VARIATION IN COLONIZATION OF FIELD SOIL AND ASSOCIATED ROOTS BY THE NEMATOPHAGOUS FUNGUS *POCHONIA CHLAMYDOSPORIA*

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Summary. The establishment of the nematophagous fungus *Pochonia chlamydosporia* in soil and roots was assessed in pots filled with field soil. In addition, the survival and dynamics of the fungus in multi-season greenhouse experiments for four cropping cycles is reported. The presence of the fungus was variable, thus restricting its potential for the management of the root-knot nematode, *Meloidogyne javanica*, in vegetable production in Crete, Greece.

Keywords: *Meloidogyne javanica*, root-knot nematodes, biological control.

Pochonia chlamydosporia (Goddard) Zare, Games *et* Evans (synonym: *Verticillium chlamydosporium*) (Zare *et al*., 2001) is a facultative fungal parasite of root-knot nematode eggs, with potential as a biological control agent on vegetable crops, as demonstrated in pot (Kerry, 1995; Leij *et al*., 1992; Viaene and Abawi, 2000; Van Damme *et al*., 2005) and microplot experiments (Leij *et al*., 1993). Trials conducted in intensive vegetable production systems of the Mediterranean area indicated that this fungus colonizes efficiently soil and roots and parasitizes nematode eggs, although results on nematode reduction were inconsistent (Tzortzakakis, 2000; Ciancio *et al*., 2002; Ornat *et al*., 2003; Sorribas *et al*., 2003; Verdejo *et al*., 2003).

A multi-season greenhouse experiment was conducted at two sites in Crete, aiming to evaluate the efficacy of the fungus against *Meloidogyne javanica* (Treub) Chitw. infecting tomato in greenhouses in Greece. The experiments indicated that the fungus had a poor establishment and a minimal to zero "carry over" to the next crop, and was inefficient at reducing nematode densities (Tzortzakakis and Petsas, 2003). These authors focused mainly on nematode counts and fungus monitoring but did not provide details on the variable establishment of the fungus in soil and roots. Therefore, in this paper we present data on the fungal dynamics from the multi-season experiment and from pot experiments, showing the variations observed in the establishment of the fungus in soil and roots.

MATERIALS AND METHODS

The fungal isolates, a) *Pc* 10 (provided by Professor B.R. Kerry, Nematode Interactions Unit, Rothamsted Research, UK), b) *Pc* Sev (provided by Professor Luis Lopez-Llorca, Laboratory of Plant Pathology, Department of Marine Sciences and Applied Biology, University of Alicante, Spain) and c) *Pc* It06 and *Pc* It11 (provided by Dr A. Ciancio, Istituto per la Protezione delle Piante, Section of Bari, Italy), were maintained in Petri dishes containing 1.7% corn meal agar. Chlamydospores were produced on a mixture of sand and milled barley in conical flasks and their concentration was estimated in diluted samples (Leij and Kerry, 1991). These were further mixed with soil in a plastic bag to give appropriate concentrations for the experiments. Root and soil colonization by the fungus (cfu/g) were assessed in one g diluted samples spread on a semi-selective medium as suggested by Leij and Kerry (1991).

Effect of the fungus on root galling, egg mass number and second-stage juvenile density on tomato grown in pots containing field soil and inoculated with Meloidogyne javanica for one generation. Combined soil samples were collected from two greenhouses (site 1 and site 2) where the multi-season experiments were conducted (Tzortzakakis and Petsas, 2003). Soil characteristics were as follows: a) 59% sand, 16% clay, 25% loam, pH=7.3, 44% $CaCO_3$, 3.27 ECm^{-1} for soil 1, and b) 48% sand, 24% clay, 28% loam, pH=7.4, 47.5% $CaCO_3$, 2.7 ECm^{-1} for soil 2. Samples were sieved through a 2-mm sieve, autoclaved (120 °C for 30 minutes), left to aerate for two weeks and mixed with coarse sun dried sand (1:1). Pots were filled with 1 dm^3 of either soil type into which the isolate *Pc* 10 of the fungus was applied at the rate of 0 or 5,000 chlamydospores/g of soil. Each pot was planted with a seedling of the nematode susceptible tomato cv. ACE and allowed to establish for five days before inoculation with 6,000 eggs of *M. javanica* (the same population as that in the field experiments). Eggs for inoculation were extracted from infected roots of tomato using NaOCl (Hussey and Baker, 1973) and appropriate amounts of suspensions were

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poured with a pipette into holes around the plant.

