Production of the Antibiotic Phenazine-1-Carboxylic Acid by Fluorescent *Pseudomonas* Species in the Rhizosphere of Wheat

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Received 1 November 1989/Accepted 25 January 1990

Pseudomonas fluorescens 2-79 and *P. aureofaciens* 30-84 produce the antibiotic phenazine-1-carboxylic acid and suppress take-all, an important root disease of wheat caused by *Gaeumannomyces graminis* var. *tritici*. To determine whether the antibiotic is produced in situ, wheat seeds were treated with strain 2-79 or 30-84 or with phenazine-nonproducing mutants or were left untreated and then were sown in natural or steamed soil in the field or growth chamber. The antibiotic was isolated only from roots of wheat colonized by strain 2-79 or 30-84 in both growth chamber and field studies. No antibiotic was recovered from the roots of seedlings grown from seeds treated with phenazine-nonproducing mutants or left untreated. In natural soils, comparable amounts of antibiotic (27 to 43 ng/g of root with adhering soil) were recovered from roots colonized by strain 2-79 whether or not the pathogen was present. Roots of plants grown in steamed soil yielded larger bacterial populations and more antibiotic than roots from natural soils. In steamed and natural soils, roots from which the antibiotic was recovered had significantly less disease than roots with no antibiotic, indicating that suppression of take-all is related directly to the presence of the antibiotic in the rhizosphere.

Biological control of soilborne plant pathogens by antagonistic microorganisms may offer a practical supplement or alternative to existing disease management strategies that depend heavily on chemical pesticides. Control of take-all, an important root disease of wheat caused by *Gaeumannomyces graminis* var. *tritici*, can occur spontaneously in soils that accumulate a microflora antagonistic to the pathogen after several consecutive years of wheat monoculture (6, 7, 12, 17, 18). Fluorescent *Pseudomonas* species indigenous to the rhizosphere of wheat are thought to have a major role in this take-all decline phenomenon (6, 7, 19, 20), and certain strains isolated from wheat roots protect against the disease when applied to wheat seed (24). Disease suppression by these introduced bacteria depends on their ability both to colonize the roots (22, 23; C. T. Bull, M.S. thesis, Washington State University, Pullman, 1987) and to produce substances inhibitory to the pathogen (14, 21, 25). One such strain, *Pseudomonas fluorescens* 2-79, synthesizes phenazine-1-carboxylic acid (PCA), a pigmented antibiotic that inhibits *G. graminis* var. *tritici* and other fungal root pathogens in vitro at less than 1 µg/ml (3, 4, 14). Mutants of 2-79 defective in production of PCA (Phz⁻) fail to inhibit *G. graminis* var. *tritici* and are greatly reduced in their ability to suppress take-all, whereas mutants genetically restored for production of PCA (Phz⁺) coordinately regain antifungal activity in vitro and on wheat (21). These results strongly suggest that take-all control by strain 2-79 is due largely to PCA production in the wheat rhizosphere.

Efforts to define the role of antibiotics in biological control consistently have been impeded by a lack of direct evidence that antibiotics are present in natural soils in which disease suppression may occur. The physical and biological constraints to antibiotic production, activity, and detection in soil have been reviewed (2, 11, 13, 25a, 27), of which the most fundamental probably is nutritional inadequacy. Soils amended with organic nutrients or treated to increase nutrient availability frequently support antibiotic production.

Antibiotics also have been detected in natural soils but only in the presence of nutrient sources, such as seeds (14a, 29), roots (15), or fragments of straw (5, 28). These microhabitats are often the primary sites of interaction between pathogens and antagonists. In one recent study (14a), the expression of *afuE*, a biosynthetic gene required for production of the antibiotic oomycin A by *P. fluorescens* HV37a, was monitored in the spermosphere of cotton and correlated with control of damping-off disease caused by *Pythium ultimum*.

From 5 to 18% of carbon assimilated as photosynthate is released by wheat roots (1) in forms such as exudates and cell lysates that are readily metabolized by rhizosphere bacteria. Because these nutrients support microbial activity at a higher level than occurs in bulk soil, it is plausible that PCA might be present in the rhizosphere in quantities sufficient for detection by direct methods. We report here the isolation of PCA from roots of wheat grown from bacterium-treated seeds in unamended steamed and natural soils. The presence of the antibiotic was correlated with suppression of take-all.

