Journal of Biological Control, 32(3): 193-202, 2018, DOI: 10.18311/jbc/2018/21359

Research Article

Assessment of nematicidal properties of fluorescent pseudomonads using peanut root-knot nematode, *Meloidogyne arenaria*

PRASANNA HOLAJJER[*], R. DEY, K. K. PAL, K. CHAKRABORTY, G. HARISH, M. V. NATARAJA and E. DEEPAK

ICAR-Directorate of Groundnut Research, Junagadh - 362001, Gujarat, India

[*]*Corresponding author E-mail:* prasannaiari@gmail.com

ABSTRACT: The primary objective of this study was to identify natural isolates of fluorescent pseudomonads with superior antagonistic activity towards plant parasitic nematodes. Nematicidal potential of eighteen isolates of fluorescent pseudomonads were compared against peanut root-knot nematode, *Meloidogyne arenaria*. Cell-free culture filtrate of DAPG-producing isolates of *Pseudomonas putida* caused significantly higher mortality of *M. arenaria* (J2) with highest in isolate DAPG3 (87.36%), followed by DAPG1 (84.16%) compared to other isolates of fluorescent pseudomonads, i.e., *P. gessardii* BHU1 and *P. aeruginosa* BM6 after exposure period of 72h at 100% concentration. The selected DAPG-producing isolates of *P. putida* caused significant inhibition in egg hatching. The lowest cumulative per cent hatch of *M. arenaria* was observed in the isolate DAPG3 (17.84%) followed by DAPG1 (18.10%). The isolates DAPG1 and DAPG3 also inhibited the nematode invasion in the roots of peanut by 41.30% and 36.34%, respectively. Significant reduction in number of galls/plant in peanut roots was recorded. The maximum reduction (51.30%) in root galling was recorded with combination of seed treatment and soil application of *P. putida* DAPG1 followed by 41.73% in combined treatment of seed treatment and soil application of *P. putida* DAPG3. The levels of Peroxidase (POD), Catalase (CAT) and Polyphenol Oxidase (PPO) were non-significant in the leaves of peanut in the treatment that received *P. putida* DAPG1 and DAPG3, either as seed treatment and/or soil application, compared to inoculated and un-inoculated control. However, significantly enhanced phenol content was recorded in the leaves of peanut in the treatment that received combination of seed treatment and soil application of *P. putida* DAPG1 and seed treatment alone.

KEY WORDS: Fluorescent pseudomonads, *Meloidogyne arenaria*, nematicidal activity, peanut

(Article chronicle: Received: 04-03-2018; Revised: 10-07-2018; Accepted: 06-09-2018)

INTRODUCTION

Microbial metabolites that can cause immobility or mortality of the infective stage of the nematode, or reduce emergence from the eggs has been studied intensively over the past three decades for the biological control of plant parasitic nematodes. Plant growth-promoting rhizobacteria are considered as ideal candidates for nematode management, because of their significant role in suppression of nematodes via production of secondary metabolites that are inhibitory to nematodes and induce systemic resistance against root-knot nematodes (Siddiqui and Shaukat, 2002; 2003). However, most of the biocontrol agents including fluorescent pseudomonads are variable in their performance due to variable expression of genes involved in disease suppression (Raaijmakers and Weller, 2001) and variable disease suppressive mechanisms. Production of secondary metabolites such as 2,4-diacetylphloroglucinol (DAPG) and HCN is most often associated with nematode suppression by fluorescent pseudomonads in the rhizosphere of potato and tomato (Cronin *et al*., 1997; Siddiqui and Shaukat, 2003). In the rhizoplane, however, production and accumulation of 2,4-DAPG depends on the outcome of interaction of cultivar–bacterial isolate (Okubara and Bonsall, 2008) and its adaptation to the new environmental conditions. Therefore, in present study, our aim was to identify natural isolates of fluorescent pseudomonads with superior antagonistic activity towards plant parasitic nematodes. The isolates of nematodes used for bioassay were obtained from broad range of geographical regions and crops managed at very different levels of intensity for ensuring that the collection represents a broad biodiversity common in the targeted niche. In present study, nematicidal potential of eighteen isolates of fluorescent pseudomonads were compared against peanut root-knot nematode, *Meloidogyne arenaria* in terms of juvenile mortality. Further, few selected and potential isolates were further evaluated for their ability to suppress hatching of eggs of nematode. The capacity of fluorescent pseudomonads to suppress root-knot disease and induction of systemic resistance was also evaluated.

MATERIALS AND METHODS

Fluorescent pseudomonads isolates

Multiple plant growth promoting and DAPG-producing fluorescent pseudomonads isolates (Table 1) viz., *Pseudomonas putida* DAPG1, *P. putida* DAPG2,

P. putida DAPG3, *P. putida* DAPG4, *P. putida* DAPG5, *P. putida* DAPG6, *P. putida* DAPG7, *Pseudomonas aeruginosa* FP82, *Pseudomonas fluorescens* FP93, *P. putida* S1(6), *Pseudomonas* sp. FP20, *Pseudomonas* sp. FP46, *P. putida* FP86, *P. fluorescens* FP94, *Pseudomonas monteilii* FP133, *Pseudomonas gessardii* BHU1, *P. aeruginosa* ACC7 and *P. aeruginosa* BM6 isolated from peanut rhizosphere and maintained in Microbiology Section, ICAR- Directorate of Groundnut Research, Junagadh were used in the present experiment. The DAPG-producing fluorescent pseudomonads were identified by PCR amplification of *phlD* gene. The fluorescent pseudomonads which showed antifungal activity against major fungal pathogens of peanut were screened for the presence of *phlD* activity in a PCR based assay. The method of Ahmadzadeh *et al.,* (2006) was followed for the detection of *phlD/phlA* gene(s). Bacterial DNA was isolated using Axygen Bacterial DNA isolation kit (Axy PrepTM Bacterial Genomic DNA Mini prep Kit, Axygen, USA) following manufacturer's protocol. The DNA, thus isolated, was used as template for the detection of *phlD*/ *phlA* genes in the fluorescent pseudomonads. PCR amplification of *phlD* gene was performed using forward primer phl2a (5'-GAG GAC GTC GAA GAC CAC CA-3') and reverse primer phl2b (5'-ACC GCA GCA TCG TGT ATG AG-3'), which were developed from the *phlD* sequence of *Pseudomonas fluorescens* Q2-87 (Raaijmakers *et al*., 1997). PCR amplification was carried out in 20 µl reaction mixtures. PCR cycling program was used as described by Wang *et al*., (2001), with minor modification. The cycling program included an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 60s, 53.2°C for 60s, 72°C for 60s, and then a final extension at 72°C for 5 min. Amplification was performed using a Takara PCR thermal cycler (Takara TP600 gradient, Takara, Japan). The primers phlA-1f (5'-TCA GAT CGA AGC CCT GTA CC-3') and phlA-1r (5'- GAT GCT GTT CTT GTC CGA GC-3') were used to amplify a 418 bp fragment of *phlA* as described by Rezzonico *et al.,* (2003) with slight modification. The cycling program included an initial denaturation at 94°C for 5 min followed by 25 cycles of 94°C for 30s, 62°C for 30s, 72°C for 45s, and final extension for 5 min at 72°C. The PCR amplified products were resolved in 1.2% (w/v) agarose gel using 100 bp DNA step ladder as molecular size marker. *PhlD* amplified product of *Pseudomonas aeruginosa* AMAAS 57 (NCBI accession no. JN391537) was used as positive control.

