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INFLUENCE OF ANTAGONIST RHIZOBACTERIA ON ATTACHMENT OF *PASTEURIA PENETRANS* AND THEIR NEMATICIDAL POTENTIAL AGAINST ROOT KNOT NEMATODE (*M. INCOGNITA*)

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A B S T R A C T

Root-knot nematodes have wide host range, causing damage to many annual and perennial crops. Therefore the present study was planned to check the effect of PGPRs on juvenile's mortality and egg hatching inhibition *of M. incognita* and attachment of *Pasteuria penetrans* with second stage Juveniles under lab conditions. All PGPR increased the spores attachment with second stage juveniles, maximum spores attachment (194.23 %) was found in *E. cloacae* and minimum spores attachment (142.3 %) was found in *E. areogenis* after 24 hours. All the PGPRs caused larval mortality and inhibition in egg hatching with varying degrees. Maximum eggs inhibition (75.48 %) was found in *P. flourescencs* and minimum eggs inhibition (69.47 %) was found in *E. areogenis* after six days and maximum juvenile's mortality (88%) was found in *P. flourescencs* and minimum juvenile's mortality (78%) was found in *E. areogenis* after two days.

Keywords: PGPR; RKN; Eggs; Juveniles; Mortality; Hatching; *Meloidogyne incognita*

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INTRODUCTION

Meloidogyne spp. is obligate sedentary endoparasites of host plants which attack plant roots. Five root-knot species viz. *M. arenaria, M. graminicola, M. hapla, M. incognita,* and *M. javanica* out of more than 100 known *Meloidogyne* spp. are found more frequently in Pakistan as well as all over the world as major pests of vegetables, fruit plants and field crops (Anwar, 1989; Anwar et al., 1991; Anwar and Khan, 1992; Anwar and McKenry, 2012; Eisenback et al., 1981; Fourie and McDonald, 2000; Hunt and Handoo, 2009; Maqbool et al., 1988; Maqbool, 1986; Mateille et al., 2000; Menjivar et al., 2011; Moens et al., 2009; Sasser, 1979, 1980; Sasser and Freckman, 1987). Root knot nematodes are polyphagous and more than 3000 plant species have been reported as hosts of these nematodes (Abad et al., 2003; Agrios, 2005). Due to such wide host arrange, root knot nematodes cause major economic damage to vegetables, fruit plants and field crops and an estimated loss of 125 billion $ occurs annually worldwide (Chitwood, 2003; Collange et al., 2011; Dodzia et al., 2012; Koenning et al., 1999; Williamson and Hussey, 1996). In Pakistan as well as worldwide, 10-100% yield losses on vegetables were reported by many scientists (Anwar and McKenry, 2012; Kamran et al., 2010; Shahid et al., 2007).

Many management strategies i.e. host plant resistance; cultural practices, physical, biological and chemical methods are used for the management of root knot nematodes but chemicals have given relatively quick and better results to farmers. Therefore, some microbial antagonists were potentially used in the replacement of chemical nematicides against root knot nematodes (Barker and Koenning, 1998; Brand et al., 2010; Hussain et al., 2014; Jairajpuri et al., 1990; Mukhtar et al., 2013; Mukhtar et al., 2017; Nico et al., 2004; Siddiqui and Shaukat, 2003; Sikora and Fernandez, 2005; Veremis and Roberts, 1996; Whitehead, 1998). Plant growth promoting rhizobacteria

(PGPR) have the potential as bio-control agents to substitute chemicals because they are ecofriendly and significantly reduce the disease.

MATERIALS AND METHODS

Single egg mass culture of *Meloidogyne incognita*

Infected plants with KN were collected during survey. Plant roots were washed under tap water and cut in to large pieces to avoid egg masses from damage. Seven weeks old singly grown plants were inoculated with an egg mass in root zone which was isolated from infected roots with the help of needle under stero microscope. The plants root were covered again with soil and watered.

Identification of *Meloidogyne* spp.

