The Bioscan 12(1): 71-74, 2017
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.com

ANTIBIOTICS MEDIATED SUPPRESSION OF *PSEUDOMONAS FLUORESCENS* AGAINST *MELOIDOGYNE INCOGNITA*

M. KALAIVANI*, A. SHANTHI, N. SWARNA KUMARI AND K. KALAIMANI
Department of Nematology,
RVS Agricultural College, Thanjavur - 613 402, INDIA
e-mail: florikalai@gmail.com

KEYWORDS
Antibiotics
Pseudomonas
fluorescens
Meloidogyne incognita

Received on :
21.11.2016

Accepted on :
27.01.2017

***Corresponding author**

ABSTRACT
Isolated the antibiotics produced by *Pseudomonas fluorescens viz*., 2, 4-diacetyl phloroglucinol (DAPG) and Phenazine 1- carboxylic acid (PCA) and it was confirmed using Thin Layer Chromotography (TLC). The crude antibiotic of 2, 4 - DAPG and PCA of *P. fluorescens* at different concentrations *viz*., 1, 25, 50, 75 and 100 μl/ ml of distilled water were tested against hatching and mortality of *M. incognita* at 24,48 and 72 h intervals. Among the different concentrations of DAPG and PCA, 100 μl/ ml of distilled water was found to be very effective in the suppression of egg hatching and enhancing juvenile mortality of *M. incognita* . From this study, it is concluded that the ovicidal and larvicidal effect of antibiotics were directly proportional to their concentrations and time of exposure.

INTRODUCTION

Plant parasitic nematodes are destructive parasites of crop plants in agricultural and horticultural crops and they cause yield losses of approximately $ 100 billion world wide each year and 70% of the damage is due to root knot nematodes, *Meloidogyne* spp. (Khan *et al*., 2012). *Meloidogyne* spp. are generally polyphagous and can attack different types of plant species from grasses to trees causing root galls (Bashour *et al*., 2013). These nematodes have world wide distribution but they are more abundant in warm and tropical regions where species including *Meloidogyne incognita* and *Meloidogyne javanica* are mainly responsible for most crop damage (Khan *et al*., 2012).

In order to overcome these problems, interest in biological control has increased recently due to public concern about the use of chemicals in the environment in general. Studies on a number of plant-microbe interactions have shown that some antagonistic rhizobacteria protect plants from soil - borne pathogens either directly, by competition and antibiosis or indirectly, by induced systemic resistance. Rhizobacteria - mediated systemic resistance to control plant - parasitic nematodes is a fairly new research area, and studies on exploiting this technique have started only recently (Siddiqui and Shaukat,2003a). The antibiotic 2, 4 - diacetyl phloroglucinol (DAPG) has received considerable attention and has been implicated in biological control of many plant pathogens by fluorescent *Pseudomonas* strains. The activity of antibiotics against bacteria, fungi and nematodes has been well documented (Vlami *et al*., 2005).

With this background, the present study has been taken up with the objective to study an antibiotic mediated suppression of *Pseudomonas fluorescens* against root knot nematode, *M.incognita*.

MATERIALS AND METHODS

Detection of antibiotics (2,4 - diacetyl phloroglucinol and Phenazine - 1 carboxylic acid) by Thin layer chromatography (TLC)

Extraction of 2, 4 - diacetyl phloro glucinol (DAPG)

Pseudomonas fluorescens strain-1 isolates were grown separately in 20ml of pigment production broth (peptone, 20g; glycerol, 20ml; NaCl, 5g; KNO_3 1g; distilled water 1 litre; pH 7.2) for four days on a rotary shaker at 30ºc. The fermentation broth was centrifuged at 3500 rpm for 5 minutes and supernatant was collected. It was acidified to pH 2.0 with 1N HCL and then extracted with equal volume of ethyl acetate. The ethyl acetate extract was reduced to dryness *in vacuo*. The residues were dissolved in methanol and kept at 4ºC until utilize for TLC (Rossales *et al*., 1995).

Detection of 2, 4-diacetyl phloroglucinol on Thin Layer Chromotography (TLC)

For the detection of an antibiotic, 2,4 – diacetyl phloroglucinol (DAPG) a volume of 5μl sample was spotted on to the aluminum coated sheet with silica gel (Merck, Silica Gel 60 F_{254}, Germany) separation was performed with acetonitrile/ methanol/ water(1:1:1) as solvent system and visualized by short wavelength (245nm) and sprayed with diazotized sulphanilic acid. R_f value for the spot confirming 2,4 DAPG was calculated and compared with migration of synthetic 2,4

DAPG and for identical colour (Rossales *et al*., 1995).