MATERIALS AND METHODS

Organisms and culture conditions. *P. fluorescens* 2-79RN₁₀, a rifampin-resistant derivative of strain 2-79 (24), was used as the wild-type strain. The 2-79 derivative strains 2-79-B46, a Phz⁻ Tn5 mutant, and 2-79-B46R, restored to Phz⁺ by genetic complementation, were previously described (21). *Pseudomonas aureofaciens* 30-84 is a biocontrol strain that produces mainly PCA but also small amounts of at least two additional hydroxylated phenazine antibiotics in vitro (L. S. Pierson III, unpublished results). Strain 30-84-44-8 is a Phz⁻ Tn5 derivative of 30-84.

Bacterial cultures used for seed treatment were grown on King medium B (16) agar plates, harvested into water, and washed twice by centrifugation to remove residual phenazine antibiotics. Sedimented bacteria were mixed with 0.5% (wt/vol) methylcellulose and applied to wheat seeds (cv. Fielder) to give a final concentration of 1 × 10⁸ to 4 × 10⁸ CFU per seed.

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The isolate of *G. graminis* var. *tritici* used in this study originated from a single ascospore and was maintained at 4°C. Oat kernel inoculum infested with the pathogen was prepared as described previously (26).

Wheat seedlings. Wheat seedlings were grown from bacterium-treated seeds either in the field or in conical plastic tubes (26.5 by 2.5 cm) in a growth chamber, using a modification of the take-all suppression tube assay (21). For the growth chamber study, the tubes were first filled with a 2-cm layer of sterile vermiculite and 15 g of soil. The soil was watered with 25 ml of one-third-strength modified Hoagland solution (macroelements only) containing metalaxyl [*N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)-DL-alanine methyl ester] to control pythium root rot. Three seeds were sown per tube, covered with 5 g of noninfested soil and a 1-cm-thick layer of sterile vermiculite, and watered (5 ml). Tubes were again watered (5 ml) on days 9 and 15. Studies were conducted in Ritzville silt loam or Puget silt loam that was left natural or steamed for 1 h to reduce the initial population of soil microorganisms. Soils infested with *G. graminis* var. *tritici* were amended with 0.45% (wt/wt) pulverized, pathogen-colonized oat kernels (26). The seedlings were removed by pushing out the contents of the tubes after 18 to 22 days. Loosely adhering soil was shaken gently from the roots, and the seeds and the portions of the roots that had grown into the lower vermiculite layer were excised and discarded. Roots from the soil layer were washed to isolate the antibiotic as described below, and the seedlings were then evaluated for disease on a scale of 0 to 8 (21). In each experiment, the treatments were replicated at least four times, with a replicate consisting of 10 tubes. A randomized complete block design was used.

Ritzville silt loam was from a site on the Washington State University Dryland Field Station at Lind, Washington, that had not been cropped previously and supported native grasses. The pH (measured in 0.01 M CaCl₂) was 7.6; available Fe was 6.75 µg/g; and the soil contained 60% silt, 6.4% clay, and 33.6% sand. Puget silt loam was from the Washington State University Northwestern Research and Extension Unit at Mount Vernon, Washington, and had been in a wheat-pea rotation. The pH was 5.5; available Fe was 93.7 µg/g; and the soil contained 64% silt, 22.4% clay, and 13.6% sand.

Field studies were conducted at two sites on the Dryland Field Station at Lind. Seeds treated as described above were sown in the Ritzville silt loam both at a site not previously cropped (virgin soil; pH 7.0, with 13 µg of available iron per g and 60% silt, 7.2% clay, and 32.8% sand) and at a site where wheat had been grown consecutively for many years (pH 6.1, with 21.9 µg of available iron per g, and 56% silt, 9.2% clay, and 34.8% sand). No inoculum of *G. graminis* var. *tritici* was introduced, and no symptoms of take-all were apparent on the wheat seedlings at either site. The plots were irrigated weekly by overhead sprinklers. After 21 days, the seedlings and surrounding soil were removed with a shovel and transported to the laboratory. Excess soil and the remnants of the seeds were removed from each plant, and PCA was isolated from the roots as described below.

Bacterial populations. Populations of introduced strains, total fluorescent pseudomonads, and total aerobic bacteria were determined as described previously (22). A single root system was weighed and macerated in 0.01 M phosphate buffer with a mortar and pestle, and appropriate dilutions were plated as described elsewhere (22). Reported values are the means from two (steamed soil) or three (natural soil)

replicates, with the roots and adhering rhizosphere soil of a single seedling constituting a replicate.

