Wet-Sieving Floatation Technique for Isolation of Sclerotia of Sclerotium cepivorum from Muck Soil

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ABSTRACT

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A wet-sieving floatation technique that facilitates the rapid isolation of sclerotia of *Sclerotium cepivorum* from muck soils was developed. Soil samples (20 g) were washed through two stacked sieves (0.595 mm openings over 0.210 mm openings) for at least 5 min, and the residues on the 0.210-mm sieves were transferred to columns containing 2.5 M sucrose solution (1.330 sp gr). After 2 hr, the soil fractions suspended in the upper portions of the columns were collected, washed with water on 0.210-mm sieves, and examined with a dissecting microscope. Sclerotia were removed with forceps, surface sterilized in 0.25% sodium hypochlorite for 2.5 min, washed in distilled water, and cut in half. The two halves were placed on potato-dextrose agar in petri dishes and kept at room temperature (22–25 C) for 2 wk to allow identification of *S. cepivorum*. Approximately 82% of sclerotia recovered from naturally-infested soils were confirmed to be *S. cepivorum*. The specific gravity of laboratory-reared sclerotia was greater than that of sclerotia produced in the field.

Additional key words: soilborne pathogen, soil populations.

White rot, which is caused by *Sclerotium cepivorum* Berk., is a serious disease of onion and other *Allium* spp. and is of worldwide distribution (8). The pathogen survives in the soil as sclerotia (1). Techniques for isolation of sclerotia of *S. cepivorum* from soil have been described (3,4,6) but in our experience none of these was useful for isolations from muck soils. The wet-sieving and dilution-plate technique (6) required in excess of 100 plates for each 20-g sample, and growth of unwanted organisms, even on the prescribed selective medium, totally precluded recovery of *S. cepivorum*. Reliable recovery of sclerotia by direct wet-sieving methods (3,4) was also precluded in muck soils by the large amount of particulate matter in the size range of sclerotia. We describe here the development of a practical technique for direct enumeration of sclerotia from samples of both mineral and organic field soils.

MATERIALS AND METHODS

The initial stages of development of a technique for isolation and enumeration of *S. cepivorum* from field soils were carried out with laboratory-reared sclerotia produced by an isolate of *S. cepivorum* obtained in 1975 from infected onions in a field at Cloverdale, British Columbia. Sclerotia were produced in sterilized sand-corn meal medium (2) inoculated with a 1 cm^2 disk of potato-dextrose agar (PDA) containing mycelium and sclerotia of *S. cepivorum*, and incubated in the dark at 22 to 24 C for 30 days. Initial tests revealed that all such sclerotia passed through a sieve with 0.500 mm openings, and were retained on one with 0.210 mm openings. Subsequent observations indicated that all of these sclerotia sank in water, but floated on an aqueous sucrose solution of 1.330 sp gr (2.5 M). These findings suggested that a combination of sieving and floatation could be used to facilitate enumeration and recovery of sclerotia from naturally-infested field soils.

The specific gravity of laboratory-reared and natural sclerotia (produced on field-grown onions) was estimated by observing their behavior in water and aqueous sucrose solutions ranging from 0.25 M to 2.50 M in 0.25 M increments. Test solutions were placed in petri dishes and separate samples of 300 sclerotia were added to each test solution. Each dish was agitated to free trapped air bubbles and minimize floatation due to surface tension effects. The number of floating sclerotia in each solution was counted after 1,2,3,4,5,6, and 24 hr. Two separate experiments were conducted to compare laboratory-reared and natural sclerotia. One experiment involved dried sclerotia (air-dried in laboratory for at least 90 days); the other dried sclerotia that were pre-soaked in water for 12 hr prior to addition to the test solutions. Ninety-three to 100% of sclerotia, regardless of origin of pretreatment, floated on a 2.5 M sucrose solution (1.330 sp gr) for at least 2 hr.

Numerous comparative evaluations of various other factors (sequence of sieving and floatation, sieve size, dry sieving vs wet sieving, etc.) ultimately led to adoption of the following technique for recovering sclerotia of *S. cepivorum* from soil samples. Composite samples were mixed thoroughly for at least 5 min, then two weighed subsamples of 20 to 25 g were removed. One of these was used for a dry weight determination. The other was washed for 5 to 10 min (organic soils - 10 min, sandy soils - 5 min) with running tap water through two stacked brass sieves (20 cm diameter × 5 cm deep, Tyler Co. of Canada, St. Catharines, Ontario), an upper one with 0.595 mm openings (28-mesh) and a lower one with 0.210 mm openings (65-mesh). The residue on the 0.595 mm sieve was discarded. The soil fraction on the bottom sieve (0.210 mm) was rinsed with tap water for an additional 5 min and then collected as a small clump along one wall of the sieve. Excess water was removed by touching a sponge to the bottom side of the sieve mesh beneath the soil. The soil then was transferred quantitatively to a glass column (200 mm long × 28 mm ID) containing about 20 ml of 2.5 M sucrose solution. The column then was filled to approximately 100 ml with 2.5 M sucrose. The soil suspension was mixed thoroughly with the sucrose solution by inverting the column several times, after which particles adhering to the rim of the column were washed down with additional sucrose solution delivered by pipet. After standing for 2 hr, the soil particles in the column had separated into a "floating" fraction and a much larger fraction which had settled to the bottom of the column. The lower fraction was drained carefully until only 20 to 25 ml of sucrose solution containing the floating fraction remained. This fraction, which contained sclerotia and other particles of 0.2 to 0.6 mm diameter and $<$1.330 sp gr, was washed onto a 0.210 mm sieve and

