Biological Control of Geranium Rust by *Bacillus subtilis*

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ABSTRACT

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Twelve strains of *Bacillus* were isolated from the leaves of geranium cultivars and tested for their effect on spore germination of *Puccinia pelargonii-zonalis*, the causal agent of geranium rust. Of these, strain 3 of *B. subtilis*, isolated from a rust-infected geranium leaf, inhibited spore germination and reduced the incidence of rust pustules on inoculated leaves in the greenhouse. The inhibitory agent(s) was present in the culture filtrate of strain 3 and was retained in or on washed bacterial cells. The culture filtrate was most inhibitory in decreasing the amount of pustules per leaf area, followed by the washed bacterial cell treatment. Nutrients affected the pathogen/antagonist interaction. Cells cultured and applied to leaves in nutrient broth were more effective in reducing rust development compared with a culture filtrate. Eugon broth as a culture medium enhanced the antagonistic effect compared with cultures produced in nutrient broth. When bacteria were applied for different periods before inoculation with rust spores, the antagonistic effect persisted for at least 4 days after application.

Geranium rust, *Puccinia pelargonii-zonalis* Doidge, is a pathogen of the cultivated garden geranium, *Pelargonium* × *hortorum* Bailey. In 1926, rust was first discovered on geranium by E. M. Doidge in South Africa (8). Since then, the pathogen spread throughout Africa, New Zealand, and Europe. In 1955, rust was intercepted on plants entering mainland United States from Hawaii, and by 1967, it was found in greenhouses within the United States and Canada, resulting in an immediate quarantine of such facilities and the destruction of infected plants. The exception has been California where the rust is endemic.

P. pelargonii-zonalis is an autoecious, microcyclic rust characterized by rust-colored uredial pustules on the upper and lower leaf surfaces, which are more predominant on the underside of the leaf. As the disease progresses, a ring of secondary pustules develop around the primary pustule, resulting in cosmetically disfigured plants that are unsaleable.

Control measures include inspection and quarantine procedures, fungicide applications, and cultural methods, e. g., hot air and hot water treatments (11). The incorporation of genetic resistance into the garden geranium has been presented as a possible control (13).

These control methods are effective but have their disadvantages. Fungicides are costly and their application is time consuming. Cultural methods can injure plants, are labor intensive, and are not acceptable to commercial growers. Inspection and quarantine procedures impose harsh economic pressure on growers. Resistant germ plasm would be an excellent alternative; however, years of breeding may be required to develop an acceptable cultivar. Even with these combined control efforts, the appearance of rusted plants in greenhouses within the United States still occurs. The purpose of this research was to evaluate the feasibity of biological control for geranium rust by the use of a bacterial antagonist in the genus *Bacillus*. This genus was chosen because species are ubiquitious, possess a resistant spore stage, and produce a multitude of broad-spectrum antibiotic compounds; and greenhouse and field control of rust diseases has been achieved with applications of *Bacillus* spp. (1,2,7,15,16). Objectives were to isolate a bacterial antagonist(s) and determine if the antagonist(s) or its metabolites can reduce geranium rust in the greenhouse.

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MATERIALS AND METHODS

Isolation of antagonist. Isolations were made from the geranium cultivars whose reactions to rust ranged from immune to highly susceptible, including Salmon Queen, Schone von Grenchen, Pink Camellia, Springtime Irene, and Snowmass, respectively. Additional isolations were made from a rust-infected plant of the cultivar Snowmass. Leaves were pressed onto nutrient agar (NA, Difco Laboratories, Detroit, MI) for 1 min and then removed. Plates were incubated for 24 hr at 41 C. Colonies with characteristics of *Bacillus* spp. were isolated individually and purified by streaking them on NA and yeast-dextrose calcium carbonate (YDC) agar (25).

Preliminary tests confirmed that 12 isolates were *Bacillus* spp. These included the presence or absence of a fermentative metabolism, Gram and spore stains, the presence of oxidase or catalase, and colony morphology on NA and YDC agar (10,20).

Bioassay. A spore germination bioassay similar to that of Spurr's (21) was used. Urediniospores of *P. pelargonii-zonalis* were collected from infected geraniums, air-dried for 24–72 hr, and stored in liquid nitrogen. Before each assay, spores were withdrawn from the liquid nitrogen, heat shocked for 6 min at 42 C, and hydrated 4–5 hr in sterile tap water.

