Erwinia herbicola as a Biocontrol Agent of *Fusarium culmorum* and *Puccinia recondita* f. sp. *tritici* on Wheat

H.-J. Kempf and G. Wolf

Graduate research assistant and professor, respectively, Institute of Plant Pathology, University of Göttingen, Grisebachstrasse 6, D-3400 Göttingen, West Germany.

Present title and address of first author: Postdoctoral fellow, Department of Plant Pathology, University of California, Berkeley 94720.

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ABSTRACT

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Antibiotic-producing microorganisms (15 bacteria and 54 actinomycetes) were isolated from the rhizosphere and rhizoplane of Gramineae species. Their in vitro activity against *Fusarium culmorum* was correlated with their ability to suppress the soilborne pathogen on wheat seedlings in the greenhouse after seed coating. At least 40% disease suppression was caused by 22% of the strains. Seed treatment with one of the most active strains, *Erwinia herbicola* B247, resulted in about 90% disease suppression, detected by enzyme-linked immunosorbent assay, even with high pathogen inoculum concentrations in the soil (10^4–10^5 cfu/g). The antagonist spread from the treated seed onto the root and shoot, although population densities declined with time and distance from the seed. An antibiosis-negative Tn*5* mutant of *E. herbicola* still suppressed *F. culmorum* in vivo to some extent, suggesting that mechanisms other than antibiosis also are involved in disease reduction. The antagonist *E. herbicola* B247 also was suppressive to *Puccinia recondita* f. sp. *tritici* on wheat leaves. In this case, antibiosis was the mechanism of control, because the Tn*5* mutant was inactive, and nearly complete protection was attained by application of cultural filtrate from the wild-type bacterium.

Fusarium culmorum (W. G. Smith) Sacc. is a soilborne pathogen that causes seedling and head blight and foot and root rot of wheat. The use of microorganisms as a biological control for this disease is of interest because there are no chemical means to effectively control this fungus and resistant cultivars are not available (21). However, there are few reports of biocontrol agents against *F. culmorum* (20,23,26). *Trichoderma harzianum* has been reported to protect wheat seedlings from infection by *F.*

culmorum; seed treatment in greenhouse pot tests reduced disease incidence up to 70% (23).

Biocontrol agents investigated in the past include strains of *Erwinia herbicola* (Löhnis) Dye, a saprophytic bacterium occurring on aerial plant parts and roots (10). It has been reported to be antagonistic to pathogens on the phylloplane, such as *E. amylovora* (28).

We report herein the isolation of several antibiotic-producing bacteria and actinomycetes antagonistic to *F. culmorum* and their application to wheat seeds to control seedling blight in a greenhouse assay. A highly active strain of *E. herbicola* was selected for further study and the mechanism of action was investigated. In addition, this bacterium was tested as a biocontrol of leaf rust caused by *Puccinia recondita* Rob. ex Desm. f. sp. *tritici*.

MATERIALS AND METHODS

Screening for antibiotic-producing microorganisms. Roots of Gramineae species from different locations in West Germany were excavated and shaken to remove loosely adhering soil. Roots then were dipped several times into a beaker of sterile tap water to suspend the tightly adhering soil with rhizosphere microorganisms. Washed roots were immersed in a second container of sterile tap water and agitated with an 'Ultra Turrax' mixer (TP 18/2, Janke and Kunkel, Staufen, West Germany) for 20 sec to remove microorganisms from the rhizoplane. Microorganisms in both suspensions were screened for antibiotic production by the triple-agar-layer plate technique of Herr (13), with *F. culmorum* (isolate 102 from Landespflanzenschutzamt Rheinland-Pfalz, Mainz, West Germany) as a test organism. Dilutions of both suspensions were made in sterile tap water so that isolated colonies developed when 1 ml of each sample dilution was incorporated in 9 ml of molten 2% tryptic soy agar (TSA, Difco Laboratories, Detroit, MI), pH 7.0, or soil extract agar at pH 5.0 or 7.0 contained in petri dishes. For the preparation of soil extract agar, 300 g of a sandy loam field soil was autoclaved in 500 ml of tap water, filtered, diluted 1:1 (v/v) with tap water, and 1.5% Bacto agar (Difco) was added. When the agar medium had solidified, 4 ml of sterile agar were poured over the first layer. After incubation for 48 hr at 20 C, 5 ml of pH 6.5 Czapek dox agar (Oxoid Ltd., Basingstoke, England) containing 0.2% (w/v) peptone and macroconidia of *F. culmorum* (3×10^4/ml) were added for the third layer. After a further 48 hr of incubation at 20 C, colonies surrounded by an inhibition zone were transferred to TSA.