An egg mass inoculated plant was harvested and 12 weeks after inoculation of egg mass, some root pieces were select having egg masses. Under microscope egg were removed and female were isolated by teasing with the help of inoculating needle and placed the mature females in 45% lactic acid to Harding cuticles of females. Adult female was placed on cavity slide having one drop of water. The interior part of female was cut and removed the debris from female body with the help of fine camel hair brush and then shifted the cut portion of female to the next cavity slide which had only one drop of glycerin and placed a cover slip. Under microscope pattern was examined and identified on base of perineal pattern of M. incognita (Eisenback et al., 1981; Jepson, 1987). Cover slip was sealed with wax for further use in feature.

Mass culturing of root-knot nematode (*Meloidogyne incognita*)

The susceptible eggplant variety Dilnasheeen was used for KN mass culturing. 42 days old eggplant singly transplanted seedling were inoculated with 3000 juveniles in root zone by making three holes with sharp pointed wooden stick to avoid the root damage by stick (Campos and Campos, 2005). The holes were enclosed with steam sterilized soil to avoid drying. After inoculation, watered the pots carefully and avoided excessive watering and pots were kept at 30 + 4 ^{0}C temperature in green house.

Extraction of M. incognita eggs to obtain the juveniles

Ninety days old RKN infected egg plants were harvested and washed carefully under the tap water to avoid the loss of egg masses from roots. For the collection of eggs from infected plants roots were cut in to pieces and put in flask having 200 ml 1% NaOCI. Then this flask was shaked well for 1-2 min for the releasing of eggs from egg mass matrix genitial material (Hussy and Barker, 1973). The mixture was passed through to 75 um sieve followed by 38 um sieve to collect the eggs. Plant material remained on 75 um sieve and eggs were on the 38 um sieve which was rinsed in cool tape water to overcome the effect of NaOCI for many min. Eggs were collected in a baker and incubated at 30+ 2 °C for 4 day on the Bearmann funnel method for eggs hatching to collect the juveniles. The J2 suspension was collected after every 24 h. The first collection was discarded because:

1) It was mixture of few older juveniles and eggs
2) Uniform and larger number of juvenile was available within the next 24 hours
3) If juvenile's suspension was to be required to stay in the laboratory for more than 24 hours then volume was reduced and oxygen was provided by an air pump.

Concentration of juveniles

Juvenile's suspension was settled for 3-4 hours and then excess water was siphoned off without disturbing the nematodes in the bottom with the help of saffin. This process is slow but juveniles loss was less.

Counting and standardization of juveniles

The juvenile's suspension was poured into a beaker to estimate the inoculum density The suspension was mixed thoroughly by blowing and stirring with pipette. The no of J2s were estimated in I ml suspension in a counting chamber under a stereo microscope. The total juveniles population was estimated by multiplying the mean of five aliquots with the total volume.

Concentration of eggs suspension

Egg suspension was allowed to stay for 3-4 hours and then excess water was poured off without disturbing the eggs in the bottom with the help of saffin. This process was slow but eggs loss was less.

Counting and standardization of eggs

The eggs suspension was poured into a measuring cylinder to estimate the inoculums density. The suspension was mixed thoroughly by blowing and stirring with pipette. The no of eggs were estimated in 1-ml suspension in a counting chamber under a stereo-microscope. The method was repeated five times and the total no. of eggs were estimated by multiplying the mean of five aliquots with the total volume.

Collection of PGPR

The cultures of PGPR were collected from Institute of Agriculture, The Punjab University, Lahore. Plant growth promoting rhizobacteria included *B. subtilis, E. cloacae, E. aerogenes and P. fluorescens*.

Multiplication of PGPR

Plant growth promoting rhizobacteria were multiplied on LB broth. Prepared the LB broth and picked the pure healthy colony of each PGPR with the help of inoculating

needle. The LB broth was inoculated and the flask was placed on shaker at 100 rpm at 25 °C for 24hrs.

Sources of *P. penetrans* utilized in experiments

Pasteuria penetrans J (a commercial product of Japan) was utilized in experiment. It was received from Dr. S. R. Gowen, School of Agriculture, Policy University of Reading U.K.

