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RESEARCH ARTICLE

Effect of Iranian strains of *Pseudomonas* spp. on the control of root-knot nematodes on Pistachios

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The role of some Iranian strains of *Pseudomonas* spp. as biocontrol agents against *Meloidogyne incognita* and their ability to colonise pistachio roots was investigated. The results of *in vitro* experiments indicated that all tested bacteria produced significant suppression of *M. incognita* and showed that all strains were able to kill *M. incognita* juveniles with strain VUPf428 achieving about 99% mortality at 72 h. The results of *in vivo* treatments indicated that the best strains that could build high populations in soil infested with nematodes were VUPf5, VUPf52 and VUPf205. These isolates also caused highest reduction in galling and nematode multiplication in a greenhouse test although all strains native to Iran could colonise pistachio roots in pots. Some strains could produce secondary metabolites such as siderophores, proteases and volatile metabolites at high population levels.

Keywords: *Meloidogyne incognita*; *Pseudomonas fluorescens*; *Pistacia vera*; root-knot nematodes

Introduction

Pistachio *Pistacia vera* (L.) is an economically important export product in Iran. Root-knot nematode damage is one of the major challenges in Iranian pistachio production. The management of nematodes is more difficult than that of other pests because nematodes mostly inhabit the soil and usually attack the underground parts of the plants (Stirling, 1991).

The presence of bacteria and nematodes in soil and their relation to each other creates the possibility of using soil microorganisms to control plant parasitic nematodes (Jatala, 1986; Mankau, 1980). The efficiency of rhizobacteria has been reported in some crops such as potato, sugar beet, radish and sweet potato (Farzana, Saad, & Kamaruzaman, 2009). Root-colonising fluorescent pseudomonads have potential to control diseases caused by root-knot nematodes (Rainey, 1999). Also, fluorescent pseudomonads can produce toxic secondary metabolites such as proteases, hydrogen cyanide (HCN) and siderophores.

Take-all disease (*Gaeumannomyces graminis* var. *tritici*) can be suppressed by the *Pseudomonas fluorescens* VUPf5 strain up to 85% by producing siderophores,

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HCN, protease, phenazine and volatile metabolites (Lagzian, Saberi Riseh, Khodaygan, Sedaghati, & Dashti, 2013). Pseudomonads affect nematodes by a variety of modes such as parasitisation, production of toxins, antibiotics or enzymes and interfering with nematode plant host recognition (Siddiqui & Mahmood, 1999). These bacteria affect a wide range of organisms, including free living and predatory nematodes and plant parasitic nematodes (Mankau, 1980). They act in a complex interaction with bacteria, nematodes and plants (Kerry 2000). Rhizobacteria have the ability to colonise the developing root system in the presence of competing soil microflora (Kloepper, Rodeigez-Kabana, Mcilnroy, & Young, 1992). Certain root-associated strains of fluorescent pseudomonads have an inhibitory role against soil-borne plant pathogens through the production of metabolites (Dowling & O'Gara, 1994; Keel et al., 2000). An investigation to evaluate *Pseudomonas* spp. for the control of *Meloidogyne incognita* on tomato plants (Hanna, Riad, & Tawfik, 1999) found that the number of gall formation and root gall index decreased when plants were inoculated with the bacteria. Some researchers noted that *P. fluorescens* and *Pseudomonas aeruginosa* reduced *Meloidogyne javanica* juvenile penetration into tomato plants. Also, they affected *M. incognita* juveniles' survival *in vitro*, and nematode mortality depended on the bacterial concentration and exposure time (Siddiqui & Shaukat, 2002). The aim of this research was to test Iranian strains of fluorescent pseudomonads for controlling root-knot nematode on Pistachio.

Materials and methods

Bacterial strains and culture conditions

Thirty fluorescent *Pseudomonas* strains isolated from different plant species throughout Iran were used (Table 1). Single colonies were used for all strains (Anderson, Liberta, & Neville, 1980). For short-term storage, bacterial strains were cultured on nutrient agar (NA) in test tubes, after 24 h of growth, the cultures were over layered by sterilised paraffin and stored at 4°C.

Preparation of M. incognita *inoculum*

Soil and root samples were collected from Badami cultivar pistachio orchard in Rafsanjan. Samples were kept in bags and transported to the laboratory for further analysis. In order to identify large-scale inoculum production of *Meloidogyne*, pure cultures of egg masses were maintained on tomato plants (*Lycopersicon esculetom* cv. Rutgers) grown in pots for 4 months.