Preparation of Diazotized sulphanilic acid

Diazotized sulphanilic acid 50 mg was dissolved in 20 ml 20% Na_2CO_3 solution. Diazotization was done by dissolving 25 g sulphanilicacid in 125 ml 10% sodium nitrite solution. The mixture was added drop by drop to 60ml of 8M HCL in ice cold condition. After 10 minutes it was filtered under ice cold condition. It was washed extensively with the cold water and then ethanol followed by ether. The crystals were air dried and stored at 4ºC.

Extraction and Isolation of Phenazine - 1 Carboxylic acid

The *Pseudomonas fluorescens* strain-1 isolates were grown separately in 20ml of pigment production broth (peptone, 20g; glycerol, 20ml; NaCl, 5g; KNO_3 1g; distilled water 1 litre; pH 7.2) for four days on a rotary shaker at 30 º C. The extraction was done by acidifying the culture with an equal volume of benzene (Phenazine in benzene layer) and then extraction of benezene phase with 5% $NaHCO_3$. Phenzine - 1 carboxylic acid was recovered from the bicarbonate layer. The bicarbonate fraction was extracted once with benzene to recover the phenazine. The pigment was air dried, dissolved in methanol and purified by TLC on silica gel with 250 μm layer thickness. The solvent system containing isopropanol/ ammonia/ water (8:1:1). Plates were viewed under UV light at 254 nm. R_f value for the spots was calculated (Rossales *et al*., 1995).

In vitro bioassay of bacterial antagonists, P. fluorescens against root knot nematode,

M. incognita

Crude antibiotics of *Pseudomonas fluorescens* strain-1 with 2,4- diacetyl phloroglucinol and Phenazine - 1 carboxylic acid were selected for assessing its efficacy against *M. incognita* by conducting hatching and mortality tests.

Effect on Hatching test

For hatching test, egg mass of *M. incognita* were obtained from the pure culure maintained in the glasshouse on tomato. One egg mass was placed in cavity block which contained 2ml of crude antibiotics of the effective *P. fluorescens* strain-1 at different concentrations of 100, 75, 50, 25, and 1 μl/ ml of distilled water and kept at room temperature (28 $\pm$ 1ºC). Cavity block maintained with distilled water served as a control. Each treatment was replicated four times. The number of juveniles hatched out was counted at 24, 48 and 72 hrs intervals (Sankari Meena *et al*., 2013).

Mortality test

About 2ml of crude antibiotic of *P. fluorescens* strain-1 was taken at different concentrations *viz*.,100, 75, 50, 25 and 1μl/ ml of distilled water and along with the distilled water served as control. Freshly hatched 100 second stage juveniles (J_2) of *M. incognita* were suspended in each cavity block and incubated at room temperature (28 $\pm$ 1ºc). Four replications were maintained for each treatment. Observation on the mortality of juveniles was recorded at 24, 48 and 72 hrs intervals using a stereozoom microscope (Sankari Meena *et al.,* 2013).

RESULTS AND DISCUSSION

Production of different antibiotics *viz*., 2, 4-diacetyl phloroglucinol (DAPG) and Phenazine 1- carboxylic acid (PCA) by *P. fluorescens* was confirmed using TLC. The presence of 2, 4 - DAPG was detected by spraying dinitrosalicylic acid (DNS) on the TLC plate which showed the R*f* value of 0.88. Phenazine was detected with the R*f* value of 0.60 (Plate a and b). It is in accordance with the findings of Rosales *et al*. (1995) who reported that the antibiotics *viz*., phenazine, pyocyanine and 2, 4 - diacetyl phloroglucinol were produced by the different isolates of *Pseudomonas* spp. Similar results was obtained by Kavitha *et al*. (2005) who identified an antifungal antibiotic phenazine from

Table 1: Effect of 2, 4 - diacetyl phloroglucinol on hatching of *M. incognita* eggs