Effect of PGPR on attachment of *P. penetrans* endospores on cuticle with second stage juveniles of *Meloidogyne incognita*

The PGPR were multiplied in nutrient broth at 25°C temperature for Says. The PGPR suspensions were centrifuged at 2400 rpm for 20 min. The Cell free culture filtrates separated in 50ml eppendorf tubes and pellets were discarded. The spores of *P penetrans* (concentration 10^4/ml) were incubated with each bacterial supertant in 50 ml eppendorf tubes for one week at 25°C in the dark (Duponnoiis et al., 1999). Control treatments consisted of *P penetrans* only. The attachment tests were performed by adding 50 + 5 J2 of *M. incognita* in 1 ml of distilled water into each tube. The experiment was arranged in CRD with five treatments having five replications. The attachment experiment was done at room temperature and after 12 and 24 hours the no of spores per juvenile was counted under inverted microscope

Effect of PGPR on egg hatching of *Meloidogyne incognita*

Eggs were collected by using Hussey and Barker (1973) method from the egg masses which were collected from single egg mass culture. The 4 ml Cell free culture filtrates of PGPR were poured with help of pipette in 4 cm diam Petri plates and added 1 ml of Eggs suspension containing 200 +5 eggs. The experiment was arranged in CRD with six treatments and replicated 5 times. Petri plates have only distilled water were served as control. All Petri plates were kept at 30 +2 °C temperature in incubator. Data was recorded on the bases, numbers of hatched juveniles after 1, 3 and 6 days under stereo microscope.

Effect of PGPR on the mortality of juveniles of *Meloidogyne incognita*

Juveniles were collected by using Bearmann funnel method from the egg masses which were collected from single egg mass culture. The 4 ml Cell free culture filtrates of PGPR were poured with help of pipette in 4 cm diam Petri plates and added 1 ml of Juveniles suspension containing 100 +5 Juveniles. The experiment was arranged in CRD with six treatments and replicated 5 times. Petri plates have only distilled water were served as control. All Petri plates were kept at 30 +2 °C temperature in incubator. Data was recorded on the bases of % juveniles Mortality after 1, 3 and 6 days under stereo microscope. Then straight and motionless J2 were picked with needle and transferred in distilled water to check their mortality by teasing with needle. If they did not move then their death was confirmed (Mehmood et al., 1979). Percent juvenile's mortality was calculated by using the formula

$$\text{Percent juveniles mortality} = \frac{No.of\ juvenile\ skilled}{Total\ No.of\ juveniles} \times 100$$

RESULTS AND DISCUSSION

Effect of PGPR on attachment of *P. penetrans* spores on cuticle of with second stage juveniles of *M. incognita*

The results showed that all PGPR increased the spores attachment with second stage juveniles. After 12 hours the maximum number of spores attachment were found with J_2 in the *E. cloacae* suspension followed by *B. subtilis, P. Flourescencs,* Combine PGPR and *E. areogenis* as compared the control. No PGPR strain decreased the juveniles' attachment. Maximum spores attachment (200 %) was found in *E. cloacae* and minimum spores attachment (127.77 %) was found in *E. areogenis* After 12 hours (Figure 1). After 1 day, the maximum number of spores attachment were found on J2 in the *E. cloacae* suspension followed by *B. subtilis, P. flourescencs,* Combine PGPR and *E. areogenis* as compared the control (Table 1). Maximum spores attachment (194.23 %) was found in *E. cloacae* and minimum spores attachment (142.3 %) was found in *E. areogenis* After 24 hours (Figure 1).

Effect of PGPR on egg hatching of *Meloidogyne incognita*

It was noted that after one day, *P. flourescencs* was most effective in inhibition of eggs hatching and *E. areogenis* was least effective in reducing the egg hatching, while other treatments showed moderate effect in reducing egg hatching. As time passed the hatching of eggs in to juveniles was increased. Maximum eggs inhibition (65.67 %) was found in *P. flourescencs* and minimum eggs inhibition (46.67%) was found in *E. areogenis* after one day (Figure 2). At third day, *P. flourescencs* gave the best results in inhibiting eggs hatching and *E areogenis* gave the least effective results in reducing egg hatching. While other treatments showed the moderate effect in reducing egg hatching. Maximum eggs inhibition (75 %) was found in P. flourescencs and minimum eggs inhibition (65.71%) was found in E. areogenis after three days (Figure 2).

