Research Article

Suppressive Effect of Certain Bacterial Isolates of *Pseudomonas fluorescens* Suspensions on Root-Knot Nematode, *Meloidogyne incognita*

Ahmed E.A. Mahgoob[1], Wafaa M.A. El-Nagdi[2], Mahmoud M.A. Youssef[2]*, Entesar H. Taha[1], Mona M.S. Zayed[3], Hassan Abd- El-Khair[4] and Nora R.A. Saleh[2]

[1]Plant Protection Department, Faculty of Agriculture Ain Shams University, P.O. Box 68, Hadayek Shoubra 11241, Cairo, Egypt; [2]Department of Plant Pathology, Nematology Laboratory, National Research Centre, Dokki, 12622, Cairo, Egypt; [3]Microbiology Department, Faculty of Agriculture, Ain Shams University, P.O. Box 68, Hadayek Shoubra 11241, Cairo, Egypt; [4]Department of Plant Pathology, National Research Centre, Dokki, 12622, Cairo, , Egypt.

Abstract | This investigation was planned with the aim to evaluate different suspensions of certain isolates of bacterium, *Pseudomonas fluorescens* for their nematicidal potentials against root-knot nematode, *Meloidogyne incognita* under *in vitro* conditions. The present results indicated that the tested bacterial isolates at two concentrations (25 and 50%) significantly ($p \leq 0.05$) inhibited *M. incognita*- egg hatching at each exposure period (24, 48, 72 and 96 hrs) compared to untreated control. As for the percentages of the second stage juveniles mortality increased with increasing the concentrations and exposure periods. Generally, the highest egg hatching inhibition and juvenile mortality occurred after 96 and 72hrs exposure to the suspensions of the tested isolates of *P. fluorescens* depending upon their concentrations and exposure periods, respectively.

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***Correspondence** | Mahmoud M. A. Youssef, Department of Plant Pathology, Nematology Laboratory, National Research Centre, Dokki, 12622, Cairo, Egypt; **Email:** myoussef_2003@yahoo.com
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Introduction

Root-knot nematodes of the genus, *Meloidogyne* are sedentary endoparasites that induce root-knot symptoms and cause serious agricultural damage (Trudgill and Blok, 2001). Currently, various attempts have been employed to fight these pests include plant breeding, field sanitation, crop rotation, and chemicals. Although some success has been reported, the use of these methods, particularly agrochemicals, has severe limitations. Increasing interest has been directed toward nematode biological control methods by using antagonistic bacterium, P*seudomonas fluorescens* isolates (Saleh *et al.*, 2020; El-Nagdi *et al.*, 2022) and other growth promoting rhizobacteria (Youssef *et al.*, 2017; Abd-El-Khair *et al.*, 2019; Abhishek-Gowda *et al.*, 2022). Under *in vitro* conditions, El-Hamshary *et al.* (2006), when studied survival of *M. incognita*

second stage juveniles (J_2s) as affected by *P. fluorescens* and *P. aeruginosa*, they found that percentages of mortality of the J_2s occurred depending upon the bacterial concentration and exposure time. Ashoub and Amara (2010) reported that several bacteria from which *P. fluorescens in vitro* can cause *M. incognita* juveniles 100% mortality after 72 hrs. Also, the highest concentration (S) of *P. fluorescens* was shown to cause the highest nematode mortality (100%) with net mortality (71%) after 72hr compared to other concentrations (Osman *et al.*, 2013). Under greenhouse conditions, Saleh *et al.* (2020) found that *P. fluorescens* isolates, Pf_1, Pf_2, Pf_9 and Pf_{10} were the most effective in reducing *M. incognita* parameters and consequently improved plant growth criteria. El-Nagdi *et al.* (2022) proved that the ability of these mentioned isolates to reduce *M. incognita* depended on the time of addition to infected plants. Yet, little work was done on the bio-efficacy of different isolates of *P. fluorescens* on root –knot nematode under *in vitro* and *in vivo* conditions. Therefore, this research was planned with the purpose to evaluate different isolates of *P. fluorescens* for their nematicidal potentials against root-knot nematode, *M. incognita* under *in vitro* conditions.

Materials and Methods

Pure culture of root-knot nematode and its identification
Root-knot nematode, *Meloidogyne* sp. pure culture was maintained on eggplant cv. Ice using a single egg-mass in screen house at 30±5 °C. The tested species of root-knot nematode was identified to be *M. incognita* from adult females based on their morphological characteristics of perineal pattern (Taylor and Sasser 1978).

