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The effect of the fungus *Pochonia chlamydosporia* on the root-knot nematode *Meloidogyne incognita* in pots

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Summary. Two application rates of the nematophagous fungus *Pochonia chlamydosporia* (isolate 10, Rothamsted Research, UK) (5,000 and 60,000 chlamydospores g^{-1} of soil) were tested for their effect on two infection rates of the root-knot nematode *Meloidogyne incognita* (450 and 1,400 juveniles/plant) on tomato and pepper plants grown in pots. The presence of the fungus in soil and root, root gall index and juvenile densities in soil were assessed seven and 14 weeks after inoculation and compared with control treatments (free of fungus). In addition, egg production and egg parasitism were recorded at the first assessment. Although the fungus established in soil and parasitized the eggs, it did not reduce nematode population densities after seven weeks in both crop plants. However, significant reductions of root galling and juvenile density were recorded after 14 weeks, but only on pepper. Fungus colonization of roots was not influenced by the initial application rate of chlamydospores and remained stable in both assessments.

Key words: Biological control; tomato; pepper; fungal parasite.

Pochonia chlamydosporia Zare & Gams (syn. *Verticillium chlamydosporium* Goddard) (Zare *et al.*, 2001) is a soil-borne facultative fungal parasite of root-knot nematode eggs, and has shown potential as a biological control agent in vegetables as demonstrated in pot tests (De Leij *et al.*, 1992; Kerry, 1995; Viaene & Abawi, 2000; Van Damme *et al.*, 2005) and microplot experiments (De Leij *et al.*, 1993). The introduction of the fungus in intensive vegetable production systems in the Mediterranean area indicated that it efficiently colonizes the soil and roots and parasitizes nematode eggs but the effects on reduction of nematode populations were inconsistent (Ciancio *et al.*, 2002; Ornat *et al.*, 2003; Sorribas *et al.*, 2003; Verdejo *et al.*, 2003).

Root-knot nematodes (*Meloidogyne* spp.) are serious pests of vegetable production in Crete (Greece) especially under plastic-house conditions. Two species have been recorded so far, *Meloidogyne javanica* Treub and *M. incognita* (Kofoid & White) Chitwood, with a prevalence of the first species. The presence of *M. incognita* is mainly associated with pepper, which was found to be a non-host for local populations of *M. javanica* (Tzortzakakis, 1997; Tzortzakakis *et al.*, 1999).

The potential of the fungus for biological control of nematodes in vegetable crops in Crete has so far been evaluated only against *M. javanica* on tomato in plastic houses. Soil and root colonization by an exotic (non-native) strain of *P. chlamydosporia* (isolate 10, Rothamsted Research, UK) was variable and there was no effect on root galling and nematode population densities (Tzortzakakis, 2000; Tzortzakakis & Petsas, 2003). The soil colonization and biological control efficacy of *P. chlamydosporia* are affected by different factors. These factors are plant species and the exposure of the nematode egg masses on the root surface, which is determined by the susceptibility of the plant to the target nematode species (Bourne & Kerry, 1999; De Leij *et al.*, 1992) and nematode density. Therefore, two pot experiments were undertaken to evaluate the potential of the same fungal isolate to control *M. incognita* at different densities infecting pepper and tomato under controlled conditions.

MATERIAL AND METHODS

Pochonia chlamydosporia isolate 10 was provided by B. R. Kerry (Nematode Interaction Unit, Rothamsted Research, UK). It was maintained on 1.7% corn meal agar in Petri dishes (stock cultures) stored in a fridge. From these cultures, pieces of fungal colonies were used to produce chlamydospores on a mixture of coarse sand and milled barley. The viability of the chlamydospores was estimated by checking the

percentage of germination and the fungal inoculum was prepared by mixing chlamydospores with fine, autoclaved sand (De Leij & Kerry, 1991).

