Suppression of the Plant-Parasitic Nematode *Heterodera schachtii* by the Fungus *Dactylella oviparasitica*

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ABSTRACT

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This study examined the role of the fungi *Dactylella oviparasitica* and *Fusarium oxysporum* in the beet-cyst nematode (*Heterodera schachtii*) suppressiveness exhibited by a southern Californian soil. In prior research, the abundance of *D. oviparasitica* rRNA genes positively correlated with high levels of suppressiveness, whereas the abundance of *F. oxysporum* rRNA genes positively correlated with minimal to moderate

levels of suppressiveness. In this report, both fungi were added to fumigation-induced nonsuppressive soil, planted with Swiss chard, and infested with *H. schachtii* juveniles. After two nematode generations, *D. oviparasitica* strain 50 reduced the population densities of *H. schachtii* eggs and juveniles to those in the suppressive soil and *H. schachtii* cysts to levels lower than in the suppressive soil. *F. oxysporum* did not significantly reduce *H. schachtii* populations. These results suggest that *D. oviparasitica* strain 50 plays a major role in the suppression of *H. schachtii* population development in this southern Californian soil.

Prior research identified a southern California soil that suppresses the population development of the beet-cyst nematode *Heterodera schachtii*. The biological nature of this suppressiveness was demonstrated by preplant treatments with various fumigants (metam-sodium, methyl bromide, and methyl iodide) or aerated steam, which diminished the suppressiveness to undetectable levels in greenhouse and field studies (13,14). Another demonstration of its biological nature was achieved by amendment of 1% suppressive soil into a fumigation-induced, nonsuppressive field site (14). In addition, treatment of the soil at 55°C for 30 min also removed the *H. schachtii* suppressiveness (15). Even more important for our investigations, cysts that developed in the suppressive soil transferred the suppressiveness to fumigation-induced, nonsuppressive soil (15). This demonstrated that the suppressive factor or factors were in the cysts, making subsequent investigations to identify these factors a more feasible task.

To identify fungi involved in this *H. schachtii* suppressiveness, we employed a three-phased approach: (i) identification of microorganisms whose population levels correlate with the suppressiveness through extensive rRNA gene analysis, (ii) validation of the population trends with quantitative polymerase chain reaction (qPCR) analyses, and (iii) reintroduction of the microorganisms into the environment to assess their functions. In the first phase of the project, oligonucleotide fingerprinting of ribosomal RNA genes (OFRG) (2,11,12) was used to identify fungal rRNA genes found in *H. schachtii* cysts isolated from soils possessing various levels of suppressiveness (16). Five soil treatments with various levels of suppressiveness, generated by mixing different amounts of suppressive and fumigation-induced nonsuppressive soil, were planted with *Brassica juncea* and infested with second-stage juveniles (J2) of *H. schachtii*. After two nematode generations, rRNA gene sequences that were more abundant in cysts isolated from the highly suppressive soils than in cysts from the less-suppres-

sive soils were considered candidate sequences. In phase 2, sequence-selective qPCR was used to determine the amounts of the candidate sequences in cysts from soils possessing various levels of suppressiveness, which were produced by mixing various quantities of suppressive and fumigation-induced nonsuppressive soil and by biocidal treatments. Phases 1 and 2 of this project led to the identification of genes with high sequence identity to *Dactylella oviparasitica* and *Fusarium oxysporum* rRNA genes, whose levels consistently and positively correlated with high and minimal to moderate levels of *H. schachtii* suppressiveness, respectively (16).

In this report, we describe the results from phase 3 of this project. Strains of *D. oviparasitica* and *F. oxysporum* were isolated from *H. schachtii* cysts collected from the suppressive soil and added to fumigation-induced, nonsuppressive soil to determine whether they can produce *H. schachtii* suppressiveness.

MATERIALS AND METHODS

Extraction of *H. schachtii* cysts from soil. Soil from *H. schachtii*-suppressive soil, field 9E located at the University of California–Riverside Agricultural Research Station (13), was air dried for 2 days at room temperature to facilitate cyst extraction. *H. schachtii* cysts were extracted as previously described (16).