The treatments were: *a)* fungus (without nematodes) in non-autoclaved soil; *b)* fungus (without nematodes) in autoclaved soil; *c)* fungus and nematodes in autoclaved soil; *d)* nematodes (without fungus) in autoclaved soil.

The plants (five replicates/treatment) grew for seven weeks in a growth room with a 16-h photoperiod, set at 24-26 °C. At the end of the experiment, the soil of each pot was mixed and a sample of 200 g was placed in a modified Whitehead tray to extract second-stage juveniles (J2s), which were collected in water and counted after four days incubation at 25 °C. Roots were assessed for root galling index (RGI) on a 0-10 scale (Bridge and Page, 1980) and the number of egg masses exposed on root surfaces was counted under a stereoscope. Root and soil colonization by the fungus were also estimated.

Effect of different plant species on soil and root colonization by the fungus in the absence of nematodes. An experiment was set up with three fungal isolates (*Pc* Sev, *Pc* It06 and *Pc* It11) to compare root and soil colonization of different plant species in nematode-free soil. The host plants tested were tomato (cvs Durinta and Nikita), pepper (cvs Lamuyo and Lipary), cabbage (cv. Salarite) and bean (cv. Tauro). Pots were filled with 1 dm^3 of non-sterile soil from site 1, to which the fungus had been applied at the rate of 5,000 chlamydospores/g of soil. After transplanting seedlings of the test plants into the soil, the pots were put on a bench in a glasshouse and left in natural conditions (no supplementary light or heating). After seven weeks (January-March) the plants were uprooted and fungus colonization of the soil and roots were assessed.

Data from greenhouse experiments. The isolate *Pc* 10 of the fungus was used. The crop cycles were: 1st spring (March to July, 1999) – nematode resistant tomato, fungus application; 2nd autumn (September 1999 to February 2000) – nematode susceptible tomato; 3rd spring (March to July 2000) – nematode resistant tomato, fungus application; 4th autumn (August 2000 to January 2001) – nematode susceptible tomato, fungus application.

Each application of the fungus was by mixing 3 dm^3 of soil removed from each planting site with fungal inoculum at the rate of 5,000 chlamydospores/g of soil. In the 4th crop, four weeks after planting, 250 ml of a suspension containing 3×10^7 chlamydospores (double the concentration applied before planting) was applied as a drench to each plant.

Soil samples were taken from the rhizosphere and root pieces were processed to estimate fungus colonization after uprooting at the end of the crop cycle. Results of each of the eight treated plots per site are presented. Monitoring of the fungus based on the average values of the eight plots per site and data on root galling, plant yields and nematode counts from these greenhouse experiments have been already published (Tzortzakakis and Petsas, 2003).

RESULTS

Effect of the fungus on root galling, egg mass number and second-stage juveniles density on tomato grown in pots containing field soil and inoculated with Meloidogyne javanica for one generation. Shoot and root weight did not differ between treatments (data not shown). In soil from site 1 the fungus did not reduce root galling or the numbers of egg masses and J2s, while in soil from site 2 all of these variables were significantly reduced (Table I). Fungal colonisation differed between replicates of the same treatment, with high standard deviations; in some pots the fungus was not detected in soil or roots. Autoclaving the soil did not result in increased fungal colonization when nematodes were absent and root colonization was not affected by the presence of nematodes in autoclaved soil (Table II).

Effect of different plant species on soil and root colonization by the fungus in the absence of nematodes. There was much variation in root and soil colonization by the fungal isolates between replicates of the same treatment and the fungus was not recovered from some pots. Standard deviations were high and, in some cases, their values were greater than the mean value. Soil and root colonization were similar for the three fungal isolates. As the surface area of root corresponding to a standard weight differs between plant species, the dif-

Table I. The effect of *Pochonia chlamydosporia* on population densities of *Meloidogyne javanica* in two autoclaved soil types.

