Berliner (HD-1), *B. thuringiensis* (HD-521), *B. cereus* var. *mycoides* (Fluegge) Smith, Gordon, & Clark (Ox-3), and *Pseudomonas cepacia* Burkholder (Pc742). *Bacillus* strains are gram-positive spore-forming rods; Pc742 is a gram-negative non-spore-forming rod.

Production of lyophilized powders. Bacteria were grown for 24 hr at 30 C in 2-L flasks containing 1 L of Difco nutrient broth + 0.5% dextrose. Fermentation tanks (14-L) containing 9 L of nutrient broth + 0.5% dextrose were inoculated with the liquid cultures, and 50 ml of antifoam agent (Thomas Scientific Co., Swedesboro, NJ) was added to each tank. Fermenters were run for 24 hr (30 C, constant aeration [10 L/min] agitated at 350 rpm), by which time a density of about 10^9 colony-forming units (cfu) per milliliter had been reached. For the *Bacillus* spp., 95–100% of the population had formed endospores by this time. Bacterial suspensions were concentrated to a volume of 1 L with a Millipore filtration system (Millipore Filter Corp., Bedford, MA) and filters of 0.5-μm (*Bacillus* spp.) or 0.25-μm (*P. cepacia*) pore size. Suspensions were then centrifuged for 20 min at 3,000 rpm. Pellets were resuspended in 400 ml of 10% aqueous lactose solution and poured into Teflon-coated aluminum pans ($20 \times 25 \times 3$ cm; 200 ml of suspension per pan). Pans were placed in a freezer at −70 C for 2 hr, then transferred to a lyophilizer and held under vacuum for 24 hr. Lyophilized powders were subsequently stored in glass bottles at room temperature. Periodically (2- to 4-wk intervals), samples of stored powders were serially diluted and plated onto Difco nutrient agar to determine viability of the stored product.

Field application of biocontrol agents and chlorothalonil fungicide. Field plots of peanut cultivar Florigiant were established at Newsoms, VA, and Rocky Mount and Oxford, NC. Fields at Newsoms and Rocky Mount were on land where peanuts were grown for several years and were adjacent to other fields with peanuts. At Oxford, there was no recent history of peanut farming and there were no other peanut fields nearby. Plots were four rows wide and 15 m long, and seven treatments were laid out in a randomized block design with five replicates, except chlorothalonil fungicide (treatment 7) was not applied at Oxford. Treatments were as follows: 1) unsprayed control; 2) HD-1, aqueous suspension, 10^7 cfu/ml; 3) HD-521, aqueous suspension, 10^6 cfu/ml; 4) Ox-3, aqueous suspension, 10^7 cfu/ml; 5) Pc742-WP (wettable powder), aqueous suspension, 10^7 cfu/ml; 6) Pc742-D (dust), dust formulation (10% bacterial powder +

90% talc), final concentration = 10^8 cfu/g; and 7) chlorothalonil (Bravo 500; 500 g a.i./L formulated) applied at a rate equal to 2.48 L/ha. A wetting agent (0.01% Tween 20, Fisher Scientific Co., Fair Lawn, NJ) was added to aqueous formulations. The wetting agent was not fungicidal or bactericidal at this concentration (H. W. Spurr, Jr., *unpublished*). Treatments were applied with a modified backpack sprayer mounted on a wheeled cart, with three nozzles per row located about 15 cm above the plant canopy. Application pressure was 50 psi. The sprayer was moved along each row at a constant speed so that about 1 L of spray material was applied to each plot on each spray date. The dust formulation (treatment 6) was applied at a rate of 350 g/plot by shaking from a jar with holes punched in the lid. Treatments were applied at 14-day intervals starting on 2 July (Rocky Mount), 3 July (Newsoms), or 8 July (Oxford). Six sprays were applied at each location. Sprays were always applied between 9:00 and 11:00 A.M.

