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Title: Root-knot nematode-destroying microorganisms for home garden and landscape use

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INTRODUCTION
    Plant-parasitic nematodes are tiny roundworms that can cause tremendous economical damage to crop production. But they are often overlooked because of their small size, hidden activities and often non-specific damage to their hosts. The best known of these pests belong to the root-knot nematodes (RKN, *Meloidogyne* spp.), named after the symptoms they can cause. They parasitize well over 2000 different plant species including many vegetable crops and ornamentals grown in home gardens.
    In California, four species of RKN are of main concern, *M. incognita, M. javanica, M. arenaria* and *M. hapla*. RKN spend only a short part of their life in soil, either as eggs or as second-stage larvae. The latter then enter the roots and establish feeding sites in susceptible hosts. They undergo three more molts during which the roots become swollen and have a characteristic "knotty" appearance. Each female can produce several hundreds of eggs that under favorable conditions continue to develop into the next generation. Water and nutrient uptake as well as transport are severely restricted by the root galling. Increased leakage of plant metabolites from those galls attracts fungi and bacteria that can enter the weakened tissues and accelerate the decay. Limitation of normal root function is typically expressed with symptoms of malnutrition, chlorosis and stunting. Consequently, vigor and production capacity of diseased crops is noticeably reduced.
    Current guidelines for the management of plant-parasitic nematodes in production agriculture and horticulture suggest soil fumigation, application of non-fumigant nematicides, soil pasteurization, and rotations with non-host crops or the use of resistant cultivars. For California's gardeners the choices are more limited, as no fumigant or nematicide is allowed for home use. A few vegetable cultivars are available that are resistant to the dominant root-knot nematode species in Southern California. Various plant- or animal-derived materials are

sold as soil amendments with potential deleterious side effects on nematodes. Some of those products have a federal registration as a "nematicide". But none is registered for that purpose in California, as CA EPA requires proof of efficacy. Furthermore, no nematode biocontrol product, based on living microorganisms that are deleterious to plant-parasitic nematodes, is registered and commercially available in the US. For large agrochemical companies, the producers of traditional chemical nematicides, the expected return on investment is often perceived as too small to pursue research and development of nematode antagonists. Small companies typically do not have the technical expertise and/or the financial backing to research and develop such a product.

Population development of endoparasites such as the root-knot nematodes is mainly influenced by the host and the environmental temperature. That has led to crop damaging models that predict the nematode population density and the resulting crop damage based on pre-planting nematode counts. However, in some locations the nematode population does not develop as expected despite susceptible host plants and suitable environmental conditions. Such nematode-suppressive soils are rare situations in which biotic factors influence the relationship between a pest and its host in favor of the latter.

In recent years, the PI's group has identified and investigated such soils in Southern California (Westphal and Becker, 1999, 2001, Pyrowolakis *et al.*, 2001, Gao and Becker, 2002, Yin *et al.*, 2003). It has been suggested earlier that nematode-suppressive soils are a valuable resource for potential biocontrol strains (Stirling, 1991). We have isolated strains of nematode-destroying fungi from various nematode-suppressive soils and screened them under controlled laboratory and growth chamber conditions. *Pochonia chlamydosporia* (formerly *Verticillium chlamydosporium*) was one of the most effective nematode-destroying strains selected during the previous year's research. Furthermore, the biocontrol activity of this fungal species has been studied by various research groups during the past two decades (Kerry and Bourne, 2002). Its ecology is therefore better understood than most other nematode-destroying fungi.

The objective of this project was to evaluate the efficacy of this and optimize its use against root-knot nematodes. The ultimate goal is to develop a method for its potential utilization in home gardens. While the typically high application rates of such microorganisms are most likely prohibitive in production agriculture, this issue is relatively unimportant in home gardens or landscape sites where the control effort is likely to be concentrated in a very small area.

