Use of *Gaeumannomyces graminis* var. *graminis* Alone and in Combination With Fluorescent *Pseudomonas* spp. to Suppress Take-All of Wheat

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ABSTRACT

Duffy, B. K., and Weller, D. M. 1995. Use of *Gaeumannomyces graminis* var. *graminis* alone and in combination with fluorescent *Pseudomonas* spp. to suppress take-all of wheat. Plant Dis. 79:907-911.

Gaeumannomyces graminis var. *graminis*, originally isolated from rice, suppressed take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici* in growth chamber studies when applied to the soil. Furthermore, combination treatments consisting of *G. g.* var. *graminis* applied to the soil and fluorescent *Pseudomonas* strains applied to the seed, either 30-84, Q29z-80, Q69c-80, or a mixture of strains (Q2-87 plus Q1c-80 plus Q8d-80 plus Q69c-80), were significantly more suppressive of take-all than either treatment used alone. In a winter wheat field trial at Pullman, Wash., *G. g.* var. *graminis* applied to the seed furrow significantly reduced crown root infection by *G. g.* var. *tritici* and the strain mixture reduced seminal root infection suggesting differential protection at various stages of disease development. However, in contrast to growth chamber studies the combination of *G. g.* var. *graminis* and the strain mixture did not enhance take-all suppression in the field compared with the same treatments used alone.

Take-all, caused by *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier var. *tritici* J. Walker is a crown and root disease of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) that occurs worldwide. Commercial varieties with resistance to take-all are not available and the fungicides that have been tested or registered (1,7,8,17) for take-all, perform inconsistently in some soils in the Pacific Northwest of the U.S. (R. J. Cook and D. M. Weller, *unpublished results*). The application of fungal and bacterial biocontrol agents potentially is an alternative method of control (6,24,29,30). Emphasis has been on the testing of fluorescent *Pseudomonas* spp. (24,26) that produce phloroglucinol (11,13) or phenazine (21,23) antibiotics. In the Pacific Northwest, bacterial seed treatments with fluorescent pseudomonads increased the yield of wheat an average of 17% in experimental plots and 11% in commercial-scale tests (24).

Gaeumannomyces graminis Arx & Olivier var. *graminis*, hypovirulent *G. g.* var. *tritici* and *Phialophora* spp. from wheat or other grasses suppressed take-all in Australia and Europe (27,29,30,31). Because these fungi colonize the cortex of the root, they may induce host resistance mechanisms in wheat and barley, causing greater lignification and suberization of the endodermis and xylem vessels. Furthermore, they may compete directly with virulent *G. g.* var. *tritici* for the same substrates and favored sites in and on roots (28,30). Finally, they may increase leakage of root exudates, thus increasing populations of other antagonistic rhizosphere microorganisms, such as fluorescent *Pseudomonas* spp., that are especially well adapted to rapidly utilizing root exudates (6,24).

Inconsistent performance is a major impediment to the widespread use of biocontrol agents in commercial agriculture (24). The application of combinations of biocontrol agents may be one possible approach to increase the amount and consistency of disease control (7,9,14,15,16,20). Dandurand and Knudsen (9) found that combining *P. fluorescens* 2-79 with *T. harzianum* ThzID1 neither inhibited nor enhanced the biocontrol activity of the latter against root rot of pea caused by *Aphanomyces euteiches*. Furthermore, strain 2-79 did not reduce the ability of ThzID1 to attack sclerotia of *Sclerotinia sclerotiorum* in field tests (3). Pierson and Weller (20) showed that combinations of two to four strains of fluorescent *Pseudomonas* spp. provided significantly better control of take-all than did each strain used alone.

The objective of this research was to study the biocontrol activity of *G. g.* var. *graminis* against take-all and the compatibility of fluorescent *Pseudomonas* spp. and *G. g.* var. *graminis* when applied together. We hypothesized that combining these organisms with different biocontrol mechanisms would result in enhanced take-all suppression.

