

## Suppression of *Pythium ultimum*-Induced Damping-Off of Cotton Seedlings by *Pseudomonas fluorescens* and its Antibiotic, Pyoluteorin

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### ABSTRACT

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Strain Pf-5 of *Pseudomonas fluorescens* was antagonistic in vitro to *Pythium ultimum*. An antibiotic, pyoluteorin (4,5-dichloro-1 *H*-pyrrol-2-yl-2,6-dihydroxyphenyl ketone), was isolated from cultures of the Pf-5 strain and found to be inhibitory to *P. ultimum*, but not to *R. solani*. Treatment of cottonseed with pyoluteorin or with *P. fluorescens* at the time

of planting in *P. ultimum*-infested soil increased seedling survival from 33 to 65% and from 28 to 71%, respectively. Although effective as a seed treatment, pyoluteorin was adsorbed and inactivated by the soil colloids when added directly to soil.

During a study on biocontrol agents for cotton seedling diseases (3) we isolated a strain of *Pseudomonas fluorescens* (Pf-5) antagonistic to *Rhizoctonia solani* Kühn and found that the bacterium produced the antifungal compound pyrrolnitrin. The antibiotic was effective against *R. solani* and several other seedling disease organisms, but was not inhibitory to *Pythium ultimum* Trow. Because *P. ultimum* is an important pathogen of cotton seedlings (2,4) we subsequently surveyed for microorganisms antagonistic to *P. ultimum*. We discovered that the Pf-5 strain of *P. fluorescens* also was inhibitory to *P. ultimum*. The finding that pyrrolnitrin was not effective against *P. ultimum*, suggested that another antibiotic may have been produced.

This paper reports the isolation and identification of an antifungal compound from *P. fluorescens* effective against *P. ultimum*. *P. fluorescens* and its antibiotic were evaluated as seed treatments to prevent damping-off of cotton seedlings by *P. ultimum*.

### MATERIALS AND METHODS

The inhibition of *P. ultimum* by *P. fluorescens* strain Pf-5 was demonstrated by spot-inoculating agar plates of King's B medium (5) with the bacterium. After 4 days of incubation at 24 C, the plates

were inoculated with PDA plugs of *P. ultimum* and, after 3 days of incubation, were examined for evidence that growth of the fungus was inhibited by the bacterium.

**Isolation and identification of a *P. ultimum* growth inhibitor from *P. fluorescens*.** King's B agar plates were spread with a bacterial suspension and incubated for 7 days at 24 C. The agar cultures were cut into 1-cm squares and extracted with 200 ml of 80% aqueous acetone for each 10 plates of medium. The extracts were filtered through cheesecloth and the filtrate was centrifuged at 12,000 *g* for 10 min to remove particulate matter. The supernatant fluids were combined and condensed by removal of the acetone in vacuo. Five grams of NaCl were added to each 100 ml of the aqueous concentrate and each portion was extracted twice with 100-ml volumes of ethyl ether. The ether extracts were combined and taken to dryness in vacuo. The residue was dissolved in acetone at 1/100 the original culture volume, and 1-ml samples were streaked on thin-layer plates coated with silica gel 7 GF (Mallinkrodt, St. Louis, MO 63160). The plates were developed in chloroform-acetone (90:10, v/v) and observed under long- and shortwave UV light for fluorescing and absorbing bands. Observed bands and blank areas were scraped separately from plates and eluted with acetone.

Samples of acetone eluates diluted 1 to 10 with sterile water were assayed for activity against *P. ultimum* by placing 100- $\mu$ l aliquots in wells cut into PDA plates that had been inoculated with PDA plugs of the fungus. After 2 days of incubation, the plates were examined

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for zones free of mycelium around the wells. The eluate containing active material was again streaked on silica gel 7GF plates and developed with chloroform-ethyl acetate-formic acid (50:40:10, v/v). The procedure for locating bands and detecting zones with antibiotic activity was repeated. The eluate with inhibitory activity was concentrated in hot cyclohexane, and the antibiotic was crystallized from it after cooling. A melting point and mass, NMR, and IR spectra of the purified crystalline antibiotic were obtained and compared with those from an authentic sample of pyoluteorin, obtained from A. H. Rees (Trent University, Peterborough, Ontario, Canada).