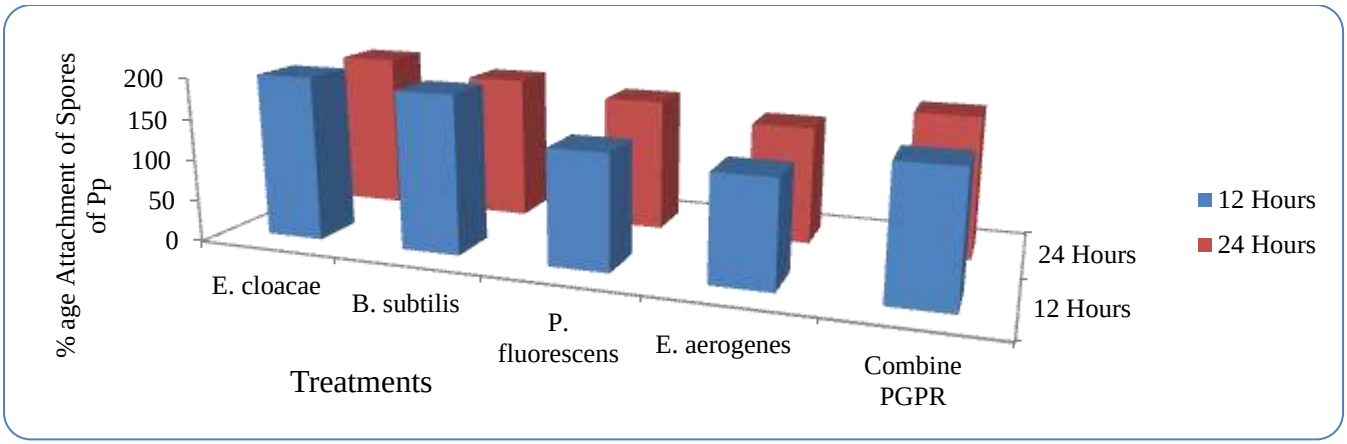

Figure 1. Effect of PGPR on attachment of *P. penetrans* spores on cuticle of with second stage juveniles of *M. incognita.*

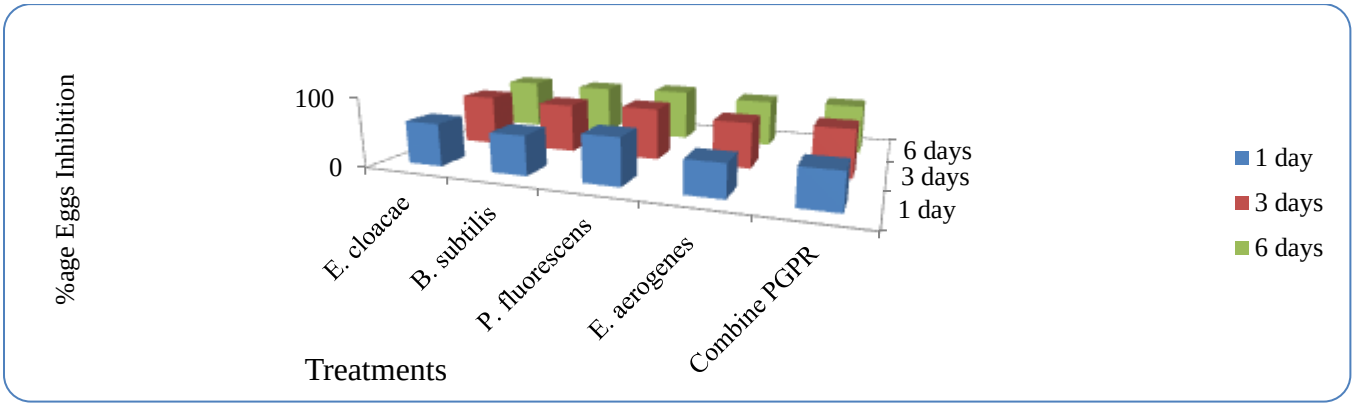

Figure 2. Effect of PGPR on egg hatching of *Meloidogyne incognita.*

Table 1. Effect of PGPR on attachment of *P. penetrans* spores on cuticle of with second stage juveniles of *M. incognita.*