Fungus	Gall index		Egg masses/root		J2s/200 g soil	
	Soil 1	Soil 2	Soil 1	Soil 2	Soil 1	Soil 2
Present	4.3	3	14	13.2	330	292
Absent	5.3	6.4	14	24.2	395	688
SED	0.97	1.2	4.31	(0.8)	(0.78)	(0.83)
P value	>0.05	<0.05	>0.05	<0.05	>0.05	<0.05

J2s = second-stage juveniles.
Means of five replicates; ( ) ANOVA on $\log_e$ x transformations.

Table II. Root and soil colonization (cfu/g) of *P. chlamydosporia* in two soil types in the presence and absence of *M. javanica*.

Autoclaved soil	Nematodes	cfu/g of soil (×10³)		cfu/g of root (×10³)	
		Soil 1	Soil 2	Soil 1	Soil 2
No	Absent	38.3 ± 31.3 (4)	4.2 ± 7.8 (3)	24.9 ± 30 (4)	22.1 ± 39.4 (5)
Yes	Absent	4 ± 1.5 (5)	9.2 ± 10.7 (5)	13 ± 29.2 (1)	22.5 ± 33.7 (4)
Yes	Present	41.9 ± 35.2 (5)	47.5 ± 37.6 (5)	60.6 ± 60.8 (5)	55.8 ± 56.4 (4)
P value		>0.05	<0.05	>0.05	>0.05

Means of five replicates; ± standard deviation; ANOVA on $\log_e$ (x+1) transformations; ( ) number out of five pots from which the fungus was recovered.

ferences in root colonization (per g of root) may not indicate different numbers of cfu/cm² root, except within tomato and pepper varieties. However, soil colonization was not influenced by the plant species (Table III).

Data from greenhouse experiments. A total of c. 25,000 chlamydospores/g of soil had been applied in 3 dm³ of soil at each planting site throughout the four cropping cycle period. However, at the end of the 4th crop, the fungus density in soil was low, with maximum values of 7,500 cfu/g at site 1 and 5,000 cfu/g at site 2. While soil colonization was high in some plots, the fungus was found at small or zero levels in the respective roots and vice versa. Therefore, a correlation between soil and root colonization for all plots cannot be made for either of the sites. Indicative data for the lack of correlation are presented for three plots per site (Fig. 1). Furthermore, the root-galling and the density of the fungus on the root were not linearly correlated (data not shown).

Considering the root colonization observed throughout the four seasons in the sixteen plots (eight plots per site) to which the fungus was applied, they were grouped as follows: *a)* in twelve plots the fungus density increased steadily between the 2nd and 4th crops; however, in eight of these plots, the final fungus density was less than 15,000 cfu/g, whereas the fungal application to the last crop alone was 15,000 chlamydospores/g of soil (Fig. 2A); *b)* in two plots the fungus density increased up to the 3rd crop and diminished thereafter (Fig 2B); *c)* in one plot the fungus density remained at very low levels while in another it failed to establish (Fig. 2C).

The respective grouping of plots for soil colonization were: *a)* in six plots the fungus density increased slightly up to the 4th crop but in all of them the final fungus density was less than 15,000 cfu/g whereas the fungal application to the last crop alone was 15,000 chlamydospores/g of soil (Fig. 3A); *b)* in eight plots the fungus density increased in the 3rd and diminished significantly in the 4th crop (Fig. 3B); *c)* in two plots the fungus density remained very low, almost zero (Fig. 3C).

Plotting means against standard deviation of fungus density. To illustrate the variation of fungus presence in tomato roots, the mean values were plotted against their standard deviations. Data were obtained from: *a)* the field sites (eight replicate plots per mean); *b)* the pot experiment with field soil (five replicates per mean); *c)* a pot experiment with commercial compost soil (3-4 replicates per mean, Tzortzakakis, 2007) and *d)* unpub-

Table III. Colony forming units (cfu) × 10³/g of three *P. chlamydosporia* isolates in soil and roots on different plant species.

