A Semiselective Medium and Procedures for Isolation and Enumeration of *Chalara elegans* from Organic Soil

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ABSTRACT

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Recovery of *Chalara elegans* from soils with high organic matter content (76–80%) and with a high population of general microbes (fungi and bacteria) was compared using three described media. Modifications were made to a basal medium composed of carrot root extract and yeast extract by adding (in milligrams per liter): ampicillin, 200; chloramphenicol, 250; nystatin, 50; oxgall, 1,000; penicillin G, 60; rose bengal, 25; dicloran, 6; metalaxyl, 45; and pentachloronitrobenzene, 750. The recovery of pathogen propagules from artificially infested soil on this semiselective medium (TBM-2RBA) was about 80%. The pathogen also was recovered from naturally infested field soil from four commercial vegetable fields sampled at various times during the growing seasons of 1990 and 1991. Diluting 5 cm^3 of field soil tenfold, then plating 0.5-ml portions of the suspension onto TBM-2RBA, either directly or following additional dilutions, and incubating dishes in the dark for 5–6 days at 22–27 C provided maximum propagule recovery. The medium proved useful for isolating *C. elegans* from muck or peat soils.

Additional keywords: black root rot, inoculum density, *Thielaviopsis basicola*

The dematiaceous hyphomycete *Chalara elegans* Nag Raj & Kendrick (synanamorph = *Thielaviopsis basicola* (Berk. & Broome) Ferraris) (8) has been isolated from agricultural and noncultivated soils in many regions of the world and can be recovered from the roots of a diverse range of plant species (20). Under favorable moisture and temperature regimes, the fungus colonizes plant roots and hypocotyls, causing black root rot. Crops such as tobacco (2), cotton (7), soybean (1), carrot (13), and many other species may be severely affected by this pathogen (20). Long-term survival of *C. elegans* in soil occurs mainly through the production of melanized, thick-walled chlamydospores (aleuriospores) (17). The hyaline cylindrical phialospores (endoconidia), which also are produced in abundance on diseased tissues and in culture (12), may permit short-term survival (3).

A commonly used technique to isolate *C. elegans* from soil utilizes carrot root disks as bait (19). Following the incubation of freshly cut disks on the soil surface, rapid (within 72–96 hr) colonization of the tissue occurs, and the pathogen can be easily recovered. Although the carrot root disk procedure is a sensitive bioassay method (15), it provides a qualitative but not quantitative assessment of pathogen population levels in soil. However, if large numbers of soil dilutions and replications are used in conjunction with the most probable number method (5), the carrot disk assay may provide some quantitative information (18). Alternatively, several semiselective agar media have been described for enumeration of populations of *C. elegans* in soil. These include RB-M2 (16), VDYA-PCNB (9), TBM-C (4), and TB-CEN (14), all of which were developed to monitor inoculum densities of the pathogen in mineral soils from various regions of the United States. The degree of recovery of *C. elegans* on semiselective media is reported to be affected by high soil microfloral populations (4,9,16).

C. elegans causes a postharvest disease on fresh market carrots grown in organic (muck or peat) soils in the Fraser Valley of British Columbia, Canada (11). Infection occurs after harvest, and subsequent fungal development on the root surface during storage makes carrots unmarketable within 7–10 days (13). Information on the distribution of infested fields and knowledge of environmental or cultural factors which influence the survival of pathogen propagules could prove useful in the management of this disease. To quantify and monitor populations of *C. elegans* in the organic soils of the Fraser Valley, the efficacy of three described media, VDYA-PCNB (9), TBM-C (4), and TB-CEN (14), were compared for recovery of the pathogen. Several modifications were necessary to enable enumeration from soils which were high in organic matter and microbial activity, and procedures had to be developed to optimize the recovery of propagules. This report describes the results of these studies, which should be applicable to the recovery of *C. elegans* from soils or soil mixtures and nonsterile greenhouse potting mixes which contain high organic matter or peat and have high populations of general fungi and bacteria.

