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Control of *Rhizoctonia solani*, causal agent of brown girdling root rot of rapeseed, by *Pseudomonas fluorescens*

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Abstract. A strain of *Pseudomonas fluorescens* antagonistic to *Rhizoctonia solani* was isolated from the rhizosphere of rapeseed seedlings. Three antibiotics inhibitory to *R. solani* were isolated from *P. fluorescens* culture and identified as pyocyanin, pyrrolnitrin and phenazine carboxamide. Pyocyanin also inhibited growth of other fungi associated with rapeseed seedling disease complex, including *Fusarium roseum* and *Pythium ultimum*. Pyrrolnitrin and phenazine carboxamide inhibited *Gaeumannomyces graminis* take-all fungus of wheat, *Alternaria brassicae* and *Botrytis cinerea*, while *Fusarium roseum* was only partially inhibited and *Pythium ultimum* was unaffected. All three antibiotics inhibited the spore germination of *Cladosporium cucumerinum* when bioassayed on silica-gel TLC plates. Treating rapeseed with *P. fluorescens* at the time of planting in *R. solani* infested peat-mix soil prevented root rot.

Key words: Brown girdling root rot; Phenazine carboxamide; *Pseudomonas fluorescens*; Pyocyanin; Pyrrolnitrin; *Rhizoctonia solani*.

Introduction

Biological control of plant pathogens using bacterial inoculants has been successful to a certain extent during recent years (Colyer and Mount, 1984; Cook and Rovira, 1976; Howell and Stipanovic, 1979, 1980; Mew and Rosales, 1984; Weller and Cook, 1983; Weller and Rovira, 1984). Pseudomonads used as inoculants are mostly *Pseudomonas fluorescens* and *P. Putida* types obtained from soils and plant surfaces. Some of these bacteria produce siderophores which strongly chelate environmental iron, making it unavailable to pathogens (Kloepper *et al.*, 1980). Brown girdling root rot in rapeseed caused by *Rhizoctonia solani* has been a serious problem in the Peace River Region of Alberta (Sippel *et al.*, 1985). In the present investigation, we report biological control of *R. solani* using an isolate of *P. fluorescens* from Alberta soils.

Materials and Methods

Isolation and Identification of an Antagonistic Bacterium from Rhizosphere Soil

Dilutions (10^{-6} and 10^{-7}, v/v) of rhizosphere soil collected from a healthy rapeseed field at Agriculture Canada, Research Station, Beaverlodge, Alberta, were spread in 0.1 ml samples

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on corn meal agar plates. Plates were incubated at 25°C for 4 days then inoculated on the surface with PDA plugs of *Rhizoctonia solani*. After 3 days of incubation at 25°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 (Pelczar and Reid, 1958) and physiological tests as described by Anonymous (1953). Identification of the species was done according to Bergey's Manual (Buchanan and Gibbons, 1974).

Isolation and Purification of Antibiotics

The bacterial isolate (*P. fluorescens*) was cultivated in potato sucrose broth for 30 days at 20°C under stationary conditions. The broth was concentrated by drying *in vacuo* at 40°C, the residue redissolved in distilled water (50 ml) and extracted with petroleum ether (50 ml; 3 x) at room temperature (20-22°C). The petroleum ether extracts were combined and dried *in vacuo* at 40°C and the residue was redissolved in methanol (5 ml). Antifungal activity of the residue in methanol was tested by using a *Cladosporium* silica-gel TLC bioassay as described earlier by Keen *et al.* (1971). Methanolic residue (100 μl) was spotted onto a silica-gel TLC plate (250 μm, thickness) and the plate developed in a solvent mixture of chloroform and methanol (85:15, v/v). The plate was dried and sprayed with a thick spore suspension of the fungus *Cladosporium cucumerinum* in double strength Czapek dox broth (7.0 g/100 ml). The plate was incubated in a humidified chamber for 72 h at 25°C. At the end of incubation period the plate was examined for an inhibitory zone as shown by a clear area of inhibition of the fungus on the TLC plate (Fig. 1).

