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Broad spectrum action of phenazine against active and dormant structures of fungal pathogens and root knot nematode

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Abstract
Antifungal antibiotic from *Pseudomonas chlororaphis* isolate PA23 was identified as Phenazine using TLC and HPLC. Phenazine recorded the highest inhibition zone of 21 mm with 35.55% percent inhibition of mycelial growth of *Pythium aphanidermatum* over control. It had a significant effect on the hyphal morphology of *P. aphanidermatum* and on spore germination of *Botryodiplodia theobromae* and *Alternaria solani*. Disorganization of hyphal morphology of *P. aphanidermatum* includes vacuolization, cell content degeneration and hyphal lysis. Similarly interaction of phenazine with *Rhizoctonia solani* resulted in abnormal swelling of hyphal tips was noticed in the hyphal tips. Similarly the germination of sclerotia of *Macrophomina phaseolina*, *R. solani* and *Sclerotium rolfsii* were completely inhibited by phenazine at a concentration 50 μl. Incubation of the eggs of the root knot nematode *Meloidogyne incognita* in 30 μl concentration of phenazine, completely suppressed the hatching of juveniles.

Keywords: Pseudomonas chlororaphis*; Phenazine; TLC; HPLC*

Introduction

Antibiotics encompass a chemically heterogeneous group of organic, low-molecular weight compounds produced by microorganisms at low concentrations; antibiotics are deleterious to the growth or metabolic activities of other microorganisms (Thomashow *et al.*, 1997). Studies on antibiotics produced by *Pseudomonas* spp. are abundant since they are common inhabitants of rhizosphere and phyllosphere. Antibiotics produced by different PGPR have a broad-spectrum activity. Slininger and Shea-Wilbur (1995) observed that in the rhizosphere *P. fluorescens* strain 2 – 79 produced an antibiotic, phenazine 1-carboxylic acid (PCA) which is the primary means of disease suppression against take-all of wheat. Raaijmakers *et al.*

(1998) focused the role of phenazine (Phz) and 2,4-diacetylphloroglucinol (PHL) produced by *Pseudomonas* spp. in soils that are naturally suppressive to take-all of wheat caused by *G. graminis* var. *tritici*. Slininger *et al.* (2000) found that *P. fluorescens* strain 2–79 (NRRL B-15132) produced phenazine-1-carboxylic acid (PCA) as its primary means of suppressing take-all disease of wheat. Chin-A-Woeng *et al.* (1998) reported that *P. chlororaphis* strain PCL1391, produced a hydrophobic compound – phenazine-1-carboxamide (PCN). Comparison of *in vitro* antifungal activity of PCN and PCA showed that the antifungal activity of PCN was at least 10 times higher at neutral pH, suggesting that this may contribute to the superior biocontrol performance of strain PCL1391 in the tomato against *F. oxysporum*.

Materials and methods

Extraction of phenazine

Strains of antagonistic bacteria were grown at $28 \pm 2°C$ in Pigment Production medium (PP) (Peptone-20g/l, Glycerol-20g/l, NaCl-5g/l, KNO_3-1g/l, pH-7.2, distilled water-1 l). The cultures were grown in PP broth for 5 days and were centrifuged at 5000 rpm and the supernatants were adjusted to pH 2.0 with conc. HCl and it was extracted with an equal volume of benzene. The benzene layer was subjected to evaporation in water bath. After evaporation, the residues were resuspended in methanol.

Bioassay of crude antibiotics by agar plate method

PDA medium was poured in sterile Petri plate and allowed to solidify. A 5 mm sterilized cork borer was placed inside the medium and wells were made in the Petri plates at the opposite ends. The pathogen *P. aphanidermatum* was cut with 5 mm cork borer and placed in the center of the well. The crude antibiotic extracted was poured into the wells at opposite ends. The effective dose for the inhibition of *P. aphanidermatum* was standardized by pouring different doses of crude antibiotics namely 100 μl, 150 μl, and 200 μl/well. The mycelial growth and inhibition zone was recorded after 72 h of incubation ($28 \pm 2°C$). Surface area of inhibition was measured by tracing the surface area of inhibition in a tracing paper and then plotting it on the graph sheet.

Detection of phenazine using TLC

For the detection of phenazine, 10 μl of samples were chromatographed on TLC aluminium sheets – silica gel 60 F 254 (Merck, Germany). The solvents used were isopropanol/ammonia/water (8 : 1 : 1). Plates were viewed under UV light were then sprayed with diazotized sulphanilic acid (DSA). Rf values of the spots were calculated.

