Control of *Rhizoctonia solani* on Cotton Seedlings with *Pseudomonas fluorescens* and With an Antibiotic Produced by the Bacterium

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This article reports the results of research only; mention of a pesticide does not constitute a recommendation by the USDA nor does it imply registration under FIFRA.
Accepted for publication 15 November 1978.

ABSTRACT

HOWELL, C. R., and R. D. STIPANOVIC. 1979. Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology 69:480-482.

A strain of *Pseudomonas fluorescens* antagonistic to *Rhizoctonia solani* was isolated from the rhizosphere of cotton seedlings. An antibiotic strongly inhibitory to *R. solani* was isolated from *P. fluorescens* cultures and identified as pyrrolnitrin (3-chloro-4-[2'-nitro-3'-chlorophenyl]-pyrrole). The antibiotic also inhibited growth of other fungi associated with the cotton seedling disease complex including: *Thielaviopsis basicola, Alternaria* sp., and *Verticillium dahliae*. A *Fusarium* sp. was only partially inhibited and *Pythium ultimum* was unaffected. Treating cottonseed with *P. fluorescens* or pyrrolnitrin at time of planting in *R. solani*-infested soil increased seedling survival from 30 to 79% and from 13 to 70%, respectively. Pyrrolnitrin persisted for up to 30 days in moist nonsterile soil with no measurable loss in activity.

Additional key words: seed treatment, biological control.

Fluorescent pseudomonads appear frequently among isolates from plant rhizospheres (11) and often comprise an important component of the bacterial population (8,12). *Pseudomonas* spp. were ineffective for control of damping-off by *Rhizoctonia solani* and *Pythium* spp. in nonsterile soil (3). However, *Pseudomonas* spp. have been used successfully to protect carnation cuttings against *Fusarium roseum* f. sp. *cerealis* (9), potato seedpieces against certain pathogenic bacteria and fungi (4), and soybean seedlings against *Phytopthora megasperma* var. *sojae* (13).

In our work on seedling diseases of cotton we have isolated fluorescent pseudomonads from the seedling rhizosphere and report here: the isolation and identification of a fluorescent pseudomonad antagonistic to *R. solani*; the isolation and identification of an antibiotic produced by the isolate; and the efficacy of that isolate or its antibiotic as a seed treatment to prevent damping-off of cotton seedlings by *R. solani*.

MATERIALS AND METHODS

Isolation and identification of an antagonistic bacterium from rhizosphere soil. Dilutions (10^{-6} and 10^{-7}, w/v) of rhizosphere soil from 7-day-old cotton seedlings were spread in 0.1-ml samples on plates of medium 523 in agar (7). Plates were incubated at 24 C for 4 days then inoculated on the surface with PDA plugs of *R. solani* (strain W-18 from cotton). After 3 days of incubation at 24 C the plates were examined for bacterial colonies antagonistic to the growth of *R. solani*. Colonies with putative antibiotic activity were isolated and challenged with the fungus after 3 days. The most active isolate, a fluorescent pseudomonad, was subjected to standard staining (10) and physiological tests (1), and identified to species according to Bergey's manual (2).

Isolation and identification of an antibiotic from the antagonistic bacterium. Plates containing medium 523 in agar were spread with a bacterial suspension, incubated for 10 days at 24 C, cut into 1-cm squares, and extracted with 200 ml of 80% aqueous acetone for each 10 plates of culture medium. The extracts were filtered through cheesecloth and centrifuged at 12,000 g for 10 min to remove particulate matter. The supernatant fluids were combined and condensed in vacuo at 40 C. 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 chloroform at a concentration 100× that in the original culture volume, and 1-ml samples were streaked on thin-layer plates coated with silica gel 7GF. The plates were developed in cyclohexane/chloroform/diethylamine (50:40:10, v/v) and observed under long and shortwave UV light to detect fluorescing and adsorbing bands. Bands and blank areas were scraped separately from the plates and eluted with acetone. Acetone eluates diluted 1:10 with sterile water were assayed for activity against *R. solani* by placing 100-μl aliquots in 7 mm diameter wells cut in PDA plates inoculated simultaneously on the surface with agar plugs of the fungus. After 2 days of incubation the plates were examined for mycelium-free zones around the wells. The eluate that contained active material again was streaked on silica gel 7GF plates and developed with chloroform and the procedure for locating bands and detecting zones with antibiotic activity then was repeated. The eluate from the band showing inhibitory activity was concentrated in hot cyclohexane, and the antibiotic was crystallized from it after cooling. Mass and NMR spectra of the purified crystaline antibiotic were made and compared with standard spectra for authentic pyrrolnitrin obtained from the Fujisawa Pharmaceutical Co., Osaka, Japan.

