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Biocontrol Efficacy Among Strains of *Pochonia chlamydosporia* Obtained from a Root-Knot Nematode Suppressive Soil

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Abstract: Three *Pochonia chlamydosporia* var. *chlamydosporia* strains were isolated from a *Meloidogyne incognita*-suppressive soil, and then genetically characterized with multiple *Pochonia*-selective typing methods based on analysis of ß-tubulin, rRNA internal transcribed spacer (ITS), rRNA small subunit (SSU), and enterobacterial repetitive intergenic consensus (ERIC) PCR. All strains exhibited different patterns with the ERIC analysis. Strains 1 and 4 were similar with PCR analysis of ß-tubulin and ITS. The strains' potential as biological control agents against root-knot nematodes were examined in greenhouse trials. All three *P. chlamydosporia* strains significantly reduced the numbers of nematode egg masses. When chlamydospores were used as inoculum, strain 4 reduced egg numbers on tomato roots by almost 50%, and showed effects on the numbers of J2 and on nematode-caused root-galling. A newly developed SSU-based PCR analysis differentiated strain 4 from the others, and could therefore potentially be used as a screening tool for identifying other effective biocontrol strains of *P. chlamydosporia* var. *chlamydosporia*.

Key words: biological control, *Meloidogyne incognita*, *Pochonia chlamydosporia*, Southern root-knot nematode, suppressive soil.

Biological control is one alternative management strategy addressing the potential environmental problems associated with chemical control of plant-parasitic nematodes. Certain fungi and bacteria can suppress plant-parasitic nematode populations directly through parasitism or indirectly by toxic metabolites (Dong and Zhang, 2006). *Pochonia chlamydosporia* var. *chlamydosporia* (syn. *Verticillium chlamydosporium*) (teleomorph = *Metacordyceps chlamydosporia*), a ubiquitous facultative hyperparasitic fungus of plant-parasitic nematodes, was first reported from the United Kingdom associated with nematode-suppressive soils (Kerry et al., 1984). It is known to parasitize several economically important nematode species in the genera *Meloidogyne*, *Globodera* and *Heterodera* (Kerry, 1990). However, strains of *P. chlamydosporia* vary in their efficacy to control nematode populations (Bourne et al., 1994; Morton et al., 2003b; Mauchline et al., 2004). Strains differ in their virulence, ability to colonize root surfaces, and chlamydospore production. Different strains of the fungus might also occupy separate niches in soil and rhizosphere possibly due to differences in their enzymatic activities (Segers et al., 1996; Mauchline et al., 2004).

Molecular biotyping techniques are useful tools for identifying and screening potential biocontrol organisms (Gil-Lamaignere et al., 2003). These methods enable the study of microorganisms at the genome level. They are reasonably rapid and economically feasible to perform, and they allow evaluation of large numbers of candidates (Gil-Lamaignere et al., 2003). Methods commonly applied for fungal typing include restriction fragment length polymorphism (RFLP) (Diguta et al., 2011), various PCR-based techniques (Cogliati et al., 2000), electrophoretic karyotyping (EK) (Usami et al., 2008), and multilocus enzyme electrophoresis (MLEE) (Tibayrenc, 2009). Application of enterobacterial repetitive intragenic consensus (ERIC) PCR on nematophagous fungi has been useful for delineating strains that are not differentiated by rRNA ITS analyses (Arora et al., 1996; Morton et al., 2003a; Manzanilla-López et al., 2009; Kerry and Hirsch, 2011). More specifically, the ß-tubulin gene of *P. chlamydosporia* var. *chlamydosporia* contains an intron not present in other fungi, and thus provides considerable utility for *Pochonia*-selective assays (Hirsch et al., 2001; Kerry and Hirsch, 2011).

Three strains of *Pochonia chlamydosporia* var. *chlamydosporia* were isolated from a *Meloidogyne incognita*-suppressive soil (Bent et al., 2008). The objectives of this study were to genetically characterize the three strains with multiple *Pochonia*-selective typing methods based on analysis of ß-tubulin, rRNA ITS, rRNA SSU, and ERIC-PCR. Furthermore, we determined the potential of the strains to act as biological control agents against the Southern root-knot nematode (*M. incognita*) in greenhouse trials.

