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Biocontrol potential of *Pochonia chlamydosporia* var. *chlamydosporia* isolates against *Meloidogyne javanica* on pistachio

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Abstract

The biocontrol potential of four *Pochonia chlamydosporia* var. *chlamydosporia* isolates on the root-knot nematode, *Meloidogyne javanica* was investigated on pistachio plants in a greenhouse experiment. Isolates were applied at 10,000, 5000 and 1000 chlamydospores per gram of soil; the latter two densities were being used for strain Pcc60 only and designated as PccB and PccA, respectively. Plants were inoculated with a suspension containing 3000 eggs of *M. javanica*. Nematode reproduction was reduced to 57% by Pcc20 and 36% by Pcc10 and Pcc60C after 4 months. Pcc20 was the most promising isolate as the final egg population of nematodes was reduced by nearly 61 and 36% by Pcc10 and Pcc60C, respectively. All strains infected eggs on the roots to varying degrees. Pcc20 was the most effective colonizer of all the isolates, infecting 37% of the eggs, while Pcc10 and Pcc60C as the second best colonizers caused an average infection rate of 17.5%. The potential of the fungus was reduced at lower densities of spore inoculum. Semi-selective medium was used to re-isolate the fungi from soil and rhizosphere but was not able to detect Pcc60A at 1000 cfu/g soil, despite the fact that 3% of the eggs were parasitized. The efficiency of the different isolates in controlling nematode reproduction parameters and their relationships in terms of survival and abundance is discussed further.

Keywords: Biological control, *Pistacia vera*, Root-knot nematode, *Meloidogyne javanica*, Survival, *Pochonia chlamydosporia* var. *chlamydosporia*

Background

Pistachio, *Pistacia vera* (Anacardiaceae) trees originated in the dry lands and desert climates of Asia Minor and are known in Iran as "Green Gold", being an important nutritional and economic commodity. Iran is the biggest producer of pistachio in the world, with approximately 346,000 ha of land under its cultivation and producing approximately 315,000 tons of yield annually (FAO 2016).

Plant parasitic nematodes are major constraints to agricultural production worldwide (Stirling 2014). In a list of the top ten rated plant parasitic nematodes, root-knot nematodes occupy the first position (Jones et al. 2013). Ten of the 98 described Meloidogyne species are agricultural pests whereas 5 species of cyst and 6 species of lesion nematodes are of economic importance. Root-knot nematodes are obligate biotrophs of a wide range of plant species, the most important species being the tropical *Meloidogyne arenaria*, *M. incognita* and *M. javanica* and the temperate *M. hapla* (Jones et al. 2013). In Iran, *M. incognita* and *M. javanica* are the major economic species found in pistachio orchards (Banihashemi and Kheiri 1995). Their damage impact and its management is one of the major challenges for the Iranian pistachio industry.

The management practices used in agriculture must be ecologically sound and promote a soil food web capable of keeping populations of plant-parasitic nematodes below the economic damage threshold (Stirling 2014). Managing biological diversity, productivity and vitality are the essence of a sustainable agriculture (Lewandowski et al. 1999). Thus, practices of pest management should protect those soil organisms that play an important part in soil health sustainability (Stirling 2014).

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Intensive use of nematicides has led to increased microbial degradation of organophosphate and carbamate nematicides in soil and outbreaks of damage caused by parasitic nematodes (Hugo et al. 2014). The urge to find alternatives to chemical nematicides and the phasing out of methyl bromide fumigant caused a vast uprising in research towards eco-friendly measures like biological control of parasitic nematodes (Stirling 2014).

The fungus, *Pochonia chlamydosporia* var. *chlamydosporia* (Goddard) Gams & Zare (Clavicipitaceae), is a facultative parasite of root-knot and cyst nematodes (Anastasiades et al. 2008; Moosavi et al. 2010; Carneiro et al. 2011 and Evans et al. 2017). The filamentous fungus is a saprotrophic in soil (Siddiqui et al. 2009) and is able to colonize roots as an endophyte of diverse species (Bordallo et al. 2002). It has been reported that *P. chlamydosporia* colonizes roots endophytically, thereby promoting plant growth and eliciting plant defenses (Lopez-Llorca et al. 2002).