Treatments	12 Hours	24 Hours
E. cloacae	72 a	101 a
B. subtilis	69 a	92 b
P. fluorescens	50 c	84 c
E. aerogenes	46 c	74 d
Combine PGPR	56 b	88 bc
Control	36 d	52 e

At six day, once again *P. flourescencs* gave the best results in inhibiting eggs hatching and *E. areogenis* gave the least effective results in reducing the egg hatching. While other treatments showed the moderate effect in reducing egg hatching (Table 2). Maximum eggs inhibition (75.48 %) was found in *P. flourescencs* and minimum eggs inhibition (69.47 %) was found in *E. areogenis* after six days (Figure 2). These results showed that while the eggs remained within the PGPR eggs did not hatch.

Effect of PGPR on the juveniles mortality of *Meloidogune incognita*

It was noted that after 12 hours *P. florescence* was the most effective and *E. preogenis* was least effective in juvenile's mortality. The other treatments showed the moderate effect in juvenile's mortality with time increasing the juvenile's mortality was increased. Maximum juvenile's mortality (68 %) was found in *P. flourescencs* and minimum juvenile's mortality (56%o) was found in *E. areogenis* after I2 hours (Figure 3). After one day, *P. flourescencs* gave the best results in juvenile's mortality and *E. areogenis* gave the least effective results in juvenile's mortality. While other treatments showed the moderate effect in juvenile's mortality. Maximum juvenile's mortality (80 %) was found in *P. flourescencs*

and minimum juvenile's mortality (70 %) was found in E. areogenis after one day (Figure 3).

After two days once again *P. flourescencs* gave the best results in juvenile's mortality followed by *B. subtilis*, E. cloacae, and Combine PGPR and *E. areogenis* (Table 3). Maximum juvenile's mortality (87 %) was found in *P. flourescencs* and minimum juvenile's mortality (78 %) was found in *E. areogenis* after two days (Figure 3). The two-thirds of the total fauna of the earth are represented by nematodes which are he multi cellular animals (Khan, 1993). Root-knot nematodes are widely distributed in vegetable crops and cause significantly yield losses in Pakistan.

Table 2. Effect of PGPR on egg hatching of *Meloidogyne incognita*

Treatments	1 day	3 days	6 days
E. cloacae	23 e	37 e	48 e
B. subtilis	26 d	41 d	51 d
P. fluorescens	20 f	35 e	46 e
E. aerogenes	32 b	48 b	58 b
Combine PGPR	29 c	44 c	54 c
Control	60 a	140 a	190 a

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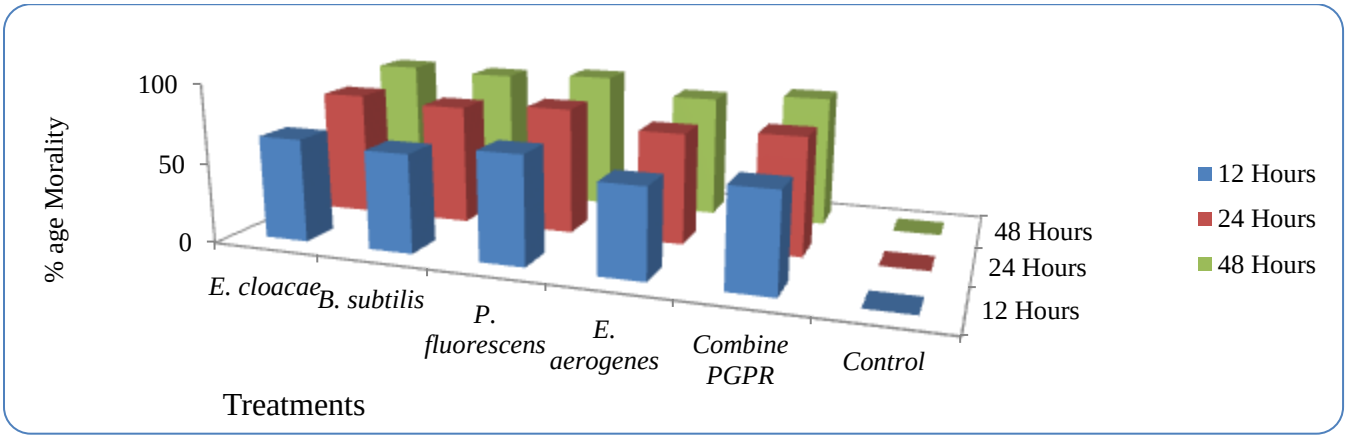