**Production sites of antibiotics from *P. fluorescens*.** Cells of *P. fluorescens* were scrubbed from 7-day-old King's B agar cultures with a moistened rubber scraper and washed three times with distilled water. The washed cells and scrubbed agar cultures were extracted and acetone concentrates were obtained as described in the second paragraph of materials and methods. Samples of the acetone concentrates were co-chromatographed with authentic pyrrolnitrin and pyoluteorin on silica gel 7GF with chloroform-acetone (90:10, v/v) and observed under shortwave UV light. Spots in the extracts with  $R_f$  values corresponding to those of the authentic antibiotics were eluted from the adsorbent with acetone and assayed for antibiotic activity against *R. solani* and *P. ultimum* by the method used to locate active bands on chromatograms.

**Antifungal spectrum of the antibiotic.** Purified antibiotic dissolved in 10% aqueous acetone was diluted to 200  $\mu\text{g/ml}$  with sterile water. The solution was assayed, by the method described previously for chromatogram eluates, against the following fungi that have been associated with cotton seedling disease: *Alternaria* sp., *Fusarium* sp., *Thielaviopsis basicola* (Berk. & Fr.) Ferr., *Rhizoctonia solani*, and *Verticillium dahliae* Kleb. Additionally, dilutions of 100  $\mu\text{g/ml}$ –1  $\mu\text{g/ml}$  at 10- $\mu\text{g/ml}$  intervals were made from the antibiotic solution and these were assayed against *P. ultimum* to determine the minimum effective concentration of the antibiotic.

**Mobility and activity of the antibiotic in soil.** One-milliliter samples containing 100 or 300  $\mu\text{g}$  of the antibiotic in aqueous solution were added to 1  $\times$  3-cm columns of 2.5 g of air-dried nonsterile soil. Soil types were loamy sand, sandy loam, and clay. The columns were eluted with distilled water under reduced pressure and the eluates were concentrated to the original 1-ml volumes in vacuo. The antibiotic solutions were assayed for activity against *P. ultimum* before and after passage through the soil columns, by the method used to locate active bands on chromatograms. However, the medium used for these assays was cotton soil extract agar (CSEA) and not PDA. *P. ultimum* is more sensitive to the antibiotic on the former less nutritious medium than on the latter. CSEA consists of equal volumes of a 10% (w/v) autoclaved cottonseed filtrate and the filtrate of an autoclaved 50% soil suspension, with 2% agar added.

Purified antibiotic was mixed into sterile sandy loam soil with acetone at a concentration of 100  $\mu\text{g/g}$  and the acetone was removed in vacuo. Five-millimeter-diameter PDA plugs of *P. ultimum* were placed in 10-mm-diameter wells cut into CSEA plates and the plugs were covered with antibiotic-treated or nontreated sterile soil. After 1–2 days at 24 C the cultures were observed for evidence of *P. ultimum* mycelium growing through the soil to the medium.

One-gram samples of pyoluteorin treated or nontreated soil were eluted with a methanolic 1%  $\text{Na}_2\text{CO}_3$  solution (80:20, v/v). The eluates were taken to dryness in vacuo and the residue was dissolved in acetone. The acetone was removed in vacuo and the residue was dissolved in 10% aqueous acetone. The eluates were then assayed for activity against *P. ultimum* on CSEA.

**Cottonseed treatment with bacterial cultures or with antibiotic to control damping-off.** Oospores of *P. ultimum* cultured and converted to germinable propagules according to a method previously described (1) were incorporated into nonsterile soil at the rate of 2,000 (40% germination) oospores per gram for the antibiotic and bacterial culture treatments. Soil for the bacterial culture component treatments was infested with 1,000 (germination 88%) oospores per gram.

Soil flats containing nonsterile oospore-infested soil or noninfested fine sandy loam were planted with four replicate 30 seed lots of cottonseed treated with approximately 10  $\mu\text{g}$  of antibiotic per seed. For this purpose, purified antibiotic in acetone was diluted to 300  $\mu\text{g/ml}$  and 1-ml samples were added to 0.1-g lots of diatomaceous earth carrier. The acetone was removed in vacuo and the carrier was coated with methyl cellulose sticker onto 30 cotton seed. Seed in another set of flats were treated at planting with 2 ml per seed of *P. fluorescens* ( $7 \times 10^8$  cells per milliliter) liquid culture obtained from 100-ml cultures of *P. fluorescens* in King's B medium shake incubated at 25 C for 5 days. The flats were placed in a growth chamber with a 14-hr photoperiod at 22 C. Flats were watered every other day with 1,200 ml of distilled water per flat. After 14 days of incubation the number of surviving seedlings was counted.

