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THE EFFECT OF INCREASED PHENAZINE ANTIBIOTIC PRODUCTION ON THE INHIBITION OF ECONOMICALLY IMPORTANT SOIL-BORNE PLANT PATHOGENS BY *PSEUDOMONAS AUREOFACIENS* 30–84

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Pseudomonas aureofaciens 30–84 produces three phenazine antibiotics which are responsible for the ability of the strain to compete with the indigenous microflora in the rhizosphere. An additional consequence of phenazine production is the inhibition of several fungal plant pathogens *in vitro*. Phenazine production is regulated by a positive activator (PhzR) that acts in conjuction with a diffusable signal produced by *PhzI*. Introduction of additional copies of *PhzR* into strain 30–84 resulted in ca. a five-fold increase in the level of phenazine production and increased the ability of strain 30–84 to inhibit the growth of nine economically important fungal pathogens *in vitro*. Six of the nine fungal strains were inhibited significantly more in the presence of the over-producing strain. *Gaeumannomyces graminis*, was inhibited greater than 50%, regardless of the level of phenazines produced. The addition of a specific carbon source in Kanner medium (medium B) enhanced pigment production and inhibition of the fungal strains as compared to 1/5 potato dextrose agar medium (medium A), consistent with the proposed role of nutrients in the regulation of phenazine biosynthesis. The sclerotia formation of *Sclerotinia sclerotiorum* was 0% in the presence of strain 30–84 and strain 30–85 on medium B. However, 30–84 could not significantly reduce sclerotia formation on medium A, where less phenazine pigments were produced. In greenhouse cone assays, 30–84 significantly reduced disease incidence and disease severity. The phenazine deficient mutant strain 30–84 Z, was significantly less effective in cone assay experiments.

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INTRODUCTION

Antibiotic production by fluorescent pseudomonads is one of the best studied areas of biological control of soilborne root pathogens. Fluorescent pseudomonads have been identified that produce many different types of antibiotics, including phenazines (Thomashow and Weller, 1988), pyoluteorin (James and Gutterson, 1986), pyrrolnitrin Kanner *et al.*, 1978), pyocyanin (Gutterson *et al.*, 1988), oomycin A (Howell and Stipanovic, 1979), 2,4-diacetylphoroglucinol (Vincent *et al.*, 1991), and Geldanamycin (Rothrock and Gottlieb, 1984).

Pseudomonas aureofaciens strain 30–84 was originally isolated from wheat roots taken from a field in which take-all disease caused by the oomycete *Gaeumannomyces graminis* var. *tritici* (*Ggt*) had declined naturally (W.W. Bockus, personal communication). Strain 30–84 produces three phenazine antibiotics; phenazine 1-carboxylic acid (PCA), 2-hydroxy-phenazine-1-carboxylic acid (2-OH-PCA) and 2-hydroxy-phenazine (Pierson III and Thomashow, 1992). Several mechanisms of action have been proposed for phenazines, including the inhibition of DNA replication and RNA synthesis (Thomashow and Weller, 1988), and the disruption of normal membrane functions resulting in the uncoupling of electron transport and energy production or the generation of toxic intercellular oxygen species. Phenazine production by strain 30–84 is responsible for its ability to inhibit *Ggt* (Cook *et al.*, 1968; Spraque, 1950). A 5.7 kb region of the 30–84 chromosome, when expressed from the *E. coli lac* promoter, was shown to be sufficient for the production of PCA and 2-OH-PCA (Pierson III and Thomashow, 1992). This region was shown to contain five open reading frames (*phzFABCD*) involved in phenazine biosynthesis (Pierson III *et al.*, 1995). Phenazines are proposed to be the primary competitive mechanism of strain 30–84 in the rhizosphere (Pierson III and Pierson, 1996). Mazzola *et al.* (1992) showed that, in contrast to populations of wildtype strain 30–84 (Phz^+) which decrease slowly over time in competition with indigenous soil microorganisms on wheat roots, populations of Phz^- mutants of strain 30–84 decrease rapidly on

roots. Further, restoration of phenazine production restored the ability of the strain to persist in the rhizosphere.