Antibiotic isolation. Seedlings from each treatment were grouped by replicate, and the replicates were pooled for extraction of the antibiotic. The roots with adhering rhizosphere soil were immersed and washed twice by sonication (2 min each time in sequential 100-ml volumes of 50 mM sodium acetate buffer, pH 4.5). The two washes, including rhizosphere soil, were combined, acidified to pH 2 with HCl, and shaken with 200-ml (for 2 h) and then 100-ml (for 2 to 6 h) volumes of benzene. The aqueous and organic phases were separated by centrifuging or by allowing the mixture to stand undisturbed. Benzene fractions from both extractions were pooled, dehydrated with Na₂SO₄, and evaporated to dryness. Phenazine antibiotics present in the residue were dissolved by gentle agitation for 8 to 12 h in 5 ml of aqueous 5% (wt/vol) NaHCO₃, extracted with an equal volume of benzene, acidified, partitioned into benzene, and dried. The efficiency of recovery by this procedure was estimated at 90 to 100% on the basis of samples in which 100 µg of purified PCA (14) was added to washes of wheat roots grown from nontreated seeds. The concentrations of the antibiotic were determined after samples were fractionated by reverse-phase high-pressure liquid chromatography (HPLC). Peak areas were converted to nanograms of PCA from a calibration curve prepared by injecting known amounts of the purified antibiotic.

Chromatography. Fractionation by thin-layer chromatography was on silica gel G plates developed with either benzene:acetic acid (95:5 by volume) or ethylacetate:*n*-butyl ether:acetic acid (150:150:10 by volume). Prior to analysis by HPLC, samples were applied in benzene to a Sep-Pak C₁₈ cartridge (Waters Associates, Inc.) and eluted with chloroform. HPLC was conducted in a chromatography system (Waters) with a Nova-Pak C₁₈ reverse-phase radial pack cartridge. The mobile phase consisted of a 30-min, 30 to 70% linear gradient of acetonitrile in water. Both solvents contained 0.1% trifluoroacetic acid. The chromatograms were monitored at 254 nm, and the spectra were analyzed with a 990 photodiode array detector (Waters).

RESULTS

Growth chamber studies. Washes of the roots of wheat grown from seeds treated with strain 2-79 contained material that comigrated with purified PCA after fractionation by thin-layer chromatography (data not shown) or HPLC (Fig. 1). Spectra measured at intervals within the peak fractionated by HPLC showed the material to be homogeneous and indistinguishable from purified PCA, whether the PCA was injected separately or coinjected with the root-derived material. No antibiotic was detected in washes of roots of plants from nontreated seeds or seeds treated with 2-79-B46, a Phz⁻ Tn5 derivative of 2-79. The antibiotic was, however, present on roots from seeds treated with 2-79-B46R, a strain restored to Phz⁺ by genetic complementation (Table 1).

Similar quantities (28 to 43 ng/g of root with adherent rhizosphere soil) of PCA were recovered from seedlings colonized by strain 2-79 or 2-79-B46R in natural Puget silt loam or Ritzville silt loam both in the presence or absence of the pathogen, even though the 2-79 population was about 10-fold smaller on roots without *G. graminis* var. *tritici* (Table 1). On roots grown in steamed soil, approximately 10-fold more antibiotic (250 to 365 ng) was detected, and the populations of the introduced strains also were about 10-fold larger than in natural soil. The introduced strains constituted

FIG. 1. Identification of PCA in washes from roots and rhizosphere soil. (A) Purified PCA (Standard), an extract of washings from roots colonized by *P. fluorescens* 2-79 (Roots), or a mixture of both samples (Coinject) was fractionated by reverse-phase HPLC. Each sample contained one major peak with a retention time of about 22.5 min. (B) Spectral analysis of the samples fractionated in panel A. Spectra were measured at the peak apex in each elution profile. (C) Spectral homogeneity of the Coinject peak. Spectra were measured at six intervals between 22.39 and 22.62 min.

the majority of the population of fluorescent pseudomonads but usually were a much smaller fraction of the total aerobic population. The populations of total aerobic bacteria on roots were comparable in size whether or not strain 2-79 or its derivatives were introduced.

PCA also was recovered from roots colonized by *P. aureofaciens* 30-84, but not its Phz^- Tn5 derivative 30-84-44-8, in steamed and natural soils (578 and 133 ng, respectively). In both soils PCA accounted for essentially all of the phenazine recovered (data not shown). The population of 30-84 on roots from the natural soil was almost 20-fold higher than that of strain 2-79, and three- to fourfold more antibiotic (133 ng compared with 36 to 43 ng) was recovered from 30-84-colonized roots. In the steamed soil, however,

the quantities of PCA recovered from roots colonized by strain 30-84 (597 ng) or 2-79 (365 ng) were more comparable, and the population sizes also were similar (Table 1).

