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The use of *Verticillium chlamydosporium* Goddard and *Pasteuria penetrans* (Thorne) Sayre & Starr alone and in combination to control *Meloidogyne incognita* on tomato plants

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Summary — The potential control of *Meloidogyne incognita* using an isolate of *Verticillium chlamydosporium* and *Pasteuria penetrans*, alone or in combination, was tested in a pot experiment at rates of 10^3 and 10^4 spores/g soil for each organism at two nematode population densities of 250 or 1500 second-stage juveniles per tomato plant. The treatments were compared with the effects of an aldicarb drench equivalent to 3.75 kg/ha. Root galling, invasion, numbers of females, egg production and infection were monitored 7 and 14 weeks after nematode inoculation. Aldicarb was the most effective treatment at reducing galling, as measured at the first harvest; by the second harvest, all treatments were giving similar root galling indices. Significantly fewer eggs and juveniles were produced after some of the treatments with biological control organisms than after treatment with aldicarb at the second harvest which gave *ca* 55 % population control. The biological control agents tended to complement each other, giving up to 92 % population control at the second harvest, although neither reduced the initial invasion significantly. *V. chlamydosporium* was most effective at infecting eggs at low nematode population densities when the galls were small and the majority of the egg-masses, produced on the root surface, were colonised by fungus resulting in population control levels of around 80 %. *P. penetrans* infected up to 65 % of first generation females, but also infected subsequent generations of nematodes produced within large galls.

Résumé — ***Utilisation de* Verticilium chlamydosporium *Goddart et de* Pasteuria penetrans *(Thorne) Sayre & Starr, seuls et combinés, pour contrôler* Meloidogyne incognita sur des plants de tomates** — Le rôle antagoniste envers *Meloidogyne incognita* d'isolats de *Verticilium chlamydosporium* et de *Pasteuria penetrans*, seuls ou combinés, est testé lors d'une expérience en pots comportant des taux de 10^3 et 10^4 spores/g de sol pour l'un et l'autre organismes et deux niveaux de population pour le nématode, soient 250 et 1500 juvéniles de deuxième stade par pied de tomate. Ces traitements sont comparés à un traitement liquide à l'aldicarbe correspondant à 3,75 kg/ha de produit. Les galles radiculaires, la pénétration, le nombre de femelles, la production d'œufs et l'infestation sont relevés 7 et 14 semaines après l'inoculation. L'aldicarbe est le traitement le plus efficace lors de la première récolte en ce qui concerne la diminution du nombre de galles; lors de la seconde, tous les traitements conduisent à des index de galles similaires. Lors de la seconde récolte, les nombres d'œufs et de juvéniles produits sont significativement plus faibles après certains traitements à l'aide des agents biologiques qu'après traitement à l'aldicarbe, le contrôle de la population étant d'environ 55 %. Les agents biologiques ont tendance à se renforcer l'un l'autre, produisant ainsi un contrôle de 92 % lors de la deuxième récolte, sans toutefois réduire significativement la pénétration initiale. *V. chlamydosporium* est le plus efficace, infestant les œufs en cas de faible densité de population de nématodes et de galles de petite taille : la majorité des masses d'œufs produites à la surface des racines sont ainsi colonisées par le champignon d'où résultent des niveaux de contrôle voisins de 80 %. *P. penetrans* infeste jusqu'à 65 % de la première génération de femelles, mais aussi les générations suivantes du nématode produites dans les grosses galles.

Key-words : *Verticillium*, nematode parasitizing fungi, *Meloidogyne*, *Pasteuria*, biological control.

Pasteuria penetrans (Thorne) Sayre & Starr is an obligate bacterial parasite of root-knot nematodes (Sayre & Starr, 1988) and has considerable potential as a biological control agent (Mankau & Imbriani, 1975; Sayre, 1980; Stirling, 1984). The natural suppression of root-knot nematodes in West Africa (Mankau, 1980), and on vines in South Australia (Stirling & White, 1982), have been associated with a large proportion of spore-encumbered juveniles in soil. *P. penetrans* reduces nematode populations when spores on the cuticle germinate and form microcolonies, which proliferate in the pseudocoelom of the developing female and eventually mature into spores (Sayre & Wergin, 1977); this prevents infected root-knot nematodes from producing eggs (Mankau, 1980; Sayre, 1980). *P. penetrans* may also reduce the numbers of second-stage juveniles that invade roots (Stirling, 1984; Brown & Smart, 1985; Davies *et al.*, 1988). Although populations of *P. penetrans* exhibit varying affinities for different populations of nematodes (Stirling, 1985; Davies *et al.*, 1988), virulent populations, with a high affinity of the spores for nematode cuticle at spore densities of between 10^4 and 10^6 spores per gram of soil, have provided very effective control of *Meloidogyne javanica* from soil (Stirling, 1984). Large numbers of second-stage juveniles may be encumbered with spores as they migrate towards the