Monitoring persistence of bacterial populations. Peanut leaves from treatments 1–6 were sampled immediately before and 1 hr after each spray at each location. At Oxford, samples were also taken at 2, 4, and 10 days after each spray. From each plot, 10 leaves (40 leaflets) were randomly sampled from throughout the canopy in the two center rows, then placed in a plastic bag and held on ice for 1–3 hr while in transit to the laboratory. This pooled sample was placed in a blender with 100 ml of 10 mM K_2HPO_4 buffer solution (pH 6.8) and blended for 10 sec. The homogenate was strained through two layers of cheesecloth and serially diluted, then 0.1-ml samples were spread onto four plates of semi-selective medium, either nutrient agar + 0.1% chlorothalonil (treatments 1–4) or *P. cepacia*-selective medium (3) (treatments 1, 5, and 6). Control (treatment 1) plates were incubated 1–3 days at room temperature. Plates from treatments 2–4 were incubated 24 hr at 30 C. Colonies of *Bacillus* spp. were easily distinguished by colony size and morphology. Randomly selected colonies were observed microscopically to verify the presence of large endospore-containing bacilli. Plates from treatments 5 and 6 were incubated for 5–7 days at room temperature, and colonies of *P. cepacia* were identified by colony morphology and color on the selective medium. Colony counts for each set of four plates were averaged. Bacteria recovered from unsprayed plots were checked for the possibility of a background microflora similar in appearance to *Bacillus* spp. or *P. cepacia*, but such colonies were rarely observed. Two parameters were used to compare bacterial populations in different treatments. First, percent survival for each treatment and spray date was calculated as the number of bacteria isolated 2 wk after spraying divided by the number isolated immediately after spraying × 100%. Arc sine transformation (11) of percent values was performed before analysis of variance. Second, the mean log population during each 2-wk interval was calculated, using two (Rocky Mount, Newsoms) or five (Oxford) observations per interval. Where analysis of variance indicated significant treatment effects, Duncan's multiple range test (11) was used to separate treatment means.

Peanut leaf spot disease progress. Naturally occurring inoculum of *C. arachidicola* caused disease at Newsoms and Rocky Mount, where leaf spot is prevalent every year. At Oxford, a leaf spot epidemic was started by inoculating plants in guard rows on 1 July with conidia of *C. arachidicola*. Conidia were obtained from 1-wk-old cultures grown on acidified potato-dextrose agar at room temperature. About 1 L of conidial suspension (10^4 conidia per milliliter) was applied per 100 m of guard row. Disease progress at each location was monitored at biweekly intervals as follows: Five stems were randomly sampled from the center two rows of each plot. The number of nodes and the number of missing leaflets or leaflets with visible leaf spot symptoms were determined, and disease severity (percent disease) was calculated as the percentage of leaflets defoliated plus those with leaf spots. Two measures were used for statistical analysis of treatments: percent disease at the last observation date (24–30 September) and area under the disease progress curve (AUDPC). AUDPC was calculated by making linear interpolations between disease levels on successive assessment dates, then calculating the area under the curve for each replicate:

$$\text{AUDPC estimates } \sum_{i=0}^{n} (\text{percent disease})_i,$$

where i = days after planting and n = last observation date. Analysis of variance was performed to determine the significance of treatment effects, and Duncan's multiple range test was used to separate treatment means.

RESULTS

Persistence of applied bacteria. Recoverable populations in all five bacterial treatments generally declined between applications (Figs. 1 and 2). Numbers of *Bacillus* spp. dropped about 90–99% during most 2-wk intervals. Populations of Pc742 (wettable powder or dust formulation) were more variable than those of *Bacillus* spp.; reductions in Pc742 populations over 2-wk intervals ranged from less than 10% to more than 99%. Treatment means for percent survival and mean log populations are shown in Table 1. Analysis of variance indicated no significant effect ($P > 0.1$) of location or spray date on means for either survival parameter. There were no significant treatment differences ($P > 0.1$) for percent survival values; however, mean log populations differed significantly ($P < 0.05$) among treatments. Mean log populations were lowest for the Pc742 dust formulation and highest for *B. thuringiensis* (HD-1). Results from the Oxford location showed that mortality of bacteria was most rapid immediately after application for both *Bacillus* spp. and *P. cepacia* formulations (Figs. 1 and 2). Several days after application, numbers of all strains often appeared to level off (at about 100 cfu/leaflet) or to