To determine what components of the bacterial culture might be contributing to the observed protective effect, four replicate 30-seed lots were planted in oospore infested soil for each of the following treatments: 2 ml per seed of a 5-day-old *P. fluorescens* culture; 2 ml per seed of filter-sterilized filtrate from a 5-day-old culture; or 2 ml per seed of washed *P. fluorescens* cells from a 5-day-old culture resuspended in fresh 10% King's B medium. The soil flats were incubated as described in the first test. After 14 days, a count of the survivors was made; and after 21 days, the fresh weight of the seedlings was measured.

Sections were taken from the roots and hypocotyls of healthy and infected seedlings from both soil flat tests, washed thoroughly with tap water, and plated on water agar containing 35 ppm each of streptomycin sulfate and penicillin G. After 2–3 days of incubation the plates were observed for the presence of fungi.

## RESULTS

Examination of the King's B agar plates inoculated with *P. fluorescens* and *P. ultimum* showed that the bacterial colonies were clearly inhibitory to the fungus. Wide zones devoid of mycelial growth were observed around bacterial colonies, while areas

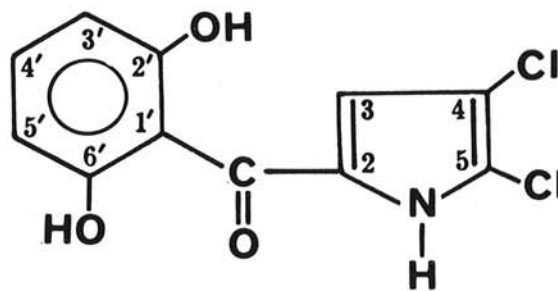


Fig. 1. The structure of pyoluteorin. The hydroxyls at 2' and 6' ortho to the carbonyl may function in a chelating reaction.

TABLE I.  $^{13}\text{C}$ -NMR shifts and coupling of pyoluteorin<sup>a</sup>

C#	$\delta$	Coupling
2	131.0	$^2J_{\text{H}3}$ : 5.1
3	117.5	$^1J_{\text{H}3}$ : 180.5
4	113.5	n <sup>b</sup>
5	119.9	$^2J_{\text{H}3}$ : 9.9
1'	110.6	n
2'6'	157.3	$^3J_{\text{H}3'}$ : 11.0
3'5'	107.5	$^1J_{\text{H}3'5'}$ : 162.4
		$^3J_{\text{H}5'3'}$ : 7.5
4'	132.5	$^1J_{\text{H}4'}$ : 160.8

<sup>a</sup>Spectra are recorded in deuterioacetone and shifts are reported in parts per million downfield from tetramethylsilane using the central resonance of deuterioacetone (29.2  $\delta$ ) as an internal standard. Specific shift assignments were based on the shifts of model compounds, long-range couplings, and catalytic reduction of pyoluteorin with deuterium and hydrogen.

<sup>b</sup>n indicates that peaks are unresolved in the proton-coupled spectrum.

without colonies were overgrown.

Solvent extraction of 7-day-old agar cultures of this bacterium yielded a concentrate that was highly toxic to *P. ultimum* when assayed against the fungus on PDA. On silica gel 7GF plates the active fraction migrated to  $R_f$  0.27 in chloroform-acetone and to  $R_f$  0.77 in chloroform-ethyl acetate-formic acid. The antibiotic appeared as a dark spot under shortwave UV light. The melting point and the mass, NMR, and IR spectra of the pure crystalline compound were identical to those of an authentic sample of pyoluteorin (4,5-dichloro-1*H*-pyrrol-2-yl-2,6-dihydroxyphenyl ketone) (Fig. 1), a compound isolated from *Pseudomonas aeruginosa* (7).  $^{13}\text{C}$ -NMR shift values, not reported previously, are included for identification purposes (Table 1).

Examination of the chromatograms made from *P. fluorescens* washed cell and scrubbed culture extracts showed that only the spot corresponding to pyrrolnitrin was present in the cell extract and only the spot corresponding to pyoluteorin was present in the scrubbed culture extract.

Bioassay of the compounds eluted from these spots against *R. solani* and *P. ultimum* showed that the spot from cell extract inhibited only *R. solani*, while the spot from scrubbed culture extract inhibited only *P. ultimum* (Fig. 2).