roots. However, those that hatch from eggs on, or in the root can reinvade without coming into contact with the soil and being exposed to spores (Stirling, 1984). Hence, control of root-knot nematodes is dependent on the infection of the first generation of juveniles (Kerry, 1987).

Verticillium chlamydosporium Goddard is a facultative parasite of cyst and root-knot nematodes (Wilcox & Tribe, 1974; Kerry, 1975; Godoy *et al.*, 1983; Freire & Bridge, 1985). Experiments monitoring the growth of a *V. chlamydosporium* isolate in soil have shown the fungus to proliferate from 10^4 to 5×10^5 cfu g^{-1} soil over a period of 30 days; increases in the rhizoplane, especially at those sites where gall-formation caused by the nematode took place, were even greater (de Leij & Kerry, 1991). When the egg-masses emerged on the root surface, one isolate of the fungus colonised the gelatinous matrix and parasitised the eggs. In a pot test, where approximately 10^4 chlamydospores and their associated hyphal fragments were introduced into soil, the numbers of *M. arenaria* were reduced by around 80 %; this significantly decreased the amount of galling during subsequent generations of nematodes (de Leij & Kerry, 1991). Because the fungus survives and continues to proliferate saprophytically on the root surface during successive nematode generations, nematode control is not limited to the first generation.

The introduction of *P. penetrans* into soil to reduce root invasion and egg production, together with *V. chlamydosporium* as a rhizosphere coloniser and facultative egg parasite, may improve nematode control compared with either of the organisms alone. In this paper the use of *V. chlamydosporium* and *P. penetrans* at different inoculum densities, singly and in combination against different densities of *M. incognita*, is described together with a report of infection of subsequent generations of nematodes. This is discussed in relation to the epidemiology of both organisms, and their use and potential in the biological control of root-knot nematodes.

Material and methods

Fungal and bacterial inocula

An isolate of *V. chlamydosporium* originally from *M. incognita* was chosen which had previously been shown to be effective against *M. arenaria* in pot tests (de Leij & Kerry, 1991). The fungus was cultured on a mixture of sand and bran (1:1 v/v) inoculated with two 5 mm agar plugs containing fungus and incubated at 18 °C. After three weeks incubation the cultures were washed through a 50 µm aperture sieve with a fine water spray to remove the sand and bran and the fungal propagules were collected on a 10 µm sieve. The deposit was further washed to remove conidia and small hyphal fragments leaving mainly chlamydospores and some hyphal fragments. The chlamydospores were counted using a haemocytometer. The inoculum was prepared by mixing the fungus with fine sand which acted as an inert carrier.

Spores of *P. penetrans* were obtained from infected females of *M. incognita* containing mature spores following the method of Stirling and Wachtel (1980); the females were homogenized with a 1 ml uniform homogeniser (Jencons) to release the endospores, which were counted using a haemocytometer. The bacterial inoculum was also mixed with fine sand as described above.

Each pot, containing 500 g unsterilised peat/sand compost (1:1 v/v) was inoculated with the fungus and/or bacterium inoculum in 10 g fine sand to produce the final concentrations of inoculum in the soil as shown in Table 1. Controls consisted of fine sand not containing the agents and an aldicarb drench of 1.5 µg active ingredient per gram compost added the day prior to nematode inoculation.

Table 1. The initial levels of *Pasteuria penetrans* and *Verticillium chlamydosporium* used in thirteen treatments used in the experiment (spores $\times 10^3$ per gram soil).