Appropriate amounts of fungal inoculum were mixed thoroughly with nematode free commercial compost soil, to obtain concentrations of 5,000 (low rate) or 60,000 (high rate) chlamydospores g^{-1} soil. The inoculated soil was used to fill 300 ml plastic pots in which a seedling at the second-leaf stage of either tomato (cv. ACE) or pepper (cv. California Wonder) was transplanted. Second-stage juveniles (J2) of the nematode were obtained by incubating egg masses in hatching dishes for four days. One day after transplanting, each pot was inoculated with 180 or 750 J2 of a local *M. incognita* population reared on tomato. After two and five days, plants were reinoculated with 150 or 250 J2 and 120 or 400 J2, respectively, to give a total of 450 (low rate) or 1,400 (high rate) J2 per plant.

Two experiments were conducted, one on tomato and the other on pepper with the following treatments: a) nematodes only (low rate); b) nematodes only (high rate); c) nematodes (low rate) + fungus (low rate); d) nematodes (low rate) + fungus (high rate); e) nematodes (high rate) + fungus (low rate); and f) nematodes (high rate) + fungus (high rate). There were nine pots per treatment for each plant species and each experiment was arranged according to a completely randomized block design on a bench in a temperature controlled room at 25 ± 2° C and 16 h photoperiod.

Previous studies indicated that egg laying of *M. incognita* on pepper and tomato starts about three weeks after juvenile inoculation at a mean temperature of 27°C (Tzortzakakis, 1997). By that time, the fungus should have colonized roots to infect the nematode eggs successfully. Therefore, three weeks after the first nematode inoculation, soil and root colonization by the fungus were assessed in one plant per treatment. This was done by dilution plating of a 1 g samples on a semi-selective medium followed by counting the colony forming units (cfu) (De Leij & Kerry, 1991). To confirm the identity of the fungus, representative colonies were transferred to Petri dishes containing corn meal agar and incubated at 25°C for 10 days until chlamydospore production. For the roots, fungal colonization was expressed as cfu cm^{-2} by calculating the surface area of 1 g of roots (De Leij et al., 1992). Seven weeks after the first nematode inoculation, four pots per treatment were harvested and the soil was mixed after uprooting of the plant. One g of soil was used to assess the fungal colonization and the remaining was put inside modified Whitehead trays. After four days of incubation at 25°C, the J2 that had migrated from the soil inside the water were collected and counted. The roots were washed carefully by gently shaking in water, the root gall index (RGI) was estimated on a 0-10 scale (Bridge & Page, 1980) and the number of egg masses on root surface was counted using a stereo-microscope. To assess the number of eggs per egg mass, 5-10 egg masses per root were shaken in a 0.01% aqueous solution of sodium hypochlorite to release eggs (Hussey & Barker, 1973), which were then counted under a stereo-microscope. The proportion of embryonated eggs was also recorded. To estimate egg infection, eggs from 5-10 egg masses were dispersed in 1 ml water with a homogenizer and plated in dishes containing 0.8% water agar solution and antibiotics (De Leij & Kerry, 1991). After two days of incubation at 25°C the dishes were observed using an inverted microscope. Eggs were considered infected when fungal hyphae developing from the inside of the eggs were visible. Percentage of infection was recorded in 50-100 eggs per replicate. The total nematode population per plant was estimated as the sum of juveniles in soil and total egg number per root (number of egg masses x average number of eggs/egg mass). Fungal colonization in root and soil was also recorded. A second assessment was made 14 weeks after nematode inoculation in four pots per teatment.

Data were statistically analysed by analysis of variance (ANOVA). The standard error of the difference between any two means (SED) was used to detect the significant differences. Data of cfu g^{-1} soil and cfu cm^{-2} root surface were transformed using *log* $(x+1)$ prior to statistical analysis when they did not meet the requirements for ANOVA.