Extraction of nematode eggs
Root-knot nematode, *M. incognita* eggs were extracted from egg-masses on infected eggplant roots as described by Hussey and Barker (1973). Then, extracted eggs were counted for bioassay.

Nematode egg hatchability bioassay
The effect of the tested 10 bacterial isolates (each contained 10^{-7}-10^{-9} colony forming unit (CFU)/ml) was determined based on the number of non- hatched eggs at two concentrations (25 and 50 %) of each bacterial isolate suspension by adding distilled water. A total of 200 nematode eggs in 1 ml of distilled water was put in plastic capsule containing 9 ml suspension of each bacterial isolate in five replicates. Treatment with eggs in distilled water only with the same replicates served as untreated control. After 24, 48, 72 and 96 hr at each concentration, the numbers of non-hatched and hatched eggs were observed by light microscope. The percentages of egg hatching inhibition were calculated according to Abbott's Formula (Finney, 1971).

$$Egg\ inhibition\ (\%) = (m - n)/(100 - n) \times 100$$

As the percentages of non-hatched eggs were represented by m and n for the treatment and control, respectively.

Extraction of Meloidogyne incognita second stage juveniles (J_2s)
M. incognita eggs were extracted from galled eggplant roots bearing their egg masses by washing thoroughly with tap water to avoid debris and cut into small pieces. Then, they were placed in plastic capsule containing sufficient water to help hatching and covered to avoid loss of water by evaporation. After eggs hatching, they were collected every 24 hrs and newly hatched second stage juveniles (J_2s) were used for bioassay.

Nematode juvenile survival bioassay
To assess the effect of 10 bacterial isolate suspensions [9 bacterial isolates +1 standard isolate (NRC)] on J_2s of *M. incognita*, the same procedures were carried, when a total of 200 J_2s in 1 ml distilled water was added to 9 ml suspension of each bacterial isolate in 5 replicates. Check control treatment in the same number of replicates served as comparison. The percentages of mortality of dead and live juveniles per each treatment were calculated after 24, 48 and 72 hrs following to Abbott's Formula (Finney, 1971) as follows:

$$Juvenile\ mortality\ (\%) = (m - n)/(100 - n) \times 100$$

As the percentages of dead juveniles in the treatment and control were represented by m and n, respectively.

Source of Pseudomonas fluorescens isolates
Soil samples, each of an aliquot of 200g were gathered from the eggplant, pepper and tomato rhizospheres. The collective samples were root-knot nematode-free. Then, they were transported to Plant Pathology Department (PPD), National Research Centre (NRC) to isolate and identify bacterial isolates.

Isolation and identification of Pseudomonas fluorescens

The total plate counts technique and dilution method were used to isolate *P. fluorescens*, as described by Ghini *et al*. (2007), Schaad (1980); Lelliot and Stead (1987) and Goszczynska *et al*. (2000). Bacterial inoculum for each isolate was justified to 10^7-10^9 colony forming unit (CFU)/ml by turbidity method (Baid *et al*., 2000). Nine *P. fluorescens* isolates were isolated and identified as reported by Saleh *et al*. (2020). Bacterial inoculum for each isolate was used as bacterial cells and cultural filtrate mixture.

Source of standard Pseudomonas fluorescens isolate

One standard *P. fluorescens* Pf_{10} (NRC isolate) was obtained from PPD, NRC. The inoculum of this isolate was prepared as mentioned before and used.

Statistical procedures

Analysis of the current data was done by using Duncan's Multiple Range Test for separation the means of treatments (Duncan, 1955). ANOVA test was also used at $p \leq 0.05$ to prove the significance of the data (Gomez and Gomez, 1984). This was done by Computer Statistical (COSTAT) software.

Table 1: *Mean numbers of non- hatched eggs of root- knot nematode, Meloidogyne incognita as influenced by certain isolates of Pseudomonas fluorescens at 25% concentrations after 24, 48, 72 and 96 hrs of exposure.*

Treatments	Exposure period (hr)			
	24	48	72	96
	No. of			
	Non-hatched eggs	Non-hatched eggs	Non-hatched eggs	Non-hatched eggs
Control water +eggs	200a	200a	164a	140b
Pf_1+Eggs	200a	200a	167a	143ab
Pf_2+Eggs	200a	200a	167a	167ab
Pf_3+Eggs	200a	200a	173a	147ab
Pf_4+Eggs	200a	200a	171a	165ab
Pf_5+Eggs	200a	200a	180a	163ab
Pf_6+Eggs	200a	200a	156a	147ab
Pf_7+Eggs	200a	200a	177a	171ab
Pf_8+Eggs	200a	200a	170a	143ab
Pf_9+Eggs	200a	200a	184a	177a

-Values are means of 5 replicates. Means followed by the same letter(s) are not significantly ($p \leq 0.05$) different according to Duncan's Multiple Range Test.