Treatments	1	2	3	4	5	6	7	8	9	10
V. chlamydosporium	1	10	0	0	1	1	10	10	0	0
P. penetrans	0	0	1	10	1	10	1	10	0	0
Aldicarb (µg g^{-1})	0	0	0	0	0	0	0	0	0	1.5

Experimental design

Ten treatments were each applied to sixteen pots. Each pot was planted with a one month old tomato seedling (cv. Roter Gnom) which was allowed to establish for two weeks before half of the pots were inoculated with 250 *M. incognita* juveniles and half with 1500 juveniles. Juveniles of less than 1 week old were obtained from infected tomato roots using a modified Baermann funnel technique (Whitehead & Hemming, 1965). The juveniles were counted and the required inoculum was introduced in 10 ml water, by pipetting the juveniles into three holes around the roots of the transplanted tomatoes. Four pots of each treatment were harvested after 7 weeks (1st harvest), while the other four were harvested after a further 7 weeks (2nd harvest). The experiment was set out as four randomised blocks, each block representing one of four replicates. The results were analysed using multivariate analysis of variance; data were transformed when necessary.

Fungal establishment

The amount of *V. chlamydosporium* present in the compost was measured immediately after inoculation of the experiment and at both harvests, using a semi-selective medium (de Leij & Kerry, 1991). Dilution

using the semi-selective medium were prepared using standard techniques. Before the root systems were processed to assess the extent of parasitism of the nematodes by both the fungus and the bacterium, root galling was determined using a gall-rating of 0-10 (Bridge & Page, 1980). Colonisation of the root surface was assessed at each harvest : root systems were washed carefully, then blotted dry, weighed and cut into small segments (*ca* 1 cm length) and mixed thoroughly. From each root system a 1 g sample was taken, and crushed with a sterilised pestle and mortar in 9 ml of agar solution (0.05 %). A dilution range of this suspension was plated onto the semi-selective medium as previously described (de Leij & Kerry, 1991).

INFECTION OF THE FIRST AND SUBSEQUENT NEMATODE GENERATIONS AND THE EFFECT OF ROOT GALLING

At both harvests, half of the chopped root segments were used for estimating the number of females and their infection by *P. penetrans*. The roots were digested in Pectinex (Novo Enzyme Products Ltd., Farnham, U.K.) for 24 h at room temperature, homogenised (Atomix, Measuring and Scientific Equipment Ltd., Crawley, U.K.), for 20 seconds, and the females extracted by washing the slurry through a 0.8 mm aperture sieve and collecting them on a 53 µm aperture sieve (Davies *et al.*, 1988). A sample of the residue was obtained and the females counted in a counting dish (Doncaster, 1962). A further ten females were individually squashed on a microscope slide and examined at × 400 for *P. penetrans* infection.

Half of the remaining root sample was used to estimate the number of eggs and juveniles using the methods described by Coolen and d'Herde (1973). The proportion of eggs in egg-masses infected with *V. chlamydosporium* was estimated by taking egg-masses from the remaining roots, dispersing the eggs in 3 ml water using a homogenizer (Jencons), and plating *ca* 0.5 ml of this egg suspension onto water agar (0.8 %) in a Petri dish (Kerry & Crump, 1977). After 2 days infected eggs could be recognised by fungal hyphae growing from the eggs; 100 eggs on each dish were examined and the proportion parasitised estimated. After 2 days the fungus had produced few conidia and so the plates were re-examined after 4 days when sporulation was more frequent, so the fungus could be identified as *V. chlamydosporium*. Plates were examined after two days because after longer periods of incubation the growth of contaminants not associated with the nematode eggs in some samples made assessment of parasitism difficult.

At the first harvest, egg-masses were picked off the surface of galled roots and examined for infection; at the second harvest, when a proportion of the egg-masses were produced within the root gall, infection of eggs on the root surface was compared with the infection of eggs and juveniles within galls. Each sample consisted of twelve randomly chosen egg-masses picked of from the root surface or picked out of dissected galls. The number of juveniles and the number of healthy and infected eggs were counted, and the presence or absence of chlamydospores in each individual egg-mass was recorded; this was done using a high power microscope (× 200). *Pasteuria* infection was studied by removing ten large galls from the *P. penetrans* treated pots and carefully dissecting each gall separately to release the second-stage juveniles from inside the gall. The juveniles were then examined microscopically (× 400) and the numbers of spores adhering to their cuticles counted.

Results

FUNGAL ESTABLISHMENT

During the experiment there was a rapid increase in *V. chlamydosporium* both in the compost and on the root surface in all the pots to which it was added (Fig. 1). Throughout the experiment there was a large difference in the establishment of *V. chlamydosporium* in the compost between the high and low rates of inoculum; however, differences in soil populations of the fungus in the compost did not result in significantly different population densities on the root surface (Fig. 1).