Preparation of diazotized sulphanilic acid

50 mg diazotized sulphanilic acid was dissolved in 20 ml 20% Na_2CO_3 solution. Diazotization is done by dissolving 25 g sulphanilic acid in 125 ml 10% sodium nitrite solution. The mixer was added drop by drop to 60 ml of 8M HCl in ice-cold condition. After 10 min, it was filtered under ice-cold condition. It was washed extensively with the cold water and then with ethanol followed by ether. The crystals were air dried and stored at 4°C.

Detection of phenazine using HPLC

The HPLC system consisted of a water pump (model 510), a rheodye injection valve with a 20 μl loop, and an analytical column – 150 mm by 4.5 mm (inner diameter) by 6 mm (outer diameter) packed with octadecyl silica (Hyperil: particle size, 5 μm). The spectrometer detector (model 440) was from Water Associates and was linked to a Phillips 8251 strip chart recorder, which plotted the detector outputs. The reagents for mobile phase preparation were of HPLC grades, and all mobile phases used were filtered and degassed on a Millipore HPLC filtration with 0.45 μm pore size membrane filters. All samples were run at a flow rate of 1.0 ml/min and detected at a wavelength of 254 nm. The peaks obtained were compared with the standards.

Effect of phenazine on the hyphal morphology and spore germination of plant pathogens

The effect of antibiotic on the hyphal morphology was studied by adding 150 μl of phenazine in the cavity slide containing the hyphae of *Pythium aphanidermatum*, *Rhizoctonia solani*, *Sclerotium rolfsii*, *M. phaseolina* and sporulating fungi like *Alternaria solani* and *Botryodiplodia theobromae*. The slides were incubated in moist chamber for 24 h and the hyphae and spores were observed under image analyzer for hyphal abnormalities and spore germination.

Results & discussion

The phenazine isolated from *P. chlororaphis* isolate PA23 recorded the maximum per cent inhibition of mycelial growth (37.77%) of *P. aphanidermatum* at 200 μl concentration over untreated control (Table I). Subsequently it was followed by 150 μl (35.55%). *P. aeruginosa* PNA1, isolated from the rhizosphere of chickpea in India, inhibited the *in vitro* mycelial growth of different phytopathogenic fungi by the production of phenazine-1-carboxylic acid (PCA) and oxychlororaphine (OCP). Strain PNA1 also protected the chickpea (cv. JG 62) plants from Fusarium wilt disease caused by *F. oxysporum* f. sp. *ciceris* (Slininger and Shea-Wilbur, 1995).

Table I. Antifungal action of phenazine against *P. aphanidermatum*.

Antagonistic bacteria	Mycelial growth of pathogen (mm)[*]			Inhibition zone (mm)			Per cent inhibition of mycelial growth over control		
	100 μl/well	150 μl/well	200 μl/well	100 μl/well	150 μl/well	200 μl/well	100 μl/well	150 μl/well	200 μl/well
PA23	63.00[b]	58.00[b]	56.00[b]	15.00[a]	21.00[a]	22.00 [a]	30.00	35.55	37.77
CBE4	70.00[b]	70.00[b]	69.00[b]	0.00[b]	0.00 [b]	0.00 [b]	22.22	22.22	23.33
M3	90.00[a]	90.00[a]	90.00[a]	0.00 [b]	0.00 [b]	0.00 [b]	0.00	0.00	0.00
KPf1	90.00[a]	90.00[a]	90.00[a]	0.00 [b]	0.00 [b]	0.00 [b]	0.00	0.00	0.00
KPf2	90.00[a]	90.00[a]	90.00[a]	0.00 [b]	0.00 [b]	0.00 [b]	0.00	0.00	0.00
Control	90.00[a]	90.00[a]	90.00[a]	0.00 [b]	0.00 [b]	0.00 [b]	–	–	–

[*] Mycelial growth was recorded after 72 h of incubation at $28 \pm 2°C$. [a] Values are mean of three replications. In a column, means followed by a common letter are not significantly different at the 5% levels by DMRT. [b]PA23 = *P. chlororaphis*; CBE4 = *B. subtilis*; M3 = *B. subtilis*; KPf1 = *P. fluorescens* and KPf2 = *P. fluorescens*.

Detection of phenazine and 2, 4 DAPG using TLC

The presence of phenazine was detected through TLC. The plates were developed in isopropanol : ammonia: water (8 : 1 : 1). Distinct spots of light yellow colour appeared after DSA spray on the developed TLC plate with an R_f value of 0.57 for *P. chlororaphis* isolate PA23. Rosales *et al.* (1995) reported that *P. fluorescens* 2–79 produced phenazine with an R_f value of 0.50.

