Use of Mixtures of Fluorescent Pseudomonads to Suppress Take-all and Improve the Growth of Wheat

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ABSTRACT

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Fluorescent *Pseudomonas* strains were tested alone and in combinations for the ability to suppress take-all in greenhouse and growth chamber tests and to enhance the growth and yield of wheat in fields infested with *Gaeumannomyces graminis* var. *tritici*. In general, certain combinations of strains enhanced yield, whereas other mixtures and strains used individually did not. Overall, the best combinations were Q2-87 plus Q1c-80 plus Q8d-80 plus Q65c-80, Q2-87 plus Q1c-80 plus Q8d-80 plus Q69c-80, and Q1c-80 plus Q8d-80 plus Q65c-80. For example, in a field test on spring-sown wheat at Pullman, WA, during 1989, the treatment Q2-87 plus Q1c-80 plus Q8d-80 plus Q65c-80 significantly ($P = 0.05$) increased yield 20.4% compared to a nontreated control, whereas each strain used individually had no significant effect on yield. This work demonstrates the potential benefits of using combination treatments to suppress take-all and suggests the importance of additive and interactive effects among introduced bacteria in biological control.

Additional keywords: plant growth-promoting rhizobacteria.

Although numerous microorganisms capable of suppressing plant diseases have been identified, few biocontrol agents have been commercialized, primarily due to their inconsistent performance (35). The complexity of the soil ecosystem makes biological control of root pathogens by introduced bacteria particularly challenging. Many factors contribute to the inconsistent performance of seed- or seedpiece-applied bacteria given the complex interactions among host, pathogen, bacteria, and soil environment. One major factor is variability in root colonization by introduced bacteria (35). Given that multiple steps are involved in colonization, that a variety of biotic and abiotic factors can affect the process (12,18), and that many bacterial traits may contribute to rhizosphere competence (1,7,8,20,32), it is not surprising that introduced bacteria become lognormally rather than normally distributed on root systems (19). Thus, population sizes vary from root to root by several orders of magnitude, and some roots may be completely unprotected. The demonstration by Bull et al (2) of an inverse relationship between the population size of *Pseudomonas fluorescens* 2-79 on wheat roots and the number of take-all lesions indicates that incomplete colonization can reduce the potential for biological control by introduced bacteria.

Another factor that can contribute to inconsistent performance is variable production or inactivation in situ of bacterial metabolites responsible for disease control. Antibiotics, such as phenazine-1-carboxylic acid and 2,4-diacetylphloroglucinol, and other secondary metabolites, such as hydrogen cyanide, contribute to the activity of some biocontrol agents (10,24,30,34,38). Production of these and other metabolites in vitro by *Pseudomonas* spp. depends on cultural conditions (14,27) and in situ production is likely to be even more sensitive to the physical and chemical environment in the rhizosphere. Ownley et al (21) reported that 16 of 28 soil variables examined were either directly correlated (e.g., ammonium nitrogen, sulfate sulfur, zinc, etc.) or inversely correlated (e.g., manganese, iron, percent organic matter, etc.) with the biocontrol activity of *P. fluorescens* 2-79 against take-all. Thus, even if a biocontrol agent is positioned in an infection court, the occurrence of a threshold concentration of a critical metabolite may be temporally separated from the site of infection or spread of the pathogen. For example, in the spermosphere of cotton, expression in *P. fluorescens* Hv37a (13) of the key biosynthetic gene for the antibiotic oomycin A occurred 10 h after planting bacteria-treated seed; however, infection by

Pythium ultimum, which is sensitive to the antibiotic, occurred by 6 h (9).

Inconsistent performance also may result from the occurrence of diseases caused by nontarget pathogens (35). If a biocontrol agent successfully suppresses a target pathogen but has no effect on a second nontarget pathogen that subsequently limits yield, then the treatment will appear ineffective. An accurate evaluation of biocontrol activity against a target pathogen is nearly impossible when there is both a large amount of disease caused by a nontarget pathogen(s) and yield is used as the measure of effectiveness.

Finally, spatial and temporal variability in disease incidence or severity is another factor that can interfere with the ability to evaluate disease suppression effectively. Under environmental conditions that are unsuitable for disease development, the introduction of a biocontrol agent may "fail" to provide an increase in crop yield simply because the disease was not severe enough to limit yield. Further, for many root diseases, areas in a field where severe disease occurs may be unpredictable. Evaluation procedures that do not take into account variability in disease incidence and severity may be ineffective in detecting significant differences.

Take-all of wheat (*Triticum aestivum* L.) caused by *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier var. *tritici* J. Walker (5) is an important root rot disease of wheat and barley. Biological control of take-all of wheat has been studied intensively because of the economic importance of the disease and the frequent absence of economically viable methods of control. One approach has been the application of fluorescent pseudomonads as seed treatments. In Washington state, they have provided significant control of take-all and increased yield up to 26% (relative to nontreated wheat). However, these bacteria were effective only about 60% of the time (35). Biological control of take-all is particularly challenging due to the long duration of host susceptibility to the pathogen, the occurrence of the disease in patches in the field, and the frequent co-occurrence of other pathogens such as *Rhizoctonia solani* and *Pythium* spp. on the same wheat roots.

Classic selection of biocontrol agents for the suppression of take-all and other root pathogens has emphasized the use of individual agents. However, a more ecologically sound approach that addresses the complexity of the environmental interactions that affect root colonization and disease suppression may involve the use of multiple microbial strains. Increasing the genetic diversity of the biological control system through the use of mixtures may result in treatments that persist longer in the rhizosphere and utilize a wider array of biocontrol mechanisms under a broader range of environmental conditions. No doubt the natural biological control that occurs in fields exhibiting take-all decline (28) involves a community of microorganisms (5,26).

In this study, we tested the hypothesis that the use of bacterial strains in combination will improve the consistency and effectiveness of biological control treatments against take-all of wheat. Further, we present an experimental design for testing large numbers of bacterial treatments in field studies that addresses the problem of the patchy distribution of the take-all disease. A preliminary account of this work was published (23).