Few studies on potential biocontrol agents extend beyond the initial target pathogen to other fungal pathogens causing similar symptoms on crop plants. The development of a broad spectrum biocontrol agent may be more desirable on a commercial basis. By genetic manipulation we can regulate a biocontrol agent to produce a larger variety of antibiotics or larger amounts of their endogenous antibiotics and thus become a more efficient biocontrol organism (Thomashow and Weller, 1988). However, unregulated overexpression at inappropriate times may be undesirable, both in terms of the host plant and the bacterium itself (Pierson and Thomashow, 1991). Several other researchers have looked at antibiotic overproducing biocontrol agents (Maurhofer *et al.*, 1992; Schnider *et al.*, 1995 and Chatterjee *et al.*, 1996). Schnider *et al.*, 1995 and Chatterjee *et al.*, 1996 derived their overproducing strains from mutational inactivation of genes. Maurhofer *et al.*, 1992 developed a strain of *P. fluorescens* CHA0, which protects cucumber, cress and sweet corn from *Pythium ultimum*, that contained a cosmid that carried additional copies of part of the CHA0 genome. This derivative overproduced two antibiotics, pyoluteorin and phloroglucinol. However, the overproducing strain reduced the fresh weights of cress and sweet corn (but not cucumber) in the presence and absence of the pathogen. They concluded that, depending on the host-pathogen system, enhanced antibiotic production by *P. fluorescens* may result in improved disease suppression or, in contrast, in a toxic effect on the plant.

The objective of this work was to compare the ability of *Pseudomonas aureofaciens* strain 30–84 with a strain that over-produced phenazines (strain 30–85) to inhibit mycelial growth and/or sclerotial formation of several important soil-borne fungal pathogens (Tab. I).

TABLE I Plant pathogenic fungi used for inhibition assays on agar media

Pathogenic fungus	*Common disease*
Gaeumannomyces graminis f.sp. *tritici*	foot rot and root rot of wheat (take-all)
Monosporascus canonballus	vine decline of cucurbits
Verticillium dahliae	wilt on dahlias and some cereals
Pythium aphanidermatum	damping-off of cucumbers and beans
Fusarium sp.	wilting lesions on beans
Sclerotinia sclerotiorum	white mold of soybean, canola and beans
Sclerotinia minor	stem rot of lettuce, celery and carrot
Pythium ultimum	damping-off and root rot of many seedlings

MATERIALS AND METHODS

Bacterial Strains and Media

All bacteria were grown on either Luria Bertani (LB) + 5 g/l NaCL, AB minimal medium (Schleif and Wensink, 1981), or pigment production medium (PPM) + Tc^{50} (Levitch and Stadtman, 1964). Medium as described (Pierson III and Thomashow, 1992). Antibiotics used included tetracycline (Tc) at 50 $\mu g/ml^{-1}$, ampicillin (Ap) at 100 $\mu g/ml^{-1}$, and rifampicin (Rif) at 100 $\mu g/ml^{-1}$.

The bacterium *Pseudomonas aureofaciens* strain 30–84 contains an intact phenazine biosynthetic operon (*phz FABCD*) and produces three phenazine antibiotics (Pierson III *et al.*, 1995; Pierson III and Thomashow, 1992). Strain 30–84Z contains a genomic *phzB*::*lacZ* fusion and is Phz^- (Pierson and Thomashow, 1991). Strain 30–85 differs from wildtype strain 30–84 in that it contains additional copies *in trans* of the transcriptional activator *phzR* (Pierson III *et al.*, 1994) on the broad host range vector pLAFR3. Strain 30–85 was constructed as follows. The plasmid pLSP20H-2.7#12, which contains the regulatory genes *phzR* and *phzI*, was digested with *Bg*lII followed by the addition of T4 DNA Ligase. The ligation products were transformed into *E. coli* DH5α and plated on LB + Ap^{100}. The resulting plasmid (pLSP20H-2.7 *Bg*ldel) contained *phzR* but not *phzI*. The 1.7 kb fragment that contained *phzR* was cloned as *Hind*III fragment into site within pLAFR3 and introduced into strain 30–84 *via* triparental mating as described (Pierson III and Thomashow, 1992). Exconjugants were selected for resistance to tetracycline (Tc^R) and maintained on AB + Tc^{50} plates.

All three strains used contained the plasmid pLAFR3 (Tc^R). Strains were streaked onto LB agar + Tc^{25} directly from −80°C stock cultures and were incubated at 28°C for 48h. A loop of the bacteria was transferred to 3 ml of LB broth in 10 ml tubes and incubated on a shaker overnight at 28°C at 150 rpm.

Fungal Strains and Medium

Nine economically important plant pathogenic fungi (Tab. I) were grown for 1–2 wk on full strength potato dextrose agar (PDA) [DIFCO] amended with Tc^{25} in a 28°C incubator. In some experiments, 1/5 strength PDA (1/5 PDA) was used for fungal growth.