RESULTS

The percentage of germinating chlamydospores was high (90%). Twenty-one days after the first nematode inoculation, the fungus was detected in soil of all treatments. At the low inoculation rate the fungus had not colonized the roots, whilst when applied at the high rate it was found in roots of tomato at both nematode densities (ca 23 cfu cm^{-2} root) and in pepper only at low nematode density (1,045 cfu cm^{-2} root). Treatment comparisons were not made as only one replicate per treatment was assessed and conclusions are not drawn (data not shown).

The total nematode population (Table 1) increased from 7 to 21 times on tomato and from 11 to 21 times on pepper over the initial

inoculation rate after seven weeks. The fungus had not suppressed the nematode population increase (Table 1) nor had it reduced the RGI (Table 2) of both plants. Embryonated eggs are much more resistant to fungal infection than non - embryonated eggs. The effect of the fungus can be estimated by comparing the percentages of the embryonated eggs in treated plants with those of eggs from the untreated control (De Leij *et al.*, 1992). There was no difference in percentage of embryonated eggs in all treatments and untreated control ($P>0.05$, data not shown) indicating absence of significant fungal effect on egg destruction. The percentage of infected eggs was 10-30% for tomato and 8-21% for pepper and did not differ between rates of fungal inoculum ($P>0.05$, data not shown).

The number of cfu in soil was higher than the initial concentration of the 5,000 chlamydospores g^{-1} soil by between 1.5 to 4 times for tomato and between 1 to 1.5 times for pepper. By contrast, in the case of the highest initial application rate of the fungus, for both crops, the cfu per g of soil was less than half (from 0.3 to 0.4 times) (Tables 4 and 5). Root colonization of tomato and pepper plants did not differ when initial application rate of chlamydospores was 5,000 or 60,000 chlamydospores g^{-1} of soil. Fungus establishment in soil and root was not influenced by nematode population density (Tables 4 and 5).

In the assessment made after 14 weeks, roots of tomato were heavily galled and partly rotted. Data for egg masses were not recorded because of root rot and the low number of egg masses that remained on root surface; for all the other parameters data were recorded (three replicates per treatment as one plant from the control treatment died). The fungus had no effect on the RGI and soil juvenile density (Tables 2 and 3), while its soil colonization for high and low fungus application rates was 3.5 and 5 times higher, respectively, than that recorded in the first assessment. Fungal colonization in soil and root of tomato plants was not influenced by the nematode inoculation dose (Table 4). Pepper roots were less galled and rotted than tomato roots and data on J2 in soil and fungus colonization in soil and roots were recorded. The fungus significantly suppressed RGI ($P<0.05$) and J2 ($P<0.01$) (Tables 2 and 3) and its soil colonization was from 4.5 to 7 times higher for the high and low initial fungal rates, respectively, while root colonization was lower (0.4 times) than that of the previous estimate (Table 5).

Table 1. Total nematode population ($x10^3$) per root (number of eggs) and in soil (number of juveniles) of *Meloidogyne incognita* in pots planted to tomato and pepper, seven weeks after nematode inoculation.

Nematode rate	Tomato		Pepper	
	Low	High	Low	High
No fungus	9.74*	10.89	18.09	13.81
Low fungal rate	12.30	11.93	7.66	24.44
High fungal rate	6.79	8.07	13.48	10.31
SED	2.67		4.15	
Block	NS		NS	
Fungus rate	NS		NS	
Nematode rate	NS		NS	
Interaction	NS		<0.01	

* Average of four replications per treatment; nematode rate: low 450 and high 1,400 J2s /plant; fungal rate: low 5,000 and high 60,000 chlamydospores g^{-1} soil; NS: non significant at 5% level.