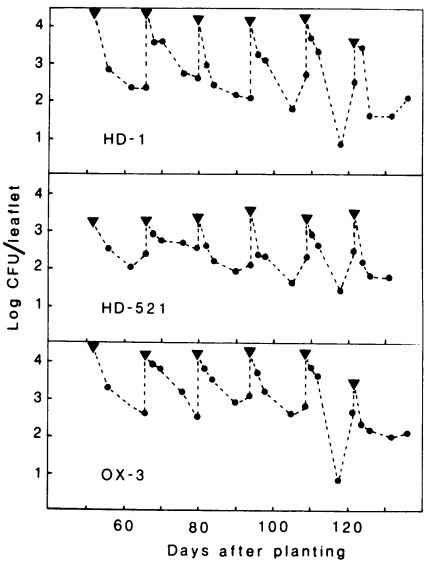

Fig. 1. Persistence of *Bacillus* spp. sprayed onto foliage of Florigiant peanut at Oxford, NC. Treatments: HD-1 = *Bacillus thuringiensis* strain HD-1, 10^7 cfu/ml; HD-521 = *B. thuringiensis* strain HD-521, 10^6 cfu/ml; and Ox-3 = *B. cereus* var. *mycoides* strain Ox-3, 10^7 cfu/ml. Mean bacterial populations monitored immediately after spraying (triangles) and at subsequent sampling dates (dots) are shown.

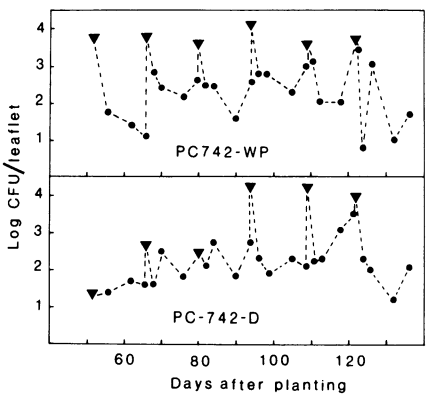

Fig. 2. Persistence of *Pseudomonas cepacia* strain Pc742 sprayed onto foliage of Florigiant peanut at Oxford, NC. Treatments: PC742-WP = wettable powder, 10^7 cfu/ml, and Pc742-D = dust formulation, 10^8 cfu/g. Mean bacterial populations monitored immediately after spraying (triangles) and at subsequent sampling dates (dots) are shown.

increase; however, populations generally did not return to initial levels. Mean populations of bacteria in unsprayed plots, averaged over all sampling dates and locations, were 1.2×10^4 cfu/leaflet (4×10^4 cfu/g). There were no significant ($P > 0.1$) differences among locations. Background populations consisted primarily of nonpigmented or yellow gram-negative rod-shaped bacteria. We did not detect seasonal trends in background populations.

Table 1. Persistence of five bacterial preparations on peanut foliage

Treatments[x]	Mean percent remaining after 14 days[y]	Mean log cfu/leaflet[z]
HD-1	0.32	3.14 a
Ox-3	0.21	3.11 ab
HD-521	2.50	2.64 bc
Pc742-WP	4.56	2.23 cd
Pc742-D	3.20	2.08 d

[x] Treatments: *Bacillus thuringiensis* strain HD-1, 10^7 cfu/ml; *B. thuringiensis* strain HD-521, 10^6 cfu/ml; *B. cereus* var. *mycoides* strain Ox-3, 10^7 cfu/ml; *Pseudomonas cepacia* strain Pc742-WP (wettable powder), 10^7 cfu/ml; and *P. cepacia* strain Pc742-D (dust), 10^8 cfu/g.

[y] Percentage of bacteria persisting after each 14-day period was calculated as the number of bacteria isolated 2 wk after spraying divided by the number isolated immediately after spraying × 100%. Percentages were transformed (arc sine transformation) before statistical analysis. Values shown are detransformed means of arc sine values over the season for all locations. There were no significant differences ($P > 0.1$) among dates of spraying, locations, or treatment means.

[z] Means of log (cfu/leaflet) for all samples over the season of all locations. There were no significant differences ($P > 0.05$) among dates of spraying or locations. Means followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test for separation of treatment means.