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MATERIALS AND METHODS

Media. The ingredients (per liter) of the three semiselective media used in this study are 1) VDYA-PCNB (9): V8 juice, 200 ml; glucose, 2 g; yeast extract, 2 g; pentachloronitrobenzene (PCNB), 0.5 g (a.i.); oxgall, 1 g; nystatin, 30 mg; streptomycin sulfate, 100 mg; chlorotetracycline HCl, 2 mg; $CaCO_3$, 1 g; and agar, 20 g (pH 5.2); 2) TBM-C (4): carrot juice (extract from 200 g autoclaved roots in 1 L of water), 970 ml; yeast extract, 2 g; PCNB, 0.75 g (a.i.); oxgall, 1 g; nystatin, 50 mg; chloramphenicol, 250 mg; penicillin G, 60 mg; and agar, 20 g (pH 5.2); and 3) TB-CEN (14): carrot juice (extract from 100 g of blended and filtered tissue in 100 ml of water [unautoclaved]), 80 ml; etridiazole, 400 mg; nystatin, 250,000 units; streptomycin sulfate, 500 mg; chlorotetracycline HCl, 30 mg; $CaCO_3$, 1 g; and agar, 15 g (pH 5.3). The antibiotics and fungicides were added after autoclaving (except in TB-CEN, which was not sterilized), and the media were poured into 100 × 15 mm plastic petri dishes and used within 24–48 hr. PCNB and nystatin had to be dissolved in small volumes of chloroform and methanol, respectively, prior to adding to the medium.

Inoculum preparation. For all experiments, an isolate of *C. elegans* obtained from diseased carrot roots was grown on V8 agar (V8 juice, 150 ml; agar, 15 g; ampicillin, 100 mg; and distilled water, 850 ml) for 14 days at 20 C. The colony was flooded with 10 ml of sterile distilled water, and the resulting phialospore suspension was diluted to the desired concentration following counts in a hemacytometer.

Evaluation of media. *Colony growth.* To evaluate spore germination and colony development of *C. elegans* on the various media, 0.5-ml portions of several dilutions of the phialospore suspension were plated. The number of colonies that developed and the rate and extent of colony growth were determined at times ranging from 5 days to 2 wk. For these experiments and those described below, two replicate dishes of each medium were used at each dilution, and the experiment

was repeated at least twice. Two replicate plates were chosen because earlier experiments indicated that mean propagule recovery was not significantly different from that achieved with four replicate plates (S. Chittaranjan and Z. K. Punja, *unpublished*). The data presented are the averages of all replications and repetitions of each experiment ($n = 6$). The data were subjected to analysis of variance, and means were compared using Fisher's LSD test.

Field soil. To assay for the presence of *C. elegans* in natural soil, soil samples were obtained from four commercial carrot fields in the Fraser Valley in 1990 and 1991 at various times during crop growth. Each field was sampled by taking about 50 g of soil from 12–15 different locations and bulking them to make a composite sample per field. The samples were brought back to the laboratory and stored for 1–10 days at 4 C prior to use. A volume of 1 or 5 cm^3 of soil was suspended in 9 or 45 ml of sterile distilled water (10^{-1}), respectively, thoroughly mixed, and 0.5 ml of the suspension plated on each of the media; or further dilutions (up to 10^{-3}) were prepared and plated. An estimate of the initial soil inoculum level in each field was made using the carrot root disk baiting assay (19). Recovery was expressed as a percentage of this inoculum estimate.

Artificially infested soil. To recover *C. elegans* from artificially infested soil, samples were taken from a field in which the fungus had not been previously detected with the carrot root disk assay. The soil had an organic matter content of 76%, a soil moisture holding capacity at saturation of 65%, and pH 5.2. To 150 cm^3 of soil, 10 ml of a phialospore suspension (2.5×10^6 ml^{-1}) was added, and the soil was mixed thoroughly. The final moisture content of the soil was 57%. Dilution platings were made (up to 10^{-4}) from 1 cm^3 of soil suspended in 9 ml of water. Soil samples were assayed 1 mo after infestation to allow the population levels to stabilize. The change in inoculum levels during this time was found to be less than 5% (S. Chittaranjan and Z. K. Punja, *unpublished*). Petri dishes were incubated on the laboratory bench (22–27 C) either in the light or covered with aluminum foil.