MATERIALS AND METHODS

Cultures of *G. g. tritici* and preparation of inoculum. Two virulent isolates of *G. g. tritici*, isolated from wheat near Moses Lake and Pullman, WA, and designated R3-111a-1 (39) and SCS, respectively, were used throughout this study. Both isolates were stored on dilute potato dextrose agar (dPDA) (37) supplemented with rifampicin at 100 μg/ml (Sigma Chemical Co., St. Louis) at 4 C and transferred yearly. The *G. g. tritici* isolates were routinely cultured on dPDA at room temperature. Oat kernel inoculum of the pathogen was prepared as previously described (39).

Bacteria. *P. fluorescens* 2-79 (NRRL B-15132) (36) was used as a standard strain for comparison of the biological control activity of other test strains because it has been tested extensively (35,36,39). Strain 2-79 was isolated in 1979 from the roots of wheat grown in pots containing Ritzville silt loam from a field at the Washington State University Dry Land Research Unit at Lind that was in the early stages of take-all decline after 7 yr of continuous wheat.

Bacteria tested for biocontrol activity were isolated from several sources. Strains designated by the prefix Q or R and the suffix 80 were isolated from roots of wheat grown in a pot assay conducted in 1980 (3 [Fig. 7.1]) that had been used to show the transferability of the take-all suppressive factor between soils. Q and R indicate that the soil was from Quincy and Moses Lake, WA (both Shano silt loams), respectively. The two soils were collected during 1979 from fields that had been cropped to wheat for over 20 yr and in which take-all decline was evident. Strains designated by the prefix Q and the suffix 87 were isolated during 1987 from roots of wheat seedlings grown in Shano silt loam collected during 1984 from the Quincy field described above. Before planting, the soil had been amended with root tips from wheat to enrich for bacteria with high rhizosphere competence (25). For long-term storage, the bacteria were either lyophilized, placed in glycerol at −20 or −80 C, or mixed with 1.0% methyl cellulose and dried onto wheat seeds and stored at −20 C.

Strains were identified by standard bacteriological tests (11) and by whole cell fatty acid analyses conducted by Microbial ID, Inc., Newark, DE. Strains also were screened for the production of metabolites that have been shown to play a role in disease suppression, including fluorescent siderophores (pyoverdins) (35), phenazines (24,30), hydrogen cyanide (38), and 2,4-diacetylphloroglucinol (10,15).

Seed treatments. For greenhouse and growth chamber studies, seeds of wheat (cv. Fielder) were surface-disinfested with 1% NaOCl for 2 min and dried on paper in a laminar flow hood. Wheat seeds (cvs. Penawawa and Hill 81) used in field studies were not surface-disinfested. Seeds were coated with bacteria by methods similar to those described previously (39). Briefly, bacteria were cultured on nutrient broth yeast extract agar (NBY) (33) for 48 h at 27 C. The bacteria were suspended in sterile deionized water, and 1.0- to 1.5-ml aliquots of the suspension were inoculated onto plates of King's medium B (KMB) (17) or KMB amended with 100 μM $FeCl_3$. Plates were incubated for 48–72 h at 27 C, and the bacteria were scraped from the plates and suspended in 1.0% methylcellulose. Bacteria were mixed with seed at a rate of approximately one petri plate of bacteria and 3.4 ml of methylcellulose per 14 g of seed. In a combination treatment, each strain was added as the fraction of the total amount of the mixture that it comprised. Coated seeds were dried under a stream of sterile air and populations among treatments generally ranged from 6×10^7 to 8×10^8 cfu per seed. In strain combinations, the relative population size of each strain remained similar after drying. Control seeds either were coated only with methylcellulose or were not treated. In some experiments, seed was treated with Apron FL (metalaxyl; Gustafson, Inc., Dallas) at 1.2 ml/kg of seed, Baytan 30 FL (triadimenol; Gustafson) at 0.76 ml/kg, or a combination of Apron and Baytan at the above rates. Triadimenol and metalaxyl are registered for use on wheat for control of take-all and Pythium root rot, respectively.

Growth chamber tube assay. All strains were tested either individually or in combination in the take-all tube assay described previously (39) with minor modifications. The assay used plastic tubes (2.5-cm diameter × 16.5-cm long) held upright in racks. The bottom of each tube was plugged with cotton and filled with a 6.5-cm-deep layer of sterile vermiculite overlaid with 10 g of soil amended with *G. g. tritici* in the form of colonized oat kernels fragmented and sieved into fractions of known particle sizes. Particles 0.25–0.5 mm in diameter were collected and added to the soil at 0.45 or 0.90% (w/w) (40). Two seeds (cv. Fielder) treated with bacteria were sown per tube and covered with vermiculite. Each tube received 10 ml of water with metalaxyl (0.05 g of wettable powder per 1,000 ml; Ciba-Geigy Corp., Greensboro, NC) to inhibit *Pythium* spp. The rack of tubes was covered with plastic to retain soil moisture and incubated on a laboratory bench at room temperature (21–24 C) to facilitate germination. After 1–2 days the racks were transferred to a growth

chamber and incubated at 15–18 C in a 12-h dark-light cycle. Four days after planting the plastic was removed and each tube received 5 ml of dilute (1:3, v/v) Hoagland's solution (macroelements only) two times per week. Each treatment was replicated three times, and each replicate consisted of a row of 10 separate tubes (20 individual plants). Treatments were arranged in racks in a randomized complete block design. After 3–4 wk, the seedlings were evaluated for disease severity on a scale of 0–8, where 0 = no disease and 8 = plant dead or nearly so (22). Disease rating was compared statistically among treatments by an analysis of variance (ANOVA). Treatment means were compared by a least significant different test (LSD) when significant differences among treatments was indicated by the ANOVA (Fisher's protected LSD). Strains that significantly ($P = 0.05$) decreased the severity of take-all compared to the nontreated control were considered suppressive. Strains that appeared promising were retested at least two times.

Two soils were used in the assay. Most strains initially were screened with Ritzville silt loam from a field containing only native grasses located at the Washington State University Dryland Research Station, Lind. Strains subsequently were tested with Thatuna silt loam (12), collected from a wheat field on the Washington State University Plant Pathology Farm, Pullman. Both soils were collected during the fall of each year, air dried, sieved, and stored in metal cans or cement bins in an unheated shed.