Roots from which PCA was recovered had significantly less take-all than control roots or roots colonized by Phz^- mutants (Table 1). Disease suppression occurred both in Puget silt loam (pH 5.5) and Ritzville silt loam (pH 7.6). In both steamed and natural soils, roots colonized by strain 30-84 had the least amount of disease. Strain 30-84 produced less PCA per bacterium than did strain 2-79, but more antibiotic was present per gram of 30-84-colonized root (Table 1) because the populations of 30-84 were larger and, in steamed soil, roots colonized by 30-84 weighed less. However, additional replicated trials are needed to deter-

TABLE 1. Production of PCA by *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 in the rhizosphere of wheat and relationship between antibiotic production and biological control of take-all by the bacteria

Seed treatment	Soil ^a	<i>G. graminis</i> var. <i>tritici</i>	Ng of PCA ^b per:		Bacterial population (CFU/g of root) ^c			
			Plant	g of root ^c	Introduced strain (10 ⁷)	Total fluorescent pseudomonads (10 ⁷)	Total aerobic bacteria (10 ⁶)	Disease rating ^d
2-79 (Phz ⁺)	RSL (NAT)	+	30,36	36,43	1.60	6.21	6.57	3.64 B
2-79-B46 (Phz ⁻)	RSL (NAT)	+	0	0	1.51	2.12	6.99	4.08 A
2-79-B46R (Phz ⁺)	RSL (NAT)	+	22	29	2.51	5.41	5.07	3.60 B
30-84 (Phz ⁺)	RSL (NAT)	+	101	133	29.30	29.30	8.36	2.08 C
30-84-44-8 (Phz ⁻)	RSL (NAT)	+	0	0	0.56	6.77	7.50	4.22 A
None	RSL (NAT)	+	0	0		7.39	5.16	4.42 A
2-79 (Phz ⁺)	RSL (STM)	+	292	365	14.30	31.90	32.00	3.40 C
2-79-B46 (Phz ⁻)	RSL (STM)	+	0	0	2.13	8.57	13.80	4.63 B
2-79-B46R (Phz ⁺)	RSL (STM)	+	245	250	37.40	49.60	56.40	3.03 C
30-84 (Phz ⁺)	RSL (STM)	+	283	578	45.60	113.00	110.00	0.80 D
30-84-44-8 (Phz ⁻)	RSL (STM)	+	0	0	96.90	96.90	9.69	4.41 B
None	RSL (STM)	+	0	0		60.60	31.80	5.81 A
2-79 (Phz ⁺)	RSL (NAT)	-	30	28	0.19	0.26	0.71	
None	RSL (NAT)	-	0	0		0.89	0.31	
2-79 (Phz ⁺)	PSL (NAT)	+	30	34	0.16	0.43	3.18	3.83 B
None	PSL (NAT)	+	0	0		0.42	3.42	4.32 A

^a Ritzville silt loam (RSL) or Puget silt loam (PSL) was left natural (NAT) or steamed (STM).

^b PCA was isolated from approximately 140 seedlings in natural soil or 70 seedlings from steamed soil. The reported data are from one experiment, except for strain 2-79 in natural Ritzville silt loam, for which duplicate sets of seedlings were extracted.

^c Root weight included the fresh weight of the roots and adhering rhizosphere soil. Populations were determined as described in the text. Reported values are the means from two (steamed soil) or three (natural soil) replicates, with one seedling per replicate.

^d Tube bioassays were conducted as described in the text. Disease ratings are the means of 6, 4, and 5 replicates with approximately 23, 18, and 28 plants per replicate in natural Ritzville silt loam, steamed Ritzville silt loam, and natural Puget silt loam, respectively. Treatments were arranged in a randomized complete block. Following a significant *F* test, a least significant difference analysis was performed. Means in the same experiment followed by the same capital letter are not significantly different by least significant difference analysis (*P* = 0.05). Least significant difference values for the three experiments were 0.43, 0.88, and 0.18, respectively.

TABLE 2. Production of PCA by *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 in the rhizosphere of wheat grown in the field^a

Seed treatment	Ng of PCA ^b per:		Bacterial population (CFU/g of root) ^c		
	Plant	g of root ^c	Introduced strain (10 ⁷)	Total fluorescent pseudomonads (10 ⁷)	Total aerobic bacteria (10 ⁸)
Wheat field					
2-79 (Phz ⁺)	12	12	2.19	5.57	14.30
30-84 (Phz ⁺)	6	5	3.02	3.04	10.10
None	0	0		0.82	5.67
Virgin field					
2-79 (Phz ⁺)	23	27	0.35	0.44	7.63
30-84 (Phz ⁺)	22	19	0.26	0.28	10.80
None	0	0		0.01	5.59

^a Field studies were conducted at two sites as described in Materials and Methods.

