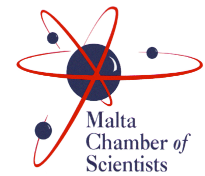
Bashiri, S., Llop, P., Davino, M., Golmohammadi, M., and Scuderi, G. (2015). *Xjenza Online*, 3:165–173.

Xjenza Online - Journal of The Malta Chamber of Scientists
www.xjenza.org
DOI: 10.7423/XJENZA.2015.2.07



Research Article

Efficacy of *Pseudomonas chlororaphis* subsp. *aureofaciens* SH2 and *Pseudomonas fluorescens* RH43 isolates against root-knot nematodes (*Meloidogyne* spp.) in kiwifruit

S. Bashiri[1], P. Llop[2], M. Davino[3], M. Golmohammadi[4], G. Scuderi[3,5]*

[1] *Department of Plant Protection, Faculty of Agriculture Science, Guilan University, Rasht, Islamic Republic of Iran*

[2] *Department of Agroecology, Faculty of Agricultural Sciences, Aarhus University, Flakkebjerg, Denmark*

[3] *Dipartimento di Agricoltura, Alimentazione e Ambiente (Di3A), Sezione di Patologia Vegetale, University of Catania, Via S. Sofia 100, 95123 Catania, Italy*

[4] *Department of Plant Protection, Citrus Research Institute, Mazandaran, Ramsar, Islamic Republic of Iran*

[5] *I.E.ME.S.T., Istituto Euro Mediterraneo di Scienza e Tecnologia, Via Emerico Amari, 123, 90139 Palermo, Italy*

Abstract. The Root-knot nematodes, *Meloidogyne* spp., are parasites of many crops and orchards, including kiwifruit trees. The Islamic Republic of Iran is among the leading kiwifruit producers in the world and *M. incognita* has been found as the dominant species responsible for severe loss of this crop. In order to evaluate the effectiveness of antagonistic bacteria on larval mortality, number of galls per plant and egg masses of nematode reduction, fifty local bacterial strains were isolated from root surrounding soils of kiwifruit plants in the northern production areas in Iran. Bacterial antagonists were characterized by morphological, physiological, biochemical and molecular methods. Two representative strains, showing the best nematicidal activity, were identified as *Pseudomonas chlororaphis* subsp. *aureofaciens* (isolate Sh2) and *Pseudomonas fluorescens* (isolate Rh43). They increased the percentage of larval mortality to 56.38% and 54.28% respectively in assays *in vitro* and showed excellent performance also *in vivo* with consistent reduction of number of galls (67.31% and 55.63%, respectively) and egg mass (86.46% and 84.29%, respectively) in plants. This study indicates that *Pseudomonas chlororaphis* subsp. *aureofaciens* isolate Sh2 and *Pseudomonas fluorescens* isolate Rh43 are good potential biocontrol agents for containing root-knot nematodes in kiwifruit trees.

Keywords: Biocontrol; Bacteria; Nematicide; kiwifruit; 16S rRNA

1 Introduction

The Islamic Republic of Iran is among the world's leading kiwifruit producers with 2.816 ha cultivated and 32.000 tonnes production (FAOSTAT, 2012). The Mazandaran, Guilan and Golestan provinces have suitable conditions for growing this crop (i.e. altitude, temperature and soil). Plant parasitic nematodes cause damages to a variety of agricultural crops throughout the world. By themselves, the root-knot nematodes (*Meloidogyne* spp.) cause an annual loss of about US $100 billion to a wide variety of crops worldwide (Oka, Shuker & Tkachi, 2009). Four major species, *M. javanica*, *M. incognita*, *M. hapla* and *M. arenaria*, have been reported in Iran and *M. incognita* has been found as the dominant species and major limiting factor in kiwifruit orchards in the main production areas of Guilan and Mazandaran provinces (Tanhamaafi & Mahdavian, 1997).

Currently, the application of chemical nematicides and fumigants are still the main strategy for managing the disease caused by root-knot nematode (Giné et al., 2013). Commonly, nematicides are used to control parasites and reduce loss of production. Although chemical nematicides are effective, easy to apply, and act rapidly, they have begun to be withdrawn from the market in some countries due to public health and environmental safety concerns (Schneider et al., 2003). The need for al-

**Correspondence to*: G. Scuderi (gscuderi@unict.it)

© 2015 Xjenza Online

ternative and more natural systems for controlling these pathogens is being reflected in numerous studies over a long period, demonstrating the concerns and efforts made in this field and the difficulties they represent in many different crops (Akhtar & Alam, 1993; Barker & Koenning, 1998; Akhtar & Malik, 2000; Oka et al., 2000; Kratochvil, Sardanelli, Everts & Gallagher, 2004; Tsay, Wu & Lin, 2004; Westphal & Scott Jr, 2005; Abbasi, Riga, Conn & Lazarovits, 2005; Felde et al., 2006; Korayem, Youssef & Mohamed, 2008; B. K. Mishra et al., 2011; Knudsen & Dandurand, 2014).

The application of microorganisms antagonistic to *Meloidogyne* spp. or the use of microbial metabolites could provide additional opportunities for managing this disease (Lamovšek, Urek & Trdan, 2013).

