Isolation and Enumeration of Propagules of Sclerotium cepivorum from Soil

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I express appreciation to R. W. Bolgiano for technical assistance and for preparing the illustration; also to A. H. McCain for providing the soil sample from Tulelake, Calif., and to W. Stanbaugh for the Walla Walla, Wash. soils.

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Accepted for publication 20 December 1971.

ABSTRACT

A selective medium and a technique were developed for the direct isolation of *Sclerotium cepivorum* from soil, and for the quantitative estimation of the inoculum density of the pathogen in soil. The selective agar medium contained inulin and $NaNO_3$ as C and N sources, respectively, and pentachloronitrobenzene, chlortetracycline HCl, and streptomycin sulfate. Sclerotia in soil were mechanically concentrated by wet-sieving; competitive soil saprophytes were reduced by washing the soil residue retained on a 0.177-mm sieve and by exposing the concentrate to 0.25% NaClO solution. The average recovery of sclerotia by this technique from several artificially infested soil samples was approximately 80%. Soils artificially infested with the pathogen, cropped to onions, and subsequently assayed with this technique contained from 6 to 15 sclerotia/g soil. Naturally infested soils from various parts of the United States averaged 0-3 sclerotia/g soil.

Phytopathology 62:545-549.

Additional key words: inoculum density, white rot.

White rot, caused by *Sclerotium cepivorum* Berk., is one of the most serious diseases of *Allium* spp., and is widely distributed in the United States and elsewhere. The pathogen, a poor competitor with soil saprophytes, does not survive in soil as active mycelium (10), but as small black sclerotia of ca. 0.2-0.5 mm in diam (2). These are produced in large numbers on the bulbs of infected plants.

Although *S. cepivorum* exists in soil in the form of distinct, separate entities with no capacity to proliferate in the absence of a host, it is difficult to isolate and enumerate propagules of this pathogen on

dilution plates because of its inherent inability to compete with soil saprophytes (10) and the sensitivity of its sclerotia to various antimicrobial agents used frequently in modern isolation media (11).

There are only two reports on the quantitative isolation of *S. cepivorum* from soil. Hickman & Coley-Smith (5) stated that, "by washing soil through two sieves of mesh size 0.5 and 0.2 mm, respectively, the sclerotium population of soil can readily be assessed". However, they did not indicate the viability of sclerotia recovered or whether their method could be used with naturally infested soils. Wet-sieving was also used by McCain (7), who isolated four to eight sclerotia from 50 g of naturally infested soil.

The present investigation describes a selective medium and dilution-plate technique for estimating the inoculum density of *S. cepivorum* in soil.

MATERIALS AND METHODS.—Isolates J-3, J-9, J-11, and J-18 were obtained from J. R. Coley-Smith, University of Hull, England; and Sc-1, from C. A. Martinson, Iowa State University, Ames. Isolate NJ-1 was obtained with the dilution-plate method by the author from infested soil collected near Sicklerville, N.J. Sclerotia of all isolates were obtained by a method previously described (1).

For infection studies, I used *Allium cepa* L. 'Yellow Globe Danvers'. Five-week-old seedlings were transplanted into infested soil in No. 4.5 plastic pots (10 seedlings/pot) and grown in a growth chamber at 15 C with a 12-hr day-length (1,200 ft-c, Sylvania VHO cool-white). After a 5-week growing period, the seedlings were removed from the soil and percentages of plants infected with *S. cepivorum* recorded.

Four replications were used for all experiments discussed in this paper. For the soil isolation experiments, a complete extraction from a given soil sample constituted a replication. All experiments were repeated at least 3 times.

RESULTS.—*The selective medium.*—Inulin and soluble starch used as sole C sources induce production of sclerotia (9) which are very useful for identification of *S. cepivorum*. Coalescence of colonies, a problem on inulin agar plates, was prevented by adding pentachloronitrobenzene (PCNB) to the medium at concentrations from 0.25 to 2.0 g/liter (active ingredient) to restrict colony size (Fig. 1). Colonies originating from single sclerotia produced abundant sclerotia within 10 to 15 days of incubation at 20 C.