Detection of phenazine by HPLC

HPLC analysis of Phenazine from *P. chlororaphis* isolate revealed the presence of typical peak maxima at 248 and 367 nm. The results revealed presence of the typical peak maxima at 248 and 367 nm. Presence of three peaks with a retention time of 18.3 min, 20.3 min and 22.8 min were obtained (Figure 1) which showed the presence of three phenazine derivatives viz., 2-OH-PHZ, PCA and 2-OH-PCA. *P. aeruginosa* produced besides pyocyanine, other phenazines such as dihydoxy-phenazine-1-carboxylic acid, PCA, chloraphine, oxychloror-aphine and aeruginosin (Chang and Blackwood, 1969). Delaney *et al.* (2001) focused the ability of *P. aureofaciens* (*P. chlororaphis*) 30–84 to produce 2-hydroxyphenazine-1-carboxylic acid (2-OH-PCA) and 2-hydroxyphenazine from the common phenazine metabolite Phenazine-1-carboxlic acid (PCA). Fernando and Pierson (1999) reported that P. *aureofaciens* 30–84 produced three phenazine antibiotics, which are responsible for the ability of the strain to compete with the indigenous microflora in the rhizosphere. *G. graminis*, was inhibited > 50%, regardless of the level of phenazines produced.

Effect of 2, 4-DAPG and phenazine on the hyphal morphology and spore germination of plant pathogens

The antibiotic phenazine had a significant effect on spore germination and hyphal morphology. All the fungal pathogens tested were highly sensitive to phenazine at a concentration of 100 μl (87 μg/100 μl). Phenazine adversely affected the hyphal morphology of *P. aphanidermatum* after 24 h of incubation at room temperature 28 $\pm$ 2°C. Different types

a) *P. chlororaphis* PA23: 3 phenazines (RT=18.3, 20.3 and 22.8 min)   b) Phenazine standard (RT= 22.8 min.)
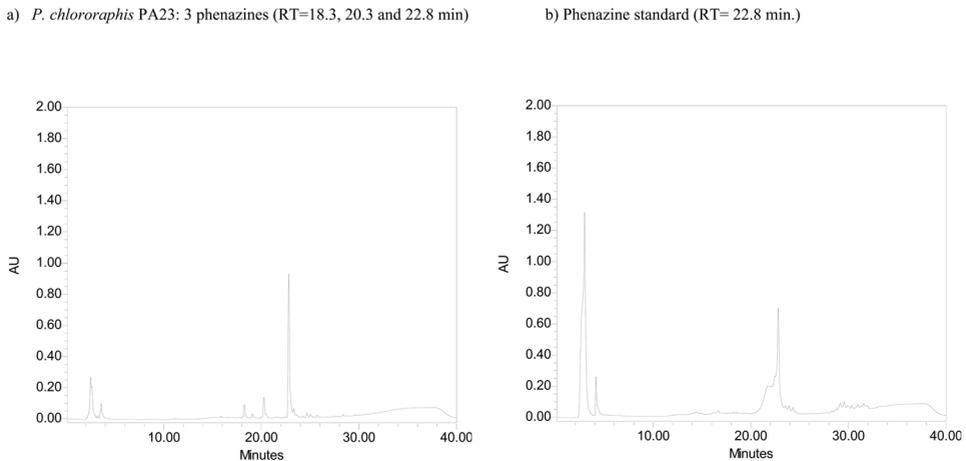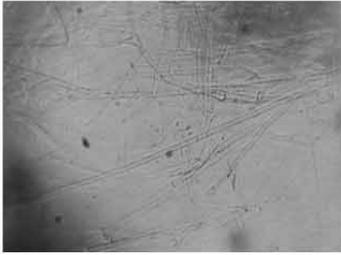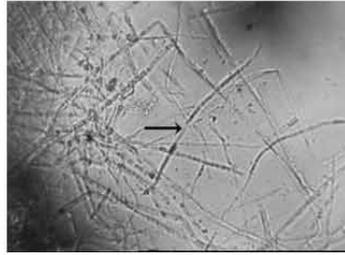

Figure 1. HPLC based detection of phenazine produced by *P. chlororaphis* PA23.

