Isolation, Selection, and Efficacy of *Pochonia chlamydosporia* for Control of *Rotylenchulus reniformis* on Cotton

Kening Wang, Robert D. Riggs, and Devany Crippen

217 Plant Sciences, Department of Plant Pathology, University of Arkansas, Fayetteville 72701.
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ABSTRACT

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The reniform nematode, *Rotylenchulus reniformis*, is a serious threat to cotton (*Gossypium hirsutum*) production in the United States, causing an annual loss of about $80 million. The objective of this study was to isolate fungi from eggs of *R. reniformis* and select potential biocontrol agents for *R. reniformis* on cotton. We focused on the fungus *Pochonia chlamydosporia* because it suppresses root-knot and cyst nematodes and because preliminary data indicated that it was present in Arkansas cotton fields. Soil samples were collected from six cotton fields in Jefferson County, Arkansas. A total of 117 isolates of the nematophagous fungus *P. chlamydosporia* were obtained. In an in vitro test, 105 of the 117 isolates parasitized fewer than 15% of *R. reniformis* eggs, but 12 isolates parasitized between 16 and 35% of the eggs. These 12 isolates produced from 6.8×10^4 to 6.9×10^5 chlamydospores per gram of medium in vitro, and chlamydospore production was similar on rice grain and corn grain media. In two greenhouse experiments, a single application of isolate 37 (5,000 chlamydospores per gram of soil) significantly reduced the numbers of *R. reniformis* on cotton roots and in soil. The three isolates (37, 26, and 14) that parasitized the most eggs in vitro were also the most effective in suppressing numbers of *R. reniformis* and in increasing cotton growth in the greenhouse.

The reniform nematode, *Rotylenchulus reniformis* Linford & Oliveira, was first reported as a pest of cotton (*Gossypium hirsutum* L.) in Georgia in 1941 (21), and subsequently has been found in 11 cotton-producing states in the southern United States (18). Approximately 350,000 bales of cotton valued at about $80 million are considered lost to *R. reniformis* each year (15). *R. reniformis* is managed mainly by rotation and nematicides because no commercial cotton cultivar has resistance to *R. reniformis*. Concerns about the environmental hazards of using nematicides and the limited alternative crops for rotation with cotton have increased the need for alternative control methods. Biological control could be integrated with other methods to control this nematode, because it may shorten rotations and prolong the useful life of resistant soybean cultivars, which is often used in rotation with cotton. Biological control of the reniform nematode, however, has not been studied in detail. Although the isolation of egg-parasitic fungi from *R. reniformis* has not been reported, the efficacy of the egg-parasitic fungus *Paecilomyces lilacinus* in suppressing *R. reniformis* on tomato has been examined twice (19,24). In both cases, *Paecilomyces lilacinus* significantly reduced numbers of reniform nematodes.

The objectives of the current study were to isolate fungi from eggs of *R. reniformis* that might have potential for biological control, and to evaluate the biocontrol potential of *Pochonia chlamydosporia* obtained from *R. reniformis*. The current study focused on the egg-parasitic fungus *P. chlamydosporia*, because it was isolated from Arkansas cotton fields (K. Wang, *unpublished data*) and because the fungus had suppressed cyst and root-knot nematodes in previous studies (1,5–7,16). We also evaluated the ability of isolates to produce chlamydospores because chlamydospores require no additional energy source and therefore are useful for adding *P. chlamydosporia* to soil (5).

Corresponding author: R. D. Riggs; E-mail address: rdriggs@uark.edu

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MATERIALS AND METHODS

Isolation and selection. Soil samples were collected from six growing cotton fields in Jefferson County, Arkansas. The fields were selected because they were known to have damaging infestations of *R. reniformis* and because cotton had been the primary crop for a number of years. Although particle analyses were not obtained, these fields were in the Arkansas River bottoms and were sandy loam soils. In some cases, root-knot nematodes, *Meloidogyne incognita*, were found in the same fields.

In June 1999, 20 soil samples were collected from each field. Each soil sample was collected to a depth of 10 cm and adjacent to randomly selected cotton plants. Each of the 120 samples (about 500 g of soil per sample) was placed in a 10-cm-diameter clay pot and further infested by adding 3,000 *R. reniformis* eggs. Samples were collected only one time and the nematode eggs were added to the soil only one time. Three seeds of cotton cv. Sure Grow 125, which is susceptible to *R. reniformis*, were planted in each pot, and the pots were placed in a greenhouse maintained as near to 27°C as possible without air conditioning. Cotton plants were replaced in these pots at 3-month intervals for about 2 years. The continuous culture on cotton was intended to provide a continuous supply of host nematodes, which would presumably increase the numbers of biocontrol agents against *R. reniformis* and make them easier to detect.