In vitro assay. Antibiotic-producing microorganisms isolated by the triple-agar-layer plate technique were streaked on 2% TSA (pH 6.5) in separate petri dishes and incubated for 72 hr at 20 C. An agar disk, 5 mm in diameter, was cut from the margin of a growing culture of *F. culmorum* and placed on the agar, 30 mm from the antagonist. The inhibition zone was measured after incubation for 7 days at 20 C.

Identification of strain B247 and its antibiotic. One antibiotic-producing bacterium, designated as B247, was identified as *E. herbicola* on the basis of standard tests (17) and by DNA-DNA-hybridization (8) with its type strain NCPPB 2971. The antibiotic produced by *E. herbicola* B247 was identified by co-chromatography on a thin-layer chromatography (TLC) plate (Silica gel 60, Merck, Darmstadt, West Germany) with a mixture of cultural filtrate and pure herbicolin A (1,29). The location of herbicolin A was bioassayed on the plate by embedding it in TSA containing conidia of *F. culmorum* (3×10^4/ml).

Isolation of a mutant lacking in vitro antibiosis. A transposon Tn*5* mutant of *E. herbicola* B247 was generated by conjugative transfer of the plasmid pSUP 5011 (pBR 325::Tn*5*-Mob; Tn*5* carries a kanamycin resistance gene) from *Escherichia coli* S17-1 (22). The spot-agar-mating technique (9) was employed with a spontaneous rifampicin-resistant mutant of *E. herbicola* B247 as the acceptor. Transconjugants were transferred to petri dishes of 2% TSA (pH 6.5) containing kanamycin (Serva, Heidelberg, West Germany) at 100 μg/ml and rifampicin (Serva) at 50 μg/ml. A suspension of *Candida albicans* (No. 1386, Deutsche Sammlung für Mikroorganismen, Braunschweig, West Germany), used as a test organism, was atomized onto the agar surface. Transconjugants not showing an inhibition zone were selected and retested against *F. culmorum* by the in vitro assay previously described. One mutant lacking in vitro antibiosis was used during this investigation. This mutant, *E. herbicola* Tn247, had a single Tn*5* insertion, which was proven by digestion of chromosomal DNA with three different restriction enzymes, followed by Southern transfer and probing with Tn*5* (19).

In vivo assay with *F. culmorum*. Antagonists were tested as seed treatments for their ability to suppress *F. culmorum* in greenhouse pot tests. Seeds of winter wheat *Triticum aestivum* L. 'Okapi' (surface-disinfested in 1% NaOCl for 3 min) were shaken for 15 min in a suspension of the antagonist (bacteria $A_{660} = 0.15$; actinomycetes $A_{660} = 0.3$) with 1% (w/v) methylcellulose 400 (mol wt 41,000; Serva) and then dried on filter paper.

Inoculum was prepared by incubating 30 g of triturated wheat straw in 400 ml of tap water with 10^7 macroconidia of *F. culmorum* for 4 days at 20 C on a rotary shaker. The straw was separated and dried and could be stored for at least 2 mo without the fungus losing virulence.

Nonsterile sand/peat (1,300–1,400 g) was blended with 3 g (unless otherwise indicated) of straw colonized by *F. culmorum* to give a pathogen concentration of 10^4–10^5 colony-forming units (cfu)/g, placed in five plastic pots ($6 \times 6 \times 7$ cm) with nine seeds each (45 replicates per treatment), and incubated for 14 days in a growth chamber (18–24 C). Pots were completely randomized. The soil was held at 30–50% water-holding capacity by weighing pots and adding lost water every 2–3 days.

To assess disease incidence, shoot lengths of all 45 plants per treatment were measured. An enzyme-linked immunosorbent assay (ELISA) with rabbit antibodies specific for *F. culmorum* also was used for quantification (25). Crowns of all plants of a treatment were ground for 5 min with mortar and pestle in 1 ml of PPK buffer (140 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl; pH 7.4) supplemented by 0.15% (w/v) polyvinylpyrrolidon (Fluka AG, Buchs, Switzerland) and 0.05% Tween 20 per gram fresh weight. The homogenate was centrifuged in Eppendorf tubes (20,000 *g*; 10 min). The supernatant was diluted 1:3 (v/v) in PPK buffer supplemented with 0.1 μg of ovalbumin (Sigma Chemical Co., St. Louis, MO) per milliliter, and direct ELISA was conducted in microtiter plates (2). After substrate incubation for 60 min, differences between measurements were read at 405 and 600 nm with a photometer (EAR 400 FW, SLT Labinstruments GmbH, Groedig, Austria). In another experiment, surface-disinfested seeds were treated with a cultural filtrate of *E. herbicola* B247 containing 1% methylcellulose, as previously described for seed coating with antagonists.