Bioassay of pyoluteorin at a concentration of 200  $\mu\text{g}/\text{ml}$  showed that it was highly inhibitory to *P. ultimum*, but noninhibitory to any of the other fungal pathogens associated with cotton seedling diseases. The minimum effective concentration of pyoluteorin against *P. ultimum* on PDA was 70  $\mu\text{g}/\text{ml}$ . When pyoluteorin solution was percolated through the soil columns, antibiotic activity at both the 100 and 300  $\mu\text{g}/\text{ml}$  levels was completely removed by the clay. Sandy loam removed antibiotic activity from the 100  $\mu\text{g}/\text{ml}$  solution, but failed to remove it from the 300  $\mu\text{g}/\text{ml}$  sample. Passage through the loamy sand caused a 50% reduction in

antibiotic activity at the 100  $\mu\text{g}/\text{ml}$  level, but no reduction at 300  $\mu\text{g}/\text{ml}$ .

CSEA plates containing *P. ultimum* plugs covered with pyoluteorin treated or nontreated sterile soil, showed mycelial growth through the soil after only 1 day of incubation. Growth through treated soil was as rapid as that through control soil. Elution of the soil with methanolic  $\text{Na}_2\text{CO}_3$  solution did not recover extracts with inhibitory activity.

Treatment with pyoluteorin of cottonseed planted in infested soil resulted in an increase in the number of surviving seedlings from 33 to 65% (Table 2). Treatment with bacterial culture resulted in an increase from 28 to 71% in the number of surviving seedlings. None of the treatments appeared to have an adverse effect on the seedlings in noninfested soil.

Treatments of cottonseed with *P. fluorescens* culture, culture filtrate or washed bacterial cells all resulted in increases in both seedling survival and seedling weights (Table 3). Washed bacterial cells were as efficacious as whole culture or culture filtrate, even though no pyoluteorin was present when the seeds were treated.

Sections taken from seedlings damped-off before emergence produced mostly mycelium of *P. ultimum* when cultured on antibiotic water agar. No mycelium from *R. solani* was observed. Sections from seedlings damped-off after emergence had the mycelium of *P. ultimum* growing from them or combinations of *P. ultimum* and *R. solani*. Seedling sections taken from the same soil, but without *P. ultimum* infestation, showed no symptoms of damping-off, and the mycelium of neither fungus was observed growing from them.

## DISCUSSION

Strain Pf-5 of *Pseudomonas fluorescens* produced two chlorinated phenyl pyrrole antibiotics. Pyrrolnitrin (3) is active against *R. solani* and other nonphycomycetous fungi, but does not inhibit the growth of *P. ultimum*. Pyoluteorin was active against *P.*

TABLE 2. Survival of cotton seedlings from seed treated with pyoluteorin or *Pseudomonas fluorescens* culture and planted in *Pythium ultimum*-infested or noninfested soil flats<sup>1</sup>

Treatment	Survival (%) <sup>2</sup>
<b>Pyoluteorin</b>	
Nontreated seed in noninfested soil	93 c
Treated seed in noninfested soil	95 c
Nontreated seed in infested soil	33 a
Treated seed in infested soil	65 b
<b><i>P. fluorescens</i> culture</b>	
Nontreated seed in noninfested soil	93 c
Treated seed in noninfested soil	95 c
Nontreated seed in infested soil	28 a
Treated seed in infested soil	71 bc

<sup>1</sup> Pyoluteorin, 10  $\mu\text{g}/\text{seed}$ ; *P. fluorescens* culture, 2 ml/seed and containing  $7 \times 10^8$  cells per milliliter; and *P. ultimum*-infested soil containing 2,000 oospores (40% germinable) in flats.

<sup>2</sup> Values are the means of four replications of 30 seeds each. Values followed by the same letter do not differ significantly ( $P < 0.05$ ) according to Duncan's multiple range test.

TABLE 3. Survival and fresh weight of cotton seedlings grown from seeds treated with *Pseudomonas fluorescens* culture, washed cells, or culture filtrate and planted in *Pythium ultimum*-infested soil<sup>1</sup>

Treatment	Survival (%) <sup>2</sup>	Seedling weight (%) <sup>2</sup>
Distilled water	46 a	1.30 a
Washed cells	78 b	1.58 b
Culture filtrate	89 b	1.62 b
<i>P. fluorescens</i> culture	89 b	1.77 b

<sup>1</sup> *P. fluorescens* culture 2 ml per seed and containing  $7 \times 10^8$  cells per milliliter and *P. ultimum*-infested soil containing 1,000 oospores per gram (88% germinable) in flats.