Integrated Pest Management (IPM) principles and the need for alternative procedures to nematicides have increased the interest for biocontrol strategies. The biological control of root-knot nematode *Meloidogyne* spp. was investigated using several fungi, including *Trichoderma harzianum*, *Pochonia clamydosporia*, *Metarhizium anisopliae* and *Beauvaria* sp. (Sharon et al., 2001; Small & Bidochka, 2005; Bent, Loffredo, Mckenry, Becker & Borneman, 2008). Bacterial species with nematicidal activity have also been used with some success for controlling root-knot diseases, including *Streptomyces* spp., *Serratia* spp., *Bacillus* spp., *Azotobacter chroococcum*, *Rhizobium* spp., *Corynebacterium* spp. and *Pseudomonas* spp. (Hallmann, Quadt-Hallmann, Miller, Sikora & Lindow, 2001; Jayakumar, 2009; Mohamed, El-Sayed, Radwan & El-Wahab, 2009; Sikora, Schafer & Dababat, 2007). I. A. Siddiqui and Shaukat (2002) demonstrated that *Pseudomonas fluorescens* and *P. aeruginosa* decreased the penetration of *M. javanica* juveniles into *Solanum lycopersicum* plants. On the same host plant, Hanna, Riad and Tawfik (1999) assayed *P. fluorescens* for the management of *Meloidogyne incognita* and showed that the percentage of gall formation and root gall index decreased when the bacteria were introduced prior to nematodes. Furthermore, El-Hamshary, El-Nagdi and Youssef (2006) reported results of an *in vitro* study in which both *P. fluorescens* and *P. aeruginosa* reduced survival rates of *M. incognita* juveniles, exhibiting nematode mortality rates dependent on bacterial inoculum and exposure time.

The goal of the current study was to isolate, identify and analyze the potential of locally-occurring bacteria for controlling and/or reducing root-knot nematode population in the kiwifruit in northern Iran.

2 Materials and Methods

2.1 Isolation of local antagonistic bacterial strains

Fifty bacterial strains were isolated, by dilution-plate method, from 250 soil samples collected from soils surrounding the roots of 250 symptomatic and asymptomatic kiwifruit plants in the north of Iran, during 2011 (Table 1). Five to ten samples were collected from each of most cultivated areas (Provinces).

From each sample, one gram of soil was aseptically mixed with 100 mL of distilled water. Homogenous soil suspensions and respective dilutions (10^{-2}) were used. 1 mL of each suspension was plated on NA (Nutrient Agar; Merck®) containing pentachloronitrobenzene (PCNB; Sigma-Aldrich) and incubated at 25 °C. Selected bacterial colonies were purified by streaking twice on plates (NA+PCNB) and cultures were stored in 20% (wt/vol) glycerol in cryotubes at −80 °C.

2.2 Juvenile nematode extraction

Soil samples (100 g) were collected and then processed for juvenile nematode extraction using the Whitehead and Hussey methods (Whitehead & Hemming, 1965; Hussey & Barker, 1973). The resulting suspension was collected through 400 mesh sieves. 1 ml suspension was pipetted into Petri plates for counting, and examined under stereomicroscope. Nematode juveniles were counted and identified following the procedure of Eisenbeck, Hirschmann, Sasser and Triantaphyllou (1981).

2.3 Evaluation of local bacterial isolates against root-knot nematode *in vitro*

Bacterial isolates were screened for antagonistic activity against juvenile *Meloidogyne* spp. (Ashoub & Amara, 2012). Morphological comparison tests (based on perineal patterns) and identification keys allowed the identification of most as *Meloidogyne incognita*. For *in vitro* testing, nematodes were collected and maintained on Tomato ‘Rutgers’. 1 ml of each bacterial isolate suspension (1.8×10^8 cfu/mL) cultured in LB broth was mixed with 1 mL of distilled water suspension containing 50 root-knot juvenile nematodes (same age J2 juvenile treated with $50\,\mathrm{mg\,L^{-1}}$ Tetracycline, $44\,\mathrm{mg\,L^{-1}}$ Streptomycin sulfate), dispensed into Petri plates and incubated at 28–30 °C for 72 h. Counts of dead nematodes were performed daily (24, 48 and 72 h after incubation) under a stereomicroscope by touching the nematodes with a sharp tip; nematodes that did not respond with movement were considered to be dead (Lee et al., 2011). Each treatment was repeated three times. Mortality rate was calculated as [mean number of dead juveniles in treatment/total number of juveniles in treatment]×100 (Ashoub & Amara, 2012).

Table 1: List of isolates obtained from kiwifruit crops used in this study.

Isolates/Age of plant (years)	Origin (Iran Province)
B1 (8), B3 (8), B14 (10), B24 (10)	Mazandaran, Babol
B38 (12), B40 (12), B42 (10)	Mazandaran, Babolsar
S1 (5), S5 (5), S12 (8)	Mazandaran, Sari
S15 (7), S19 (7), S20 (12), S42 (12)	Mazandaran, Amol
K10 (10), K12 (10), K20 (18)	Guilan, Kelachay
K31 (6), K35 (6), K37 (5)	Gorgan, Kordkuy
R1 (8), R2 (8), R3 (6), R5 (6)	Guilan, Rhasht
R6 (18), R8 (18), R10 (8)	Guilan, Astara
Rs11 (10), Rs13 (12), Rs15 (15)	Guilan, Roudsar
Rs17 (10), Rs20 (10), Rs24 (9)	Mazandaran, Ramsar
Rh34 (15), Rh36 (18), Rh40 (17)	Guilan, Talesh
Rh43 (7), Rh45 (7)	Guilan, Lahijan
Ch40 (14), Ch42 (6), Ch44 (6)	Mazandaran, Chalus
Ch45 (10), Ch48 (10), Ch50 (7)	Mazandaran, Nour
Sh2 (15), Sh6 (15), Sh7 (25), Sh10 (20), Sh15 (12), Sh20 (10)	Mazandaran, Tonekabon

