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Determinants of *Pseudomonas putida* IIHR PP 17 against nematode management in gherkin

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Abstract
The potential of biocontrol agents to control root knot nematode *Meloidogyne incognita* was scrutinize under the *in vitro* studies. Treatments using culture free filtrates of *Pseudomonas putida* was recorded higher nematode egg hatching and juvenile mortality at the rate of 83.67% and 81.34%, respectively, compared to the control. Further studied the repellence of *P. putida* against *M. incognita* in gherkin was recorded by twin pot set-up, wherever the penetration of juveniles was decreases compared to un-inoculated control and supressed the production of egg masses. Hence, dual action of this bacteria could deliver better control management of nematode in gherkin.

Keywords: *Pseudomonas putida* IIHR, *Meloidogyne incognita*

Introduction
Root knot Nematode, *Meloidegyne incognita* is one of the major devastating pathogen in gherkin which results in substantial decrease in its yield. Recent reports agree that root knot nematodes are causing more destruction in horticultural crops and projected yield loss due to this tiny unseen pest was 12.3 % ($157 billion) world-wide. India suffers $40.3 dollars yield loss due to nematodes alone (Singh *et al*., 2015) [14].
Nematodes enter the vascular tissue of the host root resulting in severe root galling, damage the water utilization, reduce the nutrient uptake and affect greatly the photosynthetic pathway (Mcclure, 1977) [7]. Subsequently the root knot infection leads to the foliage symptoms like wilting, poor yield and stunted growth.
In India, the nematode management strategies on gherkin mainly rely on chemical nematicides, fallow, crop rotation and biological control. Since chemical nematicides have an environmental concern, more emphasis is given to biocontrol agents.
Biocontrol agents are better alternatives to chemical nematicides, exclusively because of the environmental and health risks posed by chemicals. Among several biocontrol agents, *Pseudomonas putida*, a plant growth promoting rhizobacteria (PGPR) plays an important role in the management of nematodes by secretion of different secondary metabolites, antibiotic and volatile compounds (Giles *et al*., 2014) [3].
Tang *et al*. 2014 [17] reported that *P. putida* 1A00316 exhibited 71.67 % inhibition of *M. incognita* under *in vitro*. *P. putida* DAPG1 and DAPG3 showed significant inhibition in egg hatching of *M. arenaria in vivo* (Holajjer *et al*., 2018) [4].
The current study was aimed to study the potential effect of *P. putida* IIHR PP-17 against *M. incognita* under *in vivo* in gherkin.

Materials and Methods
Maintenance of Culture
The rhizospheric strain of *Pseudomonas putida* IIHR PP 17 was maintained in King's B media at Nematology lab, Division of Entomology & Nematology, ICAR- Indian Institute of Horticultural Research, Bengaluru.

Preparation of culture filtrate concentration
Cultures of IIHR PP 17 was grown in Nutrient broth (100 ml) and incubated at 26±2 °C for 36 hours. After the period of incubation, the well grown broth was centrifuged at 10,000 rpm for 15 minutes and supernatant was collected, filtered through 0.22 µ syringe filters (Whatmann filters). The filtrate was diluted with sterile distilled water into different concentration such as 25 %, 50 %, 75% and 100%.

Sterile distilled water served as control. Un-inoculated sterile nutrient broth served as media control.

Nematode maintenance

M. incognita was collected from nematode diseased roots of tomato (cv. PKM-1) maintained in Nematology lab, ICAR-IIHR. As per Sasser and Carter (1982), the identity of female was confirmed through the perineal cuticular pattern. Egg masses were handpicked from the sick roots and allowed to hatch in distilled water for 3 days at 28±2°C. Using stereoscopic microscope (Motic, Hong-kong), emergence of the second stage juveniles (J_2) was observed which were used for further nematode assays.

***In vitro* egg hatch**

Effect of bioagent on egg hatching was evaluated by modified method of Su and Mulla (1998). Fifty eggs were handpicked carefully and transferred to each Petri plate (3 cm). In all test petri plates, 3 ml of cell free culture filtrate at different concentration such as 25%, 50%, 75% and 100% was added to corresponding treatments. All the test plates were replicated thrice. Sterile broth and autoclaved water served as respective control. Test replicates were incubated at 27° C for 5 days. Lugol's iodine solution was additionally added to individually plate to stop further egg hatching (Saikia *et al*., 2013) [11]. Unhatched eggs were carefully examined and counted microscopically.