These isolates were grown axenically in 250 ml of King's B broth medium in 500ml conical flasks for 48 h, 96 h, and 144 h at 28 ± 2ºC in an orbital shaker at 120 rpm. The medium was sterilized at 121ºC for 20 min followed by inoculation with 1 ml of 2.0×10^8 cfu ml^{-1} of pure culture and incubated.

Eighteen isolates of fluorescent pseudomonads were screened for nematicidal activity in terms of mortality of peanut root-knot nematode, *Meloidogyne arenaria* (J2). Four potential isolates (selected on the basis of maximum mortality of nematode juveniles) were evaluated to understand the effect on hatchability of the nematodes. The effect of DAPG-producing fluorescent pseudomonads on nematode invasion, reproduction factor, and induction of systemic resistance were further studied with two potential fluorescent pseudomonads, *P. putida* DAPG1 and *P. putida* DAPG3.

Table 1. Identity of the cultures used in the experiments

Sl. No.	GenBank Accession no.	Identity
1	JX514425	*Pseudomonas putida* DAPG1
2	JX514408	*Pseudomonas putida* DAPG2
3	JX514404	*Pseudomonas putida* DAPG3
4	JX514407	*Pseudomonas putida* DAPG4
5	JX514414	*Pseudomonas putida* DAPG5
6	JX514412	*Pseudomonas putida* DAPG6
7	JX514402	*Pseudomonas putida* DAPG7
8	-	*Pseudomonas aeruginosa* FP82
9	-	*Pseudomonas fluorescens* FP93
10	JX514426	*Pseudomonas putida* S1(6)
11	-	*Pseudomonas* sp. FP20
12	-	*Pseudomonas* sp. FP46
13	-	*Pseudomonas putida* FP86
14	-	*Pseudomonas fluorescens* FP94
15	-	*Pseudomonas monteilii* FP133
16	JX514417	*Pseudomonas aeruginosa* BM6
17	JX514422	*Pseudomonas aeruginosa* ACC7
18	JX514410	*Pseudomonas gessardii* BHU1

Preparation of Cell-Free Extracts

The cultural broth of fluorescent psuedomonads was centrifuged individually at 8000rpm for 10 min. The supernatant was collected and passed through a Millipore filter of 0.22 µm to remove the bacterial cell, if any. The cell-free filtrate, thus obtained, was used for experimental purpose.

In-vitro nematicidal activity

The cell-free filtrate was used to observe the *in vitro* mortality of juveniles of infective stages of peanut root-knot nematode, *Meloidogyne arenaria* in plastic multi-well plates. Freshly hatched second stage juveniles (J2) of *M. arenaria* were obtained from pot cultures maintained in the glasshouse. At least 100 nematodes in 0.5ml sterile distilled water were poured into each well of the multi-well plate containing 1ml cell-free filtrate, distilled water (control) and medium (control). There were four replications for each treatment. After 48 and 72h of exposure, the filtrate of each exposure period was replaced with distilled water, rinsing five times. After the final rinse, nematodes were kept in distilled water for 24h to observe if nematode activity resumed. The numbers of active and inactive nematodes were counted to calculate the per cent mortality.

Evaluation of the effect on hatchability

Based on the result of *in vitro* mortality, effect on hatchability of nematode eggs was carried out with four selected isolates of fluorescent pseudomonads viz. *Pseudomonas putida* DAPG1, *P. putida* DAPG3, *P. putida* DAPG5 and *P. putida* DAPG6. Healthy egg masses of *M. arenaria* were hand-picked with forceps from infected plants. Ten egg masses were placed in each well of a multi-well plate containing 0.5ml of sterile distilled water, to which 1ml of cell-free filtrate of fluorescent pseudomonads (100% concentration) was added. Sterile distilled water and medium were used as controls. Each treatment was replicated four times. The multi-well plates were incubated at room temperature (17-25 C) for 12 days. The hatched J2 were removed and counted after 1, 2, 4, 6, 9 and 12 days, and hatching medium was replaced with a fresh medium after each observation. On day 12, as there was minimal hatch in the treatments including water control, the hatching medium was replaced with sterile distilled water. Further, hatch was recorded on days 14 and 17 in order to observe resumption of hatching after removal of cell-free filtrate and medium. The experiment was terminated after day 17 as no further hatching occurred in the medium or in sterile distilled water. At the termination of the experiment, 2 ml of 2% NaOCl was added to each well to separate the eggs from the gelatinous matrix of *M. arenaria* to count the total number of embryonated but unhatched J2. The cumulative percentage hatch was calculated by dividing the total number of hatched J2 by the total number of hatched and unhatched J2.