Each assay consisted of three treatments, including rust spores in sterile tap water (control), a known strain of *B. subtilis* (Ehenberg) Cohn (strain 13; The Pennsylvania State University, Department of Plant Pathology), and one of the 12 isolates from geranium leaves. A water suspension of the candidate antagonist and the known strain of *B. subtilis* was made from 48-hr cultures maintained on NA slants. Bacterial densities were standardized by adjusting the suspension to 50% transmittance as measured with a spectrophotometer at 640 nm.

Approximately 0.2 ml of the control, *B. subtilis*, and the candidate antagonist were added separately to depression wells of double-depression microscope slides. Urediniospores (0.05 mg) were added to all wells, and slides were incubated for 24 hr in a sterile petri dish containing filter paper moistened with distilled water. Fifty spores per well per treatment were counted, and the number germinating was recorded. Each candidate antagonist was replicated three to six times.

Several strains proved to be antagonistic in the bioassays; however, only one isolate (strain 3) consistently inhibited urediniospore germination when compared with water controls. This antagonist was identified as *B. subtilis* based on the following

characteristics: Gram positive, presence of an endospore, catalase positive, oxidase negative, growth in anaerobic agar negative, Voges-Proskauer positive, hydrolysis of starch, colonies rough, wrinkled, and dull on YDC agar.

Biological control of *P. pelargonii-zonalis*. The most promising antagonist, *B. subtilis*, strain 3, was tested for its ability to control rust on geranium. Test plants included cultivars Snowmass (diploid) and Springtime Irene (tetraploid) and were chosen because of their high susceptibility to geranium rust (11,13). Culture-virus-indexed plants (18) from Oglevee Associates Inc., Connellsville, PA, were maintained in the containment facility at the Foreign Disease-Weed Science Research Unit, Frederick, MD. Six to eight weeks after transplanting, fully expanded leaves in positions two, three, and four (counting from the apex downward) were inoculated. Treatments consisted of a sterile distilled water control (C), sterile nutrient broth (NB; Difco), washed bacterial cells (WC), and a cell-free culture filtrate (CF). All treatments were applied to leaves before inoculation with rust spores.

The WC treatment was prepared by centrifuging a 48-hr shake culture (50 ml in 250-ml flask, 21 C) of the antagonist to obtain a pellet of bacterial cells. These cells were washed twice by centrifugation and resuspended in 250 ml of sterile distilled water, rendering a population density of approximately 10^6 colony-forming units (cfu)/ml (verified by dilution plating on NA). The CF treatment consisted of the filter-sterilized supernatant from the washed cells; sterility was confirmed by plating on NA. Four plants of each cultivar, three leaf positions each, were sprayed with each treatment. Treatments were applied to the upper and lower surfaces until runoff. When dry, approximately 30 min later, plants were inoculated with *P. pelargonii-zonalis*.

Inoculum consisted of 50 mg of urediniospores in 500 ml of sterile distilled water containing 0.1 ml of Tween 20 (2.0×10^4 spores/ml). Inoculum was sprayed to the upper and lower surfaces of the treated geranium leaves. After inoculation, plants were placed in a dew chamber for 17 hr at 100% relative humidity (RH) and at 19–20 C, after which they were removed and placed on a greenhouse bench. The number of pustules per leaf position per plant for all treatments was counted and the total leaf area (square centimeter) measured by a Li-Cor portable area meter (Lambda Instruments, Lincoln, NE) 20 days after inoculation.

Nutrient effect on the antagonist and the development of rust. A cell-free culture filtrate (CF) was compared with bacterial cells cultured and applied in nutrient broth (NB & B) to determine the effect of live cells in a nutrient solution on the development of geranium rust. Both treatments consisted of 48-hr shake cultures (50 ml in 250-ml flask, 21 C) of the antagonist. Bacterial cells were removed from the CF as previously described. A water (C) and a nutrient broth (NB) control also were included. At treatment application time, the bacterial cell suspension (6.0×10^6 cfu/ml), the CF, and controls were sprayed onto leaf surfaces of cultivars Snowmass and Springtime Irene.

In a separate study, the antagonist was grown in NB and in eugon broth (EB, Difco) to further explore the effect of nutrients on biocontrol efficiency. Treatments consisted of a sterile distilled water control (C), NB, EB, a 48-hr NB shake culture (NB and B) and a 48-hr EB shake culture (EB and B) of the antagonist (50 ml each in 250-ml flask, 21 C). The EB and B treatment yielded population densities of 6.0×10^6 cfu/ml, and the NB and B treatment yielded 2.0×10^7 cfu/ml. Leaf position two, three, four, and five of the cultivar Snowmass were inoculated in this experiment.