Percentage of egg hatching was calculated by formula = number of hatched eggs/total no of eggsx100.

***In vitro* mortality test**

Freshly hatched second stage juveniles (approx.50 J2/3ml) in sterile water suspension were carefully pipetted out and added in to corresponding test petri plates with concentration of 25% to 100% as mentioned in the previous section. Experiment plates were incubated at 27 ^{0}C for 5 days. Second stage juveniles were examined after 120 h exposure period and percentage of mortality was calculated. Live and dead second stage juveniles were detected by stereoscopic microscope and experiment was repeated thrice.

Effect of antagonistic strains on Juvenile repellence

Repellence experiment was evaluated by twin-pot technology as suggested by Adam *et al*. (2014). Two plastic covers of 7 x 7 x 8 cm^3 were filled with 2 kg of soil and connected by 2 cm diameter and 5 cm length PVC tube with soil (Fig 6). Gherkin seeds were coated with *P. putida* IIHR PP 17 culture and sown in right side and uncoated seeds were sown in left side. The bacterial culture was prepared by centrifugation at 7500 rpm for 20 min and supernatant was discarded and resulting pellet was suspended with sterile water. The density of the bacteria was adjusted to 4x10^7 CFU/ml. After 21 days, 10 ml of bacterial solution was inoculated into treatment plant near the root zone. After 72 h, approx.1000 J2 suspended in 5 ml of water was inoculated through the small hole in the centre of the tube. The hole was sealed with aluminium foil cover to maintain the moisture. Each treatment was replicated thrice.

After 45 days, the plants were uprooted, and the roots were recorded for root knot index (RKI). The RKI was determined on scale of 1–5 (1= no galls, 2=25% galls, 3=50%, 4=75%, 5=100%) as per Heald *et al*., 1989). Nematode population in soil was assessed in each pot as per Cobb's sieving and decanting method (Cobb, 1918). Plant growth parameters were also recorded *viz*., root length (cm), root weight (g), shoot weight (g) and shoot length (cm). Data of Mean and Standard deviation were analysed using the student T test (P<0.01) and regression analysis. All statistical analysis performed using a software SAS 9.3.

Results and Discussion

The nematicidal properties of *P. putida* IIHR PP 17 were investigated through *in vitro* egg hatching and mortality assays against *M. incognita* (Fig 1 & Fig 2). It was detected that bio-agents were able to significantly decrease the egg hatch of *M. incognita* and increase the mortality of the second stage juveniles in all the concentrations. Maximum egg hatch (Fig 1 & 4) and mortality (Fig 2 & 5) was recorded in 100 % concentration as 83.67% and 81.34%, respectively followed by other concentrations. Egg masses and juveniles kept in sterile distilled water stayed healthy and stable (Fig 3). Siddiqi and Shaukat (2003) showed the culture filtrate of *P. fluorescens* CHA0 efficiently reduced the egg hatching and elevated the second stage juvenile mortality.

In the present study, the isolate *P. putida* IIHR PP17 showed significant egg parasitism and juvenile mortality which might be because of the production of DAPG and various antibiotic compounds (Loeschcke and Thies, 2015) [6]. Timper *et al*. (2009) [18] recorded lower nematode population of *M. incognita* on cotton, corn and soyabean by seed treatment with DAPG producing *P. fluorescens*.

Due to polyphagous nature of root knot nematode, complete eradication is impossible, but suppression of *M. incognita* egg hatching is helpful in decreasing nematode population in root and soil (Meyer *et al*., 2004) [8].

Rao *et al*. (2016) and Sowmya *et al*. (2006) proved that *P. putida* IIHR PP 17 was effective in the management of nematode in carrot and okra, respectively. *P. putida* 1A00316 isolated from Antarctic soil produced effective volatile organic compounds (VOCs) such as dimethyl-disulfide, 2-nonanone, 2-octanone, (Z)-hexen-1-ol acetate, and 2-undecanone that exhibited strong nematicidal potential against root knot nematodes *M. incognita* (Zhai *et al*., 2018) [19].