Fungal isolation from *H. schachtii* cysts. Individual cysts were placed in 1.5-ml microcentrifuge tubes and washed by adding 100 µl of sterile distilled water, mixing with a vortex mixer for 5 s, and removing the water by pipetting. Cysts were surface sterilized by adding 100 µl of 0.6% NaOCl, mixing with a vortex mixer for 90 s, and then removing the bleach solution by pipetting. Individual cysts were placed in separate microcentrifuge tubes containing 100 µl of sterile distilled water and crushed with a pipette tip. Crushed cyst mixtures were spread with a sterile glass rod on water agar containing rifampicin (10 µg/ml). After 24 h at room temperature, individual colonies were examined as described below.

Fungal identification and the fungal strains used in this study. Portions of each fungal colony were collected with a sterile

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toothpick and placed in 1.5-ml microcentrifuge tubes containing 10 μ l of sterile water. Mycelia were crushed with a pipette tip and homogenized by mixing with a vortex mixer for 30 s. The resulting mixtures were centrifuged for 1 min at 14,000 $\times$ g. Supernatants from individual colonies were subjected to *F. oxysporum*- and *D. oviparasitica*-selective PCR analysis. Amplification reactions (10 μ l total volume) were performed in glass capillary tubes containing the following reagents: 50 mM Tris (pH 8.3), bovine serum albumen at 500 μ g/ml, 2.5 mM MgCl₂, 250 μ M each dNTP, 400 nM each primer, 1 μ l of supernatant from the fungal colonies, and 0.5 units of *Taq* DNA polymerase. For the *F. oxysporum*-selective PCR, which targeted the small-subunit (SSU) rRNA gene, the primers were FusF, GTATTGCTTTGGCAGTA, and FusR, TTTCGGACAAGGAAGTA, and the cycling parameters were 94°C for 2 min; 35 cycles of 94°C for 10 s, 55°C for 15 s, and 72°C for 20 s; followed by 72°C for 2 min (16). *D. oviparasitica*-selective PCR was performed with one of two different primer sets. For the *D. oviparasitica*-selective PCR assay targeting the SSU rRNA gene, the primers were DactF, CAGGTCCA-GACACATTA, and DactR, CTTTCGGCCAAGGATT, and the cycling parameters were 94°C for 2 min; 35 cycles of 94°C for 10 s, 60°C for 20 s, and 72°C for 30 s; followed by 72°C for 2 min (16). For the *D. oviparasitica*-selective PCR assay targeting the internal transcribed spacer (ITS) region, the primers were DactITSF, AAAACCCCCAAGTTGCTTC, and DactITSR, CCA-CTGTGCTGTTACAACC, and the cycling parameters were 94°C for 2 min; 35 cycles of 94°C for 15 s, 64°C for 15 s, and 72°C for 20 s; followed by 72°C for 2 min. Thermal cycling was performed using a 1002 RapidCycler (Idaho Technology Inc., Salt Lake City, UT). Nucleotide sequences of the amplified PCR products were obtained using the PRISM BigDye Terminators Cycle Sequencing Kit (version 3.0; ABI, Foster City, CA) and a 3100 Genetic Analyzer (ABI). Colonies whose SSU rRNA gene sequences had 100% identity to the *F. oxysporum* and *D. oviparasitica* rRNA genes identified in a previous study (16) were saved for further study and subcultured on potato dextrose agar (PDA). Two of these colonies, *F. oxysporum* strain 471 and *D. oviparasitica* strain 50, were used in this study. *D. oviparasitica* strain Stirling (9), which was obtained from the University of California–Riverside Culture Collection of Nematode-Destroying Fungi (J. O. Becker, Curator), also was used in this study.

Fungus production. Petri plates (10 cm in diameter) containing PDA were inoculated with individual 5-mm-diameter agar plugs containing one of the three following fungi: *F. oxysporum* strain 471, *D. oviparasitica* strain 50, or *D. oviparasitica* strain Stirling. These cultures were grown at room temperature for approximately 10 (*F. oxysporum*) and 21 (*D. oviparasitica* strains) days. For each plate, after the incubation period, the fungal culture and PDA medium were transferred to a Sunbeam 6 Speed Blender (Model 4142; Sunbeam Products Inc., Boca Raton, FL) containing 50 ml of sterile water. The components were blended for 30 s using the “blend” setting. Then, 0.5 ml of each mixture was used to estimate the inoculum concentrations by determining the number of CFU from a dilution series. The remaining portions of the fungal mixtures were added to soil (within 1 h of blending the fungi) as described below.