<sup>2</sup> Values are the means of four replications of 30 seeds each. Values followed by the same letter do not differ significantly ( $P < 0.05$ ) according to Duncan's multiple range test.

<sup>3</sup> Values are the mean weights of 30 seedlings.

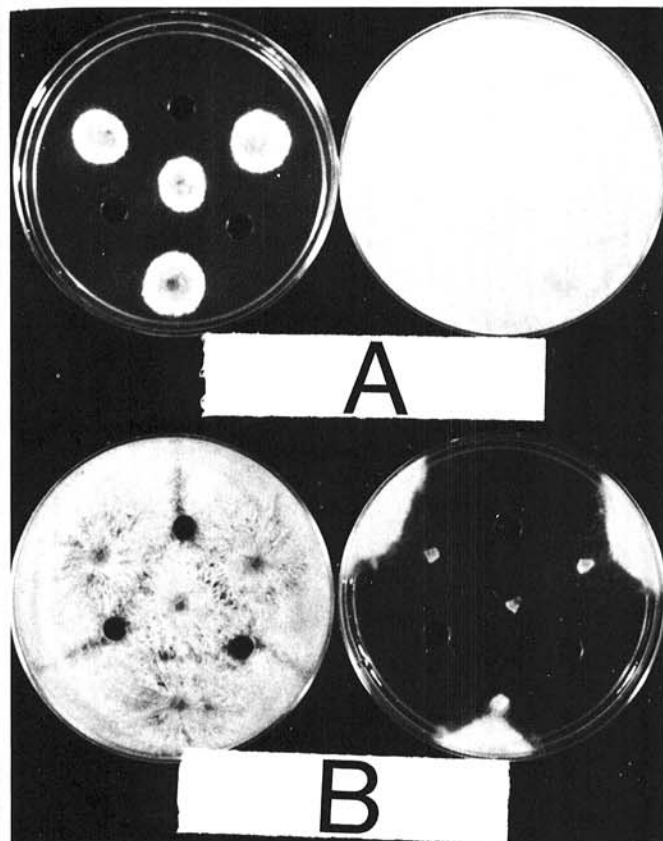


Fig. 2. Antibiotic effect on, from left to right, *Rhizoctonia solani* and *Pythium ultimum* of: A, pyrrolnitrin; B, pyoluteorin.

*ultimum*, but did not affect *R. solani* (Fig. 2). Both antibiotics were produced in the same culture, however, pyrrolnitrin was retained within the cells until lysis, whereas chromatography and assay of culture and cell extracts has shown that pyoluteorin is released to the environment upon production.

The results of the soil column and soil penetration experiments indicate that pyoluteorin is adsorbed and inactivated by the soil. Its movement and effectiveness, is restricted and appears to be inversely related to the clay content of the soil. The difficulty encountered in eluting pyoluteorin from the soil and the presence of hydroxyls ortho to the carbonyl in its structure (Fig. 1) indicate that a chelation reaction between pyoluteorin and soil metals is possible and may be responsible for the loss of mobility and activity observed. In spite of this obvious drawback, pyoluteorin still functioned as an effective seed protectant when coated onto seed in diatomaceous earth. Perhaps slow release from the carrier provides an adequate concentration of the antibiotic during the critical period.

Treatment with washed bacterial cells appears to be an effective seed protectant, and it indicates that the bacterium can produce pyoluteorin in nonsterile soil when the cells are present in large numbers and have an available nutrient source.

We have concluded from the results of our experiments that strain Pf-5 of *P. fluorescens* is antagonistic to *P. ultimum* and that this antagonism may be due to the production of pyoluteorin by the bacterium. This conclusion is supported by the observation that loss of antibiotic activity by fluorescent pseudomonads is accompanied by a loss in their effectiveness as plant growth

promoters (6).

Strain Pf-5 helps protect cotton seedlings against damping-off by both *P. ultimum* and *R. solani*. Treatment of cottonseed with this bacterium or with a combination of the two antibiotics may facilitate the establishment of stands of healthy cotton seedlings.

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