Experiment results of twin pot set up demonstrated the nematicidal and plant growth promotion potential of the *P. putida*. Nematode population was reduced by 39.7 % in soil and 29.5 % in root and root knot index was 2.8 in scale 1-5 and in control was 4.9 (Table 1). In terms of plant growth promotion, *P. putida* IIHR PP17 showed significantly higher growth parameters root length (20 cm), root weight (4 g), shoot weight (55. 67g) and shoot length (142 cm) compared to control. Similar repellence of *Meloidogyne graminicola* was evidenced in rice roots by treatment with *Bacillus megaterium* (Padgham and Sikora, 2007) [9]. *P. putida* MCCC 1A00316 isolated form Antarctic soil reduced the nematicidal activity of *M. incognita* by the production of nematicidal compound as cyclo (L-Pro–L-Leu) under controlled conditions (Zhai *et al*., 2019). Likewise, several researchers were showed the PGPR's were effective and decreases the number of galls and egg masses of *M. incognita* (Siddiqui *et al*., 2001, Ali *et al*., 2002, Li *et al*., 2002) [13, 1, 5].

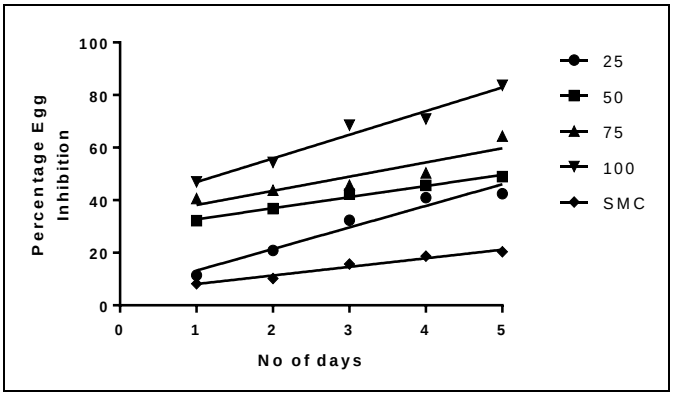

Fig 1: Percentage of Egg hatching of different cell extracts of *P. Putida* IIHR PP-17 on *M. incognita* exposure to 25 to 100% concentrations for various time interval. Regression equation for curves on 25%, Y = 8.207*X + 5.001(R^2 = 0.95, *P*< 0.001, df = 5); 50%, Y = 4.229*X + 28.48 (R^2 = 0.98, *P*< 0.001, df = 5); 75%, Y = 5.398*X + 32.76(R^2 = 0.84, *P*< 0.001, df = 5); 100%, Y = 9*X + 37.88(R^2 = 0.96, *P*< 0.001, df = 5); SMC,Y = 3.266*X + 4.832(R^2 = 0.95, *P*< 0.001, df = 5); where Y = Percentage of Egg hatching, x is the hours of exposure.

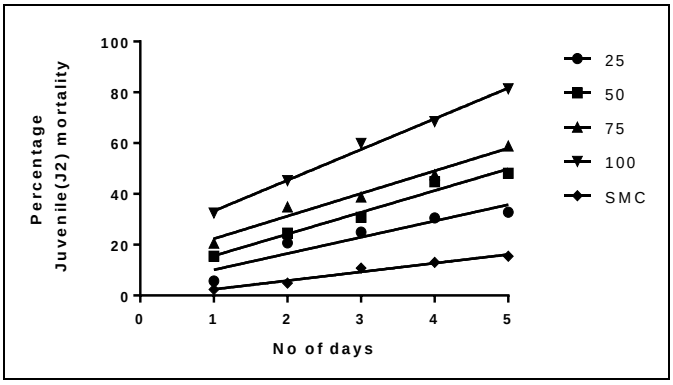

Fig 2: Percentage of Juvenile mortality of different cell extracts of *P. Putida* IIHR PP-17 on *M. incognita* exposure to 25 to 100% concentrations for various time interval. Regression equation for curves on 25%, Y= Y = 6.411*X + 3.691 (R^2 = 0.88, *P*< 0.001, df = 5); 50%, Y = 8.554*X + 7.066 (R^2 = 0.97, *P*< 0.001, df = 5); 75%, Y = 8.915*X + 13.41 (R^2 = 0.97, *P*< 0.001, df = 5); 100%, Y = 12.1*X + 21.17 (R^2 = 0.99, *P*< 0.001, df = 5); SMC, Y = 3.429*X - 0.999 (R^2 = 0.96, *P*< 0.001, df = 5); where Y = Percentage of Juvenile mortality, x is the hours of exposure.