Disease control. Disease progress curves for treatments at the three locations are shown in Figure 3. Although patterns of disease increase were similar at different locations, the onset of rapid disease increase at Oxford was 30–40 days later than at Rocky Mount or Newsoms. Analysis of variance, with treatments blocked by location, indicated that both disease severity (percent disease) and AUDPC differed significantly ($P < 0.01$) among treatments and among locations. There was no significant treatment × location interaction ($P > 0.5$). Disease severity and AUDPC were similar at Rocky Mount and Newsoms but significantly less at Oxford. Duncan's multiple range test indicated that disease severity was significantly less ($P < 0.05$) in plots treated with fungicide or three of the biocontrol agents (HD-1, Pc742-WP, and Pc742-D) than in unsprayed plots (Table 2). All biocontrol agent and fungicide treatment means for AUDPC were significantly lower than in unsprayed plots. Chlorothalonil was significantly better than all other treatments in reducing both percent disease and AUDPC, and Pc742-WP was intermediate between chlorothalonil and the other biocontrol agents.

DISCUSSION

Reports on field studies of foliar disease biocontrol with antagonistic bacteria include trials on tomatoes, tobacco, peanut, pine seedlings, and other crops (1,6,10,13). Only a few of these field trials included monitoring populations of the biocontrol agent at periodic intervals during the experiment. Leben et al (10) reported a 99% reduction after 1 day in the viability of bacterial antagonists on apple leaves. During a 24-hr period, Knudsen and Hudler (5) observed less then 0.01% survival of *P. fluorescens* cells applied in a water suspension to pine foliage, but survival was higher than 10% when bacteria were applied in a dilute nutrient solution. Because candidate biocontrol agents are usually selected on the basis of some dose-response relationship observed in vitro or in the greenhouse, information about population levels during a field experiment is part of the minimum data set needed to adequately evaluate potential control agents. In addition, such data serve as a basis to compare persistence characteristics of different biocontrol agents, to evaluate different formulations for their effects on persistence, and eventually to optimize application schedules to ensure that adequate population levels are maintained on foliar surfaces.

Interactions among microorganisms, nutrient availability, and a dynamic microenvironment ensure that populations of bacteria on leaf surfaces will fluctuate. In the field experiments described here, populations of each of the four bacterial strains declined rapidly during the 14-day periods between applications. Populations of *P. cepacia*,

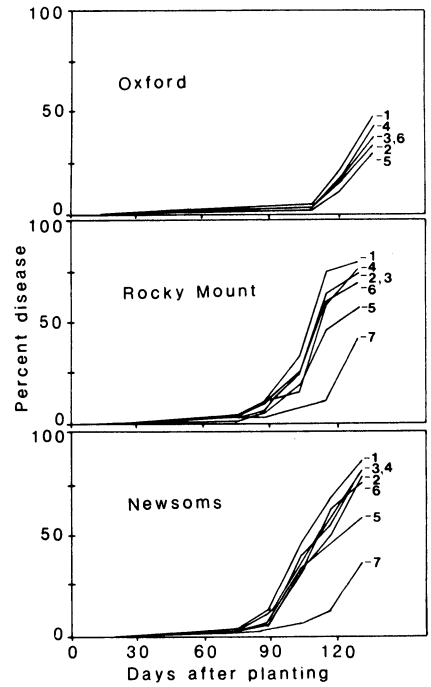

Fig. 3. Disease progression of peanut leaf spot on Florigiant peanut treated biweekly with biocontrol agents or chlorothalonil fungicide. Treatments: 1) unsprayed; 2) *Bacillus thuringiensis* strain HD-1, 10^7 cfu/ml; 3) *B. thuringiensis* strain HD-521, 10^6 cfu/ml; 4) *B. cereus* var. *mycoides* strain Ox-3, 10^7 cfu/ml; 5) *Pseudomonas cepacia* strain Pc742 (wettable powder), 10^7 cfu/ml; 6) *P. cepacia* strain Pc742 (dust), 10^8 cfu/g; and 7) chlorothalonil fungicide, 2.48 L/ha. Disease progression was monitored at 2-wk intervals by sampling five stems per replicate (five replicates per treatment per location) and determining the mean percentage of leaflets defoliated or with leaf spots.