2.4 Biochemical identification of bacterial isolates antagonistic to root-knot nematode

Isolates selected for antagonistic activity were biochemically characterized and identified according to Bergey's Manual of Determinative Bacteriology (Holt, Krieg, Sneath, Staley & Williams, 1994). The Gram reaction was determined. Morphological (shape, size, endospore, fluorescent pigmentation) and cultural characteristics were examined on NA and King's B medium (King, Ward & Raney, 1954) and isolates subsequently analysed by LOPAT test characteristics following the procedures described by Lelliot and Stead (1987). Other biochemical characteristics, including Gelatin liquefaction, Starch hydrolysis, growth on NA at 41 °C, Oxidative and Fermentative test, were also studied (Schaad, Jones & Chun, 2001).

2.5 Molecular identification of bacterial isolates antagonistic to root-knot nematode

Molecular identification of isolates, selected on the basis of their antagonistic activity, was performed using PCR. DNA isolation was performed according to Llop, Caruso, Cubero, Morente and López (1999). 1 mL of the bacterial suspension (10^8 cfu/mL), grown in LB broth, was centrifuged at 8.000 g for 5 min. The pellet was resuspended in 500 µL extraction buffer and the suspension was shaken for 60 min at room temperature. The sample was centrifuged at 1.000 g for 5 min and 450 µL of the supernatant was mixed with 450 µL isopropanol and kept for 30 min at room temperature. The mixture was then centrifuged at 8.000 g for 10 min, the supernatant was removed and the pellet resuspended in 100 µL of sterile water. DNAs were either used immediately for PCR or stored at −20 °C until further use.

For the identification of genus *Pseudomonas*, the amplification of subunit 16S rRNA gene was performed using Ps-for/Ps-rev primer set, as described by Widmer, Seidler, Gillevet, Watrud and Di Giovanni (1998). PCR cocktails for 100 µL reaction mixtures contained 1× reaction buffer (Boehringer Mannheim), 1 U of Taq DNA polymerase (Boehringer Mannheim), 200 nM of each deoxynucleoside (Boehringer Mannheim), 5 mg of bovine serum albumin (Sigma Co.) per mL, 200 nM of each oligonucleotide primer (Sigma Co.) and DNA sample. PCR amplification programme was performed as follows: denaturation for 5 min at 95 °C and then 40 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 64 °C, extension for 1 min at 72 °C, and a final extension for 10 min at 72 °C.

Specific amplification of *Pseudomonas fluorescens* 16S rRNA gene partial region was performed using the primer set 16SPSEfluF/16SPSER (Scarpellini, Franzetti & Galli, 2004). Positive control consisted of strain CHAO of *P. fluorescens*, and negative controls consisted of water. All oligos used in this study were synthesized by Fermentas and are listed in Table 2.

All PCR reactions were performed twice in a DNA thermal cycler Engine PTC-200 (MJ Research Inc.). Following amplification, 7 µL of each PCR product was analysed by electrophoresis at 100 V (1% agarose gel, 0.2 µg of ethidium bromide mL^{-1}) in TBE buffer. Gels were visualized under UV light and then photographed.

For sequencing analysis, F27/R1492 universal primer set was used as reported by Lagacé, Pitre, Jacques and Roy (2004). Briefly, reaction mix (25 µL) contained 25 ng genomic DNA, 0.2 µM primer, 0.2 mM dNTP (Fermentas), 1.5 units of Taq DNA polymerase (Fermentas), 10× buffer and 1.25 mM $MgCl_2$. Amplification was checked by electrophoresis on a 1.0% agarose gel. PCR product was purified with the AccuPrep® PCR Puri-

Table 2: Nucleotide sequences of primers used in this study.

Primer	Sequence (5′-3′)	Amplicon size (bp)	Reference
Ps-for	GGTCTGAGAGGATGATCAGT	1200	Widmer, Seidler, Gillevet, Watrud and Di Giovanni (1998)
Ps-rev	TTAGCTCCACCTCGCGGC		
16SPSEfluF	TGCATTCAAAACTGACTG	850	Scarpellini, Franzetti and Galli (2004)
16SPSER	AATCACACCGTGGTAACCG		
F27	AGAGTTTGATCCTGGCTCAG	1500	Lagacé, Pitre, Jacques and Roy (2004)
R1492	TACGGYTACCTTGTTACGACTT		

fication Kit (Bioneer Corporation). The purified DNA concentration was measured spectrophotometrically and sent to the Sequencing Core Facility of Bioneer Corporation.

The resulting electrophorograms were analyzed with the software Chromas (version 1.43; Techelysium Pty Ltd.) and exported to FASTA format. Similarity searches of sequence data were carried out using the National Center for Biotechnology Information Blast Network Server (http://www.ncbi.nlm.nih.gov/BLAST) for comparison with known gene sequences in GenBank (Altschul, Gish, Miller, Myers & Lipman, 1990). Among all samples analysed, two isolates were chosen for further experiments of nematode control in *in vivo* analyses.

