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ISOLATION, IDENTIFICATION AND POTENTIAL BIOLOGICAL CONTROL OF SOME RHIZOBACTERIA AGAINST *Meloidogyne incognita*

[175]

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ABSTRACT

The plant-parasitic nematodes seriously affect the growth of many crops and are responsible for agricultural losses worldwide. The losses range from 8 to 20% of major crops around the world. Root-knot nematode *Meloidogyne* spp. infect and damage a wide range of important crops particularly vegetables in tropical and subtropical countries.

The main way of controlling the plant parasitic nematodes is the use of chemical nematicides. Although the nematicides are quickly effective, they are usually expensive and not available and also cause a lot of risk to humans and inflict injury to the environment. Due to the environmental hazards associated with their application, identifying alternatives for nematode control and developing effective and safe application techniques is urgent strategy for alleviating the nematode induced damage**.** The biological controls are alternatives eco-friendly agricultural systems and safer for environments and humans and cheaper than chemical control

This study was conducted to isolate and estimate the potential of some native rhizobacteria from roots in agriculture soil against root-knot nematode, *Meloidogyne incognita* J_2 to evaluate their efficiency as eco-friendly control alternatives for controlling root- knot nematode *M. incognita* under laboratory conditions. Nine rhizobacteria were isolated from soil (R1 to R9). The best mortality was recorded by isolate no. R6 (77.55%) followed by isolated no. R2 (75.59%) followed for isolate no. R7 (71.43%) as compared with zero% for the control (water only) after 72h exposure periods. R6, R2, and R7 were identified as *Lysinibacillus sphaericus, Bacillus pumilus* and *Pseudomonas flourescens*, respectively based on the analysis of the 16S rRNA gene sequence. The partial 16S rRNA gene sequence of these bacterial isolates were deposited in GenBank under accession numbers of MF000302, MF000303 and MF000304 for the previously mentioned bacterial species.

Keywords: 16s rDNA, *Meloidogyne incognita, Bacillus pumilus, Lysinibacillus sphaericus, Pseudomonas fluorescence*

INTRODUCTION

Plant-parasitic nematodes seriously affect the growth of many crops **(Ma et al 2017)** and are responsible for agricultural losses worldwide **(Abad et al 2008)**. Losses range from 8 to 20% in major crops around the world. US $157 billion loss globally on an annual basis is estimated by these infections **(Sikora and Fernandez, 2005 and Abad et al 2008),** in turn it has an impact on international trade social and economic development **(Perry and Maurice, 2013).**

Control of root-knot nematode is more difficult, due to their life underground and into plant, high reproductive rates, wide host range and short generation times, (**Trudgill and Blok, 2001**). Many preventive measures have been tried with different levels of successes for controlling plant nematodes such as chemical control, rotation, host plant resistance, fumigants, nematicides, resistant cultivars, solarization of soil and biological control **(Mukhtar et al 2014, Xiao et al 2016, Zhang et al 2015 and Ma et al 2017).** Although nematicides are quickly effective, they are usually expensive and not available and also cause a lot of risk to humans and inflict injury to the environment **(Nyczepir and Thomas, 2009)**. Due to environmental hazards associated with their application,

finding alternatives for nematode control by effective and safe applicable techniques is both urgent strategies for Mitigation of damage caused by nematodes (**Kerry, 1990).** Biological controls are alternatives eco-friendly agricultural systems and safer for environments and humans and cheaper than chemical control (**Gowen and Ahmad, 1990, Padgham and Sikora, 2006, Ashoub and Amara, 2010 and Ahmed et al 2018).**

Recently, attention has been placed on biological control of plant-parasitic nematodes using bacteria. Many studies have reported nematicidal activity of various bacterial strains of *Bacillus* spp. against plant-parasitic nematodes such as *B. subtilis*, *B. thuringiensis* and *B. pumilus*. *P. fluorescens* as an effective bacterial agent for various soil borne plant diseases including plant parasitic nematodes **(Abdel Razik et al 2016).** Seven strains were isolated and identified as *P. aeruginosa* and *P. fluorescens* by **Rahanandeh et al 2013**, he found that *P. fluorescens* Rh-19 achieved the best mortality percentage of 95.24% for *M. incognita* J_2.