MATERIALS AND METHODS
The *P. chlamydosporia* strain was evaluated for its ability to cause reductions in the RKN population and mitigation of RKN damage. Tests were conducted with cucumber (*Cucumis sativa* cv. Straight Eight), tomato (*Lycopersicum esculentum* cv. Tiny Tim), floss flower (*Ageratum houstonianum*) and *Chrysanthemum paludosa* in greenhouse trials. All test plants were susceptible to RKN. The fungal strain was preserved in sand cultures stored at 4°C. The fungal inoculum was produced on autoclaved millet seeds in culture pouches (Han and Becker, 2003). Four-week-old cultures were air-dried in a

laminar flow hood and were then stored at 4°C in the dark. Initially, this millet inoculum was used as a 2% or 4% (v/v) amendment. However, the millet itself sometimes caused plant growth problems. In subsequent trials, the millet was washed with sterile water to separate chlamydospores from the growth substance.

The soil was infested with 2000 or 4000 chlamydospores of *P. chlamydosporia* per cc soil. RKN (*M. incognita*) were reared on tomato (cv. Tropic) in greenhouse pot cultures. Nematode eggs were extracted and used to infest pasteurized sandy or non-treated sandy loam field soil at approximately 4000 eggs per 1000-cc soil. The hatching rate within the first 5 days was approximately 10%.

The pots were either seeded (cucumber) or 3 wk-old transplants (tomato, floss flower, chrysanthemum) were used. Each pot received slow-release fertilizer (Osmocote 14-14-14). The pots were arranged as a randomized complete block with 6 replications and incubated at 24±2°C and ambient light.

Plant height or main vine length was determined after 3 weeks (cucumber) and 8 weeks (both cucumber and tomato). Eight weeks after seeding or planting the trials were terminated. Two tomato trials were maintained until fruit production. The plant shoots were cut off and their dry weight was determined. The root system of each plant was rated for galling (Bridge and Page, 1980) and nematode population densities were determined by counting egg masses, eggs and second-stage juvenile (J2).

Soil subsamples were extracted on a modified Baermann funnel at 26°C for 5 days. Second-stage juveniles were collected and counted under a dissecting scope. *P. chlamydosporia* was enumerated by using a semi-selective medium (de Leij and Kerry, 1991) that was modified by substitution of the fungicides and antibiotics. Percentage parasitized eggs were determined by staining with rose bengal solution. The tomato trials were harvested each week until production ceased.

A miniplot trial was conducted at the UC South Coast Research & Extension Center. The use of nematode-destroying fungi in an area dedicated to plant-parasitic nematode research created some concern about a potential spread of these organisms. In order to mitigate those concerns we utilized soil-filled plastic pools as miniplots that were placed in larger polyvinyl pools to allow containment of run-off and drainage water. Nine pools (ca. 3-m diameter, 350000-cc soil) were filled with RKN-infested field soil. Each miniplot was planted with 3 wk-old tomato plants that had been grown in Sunshine mix with or without the biocontrol fungi. We report here on preliminary data as this trial is still ongoing. Yield data, root galling and nematode population densities will be determined at the end of the growing season.

All the data were subjected to ANOVA and, if appropriate, mean separation with Fisher's protected LSD ($P \leq 0.05$).

RESULTS AND DISCUSSION
In our greenhouse trials, *P. chlamydosporia* was effective in reducing the negative influence of RKN on cucumber, tomato, floss flower and

chrysanthemum. The protection of the root systems against the nematodes resulted in reduced galling with significant growth increases after 3 to 6 weeks compared to the non-treated control (Fig. 1-5). Tomato yields were increased, both in terms of number of fruits and total weight (Table 2, Fig. 6-7, 9). The population densities of *M. incognita* at the end of the season were not different (Table 1, 2). The treated plants had a larger, healthier root system that provided more feeding sites for the nematodes than non-protected plants. Overall, the tests confirmed that the fungal strain is able to mitigate the negative effect of the nematodes in previously pasteurized soil. There was no consistent advantage using the higher application rate. The use of chlamydospores as inoculum was considerably better than the millet inoculum. It is assumed that the colonized millet still contained sufficient nutrients to cause a localized buildup of bacteria that led occasionally to poor root health. In contrast, no negative effect of the chlamydospore infestation on plant growth was observed. The fungus was recovered at the end of the trials but at very low densities.