2.6 Biocontrol activity of local bacterial isolates against *Meloidogyne* spp. in greenhouse experiments

A pot experiment was set up to explore efficacy of isolates to control population density of root-knot nematode juveniles in greenhouse conditions. Seedlings (six months old) of kiwifruit (*Actinidia deliciosa*) were sown in 30 cm^3 pots containing autoclaved sandy loam soil (1:1). Pots were divided into three groups each containing three replicates. Two isolates were selected on the basis of best antagonistic activity: *P. chlororaphis* subsp. *aureofaciens* (Sh2) and *P. fluorescens* (Rh43). Bacterial suspensions were prepared from them in sterile distilled water (1.8×10^8 cfu/mL) and were mixed with the soil up to a final concentration of 1.8×10^7 cells/cm^3. After seven days, plants were inoculated with 2.000 freshly hatched second stage juveniles (J2) of *Meloidogyne* spp. (same age J2 juvenile treated with 50 mg/L Tetracycline, 44 mg/L Streptomycin sulfate). Pots were fertilized with recommended dose and kept at 25 ± 3 °C in complete randomized design. After two months plants were uprooted, galls and egg masses were recorded and indexed, following the method reported by Zeck (1971).

Additionally, efficacy of *P. chlororaphis* subsp. *aureofaciens* Sh2 and *P. fluorescens* Rh43 bacterial suspensions in controlling *Meloidogyne* spp. was compared to Fenamiphos treatment (Nemacur® 10G, Bayer Crop-Science, Research Triangle Park, NC), one of most used chemical products for managing the effect of plant-parasitic nematodes.

Both infected (positive control) and uninfected (negative control) plants exposed to no treatments were included in the tests.

2.6.1 Statistical analysis

Statistical analysis was performed using ANOVA. Mortality data were subjected to one-way Analysis of Variance and the means separated by Duncan's multiple range test. All statistical analysis were performed using STATGRAPHICS Plus, Version 5.1. (Copyright Manugistics Inc.)

3 Results and Discussion

3.1 Isolation and identification of bacterial isolates antagonistic to root-knot nematode

Antagonistic bacteria were isolated from nematodes conductive and suppressive soils. Fifty bacterial isolates (Table 1) were screened for their ability to reduce nematode juvenile population density *in vitro* (data not shown). Based on this preliminary screening, ten isolates were selected on the basis of pronounced activity and efficacy to reduce nematode juveniles belonging to *Meloidogyne* spp.: S1, R5, K12, B14, Sh2, Rs20, B24, B38, Rh43, Ch50. Percentage of population juvenile reduction ranged from 20.22–27.12% at 24 h; 41.08–49.24% at 48 h and 50.14–56.38% after 72 h, being the strain Sh2 who produced the highest reduction at all recording times (Table 3).

These bacterial strains were arranged into two main groups depending on morphological and biochemical characteristics.

The first group included isolates S1, R5, K12, Sh2,

Table 3: Effect of the selected isolates on the population density of root-knot juveniles of *Meloidogyne* spp. (*in vitro*). Mean comparisons were performed with each treatment-time separately. The significance of the effect of bacterial treatment was evaluated by Duncan's multiple range test ($P < 0.05$).

Bacterial isolates	Juveniles (J2) mortality percentages after 24 h	Juveniles (J2) mortality percentages after 48 h	Juveniles (J2) mortality percentages after 72 h
S1	26.45 gh	48.37 f	53.05 c
R5	20.46 b	41.08 b	50.14 b
K12	25.73 f	47.42 e	51.20 c
B14	21.22 c	44.52 c	52.22 d
Sh2	27.12 h	49.24 g	56.38 e
Rs20	23.49 e	48.31 f	53.01 d
B24	21.57 c	48.56 fg	52.46 d
B38	26.04 g	47.38 e	53.12 d
Rh43	22.38 d	47.33 e	54.28 e
Ch50	20.22 b	46.25 d	51.03 cd
Control (-)	0.00 a	0.00 a	0.00 a

B24 and B38. They were rod shape, Gram negative, non-spore forming organisms. All strains produced fluorescence on King's B medium and were positive for levan production, oxidase, arginine dihydrolase, catalase, and gelatin liquefaction. They resulted negative in hypersensitive reaction on tobacco leaves (HR), potato rot (pectolytic activity), growth at 41 °C, and starch hydrolysis. According to the reference controls, isolates could be identified as *Pseudomonas chlororaphis* (Peix et al., 2007).

The second group included isolates B14, Rs20, Rh43 and Ch50. They were also rod shape, Gram negative, non-spore forming bacteria. All strains produced fluorescence on King's B medium and were positive for levan production, oxidase, arginine dihydrolase, catalase, gelatin liquefaction, starch hydrolysis. They resulted negative for hypersensitive reaction on tobacco leaves (HR), potato rot (pectolytic activity), and growth at 41 °C. Therefore, these isolates could be identified as *Pseudomonas fluorescens* (Schaad et al., 2001).