Greenhouse soil-bed assay. Some strains were tested either individually or in combination in a soil bed (1.2 m × 2.25 m × 45 cm) constructed in the floor of a greenhouse. This assay enabled us to test the effectiveness of biocontrol treatments on plants that could be grown to maturity and in a less restrictive environment than in the growth chamber tube bioassay. The bed was filled with a 7-cm-deep layer of crushed rock in the bottom to promote drainage followed by a 34-cm-deep layer of Thatuna silt loam amended with pulverized oat kernel inoculum of *G. g. tritici* at 0.5% (w/w). Seeds were sown in a grid pattern with a plywood board (dimensions of the bed) with 0.5-cm-diameter holes spaced 5 cm apart as a template for planting. The board was laid on the surface of the soil, and a phillips screwdriver was pushed through each hole to create 4-cm-deep depressions in the soil. Three seeds (cv. Fielder) were sown in each hole. After planting, the plywood was removed, and Thatuna silt loam not amended with *G. g. tritici* was added to the surface to cover the holes. Approximately 4 L of full-strength Hoagland's solution (macroelements only) was applied to the plot weekly, and additional water was applied as needed. Plants were grown to the heading stage at 12–20 C under supplemental lighting and then plants along with roots were excavated, washed, and assessed for disease severity on a 0–6 scale, where 0 = healthy plant, 1 = lesions confined to seminal roots, 2 = lesions on seminal roots and the subcrown internode, 3 = lesions up to the first node, 4 = lesions up to the second node, 5 = plants severely stunted and yellow, and 6 = plants dead. Plant height and tiller number also were measured.

Treatments were replicated six times and arranged in a randomized complete block design. Each replication of a treatment consisted of a cluster of plants from six planting holes. A border of nontreated seed was sown around each treatment to minimize the possibility of bacterial contamination from one treatment to another. A border of nontreated seed also was sown around the entire experiment to minimize edge effects. Disease rating, main stem length (height), and tiller number were compared statistically among treatments by ANOVA. Treatment means were compared by Fisher's protected LSD test. Treatments that significantly ($P = 0.05$) reduced the severity of take-all relative to the untreated control were considered suppressive.

Field tests. Strains were tested in research plots established at Pullman, WA, during April and October 1989 (Plant Pathology Research Farm, Thatuna silt loam) (12) and at Mt. Vernon, WA, during May 1989 and 1990 (Washington State University, Northwest Research and Extension Station, Puget silt loam) (2). These sites represent two distinct agroecosystems. Pullman, located in the Palouse region of eastern Washington, has an average annual rainfall of approximately 51 cm. Throughout the Palouse, the severity of take-all is moderate (lesions generally confined to the roots) and becomes severe (lesions common on the base of tillers) when wheat is grown without rotation and with reduced tillage or with irrigation. Mt. Vernon, approximately 300 miles northwest of Pullman in the Skagit Valley, has an average annual rainfall of approximately 99 cm. Throughout the valley, take-all is severe after only one or two crops of wheat. At each field site, 2–3 wk before planting the soil was disced two or three times. Rows (40.6 cm apart) were opened the day of planting with a row opener to a depth of about 6–8 cm.

Treatments were arranged in a highly modified randomized complete block design. The field was divided into five or six replicate blocks. Each replicate block was subdivided into smaller units called "miniblocks." Thus, the resulting design was a series of randomized complete blocks nested within a large randomized complete block design. Each miniblock contained three bacterial seed treatments and three control treatments, and each treatment was sown in three 1.5- to 3.1-m rows (depending on the site). *G. g. tritici*, as whole oat kernel inoculum, was introduced into the furrow immediately before the seed was sown by hand. Controls included methylcellulose-treated seed with *G. g. tritici* inoculum in the furrow, nontreated seed with *G. g. tritici* inoculum (NT+), and nontreated seed without *G. g. tritici* inoculum (NT−). The second control (NT+) was used as the standard against which the other treatments in the miniblock were compared. The third control (NT−) was used to estimate the potential yield in the miniblock in the absence of added inoculum of *G. g. tritici*. Treatments within a miniblock were randomized and miniblocks were positioned randomly within replicate blocks. By partitioning bacterial seed treatments with controls in a miniblock, the design accommodated the patchy nature of the disease yet still allowed for testing of many different treatments over a large test area. As many as 42 treatments were tested in a single field experiment; data for all treatments are not given. Plant height, number of heads, and yield were measured. These fitness variables were compared statistically among treatments within each miniblock by ANOVA and Fisher's protected LSD test. All statistical conclusions were made only within miniblocks. Seed treatments in different miniblocks were compared by calculating the increase or decrease in height, heads, or yield of a treatment relative to that of the NT+ control in the same miniblock. For example:

$$RELATIVE\ YIELD = [(TREATMENT_{YIELD} - NT + CONTROL_{YIELD}) \times 100]/(NT + CONTROL_{YIELD}).$$

However, no statistical conclusions were drawn from these relative comparisons across miniblocks.

In vitro inhibition. Bacterial strains were tested for their ability to inhibit *G. g. tritici* in vitro. Each strain was incubated with shaking in 30 ml of NBY for 24 h at room temperature. Aliquots (5 μl) of a culture were spotted on opposite sides of a petri plate (1.5 cm from the edge) containing either potato dextrose agar (37) (PDA) or KMB. Tests were performed on both media to determine whether a medium favoring *G. g. tritici* (PDA) or a medium favoring the bacteria (KMB) had any effect on inhibition. Sterile, noninoculated NBY (5 μl) was spotted on opposite sides of the plate at right angles to the bacterial treatments as a control. Plates were incubated at 27 C, and after 48 h a 4-mm-diameter plug of *G. g. tritici*, taken from the leading edge of a culture grown on dPDA, was placed in the center of each plate. Plates were incubated for about five more days or until the leading edge of the fungus reached the edge of the plate. The size of the zone of inhibition of fungal growth around each bacterial strain was used as a measure of the ability of that strain to inhibit *G. g. tritici* and was scored as previously described (37): − = no zone of inhibition and the fungus overgrew the bacterial colony; + = a distinct zone of inhibition with fungal growth inhibited less than 6 mm from the point where the bacteria were spotted; ++ = a distinct zone of inhibition with fungal growth inhibited 6–10 mm from the point where the bacteria were spotted; +++

= a distinct zone of inhibition with fungal growth inhibited greater than 10 mm from the point where the bacteria were spotted. Each treatment was replicated six times, and the entire experiment was performed three times.