After considerable experimentation on kinds of basal media, pH values of media, kinds and quantities of N, inulin and PCNB concentrations, and kinds and

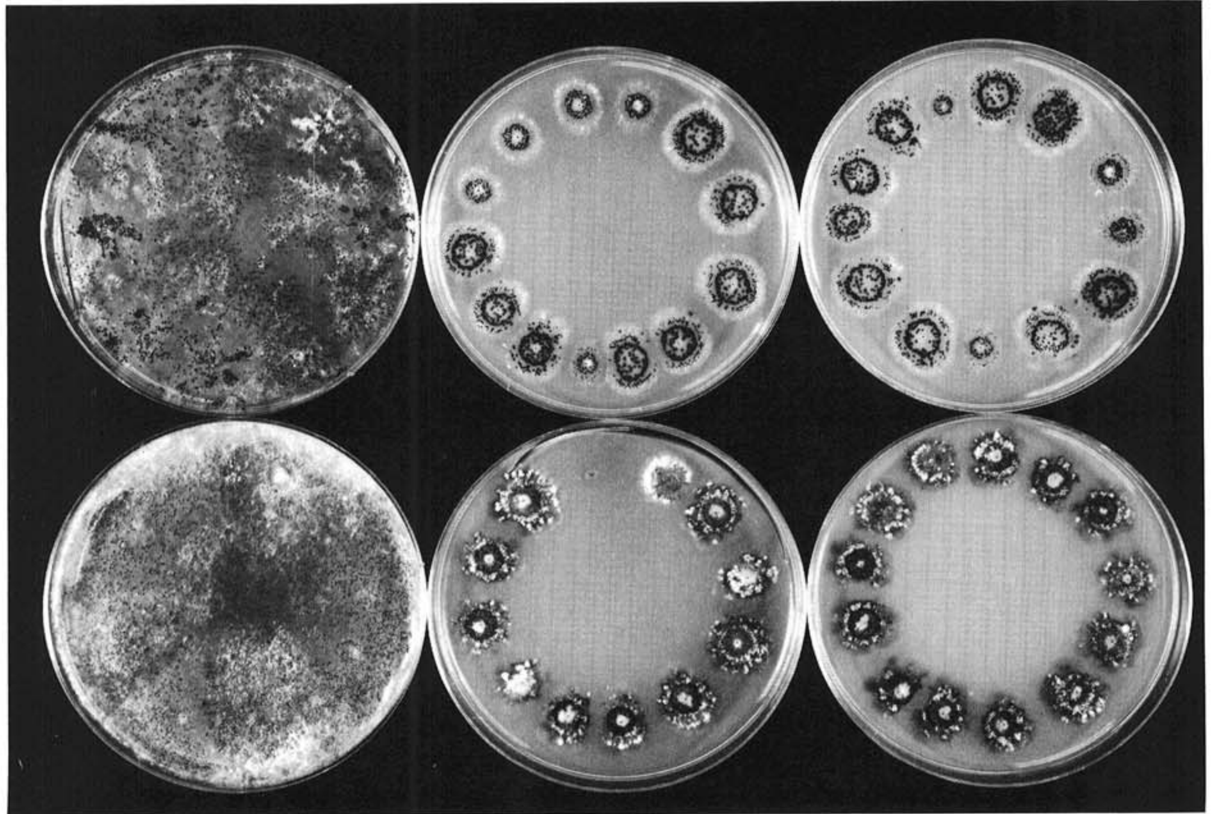

Fig. 1. Effect of three concentrations of pentachloronitrobenzene (PCNB) on size and appearance of colonies of *Sclerotium cepivorum* which started from single sclerotia cultured on the inulin-$NaNO_3$ medium. Thirteen surface-disinfected sclerotia were placed around the periphery of the dishes 3 weeks before the photograph was taken. Upper row, isolate J-11; lower row, isolate NJ-1. Left, no PCNB; middle, 0.5 g/liter PCNB; right, 1.0 g/liter PCNB.

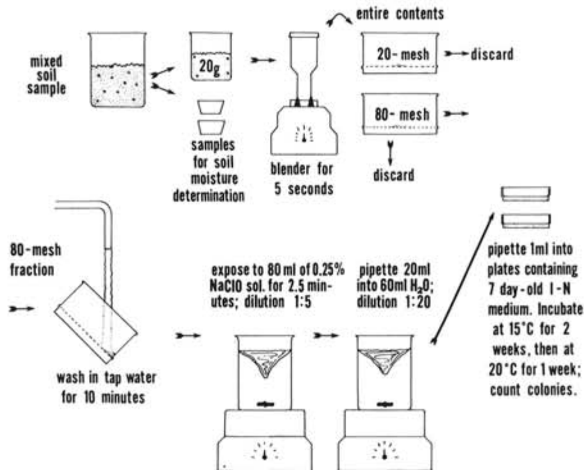

Fig. 2. Schematic diagram of the process used for the isolation of *Sclerotium cepivorum* from soil with the dilution-plate method.

