Inhibition of *Tilletia laevis* Teliospore Germination and Suppression of Common Bunt of Wheat by *Pseudomonas fluorescens* 2-79

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ABSTRACT

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A rifampicin-resistant derivative of *Pseudomonas fluorescens* strain 2-79 (Pf2-79r) inhibited teliospore germination of *Tilletia laevis*. A rifampicin-resistant derivative of a spontaneous mutant of strain 2-79 deficient in phenazine-1-carboxylic acid production (2-79PCA$^-$r) had no effect on germination of *T. laevis*. Inhibition of germination by Pf2-79r was not overcome by supplementing culture media with iron. Common bunt incidence was reduced by 65 and 50% during consecutive seasons when wheat seeds and 2-wk-old seedlings were treated with Pf2-79r. Similar treatment of seeds and seedlings with 2-79PCA$^-$r afforded no protection against common bunt.

Additional keywords: biological control, smut, *Tilletia caries, Tilletia foetida, Tilletia tritici, Triticum aestivum*

Tilletia laevis Kühn in Rabenh. (= *T. foetida* (Wallr.) Liro) and *T. tritici* (Bjerk.) G. Wint. in Rabenh. (= *T. caries* (DC.) Tul. & C. Tul.) are the causal organisms of common bunt of winter wheat (*Triticum aestivum* L.). While *T. tritici* is found worldwide wherever wheat is grown, *T. laevis* has a somewhat narrower distribution but is prevalent throughout the European mainland and central and eastern North America (9,22). Common bunt is characterized by masses of teliospores that form sori or "bunt balls" in place of kernels. Harvesting procedures rupture sori, and the liberated teliospores contaminate the soil and healthy seeds. Teliospores germinate within a few days to a few weeks after exposure to moisture. Germ tubes bear monokaryotic primary sporidia that fuse and in turn yield dikaryotic secondary sporidia. When germination of wheat seed coincides with germination of secondary sporidia, the infectious hyphae can penetrate young seedlings and grow systemically in the plant.

Seed treatment with fungicides remains the most effective deterrent of the common bunt fungi (7,11). However, the trend toward alternate disease management strategies is growing, partly because of the perceived health and environmental risks associated with chemical pesticides (8). Biological control of bunt could be achieved if an organism capable of disrupting the initial infection of wheat seedlings by *T. laevis* were found.

Bacteria-produced antifungal compounds, siderophores, or both, have been implicated in the control of soilborne fungal pathogens by fluorescent *Pseudomonas* spp. (6,18,20). Weller and Cook (21) isolated *P. fluorescens* (Trevisan) Migula strain 2-79 from soil that had been cropped to wheat for several consecutive years. Strain 2-79 proved inhibitory to the take-all pathogen, *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier var. *tritici* J. Walker, in vitro and suppressed take-all in the field when seeds were coated with bacteria prior to planting (21). Subsequently, this strain was shown to produce the antibiotic phenazine-1-carboxylic acid (PCA) as the primary mechanism of take-all suppression, whereas a fluorescent siderophore and anthranilic acid played minor roles (5,17,19). Mutants of strain 2-79 deficient in PCA production were consistently less inhibitory to *G. g. tritici* in vitro and less suppressive to take-all (5,13,17), whereas siderophore-deficient mutants were generally as effective as their fluorescent parental strains in suppressing take-all (5,13).

The goals of this study were to determine if *P. fluorescens* strain 2-79 inhibits germination of *T. laevis* teliospores and to evaluate strain 2-79 as a biological control agent for common bunt. In addition, a mutant strain of 2-79 deficient in PCA production was studied to test the role of PCA in fungal inhibition and disease suppression.

MATERIALS AND METHODS

Preparation of *T. laevis* inoculum. *T. laevis* teliospores were harvested from bunted wheat at the Botany and Plant Pathology Research Farm of Michigan State University (MSU) at East Lansing. For in vitro studies, mature sori were surface-sterilized for 2 min in 1% sodium hypochlorite, rinsed with sterile distilled water, and crushed with a glass rod in a funnel lined with Miracloth. Teliospores were washed through the cloth with sterile distilled water and collected in a sterile flask. The suspension was adjusted with water to approximately 10^6 teliospores per milliliter. For field experiments, mature bunted wheat heads were crushed and the teliospores were passed through a series of nylon screens to exclude most plant tissue. Seeds of the highly susceptible soft white winter wheat cultivar Frankenmuth were inoculated by shaking 0.75 g of teliospores with 1 kg of seed in a plastic bag, resulting in approximately 10^4 teliospores per seed.