Quantitation of Phenazine Production

Strains 30–84 and 30–85 were grown at 28°C in PPM + Tc^{50}. The cultures were centrifuged (5,000 × g), the culture supernatants were adjusted to ca. pH 2.0 with concentrated HCL, and extracted with an equal volume of benzene. After evaporation (Thomashow *et al.*, 1990), the phenazine residues were resuspended in 0.1 N NaOH and the total amount of phenazine was determined by scanning UV-VIS spectroscopy as described previously (Pierson III and Thomashow, 1992).

Plate Assays

Two different Tc^{25} media were used for the *in vitro* antagonism tests. Medium A was 1/5 strength potato dextrose agar (1/5 PDA) made from 40 g potatoes, 4 g dextrose, 18 g agar in 1000 ml distilled water. Medium B was 1/5 PDA supplemented with mineral salts medium (8)- 4g of Na_2HPo_4, 1.5 g KH_2PO_4, 1 g NH_4Cl, 0.2 g $MgSO_4 \cdot 7H_2O$, and 0.005 g $FeNH_4$- citrate, and 20 ml glucose solution (20% carbon source) was added.

Five microlitres of each bacterial strain was dispensed carefully near the perimeter along the center line of a petri plate containing Medium A or Medium B. The plates were incubated for 24 h at 28°C. A 0.3 cm plug of each fungal isolate was removed from the actively growing margin of a fungal stock plate and placed at the centre of the plate. The plates were incubated at 28°C and allowed to grow until the fungal inoculum on the control plates (no bacteria added) grew to the edge. The experiment contained 5 replicate plates per treatment and was repeated twice. The percentage mycelial inhibition was calculated using the equation $100 \times (R_1 - R_2)/R_1$ where R_1 is the maximum radius of growth (40 mm) and R_2 is the radius directly opposite the bacterial spots.

Plant Assay

Greenhouse assays were carried out with strains 30–84 and 30–84 Z. Strain 30–85 was not included in the experiment as the pLAFR3 plasmid containing extra copies of *phzR* is unstable *in vivo* without direct antibiotic selection. The strains were tested for the inhibition of *Ggt* and possible disease reduction in Super cell conetainers (155 cc; Ray

Leach Nursery, Canby, OR). Two hundred and fifty ml of twice-autoclaved oats in a 1000 ml flask was inoculated with seven plugs of *Ggt* (0.5 cm) cut from an actively growing margin of a seven-day-old PDA plate. After 21 days, the inoculum was dried under an air flow hood. Inoculum was ground in a blender and sieved (1 mm sieve). Unsterilized sandy loam soil was mixed with inoculum (0.45% inoculum w/w) and placed in plastic Super cell tubes. Wheat seeds var. 'Fielder' were surface sterilized in Chlorox for 2 min. and coated with the bacterial treatment (30–84 or 30–84 Z) in 0.5% carboxy methyl cellulose. The bacterial suspension was prepared as described by Fernando and Linderman (1995). The cell density was approximately 10^9 colony forming units (cfu)/ml calibrated with a spectrophotometer to $A_{640\,nm} = 0.5$. The bacterized seeds were dried under a laminar flow hood for 30 min and planted. There were 80 cones per treatment. The cones were kept on a greenhouse bench in complete randomized block design using a 16/8 h light/dark cycle with 28/20°C day/night temperature and daily watering. After 21 days, the plant roots were removed from the soil, washed, and scored for disease severity and incidence. Disease severity was rated on a scale of 0 to 3, where 0 = healthy plants, 1 = one to multiple lesions on roots, 2 = *Ggt* lesions visible on stem and root with or without chlorosis, 3 = dwarfed and/or dead plants.

Data Analysis

All experiments were arranged in a complete randomized block design with the number of replicates relevant to the experiment, as mentioned above. All experiments were repeated at least once. Data were analyzed using analysis of variance (ANOVA) and mean separation test (Duncan Multiple Range Test) was performed at $P < 0.05$.

RESULTS AND DISCUSSION

The introduction of *phzR in trans* into strain 30–84 (30–85) resulted in ca. a five-fold increase in the level of total phenazine produced by strain 30–84 (Fig. 1). The plates containing strain 30–85 showed the characteristic orange colour of production of the phenazine pigments to be greater than in plates with strain 30–84. This was more evident in