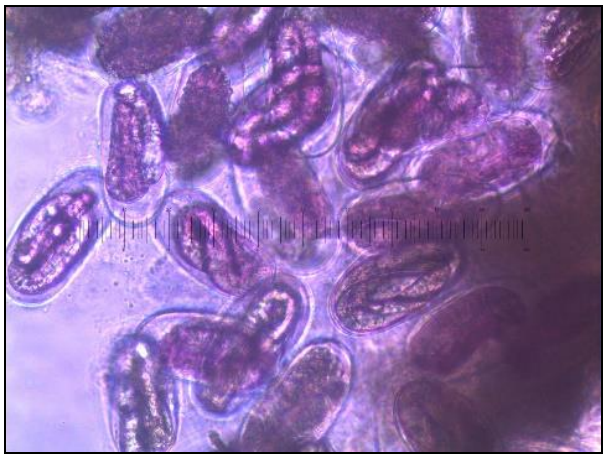

Fig 3: Control – healthy eggs

Fig 4: *P. putida* treated – nematode development affected inside the egg shell

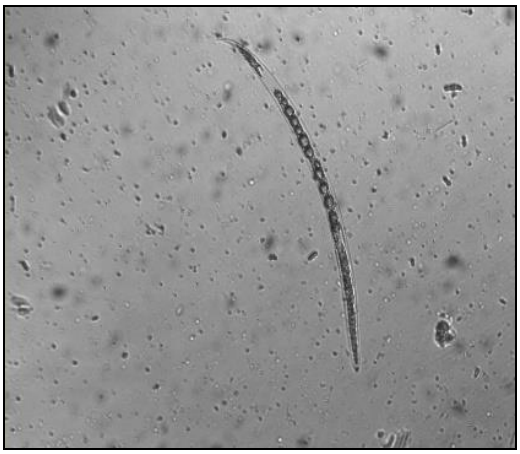

Fig 5: Dead nematode

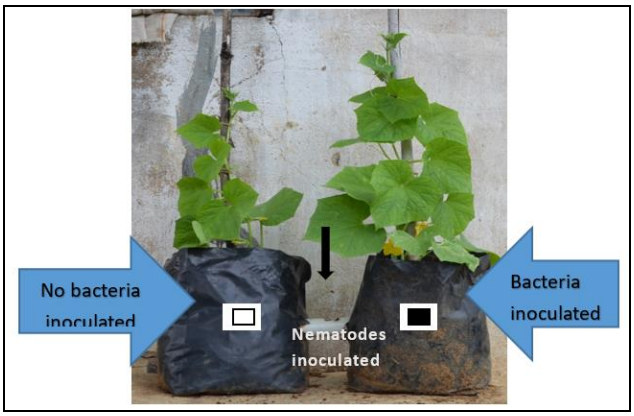

Fig 6: Effect of bacterial antagonists on repellence of *M. incognita* (J2). Juveniles were attracted and repelled from a tube connecting two pots either to the side inoculated with an antagonistic strain or to the opposite side into gherkin roots. Error bars indicates standard error.

Table 1: Evaluation of IIHR PP-17 on *M. incognita* under Twin pot set-up. All the data were mean of thirteen replicates. Indicated results were significantly (*P*<0.01) different from the control.

Treatments	Nematode population Root/g	Nematode population Soil/100 cc	RKI
Control	60.0 ±2.77	199±0.83	4.9±0.19
IIHR PP 17**	42.3±4.03	120±3	2.8±0.27

Conclusion

In summary, *P. putida* IIHR PP 17 showed antagonistic activity against *M. incognita* by ovicidal, larvicidal action *in vitro* and repellent action *in vivo.* This might be due to production of various antibiotic compounds, nematicidal volatiles with different modes of action against nematodes. Moreover, the further research is necessary to understand the molecular pattern and volatile compounds produced by the strain and also evaluate the effectiveness of the strain in greenhouse and field conditions.

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