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Pyrrolnitrin and phenazine production by *Pseudomonas cepacia*, strain 5.5B, a biocontrol agent of *Rhizoctonia solani*

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Abstract Rhizoctonia stem rot of poinsettia caused by *Rhizoctonia solani* is controlled by strain 5.5B of *Pseudomonas cepacia* when poinsettia cuttings are rooted in polyfoam rooting cubes. Experiments were conducted to isolate and characterize secondary metabolites from strain 5.5B that were inhibitory towards *R. solani*. Inhibitory compounds were detected in fractions processed from liquid cultures of strain 5.5B. The most inhibitory compound isolated was pyrrolnitrin. A purple pigment consistently produced in culture by strain 5.5B was isolated and identified as 4,9-dihydroxyphenazine-1,6-dicarboxylic acid dimethyl ester, a phenazine. In vitro inhibition of *R. solani* occurred with the phenazine.

Introduction

The soil-borne pathogen, *Rhizoctonia solani* Kuhn [teleomorph = *Thanatephorus cucumeris* (Frank) Donk], causes stem rot of poinsettia (*Euphorbia pulcherrima* Willd. ex Klotzsch), a serious disease occurring during all stages of poinsettia production (Ecke et al. 1990; Strider and Jones 1985). Control methods have traditionally involved cultural practices and the application of chemical fungicides (Bailey 1990; Jones 1990; Powell 1988). Stem rot, however, persists as an endemic and occasionally epidemic problem (Jones 1990). This is attributed, in part, to the continued restrictions on chemical fungicides (Cook and Baker 1983; Staub and Sozzi 1984; Tweedy 1983), particularly in the greenhouse environment as shown by the recent removal of Benlate, one of the most effective fungicides for stem rot control, from the market. Alternative measures, such as biological control tactics utilizing antagonistic microorganisms, are attractive for plant diseases such as stem rot.

Strains of *Pseudomonas* species have been studied extensively as biological control agents of plant diseases (Campbell 1989; Jayaswal et al. 1990; Loper 1988). *Pseudomonas* strains can often be grown using conventional methods, be easily distributed to a target area, and typically survive and colonize well in the cropping system to which they are applied. In addition, *Pseudomonas* species may employ a wide range of antagonistic mechanisms such as antibiosis (the production of inhibitory compounds), siderophore production (iron-sequestering compounds), and nutrient or site competition (Bull et al. 1991; Thomashow and Weller 1988; Thomashow et al. 1990; Weller 1988). The production of antibiotics such as pyrrolnitrin and phenazine-1-carboxylic acid is important in the biocontrol of several soil-borne plant pathogens by *Pseudomonas* species (Howell and Stipanovic 1979; Thomashow et al. 1990).

Pseudomonas cepacia is a ubiquitous soil-borne bacterium with potential for use in biocontrol. The bacterium is capable of utilizing a wide array of carbon compounds and is known to produce a broad range of secondary metabolites, which may be volatile or non-volatile and antibiotic in nature (Homma and Suzui 1989; Howell and Stipanovic 1979; Janisiewicz and Roitman 1988; Roitman et al. 1990; Smilanick and Denis-Arrue 1992).

Strain 5.5B of *P. cepacia* (ATCC 55344) was isolated from a soil sample collected in North Carolina. This strain has proven effective for biocontrol of Rhizoctonia stem rot of poinsettia in polyfoam rooting cubes during the early stages of production (Cartwright and Benson 1992, 1994). Little is understood about the antagonistic mechanisms exhibited by strain 5.5B towards *R. solani* in rooting cubes. However, on the basis of previous in vitro studies on agar, *R. solani* is inhibited by strain 5.5B (Cartwright and Benson, unpublished).

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In addition, this strain consistently produces a distinct, purple pigment in solid and liquid culture, which appears to be soluble or insoluble depending on the type of medium used. This compound and/or additional compounds may be important in control of *R. solani* in the rooting cube environment by strain 5.5B.

This study was undertaken to isolate and characterize metabolites from strain 5.5B, including the purple pigment, and determine the inhibitory activity of these towards *R. solani in vitro*.