Results and Discussion

Effect of Pseudomonas fluorescens on egg hatchability of root-knot nematode, Meloidogyne incognita.

The tested bacterial isolates of *P. fluorescens* at two concentrations (25 and 50%) significantly ($p \leq 0.05$) inhibited *M. incognita* egg hatching at each exposure time compared to that of untreated control as shown in Tables 1 and 2. Generally, In Table 3, egg hatching occurred at 72 and 96 hrs. But after 24 and 48 hrs, no egg hatching occurred. By using 25% concentration, it was noticed that Pf_9 recorded the highest percentage of egg inhibition (55.6%) after 72 hrs followed by Pf_5 (44.4%) and increased to 61.7 and 70.0% at 96 hrs, respectively. At 50%, the highest percentages of egg inhibition (83.3 and 72.2%) were achieved after 72hr by Pf_9 and Pf_7, but they decreased to 70 and 58.3%, after 96hr, respectively.

Table 2: *Mean numbers of non-hatched eggs of root-knot nematode, Meloidogyne incognita as influenced by certain isolates of Pseudomonas fluorescens at concentrations of 50% after 24, 48, 72 and 96 hrs of exposure.*

Treatments	Exposure period (hr)			
	24	48	72	96
	No. of			
	Non-hatched eggs	Non-hatched eggs	Non-hatched eggs	Non-hatched eggs
Control (eggs + water)	200a	200a	164b	140d
Pf_1+Eggs	200a	200a	177ab	168a-c
Pf_2 +Eggs	200a	200a	177ab	175a-c
Pf_3+Eggs	200a	200a	175ab	167a-c
Pf_4+Eggs	200a	200a	173ab	167a-c
Pf_5+Eggs	200a	200a	182ab	181a
Pf_6+Eggs	200a	200a	177ab	152cd
Pf_7+Eggs	200a	200a	190a	175a-c
Pf_8+Eggs	200a	200a	182ab	156b-d
Pf_9+Eggs	200a	200a	194a	182a

-Values are means of 5 replicates. Means followed by same letter(s) are not significantly ($p \leq 0.05$) different according to Duncan's Multiple Range Test.

Effect of Pf isolates on nematode juvenile's mortality

Data in Tables 4, 5 and 6 showed the mean numbers of *M. incognita*, when treated with different 10 bacterial isolate suspensions of *P. fluorescens* at 25% and 50% concentrations after different durations (24, 48 and 72 hrs). The results indicated that, the number of dead J_2s significantly ($p \leq 0.05$) increased with increasing the concentrations of the tested isolates and exposure periods. Table 7 illustrated the mortality percent of

Table 3: *Egg inhibition % of root-knot nematode, Meloidogyne incognita as influenced by certain isolates of Pseudomonas fluorescens at 25 and 50% concentrations after 24, 48, 72 and 96 hrs of exposure.*

Treatments	Egg inhibition% at 25% concentration			
	Exposure period (hr)			
	24	48	72	96
Control (water+eggs)	0.0	0.0	0.0	0.0
Pf_1+eggs	0.0	0.0	8.3	5.0
Pf_2+eggs	0.0	0.0	8.3	45.0
Pf_3+eggs	0.0	0.0	25.0	45.0
Pf_4+eggs	0.0	0.0	19.4	41.7
Pf_5+eggs	0.0	0.0	44.4	70.0
Pf_6+eggs	0.0	0.0	0.0	11.7
Pf_7+eggs	0.0	0.0	36.1	51.7
Pf_8+eggs	0.0	0.0	16.7	5.0
Pf_9+eggs	0.0	0.0	55.6	61.7
% Egg inhibition at 50%				
Control (water+eggs)	0.0	0.0	0.0	0.0
Pf_1+eggs	0.0	0.0	36.1	46.7
Pf_2+eggs	0.0	0.0	36.1	58.3
Pf_3+eggs	0.0	0.0	30.5	45.0
Pf_4+eggs	0.0	0.0	25.0	45.0
Pf_5+eggs	0.0	0.0	50.0	68.3
Pf_6+eggs	0.0	0.0	36.1	20.0
Pf_7+eggs	0.0	0.0	72.2	58.3
Pf_8+eggs	0.0	0.0	50.0	26.7
Pf_9+eggs	0.0	0.0	83.3	70.0

Table 4: *Mean numbers of root-knot nematode, Meloidogyne incognita juveniles (J_2s) as affected by certain isolates of Pseudomonas fluorescens at 25 and 50% concentrations, after 24hr of exposure.*