Treatments	Number of hatched juveniles at different period of exposure					
	24 h	Per cent inhibition	48 h	Per cent inhibition	72 h	Per cent inhibition
T_1- Concentration of DAPG @ 1 ì l/ml of distilled water	25.52(5.05)	51.0	35.00(5.92)	50.0	45.89 (6.77)	49.0
T_2- Concentration of DAPG @ 25 ì l/ml of distilled water	23.38(4.84)	55.0	34.00(5.83)	52.0	44.65(6.68)	51.0
T_3- Concentration of DAPG @ 50 ì l/ml of distilled water	21.43(4.63)	59.0	32.00 (5.66)	55.0	43.56(6.60)	52.0
T_4- Concentration of DAPG @ 75 ì l/ml of distilled water	20.09(4.48)	61.0	29.64(5.44)	58.0	39.82(6.31)	56.0
T_5- Concentration of DAPG @ 100 ì l/ml of distilled water	17.50(4.18)	66.0	24.60(4.96)	65.0	32.33(5.69)	64.0
T_6- Untreated control (Distilled water)	51.93(7.21)	-	70.42(8.39)	-	90.65(9.52)	-
SEd	0.09		0.08		0.04	
CD (p = 0.05)	0.19	-	0.17	-	0.09	-

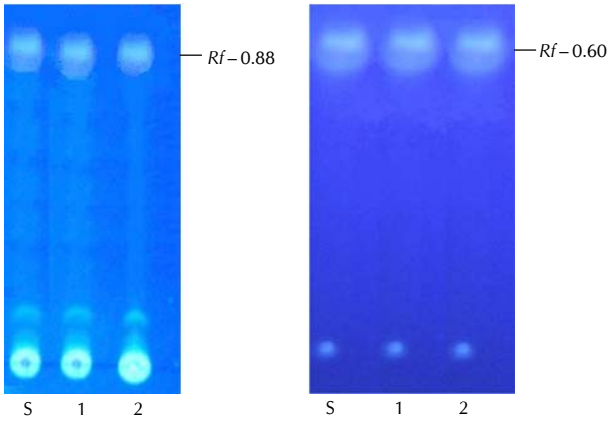
Table 2: Effect of 2, 4 - diacetyl phloroglucinol on mortality of juveniles of *M. incognita*

Treatments	Per cent mortality of juveniles at different period of exposure					
	24 h	Per cent mortality	48 h	Per cent mortality	72 h	Per cent mortality
T_1- Concentration of DAPG @ 1 ì l/ml of distilled water	15.74(23.37)	49.0	25.13(30.09)	50.0	34.72(36.10)	54.0
T_2- Concentration of DAPG @ 25 ì l/ml of distilled water	18.00(25.10)	55.0	29.11(32.65)	57.0	38.52(38.36)	58.0
T_3- Concentration of DAPG @ 50 ì l/ml of distilled water	19.50 (26.21)	59.0	31.08(33.88)	60.0	40.78(39.69)	61.0
T_4- Concentration of DAPG @ 75 ì l/ml of distilled water	20.26 (26.75)	61.0	33.04(35.09)	62.0	43.70 (41.38)	63.0
T_5- Concentration of DAPG @ 100 ì l/ml of distilled water	22.30(28.18)	64.0	35.30(36.45)	65.0	46.92(43.23)	66.0
T_6- Untreated control (Distilled water)	8.00(16.43)	-	12.50(20.70)	-	16.10 (23.66)	-
SEd	0.80	-	0.49	-	0.21	-
CD (p = 0.05)	1.7	-	1.03	-	0.43	-

Pseudomonas chlororaphis isolate PA 23 using TLC. In support of the present study, Sankari Meena *et al*. (2013) reported that the four antagonistic isolates of *P. fluorescens viz*., Pf 128, Pf 223, Pfbv 22 and Pf1 produced phenazine.

Effect of antibiotics on hatching of *M.incognita*

Observations recorded in the hatching studies indicated that among the different concentrations of DAPG and PCA tested, 100 µl showed the least number of hatched out juveniles of 32.33 and 34.85 after 72 h exposure respectively over control (Table 1 and Fig. 1). It was similar with the findings of Meyer *et al*. (2009), who reported that the 2,4 - DAPG produced by *P. fluorescens* showed constant decrease in egg hatch of *M. incognita* as concentrations of 1, 10, 25, 50, 75 and 100 µg/ml DAPG increased. There was a significant increase in rate of mortality of juveniles of *M. incognita* on exposure to different periods and the per cent mortality was found positively correlated with increase in the concentration and period of exposure. The study conducted by Siddiqui and Shaukat (2003b) also proved the effectiveness of 2, 4- DAPG which caused substantial mortality of *M. javanica* juveniles and reduced the egg hatch. Cronin *et al*. (1997) proved that the antibiotic DAPG was responsible for the increase in egg hatch and the reduction in juveniles mobility of potato cyst nematode, *Globodera rostochiensis*. The antibiotic 2,4 - DAPG produced by some strains of *Pseudomonas* spp. were found to suppress the plant parasitic nematodes *viz*., *Heterodera glycines*, *Paratrichodorus minor* and *M. incognita* has been observed by Timper *et al*. (2009). In support of the present study, Hultberg *et al*. (2000) reported that *P. fluorescens* produced the maximum amount of 10µg/ml 2, 4 – DAPG and protected tomato seedling from damping off incidence caused by *P. ultimum*.