Isolates of *P. chlamydosporia* var. *chlamydosporia* differ in their pathogenicity to nematodes, their ability to grow in the rhizosphere, in production of chlamydospores and in their temperature optima for growth (Irving and Kerry 1986). In addition, the host plant has a significant effect on the growth of the fungus in the rhizosphere (Bourne et al. 1996).

One of the features of egg-parasitic fungi is their ability to penetrate the chitin layer in the eggs. Further proof of this has been obtained in recent studies when chitosan was found during nematode egg infection, an advantage for the adaptation of biocontrol fungi to their natural environment (Aranda-Martinez et al. 2016). This feature of chitosan production has been taken further by Aranda-Martineza et al. (2017), showing that ethylene can be produced from chitosan using nematophagous and entomopathogenic fungi.

Most studies of the biocontrol ability of *P. chlamydosporia* against parasitic nematodes have been on annual crops, however, and reports on perennial crops are few (Stirling 2014).

The main goal of this study was to characterize four isolates of *P. chlamydosporia* var. *chlamydosporia* for future selection as a biocontrol agent against *M. javanica* on pistachio. In greenhouse conditions, whether these isolates are able to colonize roots, the following criteria were evaluated:

1) Their competence in colonizing egg masses on the roots; the degree to which they were able to reduce reproduction of *M. javanica;*
2) Their capability for establishment, survival and multiplication whether they could promote growth of pistachio plants.

Methods

Nematode inoculum

Nematodes used for the experiment were reared on tomato seedlings of cv Early Urbana inoculated with 5000 second-stage juveniles (J2) as explained in Ebadi et al. (2009). At harvest, plant roots were washed, cut into pieces, placed in a jar of 0.5% commercial NaOCl and shaken for 4 min. The suspension was washed with tap water through 75- and 20-μm sieves and their numbers were counted with a counting slide under a light microscope (Hussey and Barker 1973).

Fungal production

The four strains of *P. chlamydosporia* var. *chlamydosporia* (Pcc isolated with the accession numbers of Pcc10, Pcc20, Pcc30 and Pcc60) used in this study had been maintained on corn meal agar at 5 °C in the Nematology Department Collection, Iranian Research Institute of Plant Protection, Tehran.

The fungal inoculum preparations were made according to the procedure of De Leij et al. (1993). Conical flasks were filled with a mixture of sand + milled barley (1:1 *v*/*v*) and 30 ml distilled water was added for each 100 g of mixture and autoclaved at 121 °C for 20 min on two consecutive days. Flasks were inoculated with plugs of isolates, kept at 25 °C and occasionally shaken for even growth. After 3 weeks, 5 g of the sand/barley substrate, mixed well with 100 ml distilled water, was transferred to a blender (Waring) and blended for 2 min. The contents were washed onto a 45-μm aperture sieve, and the chlamydospores were collected on a nested 10-μm sieve (De Leij et al. 1993). The chlamydospores were counted by a hemocytometer.

Greenhouse experiment

A suspension of washed chlamydospores was mixed with 50 g sterilized sand, added to 1 kg natural soil collected from a pistachio orchard, and the resulting mixture was used to fill a 14-cm diameter plastic pot, to give a final count of 1×10^4 chlamydospores/g soil. This spore density was used for all four fungal isolates and two further sets of chlamydospore densities were prepared to give 1×10^3 and 5×10^3 chlamydospores/g soil for isolate Pcc60 only and designated Pcc60A and Pcc60B respectively and Pcc60C (1×10^4 chlamydospores/g soil).

The nematode genera present in soil were mostly non-parasitic nematodes with very few Tylenchidae, and there were no root-knot nematodes present.

Pistachio cv Kaleghochi was chosen for the experiment. Prior to planting, seeds were disinfested for 4 min in 1% NaOCl, rinsed with sterile distilled water, immersed in 1% pentachloronitrobenzen, followed by soaking overnight in sterilized water and then pre-

germinated in the dark on moist filter paper in Petri dishes.