Table 2. Root gall index (RGI) of tomato and pepper plants assessed 7 and 14 weeks after inoculation with *Meloidogyne incognita* juveniles

Nematode rate	After 7 weeks				After 14 weeks			
	Tomato		Pepper		Tomato		Pepper	
	Low	High	Low	High	Low	High	Low	High
No fungus	5.5*	5	4	4	7.6**	8	4.75	4.75
Low fungal rate	5.25	5.5	3.75	4	8.3	7.6	4.75	4.75
High fungal rate	5.25	5.5	4	3.75	7.3	7.6	4.25	4
SED	0.47		0.18		0.58		0.29	
Block	<0.01		NS		NS		<0.05	
Fungus rate	NS		NS		NS		<0.05	
Nematode rate	NS		NS		NS		NS	
Interaction	NS		NS		NS		NS	

* Average of four replications per treatment; ** average of three replication per treatment for tomato and four for pepper; nematode rate: low 450 and high 1,400 J2s/plant; fungal rate: low 5,000 and high 60,000 chlamydospores g^{-1} soil; NS: non significant at 5% level.

DISCUSSION

If the fungus is successfully established in the rhizosphere, it invades egg masses exposed on the root surface and infects eggs. Ideally, the reduction of eggs will result in a lower number of first generation J2 and, consequently, of second generation females. However, *Meloidogyne* species develop several generations per crop cycle; therefore, the effect of a treatment in reducing the nematode population density of the first generation may be masked at a later stage. Failure of the same fungal isolate to suppress *M. javanica* on tomato previously in Crete (Tzortzakakis, 2000; Tzortzakakis & Petsas, 2003), could be attributed to a too large nematode population and the formation of big compacted galls (values of RGI higher than 6 in plots treated with the fungus) with the majority of egg masses embedded inside the plant tissue and protected from infection.

Table 3. Juveniles ($x10^3$) in soil on tomato and pepper plants 14 weeks after inoculation with *Meloidogyne incognita*.

	Tomato		Pepper	
Nematode rate	Low	High	Low	High
No fungus	0.840*	0.440	4.425	3.200
Low fungal rate	0.717	2.613	1.623	0.590
High fungal rate	1.467	1.720	0.738	0.423
SED	1.09		1.30	
Block	NS		NS	
Fungus rate	NS		<0.01	
Nematode rate	NS		NS	
Interaction	NS		NS	

* Average of three replications per treatment for tomato and four for pepper; nematode rate: low 450 and high 1,400 J2s / plant; fungal rate: low 5,000 and high 60,000 chlamydospores g^{-1} soil; NS: non significant at 5% level.

Our results of the pot experiments indicate that fungus was established successfully in roots and soil of both tomato and pepper, and parasitized moderately the eggs laid by the first generation females, as assessed seven weeks after inoculation. A commercial compost soil was used for plant growing and in such soil types containing organic material, the fungus is better established compared with mineral soils (Kerry *et al.*, 1993). However the fungus did not have any effect in reducing nematode population densities in both tomato and pepper. By contrast, in another pot experiment in UK, the same fungal isolate controlled a different population of *M. incognita* on tomato (De Leij *et al.*, 1992). This would indicate that the efficacy of the fungus could vary when experiments are conducted under different conditions or when different nematode populations are involved.

When 60,000 chlamydospores g^{-1} soil were inoculated, soil colonization was larger than that at 5,000 chlamydospores g^{-1} soil after seven weeks but similar after 14 weeks. However, root colonization, which is important for egg parasitism, was not influenced by the fungal application rate.

In the second assessment made after the development of more than one nematode generation, the fungus did not have any effect on tomato but in pepper it significantly reduced root galling and number of juveniles in soil. However, no observations could be made on egg production and parasitism. Probably infected egg masses decompose quickly and are not found if the crop is assessed a long time after their formation (Van Damme et al., 2005). Furthermore, growing plants in small pots for 14 weeks and the extended rotting in tomato roots, which limited the number of replications, may have influenced the results. The only possible explanation for the effect of the fungus on pepper but not on tomato is the

Table 4. Fungal colonization in soil and root of tomato plants.