To identify the *Meloidogyne* sp., single root galls (n = 10–20) were dissected and adult females were removed and used for the preparation of the perineal patterns for their identification according to species (Eisenback & Hirschmann, 1991; Onyeke & Akueshi, 2012). Detailed observations of 10–20 female nematodes indicated that the perineal patterns corresponded to *M. incognita* (Jepson, 1987).

For preparation of second-stage juveniles, egg masses were carefully picked from the roots with the help of forceps and rinsed with sterile water, placed in sodium hypochlorite solution (NaOCl = 0.5%), agitated for 4 min and rinsed with sterile water on a sieve (500 mesh, aperture = 0.025 mm) (Hussey & Barker, 1998). The extracted eggs were transferred to a small coarse sieve covered with tissue paper in a

Table 1. Source and code of bacterial strains from the rhizosphere of several plants.

Isolates	Source of isolates	Code of isolates
P. fluorescens	Tobacco (Switzerland)	CHA0
	Tobacco (Switzerland)	CHA89
	Berry (Iran North)	VUPf 93
	Cherry (Jiroft, Iran)	VUPf 428
	Potato (Ardebil, Iran)	VUPf 506
	Wheat (Baft, Iran)	VUPf 49
	Wheat (Baft, Iran)	VUPf 44
	Wheat (Iran North)	VUPf 205
	Peach (Iran North)	VUPf 5
	Plantain (Tehran, Iran)	T29-z
	Wheat (Washington, USA)	2-79
	Alfalfa (Shahdad, Iran)	VUPf 759
	Peach (Iran North)	VUPf 52
Pseudomonas sp.	Plantain (Tehran, Iran)	T17-4
	Orange (Bandar abbas, Iran)	VUPf 407
	Pine (Tehran, Iran)	VUPf 760
	Orange (Bandar abbas, Iran)	VUPf 409
	Pistachio (Rafsanjan, Iran)	BP1
	Ryegrass (Bam, Iran)	VUPf 354
	Peach (Iran North)	VUPf 60
	Plantain (Esfahan, Iran)	VUPf 680
	Orange (Iran North)	VUPf 432
	Pine (Iran North)	VUPf 240
	Alfalfa (Shahdad, Iran)	VUPf 738
	Rattler (Shahdad, Iran)	VUPf 747
	Peach (Iran North)	VUPf 58
	Wheat (Baft, Iran)	VUPf 50
	Cypress (Esfahan, Iran)	VUPf 673
	Plantain (Esfahan, Iran)	VUPf 686
	(Tehran, Iran)	F140

Petri plate (18 × 2.5 cm, diameter × height, ca. 70 egg masses per dish) containing sufficient amount of water. The Petri plates were kept at 27 ± 5°C for 2 days and second-stage juveniles were collected in sterile water. Suspensions containing nematode larvae were disinfected with tetracycline (50 mg/l), and streptomycin sulphate (0.044 g/l) for 15–20 min and then washed three times with sterile distilled water. Seedlings of pistachio Badami-Zarandi cultivar grown in autoclaved soil were inoculated with the second-stage juveniles for greenhouse experiments.

Effects of bacterial strains on mortality of Meloidogyne *juvenile under in vitro conditions*

Fluorescent *Pseudomonas* strains were cultured on NA. Bacterial strains were suspended in sterile distilled water and the concentration was adjusted to 10^{10} cfu/ml (optical density = 0.50 at 540 nm) using a UV-visible spectrophotometer (Spectronic®, UK). In order to evaluate the effectiveness of bacterial strains on *M. incognita*

juvenile mortality, 2 ml of water containing 50 freshly sterile juveniles of *M. incognita* were first added to each Petri dish (6 cm diameter) followed by 2 ml of the bacterial suspensions. The plates were kept at room temperature in a completely randomised design. Each strain was tested three times and experiments were replicated thrice. Control *M. incognita* juveniles were treated with sterile distilled water in the same way. Mortality was recorded after 24, 48 and 72 h. Mortality was checked by poking the juveniles with a sterile needle. At the end of the exposure times (72 h), the juveniles were transferred to distilled water (24 h) to ensure that no recovery occurred (Ashoub & Amara, 2010).

The production of volatile metabolites

A fresh culture of bacterial strains on NA was spread onto King's B (KB) containing glycine (4.4 g/l). Whatman papers soaked in picric acid (0.50% V/V) and sodium carbonate (2% W/V) were placed in the lid of plates which were then incubated at 27°C for 48–72 h. Any positive response caused the indicator paper to turn from yellow to cream, light brown, dark brown and brick (Alstrom & Burns, 1989).