One seedling was planted in each pot and allowed to establish for a month, when each relevant pot was inoculated with a suspension of nearly 3000 eggs of *M. javanica* added to three holes around each plant. Treatments included: nematode + isolate of Pcc10, Pcc20, Pcc30 and Pcc60C (10,000 cfu/g soil); nematode + isolate Pcc60B (5000 cfu/g soil), nematode + isolate Pcc60A (1000 cfu/g soil), nematodes alone and pistachio alone. Each treatment was replicated five times, and the pots were arranged in a completely randomized design on a glasshouse bench, with average temperature of 27.5 °C, and irrigated as required.

Plants were harvested after 4 months. Roots were washed in water, blotted gently dry and the fresh weights of the pistachio shoots and roots were taken. The numbers of galls or egg masses on roots were rated based on the 0–5 scale of Hartman and Sasser (1985).

To determine nematode multiplication rates, eggs were extracted from the roots by the NaOCl, using the same procedure as described above (Hussey and Barker 1973), treated roots were further processed in a blender to extract any possibly hidden eggs within the roots. The extracts were filtered through 75- and 20-μm sieves, and the numbers of eggs were estimated from the contents of the latter.

The populations of J2 were measured in 200 g soil from each pot of each treatment combination by means of modified Whitehead trays (Whitehead and Hemming 1965). The final total population density of healthy nematodes was calculated by combining the total numbers of J2 and healthy eggs. For estimation of the numbers of healthy eggs, the total numbers of infected eggs were subtracted from the total numbers of eggs. A reproduction factor was estimated by dividing the final nematode population density by the initial population density (Pf/Pi) (Ebadi et al. 2009).

To verify the percentage of egg infection, 10 egg masses/replicate (there were few egg masses at the time) were incubated in 0.05% NaOCl between a glass slide and coverslip and observed under × 400 magnification. To re-isolate and confirm identification of these infected eggs, a 0.2-ml sub-sample of a suspension made from these eggs was taken, spread over the surface of 0.8% water agar, containing 50 ppm each of tetracycline, chloramphenicol and streptomycin, and the grown hyphae were sub-cultured on PDA for further examination.

Viability and abundance of the fungi in soil and on the roots at the end of the experiment were checked, using a SSM (semi-selective medium) (De Leij and Kerry 1991). A sub-sample of 1 g soil from each replicate was added to 9 ml of 0.05% sterile water agar and used to prepare dilution series of 10^{-1} to 10^{-4}. An aliquot of 0.2 ml of each dilution was transferred onto a 9-cm Petri dish containing SSM, with three replicates per dilution. Dishes were kept in an incubator at 25 °C for 1 or 2 weeks and the final numbers of cfu were counted.

Semi-selective medium contained the following: 37.5 mg carbendazim, 37.5 mg thiabendazole, 75 mg rose bengal, 17.5 mg NaCl, 50 mg each of streptomycin sulphate, chlortetracycline hydrochloride and chloramphenicol, 3 ml Triton X-100, and 17 g corn meal agar (Difco) in a liter of distilled water.

Roots of each replicate were cut into small pieces and then a 1 g sub-sample was rinsed with sterile distilled water, crushed with a sterilized pestle and mortar and added to 9 ml of 0.05% sterile water agar solution. As in the soil test, a dilution series was prepared and the suspensions were transferred onto the SSM. Colonies were counted after 1 week (Bourne et al. 1996).

Statistical analysis

All data were subjected to analysis of variance (ANOVA), using SPSS version 16. Data were checked for homogeneity of variance before being pooled. Data for percent egg infection, the reproduction factor and number of healthy eggs and J2/pot were transformed to arcsin (x), sqrt $(x + 0.5)$ and log $(x + 1)$, respectively, before ANOVA. Means were separated using the least significant difference (LSD) test ($P \leq 0.05$).