EFFECTS OF *V. CHLAMYDOSPORIUM* AND *P. PENETRANS* ON NEMATODE POPULATIONS AND ROOT GALLING

After the first harvest, galling was reduced by almost all treatments compared with the untreated controls (Table 2). Aldicarb was the most effective treatment, producing a significant reduction in galling in both the

Table 2. Galling index (1-10; Bridge & Page, 1980) of tomato roots grown in compost inoculated 250 or 1500 with juveniles of *Meloidogyne incognita* pot and treated with *Verticillium chlamydosporium* at 10^3 (VL) and 10^4 (VH) chlamydospores/g soil, *Pasteuria penetrans* at 10^1 (PL) and 10^4 (PH) spores/g soil, the two in combination (VL + PL; VL + PH; VH + PL; VH + PH), or aldicarb (1.5 ppm) and an untreated control.

	Harvest time			
	7 weeks		14 weeks	
Treatment/Pi	250	1500	250	1500
Control	1.3	3.3	7.3	8.0
Aldicarb	0.4	0.6	4.3	5.8
VL	1.0	2.5	4.5	6.3
VH	0.9	2.5	4.0	5.0
PL	1.3	2.8	5.8	7.0
PH	1.1	2.0	5.3	7.0
VL + PL	0.6	2.5	5.3	5.8
VL + PH	1.0	1.8	6.0	5.3
VH + PL	0.8	3.3	4.3	4.8
VH + PH	0.6	2.3	2.5	4.0
SED	0.3*	0.3***	0.6***	0.5***

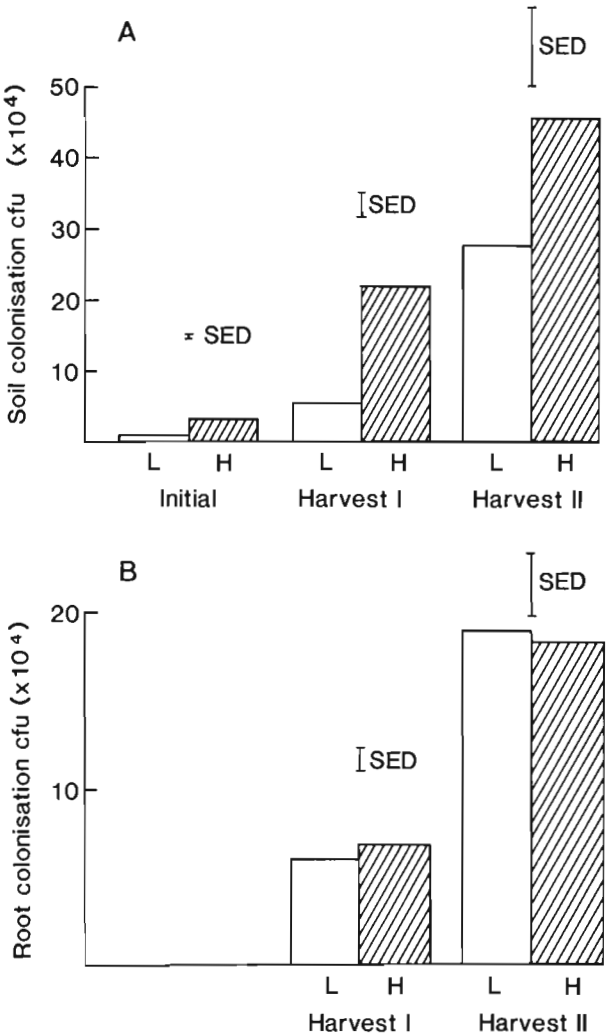

Fig. 1. Colonisation of soil and rhizosphere by *Verticillium chlamydosporium* inoculated at rates of 1000 (L) and 10 000 (H) chlamydospores g soil (n = 4).

low and high rates of nematode inoculation. At the second harvest, all treatments reduced galling rates significantly irrespective of the initial nematode level. *V. chlamydosporium* applied at 10^3 chlamydospores/g soil was significantly better than *P. penetrans* and comparable to aldicarb in reducing galling. The combined high rates of *V. chlamydosporium* and *P. penetrans* caused the greatest reduction in root galling index, at the second harvest.