Periodically over the 2-year period, cotton roots were collected from each pot, and a total of 20 egg masses of *R. reniformis* were randomly picked off the root surface with forceps and a stereomicroscope (×10 magnification). Each egg mass was rinsed in sterilized water three times, placed in a drop of sterilized water on a sterilized glass slide, and crushed to release the eggs, which were examined with a stereomicroscope (×20 magnification). Individual eggs containing hyphae were transferred with a fine glass pipette to petri dishes containing 1.5% water agar supplemented with 12.5 mg of chlortetracycline-HCl and 300 mg of streptomycin sulfate per liter. The dishes were incubated at 25°C for 3 days, and eggs were inspected daily (×20 magnification). Fungi

that grew from eggs were isolated by aseptic transfer of hyphal tips to potato dextrose agar (PDA) supplemented with 300 mg of streptomycin sulfate per liter. When the fungal colonies had grown sufficiently for identification, one isolate of *P. chlamydosporia* from each soil sample, if available (*P. chlamydosporia* isolates were not obtained from all samples), was selected for testing. Identification of *P. chlamydosporia* was based on the abundance and location of dictyochlamydospores, the conidiophores, and whether conidia were borne in chains or in heads (27). *P. chlamydosporia* was selected for extended study because of its frequent occurrence in samples from cotton fields (K. Wang, *unpublished data*), reports of its effectiveness against cyst and root-knot nematodes and because early laboratory studies indicated that it had some potential to suppress *R. reniformis* (25).

Fungal isolates that were identified as *P. chlamydosporia* were tested in the laboratory for pathogenicity to *R. reniformis* in vitro. A 1-cm-diameter plug was cut at the edge of a fungal colony growing on PDA or from PDA without fungus and transferred to a petri dish containing 1.5% water agar (one plug per dish). Egg masses of *R. reniformis* obtained from stock cultures were surface-disinfested with 0.01% $HgCl_2$ for about 1 min and rinsed in sterilized water three times. Thirty disinfested egg masses were placed on each PDA plug with or without fungus. Each fungal isolate and the control were replicated three times. Egg masses were incubated at 24 to 26°C. After 10 days, the egg masses from each replicate were crushed in a glass tissue grinder to release the eggs. The egg suspension was poured through nested 75- and 25-µm-pore sieves. The eggs retained on the 25-µm-pore sieve were washed into a conical centrifuge tube and concentrated by centrifugation. The percentage of parasitized eggs was determined by examining 100 arbitrarily selected eggs (per replicate, per isolate) with a compound microscope (×100 magnification). This test was performed once, and isolates that parasitized fewer than 15% of the eggs were discarded.

Chlamydospore production. Twelve isolates of *P. chlamydosporia* that had parasitized >15% of *R. reniformis* eggs in vitro were compared for chlamydospore production on sand/rice grain (1:1, vol/vol) medium. The rice grains were milled through a 1-mm-aperture mill. Coarse sand was used to improve aeration and prevent clogging. Approximately 80 cm^3 of milled rice grain, 80 cm^3 of coarse sand, and 100 ml of water were mixed in a 500-ml flask and autoclaved for 15 min. Two 1-cm-diameter plugs of isolates growing on PDA were added to each flask. Each fungal isolate was replicated four times. After 30 days at room temperature (24 to 26°C), the cultures were washed through a 50-µm-pore sieve with a fine water spray. The suspension of conidia, chlamydospores, and some hyphal fragments was poured through a 10-µm-pore sieve. The material retained on the 10-µm-pore sieve was mainly chlamydospores, which were washed into a beaker and counted with a hemacytometer. This experiment was performed once.

Because corn grain is more economical and readily available than rice grain, three isolates (14, 26, and 37) that showed the greatest control of the reniform nematode on cotton in the greenhouse tests were compared for chlamydospore production on rice grain culture medium and corn grain culture medium. The culture media were made, and the chlamydospores were collected and counted by the procedures described previously. This test was performed once.