Mokbel and Alharbi, 2014, evaluated the effectiveness of four bacterial isolates, *P. fluorescens, Serratia marcescens*, *B. subtilis* and *B. thuringiensis*, against *M. javanica*. and reported 64.9%, 79.1%, 50.5, 62.0% inhibition in egg-hatch and *M. javanica* J_2 activity caused by *B. thuringiensis*, *P. fluorescens*, *B. subtilis* and *S. marcescens*, respectively. The lowest inhibition percentage 33.7- 48.8% of egg-hatch and *M. javanica* J_2 activity were observed in *B. megaterium* under laboratory condition.

With respect to bacterial taxonomy, 16S rRNA gene sequences are the most commonly used for many reasons, 1) its presence in all bacteria; 2) its function has not changed over time, and 3) its informatics purposes, about 1,500 bp **(Patel, 2001).** Bacterial identification and phylogeny have been achieved by large public-domain databases established via molecular approaches (**Drancourt et al 2000).** The present study aims to obtain new local bacterial isolates which are potent in killing nematodes juveniles under laboratory conditions and identifying these isolates by 16s rDNA PCR amplification technique.

MATERIALS AND METHODS

The present study was carried out in Ain shams center of Genetic Engineering and Biotechnology, Fac. of Agric. (ACGEB), Plant Pathology Dept., Microbial genetic Dept., National Research Centre (NRC).

Collection of soil samples and bacterial strains isolation

Different soil samples were collected from various agriculture soils. Soil samples were put in sterile plastic bags and transferred to the laboratory for bacterial isolation. One gram from previously collected soils was used to inoculate 50 ml autoclaved distilled water. Cultures were then incubated on an orbital shaker at 30ºC for 5 days at 150 rpm.

All cultures were allowed to settle for 2 h and 5ml of each supernatant was used to inoculate 45 ml fresh MSM media. Serial dilutions of cultures 10^{-4} to 10^{-6} were plated on LB agar plates. Plates were incubated at 30ºc for 18 h.

Extraction of *M. incognita* J_2

Meloidogyne incognita eggs were extracted from the infected tomato roots; according to **Hussey and Barker (1973)**, the egg suspension was incubated at room temperature for egg hatching. Hatched, second-stage juveniles were collected after 4 days and rinsed with sterile distilled water, and the inoculum concentrations of J2 were adjusted to 50 juvenile's ml− 1. In a 6-cmdiam Petri dish, 4 ml of nematode suspension was added to 1 ml (2 × 106 cfu ml− 1) from the isolated bacterial strains.

Evaluating the nematicidal effect of bacterial isolates against *Meloidogyne incognita* J_2 under laboratory conditions

For bioassay test, *M. incognita* eggs were extracted from the infected tomato roots that carry egg masses **(Hussey and Barker, 1973)** and then incubated in egg hatching plastic cups at laboratory temperature of 24±3°C for 72 h. to provide *M. incognita* J_2. Petri dishes 6 cm in diameter were supplied separately with one ml from the bacterial suspensions plus 4 ml of nematode suspension in distilled water containing 100±5 freshly hatched M. incognita J_2. A volume of 5 ml of distilled water containing 100±5 freshly hatched M. incognita J_2 served as control. All treatments and control were replicated five times. All dishes were kept in incubator at 35°C. Dishes were loosely covered to permit aeration and lessen evaporation. Number of live and dead individuals was counted after 24 h for 3 days using 1 ml nematode counting slide. After the exposure periods, the nematodes in each treatment were transferred to distilled water and

left for 24 h to observe whether immobile nematodes resumed activity or not. The corrected percentages of nematode mortality were calculated according to the following equation: mortality (%) = (m–n)/ (100–n)×100, where m and n indicate the percentages of mortality in treatments and control, according to **(Abbott, 1952).**

Extraction of genomic DNA from bacterial isolates

The isolated bacteria were cultured in conical flasks (Pyrex, USA) containing 20 ml LB medium by shaking in an orbital shaker (Thermo fisher scientific, UK) at 180 rpm for 18h. The cultures were centrifuged at 13,000 rpm for 5min at 4ºC. The pellets were subjected to genomic DNA extraction using the QIAamp DNA Mini Kit, (QIAGEN, Germany). The extracted DNA was used as a template for PCR to amplify 16S rRNA gene.