Proteases

Production of extracellular protease was determined by culturing bacterial strains on skim milk agar (SMA) plates (Maurhofer, Keel, Haas, & Défago, 1995). Semi-quantification evaluation of protease production was carried out by measuring a clear halo zone around the bacterial colonies. The experiments were performed in triplicate.

Siderophores

Evaluation of siderophore production was performed using Chrome Azurol S (CAS) medium as described by Schwyn and Neilands (1987). The diameter of the orange halo around colonies was measured after 48–72 h incubation at 27°C.

Effects of selected bacterial strains on Meloidogyne *juveniles under greenhouse conditions*

Eleven bacterial strains with the highest suppressive effects on *M. incognita* larvae in the *in vitro* experiments were used under greenhouse conditions. Seeds of Pistachio (cv. Badami) were sown in pots (25 cm in diameter) containing sterilised soil (sand and clay; 2:1; V/V). Soil was collected from pistachio orchards in Rafsanjan, Iran and autoclaved for 45 min at 121°C. When pistachio seedlings reached to 8–10 leaves, 10 ml suspensions of bacterial strains were added as a soil drench (10^8 cfu/ml for each pot). Two months later, an estimated 2000 juveniles of *M. incognita* were used for inoculation in 20 ml sterile distilled water for each pot. The suspension was poured into a shallow trench created around the root tips of each of the test plants (Hussey & Boerma, 1981) and covered immediately with top soil (Goswami & Chenulu, 1974). Pots were kept at 24°C ± 2°C in a complete randomised design with five replicates containing one seedling. Two months after nematode inoculations, pistachio seedlings were uprooted and the number of galls (gram) and populations of *M. incognita* juveniles in root and soil (sum of different larval instars) were determined.

Populations of bacterial strains were determined in the rhizosphere of pistachio seedlings 1 month either before or after nematode inoculation. Briefly, 1 g of

rhizosphere was transferred to 1 ml of sterile distilled water and then shaken thoroughly. For each sample, a serial dilution of 10^{-1} to 10^{-3} in 0.1 ml aliquots were spread on NA medium in three replications. The populations were recorded after 24 h incubation at 28°C in darkness.

Statistical analysis

The average values of juvenile mortality, populations of bacterial strains and *M. incognita* juveniles in soil, and number of galls per gram of root were separately determined for each replication. The data were analysed using Proc GLM procedures (SAS Release Version 9.1, SAS Institute, Inc., Cary, NC) by one-way analysis of variance (ANOVA). When it was necessary, data were log-transferred prior to analysis. Mean comparison was done using a Duncan's multiple range test (ANOVA, $p > 0.05$, SAS version 6.12).

Results and discussion

In vitro *experiment*

Efficacy of the selected strains in the *in vitro* experiment under greenhouse conditions ranged from 79% to 99% with the suppressive activity increasing gradually with the increment of the exposure periods (Table 2). All strains exhibited high ability to kill second-stage juveniles after 72 h. However, after 24 h, strain VUPf428 exhibited a distinct effect, reaching 80% juvenile morality. During the two beginning exposure

Table 2. Mortality of *Meloidogyne incognita* juveniles exposed to *Pseudomonas* strains for 24, 48 and 72 h in *in vitro* tests in Petri dishes.

	Juvenile mortality (%)				Juvenile mortality (%)		
Isolate code	24 h	48 h	72 h	Isolate code	24 h	48 h	72 h
VUPf428	80.0a	93.3a	99.0a	VUPf673	72.0a–f	84.0b–e	98.0a–f
CHA0	72.0a–f	87.3c–g	94.5ab	VUPf49	74.0a–b	84.0ab	94.0ab
VUPf354	56.0c–h	59.0c–g	94.0a–c	VUPf506	68.7a–d	80.0a–c	93.3ab
VUPf93	73.3a–c	80.0a–d	90.0a–h	VUPf205	57.3b–h	68.0b–g	86.7a–d
VUPf60	66.7a–f	80.0a–c	85.3a–e	BP1	53.3c–h	70.7d–g	84.0h–l
VUPf5	48.7e–i	52.0fg	80.0b–f	VUPf52	57.3b–h	68.7b–g	80.0c–g
VUPf738	54.0c–h	70.0b–g	79.3c–g	VUPf409	54.0c–h	68.0b–g	78.7c–h
VUPf680	40.0h–j	60.1c–g	78.0d–i	VUPf407	62.7a–g	68.7b–g	76.7d–i
VUPf44	43.3g–i	72.0b–f	76.0d–i	VUPf759	70.7a–e	72.7b–g	75.3d–j
T29-Z	54.0c–h	68.0b–g	72.7e–i	VUPf747	60.7b–g	52.7d–g	67.3h–l
VUPf58	43.3g–i	61.3c–g	65.3h–l	2-79	22.7j	51.3g	64.7i–l
T17-4	48.7e–i	54.0e–g	62.0j–l	VUPf240	40.0h–j	52.0fg	61.3j–l
VUPf760	50.0d–i	57.3d–g	60.7j–l	VUPf432	49.3f–i	50.0fg	58.7kl
VUPf686	51.3c–i	56.0c–g	58.0f–k	VUPf50	26.7i–j	53.3e–g	55.3l
Control set							
Culture medium		0		0		0	
Distilled water		0		0		0	