Treatments in both studies were applied to the upper and lower leaf surfaces until runoff. When dry, 30 min later, all plants were inoculated with urediniospores (2.0×10^4 cfu/ml). All postinoculation manipulations were conducted as previously described.

Longevity of rust inhibition. Treatments consisted of five time periods. Each day, for 5 consecutive days, a 48-hr EB shake culture of the antagonist (6.0×10^7 cfu/ml) was sprayed onto separate groups consisting of four Snowmass plants, leaf positions two, three, and four until runoff. Four additional plants were included each day and were sprayed with EB to serve as controls. Treatments were applied the same time (1500 hr) each day. Plants remained in the greenhouse until the fifth day when they were inoculated with the rust fungus. Greenhouse temperatures ranged from 21 to 29 C during treatment application times and then dropped to an average of 18 C within a 3-hr period, remaining steady from 2000 to 0800 hr.

On the fifth day, all 40 plants were inoculated with spores of *P. pelargonii-zonalis* and placed in a dew chamber for 17 hr at 100% RH and 21 C, after which they were removed and placed on a greenhouse bench. Greenhouse temperatures were monitored 2 wk after inoculation. Maximum daytime temperatures ranged from 26 to 30 C, which occurred each day between 1400 and 1600 hr, then dropped to an average of 15–20 C. RH ranged from 55 to 97% each day.

Experimental design and statistical analyses. A completely randomized design was used for all experiments. Analyses of variance were used to determine significant effects (19,22). Mean separations were performed with the Waller-Duncan *K*-ratio *t*-test. All experiments were repeated once or twice as indicated. Bartlett's test of homogeneity of variances was used to compare within treatment variances in each trial. In most trials, variances were nonhomogeneous, thus raw data were transformed to log $(x + 1)$. Treatment means for individual trials were not pooled because interactions were observed between treatments and trials. The mean number of pustules per square centimeter are presented in the tables; however, analyses were performed on transformed data. Percent control was calculated with the formula $(u - t)/u$, where u is the untreated (control) and t is the treated. Percentages refer to comparisons of treatments with the water control.

RESULTS

Efficacy of the antagonist. Strain 3 of *Bacillus subtilis* significantly ($P = 0.05$) reduced disease severity when compared with a water (C) and a nutrient broth (NB) control (Table 1). The washed bacterial cells (WC) and the cell-free culture filtrate (CF) consistently reduced the number of pustules per square centimeter on both cultivars. Compared with the water control, the filtrate was most inhibitory and controlled rust by 84%, followed by the washed cell treatment, which provided 71% control across trials and cultivars. Significant differences ($P = 0.05$) were observed between the two bacterial treatments on cultivar Snowmass in each trial, with 81 and 66% reductions in rust infection for CF and WC, respectively, as compared with C across trials. A significant difference between these treatments was observed only in one trial for the cultivar Springtime Irene, with the CF reducing infection by 92% and the WC by 81% compared with C. In trials 1 and 2 combined, although not significant, the CF and WC treatments reduced rust infection 91 and 81%, respectively, as compared with

TABLE 1. Effect of washed cells (WC) and a culture filtrate (CF) of *Bacillus subtilis*, strain 3, on pustule development by *Puccinia pelargonii-zonalis* on geranium cultivars Snowmass and Springtime Irene, compared with a nutrient broth (NB) and a water control (C)

	Treatment[x]			
Trial	C	NB	WC	CF
Snowmass				
1	1.20 a[y]	1.41 a	0.23 b	0.07 c
2	1.80 a	1.44 b	0.88 c	0.52 d
3	2.94 a	3.31 a	0.92 b	0.54 c
Mean	1.98	2.05	0.68	0.38
Springtime Irene				
1	0.35 a	0.27 a	0.04 b	0.01 b
2	0.51 a	0.23 b	0.11 b	0.07 b
3	1.86 a	2.00 a	0.35 b	0.15 c
Mean	0.91	0.83	0.17	0.08
Overall mean	1.44	1.44	0.42	0.23

[x] Each value represents the mean (pustules per square centimeter) of 12 observations representing four plants with three leaves per plant.