Assay on nematode invasion

The experiment was conducted to assess the influence of two selected efficient isolates of DAPG-producing fluorescent pseudomonads viz. *P. putida* DAPG1, and *P. putida* DAPG3 on invasion of *M. arenaria* in roots of peanut, cultivar GG 20. The 200 cm^3 cups were filled with sterilized soil and drenched with 5 ml suspension of each fluorescent pseudomonad at a concentration of 3×10^8 cfu/ml and control received sterile water only. The peanut root-knot nematodes were inoculated @ 2 J2/cc soil. Eight such cups were maintained for each treatment. The plants were harvested 15 days after sowing and root systems were washed to remove the adhering soil particles and stained with acid fuchsin (Byrd *et al*., 1983). The root systems were evaluated for numbers of juveniles (J2) in roots under microscope.

Evaluation of the effects of two selected fluorescent pseudomonads on nematode reproduction and root galling

Effect of two selected isolates of fluorescent pseudomonads on nematode reproduction and galling in groundnut was studied. The treatment included T1 - Seed treatment of *P. putida* isolate DAPG1; T2 - Soil application of *P. putida* isolate DAPG1; T3 - Seed treatment + Soil application of *P. putida* isolate DAPG1; T4 - Seed treatment of *P. putida* isolate DAPG3; T5 – Soil application of *P. putida* isolate DAPG3; T6 - Seed treatment + Soil application of *P. putida* isolate DAPG3; T7-Inoculated control and T8-Uninoculated control. The cups (200 cm^3) were filled with sterilised soil. Seed treatment was done by placing the peanut seeds in fluorescent pseudomonads suspension containing $3x10^8$ cfu/ml for ½ hr. Soil application was done by drenching with 5 ml fluorescent pseudomonads suspension at a concentration of $3x10^8$ cfu/ml. Inoculated control received nematodes only and untreated control received water only. The peanut root-knot nematodes were inoculated @ 2 J2/cc soil. The plants were harvested 56 days after nematode inoculation and root systems were evaluated for number of galls.

Induction of induced systemic resistance by DAPG-producing fluorescent pseudomonads

The experiment was setup with inoculation of nematode @ 2J2/cc soil after 15 days of sowing. The enzymes such as catalase, peroxidase, polyphenol oxidase activity and total phenol content were estimated in the leaves of peanut after 24, 48 and 72h post inoculation of nematodes.

The extract for antioxidant enzymes (POX and CAT) was prepared by freezing 1 g of root tissue in liquid nitrogen followed by grinding in 10 mL of extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA) and filtered through 4 layers of cheese cloth (Chakraborty *et al*., 2015). The filtrate was centrifuged for 20 min at 15000 × g and supernatant was used for enzymes assay.

Catalase (EC 1.11.1.6) activity was assayed by measuring the loss of H_2O_2 (Aebi, 1984) in a reaction mixture (3 mL) consisting of 0.5 mL of 75 mM H_2O_2 and 1.5 mL of 0.1 M phosphate buffer (pH 7) and 50 µL of diluted enzyme extract. The decrease in absorbance at 240 nm was observed for 1 min and enzyme activity was calculated in terms of the amount of H_2O_2 decomposed.

Peroxidase (EC 1.11.1.7) activity was measured spectrophotometrically by recording the increase in absorbance at 470 nm, due to formation of tetra-guaiacol in a reaction mixture containing 50 mM phosphate buffer (pH 6.1), 16 mM guaiacol, 2 mM H_2O_2 and 0.1 mL enzyme extract diluted to a final volume of 3 mL. The enzyme activity was expressed as µmol tetra-guaiacol formed per min per gram fresh weight, was calculated using the extinction coefficient of its oxidation product, tetra-guaiacol $\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$ (Castillo *et al*., 1984).

Polyphenol oxidase (EC 1.14.18.1) activity was measured by grinding three hundred milligram of leaves in 3 ml of 0.1 M sodium phosphate buffer, pH 6.0. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was used for enzyme assay. The reaction mixture contained 2.9 ml of catechol (0.01 M catechol in 10 mM phosphate buffer pH 6.0) and reaction was initiated by the addition of 0.1 ml of enzyme extract. The change in the colour due to the oxidized catechol was read at 490 nm for one minute. The enzyme activity was expressed as change in O.D. per min per g fresh weight (Malik and Singh, 1980).

Total phenol was extracted with 80% ethanol and estimated with Folin and Ciocalteu reagent as described by Malik and Singh (1980).

Statistical analysis

Statistical analysis of data was carried out by a single factor ANOVA (Gomez and Gomez, 1984). The data on percent mortality and hatching were arcsine transformed before analysis. Analyses of variance were carried out using DSAASTAT, version, 1.1 statistical package (Onofri, 2007) available at *http://www.unipg.it/~onofri/DSAASTAT/DSAASTAT.htm*. The Least Significant Difference (LSD) values at P = 0.05 and P = 0.01 were used to determine the significance of treatment mean differences.

RESULTS AND DISCUSSION

Effect on mortality of nematode

The cell-free filtrate of 4-day-old cultures of four isolates of fluorescent pseudomonads showed maximum nematicidal activity compared to 2- and 6-day-old culture, as the toxic effects of fluorescent pseudomonads are influenced by the age of the culture (data not shown). Therefore, *in vitro* study was carried out using the cell-free filtrate of 4-day-old cultures to evaluate eighteen isolates of fluorescent pseudomonads for their nematicidal potential against peanut root-knot nematode, *Meloidogyne arenaria.* The study revealed significant differences in mortality, after an exposure period of 48h and 72h, in all concentrations of cell-free filtrate, compared to medium (control) and water (control). Mortality was directly related to concentration of cell-free filtrate and exposure period i.e., with increasing concentration, there was a corresponding increase in percent mortality. After 48h of exposure, maximum juvenile (J2) mortality (63.71%) was observed in *P. putida* DAPG3 followed by *P. putida* DAPG5 (60.01%) at 50% concentration, which increased to 77.23 and 75.16%, respectively, at 100% concentration. After 72h of exposure at 100% concentration, five isolates (*P. putida* isolates: DAPG1, DAPG3, DAPG5, DAPG6 and DAPG7) caused percent mortality in the range of 81.51 to 87.36%; five isolates (*P. putida* isolates: DAPG2 and DAPG4; *Pseudomonas* sp. FP46, *P. fluorescens* FP94, and *P. monteilii* FP133) caused percent mortality in the range of 50.01 to 67.32%; eight isolates (*P. aeruginosa* isolate FP82, *P. fluorescens* FP93, *Pseudomonas*. sp. FP20 and *P. putida* FP86; *P. aeruginosa* isolates: ACC7 and BM6; *P. putida* S1(6) and *P. gessardii* BHU1 caused mortality of *M. arenaria* (J2) in the range of 34.81 to 47.31%. Thus, highest mortality was observed with *P. putida* DAPG3 (87.36 %) followed by *P. putida* DAPG1 (84.16%), *P. putida* DAPG6 (82.37%) and *P. putida* DAPG5 (81.79%) after exposure period of 72h at 100% concentration (Table 2).