Treatments	After 24 hrs exposure			
	25%		50%	
	No. of			
	Live J_2s	Dead J_2s	Live J_2s	Dead J_2s
Control (water + J_2s)	170a	30d	170a	30g
Pf_1+ J_2s	60bc	140b-d	10efg	190a-c
Pf_2+ J_2s	87b	113c	37c-e	163c-e
Pf_3+ J_2s	40b-d	160a-c	33c-f	167b-e
Pf_4+ J_2s	163a	37d	140b	60f
Pf_5+ J_2s	17cd	183ab	3g	197a
Pf_6+ J_2s	20cd	180ab	17d-f	183a-d
Pf_7+ J_2s	7d	193a	7fg	193ab
Pf_8+ J_2s	43b-d	157a-c	30d-g	170a-d
Pf_9+ J_2s	47b-d	153a-c	43cd	157de
Pf_{10} (standard) + J_2s	60bc	140b-d	30d-g	170a-d

-Values are means of 5 replicates. Means followed by same letter(s) are not significantly ($p \leq 0.05$) different according to Duncan's Multiple Range Test.

Table 5: *Mean numbers of root-knot nematode, Meloidogyne incognita juveniles (J_2s) as affected by certain isolates of Pseudomonas fluorescens at 25 and 50% concentrations, after 48hr of exposure.*

Treatments	After 48hrs exposure			
	25%		50%	
	No. of			
	Live J_2s	Dead J_2s	Live J_2s	Dead J_2s
Control (water + J_2s)	140a	60c	140a	60b
Pf_1+ J_2s	0c	200a	0b	200a
Pf_2+ J_2s	0c	200a	0b	200a
Pf_3+ J_2s	0c	200a	0b	200a
Pf_4+ J_2s	77b	123b	0b	200a
Pf_5+ J_2s	10c	190a	0b	200a
Pf_6+ J_2s	0c	200a	0b	200a
Pf_7+ J_2s	0c	200a	0b	200a
Pf_8+ J_2s	0c	200a	0b	200a
Pf_9+ J_2s	0c	200a	0b	200a
Pf_{10}(standard) + J_2s	0c	200a	0b	200a

-Values are means of 5 replicates. Means followed by same letter(s) are not significantly ($p \leq 0.05$) different according to Duncan's Multiple Range Test.

Table 6: *Mean numbers of root-knot nematode, Meloidogyne incognita juveniles (J_2s) as affected by certain isolates of Pseudomonas fluorescens at 50 and 25% concentrations after 72hr exposure.*

Treatments	After 72hrs exposure			
	25%		50%	
	No. of			
	Live J_2s	Dead J_2s	Live J_2s	Dead J_2s
Control (Water + J_2s)	140a	60b	140a	60b
Pf_1+ J_2s	0b	200a	0b	200a
Pf_2+ J_2s	0b	200a	0b	200a
Pf_3+ J_2s	0b	200a	0b	200a
Pf_4+ J_2s	0b	200a	0b	200a
Pf_5+ J_2s	0b	200a	0b	200a
Pf_6+ J_2s	0b	200a	0b	200a
Pf_7+ J_2s	0b	200a	0b	200a
Pf_8+ J_2s	0b	200a	0b	200a
Pf_9+ J_2s	0b	200a	0b	200a
Pf_{10}(standard) + J_2s	0b	200a	0b	200a

-Values are means of 5 replicates. Means followed by same letter(s) are not significantly ($p \leq 0.05$) different according to Duncan's Multiple Range Test.

M. incognita J_2s. Generally, their mortality percent was increased by increasing concentration and exposure time. By using the concentration of 25%, Pf_7 induced the highest percentage mortality (95.9%)

after exposure time of 24 hrs and reached 100% after 48 and 72 hrs, followed by 90.0% occurred by Pf_5 which reached 92.2% after 48 hrs, then 100% after 72 hrs. At 50%, the highest percentage nematode mortality (98.2%) was achieved by using Pf_5 after 24 hrs followed by 95.9, 94.1 and 90.0% caused by Pf_7, Pf_1 and Pf_9 at the same period, respectively. However, at 48 and 72 hrs all bacterial treatments caused full mortality of J_2s.