Crop plant (cultivar)	Fungal isolate							
	Pc Sev		*Pc* It11		*Pc* It06		Mean (transformed)	
	Soil	Root	Soil	Root	Soil	Root	Soil	Root
Bean (Tauro)	42 ± 48 (4)	128 ± 45 (4)	4 ± 5 (2)	54 ± 44 (4)	97 ± 72 (4)	149 ± 62 (4)	1.19	1.94
Cabbage (Salarite)	38 ± 52 (3)	72 ± 49 (4)	20 ± 24 (2)	32 ± 31 (4)	0.9 ± 1 (2)	0.4 ± 0.8 (1)	0.68	1.07
Tomato (Durinta)	14±26 (2)	24±8 (4)	22±30 (3)	36±35 (4)	47±62 (4)	30±31 (4)	0.88	1.31
Tomato (Nikita)	13 ± 14 (3)	0.2 ± 0.3 (2)	17 ± 15 (4)	18 ± 28 (3)	55 ±46 (3)	19 ±15 (4)	1.09	0.66
Pepper (Lamuyo)	4 ± 5 (2)	6 ± 7 (3)	17 ± 31 (4)	9 ±10 (4)	23 ±36 (3)	21 ±26 (3)	0.73	0.81
Pepper (Lipary)	2 ±2 (3)	8 ± 7 (3)	23 ± 25 (4)	47 ±27 (4)	4 ±7 (3)	4 ±4 (3)	0.62	1.04
Mean (transformed)	0.71	0.66	0.88	0.33	1	0.60		
	Soil				Root			
LSD 5%	0.94				0.20			
LSD 1%	1.25				0.33			
P fungal isolate	>0.05				>0.05			
P plant species	>0.05				<0.001			
P interaction	>0.05				<0.001			

Means of four replicates; ± standard deviation; P values of ANOVA on $\log_e$ (x+1) transformed data; ( ) number out of five pots from which the fungus was recovered.

lished data from pot tests with field soil or commercial compost soil (five replicates per mean). The cfu/root counts of the fungus were more variable than the cfu/soil counts; furthermore, in the case of roots, the plants in pots filled with compost soil showed the least variation (Figs 4 and 5).

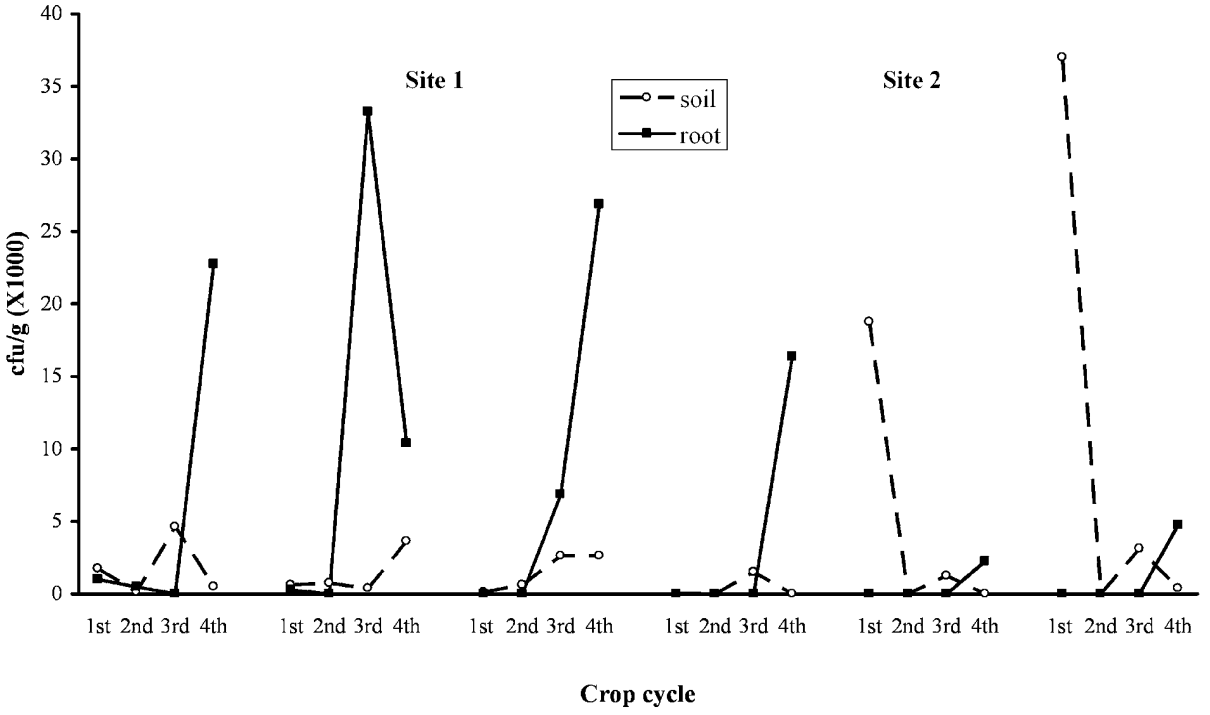

Fig. 1. Density of fungus (cfu/g × 10^3) in soil and roots of tomato at the end of each of the four cropping cycles in three plots per site.