[y] Means within a trial followed by the same letter within a cultivar are not significantly different according to the Waller-Duncan *k*-ratio *t*-test ($k = 100$, $P = 0.05$).

C for this cultivar. In all trials, cultivar Springtime Irene had fewer pustules per square centimeter than Snowmass for all treatments.

Effect of nutrients on antagonism. When the antagonist was applied in a nutrient solution to leaf surfaces, rust control generally was improved when compared with the cell-free culture filtrate (CF) (Table 2). Nutrient broth plus bacterial cell suspension (NB and B) was most inhibitory and, compared with the water control (C), controlled rust by 93% followed by the CF, which provided 75% control across trials and cultivars. Significant differences ($P = 0.05$) occurred between these treatments in each trial for cultivar Snowmass, where NB and B reduced the number of pustules by 92% and the CF by 70% for this cultivar compared with C. Only in one trial was the difference between these two treatments significant for cultivar Springrime Irene; however, trends were similar in all trials. In this cultivar, the bacterial culture reduced rust infection by 95%, compared with the filtrate, which provided 84% control across trials. Cultivar Springtime Irene had less infection when compared to Snowmass for all treatments in each trial.

When the antagonist was cultured in eugon broth and nutrient broth, significant differences in rust pustule development were observed between the two treatments (Table 3). Bacterial cells cultured in eugon broth (EB and B) inhibited geranium rust by 67% and those cultured in nutrient broth (NB and B) by 24% when compared with water controls (C).

Persistence of the antagonist. Strain 3 of *Bacillus subtilis* cultured in eugon broth significantly inhibited the development of geranium rust at all time periods. (Table 4). Rust inhibition persisted over a 4-day period and was approximately 96% compared with eugon broth controls. Whereas differences occurred among time periods, the inhibitory effect of the bacterium remained stable over time.

TABLE 2. Effect of nutrient broth plus cells (NB and B) and a culture filtrate (CF) of *Bacillus subtilis*, strain 3, on pustule development by *Puccinia pelargonii-zonalis* on geranium cultivars Snowmass and Springtime Irene, compared with a nutrient broth (NB) and a water control (C)

	Treatment[x]			
Trial	C	NB	NB and B	CF
Snowmass				
1	2.94 a[y]	3.48 a	0.14 b	0.54 c
2	2.00 a	1.66 a	0.23 b	1.03 c
3	5.36 a	8.30 b	0.51 c	1.51 d
Mean	3.43	4.48	0.29	1.03
Springtime Irene				
1	1.86 a	2.00 a	0.04 b	0.15 b
2	0.93 a	0.88 a	0.07 b	0.51 c
3	2.46 a	2.98 a	0.13 b	0.17 b
Mean	1.75	1.95	0.08	0.28
Overall mean	2.59	3.22	0.19	0.65

[x]Each value represents the mean (pustules per square centimeter) of 12 observations representing four plants with three leaves per plant.
[y]Means within a trial followed by the same letter within a cultivar are not significantly different according to the Waller-Duncan k-ratio t-test ($k = 100$, $P = 0.05$).

TABLE 3. Effect of *Bacillus subtilis*, strain 3, cultured in nutrient broth (NB and B) and Eugon broth (EB and B) on pustule development by *Puccinia pelargonii-zonalis* on the geranium cultivar Snowmass compared with nutrient broth (NB), Eugon broth (EB), and water controls (C)

	Trial		
Treatment[x]	1	2	Mean
C	3.95 a[y]	1.56 a	2.76
NB	5.96 b	1.63 a	3.80
EB	4.64 c	2.63 b	3.64
NB and B	3.48 a	0.72 c	2.10
EB and B	1.53 d	0.28 d	0.91

[x]Each value represents the mean (pustules per square centimeter) of 12 observations representing four plants with three leaves per plant.
[y]Means within a trial followed by the same letter within a cultivar are not significantly different according to the Waller-Duncan k-ratio t-test ($k = 100$, $P = 0.05$).