Effect on hatchability of nematode eggs

Effect of cell-free filtrate (100% concentration) of four isolates (*Pseudomonas putida* DAPG1, *P. putida* DAPG3, *P. putida* DAPG5 and *P. putida* DAPG6) of fluorescent pseudomonads which had shown greater nematicidal activity were used to study the effect of DAPG-producing fluorescent pseudomonads on hatchability of eggs. The result revealed significant inhibition in hatching of peanut root-knot nematode, *Meloidogyne arenaria* in cell-free filtrate of all four isolates, compared to medium (Control) and water (Control) (Table 3). The cumulative percentage hatch of *M. arenaria* in cell-free filtrate was slow and gradual during the entire period of incubation. Only 18.10%, 17.84%, 25.58%, and 20.99% of the J2 hatched upto day 12, with *P. putida* DAPG1, *P. putida* DAPG3, *P. putida* DAPG5 and *P. putida* DAPG6, respectively, compared to 67.11% in medium (control) and 87.71% in water (control). Transferring the egg masses to water after 12 days resulted in 20.04%, 18.19%, 27.52%, and 31.77 % cumulative hatching with *P. putida* DAPG1, *P. putida* DAPG3, *P. putida* DAPG5, and *P. putida* DAPG6 respectively, compared to 70.12 % in medium (Control) and 89.46% in water (Control) (Table 3).

Effect on nematode invasion

The *Pseudomonas putida* DAPG1 and *P. putida* DAPG3, which have greater nematicidal activity (mortality of juveniles and inhibition of egg hatching), were used for studying the invasion of roots of peanut by nematodes. The study revealed that *P. putida* DAPG1 and *P. putida* DAPG3 inhibited the invasion of peanut root-knot nematode, *M. arenaria* in the roots of peanut by 41.30% and 36.34%, respectively (Figure 1).

Effect on reproduction and root galling of nematode

Significant reduction in number of galls/plant in peanut roots was recorded in the treatment with either *Pseudomonas putida* DAPG1 and/or *P. putida* DAPG3 compared to inoculated control. The maximum reduction (51.30%) in root galling was recorded in the combination of seed treatment and soil application of *P. putida* DAPG1 followed by 41.73% in seed treatment and soil application of *P. putida* DAPG3. The least reduction (33.91%) was recorded in seed treatment of *P. putida* DAPG3 (Table 4). The reproductive factor was significantly less (1.04) in combined treatment of seed treatment and soil application of *P. putida* DAPG1 compared to 2.07 in inoculated control (Table 4).

Induction in production of defense related enzymes

The *Pseudomonas putida* DAPG1 and *P. putida* DAPG3 were evaluated further for their ability to induce systemic resistance in peanut against root-knot nematode, *M. arenaria*. The levels of peroxidase (POD), catalase (CAT) and Polyphenol Oxidase (PPO) were non-significant in the treatment with *P. putida* DAPG1 and/or DAPG3, either as seed treatment and/or soil application, compared to inoculated control. However, significantly increased phenol content was recorded in the leaves of peanut after 24, 48 and 72h post-inoculation of nematodes. The phenol content after 72h of post-inoculation of nematodes in the leaves of peanut was significantly higher in combination of seed treatment and soil application of *P. putida* DAPG1 (5.71 mg/g FW) followed by seed treatment of *P. putida* DAPG3 (5.24 mg/g FW) which were

Table 2. Effect of cell-free filtrate of fluorescent pseudomonads on mortality of *Meloidogyne arenaria* (J2)

Fluorescent pseudomonas species/strains	Per cent mortality of nematodes after exposure period of 48 h			Per cent mortality of nematodes after exposure period of 72 h		
	50%	75%	100%	50%	75%	100%
Pseudomonas putida DAPG1	55.22 (48.01)*	57.46 (49.33)	59.71 (50.71)	62.30 (52.16)	70.01 (56.89)	84.16 (66.70)
P. putida DAPG2	34.19 (35.73)	35.36 (36.42)	41.10 (39.81)	44.41 (41.75)	45.10 (42.19)	50.01 (45.01)
P. putida DAPG3	63.71 (53.08)	64.77 (53.63)	77.23 (61.73)	73.43 (59.30)	86.08 (68.30)	87.36 (69.67)
P. putida DAPG4	47.57 (43.60)	50.09 (45.05)	57.39 (49.31)	54.45 (47.59)	53.04 (46.78)	63.47 (52.86)
P. putida DAPG5	60.01 (50.91)	60.34 (51.17)	75.16 (60.46)	72.40 (58.60)	75.82 (60.86)	81.79 (64.82)
P. putida DAPG6	59.57 (50.53)	60.20 (51.01)	68.61 (55.97)	67.20 (55.19)	67.25 (55.42)	82.37 (65.26)
P. putida DAPG7	51.47 (45.90)	56.40 (48.72)	71.42 (57.86)	64.88 (53.85)	73.14 (58.95)	81.51 (64.84)
P. aeruginosa FP82	26.14 (30.50)	33.91 (35.56)	37.11 (37.46)	32.44 (34.63)	33.01 (35.03)	42.73 (40.76)
P. fluorescens FP93	19.86 (26.30)	32.10 (34.48)	32.86 (34.86)	25.90 (30.41)	33.53 (35.30)	34.81 (36.11)
P. putida S1(6)	33.71 (35.41)	38.90 (38.55)	41.91 (40.33)	41.75 (40.24)	41.79 (40.24)	45.08 (42.15)
Pseudomonas sp. FP20	35.19 (36.32)	35.50 (36.45)	40.04 (39.18)	38.66 (38.36)	44.52 (41.80)	46.77 (43.12)
Pseudomonas sp. FP46	40.73 (39.53)	42.82 (40.80)	58.40 (49.90)	44.48 (41.82)	55.16 (48.01)	67.32 (55.56)
P. putida FP86	27.96 (31.73)	29.10 (32.58)	33.84 (35.43)	30.83 (33.67)	41.24 (39.91)	42.21 (40.49)
P. fluorescens FP94	40.54 (39.50)	43.62 (41.32)	52.19 (46.28)	41.06 (39.77)	44.81 (41.99)	59.96 (50.81)
P. monteilii FP133	24.07 (29.24)	34.12 (35.66)	35.37 (36.38)	41.56 (40.10)	43.54 (41.24)	54.54 (47.61)
P. gessardii BHU1	26.00 (30.38)	27.59 (31.67)	28.36 (32.13)	32.84 (34.90)	33.25 (35.09)	45.90 (42.63)
P. aeruginosa ACC7	22.22 (27.72)	28.66 (32.26)	41.49 (39.93)	39.26 (38.69)	46.69 (43.09)	47.31 (43.45)
P. aeruginosa BM6	22.27 (28.06)	29.79 (32.91)	31.01 (33.70)	30.42 (33.21)	32.40 (34.60)	42.90 (40.84)
Control (medium)	5.55 (13.56)	5.38 (13.23)	5.59 (13.60)	5.57 (13.55)	5.63 (13.36)	5.26 (13.21)
Control (water)	5.06 (12.93)	5.10 (13.01)	5.00 (12.88)	4.80 (12.55)	5.04 (12.92)	4.51 (12.10)
ANOVA	F = 22.13 df = 9, 79 P< 0.01	F = 25.32 df = 9, 79 P< 0.01	F = 27.85 df = 9, 79 P< 0.01	F = 26.29 df = 9, 79 P< 0.01	F = 36.59 df = 9, 79 P< 0.01	F = 49.65 df = 9, 79 P< 0.01
LSD	9.05	8.21	9.49	9.29	8.61	8.37