***D. oviparasitica* and *F. oxysporum* greenhouse trials.** Two replicate greenhouse trials were conducted to evaluate the ability of the fungal strains to suppress *H. schachtii* when added to non-suppressive soil. The soil used in these studies was collected from the upper 10 cm of field 9E. The 9E soil is a Hanford fine sandy loam (60.9% sand, 29.6% silt, and 9.5% clay; pH 8) (13). After large aggregates were removed from the soil using a sieve with 6-mm openings, a portion ($\approx$ 18,000 cm³) of the sieved soil was fumigated with 2 ml of methyl iodide (100%; DSM Fine Chemicals, Saddle Brook, NJ) in 19-liter polyethylene buckets for 4 days (1). Three weeks later, fumigated (nonsuppressive) and nonfumigated (suppressive) 9E soils each were mixed 10:1 with

silica sand to facilitate water drainage during the greenhouse trials. The amount of soil used per pot ($\approx$ 1,600 cm³) was standardized by filling 15-cm-diameter pots to 2.5 cm below the top. Fungal suspensions (described above) and the fumigation-induced nonsuppressive soil from each pot were combined in plastic bags, mixed by inverting the plastic bags, and returned to the pots. Two control soils (nonsuppressive and suppressive), which did not receive fungal inoculum, also were added to pots.

The treatments consisted of (i) *F. oxysporum* strain 471, (ii) *D. oviparasitica* strain 50, (iii) *D. oviparasitica* strain Stirling, (iv) 9E soil (positive control suppressive soil), (v) fumigated 9E soil (negative control nonsuppressive soil), and (vi) simultaneous addition of *D. oviparasitica* strain 50 and *H. schachtii* inoculum. For trial 1, fungal CFU/pot were *D. oviparasitica* strain 50 (treatments 2 and 6), 1.1×10^8 ; *F. oxysporum* strain 471 (treatment 1), 1.4×10^{10} ; and *D. oviparasitica* strain Stirling (treatment 3), 2.9×10^7 . For trial 2, fungal CFU/pot were *D. oviparasitica* strain 50 (treatments 2 and 6), 1.0×10^8 ; *F. oxysporum* strain 471 (treatment 1), 1.3×10^{10} ; and *D. oviparasitica* strain Stirling (treatment 3), 2.9×10^7 . All treatments were infested with J2 of *H. schachtii* 4 weeks after the addition of the fungal inoculum except for treatment (vi), where the fungus was added simultaneously with the *H. schachtii* inoculum in three holes ($\approx$ 3.0 cm deep and 1.5 cm wide) in the surface of the soil.

After fungal suspensions were added to the soil, each pot was sown with three seed of Swiss chard (*Beta vulgaris*; Lockhart Seeds, Stockton, CA). All pots were placed in a greenhouse under natural light at $23 \pm 3^\circ\text{C}$ in a randomized complete block design with six replicates. Plants were watered daily with tap water. Soil temperature was monitored using HOBO Temp devices (Onset Computer Corporation, Bourne, MA) buried in the soil. After emergence, the seedlings were thinned to one per pot and fertilized with 6 g of slow-release fertilizer (Sierra 17-6-10 plus Minors; Scotts-Sierra Horticultural Products Company, Marysville, OH). Four weeks after seeding, each pot was infested with *H. schachtii* by pipetting an aqueous suspension of 10,000 freshly hatched J2 into three holes ($\approx$ 3.0 cm deep and 1.5 cm wide) in the surface of the soil; the juvenile inoculum was collected and enumerated using a zinc chloride hatching protocol as previously described (7). Starting 3 weeks after infestation, each plant was fertilized with 50 ml of a nutrient solution (Miracle Gro 15-30-15; Scotts Miracle-Gro Products, Marysville, OH) every 2 weeks. Eleven weeks after infestation ($\approx$ 1,185 degree days and therefore at least two nematode generations) (3), plant tops were cut off at soil level and roots were removed from soil. Fresh and dry weights of shoots and roots were measured. Cysts were extracted from 350-g subsamples of the soil using the modified Fenwick flotation can method (4). Cysts were counted and then broken in a tissue homogenizer for egg counting. Egg parasitism was assessed by using a dissecting microscope to examine 100 randomly selected eggs per sample; these assessments were performed for trial 1 only. Eggs were considered parasitized if they appeared disrupted or disintegrated; nonparasitized eggs had a clear outline of a developing juvenile in the eggshell.

Data analysis. Cysts, eggs, and J2 were counted and the numbers were log ($x + 1$) transformed to normalize the data. The transformed cyst and egg data and the plant mass data were subjected to analysis of variance and Fisher's least significant difference tests (SAS Institute, Cary, NC).

