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In vitro Study on the Antagonistic Activity of Different Native Isolates of Rhizobacteria Against *Meloidogyne incognita*

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Abstract

The nematicidal properties of eight isolates of rhizobacteria as well as an isolate of *Pseudomons fluorescens* against egg hatching and juveniles mortality of *Meloidogyne incognita* after different exposure periods were investigated *in vitro*. Results indicated that all tested bacteria caused significant effects on *M. incognita* with different percentages of egg hatching inhibition and juveniles mortality. Out of bacterial isolates, 2KT (54.0%), 3SN (49.2%) and *Pseudomonas fluorescens* (46.4%) significantly induced the greatest egg hatching inhibition of *M. incognita*. Moreover, the longer the exposure to bacterial isolates the higher the juvenile mortality was revealed. The bacterial isolates 2KT and 3SN that exhibited pronounced nematicidal activity against *M. incognita* were identified as *Bacillus subtilis* and *P.aeuroginosa*, respectively.

Key words: *Bacillus subtilis, Meloidogyne incognita*, *Pseudomonas aeuroginosa, Pseudomonas fluorescens, rhizobacteria.*

Introduction

In Egypt, the root-knot nematodes, *Meloidogyne incognita* (Kofoid and White) Chitwood and *M. javanica* (Treub) are the most deleterious plant-parasitic nematodes contributing to stunting, wilting, poor plant growth and significant yield losses. Root-knot nematodes infected plants showed characteristic gall formation that affects both water and food absorption **(Sasser, 1989; Khan and Haque, 2011).** Use of nematicides is one of the most reliable and efficient means for the management of root-knot nematodes. However, the persistence of nematicides poses detrimental and negative impact on the environment, human health and kills non target species. The expensive cost and short-term nematode suppression has also led to regulatory constraints in the use of excessive toxic nematicides **(Pakeerathan et al., 2009).** Therefore, new and more alternative management strategies are urgently needed for the control of root-knot nematodes.

Strategies using biological control with microbical agents are considered as environmental alternative methods for the management of plant parasitic nematodes on various crops. The increase in the use of biological control in recent decades is attributed to its safety, species specific and long term action on target

species **(Sanda and Sunusi, 2014).** Plant growth-promoting rhizobacteria (PGPR) are naturally soil bacteria that could provide a promising tool for root-knot nematodes management **(Siddiqui and Ehteshamul-Haque, 2000; Padgham and Sikora, 2007; Senthamarai et al., 2008)**. Such microorganisms stimulate plant growth, colonize the roots, and can produce substances that may limit nematode reproduction e.g. producing antibiotics, siderophores and a variety of enzymes **(Padgham and Sikora, 2007).**

The rhizobacteria i.e. *Bacillus* spp., *Pseudomonas* spp., *Serratia marcescens* .etc. are among the biocontrol agents that have been received more attention for more than one decade and showed nematicidal activity against root-knot nematodes **(Sikora and Fernandez, 1992; Khan, 2009; Wahla et al., 2012 and Rhajkumar, et al., 2013).** Some strains of *B. subtilis* have exhibited enormous potential as biocontrol agents in the management of root-knot nematodes **(Karanja et al., 2007; Khalil et al., 2012).** The insecticidal rhizobacterium, *B*. *thuringiensis*, was evaluated for their nematicidal potential against *M. incognita* under laboratory conditions **(Khan, et al., 2010).** The cell free filtrate not only inhibited the hatching of eggs but also killed the second stage juveniles at different time intervals. On the other hand, the exposure of eggs and second stage juveniles (J_2) of *M. incognita* to different isolates of *Pseudomonas* spp., revealed that four isolates of *Pseudomonas* spp. (CRS3, CRS6, CRS8 and CRS10) significantly induced inhibition of egg hatching and mortality of *M. incognita* juveniles **(Rhajkumar, et al., 2013).** Out of 19 bacterial isolates, and three bioagents strains, two isolates (*P. fluorescens* and *B. subtilis*) and *S. marcsecens* exhibited the highest nematicidal activities against *M.incognita in vitro* **(Zaghloul et al., 2015).**

Accordingly, the present study was conducted *in vitro* in order to evaluated the nematicidal activity of *Pseudomonas fluorescens* and different isolates of native bacteria obtained from the rhizosphere of oil crops against the root-knot nematode, *Meloidogyne incognita.*

Material and Methods

Bacterial isolates

Eight strains of soil bacteria, isolated from the rhizosphere of oil crops grown in Dakahlia governorate as well as an isolate of *P. fluorescens* were used in the current study to assess their effectiveness on egg hatching and juveniles mortality of *M. incognita* at different times of exposure. Bacterial isolates used in this study are listed in table (1).