The isolates Rh43 and Sh2, exhibited the best biocontrol activity (Table 3) and were chosen for further molecular characterization. They were representative for each of the two groups obtained on the basis of previous biochemical characterization. Because of specific PCR product (1.200 bp), polymerase chain reaction with Ps-for/Ps-rev primers confirmed that all isolates belonged to the genus *Pseudomonas*, according to the results of biochemical tests. Strain Rh43 resulted positive for PCR with primer 16SPSEfluF/16SPSER (data not shown), thus confirming that it belonged to *Pseudomonas fluorescens* species (Scarpellini et al., 2004).

In order to identify isolate Sh2 that produced a negative PCR result with *P. fluorescens* specific primers, sequence analysis was performed on PCR product generated by 16rRNA gene universal primers F27/R1492 (data not shown). Blast analysis of partial 16S rRNA gene sequence exhibited high sequence homology (99%) with *P. chlororaphis* subsp. *aureofaciens* strain HN-4 (JQ267650). The nucleotide sequence was deposited as *P. chlororaphis* subsp. *aureofaciens* strain Sh2, 16S ribosomal RNA gene, partial sequence (Accession Number: JX477174). Therefore, BLASTn analysis confirmed the diagnostic results obtained by morphological, physiological and biochemical tests.

3.2 Evaluation of *P. chlororaphis* subsp. *aureofaciens* Sh2 and P. fluorescens Rh43 *in vivo* against the nematodes of kiwifruit

Data presented in Table 4 showed the effect of microbial treatments on the development of *Meloidogyne* spp. infecting kiwifruit (cv. Hyward) in greenhouse conditions. Results revealed that both *P. chlororaphis* subsp. *aureofaciens* Sh2 and *P. fluorescens* Rh43 had the ability to reduce the root-knot nematodes population. During the growing season a high percentage reduction in the number of the egg mass in the root, ranging from 83.90% to 86.46%, was recorded. Data also revealed that the aggressiveness of the nematode was tremendously affected. The number of galls per root system was significantly ($P < 0.05$) decreased from 85.66 (control treatment) to 28.0 and 38.0, with percentage reductions of 67.31% and 55.63%, when the strains *P. chlororaphis* subsp. *aureofaciens* Sh2 and *P. fluorescens* Rh43 were added to pots infested with root-knot nematode. The number of root galls in the pots treated with Fenamiphos was higher than in pots with the bacterial treatments. Regarding the effect of bacterial agents on kiwifruit growth, results in Table 4 showed that all bacterial treatments significantly increased ($P < 0.05$) root growth as compared to the control; the application of *P. chlororaphis* subsp. *aureofaciens* Sh2 and *P. fluorescens* Rh43 resulted in increased root weights of 20.28 g and 20.13 g, respect-

Table 4: Effect of bacterial isolates on the root-knot nematodes Meloidogyne spp. infecting kiwifruit in greenhouse conditions. [1]

Bacterial treatment	Fresh root system weight (g)	Galls/plant	Reduction (%)	Egg masses/Plant	Reduction (%)	G^a/E.I.^{b}
Rh43	20.13 b	38.00 c	55.63	12.30 b	84.29	6/4
Sh2	20.28 b	28.00 d	67.31	10.60 c	86.46	5/4
Untreated infected plant (control +)	11.79 d	85.66 a	–	78.30 a	–	8/8
Fenamiphos	19.76 c	36.00 b	57.91	12.60 b	83.90	6/4
Untreated uninfected plant (control −)	24.22 a	0.00 e	0.00	0.00 d	0.00	1/1

ively.

4 Conclusions

Reducing the use of chemical pesticides is an objective that has been the focus of many research groups when developing new agriculture protection systems. Application of microorganisms as antagonists of plant pathogens is one of the most widely employed approaches of environmentally-safer disease control. Many bacteria and fungi have shown potential as agents for biological control of different crops and diseases (Ashoub & Amara, 2012; Cirvilleri, Bonaccorsi, Scuderi & Scortichini, 2005; Lee et al., 2011; B. K. Mishra et al., 2011; Kim & Knudsen, 2013; Scuderi et al., 2009).

In this study we isolated *Pseudomonas* strains from soils surrounding the roots of kiwifruit plants with biocontrol activity against root-knot nematodes (*Meloidogyne* spp.). In particular, the characterized strains *P. chlororaphis* subsp. *aureofaciens* Sh2 and *P. fluorescens* Rh43, when inoculated in the soil, positively controlled root-knot nematode aggressiveness in kiwifruit trees. Moreover, both pseudomonads increased root weights in kiwifruit plants. *P. chlororaphis* Sh2 was more effective than *P. fluorescens* Rh43 in reducing population of *Meloidogyne* spp.

Biological control of nematodes with bacteria includes different modes of action: obligate parasitism and interference processes of host recognition related to root exudate pattern changes; competition for nutrients in the root and induced systemic resistance (Z. A. Siddiqui & Mahmood, 1999; Hallmann et al., 2001; Sikora et al., 2007). Furthermore, Aalten, Vitour, Blanvillain, Gowen and Sutra (1998) reported that the presence of secondary metabolites in the culture filtrates was responsible for the nematicidal action.