MATERIALS AND METHODS

Strains. A mixture of virulent *G. g.* var. *tritici* isolates was used throughout this study (20). *G. g.* var. *graminis* TX1 (our strain designation) was isolated from rice in Texas (10). It was tested for pathogenicity to wheat using methods described by Duffy and Weller (10). In vitro inhibition of *G. g.* var. *tritici* by TX1 was assayed by placing a plug of each fungus, taken from the leading edge of 4- to 7-day-old cultures, on opposite ends of plates of dilute potato-dextrose agar (dPDA) (10) and monitoring growth of the two fungi for 7 days.

The fluorescent *Pseudomonas* strains used in this study either individually or as part of a bacterial mixture are shown in Table 1. The mixture, consisting of strains Q2-87 plus Qlc-80 plus Q8d-80 plus Q69c-80, was selected for this study because it previously was shown to suppress take-all in the field (20). Bacteria and fungi (as mycelial fragments) were stored in 80% glycerol at –70°C. Fungi were grown on dPDA.

Inoculum preparation. Seeds were coated with bacteria using methods similar to those previously described (19). For growth chamber studies, bacteria were cultured on nutrient broth-yeast extract agar (NBY) (20) for 48 h at 25 to 27°C. The bacteria from two plates of NBY were suspended in 20 ml of 0.5% methylcellulose (Sigma, St. Louis, Mo.) and then mixed with 50 g surface-disinfested seed. For field trials, bacteria were grown on King's medium B (20) and suspended in 1.5% methylcellulose. Seeds were dried under a stream of sterile air and coated with approximately 10^8 cfu per seed. The mixture of bacteria was prepared by combining equivalent proportions of each strain prior to seed application as previously described (26). Controls consisted of nontreated seed and seed coated only with methylcellulose.

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Accepted for publication 17 May 1995.

Oat-kernel inoculum of *G. g.* var. *graminis* and *G. g.* var. *tritici* was prepared as previously described (20). Seeds were coated with *G. g.* var. *graminis* by mixing seeds with fragmented oat-kernel inoculum, (particles 0.25 to 0.5 mm, see below) in 1.5% methylcellulose, at a rate of 8 g of inoculum per 50 g of seed.

Growth chamber tube assay. The biocontrol agents were tested in a take-all tube assay similar to that previously described (19,23). The assay used plastic tubes (2.5 cm in diameter × 16.5 cm long) held upright in racks (200 per rack). Each tube was filled with a 6.5-cm-deep layer of sterile vermiculite overlaid with 15 g of raw Puget silt loam (20) amended with inoculum of *G. g.* var. *tritici* and/or *G. g.* var. *graminis* in the form of colonized oat kernels fragmented and sieved into fractions of known particle sizes. Particles 0.25 to 0.5 mm in diameter were added to the soil at 1.0% (wt/wt). Two seeds (cv. Hill 81) were sown per tube and covered with a layer of vermiculite. Each tube received 10 ml of water with metalaxyl (0.075 g of wettable powder per 1,000 ml; CIBA-GEIGY, Greensboro, N.C.) prior to seeding to inhibit *Pythium* spp.; 5 ml of water was added after the seeds were covered with vermiculite. Racks were covered with clear plastic, incubated at room temperature (21 to 24°C) for 2 days to promote germination, and then transferred to the growth chamber (12 to 15°C, 99% relative humidity, and 12-h photoperiod). The racks remained covered with plastic until the plants emerged. Plants were watered twice a week with dilute (1:3, vol/vol) Hoagland's nutrient solution (macroelements only). After 3 to 4 weeks, roots were washed free of soil and the severity of take-all was evaluated by rating disease symptoms on a scale of 0 to 8 (19), where 0 = no disease and 8 = plants dead or early so.

The experiment to test the compatibility of *G. g.* var. *graminis* with the fluorescent pseudomonads was arranged as a 2 × 8 factorial consisting of two fungal treatments (plus and minus *G. g.* var. *graminis*) and eight seed treatments (nontreated, methylcellulose-treated, 30-84, 2-79, Q29z-80, Q2-87, Q69c-80, and the strain mixture) in a split-plot design. Fungal treatment was the main plot and seed treatment was the subplot. Each treatment consisted of 20 seedlings (10 tubes with two seedlings each) and was replicated five times. The experiment was repeated once with similar results. The variance between trials was not significant and the data were pooled for final analysis. The main effects of *G. g.* var. *graminis* and seed treatment and the interaction were analyzed for significance by the SAS general linear model procedure (22). Results indicated that both main effects and the interaction were significant ($P = 0.0001$). Further analysis of the interaction was performed using Fisher's protected least significant difference (LSD) test ($P = 0.05$) on the response to both *G. g.* var. *graminis* and seed treatment.