Materials and methods

Assays for volatile inhibitors of *R. solani* produced by strain 5.5B

Because many bacteria produce volatile inhibitors such as hydrogen cyanide (HCN), experiments were conducted to assay for volatile material. Strain 5.5B was stored in sterile, deionized water at 4° C. A drop (approx. 0.1 ml) of suspension from water-stored cultures was placed in the center of one compartment of a 9-cm-diameter sectioned (four compartments or sections) petri plate (Fisher Scientific, Pittsburgh, Pa.) containing potato/dextrose/agar (Difco Laboratories, Detroit, Mich.) (PDA) or King's medium B agar (KB) (Dhingra and Sinclair 1985). The compartments allowed no physical contact between strain 5.5B and *R. solani* or the agar on which they were grown. The drop of bacterial suspension was streaked across the agar surface with a sterile loop. Petri plates were wrapped in Parafilm (American National Can, Greenwich, Conn.) and incubated at room temperature. After 10 days, a 5-mm mycelial agar disk taken from the leading edge of an actively growing colony of *R. solani* (isolate RS-3, AG 4, NRRL 22805) on PDA was placed at the outside edge of the compartment opposite the compartment containing the bacterial colony. The plates were wrapped in Parafilm to assist in the containment of any volatile compound produced by the bacterium. Colony growth was measured daily for 3 days from the outside edge of the petri plate to the leading edge of the colony of *R. solani*. Agar plates with *R. solani* growing in the absence of strain 5.5B were used as controls. Five plates were utilized for each agar medium. The experiment was repeated. Data were pooled and analyzed by PC SAS (SAS Institute, Cary, N.C.) with PROC GLM. Means were separated by the Waller Duncan *k*-ratio test.

To test specifically for HCN, the following method was used: chomatography paper was saturated with picric acid, allowed to dry and soaked in sodium carbonate; strips of paper were then attached to the underside of a petri dish lid and placed over viable cultures of strain 5.5B; petri dishes were sealed with Parafilm and incubated overnight at room temperature; a color shift to purple indicated the presence of HCN.

Culture of strain 5.5B for isolation of secondary metabolites

Nutrient broth (Difco Laboratories, Detroit, Mich.) was seeded with cells of strain 5.5B cultured on PDA. After 4 days growth at room temperature on a bench-top agitator, 5-ml aliquots were transferred to 500 ml nutrient broth contained in 2.8-l flasks. Flasks of nutrient broth were placed on a rotary shaker (New Brunswick Scientific, Edison, N.J.) and grown at 25° C (150 rev min^{-1}) for 4 days, turning a distinct purple color. Centrifugation (Sorvall RC2-B centrifuge, Sorvall GSA rotor, Wilmington, Del.) of the culture broth at 13200*g* (20 min) gave a purple bacterial pellet and a violet-colored, cell-free supernatant.

Purification of pyrrolnitrin and a phenazine pigment

The bacterial pellet was extracted three times by stirring with neutral methanol, which removed none of the purple pigment. The neutral methanolic extract (PM, Fig. 1) of the bacterial pellet was added to the ethyl acetate extract (SO) of the bacterial culture supernatant. Evaporation (Rotovapor RE 111, Brinkmann, Westbury, NY) of solvent from this organic-soluble fraction gave 160 mg solid. Pyrrolnitrin in this fraction was purified to homogeneity by thin-layer chromatography (TLC) on silica gel plates (aluminium-backed, pre-coated TLC sheets, Silica Gel 60 F_{254}, EM Separations, Gibbstown, NJ) developed with hexane/acetone (3:2, v/v). Ehrlich reagent (10% p-dimethylaminobenzaldehyde in concentrated HCl)/acetone (1:4, v/v) was used to locate the pyrrolnitrin zone and phosphomolybdic acid was used to detect non-pyrrole metabolites on the chromatogram.