Molecular identification of bacterial isolates by PCR amplification and sequencing of 16S rDNA

Molecular tools such as 16S rDNA PCR amplification were used to identify the bacterial isolates (**Ben Amor et al 2007**). Bacterial 16S rDNA was amplified by PCR using the universal primers; forward primer sequence (5´AGAGTTTGATCCTG GCTCAG3´) and reverse primer sequence (5´CT ACGGCTACCTTGTTACGA3´), thereby producing an amplicon of ~1500 bp. Amplification was carried out in 50µl reactions by using a PCR master mix kit (Qiagen, Germany) according to the manufacturer's instructions using a GeneAmp PCR System 2400, Thermal cycler (Perkin-Elmer Norwalk, Connecticut, USA). The following program was used: 94ºC for 3 min as initial denaturation step followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 1 min, extension at 72ºC for 2 min and final extension at 72ºC for 10 min. PCR product was purified by QIAquick Gel Extraction Kit (Qiagen, Germany) following the protocol provided by the supplier and then resolved by electrophoresis on 1.5 % agarose gel. Nucleotide sequence was determined using the same Primers with the dideoxy-chain termination method. The obtained sequences were analyzed for similarities to other known sequences found in the GenBank database using BLAST program of the NCBI database.

RESULTS AND DISCUSSION

Effect of the bacterial isolates on mortality of *M.incognita* J_2 juveniles under laboratory conditions

Nine bacterial isolates, R1 to R9 were isolated from the collected soil samples and their nematicidal effect against *M. incognita* J_2 were evaluated. As indicated in **Table (1) and Fig. (1)**, the best mortality was recorded by isolate no. R6 (77.55%) followed by isolate no. R2 (75.59%) followed for isolate no.R7 (71.43%) as compared with 0% in case of control (water only) after 72h exposure periods. This result agreed with **(Siddiqui et al 2009, Siddiqui and Mahmood, 1999, Mokbel and Alharbi, 2014 and Soliman et al 2018).** It is known that Rhizobacterial enzymes, toxins and metabolic by-products inhibit root-knot nematodes and suppress plant parasitic nematode reproduction, gall formation, egg hatching, and juvenile survival **(Siddiqui and Mahmood, 1999).** Some rhizobacteria can also act indirectly through stimulating and development of plant nematode resistance and as a result, decreasing the damage related to nematode infection **(Soliman et al 2018)**.

Siddiqui et al 2009 isolated five species of fluorescent *Pseudomonads* and five species of genus *Bacillus*. *Pseudomonade* were found to be more effective than the isolates of *Bacillus* as inhibitory effect on hatching and penetration of *M. incognita* due to produced siderophores, hydrogen cyanide (HCN) and indole acetic acid production. **Mokbel and Alharbi, (2014)**, used four bacterial isolates; *P. fluorescens, Serratia marcescens, B. subtilis* and *B. thuringiensis*, against *M. javanica* as biocontrol agents against *M. javanica* J_2, 64.9% and 79.1% egg-hatch inhibition which were recorded in *B. thuringiensis* and *P. fluorescens*, whereas, 50.5% and 62.0% were shown in *B. subtilis* and *S. marcescens* under laboratory condition.

Values are average of four replicates** Values of the same column followed by the same letter (s) are not significantly different at P ≤ 0.05 (according to Duncan's MultipleRange Test). ** Mortality (%) = (m–n)/ (100–n)×100. where m and n indicate the percentages of mortality in treatments and control. ## Net mortality= % mortality after 72 hrs - % nematode recovery in distilled water.

Table 1. Effect of bacterial isolates on mortality of *M. incognita* juveniles under laboratory conditions

Serial Number	Nematode mortality after different exposure periods Juveniles**			% Recovery	% Net mortality##
	24h	48h	72h		
R 1	57.29*f	60.82e	65.42de	0.00	65.42
R 2	61.35d	62.89d	75.59ab	0.00	75.59
R 3	60.42de	60.82e	61.35e	0.00	61.35
R4	65.42c	67.01bc	67.35cd	0.00	67.35
R 5	59.32e	65.42c	67.01cd	0.00	65.42
R 6	75.72a	77.55a	77. 78a	0.00	77.55
R 7	67.61b	67.68b	71.43bc	0.00	71.43
R 8	50.01g	53.54f	55.10f	0.00	55.10
R9	67.49b	67.49b	69.40de	0.00	69.40
Control(water only + nematodes)	0.00h	0.00g	0.00g	---	---