In the miniplot trial (Fig. 8) with RKN-infested field soil, no obvious plant growth differences were observed between the non-treated control and the *P. chlamydosporia* treatment. The yield data of the first harvest was not different between the two treatments. In the second harvest, the *P. chlamydosporium* treated plants had considerably more fruits and a larger total fruit weight than the non-treated check (Fig. 9).

CONCLUSIONS
This project has demonstrated that the use of nematode-destroying fungi can be a useful tool to help gardeners improve plant growth in root-knot nematode-infested soil. We have made considerable progress in adapting a production process for fungal biocontrol strains that relies on growing the organisms on a solid nutrient substrate in autoclavable culture bags (Han and Becker, 2003). Microfilters facilitate air exchange that is a key factor in quality inoculum production.

P. chlamydosporia has been previously described as egg and/or female parasites of root-knot nematodes (Godoy et al., 1983, de Leij and Kerry, 1991) and cyst nematodes (Bourne et al.,1996, Kerry and Crump, 1998, Kerry and Bourne, 2002). The fungus colonized in the rhizosphere of many plant species and destroyed developing nematode females. Furthermore, it proliferated on the egg masses, produced abundantly robust chlamydospores that ensured its survival during host-free seasons. Eventually, it invaded and destroyed the nematode eggs.

Previous attempts at using *P. chlamydosporia* as a biological control organism are well-documented (Kerry, 1990, Stirling, 1991, Kerry and Bourne, 2002). Similar to our results, most authors report very good efficacy in greenhouse trials but less convincing performance from field trials (Stirling and Smith, 1998, Tzortzakakis, 2000, Verdejo-Lucas et al., 2003). For successful establishment of *P. chlamydosporia* in non-pasteurized field soil, several applications might be necessary because its population often drops within a few

weeks after introduction (Kerry *et al*., 1993; Bourne *et al*., 1996; Stirling and Smith, 1998; Viaene and Abawi, 2000).

Population reduction of RKN is likely to increase overall plant health and to improve yield and/or quality in comparison to non-treated plants. In home gardens, the application rate of biological control products is of lesser importance than in commercial agriculture because the planting sites are typically fairly small. Given the lack of effective alternatives, there is a potential for nematode biocontrol products in the home garden sector.

Further studies will concentrate on strain selection among isolates of *P. chlamydosporia*. Differences in strain efficacy is a recognized phenomenon but has been so far insufficiently investigated (Kerry and Bourne, 2002). Also, the choice of the rotation crop is likely to influence the population build-up of the fungus in the rhizosphere and could influence its survival as well as its performance on the following crop. More information is needed about the ecology of the fungus in the rhizosphere of various plant species.

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Fig. 1 Floss flower (*Ageratum houstonianum*) in root-knot nematode infested soil. From left to right: non-infested control; *P. chlamydosporia* 2% (v/v), *P. chlamydosporia* 4% (v/v), non-treated check in root-knot nematode (*M. incognita*)-infested soil (400 eggs/100 cc pasteurized sandy soil).

Fig. 2 *Chrysanthemum paludosa* in root-knot nematode infested soil. From left to right: non-infested control; *P. chlamydosporia* 2% (v/v), *P. chlamydosporia* 4% (v/v), non-treated check in root-knot nematode (*M. incognita*)-infested soil (400 eggs/100 cc pasteurized sandy soil).

Fig. 3 Cucumber seeded in root-knot nematode infested soil (first trial). From left to right: non-infested control; non-treated check, *P. chlamydosporia* (2000 chlamydospores/cc soil), and *P. chlamydosporia* (4000 chlamydospores/cc soil) in *M. incognita*-infested soil (400 eggs/100 cc pasteurized sandy soil).

Table 1. Effect of *P. chlamydosporia* on seeded cucumber growth, root galling and RKN population density in infested soil. Data of two trials were pooled. Means with the same letters in the same column are not significantly different (*P*<0.05).