Table 2. Efficacy of five bacterial preparations and chlorothalonil fungicide for control of peanut leaf spot

Treatment[v]	Percent disease[w] Oxford	Rocky Mount	Newsoms	DMR[y]	AUDPC[x] Oxford	Rocky Mount	Newsoms	DMR
Unsprayed	48	80	86	a	1,011	2,415	2,570	a
HD-521	39	74	81	ab	726	2,088	2,154	b
Ox-3	43	76	81	ab	834	1,885	2,167	b
HD-1	34	74	78	b	784	1,937	1,937	b
Pc742-D	38	70	77	b	734	2,049	2,138	b
PC742-WP	31	58	59	c	553	1,443	1,763	c
Chlorothalonil	...[z]	43	36	d	...	843	667	d

[v] Treatments: unsprayed control; *Bacillus thuringiensis* strain HD-1, 10^7 cfu/ml; *B. t huringiensis* strain HD-521, 10^6 cfu/ml; *B. cereus* var. *mycoides* strain Ox-3, 10^7 cfu/ml; *Pseudomonas cepacia* strain Pc742-WP (wettable powder), 10^7 cfu/ml; *P. cepacia* strain Pc742-D (dust), 10^8 cfu/g; and chlorothalonil fungicide, 2.48 L/ha.

[w] Mean percentage of leaflets defoliated and/or with leaf spot symptoms.

[x] Area under the disease progress curve: summation of daily observed or interpolated percent disease values over the season.

[y] Means followed by the same letter are not significantly different ($P > 0.05$) according to Duncan's multiple range test for separation of treatment means.

[z] Not done at this location.

whether applied as wettable powder or dust formulations, were more variable than populations of *Bacillus* spp. Presumably, *Bacillus* strains survived largely as spores, which may be less sensitive to environmental conditions. Although populations of *P. cepacia* dropped off rapidly immediately after application (Fig. 2), rates of decline apparently slowed thereafter. Populations sometimes leveled off near a density of about 100 cfu/leaflet and increased in several instances. Thus, mean percentages of bacteria remaining after 14 days were often higher for *Pseudomonas* strains than for *Bacillus* strains; however, because of extreme variability, seasonal means were not significantly different (Table 1). Originally, we anticipated that applying *P. cepacia* as a dry (dust) formulation might reduce the initial high mortality associated with rapid rehydration of lyophilized bacteria and subsequent exposure to drying at midday temperatures. The survival data, however, do not indicate better persistence of bacteria in the dust formulation.

Because population changes were more consistent between locations and over time for the *Bacillus* strains than for formulations of *P. cepacia*, relatively simple predictive models might be used to estimate numbers of viable *Bacillus* over time and with reasonable accuracy. This predictive ability, coupled with dose-response information, could be a basis for predicting biocontrol efficacy in the field. A similar approach was taken by Brand and Pinnock (2), who used exponential decay models to predict persistence of *B. thuringiensis* spores on foliage and related those numbers to mortality of herbivorous insects (12). Knudsen and Hudler (6) described a computer simulation model to predict persistence of *P. fluorescens* on red pine foliage under varying environmental conditions and effects on conidial germination of the pathogen *Gremmeniella abietina* (Lagerb.) Morelet. A dose-response model linked persistence and efficacy of the control agent. Eventually, survival and efficacy data for biocontrol agents on foliage may be incorporated directly into a predictive model of seasonal disease progress. We have previously described a prototype of such a system (7), based on a computer simulation of Cercospora leaf spot on peanut (8).

Application of *B. thuringiensis* HD-1 and both formulations of *P. cepacia* Pc742 resulted in significantly lower percent disease and AUDPC levels than in untreated plots. Visible differences between plots treated with Pc742-WP and control plots could be readily distinguished in the field at the end of the season. Differences between HD-1 or Pc742-D treatments and controls, though statistically significant, were not readily visible in the field and may not be of practical significance. None of the biocontrol treatments performed as well as chlorothalonil fungicide at the locations (Rocky Mount or Newsoms) where fungicide was applied. Adequate models to relate yield loss to disease severity for Florigiant peanut affected by Cercospora leaf spot are not yet available. Development of models to predict the relationship between disease severity (perhaps at multiple points) or AUDPC and yield are necessary to establish benefit/cost ratios for different control measures and to determine the level of disease control that a successful biocontrol agent must provide.

It is apparent that at least one of the biocontrol agents described herein (Pc742-WP) has potential for management of peanut leaf spot, but significant improvements in performance are still needed to equal the performance of chemical fungicides. Lyophilized powder formulations have the advantage of being easy to work with and incorporate into a spray program. Monitoring population trends as described provides an information benchmark that is necessary if performance of biocontrol agents is to be improved through better formulation, adjustment of dosage, or spray scheduling. Quantification of biocontrol agent persistence will also help make biological control more predictable, thus removing a major impediment to its acceptance by growers.

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