Values are average of four replicates** Values of the same column followed by the same letter (s) are not significantly different at P ≤ 0.05 (according to Duncan's MultipleRange Test). ** Mortality (%) = (m–n)/ (100–n)×100. where m and n indicate the percentages of mortality in treatments and control. ## Net mortality= % mortality after 72 hrs - % nematode recovery in distilled water

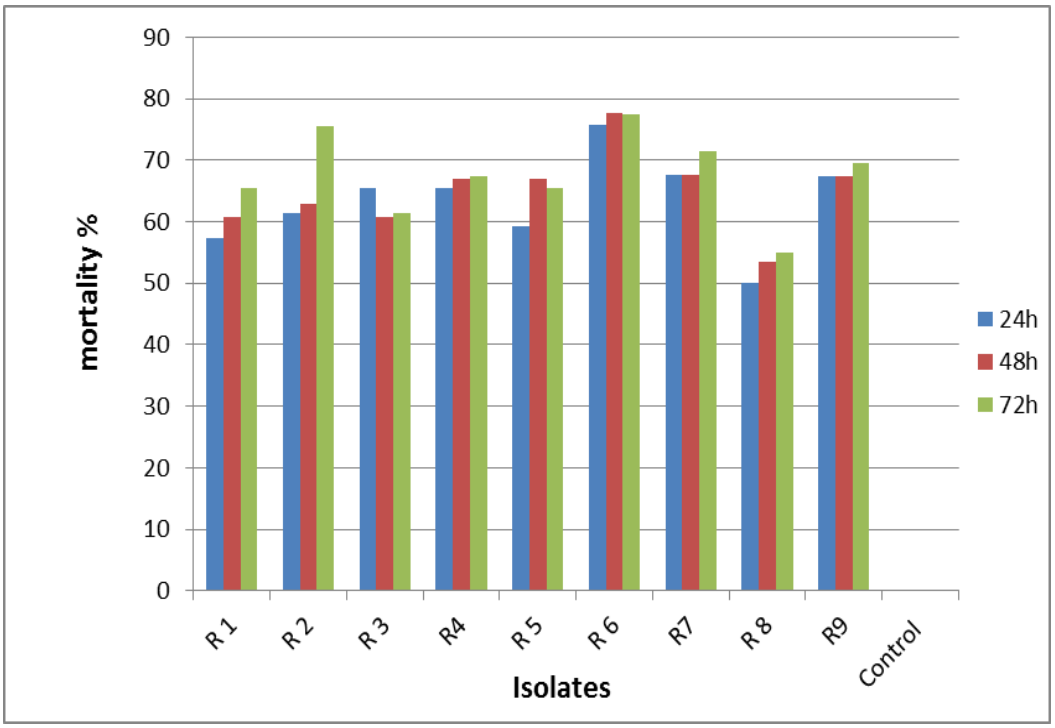

Fig. 1. mortality percentages of 9 bacterial isolates of *M. incognita* juveniles.

Molecular identification via 16S rDNA PCR amplification of the selected isolated bacterial strains

16S rDNA PCR amplification has been used extensively to identify prokaryotes as well as understanding prokaryote diversity and phylogenetic relationships **(Pace 1996, 1997 and 1999).** The most potent three isolated bacterial strains, with different morphological characteristics were selected and directed for PCR amplification of the 16S rDNA. 16s rDNA universal primers amplified ~1550 bp **(Fig. 2)** for all bacterial isolates. Partial DNA sequences of 16S rDNA were subjected to BLAST search on https://blast.ncbi.nlm.nih.gov/Blast against the sequences deposited in NCBI database. Based on BLAST, results of bacterial isolates R2, R6 and R7 were identified as *B. pumilus*, *Lysinibacillus sphaericus* and *Pseudomonas flourescens*, respectively. Their 16s rDNA partial nucleotide sequences were deposited in GenBank under accession numbers of MF000303, MF000302 and MF000304, respectively for the previous mentioned bacterial strains. No doubt, that identification of poorly described rarely isolated or phenotypically aberrant strains is better detected by 16S rDNA which can be also used for identification of mycobacteria and it can lead to the recognition of novel pathogens **Jill (2004). Barman et al (2014)** used universal primers to amplify 16s rDNA for molecular identification of isolated endophytic *Pseudomonas sp.* BF1-3.