quantities of antibacterial agents, an inulin-$NaNO_3$-PCNB (IN-PCNB) medium was adopted for isolation of *S. cepivorum* from soil. This medium contained per liter: inulin, 12 g; $NaNO_3$, 3 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; and sodium ferric diethylenetriamine pentaacetate, 5 μg/ml of Fe. The inulin was dissolved in hot water and cooled to 40 C. The other ingredients were added, and the pH of the medium was adjusted to 5.2. A pH value of 5.2 was previously found to be near optimum for germination of sclerotia of *S. cepivorum* (1). Twenty g of agar were added, and the medium was autoclaved at 121 C for 30 min. After the medium cooled to 50, 1.0 g of PCNB (active ingredient), 50 mg chlortetracycline HCl, and 100 mg streptomycin sulfate were added. The two antibiotics were prepared in 0.25% solution before being added to the medium. The medium was poured into petri dishes (15 ml/dish) 6 to 7 days before the assay, and the dishes were kept at 23 to 26 C in the dark. PCNB was also successfully used at 0.5 g/liter.

The isolation technique.—After considerable preliminary experimentation on kinds and sizes of sieves, comminuting soil suspensions for various periods in a blender, pretreating soil or sieve fractions with antimicrobial agents to reduce competitive microorganisms without affecting *S. cepivorum*, and temperatures of incubation, a process of isolation shown in Fig. 2 was adopted. The final technique is a modification of washing and wet-sieving techniques which have been described previously (4, 5, 7, 8).

Twenty g of soil in 80 ml of sterile distilled water were comminuted for 5 sec in a microblender at low speed (2,000 rpm). Longer comminution resulted in considerable loss of germinability of sclerotia. The entire contents were sieved through 0.841-mm (20-mesh) and 0.177-mm (80-mesh) sieves. Sieves of 0.595-mm and 0.250-mm were equally satisfactory. Residue on the 0.841-mm sieve was discarded, and the residue on the 0.177-mm sieve washed with running tap water for 10 min. The washed residue on the 0.177-mm sieve was transferred to 80 ml of 0.25% NaClO solution for 2.5 min. The final soil dilution was prepared by transferring (while the liquid was agitated by a magnetic stirrer) 20 ml from the 0.25% NaClO solution (1:5 dilution) to 60 ml of sterile tap water in a 400-ml beaker. One-ml aliquots were removed from the final dilution while the liquid was agitated and pipetted onto the surface of the 7-day-old IN-PCNB medium and the liquid was spread to cover the entire surface of agar. If a low dilution is desired (1:5), 1-ml aliquots can be transferred directly from the 0.25% NaClO solution to the medium without washing. It is important that the 1-ml aliquots be pipetted onto the surface of the medium because previous studies (*unpublished data*) showed that sclerotia of *S. cepivorum* do not germinate when embedded in agar. The dishes were incubated at 15 C for 2 weeks; then at 20 C for 1 week. The dishes were examined for *S. cepivorum* colonies after 3- to 4-weeks' incubation. Sclerotia associated with colonies on dilution plates normally appear within 3 weeks.

Recovery of isolates from soil as affected by length of exposure to NaClO.—Although wet-sieving, washing with tap water, and the antimicrobial agents in the IN-PCNB medium eliminated the great majority of soil competitors, some unwanted fungal colonies occasionally developed on the medium. An attempt was made to further improve the isolation technique by exposing, before plating, the final sieve fraction to a selective antimicrobial agent. Of various agents tested, 0.25% NaClO was the most satisfactory. The effect of length of exposure to 0.25% NaClO solution on the recovery of six isolates from soil was studied. Sclerotia of isolates J-3, J-9, J-11, J-18, Sc-1, and NJ-1 were added to microbially active soil (recently cropped to beans), and the 0.177-mm fraction was exposed (with slow stirring) to 0.25% NaClO for 0.0, 2.5, 5.0, 7.5, 10.0, 15.0, and 20.0 min before the final dilution as indicated by the procedure in Fig. 2.

Exposure to 0.25% for 2.5 min was the best treatment employed for recovery of most of the isolates (Table 1). Representative plates of the six isolates are shown in Fig. 3. Exposure for 5.0 and 7.5

TABLE 1. Recovery of *Sclerotium cepivorum* from soil infested with 100 sclerotia/g soil 24 hr before the assay as affected by length of exposure of final sieve fractions to 0.25% solution of sodium hypochlorite

Length of exposure (min)	% Recovery[a] from soil infested with specified isolates					
	J-3	J-9	J-11	J-18	Sc-1	NJ-1
0.0	25	44	56	6	8	44
2.5	96	80	80	44	44	78
5.0	80	84	86	12	40	74
7.5	82	60	38	4	24	28
10.0	76	16	12	8	4	34
15.0	24	7	16	0	8	24
20.0	12	0	4	0	1	12

[a]Dilutions of 1:20 were used on 20 petri dishes.