Bacterial Inoculum Preparation

Screened bacterial isolates and *P. fluorescens* were cultured on nutrient agar at 28°C, at Department of Microbiology, Faculty of Agriculture, Mansoura University. After an incubation period of 7 days, bacterial culture (cells and filtrates) was adjusted to 12 x 10^6 colony forming unit/ml (cfu/ ml).

Nematode Inoculum Preparation

Egg masses related to *M. incognita* previously identified females (**Taylor, et al. 1955)** were separately used to inoculate coleus plants, *Coleus blumei* L. grown in 25 cm-d plastic pots filled with sterilized sandy loam soil and kept on a clean bench under greenhouse conditions. For egg inocula, coleus roots were washed free of soil and cut into small pieces then nematode eggs were extracted using 0.5 % NaOCl solution **(Hussey and Barker, 1973).** However, for juveniles inocula, freshly second stage juveniles (J_2) were extracted from coleus infected roots by hatching.

Table (1): Code and source of screened native bacterial isolates.

No.	Isolate Code	Source of isolates
1	2KT	Cotton rhizosphere
2	3KT	Cotton rhizosphere
3	6KT	Cotton rhizosphere
4	2KA	Canola rhizosphere
5	3KA	Canola rhizosphere
6	1SN	Sunflower rhizosphere
7	3SN	Sunflower rhizosphere
8	5SN	Sunflower rhizosphere

Efficacy of bacterial isolates on egg hatching of *Meloidogyne incognita*

The present study was conducted in the Nematology Research Laboratory of the Nematological Research Unit (NERU), Faculty of Agriculture, Mansoura University, Egypt. One hundred freshly eggs of *M. incognita* were placed in glass Petri dishes (6 cm-d) containing 3 ml of tested bacterial cultures (cells and filtrates). Petri dishes free of bacterial isolates and filled with distilled water were served as control. There were ten treatments and each treatment was replicated five times. All treatments were kept in the laboratory at room temperature and laid out in a completely randomized design. The number of hatched juveniles was recorded after 10 days using the stereomicroscope and percentage of egg hatching inhibition were then calculated according to the following formula: [Egg inhibition number in treatment / Total number of eggs in treatment] x 100.

Efficacy of bacterial isolates on juveniles mortality of *Meloidogyne incognita*

The previous methodology was repeated with the nine bacterial isolates and 100 freshly second stage juveniles (J_2) of *M. incognita*. Treatments including bacterial isolates and distilled water were replicated five times. All Petri dishes were kept at room temperature and laid out in a completely randomized design. Dead nematodes were counted and recorded after 24, 48 and 72 hr with the help of stereomicroscope. Juveniles exhibited no movement and attained the shape of straight line were considered as dead. For each bacterial isolate, percentage mortality was calculated as: [Number of dead juveniles in treatment / Total number of juveniles in treatment] x 100.

Data analysis

Data were subjected to analysis of variance (ANOVA) **(Gomez and Gomez, 1984)** followed by Duncan's multiple range test to compare means **(Duncan, 1955).**

Bacterial identification

The most potent bacterial isolates that exhibited pronounced nematicidal activity against egg hatching as well as juveniles viability were subjected to bacterial identification at Pharmaceutical Department, Research National Center, Dokki, Egypt, according to morphological and biochemical diagnosis tests **(Skerman, 1967).**

Results and Discussion

Biological control is considered as safe alternative approach for nematode management, without negative effects on human, animals, plants health or the environment. The effectiveness of *P. fluorescens* and eight rhizobacteria isolates on egg hatching inhibition and juveniles mortality of *M. incognita* is depicted (Table 2). All bacterial isolates were found to have nematicidal activity against egg hatching after 10 days of exposure with different percentages of inhibition. The maximum egg hatching inhibition was significantly recorded with the bacterial isolate 2KT (54.0%). However, bacterial isolate 3SN ranked the second followed by isolate of *P. fluroescens* and bacterial isolates 3KT and 2KA with egg hatching inhibition reached 49.2, 46.4, 45.6 and 44.4%, respectively. Bacterial isolate 5SN (27.6%) revealed the least egg hatching inhibition of *M. incognita* compared to control (12.0%).