Bacterial strains were tested for their ability to inhibit the growth of other biocontrol strains. The bacteria were cultured and inoculated onto plates as described above, except that 5-μl aliquots of three separate test strains were spotted once onto plates (1.5 cm from the edge) equidistant from each other. After 24 h the plates were sprayed with a 24-h-old culture of a single strain using a chromatography sprayer and incubated at 27 C. After 24–48 h, the diameter of the zone of inhibition around each test strain was measured and used as a measure of the ability of each test strain to inhibit the overlay strain. Each treatment was replicated six times, the entire experiment was performed twice. All strains served as both test and overlay treatments in each experiment. Inhibition was rated as follows: − = no zone of inhibition; +− = no definite zone of inhibition but less growth around the colony; + = definite zone of inhibition of no more than 4 mm from the edge of the spotted colony to the edge of the growth of the overlay; ++ = definite zone of inhibition of 4–8 mm from the edge of the spotted colony to the edge of the growth of the overlay; and +++ = definite zone of inhibition of more than 8 mm from the edge of the spotted colony to the edge of the growth of the overlay.

RESULTS

Effectiveness of individual strains in growth chamber and greenhouse experiments. Approximately 21% of a total of 425 bacterial strains tested individually in tube bioassays showed significant ($P = 0.05$) suppression of take-all (data not shown). The most effective strains were fluorescent pseudomonads. Strain 2-79 provided significant suppression of take-all in only half of the experiments. About 20 strains of fluorescent pseudomonads were more suppressive or more consistent in their control of take-all than strain 2-79. Results from growth chamber and greenhouse experiments for nine of the strains tested most extensively in the field are shown in Table 1. On the basis of these experiments, strain Q2-87 appeared to perform the best (Table 1).

Effectiveness of individual strains and mixtures in field trials. In all four field tests, nontreated wheat sown in the presence of *G. g. tritici* (NT+), yielded significantly less than nontreated wheat without the pathogen (NT−), indicating that take-all was a major yield-limiting factor in these studies.

To allow comparison of strains across miniblocks, values for treatments in Tables 2–5 are expressed as a percent increase or decrease relative to nontreated wheat plus *G. g. tritici* in the same miniblock as the treatment. Strain combinations provided the greatest increase in yield in all four field tests conducted during 1989 and 1990 (Table 2–5). However, not all combinations were effective, and the composition of the best combinations was not always the same among trials. Generally, the most effective strain combinations increased yield more than the same strains used individually.

For example, in the 1989 spring wheat plot at Pullman, where moderate take-all developed, treatment Q2-87 plus Q1c-80 plus Q8d-80 plus Q65c-80 significantly ($P = 0.05$) increased plant height and yield 4.9 and 20.4%, respectively, compared to the nontreated control (NT+) within the same miniblock (Table 2). The second-best treatment (significant at $P = 0.1$) with a 16.4% increase in yield was Q2-87 plus Q1c-80 plus Q72a-80 plus R4a-80. None of the strains used individually significantly increased plant height or yield (Table 2) although strains Q8d-80 and Q29z-80 significantly ($P = 0.05$) increased the number of heads by 15.6 and 20.1%, respectively. Nontreated wheat in the absence of *G. g. tritici* inoculum (NT−) yielded 22.6% more than the nontreated control in the presence of the pathogen (NT+).

In the 1989–1990 winter wheat plot at Pullman, where moderate take-all developed, the combinations Q2-87 plus Q1c-80 plus Q8d-80 plus Q69c-80 and Q1c-80 plus Q8d-80 plus Q65c-80 significantly ($P = 0.05$) increased yields 17.6 and 13.1%, respectively, compared to the nontreated control (NT+) within the same miniblocks (Table 3). The mixture Q2-87 plus Q1c-80 plus Q8d-80 plus Q65c-80, which was the best treatment in the 1989 spring plot (Table 2), increased yield only 1.4%. Strain Q69c-80 was the only individual treatment to significantly improve yield (15.9%). Strain Q2-87 resulted in a nonsignificant 15.2% increase compared to the nontreated control. Nontreated wheat in the absence of *G. g. tritici* inoculum (NT−) yielded 37.5% more than the nontreated control in the presence of the pathogen (NT+).

TABLE 1. Summary of tests of fluorescent *Pseudomonas* spp. applied as seed treatments for suppression of take-all of wheat in growth chamber tube and greenhouse soil-bed assays

Seed treatment	Tube assay[a]	Greenhouse assay[a]
Q2-87	5/5	3/3
Q1c-80	1/3	1/2
Q65c-80	3/5	2/3
Q8d-80	5/5	2/3
Q29z-80	2/3	...[b]
Q72a-80	3/7	...
Q69c-80	4/5	...
Q88-87	1/3	...
Q128-87	1/3	...
2-79	4/8	1/2

[a] Number of times the strain was significantly ($P = 0.05$) better than the nontreated control/number of times the strain was tested.
[b] Strain not tested in the greenhouse assay.