The distribution of *E. herbicola* B247 on plant parts after seed treatment was determined with a spontaneous mutant resistant to streptomycin and rifampicin. Twelve pots ($11 \times 11 \times 12$ cm) containing nonsterile sand/peat (pathogen concentration 10^3–10^4 cfu/g) were sown with nine seeds each, which had been coated with the antibiotic-resistant mutant and were incubated as previously described. Six plants were randomly harvested after 10 and 32 days (one plant per pot) and cut into sections: the seed, the subterranean shoot, the first and second 2 cm above ground, and the remaining shoot length. The root was divided in upper 2 cm, the second 2 cm, the following 4 cm, and the remaining root length, including the tip. Roots were separated from soil by dry-shaking with glass beads. Bacteria were isolated by agitating plant parts with an Ultra Turrax mixer (Janke and Kunkel) in sterile tap water for 60 sec. Dilutions were plated on 2% TSA (pH 6.5) containing streptomycin sulfate (Serva) at 500 μg/ml and rifampicin at 50 μg/ml to enumerate *E. herbicola* B247 (cfu/g fresh weight), and on 0.3% TSA (pH 7.2) without antibiotics to enumerate total bacterial populations.

All experiments were repeated at least once with similar results.

In vivo assay with *P. r. tritici*. *E. herbicola* was tested for its ability to suppress *P. r. tritici* (mixture of races from Biologische Bundesanstalt für Land- und Forstwirtschaft, Braunschweig, West

Germany) on leaves of wheat plants in the greenhouse. A bacterial suspension (100 ml; A_{660} = 1.0) of *E. herbicola* B247, a Tn*5* mutant Tn247, or cultural filtrate of the wild type was atomized to runoff on winter wheat cultivar Jubilar at the one-leaf stage. All treatments contained 1% methylcellulose. Two pots of 25 plants each were used per treatment. After 2 hr, when the leaves had dried, 40 ml of a urediniospore suspension ($1–2 \times 10^5$/ml) were atomized onto the plants, which then were incubated in a dew chamber (100% relative humidity) for 20 hr at 20 C. Pots were completely randomized and after incubation for 8 days in a growth chamber (22–25 C, 85–95% relative humidity), all treated leaves were harvested, and the total number of sori per treatment (50 leaves) were counted on the abaxial surface. Leaf area was determined by an image-processing computer system (TV-camera, analog/digital-modem, image memory, and microcomputer VT101, Digital, Philips, Hannover, West Germany) to calculate the number of sori per square centimeter of leaf area. The experiment was repeated once with similar results.

RESULTS

Fifteen bacterial and 54 actinomycete strains from the rhizosphere and rhizoplane of Gramineae species were selected for their production of antibiotics inhibitory to *F. culmorum*. Fifteen antagonists (22%), applied to seed, resulted in more than 40% suppression of seedling blight on wheat caused by *F. culmorum* (data not shown). A highly significant correlation (r = 0.60; P = 0.001) occurred between the size of inhibition zone in vitro and disease suppression in vivo. One of the most active antagonists, bacterium B247 (22-mm inhibition zone, 54% of disease suppression), was investigated in detail. B247 was identified as *E. herbicola* on the basis of standard tests (17) and 89% DNA-DNA-homology with the type strain *E. herbicola* NCPPB 2971. The antimycotic substance produced by *E. herbicola* B247 was identified as herbicolin A. When co-chromatography was conducted on a TLC-plate with a mixture of cultural filtrate and pure herbicolin A, only one small inhibition zone of 2.5 mm in diameter appeared in a bioassay with *F. culmorum*.