The 16S rDNA sequences obtained were added to publicly available bacterial 16S rRNA sequences, the sequences were integrated into the database with the automatic alignment tool. Phylogenetic tree was generated by performing distance matrix analysis using the NT system. Database search and comparisons were done with the BLAST search using the National Center for Biotechnology Information (NCBI) database**. (Ola and Osama, 2007).** Phylogenetic trees of bacterial isolates were constructed and presented in **(Figs. 3, 4 and 5)** for bacterial strains R2, R6 and R7, respectively. From **Fig. (3)** R2 (query_82889) based on 16S rRNA gene sequences using neighbor joining method was identified as *Bacillus pumilus,* from **Fig. (4)** R6 (query_131723) based on 16S rRNA gene sequences using neighbor joining method was identified as *Lysinibacillus sphaericus* and from **Fig. (5)** R7 (query_182269) based on 16S rRNA gene sequences using neighbor joining method was identified as *Pseudomonas flourescens.*

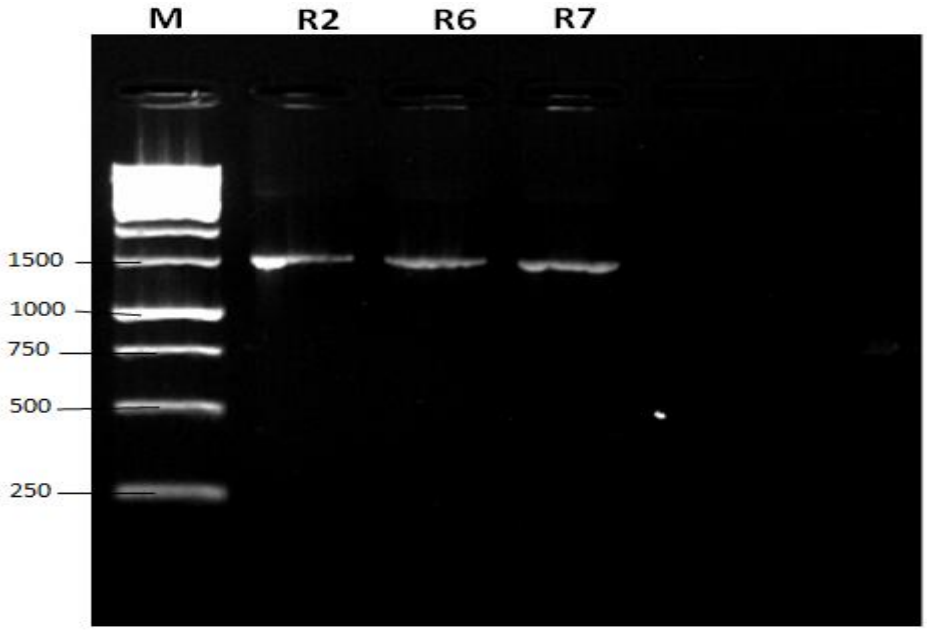

Fig. 2. Agarose gel electrophoresis of 16s rDNA PCR product in bacterial Isolates (R2, R6 and R7). 1Kb DNA ladder (thermoscientific)

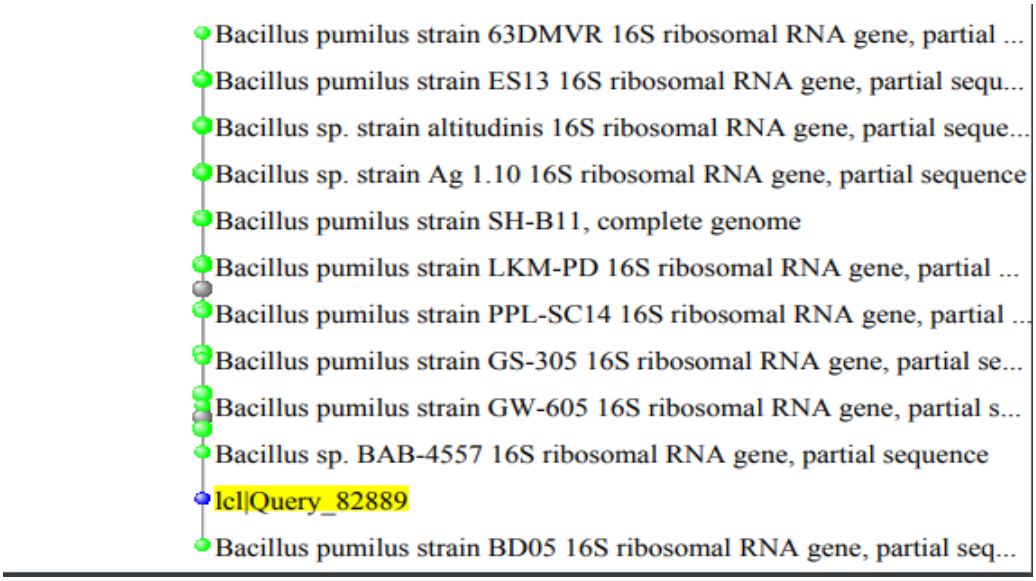

Fig. 3. Phylogenetic tree of bacterial isolate R2 (query_82889) based on 16S rRNA gene sequences using neighbor joining method.