Culture of *P. fluorescens* and selection of mutants. *P. fluorescens* strain 2-79 (21) was obtained from the Agricultural Research Culture Collection (Peoria, IL), accession NRRL B-15132. The bacterium was initially cultured on King's medium B (KB) (10) and potato-dextrose agar (PDA) at 25 C. PCA produced by strain 2-79 imparts an olive green pigment to centers of colonies when cultured on PDA (17). After approximately 40 transfers in culture, colonies of strain 2-79 arose that lacked the green pigment on PDA but fluoresced when transferred to KB. These colonies were presumed deficient in PCA production and were designated 2-79PCA$^-$. Rifampicin-resistant mutants of strains 2-79 and 2-79PCA$^-$ were obtained by plating 0.1 ml of nutrient broth yeast extract (NBY) cultures that had incubated overnight onto KB amended with 100 μg per milliliter rifampicin (KBr). Generation times in liquid NBY of Pf2-79 and 2-79PCA$^-$ and of their respective rifampicin-resistant mutants, designated Pf2-79r and 2-79PCA$^-$r, were determined by plating appropriate dilutions at 2-hr intervals for 6 hr and plotting the number of colony forming units vs. time. This procedure was repeated once. The strains were stored in 15% glycerol at −80 C, and bacteria for all experiments were grown from stored cultures.

In vitro tests. Strains Pf2-79r and 2-79PCA$^-$r were tested as inhibitors of *T. laevis* teliospore germination by spotting single colonies of each culture to the centers of 20 plates of PDA for a total of 40 plates. After incubation at 25 C for 48 hr, either bacteria were killed by inverting each plate over a chloroform-soaked cotton ball in an open glass petri dish for 10 min or bacterial growth was

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removed from the media with a sterile scalpel. Twenty uninoculated plates were similarly treated. All plates were then atomized with 0.25 ml of a teliospore suspension (10^6 teliospores per milliliter of water). After 8 days, each plate was divided into quadrants, and 50 teliospores approximately 30 mm from the center of the plate in each quadrant were rated as germinated or ungerminated. This experiment was performed once.

If sequestering of iron by bacteria-produced siderophores prevents teliospore germination, then inhibition should be overcome by supplementing the growth medium with iron. The role of siderophores in fungal inhibition was tested by amending KB with 0, 125, 250, 500, 1,000, or 2,000 μM of $FeCl_3$ prior to inoculation with bacteria. Three replicate plates for each level of $FeCl_3$ were inoculated with either Pf2-79r or 2-79PCA$^-$ or were left untreated as a control. Incubation, colony removal, teliospore application, and rating of germination were performed as described above. This experiment was performed twice.

Bacterial seed treatment. Wheat seeds were treated by a modification of the methods of Weller and Cook (21). A slurry of methylcellulose (Methocell A-15) was added to liquid NBY cultures of Pf2-79r or 2-79PCA$^-$r that had incubated overnight (final methylcellulose concentration of 1%, w/v). Seeds were rinsed with tap water, then thoroughly coated with the suspension at a rate of 200 ml/kg of seed and air-dried overnight. Each seed was coated with 10^7–10^8 cfu as determined by rinsing seeds in water and plating 0.1 ml of the appropriate dilutions onto KBr. Seeds for control treatments were coated with 1% methylcellulose without bacteria. Seeds were stored at 4 C and planted within 48 hr of treatment.

Field tests. Field tests were performed at MSU. Seeds were sown with a seven-row drill planter into sandy clay loam, pH 5.6, containing 43 μg of DPTA-extractable iron per gram of soil. Two experiments were initiated on 2 October 1990. In the first experiment, six repetitions of the following four seed treatments were arranged in a completely randomized design: 1) methylcellulose followed by *T. laevis* teliospore inoculation (10^4 teliospores per seed), 2) Pf2-79r (10^7–10^8 cfu per seed) followed by teliospore inoculation, 3) Pf2-79r only, and 4) methylcellulose control. Each treatment consisted of seven 3-m rows spaced 0.18 m apart. Preliminary field experiments indicated that enriching the soil and rhizosphere with Pf2-79r after seedlings had emerged from bacterized seeds was more effective in reducing bunt than either treatment alone. Therefore, 14 days after planting, seedlings of treatments 2 and 3 were sprayed with a suspension of Pf2-79r in water (10^7 cfu per milliliter) at a rate of 1 L/11 m of row. The second experiment was identical to the first except that seeds and seedlings were treated with 2-79PCA$^-$r. The two experiments were repeated in 1991 with planting on 4 October and spraying 15 days later.