Biocontrol of *F. culmorum* by *E. herbicola* B247. Strain B247 effectively controlled *F. culmorum* in pot tests; there was only a minor reduction in shoot length of wheat seedlings grown from treated seed compared with a marked stunting of shoots with nontreated seed (Figs. 1 and 2). Furthermore, treated plants had

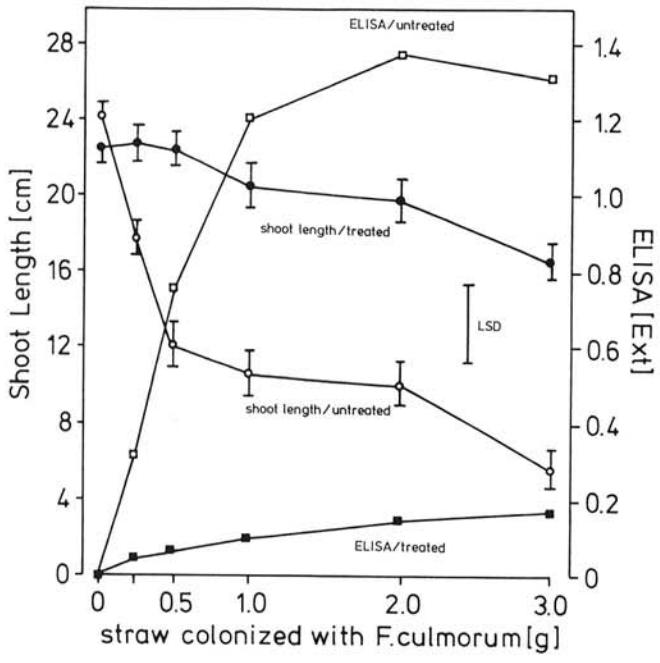

Fig. 1. Influence of seed treatment with *Erwinia herbicola* B247 on shoot length and enzyme-linked immunosorbent assay (ELISA) measurements of the biomass of *Fusarium culmorum* in crown tissue of wheat seedlings in response to different inoculum concentrations. Data represent mean shoot lengths of 45 plants per treatment (LSD = 3.8; P = 0.01; two-way analysis of variance [ANOVA]) and ELISA measurements from pooled plant crowns (45 plants per treatment). There was a highly significant inverse correlation between shoot length and ELISA values (r = −0.91 for treated plants and r = −0.95 for nontreated plants; P = 0.01). Bars indicate the standard error of the mean. Seeds were either treated or not treated with the bacterium and sown into nonsterile sand/peat mixed with straw colonized by *F. culmorum* (3 g of straw per 1,300–1,400 g of soil = 10^4–10^5 cfu/g) and incubated for 14 days at 18–24 C.

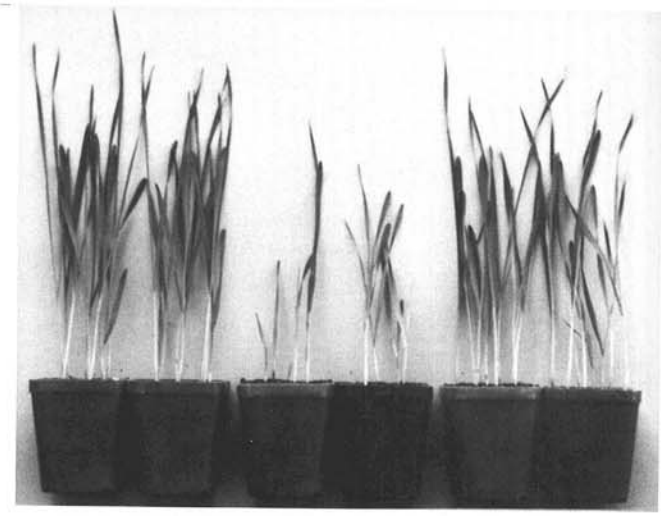

Fig. 2. Control of soilborne *Fusarium culmorum* (10^4–10^5 cfu/g soil) by seed treatment with *Erwinia herbicola* B247 (right) compared with inoculated control (center) and uninoculated control (left).