Material and Methods

Characterization of test soil: The soil used was obtained from the University of California Kearney Research and Extension Center, Parlier, CA. This soil was previously selected among six California soils for its abilities to biologically suppress a *M. incognita* population on two different crops under greenhouse conditions (Bent et al., 2008). The test soil was a sandy loam (66% sand, 23% silt, 11% clay; 0.5% organic matter; pH 7.3) mixed with pasteurized silica sand (4:1) to facilitate water drainage and aeration during the greenhouse tests. The soil was pasteurized by submerging samples in plastic bags into a 60°C water bath. Once the center of the sample reached 60°C, the soil was left immersed for 30 minutes at this temperature. The bags were then quickly cooled to room temperature under running tap water. All pasteurized soil samples were pooled and thoroughly mixed.

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Pochonia chlamydosporia phylotyping: The fungal strains were grown at 22°C on a sterilized moist mixture of two parts sandy loam, one part sand and one part compost in capped and parafilm-sealed test tubes (modified after Schneider, 1958). After the substrate had dried out, the tubes were stored at 16°C. For new starter cultures, a few crumbs of the fungal-colonized substrate were sprinkled aseptically onto PDA. Multiple *Pochonia*-selective phylotyping methods were employed based on analysis of ß-tubulin, rRNA ITS, rRNA SSU, and ERIC. The ß-tubulin, rRNA ITS and ERIC methods were used as previously described (Arora et al., 1996; Hirsch et al., 2000). The rRNA SSU method was developed in this study, and it was performed using 10-µl PCR reactions in a RapidCycler (Idaho Technologies, Salt Lake City, UT) containing 50 mM Tris (pH 8.3), 500 µg mL^{-1} bovine serum albumin (BSA), 2.5 mM MgCl2, 250 mM of each dNTP, 400 nM of each primer, 1 mL (c. 66 ng) of template DNA (agarose gel purified) and 0.5 unit *Taq* DNA polymerase. *Pochonia*-selective PCR primers PochSSUF5 (TGCTTTGGCAGTACGCC) and PochSSUR4 (CTTCCGGCCAAGGG) were used with the following thermal cycling conditions: 94°C for 5 min, followed by 42 cycles of 94°C for 20 sec, 64°C for 30 sec, 72°C for 30 sec, and a final incubation at 72°C for 2 min. The amplification product size was 149 bp. Primers used for obtaining ITS sequences were ITS1FUSER (GGGAAAGUCTTGGTCATTTAGAGGAAGTAA) and ITS4USER (TCCTCCGCTTATTGATATGC) with the following thermal cycling conditions: 94°C for 5 min, followed by 40 cycles of 94°C for 20 sec, 52°C for 20 sec, 72°C for 40 sec, and a final incubation at 72°C for 5 min. PCR amplification products were isolated and cloned as previously described using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) (Bent et al., 2008). Sequence identities were determined by BLAST analysis (Altschul et al., 1997).

Inoculum production: For the production of chlamydospores, 250-ml flasks were prepared with 20 g of barley immersed in 40 ml of deionized water. After overnight incubation, excess water was discarded. The flasks were autoclaved at 121°C for 20 min and cooled to room temperature before use. Each flask was inoculated with a different strain of *P. chlamydosporia* and closed with a sterile cotton plug. Chlamydospores were harvested from the flasks after four wk of incubation at 25°C. The content of each flask was blended separately with 50 ml water in a mixer for 1 min. The suspension was poured and washed with a fine jet spray through several sieves (300-µm-pore to 50-µm-pore). After a final rinse the chlamydospores were retained on a 10-µm-pore sieve. The concentration of chlamydospores was determined using a hemotocytometer (De Leij et al., 1993). To check the viability of chlamydospores, a known amount of inoculum was diluted in series onto 1.7% corn meal agar (CMA) with antibiotics (50 mg per liter streptomycin sulfate, chloramphenicol and chlortetracycline). The percentage of germination was determined after incubation at 25°C for 2 d. For the production of hyphae, *P. chlamydosporia* was cultured on 20 ml potato dextrose agar medium (PDA) in 90 mm petri dishes at 23 ± 2°C for 21 d prior to inoculum preparation. Each plate of fungal culture was mixed with 50 ml sterile water with a Sunbeam 6 Speed Blender (Model 4142; Sunbeam Products Inc., Boca Raton, FL) for 1 min using the "blend" setting. The number of CFUs in each mixture was determined from a dilution series. The remaining portions of the fungal mixtures were added to soil (within 1 hr of blending the fungi) as described below.