Treatment	Plant height (mm)@3 wk.	Main vine length (cm)@8wks	Plant dry weight (g)@ 8wks	Root galling @ 8 wk.
Non-treated, non-infested	108.5±5.5de	174.8±2.3d	7.5±0.2a	0.0±0.0a
non-treated, RKN infested	58.0±8.2a	104.0±9.6a	4.1±0.4bc	8.2±0.5d
P. chlamydosporia, 2000 chl.spores/cc	93.8±3.9bc	117.7±6.3ab	5.1±0.4c	5.8±0.2bc
P. chlamydosporia, 4000 chl.spores/cc	82.5±6.6b	133.2±9.6bc	5.6±0.4bc	6.7±0.5c

Treatment	Egg masses/root	Egg count/root	J2/50ml soil
Non-treated, non infested	0.0±0.0a	0.0±0.0a	0.0±0.0a
non-treated, RKN infested	44.0±8.3bc	22560±8074bc	10.0±5.8ab
P. chlamydosporia, 2000 chl.spores/cc	80.8±18.0c	38800±10524bcd	6.3±3.3ab
P. chlamydosporia, 4000 chl.spores/cc	59.2±14.3bc	32000±5868bcd	7.3±5.8ab

Fig. 4 Cucumber seeded in root-knot nematode infested soil (second trial). From left to right: non-infested control; non-treated check, *P. chlamydosporia* (2000 chlamydospores/cc soil), and *P. chlamydosporia* (4000 chlamydospores/cc soil) in *M. incognita*-infested soil (400 eggs/100 cc pasteurized sandy soil).

Table 2. Effect of *P. chlamydosporia* on tomato growth and root galling in RKN-infested soil.

Treatment	Plant height(cm)	Plant dry weight(g)	Root galling
Non-treated, non-infested	28.5±1.6cd	8.2±0.2c	0.0±0.0a
non-treated, RKN-infested	19.7±1.2a	3.2±0.4a	8.3±0.2e
P. chlamydosporia, 2000 chl.spores/cc	26.0±0.9bc	5.2±0.4b	7.3±0.3de
P. chlamydosporia, 4000 chl.spores/cc	24.0±1.3b	4.9±0.3b	6.2±0.4bc

Treatment	Egg masses/root	Egg count/root	J2/50ml soil
Non-treated, non-infested	0.0±0.0a	0.0±0.0a	0.0±0.0a
non-treated, RKN-infested	454.2±85.5c	35913±29328d	56.5±33.3bc
P. chlamydosporia, 2000 chl.spores/cc	377.5±53.5c	27680±25844cd	84.5±31.7c
P. chlamydosporia, 4000 chl.spores/cc	444.2±80.2c	26266±22880cd	22.8±13.1ab

Treatment	Numbers of fruits	fruit weight (g)	Mean weight (g)
Non-treated, non-infested	58.2±3.9f	313.2±23.9e	5.42±0.38bc
non-treated, RKN infested	9.5±2.3a	42.1±10.7a	3.72±0.80a
P. chlamydosporia, 2000 chl.spores/cc	21.5±5.7ab	106.6±27.3ab	5.05±0.29bc
P. chlamydosporia, 4000 chl.spores/cc	28.0±3.9bc	142.6±16.8b	5.16±0.13bc

Fig. 5 Tomatoes transplanted in root-knot nematode infested soil. From left to right: non-infested control; non-treated check, *P. chlamydosporia* (2000 chlamydospores/cc soil), and *P. chlamydosporia* (4000 chlamydospores/cc soil) in *M. incognita*-infested soil (400 eggs/100 cc pasteurized sandy soil).

Fig. 6 Tomatoes transplanted in root-knot nematode infested soil (first yield trial). From left to right: non-infested control; non-treated check, *P. chlamydosporia* (2000 chlamydospores/cc soil), and *P. chlamydosporia* (4000 chlamydospores/cc soil) in *M. incognita*-infested soil (400 eggs/100 cc pasteurized sandy soil).

Fig. 7 Tomatoes transplanted in root-knot nematode infested soil (second yield trial). From left to right: non-infested control; non-treated check, *P. chlamydosporia* (2000 chlamydospores/cc soil), and *P. chlamydosporia* (4000 chlamydospores/cc soil) in *M. incognita*-infested soil (400 eggs/100 cc pasteurized sandy soil).