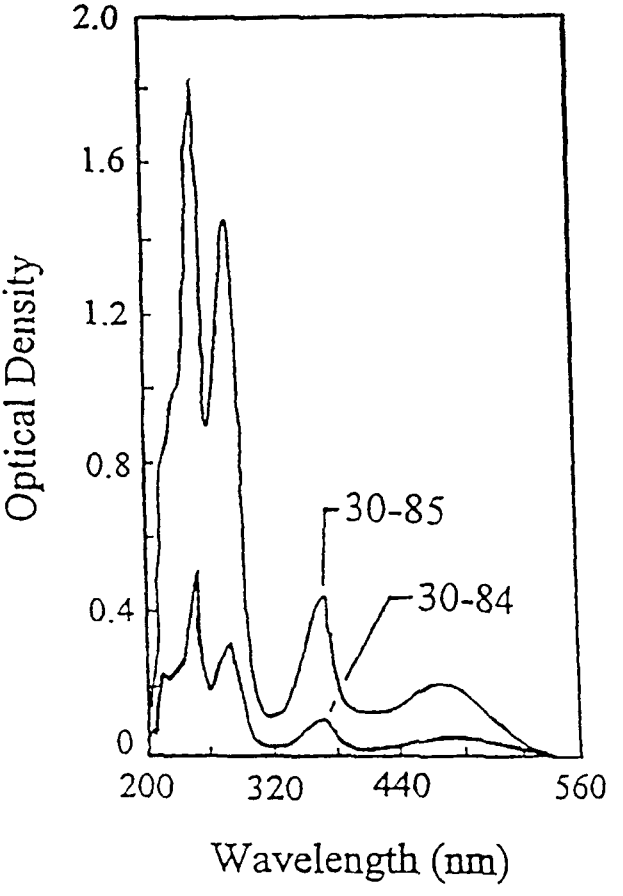

FIGURE 1 Phenazine production by *Pseudomonas aureofaciens* 30–84 and 30–85. Phenazines were extracted from 18–24 h overnight cultures of each strain as described, and total phenazine was determined by UV-VIS scanning spectroscopy. Phenazine-1-carboxylic acid has characteristic absorption peaks ca. 250 and 367 nm (Pierson and Thomashow, 1992). The 2-hydroxylated derivatives contain an additional peak ca. 484 nm.

medium B. Inhibition assays on 1/5 PDA supplemented with Kanner minimal media (medium B) showed a significant decrease in mycelial growth of several pathogenic fungi in the presence of 30–84 or the phenazine over-producing strain 30–85 (Tab. II). *Monosporascus canonballus, Verticillium dahliae, Pythium aphanidermatum, P. ultimum, Fusarium* sp. and *Sclerotinia sclerotiorum* were inhibited significantly more by 30–85 in the presence of additional phenazine. The only

TABLE II Inhibition of plant pathogenic fungi on agar media

Fungal pathogens	30–84	30–85	30–84 *Z*	*Control*
Percent inhibition of mycelial growth by *P. aureofaciens* strains in medium A				
Gaeumannomyces graminis	29.05b*	65.00a	27.50b	0.00c
Monosporascus cannonballus	33.25b	62.00a	20.00c	0.00d
Verticillium dahliae	20.00b	35.15a	17.56b	0.00b
Pythium aphanidermatum	00.00	00.00	00.00	00.00
Fusarium sp.	20.00b	36.50a	19.00b	0.00c
Sclerotinia sclerotiorum	04.56b	25.55a	1.17c	0.00c
Sclerotinia minor	30.55a	50.00a	3.33b	0.00b
Pythium ultimum	11.50b	30.25a	0.33c	0.00c
Percent inhibition of mycelial growth by *P. aureofaciens* strains in medium B				
Gaeumannomyces graminis	63.52a	65.66a	07.33b	00.00b
Monosporascus cannonballus	41.91b	58.25a	09.41c	00.00b
Verticillium dahliae	46.55b	57.50a	34.14c	00.00d
Pythium aphanidermatum	03.78b	07.51a	00.00c	00.00c
Fusarium sp.	23.66b	33.50a	00.00c	00.00c
Sclerotinia sclerotiorum	53.56b	62.46a	00.00c	00.00c
Sclerotinia minor	42.80b	60.68a	00.92c	00.00c
Pythium ultimum	13.00b	20.41a	00.00c	00.00c

*Means in the same row, followed by a common letter do not differ significantly ($P = 0.05$) as determined by Duncan multiple Range Test.
Medium A: 1/5 strength PDA amended with 25 mg/L tetracycline.
Medium B: 1/5 strength PDA supplemented with Kanner mineral solution and glucose, and amended with 25 mg/L tetracycline.

