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* (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,
Nematicidal properties of fluorescent pseudomonads

The nematicidal properties of fluorescent pseudomonads were investigated. The cycling program included an initial denaturation at 94°C for 2 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 PCR was carried out in 20μl 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 GCA GC-3') and phlA-2b (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 20μl 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 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 a 100 bp DNA step ladder as molecular size marker. PCR amplification 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 20μl 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 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 ± 2°C in an orbital shaker at 120 rpm. The medium was sterilized at 121°C for 20 min followed by inoculation with 1 ml of 2.0 × 10^8 cfu ml⁻¹ of pure culture and incubated for 48 h.

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 montelii FP133 |
| 16     | JX514417              | Pseudomonas aeruginosa BM6 |
| 17     | JX514422              | Pseudomonas aeruginosa ACC7 |
| 18     | JX514410              | Pseudomonas gessardii BHU1 |

### Preparation of Cell-Free Extracts

The cell-free broth of fluorescent pseudomonads 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 48h 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 percent 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 *Meloidogyne arenaria* were hand-picked with forceps from infected plants. Ten egg masses were placed in each well of a multi-well plate containing 0.5 ml of sterile distilled water, to which 1 ml 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 cm³ cups were filled with sterilized soil and drenched with 5 ml suspension of each fluorescent pseudomonad at a concentration of 3×10⁸ 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 cm³) were filled with sterilised soil. Seed treatment was done by placing the peanut seeds in fluorescent pseudomonads suspension containing 3×10⁸ cfu/ml for ½ hr. Soil application was done by drenching with 5 ml fluorescent pseudomonads suspension at a concentration of 3×10⁸ 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 72 h 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 H₂O₂ (Aebi, 1984) in a reaction mixture (3 mL) consisting of 0.5 mL of 75 mM H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 ε = 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).
Nematicidal properties of fluorescent pseudomonads

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/DSSASTAT/DSSASTAT.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
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

| 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)*                                               | 62.30 (52.16)                                                |
| *P. putida* DAPG2                      | 34.19 (35.73)                                                | 44.41 (41.75)                                                |
| *P. putida* DAPG3                      | 63.71 (53.08)                                                | 73.43 (59.30)                                                |
| *P. putida* DAPG4                      | 47.57 (43.60)                                                | 54.45 (47.59)                                                |
| *P. putida* DAPG5                      | 60.01 (50.91)                                                | 72.40 (58.60)                                                |
| *P. putida* DAPG6                      | 59.57 (50.53)                                                | 67.20 (55.19)                                                |
| *P. putida* DAPG7                      | 51.47 (45.90)                                                | 64.88 (53.85)                                                |
| *P. aeruginosa* FP82                   | 26.14 (30.50)                                                | 32.44 (34.63)                                                |
| *P. fluorescens* FP93                  | 19.86 (26.30)                                                | 25.90 (30.41)                                                |
| *P. putida* S1(6)                      | 33.71 (35.41)                                                | 41.75 (40.24)                                                |
| *Pseudomonas* sp. FP20                 | 35.19 (36.32)                                                | 38.66 (38.36)                                                |
| *Pseudomonas* sp. FP46                 | 40.73 (39.53)                                                | 44.48 (41.82)                                                |
| *P. putida* FP86                       | 27.96 (31.73)                                                | 30.83 (33.67)                                                |
| *P. fluorescens* FP94                  | 40.54 (39.50)                                                | 41.06 (39.77)                                                |
| *P. monteilii* FP133                   | 24.07 (29.24)                                                | 41.56 (40.10)                                                |
| *P. gessardii* BHU1                    | 26.00 (30.38)                                                | 32.84 (34.90)                                                |
| *P. aeruginosa* ACC7                   | 22.22 (27.72)                                                | 39.26 (38.69)                                                |
| *P. aeruginosa* BM6                    | 22.27 (28.06)                                                | 30.42 (33.21)                                                |
| Control (medium)                       | 5.55 (13.56)                                                 | 5.57 (13.55)                                                 |
| Control (water)                        | 5.06 (12.93)                                                 | 5.00 (12.88)                                                 |
| ANOVA                                  | *F* = 22.13                                                   | *F* = 25.32                                                   |
|                                        | df = 9, 79                                                   | df = 9, 79                                                   |
|                                        | *P*< 0.01                                                   | *P*< 0.01                                                   |
|                                        | *F* = 27.85                                                   | *F* = 26.29                                                   |
|                                        | df = 9, 79                                                   | df = 9, 79                                                   |
|                                        | *P*< 0.01                                                   | *P*< 0.01                                                   |
|                                        | *F* = 36.59                                                   | *F* = 49.65                                                   |
|                                        | df = 9, 79                                                   | df = 9, 79                                                   |
|                                        | *P*< 0.01                                                   | *P*< 0.01                                                   |
| LSD                                    | 9.05                                                        | 8.21                                                         |
|                                        | 9.49                                                         | 9.29                                                         |
|                                        | 8.61                                                         | 8.37                                                         |

*Values in parentheses are arcsine-transformed values
Nematicidal properties of fluorescent pseudomonads

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).

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

| 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*

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

![](image.png)

Fig. 1. Effect of application of *Pseudomonas putida* isolates on invasion of *Meloidogyne arenaria* in peanut roots.
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, $P<0.05$

LSD = 11.39

*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 rhizobacteria 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 with antifungal activity.

**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, Pfeuffer 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 Fl, 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, Kalaria 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, Moënne-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:16535555 PMCid:PMC1389120

Raaijmakers JM, Weller DM, Thomasow 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-Loccoz Y, Défago G. 2003. Effect of stress on the ability of a phiA-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

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

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-Loccoz Y, Defago G. 2001. Cosmopolitan distribution of phID-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

HOLAJER et al.