The effect of *G. g.* var. *graminis* on the growth of wheat in the absence of *G. g.* var. *tritici* also was evaluated in the tube assay. Steam-treated and raw Puget silt loam was amended with *G. g.* var. *graminis*; soil without the fungus served as the control. Four weeks after planting the wheat was harvested and shoot heights were measured. The experiment was arranged as a 2 soil (steamed and raw soil) × 2 fungal treatment (plus and minus *G. g.* var. *graminis*) factorial in a randomized complete block design and analyzed as described above. The experiment was repeated once with similar results.

Take-all suppression in the field. Field trials were conducted with winter wheat (cv. Hill 81) in 1990 at Pullman, Wash. (Plant Pathology Research Farm, Thatuna silt loam) and with spring wheat (cv. Penawawa) in 1991 at Mt. Vernon (Washington State University, Northwest Research and Extension Station, Puget silt loam) and at Pullman, Wash. (20). Plots were fertilized with an equivalent of 114 kg N per ha. Seed furrows (40.6 cm apart) were opened mechanically to a depth of 6 to 8 cm and seeds were sown by hand. *Gaeumannomyces g.* var. *tritici*, as whole oat-kernel inoculum, was added to the furrow immediately before the seeds were sown at a rate of 4.9 g per 2.13-m row. *G. g.* var. *graminis* was added to the furrow, as whole oat-kernel inoculum, at the same rate as the *G. g.* var. *tritici* inoculum. Treatments were arranged in a highly modified randomized complete block design as described by Pierson and Weller (20). Fields were divided into six replicate blocks and each block was subdivided into smaller units called miniblocks. These miniblocks were part of larger experiments in 1990 and 1991 designed to screen biocontrol agents of take-all. In the winter wheat trial the miniblock contained four biocontrol treatments and one control (nontreated seed with *G. g.* var. *tritici*). In the spring wheat trials, each miniblock contained three biocontrol treatments and three controls. The controls were nontreated seed with and without *G. g.* var. *tritici*, and methylcellulose-treated seed with *G. g.* var. *tritici*. Each treatment was sown in three 2.13-m rows and was replicated six times.

In the winter wheat trial, winter damage precluded accurate yield measurements,

Table 1. *Pseudomonas* strains and phenotypes

Strains		Relevant phenotypes[y]	Reference
P. chlororaphis (formerly *P. aureofaciens*)	30-84	$Flu^+HCN^+Phz^+$	(21)
P. fluorescens	Q2-87	$Flu^+HCN^+Phl^+$	(11,20)
P. fluorescens	Q65c-80	$Flu^+HCN^+Phl^+$	(11,20)
P. fluorescens-putida[z]	Q69c-80	Flu^+HCN^+	(11,20)
P. fluorescens	Q1c-80	Flu^+HCN^+	(11,20)
P. fluorescens	2-79	$Aff^+Flu^+HCN^-Phz^+$	(23,25)
P. fluorescens	Q29z-80	Flu^+	(12,20)
P. putida	Q8d-80	Flu^+	(11,20)

[y] Fluorescent siderophore (Flu^+), cyanide (HCN^+), phenazine antibiotics (Phz^+), 2,4-diacetylphloroglucinol (Phl^+), and anthranillic acid (Aff^+).
[z] Strain Q69c-80 has characteristics of both species.