*Values in parentheses are arcsine-transformed values

statistically significant (P<0.05) from each other and significantly (P<0.05) differ from inoculated (3.56 mg/g FW) and un-inoculated control (3.55 mg/g FW) (Figure 2).

The result indicates that cell-free filtrate of DAPG-producing isolates of *P. putida* resulted in significantly higher mortality of *M. arenaria* (J2) with highest in isolate DAPG3 (87.36%) followed by isolate DAPG1 (84.16%) compared to other isolates of fluorescent pseudomonads, *P. gessardii* and *P. aeruginosa.* Further, selected DAPG-producing isolates of *P. putida* resulted in significant inhibition in hatching in eggs of nematode. The lowest cumulative percent hatch of *M. arenaria* was recorded in the isolate DAPG3 (17.84%) followed by DAPG1 (18.10%), DAPG6 (20.99%) and DAPG5 (25.58%). However, significant resumption of hatch was observed in the isolate DAPG6 but not in DAPG1, DAPG3 and DAPG5, when the cell-free filtrate was replaced with distilled water. Cronin *et al*., (1997) reported that DAPG-producing *P. fluorescens* F113 increased the ability of *G. rostochiensis* to hatch and reduced the percentage of mobile juveniles of the potato cyst nematode in *in vitro* and in soil. Siddiqi and Shaukat (2003) also

Table 3. Effect of cell-free filtrate of *Pseudomonas putida* isolates on hatching of eggs of *Meloidogyne arenaria*

Fluorescent pseudomonas strains	Cumulative percentage hatch of *M. arenaria*							
	Day 1	Day 2	Day 4	Day 6	Day 9	Day 12	Day 14	Day 17
Pseudomonas putida DAPG1	9.07 (17.50)*	13.76 (21.70)	14.75 (22.53)	15.52 (23.13)	17.21 (24.45)	18.10 (25.09)	18.87 (25.64)	20.04 (26.49)
P. putida DAPG3	10.05 (18.35)	14.83 (22.50)	16.48 (23.84)	17.65 (24.75)	17.84 (24.90)	17.84 (24.90)	17.93 (24.97)	18.19 (25.18)
P. putida DAPG5	16.35 (23.50)	18.42 (25.15)	20.23 (26.42)	22.09 (27.79)	24.25 (29.26)	25.58 (30.13)	26.88 (30.96)	27.52 (31.38)
P. putida DAPG6	18.80 (25.46)	19.77 (26.16)	19.98 (26.30)	20.18 (26.44)	20.59 (26.71)	20.99 (26.98)	27.26 (31.26)	31.77 (34.16)
Control (medium)	13.92 (21.59)	24.03 (29.25)	37.31 (37.59)	52.33 (46.34)	66.89 (54.92)	67.11 (55.05)	69.10 (56.29)	70.12 (56.93)
Control (water)	24.68 (29.77)	53.12 (46.80)	73.18 (58.89)	82.25 (65.25)	84.25 (66.79)	87.71 (69.64)	88.56 (70.40)	89.46 (71.28)
ANOVA	$F = 3.96$ df = 5, 17 $P < 0.05$	$F = 14.92$ df = 5, 17 $P < 0.01$	$F = 31.50$ df = 5, 17 $P < 0.01$	$F = 49.18$ df = 5, 17 $P < 0.01$	$F = 55.94$ df = 5, 17 $P < 0.01$	$F = 57.84$ df = 5, 17 $P < 0.01$	$F = 53.76$ df = 5, 17 $P < 0.01$	$F = 54.19$ df = 5, 17 $P < 0.01$
LSD	7.11	10.42	10.73	10.35	10.57	10.81	11.11	11.04

*Values in parentheses are arcsine-transformed values

reported that culture filtrate of DAPG-producing *P. fluorescens* isolate CHA0 and its antibiotic overproducing isolate CHA0/pME3424 inhibited egg hatching and induced juvenile mortality of *M. javanica* under in vitro.