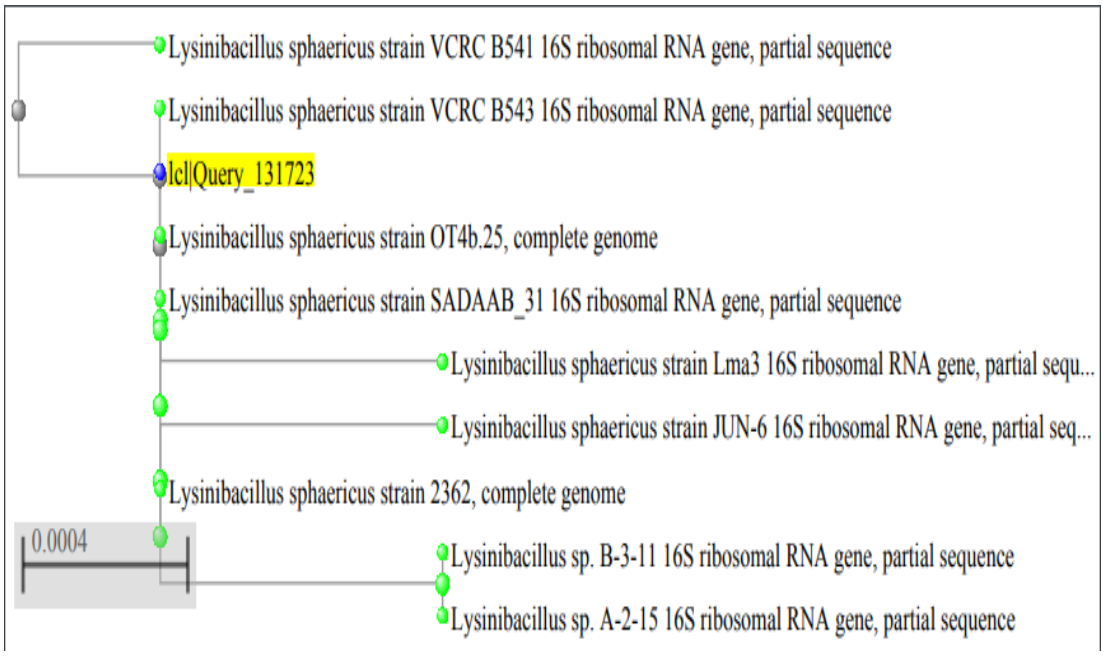

Fig. 4. Phylogenetic tree of bacterial isolate R6 (query_131723 based on 16S rRNA gene sequences using neighbor joining method.