Greenhouse trials: The efficacy trials were conducted in a greenhouse of the Department of Nematology, University of California, Riverside, CA. Tomatoes (*Solanum lycopersicum*) cv. UC 82 were sown in seedling trays filled with Sunshine mix #5 (Sun Gro Horticulture Canada Ltd) and incubated in a greenhouse at 26 ± 2°C and ambient light. After three wk the seedlings were transplanted into 800 cm^3 cups with pasteurized, infested test soil. The soil contained 5,000 chlamydospores/cm^3 or 50 ml of blended hyphae of *P. chlamydosporia* and 600 eggs/100 cm^3 of *M. incognita*. Each of the *Pochonia* strains was tested with chlamydospores as inoculum while strain 4 was also evaluated by amending the soil with a blended hyphae suspension. *Pochonia*-free soil served as a control treatment. The cups were arranged in a randomized complete block design with 5 treatments and six replications. The plants were fertilized with 2 g slow-release fertilizer (Osmocote 17-6-10, Scotts, Marysville, OH) and watered as needed. After 6 wk incubation, the plants were cut off at the soil level and dry weights of shoots were determined. The roots were removed from the soil, rinsed with water and blotted dry before weighing. Root galling was rated on a scale of 0-10 (Zeck, 1971). The roots were immersed in erioglaucine solution overnight and the stained egg masses of root-knot nematodes were counted (Omwega et al., 1988). The eggs from the roots were extracted and counted (Hussey and Barker, 1973). A soil subsample (50 cm^3) from each cup was incubated on a Baermann funnel for 5 days at 26° C. The collected J2 were counted under low power magnification (x30 - 40 magnification). The trial was repeated once with the tomato cultivar Red Gnome. Population and gall rating data were transformed $\log_{10}(x + 1)$ and by arcsin ($\sqrt{x}$), respectively before statistical analysis. The data were subjected to ANOVA and, if appropriate, mean separation by Fisher's LSD test ($P \leq 0.05$). Nontransformed data are shown.

Results

Pochonia chlamydosporia phylotyping*:* Molecular phylotyping of the *P. chlamydosporia* strains used in this study showed that they were all distinct (Fig. 1). Multiple assays based on analysis of ß-tubulin (panel A), rRNA

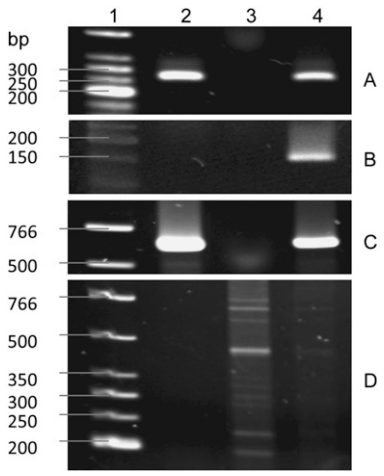

FIG. 1. Molecular phylotyping of *Pochonia chlamydosporia* strains used in this study. *Pochonia*-selective PCR-based assays targeted: A, ß-tubulin; B, rRNA SSU; C, rRNA ITS; and D, ERIC. Lanes were: Lane 1, Low molecular weight DNA ladder (New England Biolabs, Inc., Beverly, MA); Lane 2, *P. chlamydosporia* strain 1; Lane 3, *P. chlamydosporia* strain 3; Lane 4, *P. chlamydosporia* strain 4.