Nucleotide sequence data. Partial SSU rRNA gene and ITS sequences for the strains used in this study were determined: *D. oviparasitica* strain 50 SSU (AY970524) and ITS (AY970525); *F. oxysporum* strain 471 SSU (AY970526) and ITS (AY970527).

RESULTS

Isolation of *D. oviparasitica* and *F. oxysporum*. *D. oviparasitica* strain 50 and *F. oxysporum* strain 471 were obtained from

cysts extracted from the suppressive 9E soil. These strains had 100% sequence identity to SSU rRNA genes whose levels previously were shown to correlate with *H. schachtii* suppressiveness (16); the lengths of the sequences compared in these analyses were 720 and 718 nucleotides for *D. oviparasitica* and *F. oxysporum*, respectively.

***D. oviparasitica* and *F. oxysporum* greenhouse trials.** Two replicate greenhouse trials were conducted to evaluate the ability of the fungal strains to suppress *H. schachtii* when added to non-suppressive soil. As the results from both trials were similar, the data were combined for presentation (Table 1). *D. oviparasitica* strain 50 reduced *H. schachtii* population levels to those found in the suppressive 9E soil or lower. Specifically, cyst numbers were smallest in soil inoculated with *D. oviparasitica* strain 50 (treatment 2). Nematode egg numbers were smallest in soil inoculated with *D. oviparasitica* strain 50 and the suppressive soil (treatments 2 and 4). The lowest numbers of nematode juveniles were produced by *D. oviparasitica* strain 50 and the suppressive soil (treatments 2, 4, and 6). The highest percentage of egg parasitism occurred in treatments containing *D. oviparasitica* strain 50 and the suppressive soil (treatments 2, 4, and 6). *F. oxysporum* did not significantly reduce *H. schachtii* populations (treatment 1). *D. oviparasitica* strain Stirling (treatment 3) led to an increase in *H. schachtii* eggs but a decrease in J2 juveniles compared with the nonsuppressive soil. No differences were observed in the plant yield data except that the 9E soil consistently produced the lowest yields.

DISCUSSION

This study examined the role of *D. oviparasitica* and *F. oxysporum* in the *H. schachtii* suppressiveness exhibited by a southern Californian soil. Prior experimentation showed that *D. oviparasitica* and *F. oxysporum* rRNA gene levels positively correlated with high and minimal to moderate levels of suppressiveness, respectively (16). In the current study, *D. oviparasitica* strain 50 reduced the numbers of *H. schachtii* cysts, eggs, and juveniles to levels observed in the naturally suppressive soil or lower, whereas *F. oxysporum* did not significantly reduce *H. schachtii* populations. These results suggest that *D. oviparasitica* strain 50 plays a major role in the suppression of *H. schachtii* population development in this soil.

Several features of the experimental approach likely contributed to the successful outcome of these studies. The first was the culture-independent nature of the method used to identify microorganisms. Although our qPCR data showed that *D. oviparasitica* rRNA gene levels were ≈ 100 times greater than rRNA gene levels from the other major cyst fungi (16), obtaining a pure culture of *D. oviparasitica* was a considerable challenge because it represented a minor fraction of the fungal strains obtained from *H. schachtii* cysts, likely due to its relatively slow growth on agar

media. The second important feature of this approach was the attempt to identify microorganisms whose population levels correlated with the suppressiveness in soil-based experiments. This was accomplished by analyzing fungal rRNA genes from *H. schachtii* cysts obtained from a series of soil with various levels of suppressiveness (16).

Several characteristics of *D. oviparasitica* may contribute to its ability to suppress *H. schachtii* populations. *D. oviparasitica* has been described previously as an efficient egg parasite with a broad host range including several *Meloidogyne* spp., an *Acrobeloides* sp., *H. schachtii*, and *Tylenchulus semipenetrans* (10). The data presented in this report support this mode of action, because the 9E soil treatment along with all three *D. oviparasitica* strain 50 treatments generated the highest levels of egg parasitism. Certain strains of *D. oviparasitica* can produce networks and knobs in the presence of nematodes (6), suggesting that this fungus could be contributing to the suppressiveness through a predation mechanism. In addition, *D. oviparasitica* sporulates on and is frequently found near plant roots (8,10). These characteristics, along with its apparent ability to grow saprophytically on roots (8), may contribute to its long-term survival in soil and, therefore, its ability to produce the stable *H. schachtii* suppressiveness exhibited by the 9E field soil (13).