exception was *Gaeumannomyces graminis*, whose inhibition was over 50%, regardless of the amount of phenazine produced in the media. Smaller concentrations of phenazine compounds seem to be effective against only *Ggt*. This is consistent with the initial identification of strain 30–84 as a biocontrol agent against *Ggt*. Kanner *et al.*, reported that in addition to temperature and aeration, the additional carbon source available in Kanner mineral salts medium had a marked influence on pigment production (Kanner *et al.*, 1978). The phenazines produced by a strain of *P. aeruginosa* in minimal glucose medium supplemented with Kanner mineral salts differed depending on temperature (28 or 37°C) and aeration (stationary or shaking). In our experiment, the additional carbon altered the level of phenazine production resulting in a marked effect on the inhibition of the different fungal pathogens. Rosales *et al.* (1995) reported a similar media-dependent effect on the antagonism of *Rhizoctonia solani* AG1 by two *Pseudomonas cepacia* strains. Kraus and Loper (1992) observed that two mutants of *P. fluorescens* Pf-5 which were no longer able to produce pyoluteorin grew poorly on media containing glucose, suggesting a linkage between

glucose metabolism and pyoluteorin production. James and Gutterson (1986) reported that mutations in the *afu*A and *afu*B loci of *P. fluorescens* strain HV37a, are deficient in glucose dehydrogenase and in the production of the antifungal compound, oomycin A.

Some fungi on medium B were significantly inhibited by 30–84 Z (Phz^-) compared to the untreated control, but inhibition was significantly lower than inhibition by strains 30–84 and 30–85. On media A, inhibition of *G. graminis*, *Macrophomina phaseolina*, *V. dahliae*, and *Fusarium* sp. with 30–84 was not significantly different from 30–84 Z. This suggests that there are one or more other antibiotics/volatiles that control these pathogenic fungi in instances where phenazines are not produced. James and Gutterson (1986) noted that at least two additional antibiotics that inhibited *P. ultimum* were produced by *Pseudomonas fluorescens* strain HV37a on a potato agar medium (PA) without glucose. They also suggested that the antibiotics produced on PA could be precursors to the antibiotics produced on PDA or *vice versa*. Both siderophores (fluorescein) and antibiotics such as pyrrolnitrins, pyoluteorins, pyocyanins may be involved in the control of the fungi, when phenazines are not produced. On media B, both 30–84 and 30–85 reduced sclerotia formation to 0% in all plates of *Sclerotinia sclerotiorum*. On media A, where less phenazine was produced (colony not orange in colour) the sclerotia production was not significantly reduced with treatment 30–84. This suggests that formation of sclerotia is significantly affected and inhibited in the presence of phenazine. The phenazines could be inhibitory to the production of over-wintering structures of some fungi such as *Sclerotinia* spp.

Another observation with all fungi, on both media was that the mycelial growth was sparse with 30–84 and 30–85, compared to 30–84 Z. On medium B the effect of phenazine production was visible over the entire plate with equal inhibition of mycelia on the sides nearest to bacteria and sides away from the bacteria.

The greenhouse cone assays showed that phenazine production was similarly effective in the reduction of disease incidence as well as reduction of disease severity (Tab. III). The inhibition observed on plate assays could be correlated to inhibition of *Ggt* on cone assay experiments. This phenomenon is not always true, as observed by Paulitz and Loper (1991). They observed pyoverdine minus (Pvd^-)

TABLE III Biocontrol of *Gaemannomyces graminis* by *Pseudomonas aureofaciens* 30–84 and its derivatives

Treatment	*Percent disease incidence*	*Disease severity index***
Control (Non-treated)	0.00c*	0.000c
Ggt only control	35.00a	0.600a
30–84 WT	6.25c	0.075c
30–84 Z	23.75b	0.386b
30–85	NT	NT

*Values followed by a common letter (in the same column) do not differ significantly ($P = 0.005$) as determined by Duncan Multiple Range Test.
**Disease Severity Index was on a scale of 0 to 3, where 0 = healthy plants, 1 = one to multiple lesions on roots, 2 = Ggt on stem and roots with or without chlorosis, 3 = dwarfed and/or dead plants.
NT = Not tested.

mutants protecting cucumber seedlings from *Pythium ultimum* (Pythium damping-off) in three different agricultural soils to the same level as the parental strain N1R of *Pseudomonas putida*. But these same mutant derivatives, as compared to strain N1R, could not inhibit hyphal growth of *P. ultimum* on King's medium B. The next step in this work is to stably insert extra copies of *phzR* into the *P. aureofaciens* chromosome to determine the effect of phenazine over-expression on disease suppression in cone assays. Our results indicate that *P. aureofaciens* has the potential of being an effective biological control agent against several plant pathogenic fungi, and that genetic manipulation of strain 30–84 could increase its effectiveness and range of use.

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