The purple bacterial pellet was then stirred for 1 h with acidified methanol (0.1 ml 6 M HCl in 500 ml methanol), which dissolved the pigment in a yellow-orange form. Neutralization of the acid methanol by addition of saturated, aqueous $NaHCO_3$ caused an immediate shift to a clear purple solution (PAMX) from which a purple precipitate separated overnight. The filtered purple pigment (IPP) was redissolved in a small volume of acidic acetone. Orange crystals (IPPC) of a phenazine separated during partial evaporation of the acetone. The phenazine was further characterized by nuclear magnetic resonance and mass spectroscopy.

Analytical instrumentation

UV spectra were recorded on a UV/visible spectrophotometer (Perkin Elmer, model Lambda 4B). Mass spectrometry was performed on a Hewlett-Packard 5985-B mass spectrometer with electron-impact or chemical ionization with CH_4 as ionizing gas. NMR was performed on a GE Omega 300 spectrometer.

Bioassay of fractions and compounds for activity against *R. solani* in vitro

Inhibitory activity in the selected crude fractions (Fig. 1), designated as the SO (in ethyl acetate), PAMX (in methanol), and SW (in water) fractions, was initially detected as follows. A 5-mm-diameter mycelial disk taken from an actively growing colony of *R. solani* (grown on PDA) was placed in the center of a 9-cm-diameter petri plate containing PDA. A 5-mm-diameter, circular disk of Whatman no. 1 filter-paper was saturated with 50 µl crude fraction solutions and placed on the agar surface about 1 cm from the mycelial disk. For the SW fraction, a 5-mm well was cut out of the agar with a cork borer (about 1 cm from the mycelial disk) and 150 µl solution was deposited in each well. An arbitrary scale was used to rate inhibition after 2 days: 1 = no inhibition, 2 = slight inhibition, 3 = 50% inhibition, 4 = 75% inhibition, and 5 = complete inhibition.

Inhibitory activity from the SO fraction (Fig. 1) was detected in the initial bioassays prompting further testing with isolated compounds from the organic soluble fraction (fraction SO plus fraction PM, the pyrrolnitrin fraction). Silica gel residue (stored dry at –15° C) from each zone/band from the preparative chromatograph was used. Additional bioassays of selected crude fractions (fractions PAMX, PM) were also completed. For the assay, a 7-mm-diameter well was cut out of the agar with a cork borer at the inside apex of each compartment of a four-compartment petri plate containing PDA. A 100-µl aliquot from each band from the pyrrolnitrin fraction (approx. 5 mg silica gel suspended in 1 ml sterile, deionized water), from the purified pigment (crystals of the purple pigment; 10 mg crystals dissolved in 2 ml acetone), or from each crude fraction tested was deposited in the wells (one fraction per band per compound per plate). As controls, sterile, deionized water and each solvent were deposited in wells. A 5-mm mycelial disk from an actively growing colony of *R. solani*