Results and discussion

All fungal isolates were able to survive to the end of the experiment (Fig. 1). However, none of the isolates proliferated in the soil during the time of the experiment as the final density of their spores declined to less than the initial density of inoculum. For survival potential, the

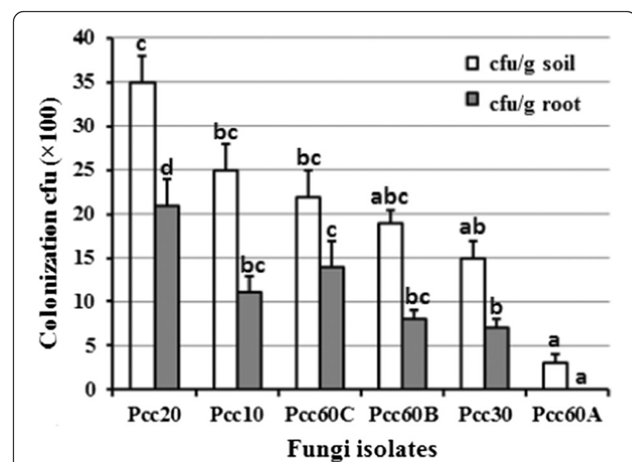

Fig. 1 Numbers of colony-forming units (cfu) of *P. chlamydosporia* var. *chlamydosporia* isolates by the end of experiment, in soil and rhizosphere of pistachio. Columns with similar letters are not significantly different at 5% live based on LSD test. Pcc10, Pcc20, Pcc30, Pcc60C = 1×10^4, Pcc60A = 1×10^3 and PccB = 5×10^3 cfu/g soil

strains are ranked as Pcc20, Pcc10, Pcc60C and Pcc30 for having the highest to lowest final cfu/g soil. The number of chlamydospores of *Pochonia* in pots, receiving 5000 cfu/g soil of Pcc60B as inoculum, was less than some isolates applied at 1×10^4 cfu/g soil, but was greater than the treatment with Pcc30. Strain Pcc60A survived during the experiment, although it had the least number of final spore/g soil of all strains.

Pochonia isolates colonized the root surface of pistachio and their numbers were greater for the strains of Pcc20 and Pcc60C (of average 1750 cfu) than other treatments (of average 650 cfu) (Fig. 1). Decreasing density of inoculum to 5×10^3 spores did not show any detrimental effect on survival of strain Pcc60B, which colonized roots and survived well till the end of the experiment. There was no colony growth on SSM from roots grown in soil treated with Pcc60A at the concentration of 1000 cfu/g.

In the pot test, pistachio plants were infected with *M. javanica* but when untreated with fungus had the greatest number of galls (69.6) on their roots (Table 1). However, the numbers of galls decreased significantly ($P \le 0.05$) in the plants treated with fungal isolates, except for those of Pcc60A and Pcc60B, and were fewest (23.6) in the plants treated with Pcc20. All strains of Pcc-parasitized eggs within egg masses on roots but to varying degrees; the lightest rate of egg infection of 3 and 9% was recorded in treatments of Pcc60A and Pcc60B respectively (Table 1). The rate of infection differed between the other treatments, although the only difference of significance was for Pcc20 which parasitized the greatest number of eggs on roots (37%).

Nematode reproduction was significantly decreased in most fungus-treated plants, being below one in all pots, except for the Pcc60A and Pcc60B treatments (Table 1).

The final numbers of healthy eggs and J2 were reduced in most plants treated with fungal isolates, but not in the Pcc60A and Pcc60B treatments, which were not different than the untreated control ($P \le 0.05$) (Table 1).

Weights of pistachio-treated plants were not significantly different from the untreated plants (data not presented).

Based on our criteria, these fungal strains showed a potential as biocontrol agents for *M. javanica* on pistachio. The numbers of galls produced were fewest in Pcc20- and Pcc10-treated plants. The final egg population of nematodes was reduced by 61% by strain Pcc20 and 36% by Pcc10 and Pcc60C. Nematode reproduction was reduced to 57 and 36% by Pcc20 and Pcc10, respectively, and also it reached to 36% by Pcc60C. In previous work, these strains have colonized more than 80% of the *M. javanica* eggs on water agar (Ebadi et al. 2009 and Moosavi et al. 2010). They also decreased infection levels of *M. javanica* on tomato between 29 and 93% (Moosavi et al. 2010). Strain of Pcc60 has lowered the *H. schachtii* population to 50% on sugar beet (Ayatollahy et al. 2008). Interesting results have also been obtained with these isolates of Pcc in recent pot trials on *Globodera rostochiensis* Woll.; isolates penetrated cysts and killed nearly 90% of the eggs in vitro (Dehghan Nasrabad and Fatemy 2016).