Greenhouse tests. The 12 isolates of *P. chlamydosporia* were tested for control of reniform nematode on cotton in a greenhouse maintained as near 27°C as possible without air conditioning. This experiment was conducted as a completely randomized block design with five replications. Chlamydospores of *P. chlamydosporia* without medium, produced and collected according to the procedure described above, were mixed at a rate of 5,000 (6) or 0 chlamydospores per gram of soil into silt loam soil (55% silt, 42% sand, and 3% clay, pH 7.6, and 0.5% organic matter), which had been steam-treated for 3 h to kill most soil organisms. Approximately 680 g of infested or noninfested soil was placed in each 10-cm-diameter clay pot and was planted with three seeds of cotton cv. Sure Grow 125. After seedlings emerged, 3,000 eggs of *R. reniformis* were added to each pot. Fifty days (approximately two nematode generations) after adding eggs, numbers of *R. reniformis* in soil were determined by extracting eggs and vermiform nematodes in 100-cm^3 soil samples using wet-sieving and sucrose centrifugation (22). Eggs on roots were extracted by the NaOCl method (3). This experiment was repeated once. Cotton shoot height and fresh shoot and root weights were measured in the second trial but not in the first.

Statistical analyses were performed using JMP (version 5.0, SAS Institute, Cary, NC). For comparison of chlamydospore production of different isolates on different media, two-way analysis of variance (ANOVA) was performed to check for interaction between fungal isolates and media. For the two greenhouse trials, two-way ANOVA was also performed to check for an interaction between trial and fungal isolate. Treatment means were separated by least significant difference after ANOVA.

RESULTS

A total of 117 isolates of *P. chlamydosporia* were selected from those obtained from eggs of *R. reniformis*. Each isolate was from a different sample of the 120 soil samples collected from the six fields. Of those 117 isolates, only 12 parasitized >15% of the eggs of *R. reniformis* in vitro, and isolates 37, 14, and 26 parasitized the greatest percentage of eggs (Table 1).

The 12 isolates produced different numbers of chlamydospores on rice grain medium, with isolate 26 producing the most and isolate 34 the least (Table 1). In a separate test with the three most virulent isolates from the in vitro experiment, chlamydospore production was similar on rice medium and on corn medium ($P > 0.05$); averaged over both media, isolates 14, 26, and 37 produced 1×10^6, 3.8×10^5, and 2.5×10^5 chlamydospores per gram of medium, respectively.

Nematode suppression by the fungal isolates differed between greenhouse trials, i.e., the interaction between trial and isolate was significant ($P < 0.05$). Therefore, the results of the two greenhouse trials are reported separately (Table 2). In greenhouse trial 1, 11 of the 12 isolates reduced ($P < 0.05$) the nematode numbers on cotton roots, but only isolates 26 and 37 reduced nematode numbers in soil. In trial 2, all isolates except isolate 79 reduced

TABLE 1. In vitro parasitism and chlamydospore production by 12 isolates of *Pochonia chlamydosporia*[y]

Isolate	Parasitized eggs (%)	Chlamydospores per g of rice medium (log_{10})[z]
37	35.3 ± 3.1	5.79 ± 0.10
14	32.7 ± 4.5	5.80 ± 0.16
26	29.3 ± 1.2	5.84 ± 0.18
29	25.7 ± 3.2	5.63 ± 0.04
34	23.3 ± 6.2	4.83 ± 0.21
36	23.0 ± 2.3	5.60 ± 0.19
79	22.3 ± 6.6	5.35 ± 0.01
2	22.3 ± 4.8	5.40 ± 0.16
78	21.7 ± 1.5	5.38 ± 0.17
27	20.7 ± 2.8	5.30 ± 0.16
28	20.3 ± 2.9	5.67 ± 0.09
33	15.7 ± 3.0	5.50 ± 0.08
Control	2.3 ± 0.9	...

[y] Data from isolates that parasitized <15% of the eggs are not shown in this table. Values are the means ± one standard error of three replications. Thirty egg masses were placed on a 1-cm-diameter plug of potato dextrose agar that had been cut from the edge of a fungal colony. The plug was placed on water agar, and parasitism of eggs was assessed after 10 days at 24 to 26°C.

[z] Chlamydospores were collected and counted after the isolates had grown for 30 days at 24 to 26°C on autoclaved rice grain medium.

($P < 0.05$) nematode numbers in soil, but only isolate 37 reduced ($P < 0.05$) the nematode numbers on roots. In trial 1, the greatest reduction in the nematode numbers on the cotton roots was 77% by isolate 26, and the nematode numbers in soil were reduced 64% by isolate 37. In trial 2, the greatest reduction of the nematode numbers on the cotton roots was 56% by isolate 37, and the greatest reduction of nematode numbers in soil was 73% by isolate 26 (Table 2).