SSU (panel B), rRNA ITS (panel C), and ERIC (panel D) were employed. Strains 1 and 4 were similar for the ß-tubulin and rRNA ITS analyses. All three strains exhibited different ERIC patterns. Analysis of the ITS sequences showed that strains 3 and 4 had greater than 98% sequence identity (GenBank accession numbers: JQ433952-433954). Strain 4 was most similar to strain Vc10 (Hirsch et al., 2000), based on the ITS sequences and the ß-tubulin and rRNA ITS PCR analyses. The ITS sequence of strain 4 is 99% identical to *P. chlamydosporia* var. *chlamydosporia*. The rRNA SSU method differentiated strain 4 from the others.

Greenhouse trials: When chlamydospores were used as inoculum, all *P. chlamydosporia* strains significantly reduced the numbers of nematode egg masses when compared with the control plants (Tables 1and 2). Egg numbers were reduced to almost 50% by *P. chlamydosporia* strain 4. The numbers of J2 were equally reduced by both inocula forms of *P. chlamydosporia* strain 4. The inoculation method did not influence the efficacy; inocula based on hyphae or chlamydospores produced similar results. As for root galling index, only the chlamydospore inoculum of *P. chlamydosporia* strain 4 showed a consistent reduction effect. In Trial 1, shoot dry weights were greatest following inoculation with chlamydospores of strain 4. However, there were no differences in shoot dry weights among treatments in Trial 2.

DISCUSSION

Pathogen-suppressive soils are a potential source of effective biological control agents. In prior research, we identified a *M. incognita*-suppressive soil, and with a series of biocidal soil treatments, we demonstrated the biological nature of the suppressiveness (Loffredo et al., 2010). Subsequent molecular population-based studies identified negative associations between the amounts of *P. chlamydosporia* and *M. incognita* through the use of a fungal rRNA gene analysis (oligonucleotide fingerprinting of rRNA genes) and qPCR validation experiments (Bent et al., 2008). We isolated and identified three genetically different *P. chlamydosporia* strains from parasitized *M. incognita* eggs and demonstrated the nematode-suppressing abilities of the fungal strains in greenhouse trials. In particular *P. chlamydosporia* strain 4 reduced nematode eggs by more than 50% and negatively affected the number of J2 extracted from the soil. Since reducing or damaging nematode females and/or eggs will be reflected in lower J2 populations, these results suggest that this strain is a contributor to the suppressiveness. This is supported by other reports about *P. chlamydosporia* strains that showed good biocontrol efficacy in lab, greenhouse or field trials (Müller, 1982; De Leij and Kerry, 1991; Crump and Irving, 1992; De Leij et al., 1993; Hay and Skipp, 1993; Siddiqui and Mahmood, 1996).

The three *P. chlamydosporium* strains possessed varying abilities to impact *M. incognita* populations, a result that has been observed in other studies. In a study of *Pochonia* species from Iranian soils, *in vitro* pathogenicity tests showed the fungal strains infected root-knot nematode eggs at varying rates between 39% and 95% (Moosavi et al., 2010). Variation in the efficacies of *P. chlamydosporium* strains are likely caused by differing traits of the fungi, including their abilities to grow rapidly in the environment, to produce chlamydospores, and to possess effective virulence factors. Varying *P. chlamydosporia* population dynamics in soil have been previously reported. In a microplot experiment, *P. chlamydosporium* survived in loamy sand and sand but did not multiply 8 wk after infestation with root-knot nematodes (De Leij et al., 1993).

TABLE 1. Effects of different *Pochonia chlamydosporia* strains on tomato (cv. UC82) plant vigor and on *Meloidogyne incognita* populations.

Strain (inoculum)	number of egg masses	number of eggs	J2/50 cm^3 soil	root galling index	shoot dry weight (g)	root fresh weight (g)
P. chlamydosporia strain 1 (chlamydospores)	421 a	646,666 b	752 c	4.7 b	12.7 ab	43.9 a
P. chlamydosporia strain 3 (chlamydospores)	420 a	652,500 b	582 bc	4.8 b	14.4 bc	46.4 a
P. chlamydosporia strain 4 (chlamydospores)	400 a	383,333 a	417 b	4.0 a	15.7 c	45.4 a
P. chlamydosporia strain 4 (hyphae)	423 a	359,166 a	156 a	4.3 ab	13.3 ab	46.4 a
Non-inoculated control	581 b	635,000 b	683 c	4.8 b	14.0 b	50.2 a

* Values means of 6 replicates. Number with same letter are not significantly different according to Fisher's protected (LSD) test at $P \leq 0.05$.