The survival of Pf2-79r and 2-79PCA$^-$r was monitored by sampling from wheat roots and crowns with adhering seed remnants approximately 4 wk after planting and in mid-May of the following year. Four 1-g samples of roots and four 1-g samples of crowns from a pool of several plants of each treatment were ground individually in 10 ml of phosphate-buffered saline or water with a mortar and pestle. Dilutions were plated onto KBr amended with penicillin (75 units per milliliter), novobiocin (45 μg/ml), and cycloheximide (75 μg/ml) to select for fluorescent pseudomonads (16). Colonies that grew on the rifampicin-amended medium and fluoresced when transferred from KBr to KB were presumed to be the marked strains.

Wheat heads were observed for common bunt in late June of 1991 and 1992. The number of bunted heads per 200 heads counted for each repetition was recorded. Often, several or all heads of a single plant were bunted; therefore, a maximum of three heads from any one plant were counted. Field experiments were analyzed by the least significant difference (LSD) test ($P = 0.05$).

RESULTS

Generation times. Mean generation times in hours ± SE of Pf2-79, Pf2-79r, 2-79PCA$^-$, and 2-79PCA$^-$r were 3.7 ± 0.1, 3.6 ± 0.1, 3.6 ± 0.2, and 3.8 ± 0.1, respectively.

In vitro tests. After 8 days, germ tubes and sporidia of *T. laevis* formed a dense white lawn on untreated PDA and PDA that had been exposed only to chloroform vapors (Fig. 1). A white lawn also formed on KBr. Pretreatment of PDA with Pf2-79r resulted in nearly complete inhibition of teliospore germination (Fig. 1). Germination ranged from 2 to 10%, with a mean rate of 5% (SD = 2.1%) compared with a mean rate of 88% (SD = 7.5%) on control plates. PDA plates treated with 2-79PCA$^-$r prior to inoculation with *T. laevis* were covered by a dense fungal lawn after 8 days and were indistinguishable from control plates. On KB without iron, teliospore germination was 6% after treatment with strain Pf2-79r, and increasing the concentration of Fe^{3+} did not overcome the inhibition imposed by this strain (Fig. 2). Pretreatment of KB with strain 2-79PCA$^-$r had no effect on teliospore germination. Germination was inhibited slightly at 500 μM $FeCl_3$ and severely at greater concentrations, regardless of bacterial pretreatment.

Field tests. Rifampicin-resistant fluorescent bacteria were recovered from all samples of roots (10^5–10^6 cfu/g) and