Plants were not measured in trial 1. Compared with the control in trial 2, shoot lengths were increased ($P < 0.05$) by isolates 2 (28%), 14 (36%), 26 (46%), 29 (37%), 33 (35%), 34 (27%), and 37 (52%); and shoot weights were increased by isolates 2 (65%), 14 (90%), 26 (111%), 33 (77%), 34 (64%), 37 (89%), and 79 (59%). Root mass was increased ($P < 0.05$) in soil treated with isolates 14 (31%), 34 (21%), and 37 (37%).

DISCUSSION

P. chlamydosporia is a facultative nematode parasite frequently isolated from cyst nematodes and root-knot nematodes (4,5,7,9, 10,13,16,17,26), but to our knowledge it has not been previously isolated from the reniform nematode *R. reniformis*. Suppression of cyst nematodes and root-knot nematodes by this fungus in greenhouse and microplot trials has been reported (1,5–7,16). Isolates of *P. chlamydosporia*, even those collected from the same soil, differ greatly in their parasitic capabilities in vitro and in their efficacy in greenhouse tests (8,11). In the current study, results of two repeated greenhouse experiments demonstrated that the selected isolates of *P. chlamydosporia* reduced the *R. reniformis* numbers on cotton roots or in soil after a single application of 5,000 chlamydospores per gram of soil. The 12 isolates, however, were not equally effective and isolates 14, 26, and 37 were superior in soil. Selecting and testing isolates in the laboratory probably only served to reduce the number of isolates that needed to be tested in the greenhouse, because isolates that perform well in vitro may not be effective in soil (12).

The chlamydospore is the preferred type of inoculum because it does not need an additional nutrient source (5), but chlamydospores are not produced in liquid culture (23). If conidia or hyphal fragments are used as inoculum, an energy base is required (14). Addition of an energy base increases competition from the residual microflora, which may reduce the survival and multiplication of *P. chlamydosporia* (2) and also would increase the cost of treatment. Chlamydospores are usually mass-produced on solid substrates, such as a barley-sand medium (5). Because barley is not readily available in the southern United States, rice and corn were used in this study. Chlamydospore production on rice grain medium was similar to that on corn grain medium. Therefore, corn, which is more economical than rice, could be used for mass production of chlamydospores.

This study demonstrates that *P. chlamydosporia* can parasitize eggs of the reniform nematode in vitro and can suppress numbers of *R. reniformis* in the greenhouse. The integration of *P. chlamydosporia* with other biocontrol agents or other management practices could make an excellent control measure for the reniform nematode. In a previous report, combining *P. chlamydosporia* with aldicarb at a rate of 2.8 kg/ha did not affect the activity of the fungus and gave better control of *M. hapla* than treatment with aldicarb or *P. chlamydosporia* alone (6). Also, *Heterodera cajani* on pigeon pea was greatly reduced when *P. chlamydosporia*, *Trichoderma harzianum*, and *Glomus mosseae* were used together (20). The reliability of such approaches needs extensive testing.

TABLE 2. Greenhouse experiment: total number of eggs and vermiform stages of *Rotylenchulus reniformis* in soil infested with *Pochonia chlamydosporia* and planted with cotton[y]

P. chlamydosporia isolates	Total number of eggs and vermiform stages per root system	Total number of eggs and vermiform stages in soil[z]
Trial 1		
2	3,994 bc	6,840 abc
14	2,957 bc	5,040 abc
26	2,055 c	3,600 bc
27	1,245 bc	8,640 abc
28	2,344 bc	9,240 abc
29	1,872 bc	6,120 abc
33	2,332 bc	6,660 abc
34	4,059 bc	7,560 abc
36	1,188 bc	6,120 abc
37	3,092 bc	3,540 c
78	3,087 bc	9,360 ab
79	3,744 ab	9,361 ab
Control	6,654 a	9,900 a
Trial 2		
2	6,576 ab	21,060 bcd
14	4,569 bcd	12,780 cd
26	5,150 abcd	10,620 d
27	3,324 abcd	9,360 d
28	5,125 abc	19,980 bcd
29	5,667 abc	20,520 bcd
33	2,217 cd	27,360 abcd
34	2,783 cd	28,800 abc
36	1,646 cd	16,560 bcd
37	2,603 d	14,760 bcd
78	6,860 a	14,760 bcd
79	3,885 abcd	31,860 ab
Control	4,283 abcd	39,240 a

[y] Cotton plants were grown in a steamed silt loam soil infested with *R. reniformis* and *P. chlamydosporia* (0 or 5,000 chlamydospores per g of soil). After 50 days in the greenhouse, nematodes were extracted from roots and soil. Values are the means ± one standard error of five replicate plots. For each trial, values within columns followed by the same letter are not significantly different at $P < 0.05$.

[z] Each pot contained 680 g of soil.

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