Tested strains, with some reservation, were able to control infections of the mentioned nematodes on different crops, despite the fact that they were initially isolated from sugar beet cyst nematode. Host preference in isolates from cyst and root-knot nematodes was reported by Mauchline et al. (2004) and Manzanilla-Lopez et al. (2011). The association has been related to specific amino acid polymorphisms in a fungal serine protease, VCP1, involved in the infection of nematode eggs (Morton et al. 2003). The enzyme degrades the proteinaceous vitelline membrane, the outer layer of the nematode eggshell, which is qualitatively different in root-knot and cyst nematodes (Morton et al. 2004). Under some circumstances, different isolates of Pcc and *P. chlamydosporia* var. *catenulata* failed to reduce gall

Table 1 The effect of *Pochonia chlamydosporia* var. *chlamydosporia* isolates on *Meloidogyne javanica* populations, survival of the fungus isolates and their colonization of roots of pistachio after 4 months in the greenhouse ($n = 5$ means ± S.E)

Treatments	Gall no./g root	Gall index	Egg infection % (arc sin)	Pf/Pi (sqrt $x + 0.5$)	J2/pot	Healthy egg and J2/pot (log $x + 1$)	% control
Nematode	69.6 ± 2.63 a	4	0 c	1.40 (1.37) ± 0.2 a	273 ± 29	4643 (3.66) ± 0.3 a	–
Nematode + Pcc10	26.6 ± 1.21 d	3	19 (0.1) ± 0.02 b	0.90 (1.18) ± 0.4 c	267 ± 18	2980 (3.47) ± 0.2 c	36
Nematode + Pcc20	23.6 ± 1.53 d	3	37 (0.4) ± 0.01 a	0.55 (1.02) ± 02 d	167 ± 24	1805 (3.25) ± 0.1 d	61
Nematode + Pcc30	33.2 ± 2.08 c	4	16 (0.1) ± 0.01 b	0.98 (1.21) ± 0.3 c	333 + 32	3253 (3.51) ± 0.2 c	30
Nematode + Pcc60A	64.0 ± 1.84 a	4	3 (0.02) ± 0.04 c	1.37 (1.35) ± 0.2 a	400 ± 36	4531 (3.62) ± 0.5 a	2.5
Nematode + Pcc60B	56.4 ± 2.14 a	4	9 (0.1) ± 0.02 b	1.20 (1.28) ± 0.3 b	300 ± 42	3969 (3.55) ± 0.3 b	15
Nematode + Pcc60C	39.4 ± 2.03 b	4	16 (0.2) ± 0.03 b	0.92 (1.19) ± 0.2 c	233 ± 26	3041 (3.47) ± 0.5 c	35
Pistachio	–	–	–	–	–	–	–
	$P < 0.05$ (SED = 3.01)	n.s.	$P < 0.05$ (SED = 0.01)	$P < 0.05$ (SED = 0.06)	n.s.	$P < 0.05$ (SED = 0.06)	

Data in parenthesis were transformed before being subjected to ANOVA; numbers followed by the same letter in the columns are not significantly different at 5% level according to LSD test; Pf/Pi = ratio of final to initial population density, % control: control–treatment/control × 100. Pcc10, Pcc20, Pcc30, Pcc60C = 1×10^4, Pcc60A = 1×10^3 and PccB = 5×10^3 cfu/g soil

and egg mass indexes of *M. enterolobii* Yang and Eisenback on tomato or banana (Silva et al. 2017).

