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Detection of the Nematophagous Fungus *Hirsutella rhossiliensis* in California Sugarbeet Fields

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The nematophagous fungus, *Hirsutella rhossiliensis*, was detected in 10 of 21 sugarbeet fields in California. Fewer than four parasitized nematodes per 100 cm^3 soil were found in any field, and all parasitized specimens were the cyst nematode, *Heterodera schachtii*. Detection of the fungus was enhanced if soil samples were periodically inoculated with large numbers of healthy juveniles of *H. schachtii* prior to assay for the fungus. Detection efficiency was measured by assaying soil seeded with known numbers of parasitized *H. schachtii*. Detection was largely unaffected by soil type but declined from 63% at Time 0 to 17% after 2 days at 22°C. Thus, the failure to detect *H. rhossiliensis* in 11 fields could reflect the absence of the fungus or limitations of the assay. The results suggest that a one-time release of *H. rhossiliensis* with the intention of establishing the fungus in sugarbeet fields is not a viable alternative for implementation of biological control of *H. schachtii*.

KEY WORDS: ***Arthrobotrys dactyloides; Heterodera schachtii; Hirsutella rhossiliensis;* cyst nematode; biological control; nematophagous fungus.**

INTRODUCTION

The sugarbeet cyst nematode, *Heterodera schachtii* Schmidt, can substantially reduce the yield of sugarbeets in the United States, Europe, and elsewhere (Cooke and Thomason, 1978; Roberts and Thomason, 1981; Steele, 1984). In California, the nematode is a widespread and serious pest and is controlled primarily through crop rotation (Roberts and Thomason, 1981). Second stage juveniles (J2) of *H. schachtii* hatch from eggs in cysts in soil. They move through soil pores, penetrate host roots, and develop into adult males or females in about 1 month at 20°C. The females are nonmotile but their swollen bodies rupture the root surface where fertilization by motile males occurs. The females become egg-filled (about 200–500 eggs per female), die, and are passively moved into the soil when the root or soil is disturbed. The dead female's body, which protects the eggs until they hatch, is called a cyst (Roberts and Thomason, 1981).

H. schachtii is a host for the nematophagous fungus, *Hirsutella rhossiliensis* Minter and Brady (= *Hirsutella heteroderae* Sturhan and Schneider) (Jaffee and Zehr, 1982; Jaffee and Muldoon, 1989). This hyphomycete produces nonmotile spores on flask-shaped structures called phialides (Sturhan and Schneider, 1980). Phialides are produced on hyphae that radiate into the soil from the host cadaver. The external network of hyphae, phialides, and spores is important for two reasons. First, it provides for local distribution of spores. Second, spores fail to adhere to nematodes if the network is destroyed by soil disturbance (McInnis and Jaffee, 1989). Spores of *H. rhossiliensis* adhere to passing vermiform nematodes, detach from the phialide, and infect and kill the host within several days (Jaffee *et al.*, 1990). The fungus can be grown in culture but has little competitive saprophytic activity (Jaffee and Zehr, 1985) and is probably an obligate parasite of nematodes in nature.

Most research on biological control of *H. schachtii* and other cyst nematodes has focused on parasites of females and eggs (e.g., Crump and Kerry, 1987; Nigh *et al.*, 1980). However, *H. rhossiliensis* is naturally present in about 25% of the sugarbeet fields in Germany and may contribute to suppression of *H. schachtii* (Muller, 1984, 1986; Juhl, 1985).

In addition to being associated with cyst nematodes in Europe, *H. rhossiliensis* is frequently associated with the ring nematode, *Criconemella xenoplax* Raski (Luc and Raski), in stone fruit orchards in the southeastern United States (Jaffee and Zehr, 1982) and California (Jaffee *et al.*, 1988). High numbers and percentages of *H. rhossiliensis*-parasitized nematodes have been found in some California peach orchards (Jaffee *et al.*, 1989). To help understand the suppressive potential of these natural infestations, Jaffee and Muldoon (1989) added healthy J2 of *H. schachtii* to soil which naturally contained the fungus but which contained no cyst nematodes; 40–60% of the J2 were infected by the fungus

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TABLE 1

Soil Characteristics and Levels of *Heterodera schachtii* (Hs) and *Hirsutella rhossiliensis* in 21 California Sugarbeet Fields