TABLE 2. Effects of different *Pochonia chlamydosporia* strains on tomato (cv. Red Gnome) plant vigor and on *Meloidogyne incognita* populations.

Strain (inoculum)	number of egg masses	number of eggs	J2/50 cm^3 soil	root galling index	shoot dry weight (g)	root fresh weight (g)
P. chlamydosporia strain 1 (chlamydospores)	485 c	605,833 b	860 a	5.6 c	9.5 a	38.0 a
P. chlamydosporia strain 3 (chlamydospores)	406 ab	537,000 b	848 a	3.6 a	11.3 a	32.8 a
P. chlamydosporia strain 4 (chlamydospores)	362 a	391,000 a	814 a	4.1 ab	10.4 a	32.4 a
P. chlamydosporia strain 4 (hyphae)	496 c	605,833 b	869 a	6.0 c	10.7 a	36.6 a
Non-inoculated control	630 d	826,250 c	1288 b	5.7 c	10.2 a	36.1 a

* Values means of 6 replicates. Number with same letter are not significantly different according to Fisher's protected (LSD) test at $P \leq 0.05$.

Others found that after soil inoculation *P. chlamydosporia* var. *catenulate* populations increased over a 5-mon period (Atkins et al., 2003). Some studies have shown that a one-time application of *P. chlamydosporia* was able to reduce *M. javanica* population densities for at least 5-7 months compared to the control (Van Damme et al., 2005), while others have observed that the fungus persisted 2 (Crump, 2004) to 5 years (Atkins et al., 2003). Strains of *P. chlamydosporia* also differed in the amount of chlamydospores produced, and the amount decreased as the culture time increased (Kerry et al., 1986). Protease studies indicated host-related genetic variation among strains of *P. chlamydosporia* might contribute to the host preference at the infra species level (Morton et al., 2003b; Mauchline et al, 2004). However, although significant differences in the in vitro production of extracellular enzymes were shown among different strains, no relationship was found between enzyme activities and parasitic or saprotrophic growth of the fungal strains (Esteves et al., 2009). Co-evolution phenomena between hosts and pathogens is commonly accepted in evolutionary and ecological functional genomics studies (Feder and Mitchell-Olds, 2003). The Red Queen Hypothesis was proposed to explain the evolutionary arms race between host pathogen populations, whereby one needs to evolve continuously to avoid being overwhelmed by the other (Clay and Kover, 1996). Virulence genes have the tendency to be kept and modified in the pathogen during gene selection and inheritance, for it to be at a selective advantage against particular host genotypes. Further studies would be needed to determine whether the differences among our *Pochonia* strains were driven by Red Queen dynamics.

Our study is concordant with others that have demonstrated the utility of molecular phylotyping for differentiating *P. chlamydosporia* strains possessing varying biological control efficacies. The greenhouse trials showed that among the test strains, only strain 4 reduced egg masses, eggs, J2, and root galling (Tables 1 and 2). Based on the analysis of ITS sequence, as well as the ß-tubulin and ITS PCR assays (Fig. 1), *P. chlamydosporia* strain 4 was most similar to *P. chlamydosporia* var. *chlamydosporia* strain Vc10 (Hirsch et al., 2000). Strain Vc10 was originally isolated from *M. incognita* eggs. Among several *P. chlamydosporia* strains tested for their ability to multiply in the rhizosphere of root-knot infested tomato plants, Vc10 had the highest proliferation rate. Among 10 tested plant species, Vc10 populations increased the most in the rhizosphere of tomato (Bourne et al., 1994). Furthermore, strain Vc10 exhibited maximum *in vitro* proteolytic activity among 5 tested *P. chlamydosporia* strains, which is a trait that might play a key role in nutrient acquisition as well as in its biocontrol activity against nematodes by degrading the outer layer of nematode eggs (Segers et al., 1994). The phylotyping method developed in this study, which targets the small subunit ribosomal gene, was able to differentiate *P. chlamydosporia* strain 4 from the other strains, making it a potentially useful screening tool for similar strains with superior biological control attributes.

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