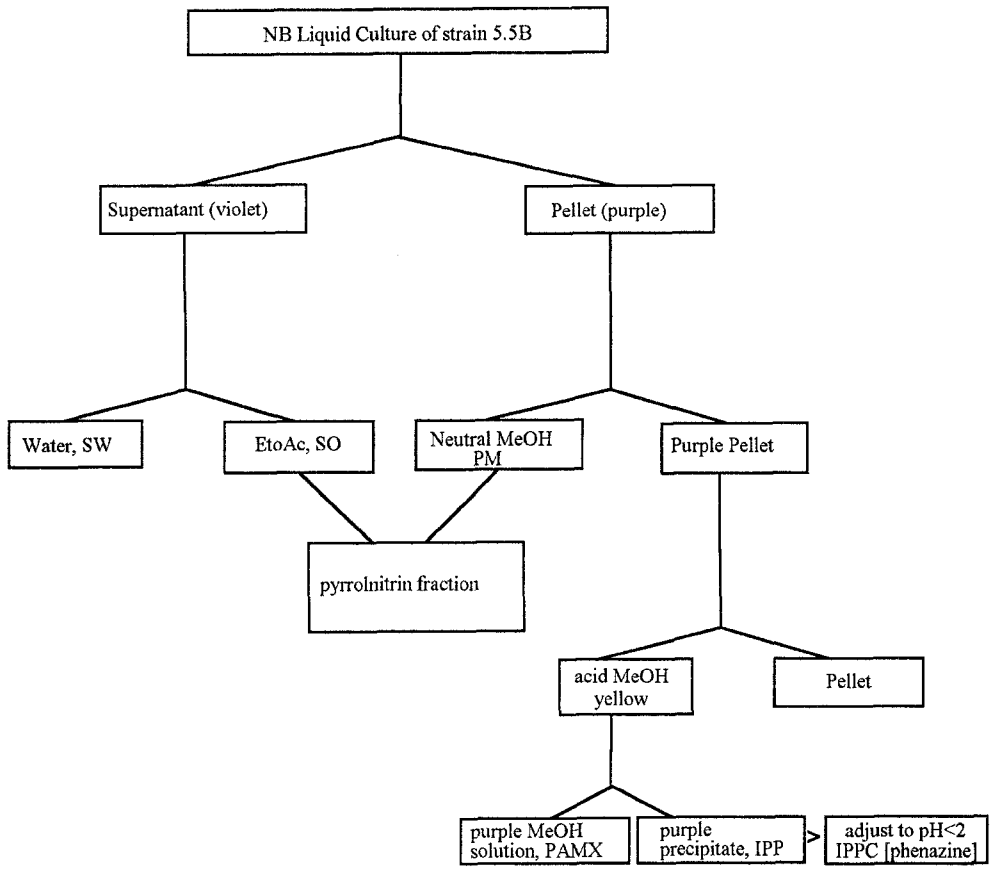

Fig. 1 Scheme of isolation procedures (showing various fractions) for compounds produced by *Pseudomonas cepacia*, strain 5.5B, in liquid culture

was placed opposite each well containing the test fractions/compounds or the water/solvents (controls) at the outside edge of each section (compartment). Plates were incubated at room temperature and colony diameter (measured from the outside of the compartment to the leading edge of the colony of *R. solani*) and inhibition zone (measured from the leading edge of the colony of *R. solani* to the outside edge of the well) were measured (in mm) for 3 days. For clarity, data in Fig. 2 are based on colony diameter and only treatments that were significantly different are shown. The experiment was repeated once. Results were similar from each experiment, so data were pooled and analyzed (PROC GLM with PC SAS). Means were separated with the Waller Duncan *k*-ratio test.

Results

Assays for volatile inhibitors of *R. solani* produced by strain 5.5B

Slight inhibition ($P=0.05$) of *R. solani* was observed after 2 days where the colony diameter was 31 mm or 34 mm on the KB or PDA agar respectively, compared to diameters of 36 mm or 38 mm on the KB or PDA control plates respectively. In all cases, *R. solani* had grown to the edge of the compartment after 3 days (42 mm), nullifying the differences. On plates where strain 5.5B of *P. cepacia* was present, however, there was noticeably less growth of *R. solani* after the third day. No HCN was produced by viable cultures of strain 5.5B, as indicated by the picric acid assay.

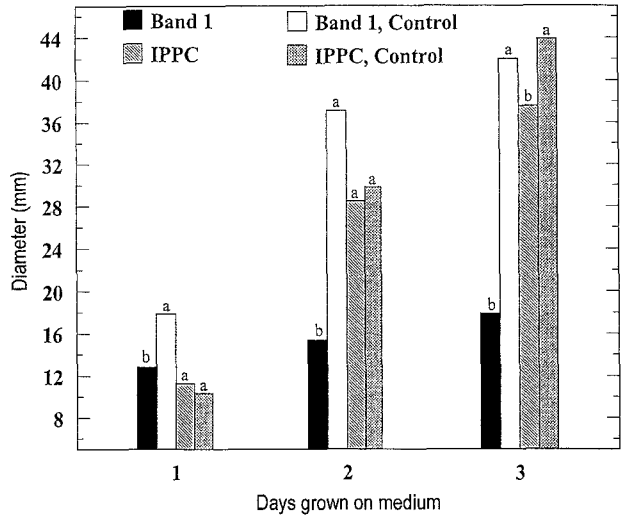

Fig. 2 In vitro inhibition of *Rhizoctonia solani* by the pyrrolnitrin fraction and purified purple phenazine obtained from liquid cultures of *Pseudomonas cepacia*, strain 5.5B. Values are means from two experiments. Values between each fraction/compound and the respective control are significantly different ($P=0.05$) if followed by a different letter, according to the Waller Duncan *k*-ratio test (IPPC purified pigment, see Fig. 1)