Having established the antifungal activity in the residue, inhibitory substances were

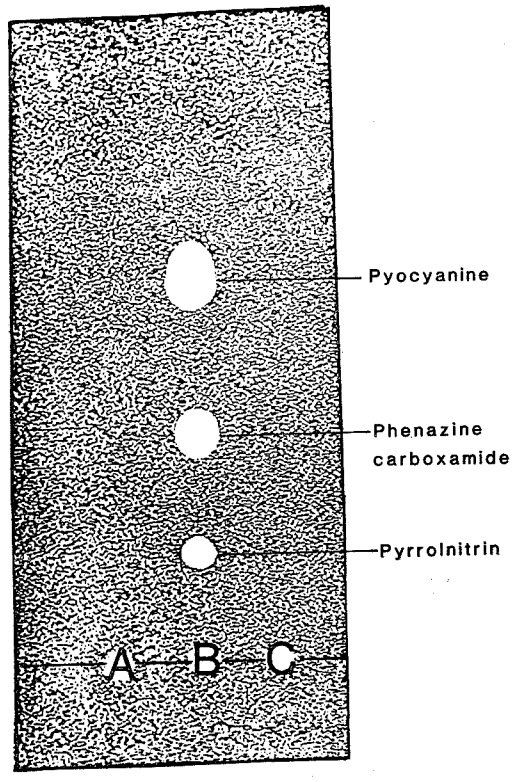

Fig. 1. *Cladosporium* silica-gel TLC bioassay. A&C: Media control; B: Extract from *Pseudomonas fluorescens* broth culture. Sovent; chloroform: methanol (85:15, v/v).

isolated by silica-gel plates (1,000 μm thickness, Terrochem Ltd., Edmonton) developed with chloroform and methanol mixture in the ratio mentioned earlier. Absorbant zones at Rf 0.2, 0.5 and 0.7 were removed and eluted with methanol. The eluates were subjected to UV, IR, NMR and mass spectral analysis.

Spectral Analysis

Mass spectra were measured with a VG-analytical 11-250J by direct probe insertion. ^{1}H-NMR spectra were recorded on a Bruker WH-90 with TMS as an internal standard. UV spectra were recorded in absolute methanol with a Varian XL-200 spectrophotometer.

For comparative studies authentic samples of phenazine carboxamide and pyocyanin were

kindly supplied by Wayne Taylor, Chemistry Department, University of Manitoba, Winnipeg, Canada and authentic pyrrolnitrin was obtained from Fujisawa Pharmaceutical Co., Osaka, Japan.

Antifungal Spectrum of the Antibiotics

The purified antibiotics in methanol were diluted to 100 μg/ml with sterile distilled water and the solutions were assayed against *Rhizoctonia solani* (AG-2 type 1), *Pythium ultimum, Fusarium roseum, Botrytis cinerea, Alternaria brassicae, Gaeumannomyces graminis* and *Cladosporium cucumerinum* by placing 100 μl into wells cut into potato dextrose agar (PDA) plates inoculated with the test fungus. The plates were incubated at 25°C for 3-5 days. The plates were then examined for evidence of clear zones around the wells. Each treatment was kept in 3 replicates and repeated thrice.

Antibiotics Stability to Test Fungi

In order to study the stability of the antibiotics, test fungi were inoculated into Erlenmeyer flasks (25 ml capacity) containing Czapek dox broth (10 ml) supplemented with the purified antibiotics (100 μg/ml). Flasks were incubated for 20 days at 25°C under stationary conditions. At the end of incubation, reisolation of the antibiotic was made and assayed for toxicity by the procedure described earlier. Each treatment was kept in four replicates and repeated thrice.

Effect of the Antibiotics on ***R. solani***

One third of the culture and media of *R. solani* on a PDA plate was removed and the space filled with PDA containing 100 μg/ml of the purified antibiotics. After 10 days incubation at 25°C samples of mycelium along the border with the new PDA were removed for microscopic examination and for reinoculation onto fresh PDA.

Efficacy of rapeseed treatment with antibiotics or bacterial culture: Nonsterile soil (peat-mix) containing 5 g of *R. solani* inoculum per 1 kg of peat-mix soil was prepared. Surface sterilized rapeseeds (*Brassica campestris* cv. Tobin) were coated with methyl cellulose slurry mixed with 100 μg/ml of the purified antibiotics and assayed by the procedure described by Howell and Stipanovic (1979). Canola seeds treated with 100 μg/ml of each antibiotic were planted in *R. solani* infested or noninfested, nonsterile soil and kept in the greenhouse for 30 days. Pots were watered every other day. Samples (20 seeds/pot, 3 x) were taken out and examined for rotted roots. Each experiment was repeated twice.