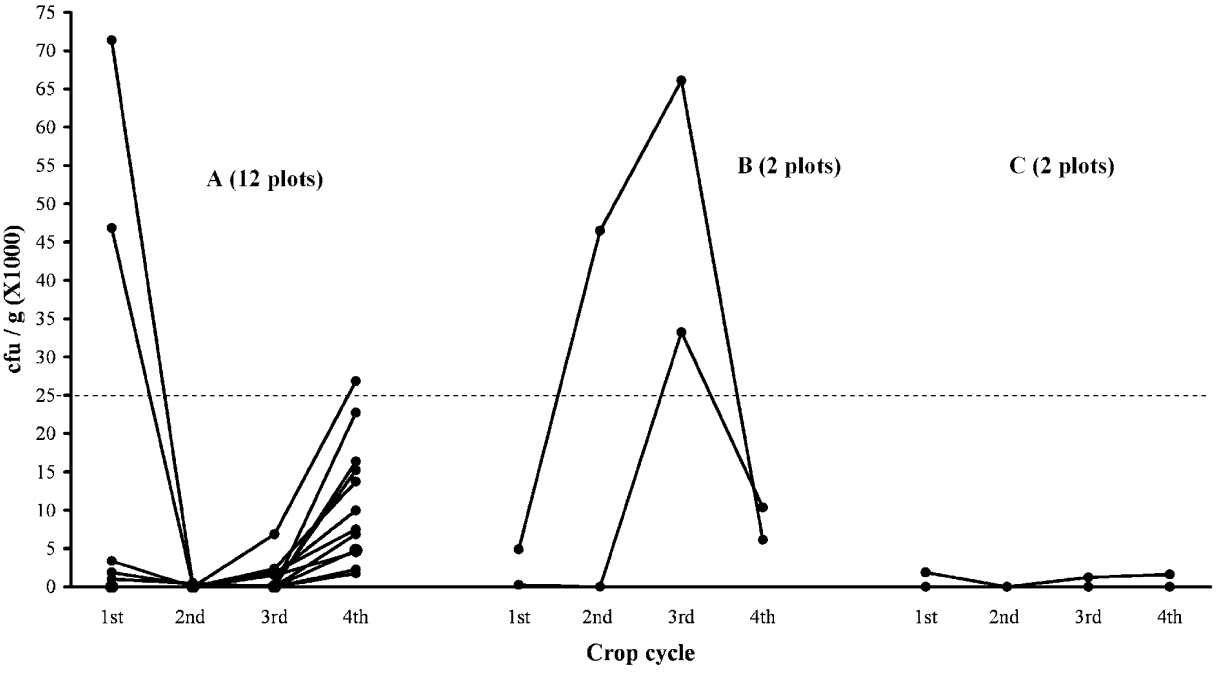

Fig. 2. Density of fungus (cfu/g × 10^3) on roots of tomato at the end of each of the four cropping cycles at both sites (data from 16 plots divided in 3 groups). The dotted line indicates the total number of chlamydospores applied per g of soil throughout the four cropping cycles.