Note: In each column, values followed by the same letter (s) are not significantly different ($P \leq 0.05$) from each other according to Duncan's multiple range test.

periods, the weakest strain was 2-79, where it resulted in juvenile mortalities of 23% after 24 h and 51% after 48 h. It was clear that the nematostatic and nematocidal effect of *P. fluorescens* 428 was highest after 24 h and 48 h resulting in juvenile mortalities of 99% and 98% consistently until 72 h.

Regarding the diversity set, the best strain was VUPf428, where it gave distinctive results at all exposure times obtaining 99% juvenile mortality after 72 h. *M. incognita* juveniles were still alive in the two control treatments (media and distilled water) at 72 h.

The production of volatile metabolites

Strains VUPf60 and VUPf5 produced higher quantities of volatile metabolites (Table 3). Other strains had little or no production of volatile metabolites in KB medium supplied with glycine. Production of these metabolites by fluorescent pseudomonads has been shown to influence efficacy against *M. incognita* juveniles (Khan, Shaukat, Islam, & Khan, 2012). Production of volatile metabolites by *Pseudomonas* spp. has been shown to improve growth in potatoes (Bakker & Schippers 1987; Bakker, Bakker, & Schippers, 1989) among other crops (Schippers, Bakker, & Bakker, 1987) and suppressing root pathogens (Défago et al., 1990).

Proteases

Extracellular protease has a key role in suppressing root-knot disease in limiting egg hatching and killing juvenile nematodes (Siddiqui, Haas, & Heeb, 2005). The strains we tested displayed proteolytic activity in SMA medium. VUPf5 yielded the highest protease activity with a clear zone of approximately 7 mm after 24 h, and 15 mm after 48 h (Table 3). The importance of exoprotease in reduced biocontrol activity of CHA0 against *M. incognita* has been previously reported (Siddiqui et al., 2005). It seems that some metabolites have positive effects on each other like exoprotease and

Table 3. The production of volatile metabolites and proteases by *Pseudomonas* strains.

Bacteria strains	Volatile metabolites[a]	Siderophore[b]	Protease (mm)[c]
CHA0	+	+	6
CHA89	–	–	0
VUPf428	+	+	2.5
VUPf60	+++	+	2.5
VUPf205	++	+	2.25
VUPf93	–	+	1.5
VUPf49	++	+	1.5
VUPf5	+++	+	7
VUPf673	++	+	3.5
VUPf738	+	–	3
VUPf506	–	–	6
VUPf52	+	+	6
VUPf354	+	+	5

[a]Colour change of indicator paper to dark brown++; +, light brown; –, no colour change.
[b]Siderophores produced (+), Siderophores NOT produced (–).
[c]Diameter of halo zone around the bacterial colonies (mm) after 24 h. Each number is mean of three replicates.

2,4-diacetylphloroglucinol (DAPG) increasing the biocontrol activity against *Pythium* (Dunne, Moenne-Loccoz, de Bruijn, & O'Gara, 2000).

Siderophores

Strain VUPf5 produced siderophores on CAS blue agar, changing the colour of the CAS medium from blue to orange. There are several strains able to induce resistance in plants by siderophore production (Höfte & Bakker, 2007). Some reports have shown that production of pyoverdines contributes to the biocontrol capacity of the fluorescent pseudomonads (Becker & Cook, 1988; Loper & Buyer, 1991). Siderophore production is one of the mechanisms of bacterial antagonism against soil-borne pathogens (Loper & Buyer, 1991).

In vivo *study*

P. fluorescens CHA0 and CHA89 strains were chosen despite their providing 94% and 79% juvenile mortality in the *in vitro* tests. We also choose them for greenhouse experiments because CHA0 colonises the rhizosphere of important crops and produces several limiting metabolite compounds which are important biocontrol factors in suppression of soil-borne diseases (Haas & Défago, 2005), but strain CHA89 has a defect in the gacA gene and does not produce a large amount of secondary metabolites in contrast to strain CHA0. Therefore, the ability of CHA89 to kill juveniles is weaker than CHA0.