^b Reported values are for one group of 200 seedlings per treatment.

^c Root weights and population sizes were determined as described in Table 1, footnote c, using three replicates, one plant per replicate.

mine whether these differences between strains occur consistently and whether they influence disease suppression.

Field studies. PCA also was produced by strains 2-79 and 30-84 on roots of wheat grown in the field (Table 2). The amounts of the antibiotic from roots grown at the virgin field site (19 to 27 ng) were similar to those from roots colonized by strain 2-79 in natural soil in the growth chamber study (28 to 43 ng). The populations of introduced bacteria (Table 2) also were comparable to those in the growth chamber treatment from which the inoculum of *G. graminis* var. *tritici* was omitted (Table 1). Less antibiotic (5 to 12 ng) was recovered from roots from the wheat field site despite the presence of larger populations of the introduced strains (Table 2). The antibiotic was not detected on roots of plants grown from nontreated seeds. Large populations of indigenous bacteria were present on the roots from both sites.

DISCUSSION

The antibiotic PCA was recovered from the roots and rhizosphere soil of wheat grown from seeds inoculated with phenazine-producing pseudomonads. If recovery was complete, then as little as 20 to 30 ng per seedling root system was sufficient to provide significant disease suppression. Extrapolating this value to wheat sown in the field at a density of 270 seeds per m², only some 55 to 80 mg of root-associated PCA per hectare would be required for significant disease suppression in seedlings at the three-leaf stage, which are comparable in development to the seedlings used in this study. In contrast, triadimenol [β -(4-chlorophenoxy)- α -(1,1-dimethylethyl)-1-*H*-1,2,4-triazole-1-ethanol], a systemic fungicide recently approved for use against take-all, is applied on seeds at about 90 g (active ingredient) per hectare and therefore is present initially at a concentration 3 orders of magnitude greater than that found for PCA in this study.

Roughly comparable amounts of PCA were recovered from roots in natural soils whether or not *G. graminis* var. *tritici* was introduced, and these amounts were sufficient to provide significant disease control in the presence of the pathogen. Nutrients in the rhizosphere apparently are ade-

quate to support a biologically meaningful level of antibiotic synthesis even in the absence of infection. Antibiotic produced on healthy roots could serve as an important first line of defense against primary infection by *G. graminis* var. *tritici*. The idea that PCA might be involved in reducing the incidence of infection was originally suggested by results from a previous study (Bull, M.S. thesis) that demonstrated an inverse relationship between the number of take-all lesions and the population size of strain 2-79 present on wheat roots. There was no such correlation when Phz⁻ mutants were present.

The effectiveness of the minute quantities of PCA detected in this study suggests that phenazine-producing biocontrol strains synthesize the antibiotic precisely where and when it is needed on the roots to suppress the pathogen. Bacteria in the rhizosphere grow in discrete microsites where nutrients are present and are thought to occupy only a fraction of the available surface area, even on well-colonized roots (9, 10). The distribution of antibiotic on the root therefore is likely to be spatially discontinuous, even allowing for diffusion, and it is difficult to imagine how disease suppression is achieved unless mechanisms exist to bring the pathogen into proximity with the antibiotic. This might occur if, as speculated by Foster (9), the sites preferred for bacterial growth also provide the pathogen physical access to the root. The occupation of such strategically important passages by PCA-producing strains would position them ideally for antibiotic delivery.

If antibiotic synthesis occurs mainly at times when the bacteria are metabolically active, then the potential for production probably also exists during or after lesion formation. Populations of strain 2-79 are up to 10-fold larger on roots with take-all lesions, and the population increase coincides with lesion development (22). The proliferation of strain 2-79 on roots with lesions is thought to occur in response to nutrient leakage, and nutrients provided by the lesions also support further infection of the root by *G. graminis* var. *tritici*. Antibiotic produced in lesions colonized by 2-79 therefore would be likely to come into contact with the pathogen and limit its spread, resulting in particularly effective control of take-all severity. This would be consistent with field and greenhouse observations (6, 8, 18, 22) indicating that take-all suppression can occur after primary lesions already have been incited. More precise studies are now needed to determine when and where in the root system the antibiotic is produced. With replicated samples and scaled-down modifications of the methods described here, it should also be possible to define the relationships between population size, the amount of antibiotic produced, its rate of turnover in the rhizosphere, and the degree of disease control attained.

ACKNOWLEDGMENTS

We thank Ian Hilton and Kay Walker Simmons for providing the space, instrumentation, and assistance necessary to complete this study and R. J. Cook and D. S. Heron for critical review of the manuscript.

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