(a)

Pythium aphanidermatum

Control

Treated

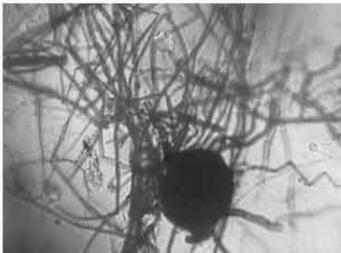*Rhizoctonia solani*

Control

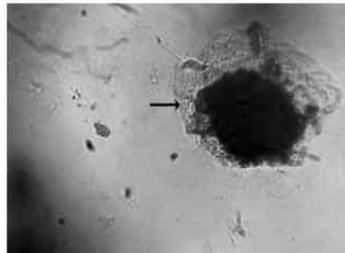Treated

Sclerotium rolfsii
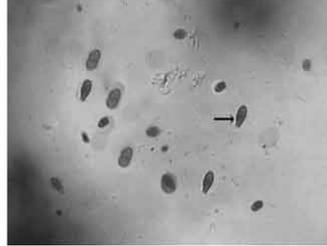

Control

Treated

Macrophomina phaseolina

Control

Treated

(b)

Alternaria solani

Control   Treated
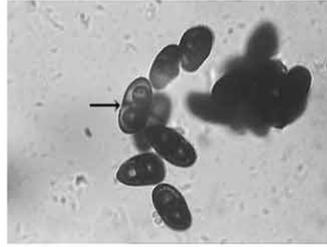
Botryodiplodia theobromae

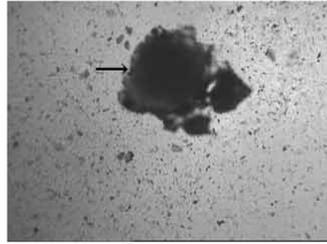Control   Treated

(c)

Meloidogyne incognita eggs
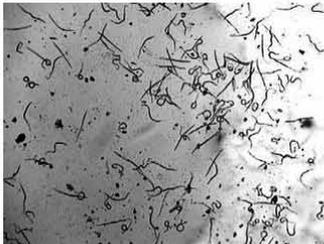

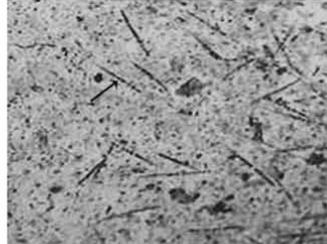Control   Treated

Meloidogyne incognita juveniles

Control   Treated

Figure 2. (a) Effect of Phenazine on hyphal abnormalities of soil borne plant pathogens. (b) Effect of Phenazine on spore germination of fungal pathogens. (c) Effect of Phenazine on *Meloidogyne incognita*.

Table II. Effect of phenazine of *P. chlororaphis* isolate PA23 on the hyphal morphology and spore germination of phytopathogens and nematode.

Phyotopathogens/nematode	Sclerotia/spore/hyphal abnormalities (phenazine)
P. aphanidermatum	Hyphal vacuolation
	Leakage of protoplasmic contents
	Hyphal lysis
B. theobromae	Inhibits spore germination
A. solani	Inhibits spore germination
M. phaseolina	Inhibits microsclerotia germination
R. solani	Inhibits sclerotial germination
	Hyphal swelling
S. rolfsii	Inhibits sclerotial germination and caused hyphal lysis
Meloidogyne incognita	Inhibits hatching of eggs
	Death of juveniles

of disorganization of the hyphae of *P. aphanidermatum* include vacuolization, cell content degeneration and hyphal lysis. But abnormal swelling of hyphal tips was noticed in *R. solani*, exposed to phenazine. However growth aberrations were not observed in the hyphal morphology of *P. aphanidermatum* and *R. solani* in untreated control (Table II, Figure 2a) Studies on the effect of 2,4-DAPG and phenazine on spore germination of *B. theobromae* and *A. solani* revealed that there was 100% inhibition of spore germination of both *B. theobromae* and *A. solani* under *in vitro*. However spore germination was not inhibited in untreated control (Figure 2b). Similarly germination of sclerotia of *M. phaseolina, R. solani* and *S. rolfsii* were also completely arrested by phenazine. Different stages of disorganization of hyphal tips with degenerated cytoplasm was observed when *P. ultimum* var *sporangiiferum* was exposed to 2,4 DAPG (de Souza *et al.*, 2003). The eggs of the root knot nematode *Meloidogyne incognita,* when exposed to phenazine completely inhibited the hatching of juveniles from the eggs and when the juveniles were exposed to phenazine it caused complete mortality of juveniles (Figure 2c). Fakhouri *et al.* 2001 found that the antifungal substance produced by fluorescent pseudomonas (pyuluteorin and phenazine derivatives) caused a collapse of the hyphae of *F. oxysporum* fsp *lycopersici*.

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