Selection of an efficient biocontrol agent is based on whether it kills nematodes or prevents them from causing damage to their hosts. Thus, the biocontrol agent must be re-isolated from soil or nematodes by appropriate and effective means to differentiate it from naturally occurring strains (Stirling 2014). After 4 months of the present experiment, fungal isolates survived in soil and established on the roots of pistachio. There were differences among strains; Pcc20 was the most efficient colonizer of roots, whereas Pcc10 and Pcc60C showed less potential in this respect. However, none of the isolates multiplied during the period of the experiment where their numbers had decreased at the end of the trial to be 65 and 85%, respectively.

The relation between nematode egg infection and abundance of *P. chlamydosporia* in the rhizosphere is complicated (Atkins et al. 2009). Isolate Pcc60A (applied at 1×10^3 cfu/g soil) did not grow on SSM from treated roots, although it infected 3% of the eggs. The reason could be that its abundance on roots was not great enough to be detected by this procedure—perhaps more advanced DNA-based techniques could have done so. Estimates of fungal density based on solid media only may not be reliable since the differentiation between colonies grown from mycelium or spore was not possible; also, the estimation is further complicated for *P. chlamydosporia*, that produced both conidia and chlamydospores (Manzanilla-Lopez et al. 2009). Quantitative PCR (Mauchline et al. 2002) and quantitative real-time PCR (Atkins et al. 2005) have provided more accurate and complementary information on abundance of filamentous nematophagous fungi. However, densities less than 10,000 cfu inoculum (as in the case of Pcc60B and Pcc60A) were less efficient in controlling nematode density.

Compared to other strains, Pcc30 was a weak biocontrol agent and colonizer of soil and rhizosphere of pistachio. Differences observed in the tested isolates' pathogenicity and abundance on roots and in soil have also been experienced by others. Isolates of *P. chlamydosporia* differ in their ability to control *Meloidogyne* (Medina-Canales et al. 2014).

Some species of *Pochonia* can penetrate roots endophytically (Lopez-Llorca et al. 2002). This type of colonization by this fungus has been found to promote growth of the host plant (Monfort et al. 2005). Most of these assessments have been done on annual plants; in the obtained findings, the weight of pistachio cv Kaleghochi plants was insignificantly increased by any of the Pcc strains and weights of treated plants did not differ from untreated plants (data not shown). Since the endophytic penetration of these isolates was not measured and the literature on the subject on perennial crops is scarce, it would be interesting to further look at this aspect of the relationship in future studies.

Involvement of different enzymes (proteases, esterases, lipases and chitinases) in the infection process was observed (Morton et al. 2004). However, in spite of high infection levels of *M. javanica* eggs in vitro by Pcc, J2s were not paralyzed when exposed to culture filtrates of the tested isolates under laboratory conditions (Ebadi et al. 2009). Esteves et al. (2009) could not correlate enzyme activity with egg parasitism or rhizosphere colonization in vitro.

Experimental data on biocontrol activity of nematophagous fungi in orchards are minimal. In a kiwifruit orchard, populations of *P. chlamydosporia* and *Purpureocillium lilacinum* were dominant species infecting root-knot nematodes (Mertens and Stirling 1993). Chlamydospores are the survival stage of fungi and their germination is triggered by nutrients leaking from roots, or by the presence of organic matter (De Leij et al. 1993). *P. chlamydosporia* is a saprotrophic in soil and could grow on crop residues (Dallemole-Giaretta et al. 2011). The continual presence of endoparasitic nematodes in an undisturbed environment such as perennial crops provides a food source for *P. chlamydosporia*, while during the unfavorable season of winter, the fungus would survive by switching to the saprophytic stage using organic matter instead of nematode hosts (Stirling 2014).

Conclusion

The ability to survive, colonize roots and restrict nematode multiplication were encouraging in some of the isolates. The isolate of Pcc20 has the most pathogenic effects, while Pcc10 and Pcc60C were the moderate ones of Pcc and these isolates could be selected for further work in the natural environment.

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Availability of data and materials
Data will not be shared, because our institute does not allow it.

Authors' contributions
First author ME is responsible for implementing the experimental work. Second author SF is responsible for designing and supervising the study, revising the paper scientifically, and checking analysis and interpretation of data. Third author HR is responsible for the general co-operation and contribution to the study. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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