In the greenhouse, the growth of kiwifruit plants treated with *P. chlororaphis* subsp. *aureofaciens* Sh2 and *P. fluorescens* Rh43 was superior in comparison to the control plants. Among the treatments, plants treated with Fenamiphos produced the lowest growth and supported the largest nematode infestations (Table 4). As previously reported, *Pseudomonas fluorescens* survives and colonizes the rhizosphere of all field crops and promotes plant growth by means of plant hormones. Reduction in multiplication of *M. incognita* by *P. fluorescens* treatment has also been reported in several other crops (Mohamed et al., 2009; Abo-Elyousr, Khan, El-Morsi Award & Abedel-Moneim, 2010; Ashoub & Amara, 2012). The present results also agree with the findings of Lee et al. (2011) reporting that the presence of secondary metabolites in the culture filtrates was responsible for the nematicidal action of *P. chlororaphis* 06. In other works similar results were obtained: I. A. Siddiqui, Haas and Heeb (2005) demonstrated that *Pseudomonas fluorescens* CHA0 mutant resulted in reduced biocontrol activity against the root-knot nematode *Meloidogyne incognita* during tomato and soybean infection; Hackenberg, Muehlchen, Forge and Vrain (2000) found nematode suppression in roots after 6 or 10 weeks by antibiotic-resistant mutant of *P. chlororaphis* Sm3.

Individual as well as mixed formulations have revealed protection against many soil pathogens (Felde et al., 2006; D. S. Mishra, Kumar, Prajapati, Singh & Sharma, 2013), and this will constitute a significant part of future research that will include the study of combined formulations of these and other microorganisms showing biocontrol features.

The present study clearly indicates that the use of *P. chlororaphis* subsp. *aureofaciens* Sh2 and *P. fluorescens* Rh43 significantly enhanced kiwifruit growth and reduced root-knot nematode populations, suggesting that it could be proposed for eco-friendly bionematicide use. However, further investigation is required to go beyond providing evidence for *in vitro*/greenhouse efficacy of the BCAs and need to involve original hypothesis testing and/or an extensive evaluation of two or more seasons testing in field studies.

[1] ^{a}Gall index; ^{b}Egg mass Index: 1 = no galls or egg masses, 2 = 1–5, 3 = 6–10, 4 = 11–20, 5 = 21–30, 6 = 31–50, 7 = 51–70, 8 = 71–100 and 9 > 100 galls or egg masses/plant (Zeck, 1971). The significance of the effect of bacterial treatment was evaluated by Duncan's multiple range test ($P < 0.05$).

Acknowledgements

We thank Jaime Cubero for the useful help in statistical analyses. This research was supported and funded by Iran Citrus Research Institute, Ramsar and Guilan University, Islamic Republic of Iran for which hereby is acknowledged.

References

Aalten, P. M., Vitour, D., Blanvillain, D., Gowen, S. R. & Sutra, L. (1998). Effect of rhizosphere fluorescent Pseudomonas strains on plant-parasitic nematodes Radopholus similis and Meloidogyne spp. *Letters in Applied Microbiology*, *27*, 357–361.

Abbasi, P. A., Riga, E., Conn, K. L. & Lazarovits, G. (2005). Effect of neem cake soil amendment on reduction of damping-off severity and population densities of plant-parasitic nematodes and soilborne plant pathogens. *Canadian Journal of Plant Pathology*, *27*(1), 38–45.

Abo-Elyousr, K. A., Khan, Z., El-Morsi Award, M. & Abedel-Moneim, M. F. (2010). Evaluation of plant extracts and Pseudomonas spp. for control of root-knot nematode, Meloidogyne incognita on tomato. *Nematropica*, *40*(2), 289–300.

Akhtar, M. & Alam, M. (1993). Utilization of waste materials in nematode control: A review. *Bioresource Technology*, *45*(1), 1–7.

Akhtar, M. & Malik, A. (2000). Roles of organic soil amendments and soil organisms in the biological control of plant-parasitic nematodes: A review. *Bioresource Technology*, *74*(1), 35–47.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410.

Ashoub, A. H. & Amara, M. T. (2012). Biocontrol Activity of Some Bacterial Genera Against Root-Knot nematode, Meloidogyne incognita. *Journal of American Science*, *6*(10), 321–328.

Barker, K. R. & Koenning, S. R. (1998). Developing sustainable systems for nematode management. *Annual Review of Phytopathology*, *36*, 165–205.

Bent, E., Loffredo, A., Mckenry, M. V., Becker, J. O. & Borneman, J. (2008). Detection and Investigation of Soil Biological Activity against Meloidogyne incognita. *Journal of Nematology*, *40*(2), 109–118.

Cirvilleri, G., Bonaccorsi, a., Scuderi, G. & Scortichini, M. (2005). Potential biological control activity and genetic diversity of Pseudomonas syringae pv. syringae Strains. *Journal of Phytopathology*, *153*(11-12), 654–666.

Eisenbeck, J. D., Hirschmann, H., Sasser, J. N. & Triantaphyllou, A. C. (1981). *A Guide to the Four Most Common Species of Root-Knot Nematodes (Meliodogyne Spp.), with Pictorial Key.* Raleigh, N.C., USA: The Departments of Plant Pathology, Genetics, and The United States Agency for International Development.

FAOSTAT. (2012). Production of Kiwi (fruit) by countries. Retrieved February 21, 2014, from http://faostat.fao.org/site/339/default.aspx

Felde, Z. A., Pocasangre, L. E., Carnizares Monteros, C. A., Sikora, R. A., Rosales, F. E. & Riveros, A. S. (2006). Effect of Combined Inoculations of Endophytic Fungi on the Biocontrol of Radopholus similis. *Info Musa*, *15*(1-2), 12–18.