Antifungal spectrum of the antibiotic. Purified antibiotic dissolved in 10% aqueous acetone was diluted to 25 μg/ml with sterile water. The solution then was assayed (by the methods described previously for the chromatogram eluates) against the following fungi that have been associated with cotton seedling diseases: *Thielaviopsis basicola, Alternaria* sp., *Verticillium dahliae, Fusarium* sp., and *Pythium ultimum*.

Efficacy of cottonseed treatment with bacterial cultures or antibiotic to control damping-off. Nonsterile soil containing 33 g of *R. solani* inoculum per 9 kg of soil was prepared and its level of infestation was determined according to methods described previously (5). Dilutions of purified antibiotic in acetone were prepared at 0, 20, 40, 60, 80, 100, 200, and 400 μg/ml. One-milliliter samples were added to 0.2-g lots of diatomaceous earth carrier, and the acetone was removed in vacuo. Each of the 0.2-g samples then was mixed with methyl cellulose, and coated onto 30 cottonseed per sample, and assayed for optimum protective effect against *R. solani* by the soil tube method described previously (5).

Cottonseed treated with 200 μg/ml antibiotic were planted in lots of 30 seed per flat in *R. solani*-infested or noninfested

nonsterile soil. 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 and incubated for 14 days. Then counts of pre-emergence and postemergence damped-off seedlings and of healthy survivors were made.

Soil flats containing nonsterile soil infested with *R. solani* or noninfested soil were planted with 30 cottonseed per flat. At planting, each seed was covered with 1 ml of liquid culture of the bacterium (7-day-old shake cultures in medium 523 containing 7×10^8 cells/ml). An additional 1 ml per seed was added to the cover soil. The flats were incubated and data taken as described for the first soil flat test.

After 14 days, seeds and seedlings from infested and noninfested flats were recovered and surface disinfested by washing them for successive 30-sec intervals in 1% sodium hypochlorite, 70% ethanol, and sterile water. Sections were plated on 2% water agar and examined after 24 and 48 hr for evidence of mycelial growth.

Persistence of antibiotic activity in nonsterile soil. The antibiotic isolated from the antagonistic pseudomonad was incorporated into moist, nonsterile soil at a concentration of 10 μg/g, and 100-g samples were packed into 9-cm-diameter glass petri dishes. Controls consisted of moist, nonsterile soil without antibiotic. Five seeds were planted in each plate, and the plates were incubated at 25 C. At the start of the experiment and at 3-day intervals thereafter, 200-mg plugs of soil were removed from around the seeds and placed in metal cylinders on the surface of soil extract agar (the filtrate of equal volumes of soil and distilled water, autoclaved with 2% agar). Sterile water (0.05 ml) was added to each cylinder and the agar surface between the cylinders was inoculated with PDA plugs of *R. solani*. The plates were incubated at 25 C for 3 days, then the widths of the clear zones around the metal cylinders were measured.

TABLE 1. Emergence and survival of cotton seedlings from seed treated with varying concentrations of pyrrolnitrin and planted in *Rhizoctonia solani* infested soil tubes

Pyrrolnitrin (μg/30 seed)	Emergence[a] (%)	Survival (%)
0	33[b] ± 3	20 ± 3
20	53 ± 0	40 ± 3
40	53 ± 0	40 ± 7
60	53 ± 3	53 ± 3
80	73 ± 7	60 ± 0
100	93 ± 3	87 ± 3
200	93 ± 3	93 ± 3
400	93 ± 0	93 ± 0

[a] Values are expressed as a percentage of the total number of seeds planted.
[b] Each value is the average of three replications of 30 seeds each; the maximum deviation from that value of the individual replicates also is shown.

RESULTS

Examination of the cotton rhizosphere dilution plates inoculated with *R. solani* revealed several yellow fluorescent bacterial colonies that inhibited the growth of the fungus. After isolation, the inhibitory effects of these bacteria were confirmed. Staining and physiological tests showed that the most active strain (PF-5) was a Gram-negative rod, motile by polar flagella, which produced a yellow, water-soluble pigment on 523 agar. It would not grow at 37 C, produced an alkaline reaction in milk, and produced acid from glucose. Nitrates were reduced to nitrite and ammonia, but indole was not produced. Gelatin stab liquefaction was infundibuliform, and the bacterium did not produce a yellow diffusible pigment in cream.

The bacterium therefore was classified as *Pseudomonas fluorescens* Migula according to Bergey's manual (2).

The antibiotic from our strain of *P. fluorescens* was readily partitioned into ethyl ether. On silica gel 7GF plates, it migrated to R_f .35 in the cyclohexane-chloroform-diethylamine solvent system and to R_f .48 in chloroform. The antibiotic appeared as a dark spot under shortwave UV light. The mass- and NMR spectra of the purified antibiotic were identical to those of an authentic sample of pyrrolnitrin (3-chloro-4-[2′-nitro-3′-chlorophenyl]-pyrrole).