DAPG-producing isolates, *Pseudomonas putida* DAPG1 and *P. putida* DAPG3 also inhibited the invasion of peanut root-knot nematode, *M. arenaria* in the roots of peanut by 41.30% and 36.34%, respectively. Reduction in nematode invasion by fluorescent pseudomonads has been reported by several workers (Becker *et al*., 1988; Aalten *et al*., 1998; Siddiqi and Shaukat, 2003). DAPG is also known to affect root morphology (Brazelton *et al*., 2008) and changes in root architecture which might alter the number of available infection sites (Timper *et al*., 2009), and this might have affected the nematode invasion in peanut roots also. In present study, greater reduction in root galling was also recorded when seed treatment was combined with soil application for both the isolates. Seed treatment and soil application of *P. putida* DAPG1 also recorded significantly less nematode reproduction factor. Similarly, suppression of population densities of *M. incognita* on cotton, soybean, and corn has been reported when Wood1R, a D-genotype of DAPG-producing *P. fluorescens* was used as seed treatment (Timper *et al*., 2009).

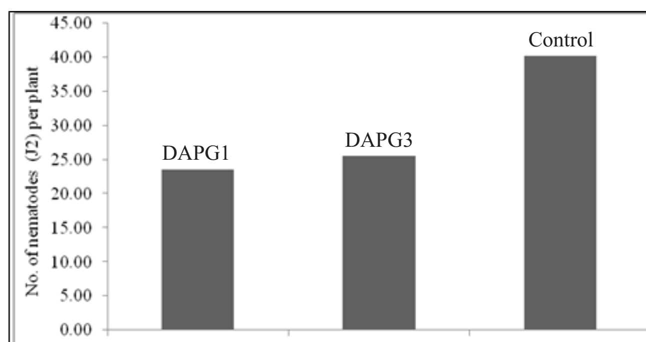

Fig. 1. Effect of application of *Pseudomonas putida* isolates on invasion of *Meloidogyne arenaria* in peanut roots.

The levels of Peroxidase (POD), Catalase (CAT) and Polyphenol Oxidase (PPO) were non-significant in the treatment that received *P. putida* DAPG1 and DAPG3, either as seed treatment and/or soil application, compared to inoculated

Table 4. Effect of application of *Pseudomonas putida* isolates on development of root-knot and reproduction of incidence of *Meloidogyne arenaria* in peanut

Treatments	No. of galls/plant	% reduction	Reproduction factor
Seed treatment of *Pseudomonas putida* DAPG1	24.67 ± 3.48*	35.65	1.37 ± 0.19
Soil application of *P. putida* DAPG1	23.67 ±3.84	38.26	1.24 ± 0.11
Seed treatment + soil application of *P. putida* DAPG1	18.67 ± 2.33	51.30	1.04 ± 0.17
Seed treatment of *P. putida* DAPG3	25.33 ± 5.70	33.91	1.51 ± 0.27
Soil application of *P. putida* DAPG3	22.33 ± 5.24	41.73	1.31 ± 0.18
Seed treatment + soil application of *P. putida* DAPG3	20.33 ± 1.86	46.95	1.20 ± 0.12
Control (nematode inoculated)	38.33 ± 1.76		2.07 ± 0.13
ANOVA	F = 2.93 df = 6, 20 P< 0.05		F = 3.60 df = 6, 20 P< 0.05
LSD	11.39		0.53