Aldicarb was the only treatment which produced a significant reduction in the number of females on the root. However this was only true at the first harvest, by the second harvest there were not significant differences between any of the treatments (Table 3). The effectiveness of the biological control agents could best be evaluated by examining the numbers of juveniles and eggs produced (Tables 4, 5). Numbers of juveniles were reduced by all treatments at the first harvest (Table 4). Greatest reductions in numbers of juveniles were achieved with those treatments where *V. chlamydosporium* was added either alone or in combination with *P. penetrans*, resulting in population control comparable with the control achieved with aldicarb (Table 4). Greatest reductions in numbers of juveniles were achieved with the combination of a high level of *P. penetrans* with a high level of *V. chlamydosporium*, resulting in 92 % control; a high dose of *V. chlamydosporium* on its own resulted in 85 % control. Both treatments gave significantly greater reductions in the number of juveniles than aldicarb (52 % population control). The data for eggs showed similar trends to the results for juveniles (Table 5).

Infection of *M. incognita* by *V. chlamydosporium* and *P. penetrans*

When *P. penetrans* was applied alone there were highly significant differences (Table 5) in the percentage infection of females between the low rate of inoculation (6 to 25 %) and the high inoculum rate (33 to 65 %). The addition of *V. chlamydosporium*, in combination with *P. penetrans*, did not significantly affect female infection; nematode density did not affect female infection by *P. penetrans* either (Table 5). After the first harvest none of the second-stage juveniles extracted from gall tissue had any *Pasteuria* spores attached to their cuticles. After the second harvest 50 % of the juveniles extracted from gall tissue treated with the high dose of *P. penetrans* had spores attached to their cuticles, with an average of more than four spores per juvenile. Approximately 15 % of the juveniles from the treatments with *P. penetrans* at 10^3 spores/g soil had spores attached at an average of little more than one spore per juvenile.

Egg-masses which did not break through the gall surface were physically protected from *V. chlamydosporium* by root tissue and contained, therefore, no infected eggs. Egg-masses produced on the outside of the gall were all colonised by the fungus; this resulted in more than 90 % of the eggs being infected (Fig. 2).

Discussion

Aldicarb was the most effective treatment at reducing galling, as measured at the first harvest. However, by the second harvest a similar degree of control of root galling was being obtained by the biological control organisms. Reductions in the numbers of eggs and juveniles after addition of biological control agents were significantly greater than after aldicarb treatment at the second harvest. Aldicarb acts by contact with the motile second-stage juvenile and reduces their ability to locate host roots and feed (Wright, 1981); its ability to control

Table 3. Number of females (FEM) per root system, and their % infection extracted from tomato roots inoculated with 250 or 1500 juveniles per pot of *Meloidogyne incognita* and treated with : *Verticillium chlamydosporium* at 10^3 (VL) and 10^4 (VH) chlamydospores/g soil, *Pasteuria penetrans* at 10^3 (PL) and 10^4 (PH) spores/g soil, the two in combination (VL + PL; VL + PH; VH + PL; VH + PH), or aldicarb (1.5 ppm) and an untreated control (n = 4).

	Harvest time											
	7 weeks						14 weeks					
Treatment/Pi	250			1500			250			1500		
	FEM	%	infection	FEM	%	infection	FEM	%	infection	FEM	%	infection
Control	88	0	—	374	0	—	1687	0	—	6161	0	—
Aldicarb	36	0	—	142	0	—	1468	0	—	2703	0	—
VL	99	0	—	364	0	—	1021	0	—	3287	0	—
VH	89	0	—	308	0	—	1037	0	—	3944	0	—
PL	106	19	(— 1.10)*	280	25	(— 0.77)	1275	6	(— 200)	4622	8	(— 1.75)
PH	64	50	(0.002)	214	65	(0.68)	1210	52	(0.15)	5655	33	(— 1.79)
VL-PL	41	17	(— 1.30)	396	19	(— 1.55)	1416	8	(— 2.14)	2854	2	(— 2.39)
VL-PH	72	38	(— 0.53)	324	31	(— 0.85)	1670	50	(0.01)	4552	52	(0.13)
VH-PL	53	13	(— 2.07)	199	17	(— 1.77)	1205	8	(— 2.14)	2580	8	(— 1.75)
VH-PH	58	59	(0.34)	360	58	(0.34)	1046	67	(0.92)	2910	50	(0.00)
SED	21		0.327	91		0.49	530		0.62	1287		0.69
Significance level p	0.05		< 0.001	NS		< 0.001	NS		< 0.001	NS		< 0.001

* Parenthesis logit x = $\log_e$ (x/1 — x).