Pots in triplicate containing nonsterile soil infested with *R. solani* or noninfested soil were planted with 1 ml of thick bacterial suspension (1×10^7 cells/ml). An additional 1 ml per seed was added to the cover soil. The pots were incubated and data recorded as described for the antibiotic treated assays (Table 2).

After 30 days, the root samples from infested and noninfested pots were recovered and surface disinfected by washing them for successive 1 minute intervals in 1% sodium hypochlorite, 70% methanol and sterile water. Root sections were plated on 2% water agar and examined after 48 hours for evidence of mycelial growth.

Persistence of the Antibiotics in Soil

Purified antibiotics (100 μg/g) were mixed with nonsterile soil maintained at 15% moisture at 25°C. Every 3 days, three replicate 100 μg samples of treated soil were transferred to penicylinders placed on the surface of PDA plates (6 penicylinders/plate). Each sample was moistened with 100 μl of sterile water and the plate center was inoculated with a PDA plug of *R. solani* (7 days old). The plate was incubated at 25°C for 5 days and the widths of clear zones around the penicylinders were recorded. The controls consisted of sterile soil

samples with antibiotics and nonsterile soil samples without antibiotics. Each experiment was kept in three replicates and repeated twice.

Results and Discussion

Examination of the rapeseed 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 of the bacterial isolate showed that it was a gram negative rod, motile by polar flagella, which produced yellow pigments in agar culture. It did not grow at 37°C, produced acid from glucose and gave alkaline reaction in milk. Nitrates were reduced to nitrite and ammonia, but indole was not produced. Gelatin stab liquefaction was infundibuliform and the bacterium did not produce yellow diffusible pigment in cream. Based on these observations, the bacterium was classified as *Pseudomonas fluorescens* according to Bergey's Manual (Buchanan and Gibbons, 1974).

It is obvious from the *Cladosporium* silica-gel TLC bioassay (Fig. 1) that three antifungal compounds were produced by the antagonist (*Pseudomonas fluorescens*) in culture broth. On mass spectral analysis the compound with Rf 0.2 gave a molecular weight of 257.09 with an empirical formula $C_{10}H_6Cl_2N_2O_2$ and an UV absorption maxima at λ_{max} 252 nm (ε 7,500). The peak at 252 nm in the ultraviolet absorption spectrum indicated the presence of a benzene ring in the structure. The presence of two bands at 1,530 and 1,376 cm^{-1} in the infrared spectrum of the compound indicated that it had a nitro group and an aromatic group. Besides, the band at 1,610 cm^{-1} in the infrared spectrum confirmed the presence of a benzene ring. The presence of a pyrrole ring was suggested by the band at 3,480 cm^{-1} in the infrared spectrum and violet colour production by the EHRLICH reaction at room temperature. In the NMR spectrum, the presence of a pyrrole ring was further confirmed by the broad singlet at about δ 1.62 and the doublet peaks at δ 3.26 and 3.38. Aromatic protons in the benzene ring were confirmed by singlets at δ 7.01, 7.28 and a multiplet at δ 7.41. Based on UV, IR, NMR and mass spectral data, the compound was identified as pyrrolnitrin, an antibiotic already reported from *P. fluorescence* (Imanaka *et al.*, 1965). Final structural configuration was established through comparison of spectral data to that of an authentic pyrrolnitrin sample.

On mass spectral analysis, the compound with Rf 0.5 gave a molecular weight M^+ 225.02 with an empirical formula $C_{13}H_{11}N_3O$ and an UV absorption maxima at λ 365, 252 and a shoulder at λ 276 nm. ^{1}H-NMR spectral analysis of the purified compound in deuterated chloroform showed proton doublets at δ 2.04 (J=9.0 Hz), 2.48 (J=9.0 Hz) and double doublets at δ 3.12 (J=9.0 Hz and 2.2 Hz) indicating the presence of a phenazine ring. Based on UV, NMR and mass spectral analysis, the purified compound was identified as phenazine carboxamide (Fig. 1) when the spectral data was compared to that of an authentic phenazine carboxamide.