TABLE 2. Influence of fluorescent *Pseudomonas* spp. applied as seed treatments on the growth and yield of wheat sown in a field infested with *Gaeumannomyces graminis* var. *tritici* at Pullman, WA, on 20 April 1989

Seed treatment[a]	Percent increase or decrease[b]		
	Plant height[c]	No. of heads	Yield
Nontreated (NT−)[b]	5.6**[d]	22.4**	22.6**
Q2-87 + Q1C-80 + Q8d-80 + Q65c-80	4.9**	9.1	20.4**
Q2-87 + Q1c-80 + Q72a-80 + R4a-80	3.4	11.8	16.4*
Q29z-80	5.4	20.1**	11.3
Q88-87	0.6	5.1	11.0
Q72a-80	2.2	14.4	9.7
Q128-87	1.2	7.0	9.4
Q69c-80	3.4	4.7	8.9
Q1c-80	1.8	10.4	5.1
Methylcellulose[b]	0.4	4.2	3.6
Q2-87 + Q29z-80 + Q72a-80	0.4	7.0	2.1
2-79 + Q2-87	2.7	6.6	1.1
Nontreated (NT+)[b]	0	0	0
Q2-87 + Q72a-80	0.4	5.2	−0.2
Q8d-80	−0.8	15.6**	−2.2
Q65c-80	−1.3	4.9	−5.5
2-79	−0.4	−0.9	−7.5
Q2-87	−2.9	3.0	−9.5

[a] Each treatment was replicated five times and consisted of three 1.52-m rows; seed (cv. Penawawa) was sown at a rate of 3.7 g per row. Populations of bacteria on seed at the time of planting ranged between 7.2×10^7 and 7.4×10^8 cfu/seed.
[b] Values for each treatment are expressed as a percent increase or decrease relative to nontreated wheat plus *G. g. tritici* (NT+) within the same miniblock as the treatment. Values for the controls represent the average across all miniblocks. *G. g. tritici* was introduced as colonized oat kernels at 3.2 g/1.52-m row, except in nontreated wheat without *G. g. tritici* added (NT−).
[c] Plants were measured 61–66 days after planting.
[d] For each treatment, statistical comparisons were made only within miniblocks by analysis of variance and Fisher's protected least significant difference test. ** = significantly better than the NT+ control within the same miniblock at $P = 0.05$; * = significant at $P = 0.1$.

In the 1989 spring wheat plot at Mt. Vernon, where severe take-all developed, the combinations Q2-87 plus Q29z-80 plus Q72a-80 and Q2-87 plus 2-79 significantly ($P = 0.05$) increased yield 15.3 and 11.5%, respectively, compared to the nontreated control (NT+) (Table 4). No individual strain had a significant effect on yield; however, Q69c-80 significantly increased plant height 3.1%. Nontreated wheat in the absence of *G. g. tritici* inoculum (NT−) yielded 51.7% more than the nontreated control in the presence of the pathogen (NT+).

In the 1990 spring wheat plot at Mt. Vernon, where severe take-all developed, wheat treated with the combination Q2-87 plus Q1c-80 plus Q65c-80 produced 12.4% more heads ($P = 0.1$) and 22.7% ($P = 0.05$) greater yield than the nontreated control (NT+) (Table 5). The combination Q2-87 plus Q29z-80 plus Q72a-80, which was the best treatment in the 1989 trial at Mt. Vernon (Table 4), increased yield 8.7%, but the increase was not significant. The combination Q2-87 plus Q1c-80 plus Q65c-80 plus Q8d-80 also increased yield 16.6%, but this was not significant. The combination chemical treatment of Baytan plus Apron was the most effective treatment and significantly ($P = 0.05$) increased the number of heads and yield 16.5 and 45.6%, respectively. Baytan alone significantly ($P = 0.05$) increased yield 17.9%. Apron increased yield 14.6%, but it was not significantly different from the nontreated control in the presence of *G. g. tritici* inoculum (NT+). No individual bacterial treatment gave a significant response.

TABLE 3. Influence of fluorescent *Pseudomonas* spp. applied as seed treatments on the growth and yield of wheat sown in a field infested with *Gaeumannomyces graminis* var. *tritici* at Pullman, WA, on 11 October 1989

Seed treatment[a]	Percent increase or decrease[b] No. of heads	Yield
Nontreated (NT−)[b]	12.1	37.5**[c]
Q2-87 + Q1c-80 + Q8d-80 + Q69c-80	6.2	17.6**
Q69c-80	4.5	15.9**
Q2-87	1.5	15.2
Q1c-80 + Q8d-80 + Q65c-80	8.2	13.1**
Q1c-80 + Q65c-80	0.4	9.0
Q29z-80	10.2	6.4
Q2-87 + Q1c-80 + Q29z-80	−1.5	6.1
Q2-87 + Q1c-80	1.5	5.5
Q2-87 + Q69c-80	1.1	4.9
Q1c-80	8.1	2.3
Q2-87 + Q1c-80 + Q8d-80 + Q65c-80	4.7	1.4
Q2-87 + Q1c-80 + Q65c-80	0.4	0.8
Nontreated (NT+)[b]	0	0
Q2-87 + Qd8-80	1.8	−0.5
Methylcellulose[b]	1.5	−0.7
Q65c-80	7.2	−1.5
Q2-87 + Q72a-80	−13.2	−5.0
Q2-87 + Q65c-80	4.5	−5.4
2-79	3.1	−5.8
Q8d-80	−1.5	−9.7

[a] Each treatment was replicated five times and consisted of three 2.13-m rows; seed (cv. Hill 81) was sown at a rate of 5.8 g per row. Populations of bacteria on seed at the time of planting ranged from 8.5×10^7 to 6.8×10^8 cfu/seed.

[b] Values for each treatment are expressed as a percent increase or decrease relative to nontreated wheat plus *G. g. tritici* (NT+) within the same miniblock as the treatment. Values for the controls represent the average across all miniblocks. *G. g. tritici* was introduced as colonized oat kernels at 4.6 g/2.13-m row, except in nontreated wheat without *G. g. tritici* (NT−).

[c] For each treatment, statistical comparisons were made only within miniblocks using analysis of variance and Fisher's protected least significant difference test. ** = significantly better than the NT+ control within the same miniblock at $P = 0.05$.