S - Standard, 1 and 2 - Pf1

Plate a & b: Detection of antibiotics of *P. fluorescens* by Thin Layer Chromatography (TLC)

Effect of antibiotics on mortality of *M.incognita*

In the mortality studies, when the juveniles were exposed to DAPG and PCA at 100 µl concentration, it caused 66.0 and 65.0 per cent mortality of juveniles after the exposure of 72 h respectively. Untreated control recorded the minimum juvenile mortality (Table 2 and Fig. 2). Kavitha *et al*. (2005) who observed that the eggs of the root knot nematode, *M. incognita* incubated in 30 *i* l concentration of phenazine completely suppressed the hatching of eggs and caused mortality of juveniles. The results of the present study is in confirmity with the findings of Sankari Meena *et al*. (2013) who revealed the effectiveness of phenazine on egg hatching and juvenile mortality of *M. incognita*. Similarly, Haas and Keel (2003) found that the roots associated with *P. fluorescens* produced and excreted secondary metabolites *viz*., phenazine, pyoluteorin, pyrrolnitrin, 2, 4 - DAPG and hydrogen cyanide which are inhibitory to plant pathogenic organisms including fungi, bacteria and nematodes. TimmsWilson *et al*. (2000) noticed that insertion of phenazine -1 carboxylic acid (PCA) gene which encodes for the synthesis of phenazine -1 carboxylic acid in wild type of *P. fluorescens* significantly improved its ability to reduce damping-off disease of pea seedlings caused by *P. ultimum* and level of phenazine -1 carboxylic acid biosynthesis was correlated with biocontrol efficacy and the persistence of bacterial colonies in soil ecosystem. Similarly, Thomashow and Weller (1988) reported that the antibiotic phenazine-1-carboxylate produced by *P. fluorescens* Strain 2-79 was active against take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici* and other fungal root pathogens.

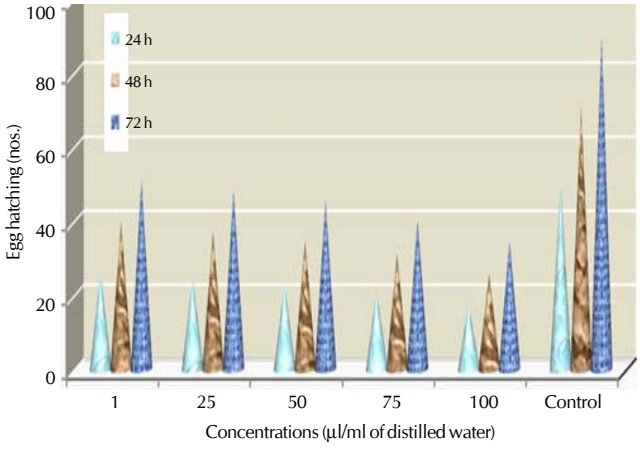


Figure 1: Effect of phenazine 1-carboxylic acid (PCA) on hatching of *M. incognita* eggs

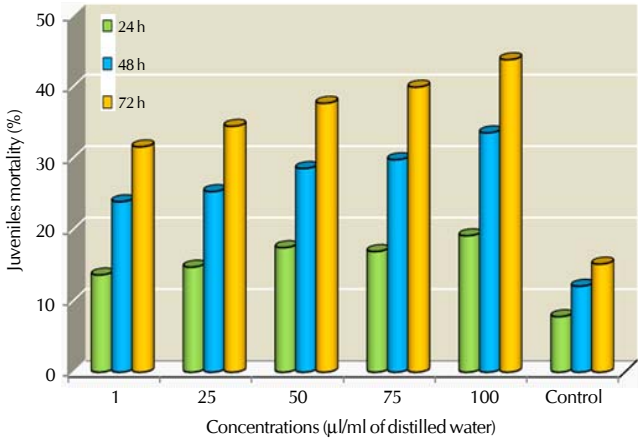


Figure 2: Effect of phenazine 1-carboxylic acid (PCA) on juveniles of *M. incognita*

REFERENCES

Bashour, I., Alameddine, A., Wehbe, L., Saad, A. & Nimah, M. 2013. The use of aqua ammonia for the control of soil borne diseases in tomato. *Lebanese Science J.* **14(1):** 41 - 47**.**

Cronin, D., Moenne-Loccoz, Y., Fenton, A., Dunne, C., Dowling, D.N. and O'gara, F. 1997. Role of 2,4-diacetylphloroglucinol in the interactions of the biocontrol Pseudomonad strain F113 with the potato cyst nematode, *Globodera rostochiensis*. Applied and Environmental Microbiology. **63(41):** 1357 - 1361.