The compound with Rf 0.7 had a molecular weight of M^+ 210.03 with an empirical formula $C_{13}H_{10}N_3O$ and UV absorption maxima at λ_{max} 292 nm (ε 7,600). ^{1}H-NMR spectral analysis of the purified compound in deuterated chloroform showed a proton singlet at δ 2.69 characteristic of a methyl group whereas phenazine group was indicated by proton doublets at δ 2.26 (J= 9.2Hz), δ 2.51 (J=9.0 Hz) and a multiplet at δ 3.36. Based on UV, NMR and mass spectral data, the compound was identified as pyocyanine (Fig. 2). Final structural confirmation was established through comparison of the spectral data to that of an authentic pyocyanine sample.

Microscopic examination of *R. solani* my-

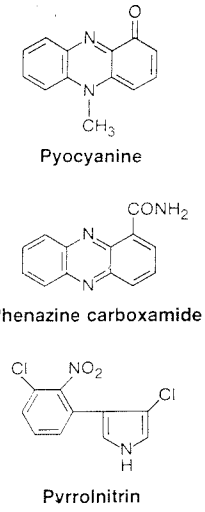

Fig. 2. Chemical structures of purified antibiotics from *Pseudomonas fluorescens*.

celium from pyrrolnitrin treated cultures showed that the cytoplasm of the hyphae had coagulated. No hyphal cytoplasm coagulation was recorded with pyocyanin and phenazine carboxamide treatment, though inhibition of mycelial growth in PDA culture was recorded. The inhibitory effect of pyocyanin and phenazine carboxamide was not clear by microscopic examination.

Washed *R. solani* mycelium transferred from purified pyrrolnitrin treated cultures to fresh PDA failed to grow even after 10 days of incubation whereas the mycelium transferred from pyocyanin and phenazine carboxamide treated cultures showed growth after 10 days. It is obvious from the experimental observations that pyrrolnitrin is a more effective inhibitor as compared to pyocyanin and phenazine carboxamide (Table. 1). Moreover, it was stable in both sterile as well as nonsterile peat-mix soil whereas no antibiotic activity was recovered for pyocyanin and phenazine carboxamide from the nonsterile soil. The loss in activity of these two antibiotics implies either adsorption on the soil particles or degradation by the soil microflora.

Exposure of the purified antibiotics to *R. solani* culture apparently does not degrade the antibiotics. No loss in activity of the purified pyrrolnitrin was noted even after prolonged exposure to *R. solani* in enriched media whereas pyocyanin and phenazine carboxamide lost their activity.

Percent survival of seedlings in *Rhizoctonia* infested soil seeded with pyrrolnitrin treated seed was 90% compared to 18% in the nontreated control. Pyocyanin and phenazine carboxamide seed treatments also increased percent survival of seedlings to 65% and 48%, respectively, compared to 20% and 23% in the non-treated

Table 1. *Antifungal activity of purified antibiotics of* ***Pseudomonas fluorescens.***

Fungi	Pyrrolnitrin[a]	Pyocyanin[a]	Phenazine carboxamide[a]
Alternaria brassicae	18.4[b]±3[c]	12.8±3	5.6±2
Botryttis cinerea	16.6 ±4	10.2±3	8.4±3
Cladosporium cucumerinum	55.6 ±6	28.9±4	16.2±2
Fusarium roseum	4.2 ±2	18.4±3	4.8±3
Gaeumannomyces graminis	14.6 ±4	10.2±2	6.8±1
Pythium ultimum	—	11.4±3	5.6±2
Rhizoctonia solani	24.6 ±5	18.4±3	16.2±2

[a] Concentration of antibiotic was 100 μg/ml.
[b] Area of inhibition in mm^2.
[c] Standard deviation value.

controls. Seeds treated with the bacterial culture gave 78% survival of the seedlings from a control value of 20% (Table 2). Both bacterial cultures and antibiotic treatments were effective in protection of rapeseed seedlings against *R. solani.*

Table 2. *Effect of seed treatment with antibiotics and bacterial (**P. fluorescens**) culture on roots of rapeseed plants grown in peat-mix soil infested with **Rhizoctonia solani.***