Media modifications. Comparison of the previously described media (Table 1) showed that the highest recovery of *C. elegans* from artificially infested soil was 46% on TBM-C prepared as initially described (4). Recovery was reduced because of contamination by numerous soil fungi, which were identified as species of *Pythium, Fusarium, Penicillium, Aspergillus, Trichoderma,* and *Mucor,* and also by various bacteria. To further enhance the recovery of *C. elegans* on TBM-C medium, the effects of each of the following modifications were evaluated. The basal component was varied to include extract from carrot roots (100, 200, 300, or 400 g/L) after blending and filtering through cheesecloth, or the carrots were incorporated after boiling and mashing; fungicides were added (metalaxyl at increasing concentrations from 10 to 45 mg a.i. at 5-mg increments, dicloran at increasing concentrations from 1 to 6 mg a.i. at 1-mg increments, or benomyl at 2 mg a.i.); other components were added (ampicillin at 100 or 200 mg, vitamin E acetate at 0.5% w/v, or rose bengal at 25 mg); and the final pH of the medium was adjusted (to 4, 4.5, 5, 5.5, or 6) with H_2SO_4 or KOH. Each of these modifications was evaluated in separate experiments conducted over a 2-yr period.

RESULTS AND DISCUSSION

Colony growth. The rate of development of colonies of *C. elegans* originating from phialospore suspensions plated onto the three described media and the modified TBM-C medium (TBM-2RBA) was most rapid on VDYA-PCNB and TBM-C, and was delayed on TB-CEN (Table 1). Individual colonies were larger on VDYA-PCNB, and recovery (number of colonies developing from spores plated with estimated viability on 1.5% water agar of 92–96%) was about 80–85% (Table 1). The presence of V8 juice and glucose in this medium has been shown to promote rapid and vigorous growth of many fungi (4,16), which would make it difficult to discern individual colonies and which also can result in high microbial contamination in semiselective media. In contrast, the percent recovery of *C. elegans* on TBM-C and TBM-2RBA was higher, and the colonies were somewhat smaller (Table 1). On TB-CEN, recovery was about 75–80%, and the colony size was 7–10 mm. The use of carrot root extract in these latter media, which provides a low-nutrient base, minimizes fungal growth and also potential microbial contamination. The delayed growth on TB-CEN necessitates an extended incubation (14). The rate and extent of colony development on TBM-2RBA was identical to that of TBM-C (Table 1).

Recovery of *C. elegans* from soil. In fields which were previously determined

Table 1. Comparison of four semiselective media for colony development and recovery of *Chalara elegans* from organic soil

	Growth from phialospores[x]					Recovery from soil (%)[y]		
	Colony size (mm)		Rate of development (days)	Recovery (%)			Artificially infested	
Medium[w]	Range	Mean		Range	Mean	Naturally infested	Range	Mean
VDYA-PCNB	8–11	8.9 b[z]	5–6	80–85	83.6 a	0	10–14	10.9 a
TBM-C	5–7	5.8 a	5–6	90–95	92.7 b	<10	40–46	42.2 b
TB-CEN	7–10	8.4 b	10–12	75–80	76.1 a	0	20–25	23.1 a
TBM-2RBA	5–7	6.2 a	5–6	90–95	93.2 b	50–60	75–80	78.6 c
LSD	...	0.8	...	...	8.7	...	...	12.9

[w] VDYA-PCNB (Papavizas, 1964), TBM-C (Maduewesi et al, 1976), TB-CEN (Specht and Griffin, 1985), and TBM-2RBA (this study).
[x] Incubated at 22–27 C. Data indicate the range of values and means from three experiments, each with two replicate dishes. Recovery is expressed as a percentage of the total number of phialospores plated onto each of the media following serial dilutions.
[y] Data indicate the range of values and means from three experiments, each with two replicate dishes. Four naturally infested soils were tested. Recovery is expressed as a percentage of the initial inoculum level, which was estimated using the carrot root disk baiting assay.
[z] Means within a column followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's LSD test.