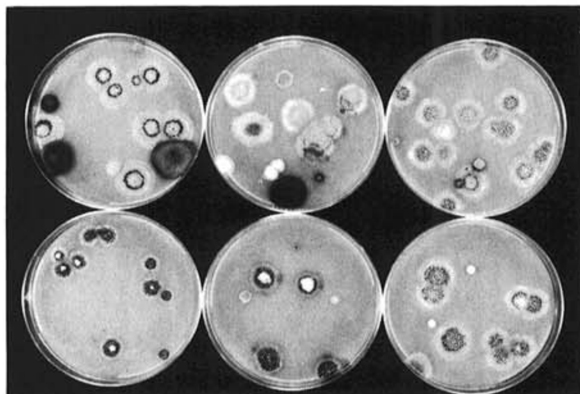

Fig. 3. Colonies of *Sclerotium cepivorum* on the inulin-$NaNO_3$-pentachloronitrobenzene medium from soil artificially infested with sclerotia. Upper row: (left to right) isolates J-9, J-11, and J-18. Lower row: (left to right) isolates Sc-1, NJ-1, and J-3.

min was satisfactory for isolates J-3, J-9, and J-11, but not for the other three. Exposures of more than 7.5 min resulted in considerable loss of germinability of sclerotia, and therefore in reduced recovery. The 2.5-min exposure was adopted for further use.

Effectiveness of the dilution-plate method.—The effectiveness of the isolation technique (Fig. 2) as a quantitative dilution-plate method was tested. Batches of 100 sclerotia of isolate J-11 were weighed with an electrical microbalance. Increasing weights of sclerotia were then added to 100-g soil samples to produce increasing concentrations of sclerotia from 3 to 96 sclerotia/g soil (Table 2).

The average percentage of sclerotia recovered was 81%, with a range at the 95% confidence level of 99-63. The percentage recovery from the 10 microbially active soil samples ranged from a low of 65% to a high of 95%.

TABLE 2. Recovery of sclerotia of *Sclerotium cepivorum* by the dilution-plate method on the inulin-$NaNO_3$-pentachloronitrobenzene medium from soil to which certain weights of sclerotia of isolate J-11 were added

Wt (mg) of sclerotia added/100 g soil	Sclerotia/g soil		% Recovery
	Estimated no. added	No. reisolated[a] on IN-PCNB	
5	3	2	67
10	6	5	83
20	12	10	83
40	24	22	92
60	36	32	89
80	48	31	65
100	60	57	95
120	72	53	74
140	84	73	87
160	96	75	78
			Avg 81 ± 18[b]

[a]Dilutions of 1:5 were used for the two lowest concentrations of sclerotia; 1:10 for soil samples receiving 20, 40, and 60 mg sclerotia/100 g soil; and 1:20 for the remainder. Average of three trials.

[b]At 95% confidence level.

Recovery from greenhouse soil samples.—Infested soils in the greenhouse were prepared in the following manner. One-month-old cultures of isolates J-9, J-11, J-18, and NJ-1 growing on a sand-cornmeal medium were added together at the rate of 1% to five soils. Onion seedlings were transplanted 3 times and allowed to grow for 1 month each time. The onions were removed and the soils were kept dry at about 25 C for 3 additional months before the assay.

Although the percentage of infected onion seedlings exceeded 70% in all soils, the maximum number of *S. cepivorum* colonies on the IN-PCNB medium did not exceed 15/g soil (Table 3). The number of colonies/g ranged from a low of six to a high of 15/g soil.

Recovery from naturally infested soils.—Six soils naturally infested with *S. cepivorum* were assayed with the dilution-plate method on the IN-PCNB medium and with McCain's wet-sieving method (7). There was considerable agreement between the two methods of isolation and enumeration (Table 4). The populations of *S. cepivorum* determined by both methods were very low, ranging from zero to three sclerotia/g soil. With the exception of a muck soil obtained from Middletown, N.Y., there was some relationship between inoculum density and percentage of infected onion seedlings. The muck soil from Middletown, N.Y., had an extremely high content of organic matter. Despite the steps taken to eliminate or reduce competitive saprophytes on the agar, considerable numbers of saprophytic fungi developed on the medium. Because of the presence of large numbers of organic particles which resemble sclerotia in size and color, sclerotia could not be observed with McCain's method.