					Before addition of Hs[a]				After addition of Hs[a]		
Field	County	Texture	pH	% Organic matter	Eggs/100 cm³ soil	J2/100 cm³ soil	Infected J2/100 cm³ soil[b]	% J2 infected	J2/100 cm³ soil	Infected J2/100 cm³ soil[b]	% J2 infected
1	San Joaquin	Sandy loam	7.6	3.7	0	0	—	—	1495 ± 358	0 ± 0	0 ± 0
2	San Joaquin	Loam	7.2	6.5	934	290	1	<1	2270 ± 282	283 ± 370	13 ± 19
3	San Joaquin	Clay loam	6.1	2.2	0	0	—	—	305 ± 96	1 ± 1	<1
4	San Joaquin	Clay loam	7.4	2.0	636	80	0	0	515 ± 146	4 ± 7	<1
5	San Joaquin	Silt loam	7.5	2.0	3488	100	2	2	475 ± 222	1 ± 1	<1
6	Solano	Silty clay loam	7.0	1.5	3008	210	3	1	1207 ± 297	6 ± 9	<1
7	Solano	Silty clay loam	7.0	1.7	2700	180	3	1	1310 ± 344	967 ± 494	71 ± 17
8	Solano	Clay loam	6.9	2.1	1431	20	1	3	1040 ± 658	595 ± 538	52 ± 22
9	Solano	Silty clay loam	6.8	2.5	154	0	—	—	867 ± 110	4 ± 4	<1
10	Solano	Silty clay	6.6	2.0	94	10	1	6	227 ± 107	121 ± 81	49 ± 23
11	Solano	Silty clay	6.6	2.2	171	3	3	100	383 ± 32	91 ± 10	24 ± 5
12	Yolo	Silty clay	6.5	2.2	9	5	0	0	2465 ± 315	0 ± 0	0 ± 0
13	Yolo	Silty clay	6.1	5.1	0	0	—	—	2655 ± 299	0 ± 0	0 ± 0
14	Yolo	Clay	7.0	3.8	531	50	0	0	1800 ± 438	0 ± 0	0 ± 0
15	Yolo	Clay	7.4	3.6	394	20	0	0	3405 ± 797	0 ± 0	0 ± 0
16	Yolo	Silty clay loam	6.9	2.6	1243	60	0	0	1900 ± 596	0 ± 0	0 ± 0
17	Imperial	Silty clay loam	7.6	1.0	0	0	—	—	535 ± 216	0 ± 0	0 ± 0
18	Imperial	Silty clay loam	7.5	1.2	0	0	—	—	737 ± 85	0 ± 0	0 ± 0
19	Imperial	Silty clay	7.5	1.1	0	0	—	—	4023 ± 1074	0 ± 0	0 ± 0
20	Imperial	Silty clay loam	7.4	1.3	17	0	—	—	1475 ± 425	0 ± 0	0 ± 0
21	Imperial	Silty clay loam	7.6	1.2	34	0	—	—	2383 ± 1201	0 ± 0	0 ± 0

[a] Samples were processed within 30 h after collection from field (before addition of Hs) or after 5000 Hs juveniles (J2) were added to soil every 2 weeks for 3 months (after addition of Hs). Values obtained before addition of Hs are means of three subsamples (not replicates). Values obtained after addition of Hs are the means ± SD of three replicates.

[b] Infected with *H. rhossiliensis*.

after 2 to 3 days at 20°C in the laboratory. This natural infestation also substantially suppressed penetration of cabbage roots by J2 of *H. schachtii*.

The suppression of *H. schachtii* by *H. rhossiliensis* in German sugarbeet fields and in soil from a California peach orchard suggests that this fungus has potential for biological control of *H. schachtii* in California sugarbeet fields. However, in order to select the appropriate method for potential implementation, information on the distribution of the fungus in California is needed. In this paper, we report that *H. rhossiliensis* is present in low numbers in many sugarbeet fields in California, and we discuss implications of these results for biological control.

MATERIALS AND METHODS

Field sampling. Sugarbeet fields located in San Joaquin, Solano, Yolo, and Imperial Counties in California were selected (Table 1). San Joaquin, Solano, and Yolo Counties are in the San Joaquin Valley in northern California, and the fields were sampled in 1989 on 7 June, 13 June, and 23 June, respectively. Imperial County is in southern California and was sampled on 28 February 1990. Sugarbeets were at least 2 months old (fields 1, 2, 6, 12–21) or had been harvested within 3 weeks (fields 3–5, 7–11). We assumed that the probability of finding *H. rhossiliensis* would be greater if fields were sampled during or shortly after the growing season. All fields but one were larger than 4 ha, but only a 4-ha section was sampled in these larger fields. Each field was sampled once. About 200 cm³ soil, 10 to 20 cm deep, was collected with a trenching shovel at each of 20 locations, about 15 m apart, along each of two diagonals per field. Locations were adjacent to sugarbeet plants if the crop was present. The soil from each field was mixed, and one 2-liter sample per field was placed in an ice chest.