Figure 3. Effect of PGPR on the juveniles mortality of *Meloidogune incognita.*

The attachment of Pp spores with second stage juveniles was found effected in the presence of different PGPR. Spores attachment increased due to structural modification of the Pp spores by the PGPR. In particular, the sporangial wall and exosporium could be changed exposing the parasporal fibres and allowing them to make contact with the juvenile's cuticle Duponnoiis et al. (1999). E. cloacae could act on the penetration and promote the vegetative growth and development of P. penetrans inside the nematode because it behaves like wall of *M. areanira*. More them 100% spores attachment was noted in the E. cloacae as compared to control which is in agreement with Duponnoiis et al. (1999).

Exposure of RKN eggs to culture filtrates of different PGPR significantly inhibited the egg hatching. These different antagonistic PGPR have been showed nematicidal effects on eggs hatching and juvenile's mortality. Many researchers investigated the effects of PGPR metabolites, toxins and suspensions against egg hatching and juvenile mortality of *Meloidogyne* spp. El-Moneim and Massoud (2009) tested the endotoxin of four isolates of *Bacillus thuringiensis* (AI, AII, AIII and AIV) against juveniles of *M. incognita*. AI and AII isolates with crystal toxin (1×10^8 crystal/ml) gave the best results against J2s. Ravari and Moghaddam (2015) tested the nematicidal activity of two *B. thuringensis* strains (ToIr 65 and ToIr 67) against eggs and juveniles of *M. javanica* in the form of bacterial suspension and spore/crystal mixture in lab. Bacterial suspensions of two strains revealed 70% nematicidal efficacy as compared to spore/crystal mixture. Meyer et al. (2009) evaluated 2,4-diacetylphloroglucinol (DAPG) antibiotic produced by various isolates of *P. fluorescens* against eggs and hatched juveniles of many plant parasitic nematodes (*Pratylenchus scribneri, Xiphinema americanum, Heterodera glycines* and *M. incognita*) and bacterial feeder nematodes (*Rhabditis rainai, Caenorhabditis elegans* and *Pristionchus pacificus*)

in different doses from 0 to 100 mg/ml of DAPG in laboratory. Antibiotic DAPG decreased the egg hatching.

The nematicidal action of culture filtrates of PGPRs against *Meloidogyne* spp. and other plant parasitic nematodes might be ascribed to the production of certain enzymes (Segers et al., 1994; Webb et al., 1972) and toxins (Minato et al., 1973) which help in weakening and dissolving the barriers of their hosts. In the current study, PGPRs strains effectively inhibited egg hatching by changing the eggshell structures due to protease, chitinase and chitosanase activities. Soliman et al. (2019) showed that eggs exposed to chitinase enzyme, more and large vacuoles in the chitin layer of eggshell resulting in an increase in percentage mortality of juveniles. These results agree with those described by Mercer et al. (1992). Soliman et al. (2019) also found that *B. subtilis* and *Paenibacillus polymyxa* produced maximum chitinase enzyme which caused egg hatching inhibition and J2s mortality. From this perspective, Sohrabi et al. (2018) reported that the rhizobacterial strains *P. polymyxa* and *B. subtilis* significantly caused reductions in the juvenile population of *M. javanica*. Batool et al. (2013) revealed that above 90% mortality of *M. javanica* in *in vitro* studies occurred due to high production of chitinase. The bacterial strains tested in the current study may be promising biocontrol agents as PGPR for the future nematode management strategies.

CONFLICTS OF INTEREST

The authors declared no conflict of interest. The funders had no part in the design, collection analyses and interpretation and writing of short communication.

AUTHOR'S CONTRIBUTION

All authors contributed and supported towards writing of this manuscript.

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