Effectiveness of mixtures of strains in growth chamber and greenhouse experiments. To verify the results from the field studies, strains comprising our best combination treatments in the field were retested singly and in combination in the growth chamber tube assays and in the greenhouse soil-bed assays. The soil used in these experiments was from the site where the Pullman field tests were conducted. In general, in the greenhouse soil-bed experiments, wheat treated with strain combinations had less disease than wheat treated with the same strains used individually, even though single-strain treatments often resulted in plants with significantly less take-all than the nontreated control. However, as in the field, not all combinations of strains provided protection against take-all. Representative data from one greenhouse trial is shown in Table 6. In this experiment, all single-strain treatments, except Q1c-80, resulted in plants with significantly ($P = 0.05$) less root disease and greater plant height than the nontreated control. Wheat treated with the combinations Q2-87 plus Q1c-80 plus Q8d-80 plus Q65c-80 or Q1c-80 plus Q8d-80 plus Q65c-80 had less root disease than wheat treated with strains Q2-87, Q1c-80, or Q8d-80 individually. In general, combinations of the same strains lacking strain Q8d-80 were not as effective as combinations in which Q8d-80 was included.

In contrast to the field and greenhouse results, in the shorter duration growth chamber tube assays no combinations were more effective than the component strains used individually (data not shown).

In vitro inhibition of *G. g. tritici* and fluorescent pseudomonads. Eleven fluorescent *Pseudomonas* spp. capable of suppressing take-all when used individually or in combination in the growth chamber, greenhouse, or field experiments were tested for their ability to inhibit *G. g. tritici* or *Pseudomonas* strains in vitro. Representative experiments are shown in Tables 7 and 8. All strains except Q8d-80 and Q1c-80 were inhibitory to *G. g. tritici* on both PDA and KMB (Table 7). Strain Q1c-80 was inhibitory

TABLE 4. Influence of fluorescent *Pseudomonas* spp. applied as seed treatments on the growth and yield of wheat sown in a field plot infested with *Gaeumannomyces graminis* var. *tritici* at Mt. Vernon, WA, on 8 May 1989

Seed treatment[a]	Percent increase or decrease[b] Plant height[c]	No. of heads	Yield
Nontreated (NT−)[b]	6.4**[d]	30.7**	51.7**
Q2-87 + Q29z-80 + Q72a-80	2.6	7.8	15.3**
Q69c-80	3.1**	10.6	13.0
2-79 + Q2-87	1.5	7.2	11.5**
Q88-87 + Q128-87	1.5	8.5	10.6
2-79	1.0	−0.9	4.7
Q1c-80	0.4	4.5	3.9
Q2-87 + Q72-80 + R39a-80 + R59a-80	2.2	10.3	2.9
Q72a-80	2.0	10.4	1.2
Nontreated (NT+)[b]	0	0	0
Methylcellulose[b]	−1.3	−3.3	−2.1
Q29z-80	−0.6	6.6	−2.6
Q2-87	−2.3	−6.1	−7.1

[a] Each treatment was replicated six times and consisted of three 3.05-m rows; seed (cv. Penawawa) was sown at a rate of 7.8 g per row. Populations of bacteria on seed at the time of planting ranged from 8.8×10^7 to 3.9×10^8 cfu/seed.

[b] Values for each treatment are expressed as a percent increase or decrease relative to nontreated wheat plus *G. g. tritici* (NT+) within the same miniblock as the treatment. Values for the controls represent the average across all miniblocks. *G. g. tritici* was introduced as colonized oat kernels at 6.8 g/3.05-m row, except in nontreated wheat without *G. g. tritici* added (NT−).

[c] Plants were measured 59 days after planting.

[d] For each treatment, statistical comparisons were made only within miniblocks using analysis of variance and Fisher's protected least significant difference test. ** = significantly better than the NT+ within the same miniblock at $P = 0.05$.

to *G. g. tritici* on PDA, but Q8d-80 was not inhibitory on either media.

Fluorescent *Pseudomonas* strains varied considerably in the number of other pseudomonads they inhibited and the intensity of the inhibition (Table 8). For example, strains 2-79, Q29z-80, and Q2-87 inhibited 10, nine, and eight of the other 10 pseudomonads (ratings of + or greater), respectively, whereas Q65c-80, Q128-87, and Q8d-80 inhibited zero, zero, and two, respectively, of the other 10 strains. Seven of the 11 strains, Q1c-80, Q2-87, Q8d-80, Q29z-80, 2-79, Q88-87, and Q69c-80, were inhibitory to themselves. Strains also varied in the number of other strains that could inhibit them. For example, Q17y-80, Q29z-80, and 2-79 were inhibited by only two, two, and four, respectively, of the other 10 strains, whereas Q128-87, Q65c-80, and Q69c-80 were inhibited by nine, eight, and eight of the other 10 strains.

Characterization and identity of biocontrol strains. The 11 strains were tested for the capacity to produce different types of secondary metabolites reported to be involved in biological control of soilborne pathogens (38). All strains produced fluorescent siderophores; however, only 2-79 produced phenazine-1-carboxylic acid (Table 7). Four strains, Q2-87, Q65c-80, Q88-87, and Q128-87, of the 10 strains from the soil near Quincy produced 2,4-diacetylphloroglucinol (15); several of the strains produced hydrogen cyanide. Strains Q2-87, Q1c-80, Q65c-80, Q69c-80, Q88-87, and Q128-87 originally were identified by Harrison et al (10) as *P. aureofaciens* (now *P. chlororaphis*) on the basis of gas chromatography of the fatty acid methyl esters in conjunction with Microbial ID Systems software. Strain Q8d-80 was identified as *P. putida*. Subsequent analyses of the same strains by Microbial ID confirmed the above results and indicated that strains Q29z-80 and Q72a-80 also were *P. aureofaciens* (now *P. chlororaphis*). Interestingly, none of these strains produced phenazine-1-carboxylic acid, which is a key characteristic of this species. Results of tests outlined in *Bergey's Manual of Determinative Bacteriology* (11), however, indicated that strains Q2-87, Q1c-80, Q65c-80, Q88-87, Q128-87, Q29z-80, and Q72a-80 should be classified as *P. fluorescens* and Q8d-80 as *P. putida*. Strain Q69c-80 was intermediate between *P. fluorescens* and *P. putida*. We feel that results obtained by classic methods are more appropriate.