DISCUSSION

Various treatment applications of strain 3 of *B. subtilis* consistently arrested the development of geranium rust under greenhouse conditions. In the first experiment, the culture filtrate was generally more inhibitory than the washed bacterial cell treatment. Possibly, this increase in inhibition was the result of inhibitory metabolites produced by the bacterium diffusing into the filtrate and, upon treatment application, exerting a detrimental effect on the pathogen. It is well known that several strains of *B. subtilis* produce a variety of antibiotics, and, in many cases, this has been the postulated mechanism of action by which this antagonist inhibits plant pathogens (3–5,9,12,14,17,23,24). When bacterial cells were washed, these inhibitory substances possibly were removed, resulting in less rust suppression; however, washed cells did control rust infection by 71%. In this case, the inhibitory substances may have been produced by the cells surviving on the leaf surface, or liberated upon death of the cells. The mechanism of action responsible for rust control in our experiments could, therefore, be the result of antibiosis defined by Cook and Baker (4) as, "the inhibition or destruction of one organism by a metabolic product of another."

Studies on the role of nutrients and their effect on the pathogen/antagonist interaction indicate that more than one geranium rust. A toxic metabolite(s) was present in the culture filtrate as well as the nutrient broth and bacterial cell suspension; however, the latter was more inhibitory. The major difference between the three treatments was the presence or absence of bacterial cells. Possibly, a nutrient effect exists that may be viewed as a competition factor, with the bacterial antagonist a better and more efficient competitor in the removal of nutrients than the pathogen.

Results from the media comparison study support the hypothesis that a nutrient effect may be occurring. Growth of the antagonist in eugon broth enhanced its ability to suppress rust compared with growth in nutrient broth. Eugon broth is composed of different nutrients than is nutrient broth (6), and the antagonist may have responded to this difference with an increased production of an inhibitory compound(s). In previous spore germination bioassays, rust urediniospores were able to germinate without external nutrient supplies (sterile tap water). The role that nutrients play is not known; however, the results of the bioassays and those of previous experiments suggest that the antagonist is more affected by the addition of nutrients than the rust pathogen. Results also suggest that competition for nutrients and the production of toxic metabolites by the bacterial antagonist may be occurring simultaneously to suppress the development of geranium rust.

TABLE 4. Effect of *Bacillus subtilis*, strain 3, on pustule development after application to leaf surfaces of geranium cultivar Snowmass 4, 3, 2, 1, and 0 days before inoculation with urediniospores of *Puccinia pelargonii-zonalis*

Time	Trial 1[w]		Trial 2	
period[x]	Control[y]	*B. subtilis*	Control	*B. subtilis*
0	4.26 ab[z]	0.35 c	2.98 c	0.25 e
1	4.21 ab	0.19 cde	3.76 b	0.11 e
2	3.71 b	0.23 cd	5.69 a	0.04 f
3	3.48 b	0.02 e	6.61 a	0.51 d
4	4.76 a	0.11 de	3.06 c	0.05 f

[w]Values represent the mean (pustules per square centimeter) of 12 observations representing four plants with three leaves per plant.
[x]Number of days *B. subtilis* remained on the plant before inoculation with *P. pelargonii-zonalis*.
[y]Eugon broth plus rust spores.
[z]Means within a trial, within time periods, followed by the same letter are not significantly different according to Waller-Duncan k-ratio t-test ($k = 100$, $P = 0.05$).

In all experiments, cultivar Springtime Irene was less susceptible than Snowmass to rust infection. This cultivar is tetraploid, whereas Snowmass is diploid. Possibly, hybrid vigor may be a factor contributing to rust resistance. Another explanation may be that the number of stomates on adaxial and abaxial leaf surfaces of Snowmass were double in number compared with Springtime Irene (Rytter, *unpublished*). This greater number of stomates on the leaf surface may explain the increase in rust infection on Snowmass since *P. pelargonii-zonalis* directly penetrates stomates. Leaf surface morphology of Springtime Irene also may be more conducive to bacterial attachment. Snowmass has a smoother, waxier surface than Springtime Irene. Bacterial adherence or retention of treatment materials may be less on this cultivar.

Our results, especially the persistence of inhibition by the antagonist, suggest that strain 3 of *B. subtilis* could be integrated with current rust control practices. Strain 3 of *B. subtilis* probably could not be used as the only control of geranium rust, because even with the most effective treatment, a few pustules remained on some plants. In commercial production, there is zero tolerance for geranium rust. Bacterial preparations possibly could be alternated with weekly fungicide applications, thereby reducing fungicide costs; however, it first would be necessary to determine the effect of the various fungicides on the bacterium.

Additional research on the mechanisms by which nutrients affect the pathogen, antagonist, or host, and interactions of additional environmental factors and bacterial survivability on the leaf surface need to be elucidated before an effective and sustainable biological control program can be developed.

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