*Values (±SE) are means of three replicates

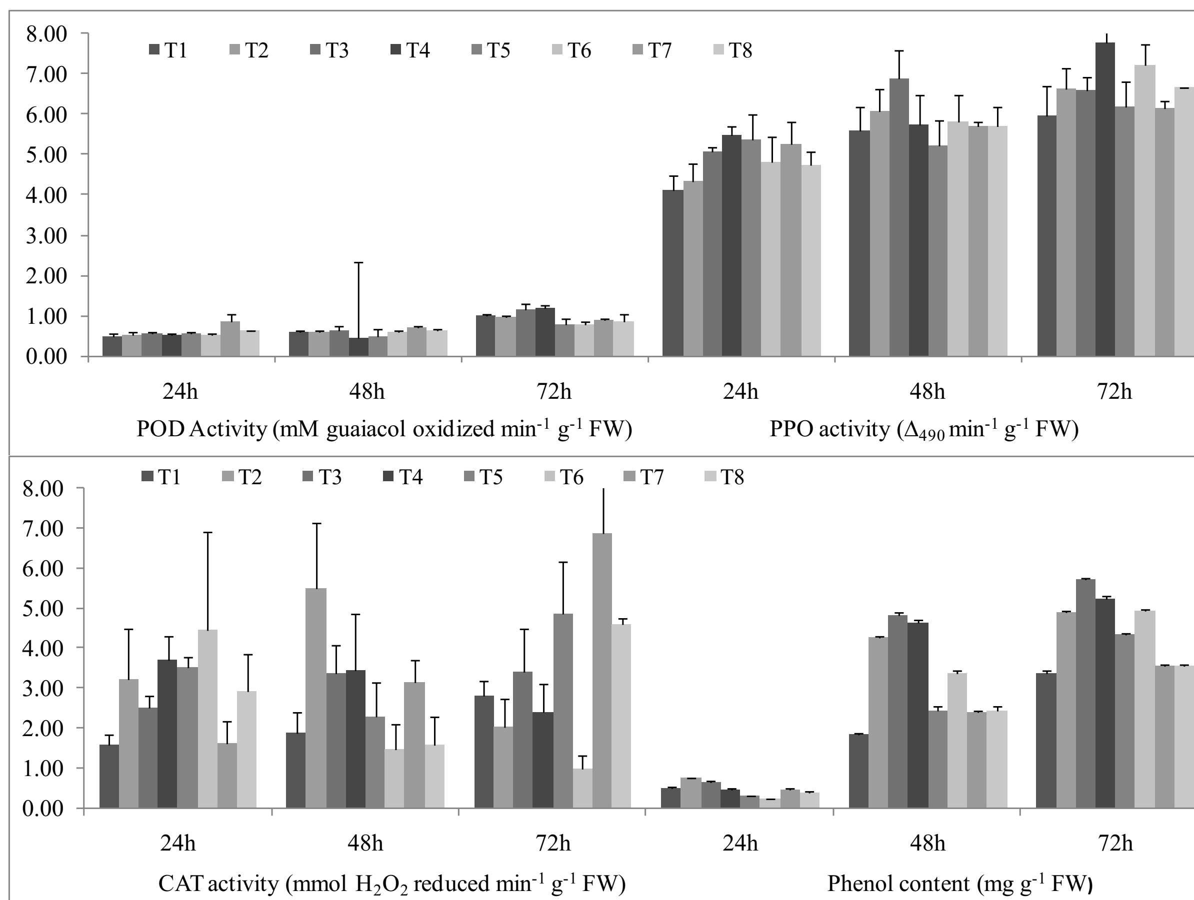

Fig. 2. Peroxidase (POD), Polyphenol Oxidase (PPO), Catalase (CAT) activity and phenol content in the leaves of peanut. Error bar indicates standard error of mean. T1= Seed treatment of *Pseudomonas putida* DAPG1; T2 = Soil application of *P. putida* DAPG1; T3 = Seed treatment + Soil application of *P. putida* DAPG1; T4 = Seed treatment of *P. putida* DAPG3; T5 = Soil application of *P. putida* strain DAPG3; T6 = Seed treatment + soil application of *P. putida* DAPG3; T7 = Inoculated (nematode) control and T8 = Uninoculated control.

and un-inoculated control. However, significantly increased phenol content was recorded in the leaves of peanut after 24, 48 and 72h post-inoculation of nematodes**.** In contrast, Chen *et al*., (2000) found increased enzyme activities of PAL, POD and PPO in bacterized cucumber roots when challenged with *P*. *aphanidermatum*. Liu *et al*., (1995) found the induction of systemic resistance by *P. putida* isolate 89B-27 and *Serratia marcescens* isolate 90-166 in cucumber against cucumber wilt. Induction of systemic resistance by DAPG-producing isolate of *P. fluorescens* CHA0 in tomato plants against *M. javanica* has also been reported by Siddiqi and Shaukat (2003). As we found, Kurabachew and Wydra (2014) also reported the non-significant increase of PAL and POD activities in rhizobateria treated tomato plants. In present study, enzyme activity has not enhanced when bacterized plants were challenged with *M. arenaria* but reduction in galling and nematode reproduction factor was recorded. It is difficult to say exactly which mechanism is responsible for induction of resistance in plants. However, the reduction in root galling might be due to reduced nematode invasion, inhibition of hatching of eggs in subsequent generation and reduction in the secondary inoculum of *M. arenaria* which were exposed to the metabolites of *P. putida* in soil.

CONCLUSION

The study demonstrated that DAPG-producing isolate of *Pseudomonas putida* DAPG1 and *P. putida* DAPG3 cause significantly higher juvenile mortality and inhibition in the hatching of eggs of *Meloidogyne arenaria* under *in vitro* conditions*.* These DAPG-producing isolates were also effective in reducing nematode invasion. Seed treatment combined with soil application of *P. putida* was found effective in reducing the root galling (51.30% reduction). The DAPG-producing fluorescent pseudomonads, discussed in this work, have been isolated from the peanut rhizosphere and exhibit multiple plant growth promoting and biocontrol abilities. These pseudomonads, besides controlling plant parasitic nematodes and soil-borne fungal diseases, also promote plant growth of peanut and enhance yield (Sherathia *et al*., 2016). In field trials, these DAPG-producing fluorescent pseudomonads have proven successful in enhancing peanut yields and reducing the incidences of major soil-borne fungal diseases (Bhayani et al., 2013; Dey *et al*., 2013). Literature is also abundant with the benefits of DAPG-producing fluorescent pseudomonads and their field applications. DAPG inhibits growth of several phytopathogenic fungi including *Thielaviopsis basicola* (tobacco black root rot), *Gaeumannomyces graminis* var. *tritici* (wheat take-all) and *Pythium ultimum* (cotton damping-off) (Howell and Stipanovic, 1980; Kwak and Weller, 2013). Suppression of few plant parasitic nematodes by DAPG- producers, has also been reported (Siddiqi and Shaukat, 2003; Timper *et al*., 2009). The present work also shows that these pseudomonads can be used for suppression of root-knot nematode, *M. arenaria* in peanut.

ACKNOWLEDGEMENTS

The authors are grateful to the Director, ICAR- Directorate of Groundnut Research, Junagadh - 362001 for the facilities provided for conducting the study.

REFERENCES

Aalten PM, Vitour D, Blanvillain D, Gowen SR, Sutra L. 1998. Effect of rhizosphere fluorescent *Pseudomonas isolates* on plant parasitic nematodes *Radopholus similis* and *Meloidogyne* spp. *Lett Appl Microbiol*. **27**: 357–361. https://doi.org/10.1046/j.1472-765X.1998.00440.x

Aebi H. 1984. Catalase *in vitro*. *Methods Enzymol*. **105**: 121–126. https://doi.org/10.1016/S0076-6879(84)05016-3

Ahmadzadeh M, Afsharmanesh HJ, Nikkhah M, Sharifi-Tehrani A. 2006. Identification of some molecular traits in fluorescent pseudomonads with antifungal activity. *Iranian J Biotechnol*. **4**: 245-253.

Becker JO, Zavaleta-Mejia E, Colbert, SF, Schroth MN, Weinhold AR, Hancock GJ, Van Gundy SD. 1988. Effects of rhizobacteria on root-knot nematodes and gall formation. *Phytopathology* **78**: 1466–1469. https://doi.org/10.1094/Phyto-78-1466

Bhayani S, Shah A, Pal KK, Dey R, Dash P, Chauhan SM, Parmar V, Chudasama A. 2013. DAPG-producing fluorescent pseudomonads for enhancing growth and yield of groundnut. National Conference of Plant Physiology on "Current Trends in Plant Biology Research", 13-16 December, Junagadh. pp. 818-819.

Brazelton JN, Pfeufer EE, Sweat TA, McSpadden Gardener BB, Coenen C. 2008. 2,4-Diacetylphloroglucinol alters plant root development. *Mol Plant Microbe Interact*. **21**: 1349–1358. https://doi.org/10.1094/MPMI-21-10-1349 PMid:18785830

Byrd DW, Kirkpatrick Jr. T, Barker KR. 1983. An improved technique for clearing and staining plant tissue for detection of nematodes. *J Nematol*. **14**: 142–143.