Extraction of parasitized juveniles from soil. Three 100-cm³ subsamples of soil from each field were wet screened (38-μm pore diameter) and centrifuged in a sucrose solution (Jenkins, 1964) within 6 h of collection for samples from San Joaquin, Solano, and Yolo Counties and within 30 h for samples from Imperial County. The suspension of nematodes from each soil sample was adjusted to 5 ml, and one 0.5-ml aliquot was spread onto each of five 9-cm-diameter petri dishes containing water agar amended with streptomycin as described previously (Jaffee *et al.*, 1988), except the suspension was not treated with NaOCl. Thus, 50% (2.5 ml) of the extract was spread onto agar. After 24–36 h at 22 ± 2°C, the entire surface of each dish was examined with a dissecting microscope at 20–60× magnification. The number of nematodes parasitized by *H. rhossiliensis* (as evidenced by *H. rhossiliensis* sporulation) was determined. The number of nematodes in the remaining 2.5 ml of suspen-

TABLE 2

Detection of *Hirsutella rhossiliensis*-Colonized *Heterodera schachtii* as Affected by Soil and Time

Soil	Particle size (%)			Organic matter (%)	Detection (%)[a]	
	Sand	Silt	Clay		Day 0	Day 2
D	14	45	41	1.8	67 ± 9	21 ± 5
F	15	44	41	1.5	63 ± 4	17 ± 2
J	37	37	26	5.2	56 ± 10	11 ± 4
M	78	13	9	<1.0	54 ± 10	18 ± 5

[a] Percentage of number added. Data for soil F were collected as part of a different experiment and are from Fig. 1. Values are the means ± SD of six to eight replications kept at 22 ± 2°C.

sion was determined. The number of eggs of *H. schachtii* per 100 cm^3 soil (Roberts and Thomason, 1981), soil texture, soil pH (soil was moistened to a paste with water), and percentage organic matter were determined for each sample. Texture and pH were measured by the Diagnostic Laboratory at the University of California, Davis.

Repeated application of many host nematodes to soil infested with an obligately parasitic fungus such as *H. rhossiliensis* may cause a density-dependent increase in the proportion of nematodes parasitized by the fungus (Jaffee *et al.*, 1989; Jaffee and McInnis, 1991). To increase the probability of detecting low levels of *H. rhossiliensis*, healthy *H. schachtii* J2 were added periodically to soil samples and then examined for parasitism as follows. Within 6 h of soil collection (or within 30 h for samples from Imperial County), 100 cm^3 of each soil sample was placed into each of three cups, moistened if necessary, and incubated at 22 ± 2°C in a moisture chamber. Each cup was inoculated with 5000 healthy J2 of *H. schachtii* in 1 ml of 3 m*M* KCl after 2, 4, 6, 8, 10, and 12 weeks. Four days after the last addition of J2, nematodes were extracted from soil, spread onto water agar plus streptomycin, incubated, and examined for *H. rhossiliensis* as described for the other part of the survey. The number of J2 was determined in the 2.5 ml of extract that was not spread onto agar.

Efficiency of fungus detection in four soils. Two experiments were conducted to quantify our ability to detect parasitized nematodes in soil. Soils D and F were from sugarbeet fields in San Joaquin County and were typical of the heavy soils in the survey (Table 2). Soil J also was from a sugarbeet field in San Joaquin County and contained a high percentage of organic matter. Soil M was from a peach orchard in Merced County and contained a high percentage of sand. The soils were heated to 60°C for 2 h to kill any *H. rhossiliensis* present (unpublished). In the first experiment, colonized J2 (i.e., J2 that have acquired spores and had their body cavities filled with *H. rhossiliensis* hyphae) were obtained as described by Jaffee *et al.* (1990) and were mixed into 100-cm^3 samples of soil F. The soil was placed in 200-ml cups in a moist chamber. Within 90 min (designated Day 0) or after 1, 2, 4, or 7 days at 22 ± 2 or 10 ± 1°C, nematodes were extracted and parasitism was assessed as for the survey. There were three replicate samples per time and temperature, and the experiment was repeated once.

In a second experiment, colonized J2 were mixed into soils D, J, and M and were extracted within 90 min or after 2 days at 22 ± 2°C. Numbers of parasitized J2 were determined as for the survey. There were four replications per soil, and the experiment was repeated once. The number of colonized nematodes added per sample ranged from 485 to 620, depending on the experiment. The detection efficiency was expressed as a percentage by multiplying the number of parasitized nematodes detected on five plates (one half of the extracted sample) by 2, dividing by the initial number added per sample, and multiplying by 100.