Table 5. Number of eggs per root system (× 100) extracted from tomato roots inoculated with 250 (NL) or 1500 (NH) juveniles of *Meloidogyne incognita* at per pot and treated with : *Verticillium chlamydosporium* at 10^3 (VL) and 10^4 (VH) chlamydospores/g soil, *Pasteuria penetrans* at 10^3 (PL) and 10^4 (PH) spores/g soil, the two in combination (VL + PL; VL + PH; VH + PL; VH + PH), or aldicarb (1.5 ppm) and an untreated control (n = 4).

	Harvest time					
	7 weeks				14 weeks	
	Total eggs		Healthy eggs		Total eggs	
Nematode inoculum	250	1500	250	1500	250	1500
Control	364	2083	364 (2.54)*	2083 (3.31)	9336 (3.88)	9703 (3.97)
Aldicarb	85	292	85 (1.92)	292 (2.41)	3759 (3.50)	4425 (3.61)
VL	189	1047	76 (0.18)	392 (2.57)	4171 (3.50)	7896 (3.77)
VH	305	1114	98 (1.93)	412 (2.60)	3562 (3.48)	5940 (3.75)
PL	209	1201	209 (2.22)	1201 (3.06)	6523 (3.74)	4040 (3.59)
PH	160	712	160 (2.15)	712 (2.83)	8734 (3.78)	7220 (3.77)
VL + PL	166	989	86 (1.93)	468 (2.62)	7643 (3.87)	7255 (3.83)
VL + PH	172	906	93 (1.93)	489 (2.55)	6704 (3.81)	4353 (3.62)
VH + PL	120	925	59 (1.70)	446 (2.64)	5218 (3.68)	3359 (3.45)
VH + PH	124	889	62 (1.79)	467 (2.64)	1043 (2.10)	2097 (3.32)
SED	64	281	0.15	0.16	0.20	0.16
Significance level (p)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.01	< 0.05

* Data not normally distributed Y = Log (X + 1).

Table 4. Number of juveniles per root system (× 100) extracted from tomato roots inoculated with 250 or 1500 *Meloidogyne incognita* juveniles per pot and treated with : *Verticillium chlamydosporium* at 10^3 (VL) and 10^4 (VH) chlamydospores/g soil, *Pasteuria penetrans* at 10^3 (PL) and 10^4 (PH) spores/g soil, the two in combination (VL + PL; VL + PH; VH + PL; VH + PH), or aldicarb (1.5 ppm) and an untreated control (n = 4).

	Harvest time			
	7 weeks		14 weeks	
Treatment/Pi	250	1500	250	1500
Control	78.0 (1.88)*	592 (2.72)	2086 (3.30)	1728 (3.20)
Aldicarb	14.0 (1.01)	73 (1.77)	894 (2.89)	814 (2.84)
VL	18.1 (1.24)	95 (1.81)	784 (2.80)	1564 (2.87)
VH	13.1 (0.98)	74 (1.74)	307 (2.46)	782 (2.01)
PL	77.5 (1.77)	219 (2.33)	768 (2.83)	541 (2.69)
PH	48.9 (1.56)	176 (2.20)	1596 (3.02)	883 (2.89)
VL + PL	17.0 (1.18)	107 (1.98)	1226 (3.05)	1493 (3.12)
VL + PH	49.2 (1.57)	180 (2.06)	955 (2.97)	401 (2.39)
VH + PL	8.6 (0.92)	153 (2.16)	682 (2.80)	893 (2.84)
VH + PH	12.4 (0.94)	89 (1.93)	177 (2.22)	293 (2.36)
SED	0.230	0.230	0.168	0.168
Significance level (p)	< 0.001	< 0.01	< 0.001	< 0.001

* Data not normally distributed Y = Log (X + 1).