Table 2. Influence of *Gaeumannomyces graminis* var. *graminis* and fluorescent *Pseudomonas* spp. on take-all of wheat in growth chamber tests in a natural Puget silt loam[v]

Seed treatment[x]		Disease ratings[w]	
		– *G. g.* var. *graminis*	+ *G. g.* var. *graminis*[y]
P. chlororaphis	30-84	4.73 Ad[z]	3.27 Bd
P. fluorescens	Q2-87	5.29 Ac	3.77 Ba
P. fluorescens-putida	Q69c-80	5.44 Abc	3.33 Bd
P. fluorescens	2-79	5.58 Ab	3.56 Bbc
P. fluorescens	Q29z-80	5.34 Abc	3.41 Bcd
Bacterial strain mixture		3.84 Ae	2.45 Be
Methylcellulose		5.53 Abc	3.43 Bcd
Nontreated		5.86 Aa	3.75 Bab

[v] Virulent *G. g.* var. *tritici* was added to soil of each treatment at planting as ground oat-kernel inoculum (1% w/w).
[w] Take-all was evaluated on a scale from 0 to 8, 3 to 4 weeks after planting.
[x] Bacteria were applied to the seed using 0.5% methylcellulose; each seed was coated with approximately 10^8 cfu after drying. The bacterial strain mixture consisted of equal proportions of Q2-87, Q1c-80, Q8d-80, and Q69c-80. Methylcellulose-treated and nontreated seeds were used for the controls.
[y] *G. g.* var. *graminis* was added to the soil at planting as ground oat-kernel inoculum (1% wt/wt).
[z] Results from two trials were similar and the data were pooled for the final analysis. Means within a column followed by the same lower case letter and means in the same row followed by the same upper case letter are not significantly different according to Fisher's protected least significant difference test ($P = 0.05$).

and thus, root infection was used exclusively as the measure of take-all severity. Plants at early heading (10 to 10.1 developmental stage on the Feekes scale) (5) were dug from the field, freed of loosely adhering soil, and transported to the laboratory. Tillers were removed leaving only the roots of the main stem. After a thorough washing, the seminal and crown roots of the main stem that had at least one black lesion characteristic of take-all were counted; these values were converted to percent roots infected by dividing by the total number of seminal or crown roots per main stem and multiplying by 100. From each treatment in a miniblock 10 plants were sampled. Data for crown and seminal root infection were analyzed independently. In spring wheat trials, take-all was evaluated as a reduction in yield compared with healthy controls, as previously described (20). Data for each miniblock (six replicates) were analyzed independent of other miniblocks. Treatment effects were tested for significance with the SAS general linear model procedure (22). Treatment means were compared by Fisher's protected LSD test.

RESULTS AND DISCUSSION

When introduced into the soil in the absence of *G. g.* var. *tritici*, *G. g.* var. *graminis* TX1 colonized the lower crown area and roots of wheat with dark mycelia; however, it did not cause lesions or other symptoms typical of take-all. In vitro, *G. g.* var. *graminis* was not inhibitory to *G. g.* var. *tritici*; however, the former grew considerably faster than the latter. On the basis of height measurements in growth chamber studies, *G. g.* var. *graminis* had no effect on the growth of wheat in the absence of the pathogen (data not shown).

In growth chamber studies, there was a highly significant ($P = 0.0001$) interaction between the presence of *G. g.* var. *graminis* and the seed treatment. Thus, seed treatments were compared only within *G. g.* var. *graminis* treatments and comparisons between plus and minus *G. g.* var. *graminis* treatments were made only within a single seed treatment. Nontreated wheat grown in soil amended with *G. g.* var. *graminis* and *G. g.* var. *tritici* had significantly less take-all (3.75 disease rating) than control wheat grown only in the presence of the pathogen (5.86 disease rating control) (Table 2). Induction of host resistance mechanisms, competition for nutrients, and occupation of infection courts are the most probable mechanisms responsible for the suppressiveness of *G. g.* var. *graminis* (29,30). *Gaeumannomyces g.* var. *graminis* is a good example of an organism that can be both beneficial, as a biocontrol agent, and deleterious, as a pathogen of rice.