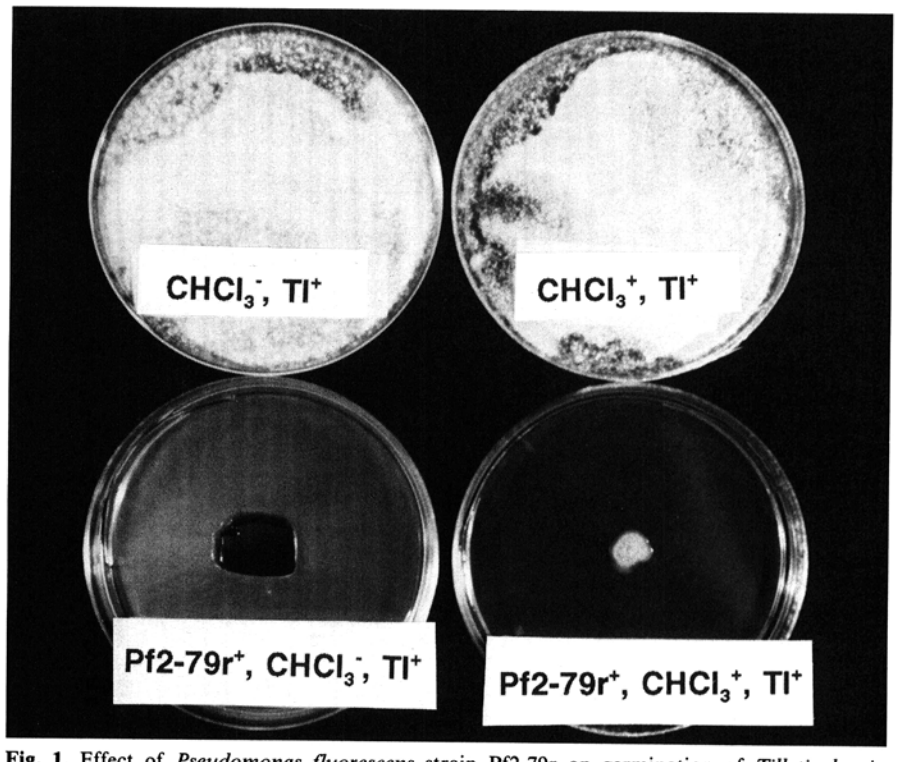

Fig. 1. Effect of *Pseudomonas fluorescens* strain Pf2-79r on germination of *Tilletia laevis* teliospores. Plates of potato-dextrose agar 8 days after being atomized with 0.25 ml of *T. laevis* (Tl) teliospores (10^6/ml of water): No treatment (upper left), a 10-min exposure to chloroform ($CHCl_3$) vapors (upper right), 48-hr incubation and subsequent removal of a colony of Pf2-79r (lower left), and 48-hr incubation and subsequent killing of a colony of Pf2-79r by a 10-min exposure to $CHCl_3$ (lower right).

crowns with seed remnants (10^6–10^8 cfu/g) 4 wk after planting in 1990 and 1991. Populations were much lower and more variable when monitored the following May, ranging from 10^1 to 10^5 cfu/g of root tissue and from 0 to 10^3 cfu/g of crown tissue. Rifampicin-resistant fluorescent bacteria were not detected among treatments for which bacteria were not applied.

The combination of treating seeds and spraying seedlings with strain Pf2-79r reduced the incidence of bunt in 1991 and 1992 by 65 and 50%, respectively (Table 1). Similar treatment of wheat with strain 2-79PCA$^-$r provided no protection against bunt (Table 1).

DISCUSSION

Strain Pf2-79r of *P. fluorescens* inhibited teliospore germination of *T. laevis* in vitro and significantly reduced the incidence of common bunt in the field. Inhibition is likely mediated in part by PCA, as a mutant strain deficient in PCA production, 2-79PCA$^-$r, did not impede the fungus. Ownley et al (13) speculated that PCA acts by interfering with energy-requiring, membrane-bound metabolic processes. This would account for the seemingly nonspecific nature of antibiosis by PCA as demonstrated by its effectiveness against the dissimilar organisms *G. g. tritici* and *T. laevis*. However, neither the study by Ownley et al (13) nor the present study focused on PCA-mediated alterations of fungal physiology. Brisbane et al (2) also reported inhibition of bacterial and fungal species by PCA isolated from strain 2-79 or by synthetic PCA. Whatever its mode of action, PCA is a potent deterrent of *T. laevis* teliospore germination, since the inhibition zone imposed by Pf2-79r encompassed the entire 100-mm-diameter petri dish. The role of iron-regulated factors, such as siderophores and anthranilic acid, is probably minor, since inhibition was not overcome when the growth medium was amended with iron. Furthermore, *T. laevis* teliospores germinated and germ tubes bore sporidia on KB, implying that endogenous iron reserves were sufficient for the brief nonparasitic phase of fungal growth. Teliospore germination was nearly completely inhibited at $FeCl_3$ concentrations in excess of 500 μM. Similar cases of iron toxicity toward fungal phytopathogens have been reported (1,14).

Fungal inhibition in vitro corresponded with disease suppression in the field. When wheat seeds and seedlings were treated with Pf2-79r, bunt was reduced by 65 and 50% in 1991 and 1992, respectively. While this degree of disease suppression was significant, it still resulted in unacceptable levels of bunt. In our experiments, seed was inoculated with *T. laevis* at a rate of approximately 10^4 teliospores per seed in order to consistently achieve infection. However, this rate was probably unnaturally high and overwhelming to Pf2-79r. For example, the equivalent of 440 teliospores of *T. tritici, T. laevis*, or both, per seed was described as "high" contamination in a survey of grain handling and transportation facilities in Montana (12). Most samples showed only "trace" or "moderate" levels of contamination, the equivalent of four to 44 teliospores per seed. Thus, we believe a higher degree of bunt control would be attained under natural field conditions.

Treatment of seeds and seedlings with 2-79PCA$^-$r resulted in levels of bunt similar to those of the control. These results agree with results of previous studies of strain 2-79 demonstrating that PCA is the primary factor governing inhibition of *G. g. tritici* and take-all suppression (5,17,19).