TABLE 5. Influence of fluorescent *Pseudomonas* spp. applied as seed treatments on the growth and yield of wheat sown in a field infested with *Gaeumannomyces graminis* var. *tritici* at Mt. Vernon, WA, on 9 May 1990

Seed treatment[a]	Percent increase or decrease[b]	
	No. of heads	Yield
Nontreated (NT−)[b]	96.5**[c]	182.0**
Baytan + Apron	16.5**	45.6**
Q2-87 + Q1c-80 + Q65c-80	12.4*	22.7**
Baytan	6.6	17.9**
Q2-87 + Q1c-80 + Q8d-80 + Q65c-80	−1.2	16.6
Apron	11.2	14.6
Q2-87 + Q29c-80 + Q72a-80	1.9	8.7
Q29z-80	6.7	8.0
Q72a-80 + Q29z-80	6.3	7.4
Methylcellulose[b]	2.0	4.3
Q2-87 + Q72a-80	5.2	1.2
Nontreated (NT+)[b]	0	0
Q2-87 + Q65C-80 + Q8d-80	1.4	−3.6
2-79	−0.2	−5.6
Q2-87	−0.4	−8.0
Q2-87 + Q29z-80 + Q65c-80	−2.4	−9.9
Q29z-80 + Q2-87	−10.2	−11.5
Q1c-80 + Q8d-80 + Q65c-80	−3.8	−13.1

[a] Each treatment was replicated five times and consisted of three 2.13-m rows; seed (cv. Penawawa) was sown at a rate of 5.5 g per row. Populations of bacteria on seed at the time of planting ranged from 6.1×10^7 to 6.5×10^8 cfu/seed.

[b] Values for each treatment are expressed as a percent increase or decrease relative to nontreated wheat plus *G. g. tritici* (NT+) within the same miniblock as the treatment. Values for the controls in this table represent the average across all miniblocks. *G. g. tritici* was introduced as colonized oat kernels at 4.8 g/2.13-m row, except in nontreated wheat without *G. g. tritici* added (NT−).

[c] For each treatment, statistical comparisons were made only within miniblocks using analysis of variance and Fisher's protected least significant difference test. ** = significantly better than the NT+ control within the same miniblock at $P = 0.05$; * = significant at $P = 0.1$.

TABLE 6. Influence of fluorescent *Pseudomons* spp. applied as seed treatments on take-all and plant height of wheat grown in the greenhouse soil-bed assays[a]

Seed treatment[b]	Disease rating[c]	Plant height[d] (cm)
Q2-87 + Q1c-80 + Q8d-80 + Q65c-80	2.7 z[e]	35.8 w[e]
Q1c-80 + Q8d-80 + Q65c-80	2.7 z	35.3 wx
Q65c-80	3.0 yz	33.7 xy
Q8d-80	3.1 y	33.7 xy
Q2-87	3.1 y	33.9 w-y
Q2-87 + Q1c-80 + Q65c-80	3.1 y	33.1 yz
Q2-87 + Q1c-80	3.2 y	32.4 yz
Q1c-80	3.3 xy	33.1 yz
Q1c-80 + Q65c	3.3 xy	32.7 yz
Nontreated	3.5 x	31.2 z

[a] Test was conducted in a 1.2 × 2.25 m section of the greenhouse floor that was excavated to a depth of 45 cm and filled with a 7-cm-deep layer of crushed rock and a 34-cm-deep layer of Thatuna silt loam amended with 0.5% oat kernel inoculum of *Gaeumannomyces graminis* var. *tritici*.

[b] Treatments were arranged in a randomized complete block design and replicated five times. Populations of bacteria on seeds at the time of planting ranged from 1×10^7 to 3.5×10^7 cfu/seed.

[c] Disease was rated on a 0–6 scale: 0 = no disease and 6 = plant dead or nearly so.

[d] Plants were rated and main stems measured 52 days after planting.

[e] Means followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's protected least significant difference test.

TABLE 7. In vitro inhibition of *Gaeumannomyces graminis* var. *tritici* and production of antimicrobial compounds by fluorescent *Pseudomonas* spp. on potato dextrose agar (PDA) and King's medium B (KMB)

Pseudomonas strain	Inhibition		Metabolites produced[a]		
	PDA	KMB	PCA	Phl	HCN
Q2-87	++[b]	+++	−	+	+
Q1c-80	+	−	−	−	+
Q8d-80	−	−	−	−	−
Q69c-80	+	+	−	−	+
Q65c-80	+++	+	−	+	+
Q72a-80	+++	+++	−	−	−
Q29z-80	++	+++	−	−	−
Q17y-80	+	+++	−	−	ND[c]
Q128-87	+++	++	−	+	+
Q88-87	+	+++	−	+	+
2-79	+++	+++	+	−	−

[a] PCA = phenazine-1-carboxylic acid; Phl = 2,4-diacetylphloroglucinol; HCN = hydrogen cyanide.

[b] − = no zone of inhibition and the fungus overgrew the bacterial colony; + = a distinct zone of inhibition with fungal growth inhibited less than 6 mm from the point where the bacteria were spotted; ++ = a distinct zone of inhibition with fungal growth inhibited 6–10 mm from the point where the bacteria were spotted; +++ = a distinct zone of inhibition with fungal growth inhibited greater than 10 mm from the point where the bacteria were spotted.

[c] ND = Not determined.

DISCUSSION

Research on biological control of *G. g. tritici*, as well as other root pathogens, has focused on the application of single microorganisms. We hypothesized that fluorescent pseudomonads when applied in combination would provide greater and more consistent disease suppression than the same strains used individually. This hypothesis was supported by the report of Weller and Cook (36) that the combination of *P. fluorescens* 2-79 and 13-79 increased wheat yields an average of 17% in experimental plots and was superior to either strain used alone in about 50% of the trials. The addition of another strain, R4a-80, further increased activity (6). Sivasithamparam and Parker (29) showed that in raw soil only a mixture of five strains of fluorescent pseudomonads reduced disease, and the strains used separately were not effective.