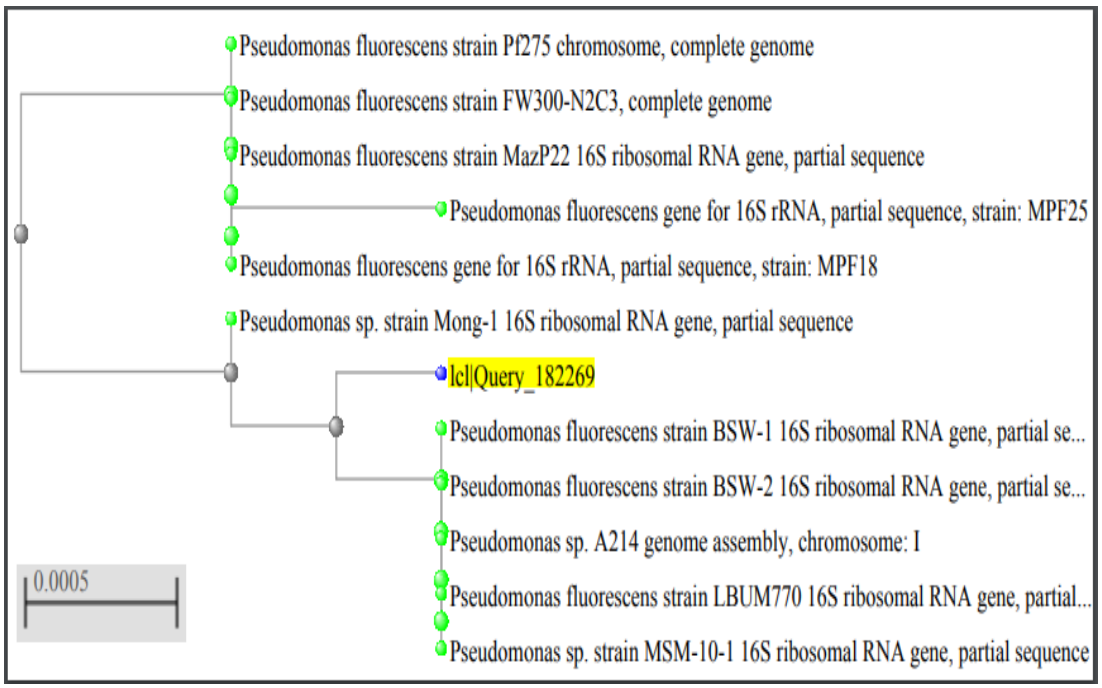

Fig. 5. Phylogenetic tree of bacterial isolate R7 (query_182269) based on 16S rRNA gene sequences using neighbor joining method.

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عزل وتعريف والتحكم البيولوجى المحتمل لبعض أنواع الريزوبكتريا لمكافحة نيماتودا تعقد الجذور *Meloidogyne incognita*

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الموجـــــز

تؤثر النيماتودا المتطفلة نباتيا تأثيراً خطيراً على نمو العديد من المحاصيل وهي مسؤلة عن الخسائر الزراعية في جميع أنحاء العالم. تتراوح الخسائر بين 8 و 20 ٪ على المحاصيل الرئيسية في جميع أنحاء العالم. تصيب نتماتودا تعقد الجذور *Meloidogyne* spp مجموعة كبيره من المحاصيل الهامة وخاصة الخضراوات في البلدان المدارية وشبه المدارية. الطريقة الرئيسية لمكافحة النيماتودا المتطفلة علي النبات هي استخدام المبيدات النيماتودية. على الرغم من التاثير السريع للمبيدات النيماتودية، إلا أنها عادة ما تكون باهظة الثمن وغير متوفرة وتتسبب أيضًا في الكثير من المخاطر التي يتعرض لها الانسان وإلحاق الأذى بالبيئة. نظرًا للأخطار البيئية المرتبطة بتطبيقها، يعد تحديد بدائل لمكافحة النيماتودا وتطوير تقنيات فعالة وآمنة للتطبيق من الاستراتيجيات الملحة لتخفيف الضرر الناجم عن النيماتودا. المكافحة البيولوجية من النظم الزراعية الصديقة للبيئة وأكثر أمانًا للبيئة والانسان وأرخص من المكافحة الكيميائية.

أجريت هذه الدراسة لعزل وتقدير إمكانات بعض انواع الريزوبكتيريا المعزولة من التربة الزراعية وتقييم كفاءتها كبدائل تحكم صديقة للبيئة لمكافحة يرقات نيماتودا تعقد الجذور *M. incognita* في ظروف المعمل. تم عزل تسعة عزلات من التربة (R1 إلى R9). وسجلت العزلة رقم R6 أفضل نسبة موت (77.5%) تليها العزلة رقم R2 (75.59٪) تليها العزلة رقم R7 (71.43 %) بالمقارنة مع صفر٪ في حالة الكنترول (الماء فقط) بعد فترات التعرض لمدة 72 ساعة. تم تعريف R6 و R2 و R7 على أنها *Lysinibacillus sphaericus* و *Bacillus pumilus* و *Pseudomonas flourescens* ، بناءً على تحليل. ترتيب التسلسل الجيني الجزيئي 16S rRNA لهذه العزلات البكتيرية تم ايداعها في بنك الجينات (GenBank) تحت ارقام MF000302 و MF000303 و MF000304 على الترتيب للأنواع البكتيرية المذكورة سابقًا.

الكلمات الداله:

16s rDNA, *Meloidogyne incognita, Bacillus pumilus, Lysinibacillus sphaericus, Pseudomonas fluorescence*

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