RESULTS

Hirsutella rhossiliensis was detected in 7 of 21 samples that received no additional *H. schachtii* (Table 1). The number of parasitized nematodes detected was low, and the fungus was not associated with any nematode other than *H. schachtii*. Other fungal or bacterial parasites of *H. schachtii* were not observed. Predacious nematodes in the Dorylaimida were observed in many samples. One of these predacious nematodes, *Mesodorylaimus bastiani* (Butchlii) Thorne & Swanger, was observed feeding on J2 and eggs in cysts on some agar plates.

After periodic additions of large numbers of *H. schachtii*, all 7 soils positive for *H. rhossiliensis* before addition of *H. schachtii* remained positive, 3 soils changed from negative to positive, and 11 soils remained negative (Table 1). High numbers and percentages of *H. rhossiliensis*-parasitized *H. schachtii* occurred in several soils (soils 2, 7, 8, 10, and 11) that received supplemental *H. schachtii*. Six of the 10 fields that contained *H. rhossiliensis* also contained damaging levels of cyst nematodes, assuming a damage threshold of 200 J2 and eggs per 100 cm^3 soil before addition of J2 (Roberts and Thomason, 1981). Eight of the 10 fields that contained *H. rhossiliensis* were sampled shortly after harvest of the crop. Constrictive rings of the nematode trapping fungus *Arthrobotrys dactyloides* Dreschler were observed on 13 ± 6% of the J2 *H. schachtii* in the extract of soil 6 after repeated addition of large numbers of *H. schachtii*. Numbers of J2 recovered after additions of J2 were low in some soils (3, 4, 5, 10, 11, 17) that contained little or no *H. rhossiliensis* (Table 1), suggesting the presence of other unknown antagonists.

Isolates of *H. rhossiliensis* (ARSEF 2788 to 2794 and 2931 to 2933) from 10 of the sugarbeet fields and the isolate of *A. dactyloides* (ARSEF 2934) were deposited in the USDA–ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, New York.

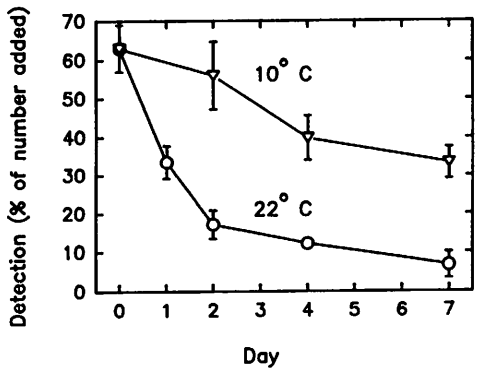

FIG. 1. Detection of *Hirsutella rhossiliensis*-parasitized *Heterodera schachtii* as affected by time and temperature. Each value is the mean ± SD of eight replications.

Detection efficiency ranged from 54 to 67% at Day 0 and was not greatly affected by soil type (Table 2). In all four soils, detection efficiency declined rapidly at room temperature (Table 2) but less rapidly at 10°C in soil F (Fig. 1).

DISCUSSION

H. rhossiliensis was found in many but not all sugarbeet fields in California. In those fields where the fungus was not detected, the fungus may never have been introduced or conditions may not have been suitable for establishment or persistence. However, it is possible that the fungus was present in these fields but was not detected due to low numbers or to limitations of our assay.

Our estimate of the prevalence of *H. rhossiliensis* may be low because the "plate assay" detects only parasitized nematodes (Jaffee *et al.*, 1988; McInnis and Jaffee, 1989). *H. rhossiliensis*-parasitized *H. schachtii*, like other parasitized nematodes (Kerry and Crump, 1977), are ephemeral when conditions are suitable for sporulation. The host "disappears" because, during sporulation, the fungus converts all of the host except the cuticle into an external network of hyphae, phialides, and spores. The rate of sporulation and host depletion is a function of temperature (Fig. 1) and host size; i.e., small nematodes such as J2 of *C. xenoplax* and *H. schachtii* degrade faster than do large nematodes (Jaffee *et al.*, 1988). The fully depleted cadaver is not recognized as parasitized because it no longer supports sporulation either in soil or on agar (Jaffee *et al.*, 1990). Thus, the decline in detection of *H. rhossiliensis* in soil seeded with parasitized nematodes (Fig. 1, Table 2) reflects a change in the form of the fungus rather than a decline in fungal population density (McInnis and Jaffee, 1989). Because total depletion of J2 of *H. schachtii* requires about 12 days at 20°C (Jaffee *et al.*, 1990), the observed decline in parasitized nematodes resulted from reduced extractability of partially depleted nematodes.