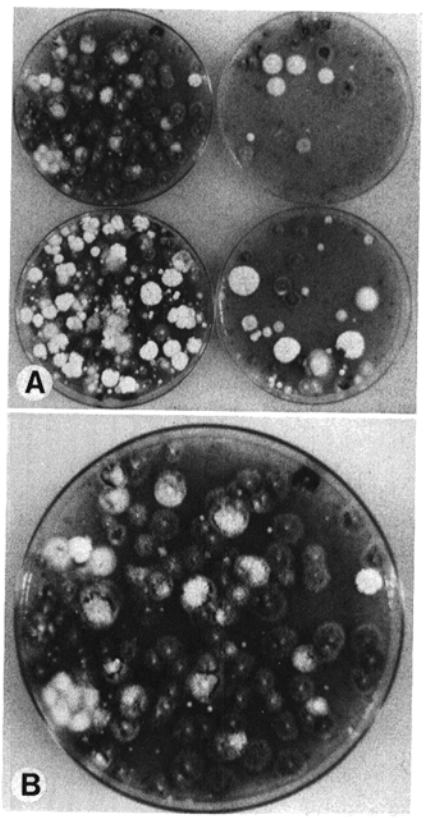

Fig. 1. Isolation of *Chalara elegans* on TBM-2RBA medium. **(A)** Recovery from dilution plating of artificially infested soil (upper row) and naturally infested soil (lower row). Petri dishes on left are at 10^{-3} dilution, on right at 10^{-4} dilution. Dark colonies are those of *C. elegans*; white colonies are *Fusarium* spp. **(B)** Close-up of *C. elegans* colonies recovered from artificially infested soil in **(A)**. Both photographs were taken after 8 days of incubation in the dark at 22–27 C.

by the carrot root disk assay (19) to be naturally infested with *C. elegans*, no colonies were recovered on VDYA-PCNB (Table 1). The growth of numerous contaminant fungi over the medium may have obscured any *C. elegans* colonies. Similar findings were reported by other investigators in studies which compared recovery of *C. elegans* from various soils, especially those with high microfloral populations (14,16). On the unautoclaved TB-CEN, contamination with bacteria was a major problem, which also prevented the detection of *C. elegans* in the organic soils used in this study (Table 1). Colonies of *C. elegans* were recovered on TBM-C, but contamination with various fungi (identified as species of *Pythium, Fusarium, Penicillium, Aspergillus, Trichoderma,* and *Mucor*) and bacteria made enumeration difficult. However, from artificially infested soil samples, the highest recovery of *C. elegans* (46%) was observed on this medium, while recovery rates were much lower on both VDYA-PCNB and TB-CEN (Table 1).

Media modifications. Of the various modifications of TBM-C tested in this study (*data not shown*), the combination which provided the highest recovery of *C. elegans* from artificially infested soil samples (80% recovery 1 mo after infestation) was the following: basal component (extract from 200 g of carrot roots) after blending and filtering; metalaxyl at 45 mg a.i./L; dicloran at 6 mg a.i./L; ampicillin at 200 mg/L; rose bengal at 25 g/L; final pH adjusted to 5.0. The presence of rose bengal (6) greatly reduced bacterial growth and made colonies of *C. elegans* much easier to see. The addition of vitamin E acetate, which was reported to stimulate the germination of chlamydospores of *C. elegans* (10), did not enhance recovery. The presence of benomyl at 2 mg a.i./L inhibited the growth of *C. elegans*.

The new semiselective medium (TBM-2RBA) provided for recovery of *C. elegans* from both naturally and artificially infested soils (Fig. 1A), and colonies were readily identified by their dark gray-to-black appearance on the medium. Incubation of dishes in the dark for 5–6 days enhanced the dark pigmentation of the colonies. The use of carrot root extract as the basal component instead of V8 juice reduced fungal contaminant growth and enhanced recovery of *C. elegans*. Since these studies were conducted over a 2-yr period, the source of carrots (age and cultivar) varied, but this did not influence the results; however, mature roots appeared to provide more consistent results (S. Chittaranjan and Z. K. Punja, *unpublished*). The medium should be used within 1 wk after preparation. More consistent recovery was obtained when 5 cm^3 of natural soil (about 1.5 g oven-dry weight) was diluted in 45 ml of water, and 0.5-ml portions were plated directly or after additional dilutions (up to 10^{-3}) (Fig. 1). The use of a 5-cm^3 volume of soil (instead of 1 cm^3) increased recovery. The major fungal contaminant on this medium was *Fusarium,* which could, however, be readily distinguished from *C. elegans* by its white colony color. By using dilutions of phialospore inoculum, the lower limit of detection of the medium was estimated to be about 10 propagules per cm^3 of organic soil (S. Chittaranjan and Z. K. Punja, *unpublished*). In comparison, the carrot root disk assay can detect the presence of propagules at a density as low as 4–5 per cm^3 (13). The TBM-2RBA medium is currently being used in studies to elucidate the environmental and cultural factors which influence survival of the pathogen in organic soils, and to determine the distribution of the pathogen in naturally infested fields.

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