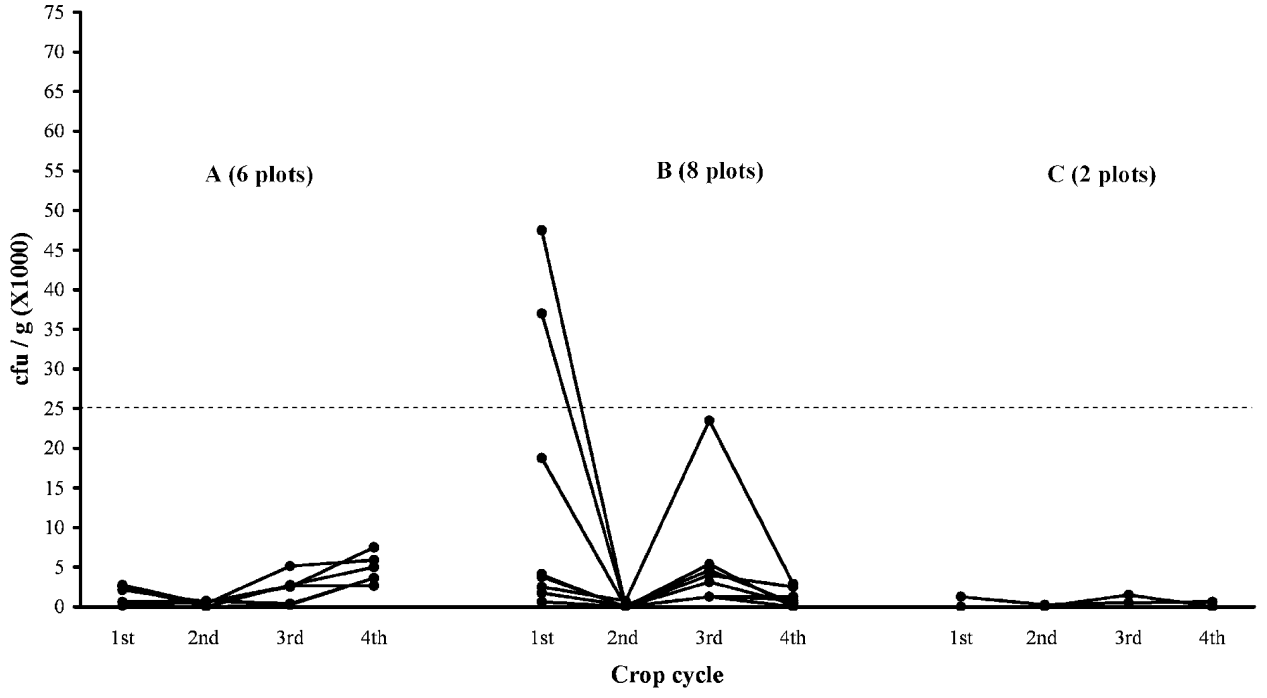

Fig. 3. Density of fungus (cfu/g × 10^3) in soil around tomato at the end of each of the four cropping cycles at both sites (data from 16 plots divided in three groups). The dotted line indicates the total number of chlamydospores applied per g of soil throughout the four cropping cycles.

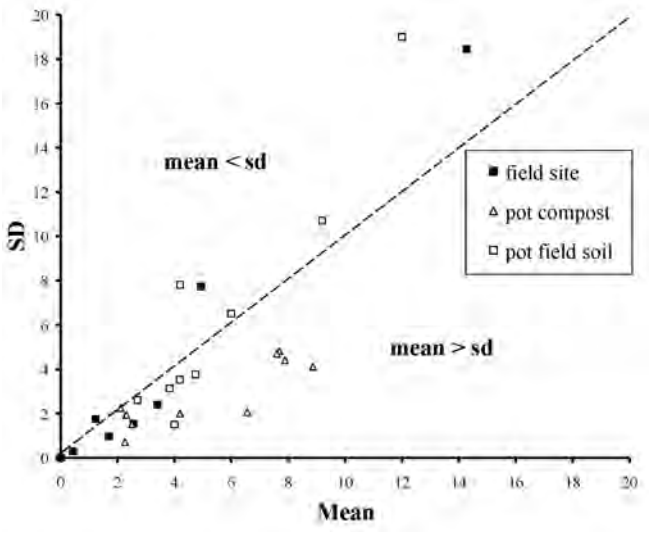

Fig. 4. Means and standard deviations of fungus density in soil at field sites (average of eight replicate plots) and in pots filled with either field soil or commercial compost (average of 3-5 replicates).