In the absence of *G. g.* var. *graminis*, the strain mixture, consisting of Q2-87 plus Qlc-80 plus Q8d-80 plus Q69c-80 (3.84 disease rating), was the most effective bacterial seed treatment against take-all. This supports the recent report of Pierson and Weller (20) that this mixture (Q2-87 plus Qlc-80 plus Q8d-80 plus Q69c-80) provided greater suppression of take-all than the same strains did when used individually. Although less effective than the mixture, all of the other bacterial seed treatments significantly reduced disease severity when compared with the nontreated control. The methylcellulose control also had a significantly lower disease rating than the nontreated control (Table 2). This effect occasionally has been observed in both growth chamber and

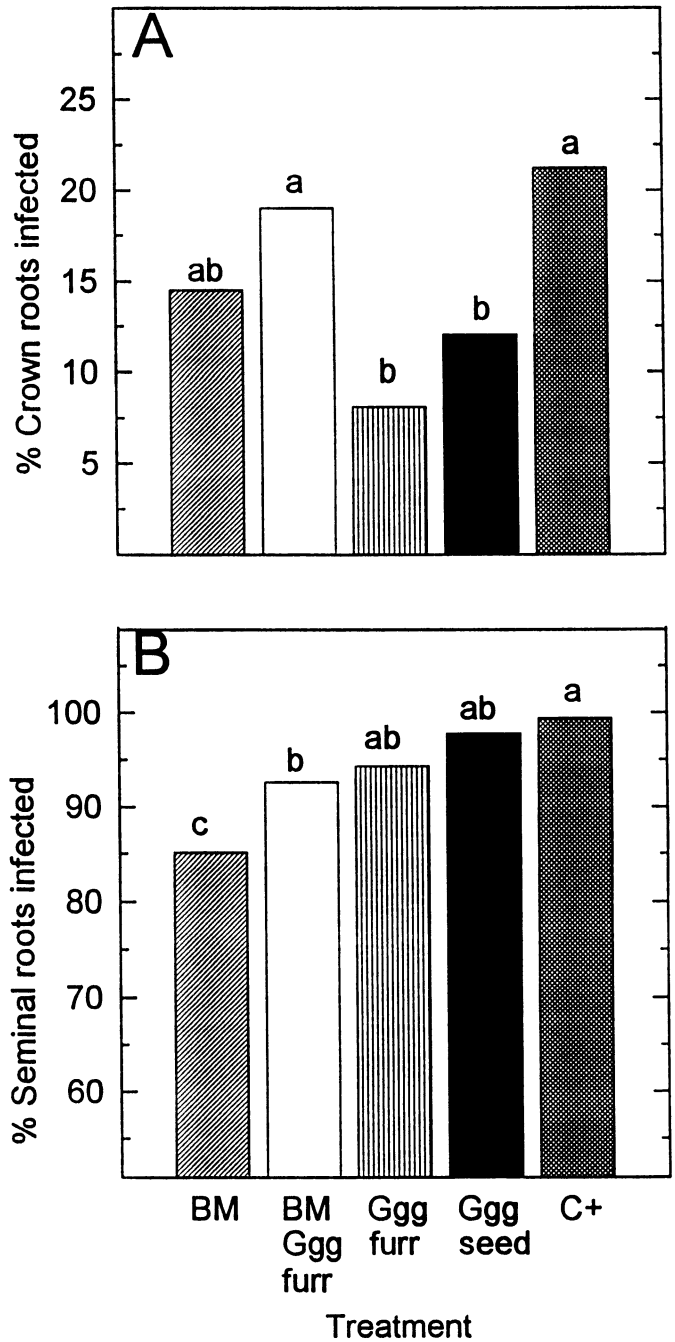

Fig. 1. Influence of *Gaeumannomyces graminis* var. *graminis* and *Pseudomonas* spp. used individually and in combination on infection of **(A)** crown roots and **(B)** seminal roots of winter wheat by *G. g.* var. *tritici* at Pullman. *Gaeumannomyces g.* var. *graminis* was applied to the seed furrow as whole oat-kernel inoculum (Ggg furr) or as a seed treatment (Ggg seed). A mixture of four bacterial strains (BM) (equal proportions of strains Q2-87, Q1c-80, Q8d-80, and Q69c-80, was applied as a seed treatment with 1.5% methyl cellulose. Nontreated seed was used for the control (C+). *Gaeumannomyces g.* var. *tritici* was added to all treatments to amend low levels of indigenous inoculum. Percentage of roots infected represents the number of crown or seminal roots on the main stem with at least one lesion divided by the total number of seminal or crown roots on the main stem and multiplied by 100. Bars with the same letter are not significantly different according to Fisher's protected least significant difference procedure ($P = 0.05$).