Giné, A., Bonmatı, M., Sarro, A., Stchiegel, A., Valero, J., Ornat, C., ... Sorribas, F. J. (2013). Natural occurrence of fungal egg parasites of root-knot nematodes, Meloidogyne spp. in organic and integrated vegetable production systems in Spain. *BioControl*, *58*, 407–416.

Hackenberg, C., Muehlchen, A., Forge, T. & Vrain, T. (2000). Pseudomonas chlororaphis strain Sm3, bacterial antagonist of Pratylenchus penetrans. *Journal of Nematology*, *32*(2), 183–189.

Hallmann, J., Quadt-Hallmann, A., Miller, W. G., Sikora, R. A. & Lindow, S. E. (2001). Endophytic Colonization of Plants by the Biocontrol Agent Rhizobium etli G12 in Relation to Meloidogyne incognita Infection. *Phytopathology*, *91*(4), 415–422.

El-Hamshary, O. I. M., El-Nagdi, W. M. A. & Youssef, M. M. A. (2006). Genetical Studies and Antagonistic Effects of a Newly Bacterial Fusant Against Meloidogyne incognita Root-knot Nematode, and Plant Pathogen Fusarium oxysporum Infecting Sunflower. *Pakistan Journal of Biotechnology*, *3*(1-2), 61–70.

Hanna, A. I., Riad, F. W. & Tawfik, A. E. (1999). Efficacy of antagonistic rhizobacteria on the control of root-knot nematode, Meloidogyne incognita in tomato plants. *Egyptian Journal of Agricultural Research*, *77*(4), 1467–1476.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. & Williams, S. T. (1994). *Bergey's Manual of Determinative Bacteriology* (9th). Baltimore, USA: Williams and Wilkins.

Hussey, R. S. & Barker, K. R. (1973). A Comparison of Methods of Collecting Inocula of Meloidogyne spp. Including a New Technique. *Plant Disease Report*, *57*, 1025–1028.

Jayakumar, J. (2009). Streptomyces avermitilis as a biopesticide for the management of root-knot nematode, Meloidogyne incognita in tomato. *Karnataka Journal of Agricultural Siences*, *22*(3), 564–566.

Kim, T. G. & Knudsen, G. R. (2013). Relationship between the biocontrol fungus Trichoderma harzianum and the phytopathogenic fungus Fusarium solani f.sp. pisi. *Applied Soil Ecology*, *68*, 57–60.

King, E. O., Ward, M. K. & Raney, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescin. *The Journal of Laboratory and Clinical Medicine*, *44*(2), 301–307.

Knudsen, G. R. & Dandurand, L.-m. C. (2014). Ecological Complexity and the Success of Fungal Biological Control Agents. *Advances in Agriculture*, *2014*, 2–11.

Korayem, A., Youssef, M. & Mohamed, M. (2008). Effect of Chitin and Abamectic on Meloidogyne incognita Infesting Rapeseed. *Journal of Plant Protection Research*, *48*(3), 365–370.

Kratochvil, R. J., Sardanelli, S., Everts, K. & Gallagher, E. (2004). Evaluation of crop rotation and other cultural practices for management of root-knot and lesion nematodes. *Agronomy Journal*, *96*, 1419–1428.

Lagacé, L., Pitre, M., Jacques, M. & Roy, D. (2004). Identification of the bacterial community of maple sap by using amplified ribosomal DNA (rDNA) restriction analysis and rDNA sequencing. *Applied Environmental Microbiology*, *70*(4), 2052–2060.

Lamovšek, J., Urek, G. & Trdan, S. (2013). Biological Control of Root-Knot Nematodes (Meloidogyne spp.): Microbes against the Pests/BIOTIČNO ZATIRANJE OGORČIC KORENINSKIH ŠIŠK (Meloidogyne spp.): MIKROORGANIZMI PROTI ŠKODLJIVCEM. *Acta Agriculturae Slovenica*, *101*(2), 263–275.

Lee, J. H., Ma, K. C., Ko, S. J., Kang, B. R., Kim, I. S. & Kim, Y. C. (2011). Nematicidal activity of a nonpathogenic biocontrol bacterium, Pseudomonas chlororaphis O6. *Current Microbiology*, *62*, 746–751.

Lelliot, R. A. & Stead, D. E. (1987). Methods for the Diagnosis of Bacterial Diseases of Plants. In T. F. Preece (Ed.), *Methods in plant pathology* (2nd, p. 216). Oxford: Blackwell Scientific Publications.

Llop, P., Caruso, P., Cubero, J., Morente, C. & López, M. M. (1999). A simple extraction procedure for efficient routine detection of pathogenic bacteria in plant material by polymerase chain reaction. *Journal of Microbiological Methods*, *37*(1), 23–31.

Mishra, B. K., Mishra, R. K., Mishra, R. C., Tiwari, A. K., Yadav, R. S. & Dikshit, A. (2011). Biocontrol efficacy of Trichoderma viride isolates against fungal plant pathogens causing disease in Vigna radiata L. *Archives of Applied Science Research*, *3*(2), 361.