Identification of pyrrolnitrin and the phenazine pigment

Pyrrolnitrin was purified by preparative thin-layer chromatography and was determined to be free of impurities detectable by UV quenching, Ehrlich reagent, or phosphomolybdic acid. Pyrrolnitrin obtained from strain 5.5B had identical mobility (in hexane/acetone 3:2) to that of a standard of pyrrolnitrin obtained from C. R. Howell (USDA/ARS, College Station, Tex.). The mass spectrum showed the proper molecular mass for pyrrolnitrin, including the $^{35}Cl/^{37}Cl$ isotopic cluster of intensity 9:3:1, characteristic of a metabolite containing two chlorine atoms ($C_{10}H_6N_2O_2Cl_2$). The purple pigment present in the bacterial cells was crystallized in its acidified, orange form. It was identified as 4,9-dihydroxyphenazine-1,6-dicarboxylic acid dimethyl ester ($C_{16}H_{12}N_2O_6$) by comparison of its UV spectrum (maxima at 276, 368, and 448 nm in acidic methanol) and mass spectrum to the spectra of the authentic compound (Korth 1978). This structure was confirmed by proton nuclear magnetic resonance.

Bioassay of fractions and compounds for activity against *R. solani* in vitro

In preliminary assays, the crude SO fraction was highly inhibitory to *R. solani* with an average rating of 5 (complete inhibition). No inhibition of *R. solani* occurred with the SW fraction. An average inhibition rating of 2 occurred with the PAMX fraction. Further assays utilized isolated compounds from the organic soluble fraction (fraction SO plus fraction PM) to detect specific inhibitory compounds in this fraction and also the PAMX, PM fractions, and the orange crystals (4,9-dihydroxyphenazine-1,6-dicarboxylic acid dimethyl ester).

Of the six compounds found in the organic soluble fraction, only the compound in band 1 (pyrrolnitrin) was inhibitory to *R. solani*. Growth of the *R. solani* exposed to this compound averaged only 18 mm after 3 days compared to 42 mm for the control (Fig. 2). The purified pigment (IPPC) also limited growth of *R. solani* after 3 days (Fig. 2). Average growth of the *R. solani* exposed to the pigment (in acetone) was about 36 mm after 3 days compared to 42 mm of growth in the acetone control (Fig. 2). Compared to their respective solvent controls, no other fractions inhibited ($P=0.05$) *R. solani* in this assay.

Discussion

Development of antagonistic microorganisms for biocontrol of plant diseases is advancing (Campbell 1989; Cook and Baker 1983; Tweedy 1983). *Pseudomonas* species are some of the most promising biocontrol microorganisms. These bacteria produce many metabolites that can be inhibitory to plant pathogens (Bakker and Schippers 1987; Flaishman et al. 1990; Gurusiddaiah et al. 1986; Harrison et al. 1993; Lockwood 1984; Roitman et al. 1990; Weller 1988).