field experiments in certain soils and under certain conditions (2; D. M. Weller, *unpublished results*); this study presents one of the most striking examples. We suspect that methylcellulose may serve as a nutrient source, stimulating populations of indigenous microorganisms, when introduced bacteria are not in the seed coating. This hypothesis is supported by the report of Kaiser et al. (12) that chickpeas coated with a suspension of 1.0% methylcellulose had a significantly greater amount of Pythium damping-off than nontreated seeds. However, an alternate explanation may be found in the work of Cheng et al. (4) in which methylcellulose prevented colonization of cellulose by cellulolytic rumen fungi. We think that in biocontrol studies the nontreated wheat is the proper control to compare the effects of biocontrol agents.

In growth chamber studies, each bacterial strain applied in combination with *G. g.* var. *graminis* resulted in less disease than the same strain used without *G. g.* var. *graminis* (Table 2). However, 30-84, Q29z-80, Q69c-80, and the mixture, used in combination with *G. g.* var. *graminis*, resulted in greater disease suppression than the nontreated control with *G. g.* var. *graminis*. *Gaeumannomyces g.* var. *graminis* plus the strain mixture (2.45 disease rating) provided significantly better protection than any of the other bacteria combined with *G. g.* var. *graminis*. None of the bacteria reduced the biocontrol activity of *G. g.* var. *graminis* (Table 2).

Lemanceau et al. (15,16) reported that Fusarium wilt of carnation, caused by *Fusarium oxysporum* f. sp. *dianthi,* was suppressed more strongly by a combination of a nonpathogenic *F. oxysporum*, isolate Fo47b10, and *Pseudomonas putida* WCS358, than by the same biocontrol agents used individually. *Pseudomonas putida* WCS358 produces the siderophore pseudobactin 358. The greater effectiveness of the combination was attributed to the pseudobactin intensifying the antagonism of *F. oxysporum* Fo47b10 against *F. o.* f. sp. *dianthi*. Pseudobactin-mediated iron limitation reduced the efficiency of glucose metabolism in both the pathogenic and nonpathogenic fusaria, but the effect was greater on the *F. o.* f. sp. *dianthi*, apparently rendering it less competitive for carbon against the nonpathogenic *F. oxysporum*. The studies of Lemanceau et al. (15,16) may provide some insight into our observation of greater suppression of take-all in the growth chamber by combinations of *G. g.* var. *graminis* and certain bacteria. All of the pseudomonads tested, except strain Q8d-80, produce inhibitory secondary metabolites, such as phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol and/or hydrogen cyanide. Interestingly, although both *G. g.* var. *tritici* and *G. g.* var. *graminis* are sensitive to these compounds, Mazzola et al. (18) demonstrated that *G. g.* var. *graminis* was much less sensitive to phenazine-1-carboxylic acid than *G. g.* var. *tritici*. Thus, in addition to direct inhibition of *G. g.* var. *tritici,* some *Pseudomonas* antibiotics may make the pathogen less competitive against *G. g.* var. *graminis*.

In the winter wheat plot, moderate take-all, as defined by Pierson and Weller (20), developed. The bacterial strain mixture used alone and in combination with *G. g.* var. *graminis* in the furrow significantly reduced the percentage of seminal roots with lesions; however, the strain mixture alone was better than the strain mixture combined with *G. g.* var. *graminis*. Surprisingly, compared with the nontreated control, *G. g.* var. *graminis* applied individually to the seed or seed furrow did not significantly reduce lesion formation on the seminal roots (Fig 1B). In contrast, however, *G. g.* var. *graminis* applied individually to the seed furrow or as a seed treatment significantly reduced the percentage of crown roots with lesions (Fig. 1A). Moreover, the strain mixture, alone or in combination with *G. g.* var. *graminis,* did not significantly reduce crown root infection. In contrast to the results from the growth chamber study, the strain mixture reduced the suppressiveness of *G. g.* var. *graminis* on crown roots (Fig. 1A). In general, it appears that the bacterial seed treatments may provide better protection against take-all in the early stages of disease development when seminal roots (the first roots produced by wheat) are attacked by *G. g.* var. *tritici*; *G. g.* var. *graminis* may provide better protection during later stages as the crown root system expands.