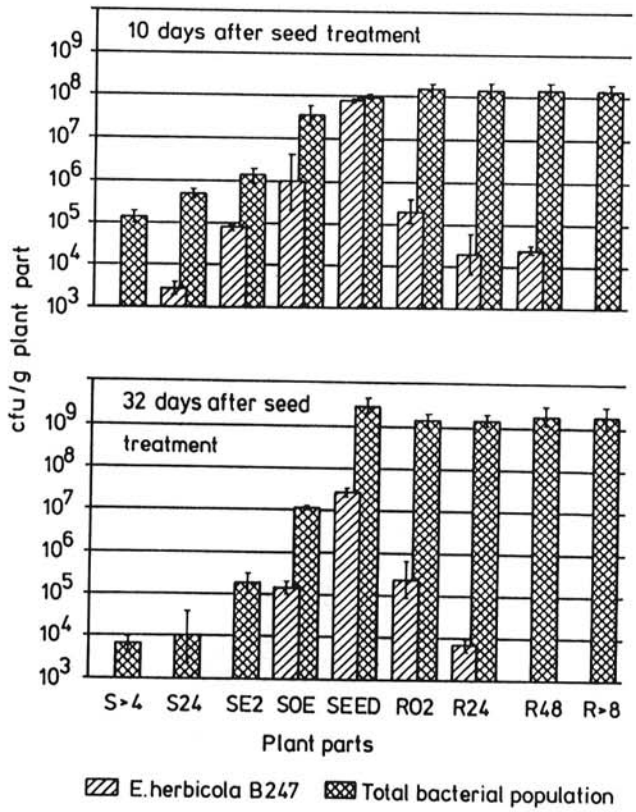

Fig. 3. Distribution and development of *E. herbicola* B247 on seed-treated plants compared with detectable total bacterial population (means of two experiments, detection limit = 10^3 cfu/g plant part), 10 and 32 days after application of $1–2 \times 10^7$ cfu/g seed. Plant sections enumerated: seed (SEED), the subterranean shoot (SOE), the first 2 cm of shoot above soil (SE2), the second 2 cm (S24), and the remaining shoot length (S > 4); the upper first 2 cm root (R02), the second 2 cm (R24), the following 4 cm (R48) and the remaining root length including the tip (R>8).

significantly higher shoot lengths than nontreated plants at all inoculum concentrations tested (LSD = 3.8; $P = 0.01$). ELISA measurements indicated that biomass of *F. culmorum* in crown tissue was inversely correlated with shoot length ($r = -0.91$ for treated plants and $r = -0.95$ for nontreated plants; $P = 0.01$). There was about a 90% suppression of biomass of *F. culmorum* at all inoculum concentrations tested; however, measurements of shoot length indicated that disease suppression decreased with increasing inoculum concentration (data not shown).

Seed treatment with the antagonist in the absence of the pathogen did not cause a plant growth-promoting effect. Application of killed cells (10 min at 80 C) to seeds did not suppress disease (data not shown).

Mechanism of action. The antibiosis-negative Tn*5* mutant gave 38% (standard deviation [SD] = 7.5) disease suppression, compared with 54% (SD = 7.4) caused by the wild type (means of six treatments each). Application of cultural filtrate containing the antibiotic to seed did not result in disease suppression.

Distribution of *E. herbicola* on young wheat plants. *E. herbicola* B247, resistant to streptomycin and rifampicin, was reisolated from roots and shoots after seed treatment, but population densities decreased with increasing distance from the seed (Fig. 3). The population densities of *E. herbicola* declined between 10 and 32 days after seed treatment, especially on plant parts remote from the seed.

Biocontrol of *P. r. tritici* by *E. herbicola* B247. Spraying a suspension of *E. herbicola* on wheat leaves before inoculation with urediniospores of *P. r. tritici* resulted in 76% disease suppression, whereas the Tn*5* mutant was ineffective (Table 1, Fig. 4). Conversely, almost complete protection was achieved by application of a cultural filtrate from the wild type. Light microscopic observations of urediniospores on wheat leaves treated with cultural filtrate of *E. herbicola* B247 showed total inhibition of germination, which also was seen in vitro.

TABLE 1. Suppression of *Puccinia recondita* f. sp. *tritici* on wheat leaves by application of the antibiotic-producing antagonist *Erwinia herbicola* B247, its cultural filtrate, or the antibiosis-negative mutant Tn247

	Number of sori[y]	Leaf area[y] (cm^2)	Density of sori[z] (cm^{-2})	Disease suppression (%)
E. herbicola B247	196	106.0	1.8 a	78
E. herbicola Tn247	809	95.9	8.3 b	−2
Cultural filtrate	6	88.4	0.1 c	99
Inoculated control	779	96.8	8.1 b	0
Uninoculated control	0	100.3	0 c	100

[y] Fifty plants per treatment at the one-leaf stage were treated with bacteria or cultural filtrate and inoculated 2 hr later with urediniospores of *P. r. tritici*. After incubation at approximately 100% relative humidity (20 C for 20 hr) and an additional 8 days at 85–95% relative humidity (22–25 C), the number of sori per treatment was counted and the corresponding leaf area was measured.