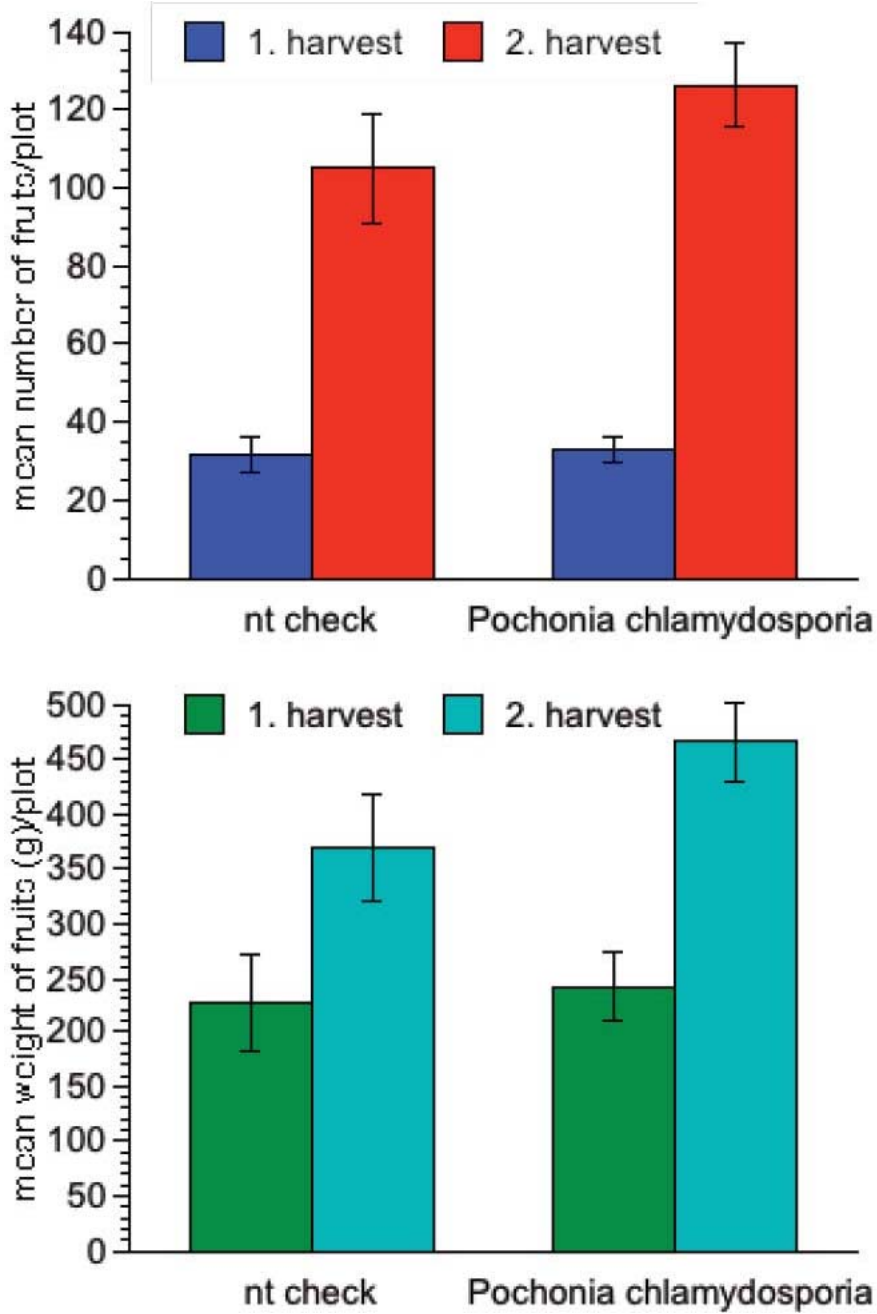Fig 8 Miniplot tomato trial at South Coast Research and Extension Center

Fig 9. Preliminary harvest data (top: number of fruits, bottom: fruit weight) from miniplot tomato trial at South Coast Research and Extension Center. The plots contained *M. incognita* infested field soil (approximately 100 J2/100cc at planting). Tomato plants were harvested at a two-week interval.