The current study demonstrates that, in general, certain strain combinations have the potential for greater biocontrol activity against take-all compared to the same strains applied individually. For example, in the 1989 spring wheat plot at Pullman, the combination treatment Q2-87 plus Q1c-80 plus Q8d-80 plus Q65c-80 increased yield 20.4%, whereas Q1c-80 increased yield only 5.1% and Q2-87, Q8d-80, and Q65c-80 reduced yields 9.5, 2.2, and 5.5%, respectively. The same combination also provided greater biological control than individual strains tested in the greenhouse soil-bed assay. However, surprisingly, greater biological control by combination treatments was never detected in any of the short-term growth chamber tube bioassays, which lasted only 3–4 wk. Thus, the benefits of certain combinations become measurable only in the later stages of wheat growth, illustrating the importance of field screening for the identification of field-effective treatments. Strain Q2-87 was particularly interesting because it was the most effective strain in growth chamber and greenhouse experiments yet did not provide a significant increase in yield in the field. Nevertheless, it was a component of the most effective combinations in all four field tests. We believe that combination treatments may be a means of achieving a higher level of take-all control that currently is unattainable with individual strains.

There are several possibilities that can account for the greater biocontrol activity of certain strain combinations compared to the individual strains that comprise them. One is that the greater diversity of introduced phenotypes results in a diverse and potentially more stable rhizosphere community able to more thoroughly colonize roots and survive the biological, chemical, and physical changes that occur in the rhizosphere throughout the growing season. Rapid colonization and persistence in the rhizosphere is especially important in biological control of take-all because wheat is susceptible to infection at all stages of development. Changes in the microbial community of the rhizosphere, the physiology and ultrastructure of the root, and the physical and chemical environment all occur over the course of the growing season. Combinations also may more closely approximate the community of microorganisms involved in the suppression of *G. g. tritici* in a soil exhibiting biological control known as take-all decline (28).

Second, the greater diversity of phenotypes within the mixture compared to single strains also is likely to result in a greater variety of traits responsible for disease suppression, including a more diverse "arsenal" of secondary metabolic products capable of suppression of both target and nontarget pathogens. Further, the biosynthetic genes responsible for the production of secondary metabolites involved in disease suppression may be regulated differently among strains. Thus, by applying several strains with multiple biocontrol mechanisms, there is a greater probability that at least some of the genes involved in biocontrol will be expressed over a wider range of environmental conditions and across a broader range of microhabitats (rhizosphere, rhizoplane, and inside the root).

Further, strain mixtures may suppress a broader range of microorganisms, reducing the potential for interference from nontarget pathogens. It is common for wheat roots to be infected with *Pythium* spp. and *R. solani* along with *G. g. tritici* (4).

Our approach to constructing strain combinations was strictly empirical and based on the premise that combining several effective strains may lead to additive or synergistic effects. Currently, there are no in vitro tests that predict which strains isolated from the rhizosphere of wheat will have biocontrol activity and which ones will be compatible in mixtures. Thus, combinations must be screened in situ just as individual strains are. We initially assumed that strains comprising effective combinations would be mutually noninhibitory because of overlapping niches in the rhizosphere and the proven ability of pseudomonads to produce inhibitory secondary metabolites in situ (15,16,31). Unexpectedly, we found that many of the strains that were components of effective mixtures were either strongly inhibitory to or strongly inhibited by other members of the mixture in our in vitro assays. For example, strain Q2-87 inhibited eight of 10 pseudomonads (rating of + or greater) and was inhibited by seven of 10 strains. Antagonism or incompatibility among strains may in fact result in earlier and greater competition among introduced bacteria in the rhizosphere and, therefore, earlier and more consistent expression of traits involved in competition and disease control, especially antibiotic production. It is clear that considerably more insight into strain interactions in the rhizosphere and particularly the molecular signaling between strains is needed.

Unfortunately, we were unable to demonstrate that strain combinations increase the consistency of biocontrol activity from site to site. The combinations that provided the greatest increase in yield were different among trials. We recognize that at least for wheat in the Pacific Northwest where soil and climate vary widely, a single biocontrol treatment will not be effective in all agroeco-

TABLE 8. In vitro inhibition of fluorescent *Pseudomonas* spp. by themselves and other strains grown on potato dextrose agar

Pseudomonas sp. inhibitor[a]	*Pseudomonas* sp. inhibited[b]										
	Q65c-80	Q1c-80	Q17y-80	Q2-87	Q8d-80	Q128-87	Q29z-80	2-79	Q72a-80	Q88-87	Q69c-80
Q65c-80	–[c]	–	–	–	–	–	–	–	–	–	–
Q1c-80	+++	+++	–	+++	+++	+++	–	+	++	+++	+++
Q17y-80	+	+–	–	+	+	+	–	+–	+	+	+
Q2-87	++	+	+–	++	++	++	+–	+	+	++	++
Q8d-80	+–	+–	–	+–	+	+	–	–	+–	+–	+
Q128-87	–	–	–	–	–	–	–	–	–	–	–
Q29z-80	+++	+++	+–	+++	+++	+++	+	++	+++	+++	+++
2-79	+++	++	+	+++	+++	+++	++	+	+++	+++	++
Q72a-80	++	–	–	+	–	++	–	–	–	–	++
Q88-87	++	++	+	++	++	+++	+–	+	++	++	++
Q69c-80	+	++	+–	+	+++	++	+	+–	+	+	++

[a]Strains were spotted on a plate and incubated for 24 h prior to being sprayed with a test strain.
[b]Cells of each strain were applied with a chromatography sprayer.
[c]– = no zone of inhibition; +– = no definite zone of inhibition but less growth around the colony; + = distinct zone of inhibition of no greater than 4 mm from the edge of the spotted colony to the edge of the zone; ++ = distinct zone of inhibition of 4–8 mm from the edge of the spotted colony to the edge of the zone; and +++ = distinct zone of inhibition greater than 8 mm from the edge of the spotted colony to the edge of the zone.

systems where take-all is a problem. Perhaps the best analogy to the development of biocontrol agents for take-all may be found in wheat breeding. Worldwide, hundreds of varieties are grown, each adapted to a given agroecosystem. Even in the Pacific Northwest, around 10 varieties commonly are grown commercially. We speculate that in the Pacific Northwest biological control of take-all may ultimately be achieved through the use of 10–15 core strains with several of these strains being mixed depending on the soil type, the use of winter or spring wheat cultivars, the level of tillage, and the inoculum potential of target and nontarget pathogens.

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