Treatment	Survival (%)
i) Pyocyanin	
a) nontreated seed in noninfested soil	90[a] ±3[b]
b) treated seed in noninfested soil	86 ±4
c) nontreated seed in infested soil	20 ±5
d) treated seed in infested soil	65 ±3
ii) Pyrrolnitrin	
a) nontreated seed in noninfested soil	90 ±3
b) treated seed in noninfested soil	95 ±2
c) nontreated seed in infested soil	18 ±4
d) treated seed in infested soil	90 ±3
iii) Phenazine carboxamide	
a) nontreated seed in noninfested soil	90 ±2
b) treated seed in noninfested soil	92 ±2
c) nontreated seed in infested soil	23 ±2
d) treated seed in infested soil	48 ±7
iv) *Pseudomonas fluorescens*[c] culture	
a) nontreated seed in noninfested soil	90 ±3
b) treated seed in noninfestted soil	95 ±2
c) nontreated seed in infested soil	20 ±3
d) treated seed in infested soil	78 ±4

[a] Each value is the average of three replications of 20 seeds each.
[b] The maximum deviation from that value of the individual replicate.
[c] The cell concentration of the *P. fluorescens* culture was 1×10^7 cell/ml.

Pyrrolnitrin was found to be stable up to 30 days in nonsterile soil with no detectable loss in fungitoxic activity. Our findings are further strengthened by similar results reported earlier by Howell and Stipanovic (1979) who found *P. fluorescens* as an effective antagonist to cotton seedling blight pathogen, i. e., *R. solani.* They found that isolates of *P. fluorescens* produced two chlorinated phenyl pyrrole antibiotics. One (pyroluteorin) decreased *Pythium* damping-off cotton seedlings but was ineffective against *Rhizoctonia solani,* and the other (pyrrolnitrin) was effective against *R. solani* but not *P. ultimum.* Pyroluteorin is released from the cells during growth, pyrrolnitrin only after lysis of the cells. Pyrrolnitrin was also effective against *Thielaviopsis brasicola, Verticillium dahliae* and *Alternaria* sps. and persisted in the moist non-sterile soil up to 30 days without any loss of activity.

Our findings suggest that *P. fluorescens* may be useful as an antagonist to *R. solani* and may facilitate establishment of the stands of healthy rapeseed plants. The antagonism exhibited by the bacterium is possibly the result of the production of antifungal substances which are themselves effective protectants against root rot.

Biological control measures which employ fluorescent rhizopseudomonads like those characterized and used in the present study have been successful against important diseases caused by soil-borne fungi such as *Pythium, Fusarium, Rhizoctonia, Ceratocystis, Gaeumannomyces, Acrocylindrium* and others (Defago *et al.*, 1984; Ganesan and Gnanmanickam, 1987; Howell and Stipanovic, 1979, 1980; Mew and Rosales, 1987; Sakthivel *et al.*, 1986; Sakthivel and Gnanamanickam, 1986a, b; Schroth and Hancock, 1982; Weller and Rovira, 1984). Besides they have the ability to enhance crop growth and yield (Schroth and Hancock, 1982). Our data presented here suggest that these fluorescent pseudomonads might prove valuable tools for future plant disease management.

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利用 *Pseudomonas fluorescenes* 控制油菜褐帶根腐病病原菌 *Rhizoctonia solani*

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自油菜幼苗之根系分離出拮抗 *Rhizoctonia solani* 之 *Pseudomonas fluorescens* 一株，此拮抗菌株產生 pyocyanin, pyrrolnitrin 和 phenazine carboxamide 等三種抗 *R. solani* 之抗生素。除 *R. solani* 外，pyocyanin 亦可抑制感染油菜幼苗之其他眞菌，如 *Fusarium roseum* 和 *Pythium ultimum*。Pyrrolnitrin 和 phenazine carboxamide 能抑制 *Gaeumannomyces graminis, Alternaria brassical* 和 *Botrytis cinerea*，但對 *Fusarium roseum* 則只部份抑制，而對 *Pythium ultimum* 則無作用。此三種抗生素均能抑制 *Cladosporium cucumerinum* 之孢子萌發。以 *P. fluorescenes* 處理油菜然後種植於有 *R. solani* 汚染之泥炭混合土中可避免根腐病之發生。