In the spring wheat plots at Mt. Vernon and Pullman where inoculum of *G. g.* var. *tritici* was added to the seed furrow, severe take-all as defined by Pierson and Weller (20) developed. *Gaeumannomyces g.* var. *graminis* applied to the seed furrow increased grain yield by 34 and 9% (not statistically significant) at the Mt. Vernon and Pullman field sites, respectively, compared with the nontreated control plus *G. g.* var. *tritici* (data not shown). At Pullman, in miniblocks where no *G. g.* var. *tritici* inoculum was added to the seed furrow, only slight to moderate take-all developed from the naturally occurring inoculum. *Gaeumannomyces g.* var. *graminis* applied to the seed furrow increased grain yield 23%; however, the effect was not statistically significant ($P = 0.05$). Although these three treatments were not statistically significant at either site due to considerable variability, there was a clear visual trend of *G. g.* var. *graminis* improving the growth of wheat. The addition of the strain mixture with *G. g.* var. *graminis* did not enhance take-all suppression in the spring wheat trials.

One approach for improving field performance of a biocontrol agent is to increase inoculum dosage. Wong (27) reported that take-all suppression by *G. g.* var. *graminis* improved as the dose of the biocontrol agent increased. Increasing the dose is an approach that is possible in small-scale biocontrol tests but it is not likely to be feasible in commercial wheat production. For example, the amount of *G. g.* var. *graminis* inoculum used in the current studies was equivalent to 75.3 kg per ha. Research in biological control needs to place greater emphasis on approaches to reduce the dose of the biocontrol agent applied. Wong (28,29) suggested that *G. g.* var. *graminis* isolates from soils with low temperatures and from wheat may be more suppressive of take-all of wheat in cooler climates than isolates originating from warmer climates and nonwheat hosts. Subsequently, he selected a strain of *G. g.* var. *graminis*, designated 90/3B, that grew more rapidly at 5°C than the parental strain and increased yield by 30 to 45% (30,31). Most importantly, isolate 90/3B appears to perform more consistently and at a lower dose than isolates of *G. g.* var. *graminis*, hypovirulent *G. g.* var. *tritici,* and *Phialophora* spp. previously tested for control of take-all.

Collectively, our results strongly suggest that larger-scale field tests of *G. g.* var. *graminis* against take-all in the Pacific Northwest are warranted. Given the findings of Wong (30), future studies should be conducted with *G. g.* var. *graminis* isolated from Pacific Northwest soils because such isolates probably are better suited to the cooler local conditions than TX1 isolated from rice and a warm climate. Our hypothesis that combinations of different types of biocontrol agents would provide enhanced take-all suppression compared with the same agents used individually was only partially validated. The positive effect of combining *G. g.* var. *graminis* and pseudomonads, observed in the growth chamber studies, was not apparent in the field. Apparently, short-term growth chamber and greenhouse tests are less useful for predicting interactions among these types of biocontrol agents in the field. Nevertheless, we think that greater emphasis on developing mixtures of biocontrol agents is needed because they represent a more ecologically sound approach to biological control. Mixtures of organisms may result in better plant colonization, be better adapted to the environmental changes that occur throughout the growing season, present a larger number of pathogen-suppressive mechanisms, and/or protect against a broader range of pathogens (20).

ACKNOWLEDGMENTS

We thank B. H. Ownley for statistical advice, M. Gano-Smith, J. Gussman, D. Krytenberg, R. Reeder, G. Stallings, and D. Wood for technical assistance, and G. Défago and P. T. W. Wong for critical evaluation of the manuscript.

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