Mishra, D. S., Kumar, A., Prajapati, C. R., Singh, A. K. & Sharma, S. D. (2013). Identification of compatible bacterial and fungal isolate and their effectiveness against plant disease. *Journal of Environmental Biology*, *34*(2), 183–189.

Mohamed, Z. K., El-Sayed, S. A., Radwan, T. E. E. & El-Wahab, S. (2009). otency Evaluation of Serratia marcescens and Pseudomonas fluorescens as Biocontrol Agents for Root-knot Nematodes in Egypt. *Journal of Applied Sciences Research*, *4*(1), 93–102.

Oka, Y., Nacar, S., Putievsky, E., Ravid, U., Yaniv, Z. & Spiegel, Y. (2000). Nematicidal activity of essential oils and their components against the root-knot nematode. *Phytopathology*, *90*, 710–715.

Oka, Y., Shuker, S. & Tkachi, N. (2009). Nematicidal efficacy of MCW-2, a new nematicide of the fluoroalkenyl group, against the root-knot nematode Meloidogyne javanica. *Pest Management Science*, *65*(November 2008), 1082–1089.

Peix, A., Valverde, A., Rivas, R., Igual, J. M., Ramırez-Bahena, M. H., Mateos, P. F., ... Velázquez, E. (2007). Reclassification of Pseudomonas aurantiaca as a synonym of Pseudomonas chlororaphis and proposal of three subspecies. *International Journal of Systematic and Evolutionary Microbiology*, *57*(Pt 6), 1286–1290.

Scarpellini, M., Franzetti, L. & Galli, A. (2004). Development of PCR assay to identify Pseudomonas fluorescens and its biotype. *FEMS Microbiology Letters*, *236*(2), 257–260.

Schaad, N. W., Jones, J. B. & Chun, W. (2001). *Laboratory Guide for Identification of Plant Pathogenic Bacteria* (3rd). St Paul, MN, USA: APS Press.

Schneider, S. M., Rosskopf, E. N., Leesch, J. G., Chellemi, D. O., Bull, C. T. & Mazzola, M. (2003). United States Department of Agriculture-Agricultural Research Service research on alternatives to methyl bromide: pre-plant and post-harvest. *Pest Management Science*, *59*(6-7), 814–826.

Scuderi, G., Bonaccorsi, A., Panebianco, S., Vitale, A., Polizzi, G. & Cirvilleri, G. (2009). Some strains of Burkholderia gladioli are potential candidates for postharvest biocontrol of fungal rots in citrus and apple fruits. *Journal of Plant Pathology*, *91*(1), 207–213.

Sharon, E., Bar-Eyal, M., Chet, I., Herrera-Estrella, A., Kleifeld, O. & Spiegel, Y. (2001). Biological Control of the Root-Knot Nematode Meloidogyne javanica by Trichoderma harzianum. *Phytopathology*, *91*(7), 687–693.

Siddiqui, I. A., Haas, D. & Heeb, S. (2005). Extracellular protease of Pseudomonas fluorescens CHA0, a biocontrol factor with activity against the root-knot nematode Meloidogyne incognita. *Applied and Environmental Microbiology*, *71*(9), 5646–5649.

Siddiqui, I. A. & Shaukat, S. S. (2002). Rhizobacteria-mediated induction of systemic resistance (ISR) in tomato against Meloidogyne javanica. *Phytopathology*, *150*(8-9), 469–473.

Siddiqui, Z. A. & Mahmood, I. (1999). Role of bacteria in the management of plant parasitic nematodes: A review. *Bioresource Technology*, *69*, 167–179.

Sikora, R. A., Schafer, K. & Dababat, A. A. (2007). Modes of action associated with microbially induced in planta suppression of plant-parasitic nematodes. *Australasian Plant Pathology*, *36*(2), 124–134.

Small, C.-L. N. & Bidochka, M. J. (2005). Up-regulation of Prl, a subtilisin-like protease, during conidiation in the insect pathogen Metarhizium anisopliae. *Mycological Research*, *109*(Pt 3), 307–313.

Tanhamaafi, Z. & Mahdavian, S. I. (1997). Identification of Species and Races of Root-knot Nematode (Melodogyne spp) on Kiwi and Effect of M. incognita on Kiwi Seedling. *Pests and Plant Diseases of Iran*, *65*, 1–11.

Tsay, T. T., Wu, S. T. & Lin, Y. Y. (2004). Evaluation of Asteraceae Plants for Control of Meloidogyne incognita. *Journal of Nematology*, *36*(1), 36–41.

Westphal, A. & Scott Jr, A. W. (2005). Implementation of soybean in cotton cropping sequences for management of reniform nematode in South Texas. *Crop Science*, *45*(1), 233–239.

Whitehead, A. G. & Hemming, J. R. (1965). A comparison of some quantitative methods of extracting small vermiform nematodes from soil. *Annuals of Applied Biology*, *55*, 25–38.

Widmer, F., Seidler, R. J., Gillevet, P. M., Watrud, L. S. & Di Giovanni, G. D. (1998). A highly selective PCR protocol for detecting 16S rRNA genes of the genus Pseudomonas (sensu stricto) in environmental samples. *Applied and Environmental Microbiology*, *64*(7), 2545–2553.

Zeck, W. M. (1971). A rating scheme for field evaluation of root-knot nematode infestations. *Pflanzenschutz-Nachrichten Bayer*, *24*, 141–144.