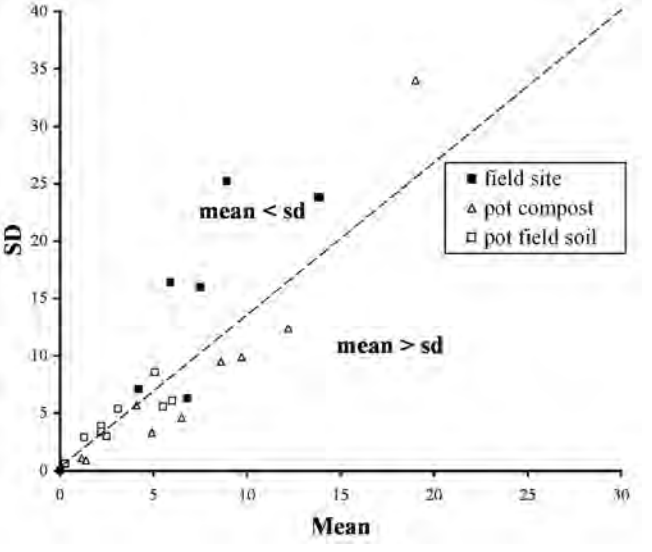

Fig. 5. Means and standard deviations of fungus density in tomato roots at field sites (average of eight replicate plots) and in pots filled with either field soil or commercial compost (average of 3-5 replicates).

DISCUSSION

The pot experiments indicated the high variation in the density of the fungus in the field soil. Even in the same treatment, great variation was observed within the limited number of five replicates; in some pots the fungus did not establish, while in others there was an up to ten-fold increase. A difference in growing conditions between pots is unlikely to occur and this great variation cannot be attributed to a block effect. Such variation has also been mentioned by Atkins *et al.* (2003), who attributed the large standard errors of fungal estimates in pots to its aggregated distribution. In our experiments one g of soil was used for estimating fungus density and, despite thorough mixing before sampling, such a small quantity may account for the variation. Also, mineral soils

support less growth of fungus than do organic soils (de Leij *et al*., 1993). The effect of the fungus in reducing nematode populations in pots with field soil for one nematode generation (soil 2) was not further confirmed in a multi-season plot experiment, indicating that results from pot tests cannot easily be extended to field conditions.

The fungus did not establish well in autoclaved soil when nematodes were absent, and nematode presence did not increase fungal colonization of the roots. These findings contradict other published results, where it has been demonstrated that root colonization is greater in plants infected with nematodes (Bourne *et al*., 1996).

Differences in root colonization between different plant species have been reported (Bourne *et al*., 1996; Bourne and Kerry, 1999) and results of this work indicate differences, even within varieties of the same plant species.

Soil colonization at the field sites was low and, at the end of the four crop cycles, the maximum value of cfu/g in individual plots was less than one third of the cumulative number of chlamydospores applied over the two years of the experiment. This indicates a failure of the fungus to build up significantly in the soil. It is interesting to note that, at the end of the 2nd crop, to which the fungus was not applied, its density decreased almost to zero, indicating an absence of "carry over" between crops. In contrast, the increase of root colonization was greater than that in the soil in 30% of the plots, but a density of cfu/g approaching the cumulative application rate of chlamydospores was found only in one plot of site 1. A simple relationship was not found between the density of fungus in soil and roots.

The fungus was detected only at a low level or not at all in the soil in two (12%) out of the sixteen tested plots. In the remaining fourteen (88%) plots, the fungus was detected in soil and roots but at levels lower than the application rates. A total of 15,000 chlamydospores/g of soil had been applied at the last crop and fungus colonization (cfu/g) was quite low, indicating that only a minority of chlamydospores survived and produced colonies. This was more apparent in the soil than the root counts.

Only in two plots per site was a build-up of fungus exceeding 15×10^3 cfu/g of root obvious at the end of the four crops, but this was not correlated with a respective increase in the density in soil. Root removal after crop harvest, as a practice to eliminate the carryover of root pathogens, would also discard a "rich" source of *P. chlamydosporia*. The amount of fungus surviving in soil was low and insufficient to ensure successful establishment in the following crop.

In the system of vegetable production in Crete, plants grow in soil of low organic matter content and are subjected to heavy nematode pressure, because there are several generations of nematode per cropping cycle due to the high temperature. The data from this work indicate that the fungus *P. chlamydosporia* may not be a viable management practice in reducing root-knot nematode densities due to the problems of establishment and increase, and the variability observed in plots of the same field site and even in small amounts of soil tested in pots. An explanation for this could be the lack of suitability of certain soils for the fungus, which, when introduced, has limited survival and multiplication ability (Ornat *et al*., 2003; Sorribas *et al*., 2003; Monfort *et al*., 2006).

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