The discovery and application of biological controls for plant diseases is a growing priority in modern agriculture. Unfortunately, biological agents seldom confer the degree of control desired in commercial agriculture. Weller (20) cites loss of ecological competence and variable root colonization by bacteria as possible reasons for the inconsistent nature of bacteria-mediated biological control. In the present study, bacterial populations remained high 4 wk after planting. The risk of infection by *T. laevis* would be low after 2 wk, since normally only seeds and very young seedlings are susceptible to common bunt (3). Thus, permanent root colonization by bacteria is probably not essential for biological control of common bunt, unlike true root diseases. Rather, a transient but timely existence on the seed coat and newly emerged coleoptile might be sufficient. The purpose of spraying young wheat seedlings with bacteria 2 wk after planting was to enrich the soil with the control

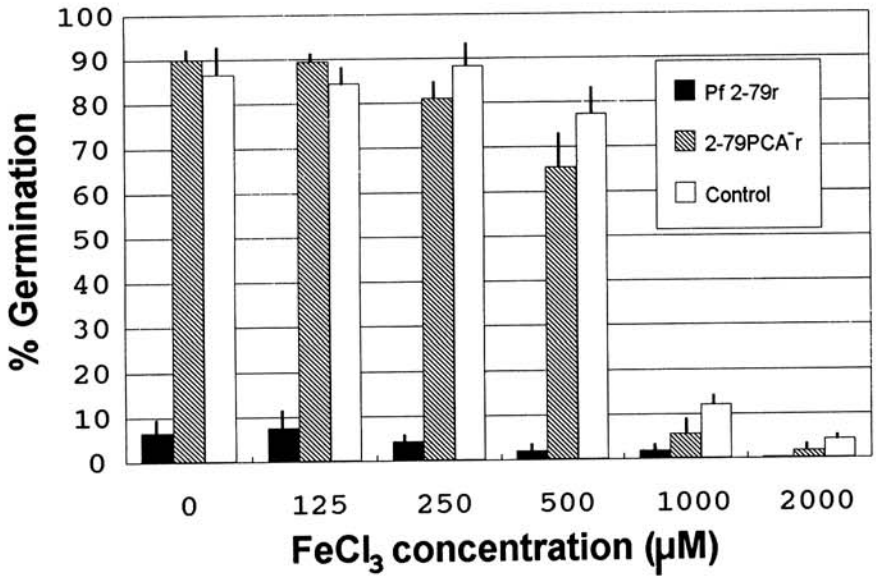

Fig. 2. Effect of $FeCl_3$ concentration and bacterial pretreatment on germination of *Tilletia laevis* teliospores. Bars represent the means of 200 teliospores from three replicate plates per $FeCl_3$ level for two experiments. Vertical lines represent the standard error of the mean.

Table 1. Effect of *Pseudomonas fluorescens* strains Pf2-79r and 2-79PCA$^-$r on percent bunted wheat heads

Strain Seed treatment[x]	Percent bunted heads[y] 1991	 1992
Pf2-79r		
Tilletia laevis only	29.7 a[z]	13.7 a
Pf2-79r + *T. laevis*	10.3 b	6.8 b
Pf2-79r only	0.0 c	0.0 c
None (control)	0.0 c	0.0 c
2-79PCA$^-$r		
T. laevis only	20.7 a	14.6 a
2-79PCA$^-$r + *T. laevis*	20.3 a	15.1 a
2-79PCA$^-$r only	0.0 b	0.0 b
None (control)	0.0 b	0.0 b

[x] *T. laevis*, the *P. fluorescens* strain, or both, were applied to wheat seeds at rates of 10^4 teliospores and 10^7–10^8 cfu per seed, respectively. Seedlings of *P. fluorescens*-treated seeds were sprayed with a 10^7 cfu/ml suspension of the indicated strain in water approximately 2 wk after planting.

[y] Determined by rating a 200-head sample for each of six repetitions per treatment.

[z] Means within the same group of four followed by the same letter are not significantly different at $P = 0.05$ by least significant difference analysis.

organism at a time we estimated would be pivotal for the establishment or prevention of disease. At this time, newly emerged seedlings would be susceptible and secondary sporidia would be producing infectious hyphae. Devising an optimal control strategy would require close monitoring of seedling emergence and teliospore germination. Both events are governed by environmental factors, including soil temperature and moisture, and would vary among years and locations. We chose to confine our experiments to the MSU campus in order to prevent the spread of *T. laevis* teliospores, which, despite relatively low numbers in Michigan soils, have resulted in common bunt outbreaks during the past two decades (23).

Seed treatments with liquid formulations of carboxin fungicides have generally been effective in controlling seedborne common bunt (7,11), although the efficacy of chemicals can vary depending on environmental conditions (4). The present study indicates that *P. fluorescens* strain Pf2-79r could be an effective biological control of seedborne bunt. When seed is sown into soil contaminated with teliospores from the previous wheat crop, however, common bunt becomes soilborne and difficult to control with chemicals (7,15,24). Additional studies are required to determine if timely application of Pf2-79r might curb soilborne common bunt, which is currently not a problem in Michigan but is of major concern elsewhere.

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