Strain VUPf5 increased its population in soil more than the other strains and had better establishment in soil and rhizosphere of pistachio roots (Table 4). Strains VUPf52, VUPf673 and CHA0 also significantly increased their populations. As expected, CHA89 had the lowest population among the strains.

After inoculation with nematodes, VUPf5, VUPf93, CHA0, VUPf93 and VUPf52 had the highest populations and VUPf506 had the lowest. However, it does not mean that their efficacy to control nematodes was affected. The plant growth promoting rhizobacteria (PGPR) characteristic of *P. fluorescens* on the growth of seedling roots is evident even if they were infected with *M. incognita*; beside disease reduction, VUPf5 increased plant growth compared to control. The mechanisms of PGPR are not completely understood but are considered to have the following: (1) the ability to produce plant hormones, such as auxins (Egamberdiyeva, 2005), cytokinins (García de Salamone, Hynes, & Nelson, 2001) and gibberellins (Gutiérrez-Mañero et al., 2001); (2) asymbiotic nitrogen fixation (Canbolat, Barik, CaKBakci, & Sahin, 2006); (3) solubilisation of inorganic phosphate and mineralisation of organic phosphate or other nutrients (Jeon, Lee, Kim, Ahn, & Song, 2003) and (4) antagonism phenomenon against plant pathogenic microorganisms by production of siderophores, antibiotics, enzymes or competition with pathogens. It has been reported that due to nitrogen fixing or phosphate solubilisation, bacterial applications increased growth rate and yield of apricot (Esitken, Karlidag, Ercisli, Turan & Sahin, 2003), peanut (Dey, Pal, Bhatt, & Chauhan, 2004) and apple (Aslantas, Cakbakci, & Sahin, 2007). VUPf5 has the capability to control phytopathogenic fungi in wheat (Lagzian et al., 2013) and root-knot nematode on pistachio seedlings. The antagonistic activities are presumably due to the production of siderophores (Kloepper, Leong, Teintze, & Schroth, 1980; Leong, 1986). The results of secondary metabolite production showed that there was a strong

Table 4. Population density of *P. fluorescens* strains before and after inoculation of pistachio seedlings with *M. incognita* and their effect on nematode populations.

Pseudomonas strains	Pseudomonas population at 30 days of inoculation	Pseudomonas population at 60 days after inoculation of 2000 larvae	Number of galls	Larvae
CHA0	2.38×10^{12}c	1.21×10^{8}d	26.40bc	99.40cd
CHA89	5.94×10^{7} l	3.64×10^{7}l	25.60bc	137.00d
VUPf5	4.97×10^{14}a	4.37×10^{11}a	10.60e–g	47.00gf
VUPf52	7.03×10^{11}d	6.79×10^{7}h	6.20fg	37.30g
VUPf93	1.75×10^{8} i	1.61×10^{8}c	24.40b–d	71.20ef
VUPf428	1.41×10^{8}j	7.27×10^{7}g	14.80c–f	80.0ed
VUPf205	9.70×10^{9}e	6.67×10^{7}i	8.00fg	33.80g
VUPf49	9.70×10^{9}e	3.64×10^{7}b	12.60d–f	56.20e–g
VUPf506	2.30×10^{9}f	5.10×10^{6}m	16.00c–f	105.30c
VUPf738	6.30×10^{12}b	8.12×10^{7}e	20.20b–e	68.30ef
VUPf60	7.28×10^{8}h	4.37×10^{7}j	30.60b	148.30b
VUPf354	9.70×10^{8}g	7.74×10^{7}f	26.40bc	56.00e–g
VUPf673	1.40×10^{8}k	3.88×10^{7}k	13.00d–f	65.80ef
Inoculated with nematode	0m	0n	62.80a	1158.00a

Note: Initial population: *Meloidogyne incognita* with 2000 J2 and *Pseudomonas* spp. 10^{8} cfu/ml. Larva: total of J2, J3 and J4 larva in soil and root. The data are the average of five repetitions. Means with the same letter are not significantly different from each other.

relationship between production of siderophores, volatile metabolites, proteases and the origin or hosts from which the fluorescent *Pseudomonas* spp. were isolated and the rate of disease inhibition. VUPf5 which had the highest root density could be used in combination with VUPf52 which had a strong ability for gall reduction (Table 4). Our study indicates that these two strains might be successfully used for the biocontrol of *M. incognita* on pistachio. However, the effects of these strains on each other as well as field studies have yet to be investigated.

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