The suppressive activity of all bacterial isolates against juveniles mobility increased gradually with the increment of the exposure periods (Table 2). All bacterial isolates were found to cause substantial mortality to J_2 with percentages in juveniles mortality ranged from 39.0 – 60.0% at the three periods of exposure. Out of the nine bacterial isolates tested, 2KT, 6KT, 1SN, 3SN and *P. fluorescens* caused maximum mortality of J_2 by 60.0%, after 72 hr of exposure. Similar trend was noticed with accumulative mean of juveniles mortality. However, bacterial

isolates i.e. 3KT (43.5%), 2KA (40.5%) and 5SN (39.0%) showed the least percentages in juveniles mortality after 24 hr of exposure. Non significant differences were noticed in juveniles mortality with bacterial isolates 2KT, 6KT, 3KA, 1SN, 3SN and *P. fluorescens* at the three periods of exposure.

Table (2): Impact of *Pseudomonas fluorescens* and eight bacterial isolates on egg hatching inhibition and juveniles mortality percentages after different exposure periods.

Bacterial isolates	Egg hatching inhibition %	Juveniles mortality %			
	10 days	24 hr	48 hr	72 hr	Mean
2KT	54.0 a	52.5 a	57.0 a	60.0 a	56.5
3KT	45.6 ab	43.5 ab	55.5 a	58.5 a	52.5
6KT	33.6 ab	52.5 a	58.5 a	60.0 a	57.0
2KA	44.4 ab	40.5 b	54.0 a	55.5 a	50.0
3KA	42.0 ab	54.5 a	55.5 a	57.0 a	56.0
1SN	39.6 ab	51.0 a	57.0 a	60.0 a	56.0
3SN	49.2 ab	52.5 a	59.5 a	60.0 a	57.3
5SN	27.6 b	39.0 b	54.0 a	57.0 a	50.0
Pseudomonas fluorescens	46.4ab	52.5 a	57.0 a	60.0 a	56.5
Distilled water	12.0 c	7.5c	7.5 b	9.0 b	8.0
LSD 0.05	14.73	6.32	3.98	3.42	---

Each value is a mean of five replicates.

Means in each column followed by the same letter(s) are not significantly different at $p<0.05$ according to Duncan's multiple-range test.

All bacterial isolates demonstrated the potential to suppress egg hatching (27.6 - 54.0%) after 10 days of exposure and increase juvenile mortality (55.5 - 60.0%) after 72 hr of exposure. The two potent bacteria i.e.2KT and 3SN exhibited strong activity against egg hatching and juvenile survival were identified as *Bacillus subtilis* and *Pseudomonas aeuroginosa.* Bacterial culture of *B. subtilis* (2KT) performed the highest nematicidal activity against egg hatching (54.0%) and juvenile mortality of *M. incognita* (60.0%). This result agreed with that reported by Kavitha, et al. (2012) who found that among the six antagonistic endophytic strains

of *B. subtilis*, Bs 5 with high surfactin and iturin activity suppressed hatching of eggs and killed second stage juveniles (J2) of *M. incognita* under *in vitro* conditions. They also indicated that more than two dozen lipopeptide antibiotics, hydrolytic enzymes and other secondary metabolites were produced by the endospore-forming rhizobacterium, *B. subtilis*.