Bioassay of pyrrolnitrin at 25 μg/ml showed that it was highly inhibitory to *T. basicola, Alternaria sp., and V. dahliae.* Growth of the *Fusarium* sp. was reduced by 30%, but *P. ultimum* was not inhibited.

The most effective concentration for optimum seedling emergence and survival was 200 μg pyrrolnitrin per 30 seed (Table 1).

Treatment of seed with pyrrolnitrin resulted in an increase in the number of surviving seedlings from 13 to 70%, while seed treatment with the bacterial culture resulted in an increase from 30 to 79% (Table 2). None of the treatments was phytotoxic to the seedlings. The mycelia growing in seeds and seedlings recovered from infested soil flats were almost exclusively *R. solani. Fusarium* sp. grew out only occasionally and *P. ultimum* was not observed. No mycelia grew from sections recovered from noninfested soil.

Assay of the soil infested with *R. solani* showed 93 propagules per 100 g of soil for the pyrrolnitrin tests and 80 propagules per 100 g of soil for the *P. fluorescens* culture test.

Soil extract plates containing pyrrolnitrin-treated soil plugs and inoculated with *R. solani*, showed 1-cm clear zones around the plugs after 3 days of incubation. Assay plates containing untreated soil plugs were overgrown by the fungus. All subsequent treated soil plugs harvested at 3-day intervals for 30 days showed 1-cm clear zones.

TABLE 2. Emergence and survival of cotton seedlings from seed treated with 200 μg/30 seed pyrrolnitrin or 2 ml/seed *P. fluorescens* culture and planted in *Rhizoctonia solani* infested or noninfested soil flats

Treatment	Emergence[a] (%)	Survival (%)
Pyrrolnitrin		
Untreated seed in noninfested soil	93[b] ± 0	93 ± 0
Treated seed in noninfested soil	97 ± 3	97 ± 3
Untreated seed in infested soil	27 ± 7	13 ± 3
Treated seed in infested soil	82 ± 3	70 ± 7
P. fluorescens culture[c]		
Untreated seed in noninfested soil	93 ± 3	93 ± 3
Treated seed in noninfested soil	93 ± 3	93 ± 3
Untreated seed in infested soil	43 ± 3	30 ± 0
Treated seed in infested soil	83 ± 3	79 ± 3

[a] Values are expressed as a percentage of the total number of seeds planted.
[b] Each value is the average of three replications of 30 seeds each; the maximum deviation from that value of the individual replicates also is shown.
[c] The cell concentration of the *P. fluorescens* culture was 7×10^8/ml.

DISCUSSION

Pyrrolnitrin is marketed in Japan for the treatment of superficial dermatophytic infections. Its use against plant pathogenic fungi has not been reported.

Our pyrrolnitrin-producing isolate of *P. fluorescens* differs in the following respects from the pyrrolnitrin-producing isolate of *P. pyrrocinia* n. sp. reported by Japanese workers (6): it does not grow at 37 C, it produces a water-soluble yellow pigment in culture, and it produces an alkaline reaction in milk.

Assay of the filtrate from *P. fluorescens* cultures showed that only a small amount of pyrrolnitrin was present in the solution. The bulk of the pyrrolnitrin appears to be retained within the cell until lysis occurs. The protective effect exhibited by treatment of cottonseed with cultures of the bacterium may be due to slow release of the antibiotic by the gradual death of the introduced cells as their numbers are reduced by the soil microflora. Even though the concentration may be low, the lysing cells may effect prolonged release and availability of the antibiotic during the critical period of seedling growth.

Pyrrolnitrin is an effective inhibitor of *R. solani* and several other seedling disease organisms in culture. It is much less effective against *Fusarium* sp., and is totally ineffective against *P. ultimum*. In nonsterile soil the antibiotic acts as an effective seed protectant against *R. solani*. However, it may be that constant suppression of *R. solani* with this antibiotic will result in a buildup of other pathogens such as *Fusarium* and *Pythium* spp.

Pyrrolnitrin is not phytotoxic to cotton seedlings at a concentration twice that required to protect them against damping-off pathogens, and it is not readily inactivated by the soil microflora, because it persisted in nonsterile soil for 30 days with no detectable loss in activity.

These results indicate that *P. fluorescens* may be useful as an antagonist to *R. solani* and may facilitate establishment of stands of healthy cotton seedlings. The antagonism exhibited by the bacterium is possibly the result of the production of the antifungal antibiotic pyrrolnitrin, which is itself an effective protectant against damping-off.

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