Table 7: *Mortality % of root-knot nematode, Meloidogyne incognita juveniles (J_2s) as affected by certain isolates of Pseudomonas fluorescens at 25 and 50% concentrations, after 24, 48 and 72hrs of exposure.*

Treatments	Exposure period (hr)					
	Mortality% at 25%			Mortality% at 50%		
	24	48	72	24	48	72
Control (Water+J_2s)	0	0	0	0	0	0
Pf_1+ J_2s	64.7	100.0	100	94.7	100	100
Pf_2+ J_2s	48.8	100.0	100	78.2	100	100
Pf_3+ J_2s	76.5	100.0	100	80.6	100	100
Pf_4+ J_2s	4.10	45.0	100	17.6	100	100
Pf_5+ J_2s	90.0	92.9	100	98.2	100	100
Pf_6+ J_2s	72.4	100.0	100	74.7	100	100
Pf_7+ J_2s	95.9	100.0	100	95.9	100	100
Pf_8+ J_2s	74.4	100.0	100	82.4	100	100
Pf_9+ J_2s	88.2	100.0	100	90.0	100	100
Pf_{10} (standard)+J_2s	64.7	100.0	100	82.4	100	100

Certain genera, species and isolates of bacteria were reported to be highly toxic to nematodes showing the most lethal activity (Ashoub and Amara, 2010; Mokbel and Alharbi, 2014; Elkelany *et al.*, 2020; Mohamed *et al.*, 2021). In the present study, there was great inhibition of egg hatching to J_2s, when fresh eggs of *M. incognita* were exposed to the tested isolates of *P. fluorescens* suspensions at various concentrations and different periods. The most obvious egg inhibition was caused by Pf_9 and Pf_5 at 25% after 96hr and by Pf_9 and Pf_7 at 50% after 72hr. The inability of the eggs to hatch is due to absorption or ingress of bacterial toxic suspensions into the eggs. The mobility of juveniles inside eggs is reduced and finally leading up to death or moribund state. As a result, these juveniles cannot penetrate through the egg shell with their stylet, and hatching will be ceased as shown by El-Gayed *et al.* (2017). This was emphasized by Hirschmann (1985) who stated that, the egg-mass which is found in posterior region of the nematode female in root-knot allows absorbing the active toxic ingredient secreted by bacterial isolates in the extracts.

As for bioassay of different *P. fluorescens* isolate suspensions on the newly hatched second stage juveniles of *M. incognita in vitro*; a great differentiation in nematicidal effects of these isolates on J_2s of *M. incognita* was achieved in the recent study. All isolates of *P. fluorescens* scored 100% mortality at 72 hrs. This result was consistent to those obtained by Ashoub and Amara (2010) and Osman *et al.* (2013). Also, the best effective isolates in reducing J_2s were Pf_7, Pf_5 and Pf_9 at all exposure times at 25% concentration. At 50% concentration, the most effective isolates were Pf_5, Pf_7, Pf_1 and Pf_9 in a descending order for their lethal effects. Hence, it was demonstrated the potentiality of all of the tested bacterial isolates to kill and immobilize J_2s of *M. incognita* at different degrees. This variation may be due to different modes of action of the isolates. In this trend, Tian *et al.* (2007) showed that certain natural bacterial antagonists of nematode pests can reduce nematodes by producing toxins, antibiotics or enzymes; causing direct inhibition of nematodes. Accordingly, Kerry (2000) explained that, rhizospheric bacteria can act by more mechanisms such as direct effect by production of toxins, enzymes and other secondary metabolites.

Conclusions and Recommendations

There was great inhibition in egg hatching of root-knot nematode to juveniles, when laid eggs of *M. incognita* were exposed to the tested isolates of *P. fluorescens* suspensions at various concentrations and exposure periods *in vitro*. Also, *P. fluorescens* isolates, when added on newly hatched juveniles of *M. incognita in vitro*, exhibited a great variation in their nematicidal effects on J_2s of *M. incognita*. Some isolates were more efficient in reducing egg hatchability and juvenile's mortality than others. More studies on the promising bacterial isolates for reducing root-knot nematodes and their role in integrated pest management under greenhouse and field conditions are needed.

Novelty Statement

The current study indicated that certain isolates of the antagonistic bacterium, *P. fluorescens* have nematicidal potentials, when used at different concentrations and exposure periods on root-knot nematode, *M. incognita* egg hatching and juveniles' mortality. These bacterial isolates proved to be acted as a biocontrol agent within sustainable pest management.

Author's Contribution

AEAM, WMAEN and MMAY supervised this work, design, writing and execution of this manuscript. EHT and MMSZ supervised the work, provided the facilities during this work and reviewed the manuscript. HAEK isolated and identified the tested bacterial isolates and NRAS carried out the experiment and examined it in the laboratory. All authors read and approved the final manuscript,

Conflict of interest

The authors have declared no conflict of interest.

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