Castillo FI, Penel I, Greppin H. 1984. Peroxidase release induced by ozone in *Sedum album* leaves. *Plant Physiol*. **74**: 846–851. https://doi.org/10.1104/pp.74.4.846 PMid:16663520 PMCid: PMC1066779

Chakraborty K, Singh AL, Kalariya KA, Goswami N, Zala PV. 2015. Physiological responses of peanut (*Arachis hypogaea* L.) cultivars to water deficit stress: status of oxidative stress and antioxidant enzyme activities. *Acta Bot Croat*. **74**. DOI: https://doi.org/10.1515/botcro-2015-0011.

Chen C, Bélanger RR, Benhamou N, Paulitz T. 2000. Defense enzymes induced in cucumber roots by treatment with plant growth promoting rhizobacteria (PGPR) and *Pythium*

aphanidermatum. *Physiol Mol Plant Pathol*. **56**: 13–23. https://doi.org/10.1006/pmpp.1999.0243

Cronin D, Moönne-loccoz Y, Fenton A, Dunne C, Dowling DN, O'Gara F. 1997. Role of 2,4-diacetylphloroglucinol in the interactions of the biocontrol pseudomonad isolate F113 with the potato cyst nematode *Globodera rostochinensis*. *Appl Environ Microbiol*. **63**: 1357–1361. PMid:16535571 PMCid:PMC1389549

Dey R, Pal KK, Singh SP, Dash P, Parmar V, Chudasma A, Chauhan SM. 2013. Application of DAPG-producing fluorescent pseudomonads for suppressing stem rot pathogen and enhancing yield of groundnut. National Conference of Plant Physiology on "Current Trends in Plant Biology Research", 13-16 December, Junagadh. pp. 329-330.

Gomez KA, Gomez AA. 1984. *Statistical Procedure for Agricultural Research*. 2nd ed. New York, USA. John Wiley and Sons. 407 pp.

Howell CR, Stipanovic RD. 1980. Suppression of Pythium ultimum damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. *Phytopathology* **70**: 712–715 https://doi.org/10.1094/Phyto-70-712

Kurabachew H, Wydra K. 2014. Induction of systemic resistance and defense-related enzymes after elicitation of resistance by rhizobacteria and silicon application against *Ralstonia solanacearum* in tomato (*Solanum lycopersicum*). *Crop Prot*. **57**: 1–7. https://doi.org/10.1016/j.cropro.2013.10.021

Kwak YS, Weller DM. 2013. Take-all of wheat and natural disease suppression: a review. *Plant Pathol J*. **29**: 125–135. https://doi.org/10.5423/PPJ.SI.07.2012.0112 PMid:25288939 PMCid:PMC4174779

Liu L, Kloepper JW, Tuzun S. 1995. Induction of systemic resistance in cucumber against Fusarium wilt by plant growth-promoting rhizobacteria. *Phytopathology* **85**: 695–698. https://doi.org/10.1094/Phyto-85-843

Malik CP, Singh MB. 1980. *Plant Enzymology and Histoenzymology: A text manual*. Kalyani Publications, New Delhi. pp. 5. PMid:6784316

Okubara PA, Bonsall RF. 2008. Accumulation of Pseudomonas-derived 2,4-diacetylphloroglucinol on wheat seedling roots is influenced by host cultivar. *Biol Control* 46: 322–331. https://doi.org/10.1016/j.biocontrol.2008.03.013

Onofri A. 2007. Routine statistical analyses of field experiments by using an Excel extension. Proceedings of 6th National Conference Italian Biometric Society: "La statisticanelle scienze della vita e dell'ambiente", Pisa. pp. 93-96.

Raaijmakers JM, Weller DM, Thomashow LS. 1997. Frequency of antibiotic producing *Pseudomonas* spp. in natural environments. *Appl Environ Microbiol*. **63**: 881–887. PMid:16535555 PMCid:PMC1389120

Raaijmakers JM, Weller DM. 2001. Exploiting genotypic diversity of 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp.: characterization of superior root-colonizing *Pseudomonas fluorescens* isolate Q8r1-96. *Appl Environ Microbiol*. **67**: 2545–2554. https://doi.org/10.1128/AEM.67.6.2545-2554.2001 PMid:11375162 PMCid: PMC92906

Rezzonico F, Moënne-LoccozY, Défago G. 2003. Effect of stress on the ability of a phlA-based quantitative competitive PCR assay to monitor biocontrol strain *Pseudomonas fluorescens* CHA0. *Appl Environ Microbiol*. **69**: 686–690. https://doi.org/10.1128/AEM.69.1.686-690.2003 PMid:12514062 PMCid:PMC152391

Sherathia D, Dey R, Thomas M, Dalsania T, Savsani K, Pal KK. 2016. Biochemical and molecular characterization of DAPG-producing Plant Growth-Promoting Rhizobacteria (PGPR) of groundnut (*Arachis hypogaea* L.). *Legume Res.* **39**: 614–622. https://doi.org/10.18805/lr.v0iOF.9389

Siddiqui IA, Shaukat SS. 2002. Rhizobacteria mediated induction of systemic resistance (ISR) in tomato against *Meloidogyne javanica*. *J Phytopathol*. **150**: 469–473. https://doi.org/10.1046/j.1439-0434.2002.00784.x

Siddiqui IA, Shaukat SS. 2003. Suppression of root-knot disease by *Pseudomonas fluorescens* CHAO in tomato: Importance of bacterial secondary metabolic 2,4-diacetylphloroglucinol. *Soil Biol Biochem*. **35**: 1615–1623. https://doi.org/10.1016/j.soilbio.2003.08.006

Timper P, Kone D, Yin J, Pingsheng JI, Brain B. 2009. Evaluation of an antibiotic-producing isolate of *Pseudomonas fluorescens* for suppression of plant-parasitic nematodes. *J Nematol*. **41**: 234–240. PMid:22736820 PMCid:PMC3380499

Wang CX, Ramette A, Punjasamarnwong P, Zala M, Natsch A, Moenne-LoccozY, Defago G. 2001. Cosmopolitan distribution of phlD-containing dicotyledonous crop-associated biocontrol pseudomonads of worldwide origin. *FEMS Microbiol Ecol.* **37**: 105–116. https://doi.org/10.1111/j.1574-6941.2001.tb00858.x