Nematode rate	After 7 weeks				After 14 weeks			
	cfu g^{-1} soil		cfu cm^{-2} root		cfu g^{-1} soil		cfu cm^{-2} root	
	Low	High	Low	High	Low	High	Low	High
Low fungal rate	7,688*	21,094	1,221	1,169	65,625**	78,958	1,986	966
High fungal rate	22,656	23,125	414	485	88,750	76,458	579	645
SED	(0.18)***		(0.64)		(0.08)		(0.38)	
Block	<0.05		NS		<0.05		NS	
Fungus rate	<0.05		NS		NS		NS	
Nematode rate	NS		NS		NS		NS	
Interaction	NS		NS		NS		NS	

* Average of four replications per treatment; ** average of three replications per treatment; *** analysis on log (X+1) transformed data; NS: non significant at 5% level; nematode rates: low 450 and high 1,400 J2s /plant; fungal rates: low 5,000 and high 60,000 chlamydospores g^{-1} soil.

Table 5. Fungal colonization in soil and roots of pepper plants.

Nematode rate	After 7 weeks				After 14 weeks			
	cfu g^{-1} soil		cfu cm^{-2} root		cfu g^{-1} soil		cfu cm^{-2} root	
	Low	High	Low	High	Low	High	Low	High
Low fungal rate	5,406*	7,906	380	2,155	61,563	33,938	566	514
High fungal rate	17,469	16,688	1,278	718	82,875	67,438	365	438
SED	(0.29)**		(0.88)		(0.11)		(0.22)	
Block	NS		NS		NS		NS	
Fungus rate	<0.05		NS		NS		NS	
Nematode rate	NS		NS		NS		NS	
Interaction	NS		NS		NS		NS	

* Average of four replications per treatment; ** analysis on log (X+1) transformed data; NS: non significant at 5% level; nematode rates: low 450 and high 1,400 J2s /plant; fungal rates: low 5,000 and high 60,000 chlamydospores g^{-1} soil.

difference in increased rate of root galling between these two plant species over time. In control treatments where fungus was absent, the root galling index between the two assessments increased on tomato (from 5 to 8) more rapidly than on pepper (from 4 to 4.75). In the case of pepper with a low root galling index, the majority of the egg masses are external to the root surface and that probably allows an easier infection by the fungal hyphae (Bourne & Kerry, 1999). A lower root galling index from these usually observed on other vegetable roots (e.g. tomato, cucumber, melon) and egg masses being exposed on root surface, has been also observed in pepper crops grown commercially in greenhouses of Crete.

Therefore, the fungus may have potential to control the nematode on pepper crop in Crete, thus warranting further testing it under field conditions.

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Tzortzakakis E.A.. Воздействие гриба *Pochonia chlamydosporia* на галлообразующих нематод *Meloidogyne incognita* в экспериментальных контейнерах.

Резюме. Проведен эксперимент по изучению воздействия двух доз нематоядного гриба *P. chlamydosporia* (изолят 10, Rothamsted Research, UK) (5,000 и 60,000 хламидоспор g^{-1} почвы) на галлообразующих нематод *Meloidogyne incognita* при двух дозах заражения нематодами растений томатов и перца в горшках (450 и 1400 личинок на растение). Присутствие гриба в почве и корнях растений, индекс поражения растений нематодой и количество личинок нематод в почве определяли черёз 7 и 14 недель после начала эксперимента и сравнивали с контролем (когда гриб не вносили). К тому же, при первом определении результатов оценивали общую продукцию яиц нематодами и уровень поражения яиц грибом. Несмотря на то, что были отмечены появление гриба в почве и поражение яиц нематод, обработка не приводила к снижению численности нематод через 7 недель после начала эксперимента на обоих видах растений. Тем не менее, существенное снижение индекса галлообразования и численности личинок наблюдали на перце через 14 недель после начала эксперимента. Уровень колонизации корней растений грибом не зависел от начальной дозы внесения хламидоспор и не различался между двумя сроками оценки (7 и 14 недель).