[z] To evaluate differences among treatments, ratios of sori number and leaf area were compared for pairs of treatments by binomial distribution (if the total number of sori of two treatments was lower than 30) and normal approximation of binomial distribution (if the total number of sori was higher than 30). Values followed by the same letter are not significantly different at $P = 0.01$.

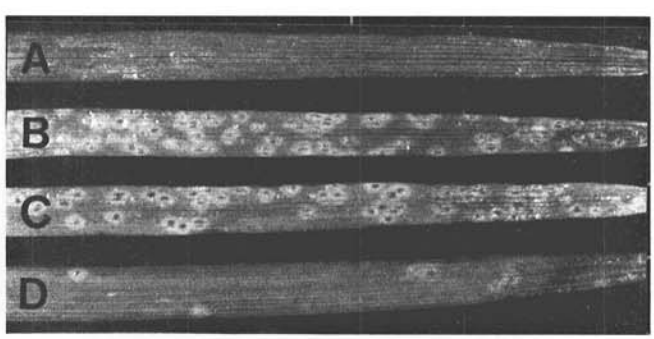

Fig. 4. *Puccinia recondita* f. sp. *tritici* infection on wheat leaves treated with **B,** an antibiosis-negative mutant *Erwinia herbicola* Tn247, **D,** the antibiotic-producing strain *E. herbicola* B247, and **A,** a cultural filtrate of B247 compared with **C,** a nontreated control.

DISCUSSION

The activity of antagonists in vitro was positively correlated to their activity against *F. culmorum* in vivo. Of the strains active against *F. culmorum* in vitro, 22% suppressed disease by more than 40%. Most of the isolated antagonists were actinomycetes, which are well known to have disease suppressing ability in soil (5). There are cases where no correlation could be established between disease control and antibiotic production, and, therefore, screening for antibiotic-producing microorganisms was not considered to be a useful strategy for finding effective biocontrol agents (15,18). On the other hand, correlations between antibiotic production and disease suppression have been reported (27,31), and antibiosis also is regarded as an important mechanism in biological control (4,16). It is apparent that it is risky to generalize about the importance of antibiotic production in disease control.

E. herbicola B247 appears to have excellent potential as a biocontrol organism since it protected wheat seedlings from infection by *F. culmorum* at all inoculum concentrations tested. ELISA appears to be an effective method for monitoring the severity of disease; biomass of the fungus in treated plants was reduced by 90%.

Antagonist colonization of plant parts prone to attack by the pathogen is essential for biological control. Because *F. culmorum* mainly infects crown tissue (25), colonization of the entire root by antagonists is not necessary, as suggested by the efficient biocontrol obtained during this investigation. Accordingly, the decline of root and shoot colonization by *E. herbicola* did not appreciably affect disease suppression. Also, protection of the crown is necessary only in the seedling stage, as adult wheat plants become resistant to *F. culmorum* (7).

Only part of the in vivo activity of *E. herbicola* against *F. culmorum* could be attributed to antibiosis. Some suppression in vivo also was achieved with the Tn*5* mutant lacking in vitro antibiosis. Antibiosis-negative and siderophore-minus mutants of different antagonists have been studied in other investigations on biological control (14,16,18,32). In most cases, the mutants still suppressed the disease to some extent (3,6,24). Wodzinski et al (30) reported that a mutant of *E. herbicola* 112Y, although incapable of producing the bacteriocin herbicolacein 112Y, still protected apple blossoms from infection by *E. amylovora*.

Competition between *F. culmorum* and *E. herbicola* B247 may play a role in biological control. Competition for nutrients and infection sites has been suggested as an explanation for antagonism of *E. herbicola* to *E. amylovora* (11,12). However, it is difficult to clearly demonstrate competition as a mechanism of biological control, except in cases where substances such as siderophores are involved (24).

The mechanism of biocontrol of *P. r. tritici* by *E. herbicola* B247 apparently differs from that of *F. culmorum*. Treatment with the bacterium resulted in 76% disease suppression, the mutant was ineffective, and application of cultural filtrate of B247 almost completely suppressed the pathogen. This suggests that antibiosis was the main mechanism of control. This is supported by the observation that urediniospore germination on agar and on leaves was inhibited by the cultural filtrate.

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