Considerable attention has been paid to plant-growth-promoting rhizobacteria (PGPR), especially the fluorescent group of *Pseudomonas* species as the best alternatives to chemicals for facilitating ecofriendly biological control of soil and seed borne plant pathogens and nematodes. Bacterial culture of the two pseudomonads*, P. fluoerescens* (46.4; 60.0%) and *P. aeuroginosa* (49.2; 60.0%), respectively, showed biocontrol activity against egg hatching and juvenile mortality of *M. incognita* after 72 hr of exposure. These results are in accordance with El Hamshary et al. (2004) who reported that *P. fluorescens* and *P. aeruginosa* affected *M. incognita* juveniles survival *in vitro*, and the mortality percentages of nematode were dependent on bacterial concentration and exposure time. The high nematicidal activity exhibited by pseudomonads could be attributed to the presence of antimetabolites e.g. 2,4-diacetylphloroglucinol, hydrogen cyanide, and pyoluteorin. Zaghloul et al. (2015) reported the high production of certain enzymes i.e. protease, chitinase and gelatinase by both *B. subtilis* B 38 and *P. fluorescens* B103.

Thus it is clear that the present *in vitro* study has aucentiated the potentials of evaluated rhizobacteria against egg hatching and juvenile mortality of *M. incognita*. However, further study is needed for the nematicidal activity of the three potent rhizobacteria i.e. *B. subtilis, P. fluoerescens* and *P.aeuroginosa* against *M. incognita* under greenhouse and field conditions.

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الملخص العربي

دراسة معملية على التأثير الأبادي لبعض العزلات المحلية من بكتريا المجال الجذري على نيماتودا تعقد الجذور (ميلودوجين إنكوجنيتا)

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تناولت الدراسة الحالية ما يلي:

١- تأثير استخدام عزلات محلية مختلفة من بكتريا المجال الجذري على معدل فقس البيض لنيماتودا تعقد الجذور

استخدمت ثماني عزلات محلية من بكتريا المجال الجذري بالإضافة إلى بكتريا *Pseudomonas fluorescens*

وتم حساب نسبة الفقس بعد ١٠ أيام من معاملة البيض وأسفرت النتائج عن الآتي:

أ- أظهرت كل العزلات خفض في نسبة فقس البيض لنيماتودا تعقد الجذور بدرجات متفاوتة مقارنة بمعاملة الكنترول.

ب- أفضل العزلات المستخدمة في خفض نسبة فقس البيض لنيماتودا تعقد الجذور هي العزلة البكتيرية *2KT* يليها العزلة البكتيرية *3SN* ثم *P. fluorescens*.

٢- تأثير استخدام عزلات مختلفة من بكتريا المجال الجذري على معدل موت يرقات الطور الثاني لنيماتودا تعقد الجذور.

تم حساب نسبة موت يرقات الطور الثاني لنيماتودا تعقد الجذور بعد تعرضها لعزلات مختلفة من البكتريا بعد ثلاث فترات زمنية مختلفة (٢٤-٤٨-٧٢ ساعة) وكانت النتائج على النحو الآتي:

أ - وجود علاقة طردية بين معدل الموت ليرقات نيماتودا تعقد الجذور وفترات التعرض.

ب - زادت معدلات موت يرقات نيماتودا تعقد الجذور كلما زادت فترة التعرض.

ج- أفضل العزلات المستخدمة في زيادة معدلات الموت لليرقات بعد ٢٤، ٤٨، ٧٢ ساعة هي العزلة البكتيرية *3SN* ويليها العزلة البكتيرية *2KT* ثم *P. fluorescens*.

٣-تعريف أفضل العزلات البكتيرية المختبرة.

تم تعريف عزلات بكتريا المجال الجذري والتي أعطت أفضل النتائج في خفض نسبة فقس البيض وزيادة معدل موت يرقات الطور الثاني لنيماتودا تعقد الجذور وجاءت النتائج على الوجه التالي:

- عرفت العزلة *3SN* ببكتريا *Pseudomonas aeuroginosa*.
- عرفت العزلة *2KT* ببكتريا *Bacillus subtilis*.
- يفسر التأثير الأبادي لكل من *P. aeuroginosa*، *B. subtilis* و*P.fluorescens* إلى احتواء البيئة البكتيرية على العديد من المركبات الأنزيمية مثل إنزيم البروتيز والكيتينيز والجيلاتينيز بالإضافة إلى الهرمونات ومركبات سيانيد الهيدروجين ...الخ، التي تعمل على خفض نسبة فقس البيض وزيادة نسبة الموت ليرقات الطور الثاني لنيماتودا تعقد الجذور.