Unfortunately, detection of the hyphae, phialides, and spores produced from the cadaver in soil is difficult. These structures break free from the cadaver when soil is disturbed, do not extract well, and do not form colonies on agar in the presence of other soil organisms (Jaffee and Zehr, 1985). It follows that detection of the fungus with the plate assay depends both on the amount of fungus present and on the proportion of the fungus present as hyphae in parasitized nematodes. If present only as external hyphae, phialides, and spores, even high densities of the fungus would not be detected.

A bioassay for *H. rhossiliensis* spores has been described by McInnis and Jaffee (1989). However, this assay is useful only for undisturbed soil because the current crop of spores loses its ability to adhere to nematodes when soil is sampled or otherwise disturbed. A direct assay is needed for spores and hyphae of *H. rhossiliensis* in disturbed or undisturbed soil.

The distribution of *H. rhossiliensis* was not random in that the positive fields were in two of the four counties sampled. We are unaware of unique features of these localities or soils that would explain these results. However, the soil temperatures in the Imperial County may be sufficiently high (Roberts and Thomason, 1981) to limit sporulation of the fungus (Jaffee and Zehr, 1983). Most of the positive fields were sampled just after rather than during the growing season. Perhaps host numbers and therefore parasitism are highest at this time, or perhaps the removal of beets from soil disperses the fungus and increases the probability of detection.

H. rhossiliensis may be common in California. In addition to *H. schachtii* in sugarbeet fields in San Joaquin and Solano Counties (Table 1), it has been isolated from *C. xenoplax* in peach and almond orchards in Merced County (Jaffee *et al.*, 1988; unpublished) and from *Rotylenchus robustus* (de Man) Filipjev in flower plantings in San Mateo County (Caswell, unpublished). The host range of *H. rhossiliensis* is relatively broad and may even include soil mites (Jaffee *et al.*, 1989), yet, in any one field, the fungus is generally isolated from only one species of host. In the present survey, for example, the fungus was observed only on *H. schachtii* although many other species of nematodes were present. The tendency to recover *H. rhossiliensis* from specific hosts may reflect the high probability of encountering the predominant host in the location or it may reflect local adaptation of the fungus to the predominant host. The latter explanation is not supported by the high virulence of peach orchard isolates to *H. schachtii* (Jaffee and Muldoon, 1989).

As expected, the addition of large numbers of healthy hosts to soil increases the probability of detecting *H. rhossiliensis*. These results are consistent with density-dependent parasitism (Jaffee *et al.*, 1989; Jaffee and McInnis, 1991). In soil 6, the density-dependent response of a trapping fungus, *A. dactyloides*, may have

been greater than that of *H. rhossiliensis*. The number of J2 recovered after the repeated addition of large numbers of J2 was very low in some soils that contained little or no *H. rhossiliensis*. This suggests that antagonists other than *H. rhossiliensis* may have been active in these soils, and the combined effect of *H. rhossiliensis* and other antagonists should be considered.

Biological control of insect pests may be achieved by habitat manipulation (if natural enemies are already present), importation of natural enemies (a one-time release with the intention of achieving establishment of the enemies and long-term suppression of the pest), or periodic (inoculative or inundative) releases of natural enemies (Cate, 1990). At present, we do not know how to manipulate the environment to favor parasitism by *H. rhossiliensis*, but we are examining the stimulation of alternate hosts (saprozoic nematodes) and the effects of soil water potential, pH, and porosity. If *H. rhossiliensis* had not been found in California sugarbeet fields, we would have considered importation into many fields in order to establish the fungus. We reject the notion of an importation for two reasons. First, the fungus is already present in many fields. Second, established populations do not appear to provide adequate control of cyst nematodes. More data on the importance of established populations are needed, however, and we are currently comparing the long-term population dynamics of *H. schachtii* in the presence and absence of *H. rhossiliensis*. We also recognize the potential for importing strains of *H. rhossiliensis* that are more effective than the indigenous strains.

Because natural infestations of *H. rhossiliensis* may not sufficiently suppress pest populations, we are investigating inundative application of the fungus for short-term control of *H. schachtii*. This approach is justified because high percentages of *H. schachtii* J2 are rapidly killed in soil microcosms naturally (Jaffee and Muldoon, 1989) or artificially (Jaffee *et al.*, 1990) infested with high densities of *H. rhossiliensis*. Furthermore, *H. schachtii*, like most other plant-parasitic nematodes, is most damaging to young seedlings (Griffin, 1981); thus, short-term protection of sugarbeet seedlings may substantially increase yield.

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