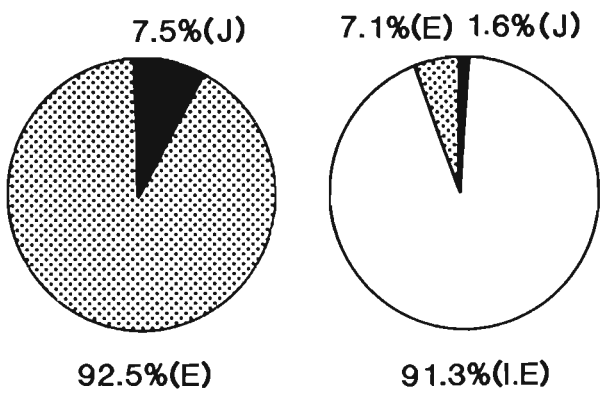

Fig. 2. Comparison of susceptibility of *Meloidogyne incognita* eggs developing inside and outside the root gall to *Verticillium chlamydosporium* (E = healthy eggs; IE = infected eggs; J = juveniles; n = 12).

second-stage juveniles is limited to about six weeks (Hague & Gowen, 1987) and, therefore, any subsequent generation of juveniles coming from nematodes which escaped its initial effect will not be controlled without further applications. Both of the biological organisms continued to act on subsequent generations of nematodes, although the amount of infection varied between the two and was dependent on the extent to which large galls had formed.

Meloidogyne spp. have a great reproductive potential (de Guiran & Ritter, 1979). Size and health of the root systems are therefore very important factors which determine to a large extent the numbers of nematodes and their reproduction in the roots at high nematode densities (Triantaphyllou, 1960). At the second harvest where restricted root growth and large numbers of nematodes prevailed, there were some treatments with more nematodes than suitable feeding sites on the roots to support the development of females. This has led to increased variation which made the interpretation of results after the second harvest difficult. Because of excessive damage caused by the nematodes in the untreated controls after the second harvest, nematode populations were in general smaller in pots receiving the large nematode inoculum than in those receiving the small inoculum. It may, therefore, be more appropriate to compare nematode control by the biological treatments at harvest two with the degree of control after aldicarb treatment.

Colonization of egg-masses by *V. chlamydosporium* was confined to those exposed to the soil on the root surface; no egg-masses embedded in the root were colonised. At the first harvest, most egg-masses had developed on the root surface as there were few large galls, resulting in more than an 80 % reduction in nematode populations, this level of control was similar to that obtained with aldicarb. Colonisation of egg-masses by *V. chlamydosporium* after the first harvest was not dependent on nematode density, making the fungus very useful in situations where nematode densities were low. As the amount of fungus detectable on the root surface more than doubled during the 7 weeks between the first and second harvest it seems likely that the effectiveness of the fungus should increase with time. However, in subsequent generations more and more nematodes developed inside the root, in large galls, making the fungus less effective as it does not penetrate the root cortex (de Leij & Kerry, 1991).

Neither *V. chlamydosporium* nor *P. penetrans* had any significant effect on the numbers of females. Although *P. penetrans* has been observed to reduce invasion (Stirling, 1984; Davies *et al.*, 1988), in this experiment, where juveniles were inoculated into pots with well established root systems, the distance the nematodes had to migrate through the soil before finding a suitable invasion site was presumably too short for them to become encumbered with sufficient spores. Therefore, for *P. penetrans* to have an effect on invasion, either the number of spores, or the length of time the second-stage juveniles are active in the soil, needs to be increased. It has been suggested that 10^5 spores per gram of soil are required to reduce invasion (Stirling, 1988). However, fewer

spores would be necessary if the distance juveniles have to migrate were lengthened.

No parasitism by *P. penetrans* could be observed on second-stage juveniles dissected from galls at the time of the first harvest. This observation alone would confirm the view that *P. penetrans* has to be sufficiently active to control the initial invasion if it is to be a useful biological control agent. At the second harvest it was expected that the percentage of females infected with *P. penetrans* would decline, because juveniles would reinvade without coming into contact with the soil and would therefore remain uninfected. However, our results showed that after the second harvest, at the high *P. penetrans* inoculation rate a relatively high percentage of the females were infected with *P. penetrans*. As spores were also observed on second-stage juveniles dissected from large galls, this suggests that either they had left the root and become encumbered with spores in the soil before reinvading, or they had picked up spores from inside the root from disintegrating females. As spores were only observed on second-stage juveniles dissected from galls at the second harvest and not at the first, the latter explanation is the most likely.

In this pot-test *V. chlamydosporium* and *P. penetrans* parasitised different stages of the nematode life-cycle and were able to produce strong complementary effects when both organisms were present in high concentrations. Both organisms reduced the reproductive potential of the female nematode. *V. chlamydosporium* was most effective after the first harvest when most egg-masses were produced in the rhizosphere and the fungus could colonise the egg-masses. Later, when egg masses were produced within galls and were protected from the fungus, *P. penetrans* liberated from disintegrating females may have produced a degree of secondary infection, hitherto unobserved.

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