Our results demonstrate that strain 5.5B of *P. cepacia* produces secondary metabolites (pyrrolnitrin and a phenazine), which are inhibitory to *R. solani* in vitro. Some evidence for the presence of a volatile inhibitor(s) other than HCN was observed, although inhibition was minimal compared to inhibition from pyrrolnitrin. On the basis of this, the most important inhibitors are non-volatile and agar-diffusible. In previous experiments, strain 5.5B significantly restricted growth of *R. solani* on an agar medium. At that time, however, specific inhibitory compounds were not identified (Cartwright and Benson 1992). This present study demonstrates that *R. solani* was inhibited by pyrrolnitrin isolated from cultures of strain 5.5B. The level of inhibition with pyrrolnitrin suggests that it may be important in the biocontrol of Rhizoctonia stem rot of poinsettia in rooting cubes. Pyrrolnitrin was first reported from *P. pyrrocinia* (Arima et al. 1964). Since then, it has been reported from a number of bacteria including strains of *P. cepacia* and *P. fluorescens* (Homma and Suzui 1989; Howell and Stipanovic 1979). Pyrrolnitrin produced by *P. fluorescens* (strain Pf-5) was critical in biocontrol of damping-off of cotton caused by *R. solani* (Howell and Stipanovic 1979). Several different strains of *P. cepacia* that produce pyrrolnitrin were effective in controlling damping-off of radish caused by *R. solani* (Homma and Suzui 1989), blue mold of apple caused by *Penicillium expansum* Lk. & Thom., gray mold of apple caused by *Botrytis cinerea* Pers. & Fr. (Janisiewicz and Roitman 1988), and green mold of lemons caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. (Smilanick and Denis-Arrue 1992). Because pyrrolnitrin production could be an important biocontrol mechanism of strain 5.5B, more studies are needed to evaluate its role fully in control of Rhizoctonia stem rot. Other important aspects of future research with strain 5.5B include pyrrolnitrin production in vivo, application of pyrrolnitrin-negative mutants of strain 5.5B in vivo, selection of growth media to enhance pyrrolnitrin production, and application of specific nutrients to the target area(s) to influence production. It is known that pyrrolnitrin production by *P. cepacia* can vary depending on the medium (Roitman et al. 1990).

Phenazines are naturally occurring pigments produced by bacteria (Korth et al. 1978; Turner and Messenger 1986). Several are known for their antibiotic properties and are important in plant disease control by *Pseudomonas* strains. Phenazine-1-carboxylic acid, produced by *Pseudomonas fluorescens* (strain 2–79), is critical in the control of take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici* (Gurusiddaiah et al. 1986; Thomashow and Weller 1988; Thomashow et al. 1990). Flaishman et al. (1990) showed that pyocyanine-deficient mutants of *Pseudomonas aeruginosa* (strain

LEC1) were less suppressive than the wild-type strain to blotch of wheat caused by *Septoria tritici* Rob. ex Desm.

Strain 5.5B produces a distinctive non-diffusable purple pigment when grown on PDA or in nutrient broth. This pigment was identified as 4,9-dihydroxyphenazine-1,6-dicarboxylic acid dimethyl ester, a phenazine. Little has been published on this phenazine. Although Morris and Roberts (1959) probably isolated this compound from *Pseudomonas multivorans* (now known as *P. cepacia*), the structure was not known until Korth et al. (1978) published that this compound was the 'missing link' in phenazine biosynthesis. Antifungal properties of this phenazine have not been published but our results show it is inhibitory to *R. solani* in vitro. More studies, including quantitative, in vivo assays are needed to determine if this compound is important in the biocontrol of stem rot; however, its very low solubility may limit toxicity. On the basis of additional observations (Cartwright D. Kelly, Chilton W. S., and Benson D. M., unpublished), there is a different purple pigment produced when strain 5.5B is grown on a different medium such as KB medium. Phenazine production can be influenced by the growth medium (Slininger and Jackson 1992). This pigment is agar-diffusable and, during fractionation, a water-soluble pigment that was similar (identical chromophores) to the insoluble pigment was detected. More studies are needed to assess fully the role of this pigment, if it has any, in stem rot control. The fraction containing this diffusable pigment did not inhibit *R. solani* in vitro compared to the solvent control but, because of the high inhibition of *R. solani* in the control, inhibitory properties could have been masked.

Our findings are significant for a number of reasons. First, the poinsettia production system is very amenable for the use of antibiotic-producing microorganisms such as strain 5.5B. In addition, to our knowledge, this is the first report of a bacterium used in biocontrol that produces both pyrrolnitrin and 4,9-dihydroxyphenazine-1,6-dicarboxylic acid dimethyl ester, and the first report of antifungal activity for this phenazine. Finally, the possibility of multiple antagonistic mechanisms provides opportunity for use of strain 5.5B in other types of plant production systems against other types of target pathogens.

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