DISCUSSION.—From several points of view, *S. cepivorum* is not a suitable organism for quantitative assays from soil by the dilution-plate method. Firstly,

TABLE 3. Average number of viable sclerotia of *Sclerotium cepivorum*/g soil from five soils that had been infested in the greenhouse with cornmeal-sand inoculum[a] 6 months before the assay and cropped to onions three times

Soil	Sclerotia/g soil enumerated[b] on the IN-PCNB medium		%[c] Infected onion seedlings
	First trial	Second trial	
Codorus loam	12	7	90
Elsinboro sandy loam	6	10	75
Hatboro loamy sand	10	15	100
Rumford loamy sand	10	12	100
Galestown-Evesboro loamy sand	8	11	80

[a]Isolates J-9, J-11, J-18, and NJ-1 were used.
[b]Dilutions of 1:20 were used on 20 petri dishes.
[c]During last cropping to onion, 3 months before assay.

TABLE 4. Average number of viable sclerotia of *Sclerotium cepivorum*/g soil in naturally infested soils as determined by the dilution-plate method on the inulin-$NaNO_3$-pentachloronitrobenzene medium and by McCain's wet-sieving method

Soil origin	Sclerotia/g soil		% Infected onion seedlings
	Dilution-plate[a] method	McCain's method (7)	
Sicklerville, N.J.	3.0	1.4	42
Middletown, N.Y.	0.0	0.0	40
Tulelake, Cal.	1.6	2.2	28
Walla Walla, Wash. (I)	1.5	1.7	15
Walla Walla, Wash. (II)	2.5	2.2	35
Walla Walla, Wash. (III)[b]	0.1	0.3	5

[a]Dilutions of 1:5 were used on 20 petri dishes. Each figure is the average of three trials.

[b]Soil sample III from Walla Walla, Wash., was obtained from a field treated with 2,6-dichloro-4-nitroaniline.

S. cepivorum is a poor competitor in soil and on agar media. A few colonies of saprophytic fungi growing on agar adjacent to *S. cepivorum* colonies normally prevent or reduce growth of the latter, or they mask its colonies or inhibit production of sclerotia vitally needed for identification. Secondly, *S. cepivorum* is extremely sensitive to antimicrobial agents (*unpublished data*). Of several antibiotics and fungicides tested in preliminary experiments, only PCNB reduced soil fungal saprophytes without appreciably reducing the ability of *S. cepivorum* to grow and produce sclerotia. Thirdly, populations of *S. cepivorum* in field soils appear to be so low (Table 4) that it is extremely difficult to isolate and enumerate propagules of the pathogen, especially since very low soil dilutions are needed to obtain a few colonies on the agar. From another viewpoint, however, *S. cepivorum* is suitable for quantitative assays with the dilution-plate method because it survives in soil as sclerotia or discrete units which can be enumerated. The often expressed argument that the dilution-plate method favors abundantly sporulating fungi does not hold true with *S. cepivorum*.

Despite the foregoing difficulties, a selective medium was developed for isolation and enumeration of *S. cepivorum* from soil. Success of soil bioassays depends on (i) mechanical concentration of sclerotia by wet-sieving; (ii) elimination of the great majority of soil saprophytes by washing; and (iii) exposing the residue to NaClO. This technique will provide a useful tool for measuring the number of *S. cepivorum* propagules in soil, and this will in turn aid in evaluating resistance to white rot and in determining the effects of biological and chemical control measures on the inoculum level.

The limited data obtained with six soils show that field soils do not have more than three sclerotia of *S. cepivorum* per gram of soil. This substantiates McCain's observations (7), which reported only four to eight sclerotia/50 g naturally infested soil. These observations on the low inoculum density of *S. cepivorum* in field soils are not surprising or new with soil-borne plant pathogens. Martinson & Horner (6) found only 10-40 viable sclerotia of *Verticillium albo-atrum*/g of soil from a potato field with history of severe Verticillium wilt. Evans et al. (3), with assays made a few months after cotton harvest, found that the population of *V. albo-atrum* had stabilized at around 40-120/g soil. Although the assays with field soil (Table 4) and with soils artificially infested with *S. cepivorum* in the greenhouse (Table 3) suggested that population of this pathogen may exist in field soils at considerably lower levels than those of *V. albo-atrum*, it was possible to detect *S. cepivorum* by the use of the IN-PCNB medium and the technique shown in Fig. 2.

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