Haas, D. and C. Keel. 2003. Regulation of antibiotic production in root colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. Annual Review of Phytopathology. **41:** 117-153.

Hultberg Malin., Beatrix Alsanius and Peter Sundin. 2000. *In vivo* and *in vitro* interactions between *Pseudomonas fluorescens* and *Pythium ultimum* in the suppression of damping - off in tomato seedlings. *Biological Control***. 19:** 1 - 8.

Kavitha, K., Mathiyazhagan, S., Sendhilvel, V., Nakkeeran, S., Chandrasekar, G. and Dilantha Fernando, W.G. 2005. Broad spectrum action of phenazine against active and dormant structures of fungal pathogens and root knot nematode. *Archieves of Phytopathology and Plant Protection*. **38(1):** 69 - 76.

Khan, A., Shaukat, S. S., Islam, S. and Adnan Khan. 2012. Evaluation of fluorescent Pseudomonad isolate for their activity against some plant – parasitic nematodes. American - *Eurasian J. Agricultural and Environmental Science*. **12(11):** 1496 - 1506**.**

Meyer Susan, L. F., Halbrendt, J. M., Carta, L. K., Skantar, A. M., Tingliu, Abdelnabby, H. M. E. and Vinyard, B. T. 2009. Toxicity of 2,4 - diacetyl phloroglucinol (DAPG) to plant - parasitic and Bacterial - feeding nematodes. *J. Nematology*. **41(4):** 274-280**.**

Rosales, A. M., Thomashow, L., Cook R. J. and Mew T. W. 1995. Isolation and identification of antifungal metabolites produced by rice - associated antagonistic *Pseudomonas* spp. Phytopathology**. 85:** 1028- 1032.

Sankari Meena, K., Jonathan, E. I. and ArdhaNareeswaran, N. 2013. Isolation of phenazine and its activity against root knot nematode, *Meloidogyne incognita*. *Indian J. Nematology*. **43:** 180-183.

Siddiqui, I. A and Shaukat,S. S. 2003a. Role of salicylic acid in *Pseudomonas aeruginosa* strain IE-6S + -mediated induction of systemic resistance against *Meloidogyne javanica* in tomato. Phytopathologia Mediterranea. **42:** 268 - 274.

Siddiqui, I. A. and Shaukat,S. S. 2003b. Suppression of root knot diseases by *Pseudomonas fluorescens* CHAO in tomato. Importance of bacterial secondary metabolite, 2,4-diacetyl phloroglucinol. *Soil biology and Biochemistry*. **35(12):** 1615-1623.

Thomashow, L. S. and Weller,D. M. 1988. Role of phenazine antibiotic from *Pseudomonas fluorescens* in biocontrol of *Gaeumannomyces graminis* var *tritici*. *J. Bacteriology*. **170:** 3499 - 3508.

Timms - Wilson, T. M., Ellis, R. J., Renwick,A., Rhodes,D. J., Mavrodi,D. V., Weller,D. M.,Thomashow,L. S. and Bailey,M.. 2000. Chromosomal insertion of PCA biosynthetic pathway enhances efficacy of damping off disease control by *Pseudomonas fluorescens*. Molecular Plant- Microbe Interactions. **13:** 1293- 1300**.**

Timper, P., Kone, D., Yin, J., Ji, P. and McSpadden Gardener, B. B. 2009. Evaluation of an antibiotic - producing strain of *Pseudomonas fluorescens* for suppression of plant - parasitic nematodes. *The J. Nematology***. 41:** 234–240.

Vlami, M. B., Mieke, E. Prins and Jos, M. Raajmakers. 2005. Influence of plant species on population dynamics, genotypic diversity and antibiotic production in the rhizosphere by indigenous *Pseudomonas spp*. Federation of European Microbiological Societies(FEMS) Microbiology Ecology. **52(1):** 59-69.