This study also provided new information concerning the potential use of *D. oviparasitica* as a biological control agent and the nature of the *H. schachtii* suppressiveness in the 9E field soil. Adding *D. oviparasitica* strain 50 to the nonsuppressive soil before *H. schachtii* infestation led to a significant decrease in *H. schachtii* cyst and egg populations compared with the simultaneous addition of *D. oviparasitica* strain 50 and *H. schachtii*; therefore, establishment of sufficient population levels of this fungus in soil likely will be important for optimal nematode suppression. In addition, *D. oviparasitica* strain Stirling, which was isolated from *Meloidogyne* eggs, was not nearly as effective in reducing *H. schachtii* populations as *D. oviparasitica* strain 50. This could have resulted from differences in host specificity between the strains or from the possible detrimental affects of long-term storage on the Stirling strain, which originally was isolated 28 years before this study (9).

Although *D. oviparasitica* strain 50 reduced *H. schachtii* numbers to those found in the suppressive 9E soil, differences in plant growth were not consistently observed. This likely was caused by several factors, including the manner in which the soils were infested with *H. schachtii* and the mode of action in which *D. oviparasitica* suppresses *H. schachtii*. First, in the greenhouse trials, all soils were infested with a large number of *H. schachtii* juveniles approximately 4 weeks after Swiss chard seed were sown. Although this increases treatment effects on *H. schachtii* numbers at the end of the trials, it also produces high nematode pressure early in the life of the plant, which may negate some of the beneficial effects of the added fungi in short-term greenhouse

TABLE 1. *Heterodera schachtii* numbers and plant mass as affected by *Dactylella oviparasitica* and *Fusarium oxysporum* in greenhouse trials^y

Treatment	Weight (g)				Number in 350 g of soil			
	Fresh shoot	Fresh root	Dry shoot	Dry root	Cysts	Eggs	J2	Parasitized eggs (%)
1. <i>F. oxysporum</i> strain 471	297 a	39 a	30 a	18 a	122 bc	9,042 b	6,300 ab	14.7 bc
2. <i>D. oviparasitica</i> strain 50	300 a	38 a	28 a	20 a	73 d	1,808 c	394 d	27.2 a
3. <i>D. oviparasitica</i> strain Stirling	295 a	35 a	27 a	17 a	194 a	22,958 a	9,727 a	12.7 c
4. 9E soil (suppressive)	81 b	4 b	9 b	1 b	116 bc	1,892 c	403 d	21.2 ab
5. Fumigated 9E (nonsuppressive)	290 a	40 a	26 a	19 a	152 ab	10,525 b	11,603 bc	3.5 d
6. <i>D. oviparasitica</i> strain 50 + J2 ^z	323 a	39 a	32 a	19 a	99 c	4,517 b	1,483 cd	20.8 abc

^y Fungi were added to fumigation-induced nonsuppressive soil (treatments 1, 2, 3, and 6). Treatments 4 (suppressive soil control) and 5 (nonsuppressive soil control) did not receive fungal inoculum. All treatments were planted with Swiss chard, infested with second-stage juveniles (J2) of *H. schachtii*, and assessed after two nematode generations. Results from two trials were similar and were combined. Values are the means of 12 replicate pots ($\approx 1,600$ cm³ of soil/pot) except for egg parasitism values, which are the means of six replicates (parasitism was not assessed in the second trial). Numbers of cysts, eggs, and J2 were log (x + 1) transformed to normalize the data. Means with the same letter are not significantly different ($P = 0.05$).

^z Simultaneous addition.

trials. Second, because we postulate that *D. oviparasitica* contributes to the suppressiveness mainly by egg parasitism, the fungus is likely to have little or no impact on the juvenile *H. schachtii* inoculum, which could lead to uniform plant damage across all treatments. This is supported by earlier trials in which no differences were observed between the numbers of J2 detected within the roots of Swiss chard grown in the suppressive 9E soil and a conducive soil 75 degree-days after soil infestation (5). Evidence supporting the egg parasitism mode of action comes from prior microscopic experimentation with another *D. oviparasitica* strain along with observations that *H. schachtii* numbers do not decrease in the suppressive 9E soil until the second nematode generation (5,10,14). The poor plant growth in suppressive 9E soil likely was due to the presence of other pathogens or deleterious microorganisms, because this was the only treatment where the plants were grown in nonfumigated soil.

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