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rinsed with tap water. The rinsed fraction was distributed into one or more 9-cm diameter petri dishes as a single layer of particles barely covered with water. Sclerotia were located visually among the soil particles viewed under a dissecting microscope at 20 to 30× with incident illumination at an angle of ~45 degrees. A black background was superior to white for visual detection of sclerotia, and a grid or spiral design drawn on the outside bottom of the petri dishes facilitated systematic search of the particle layers. Sclerotia were removed with forceps.

A medium reported to be selective for *S. cepivorum* (6), and PDA supplemented with streptomycin sulfate (5) were compared with PDA for cultural identification of recovered sclerotia. Fungal and/or bacterial contaminants present on sclerotia from muck soils prevented development of *S. cepivorum* on any of these media. After considerable testing, a simple but effective procedure was adopted. Sclerotia recovered from soil samples were surface sterilized in 0.25% sodium hypochlorite for approximately 2.5 min, rinsed in sterile distilled water, and cut in half by pressure of the tips of fine forceps. The two halves of sclerotia with firm mycelial contents were plated onto PDA and kept at room temperature (22 to 25 C) for 10 to 14 days.

RESULTS

The apparent specific gravity (ASG) of sclerotia of *S. cepivorum* was estimated from differences in the proportions floating on the series of sucrose solutions. Sclerotia in the unbounded classes (<1.00 and >1.330) were arbitrarily assigned midclass values of 0.982 and 1.343, respectively. Up to 10% of the dry but virtually none of the presoaked sclerotia floated on water during the initial 6 hr of the test period. The proportion of sclerotia with ASG>1.330 after 2 or more hr in sucrose solution was 0 to 4% except for dry laboratory-reared sclerotia in which 13 to 15% had ASG>1.330 after 5 and 24 hr, respectively, in sucrose solution. The ASG of laboratory-reared sclerotia was greater than that of natural sclerotia. The ASG of all sclerotia, irrespective of origin or pretreatment, increased during the initial 5 to 6 hr in which they were in contact with the sucrose solutions and, with the exception of presoaked natural sclerotia, little further change in ASG occurred between 6 and 24 hr (Fig. 1).

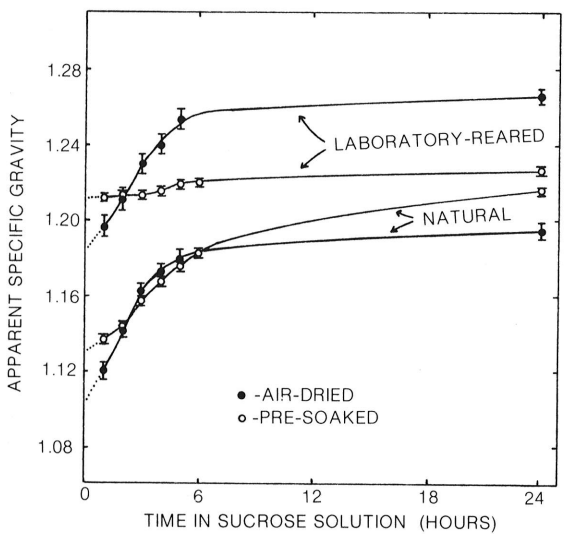

Fig. 1. Apparent specific gravity of air-dried and hydrated natural and laboratory-reared sclerotia of *Sclerotium cepivorum* in relation to time in aqueous sucrose floatation medium.

The effectiveness of the technique for recovery of sclerotia was tested by analyses of 20-g samples of noninfested organic muck soil to which had been added known numbers (25 to 30/sample) of natural sclerotia. In the first tests with 0.210-mm and 0.500-mm sieves, some sclerotia were retained on the 0.500 mm sieve. By using a sieve with 0.595-mm openings, recoveries of sclerotia increased from approximately 78% to 98% (Table 1). Tests with sclerotia produced on field-grown onions revealed that all were retained on a sieve with 0.210 mm openings. In a second test at natural population levels (6), nine samples containing zero, one, or two sclerotia/20 g of soil were randomized and analyzed by a person who did not know the number of sclerotia present in each sample. The results (recovered/added) of this test were as follows: 1/1, 0/0, 0/1, 1/2, 2/2, 2/2, 0/0, 0/1, and 0/0.

The technique was used to survey the distribution of *S. cepivorum* in all fields planted to commercial onions in 1977 in the muck soils of the Fraser Valley of British Columbia. A total of 219 composite samples representative of the top 10-cm portions of 153 fields on 53 different farms was analyzed in triplicate, and viable sclerotia of *S. cepivorum* were isolated at levels ranging from 0.02 to 0.25/g of air-dry soil from samples representing 13 fields on a total of 10 farms. *Sclerotium cepivorum*-infected onions were found on only one of these farms. The results of greenhouse pathogenicity tests indicated that 10 of 12 isolates derived from sclerotia obtained from fields in which white rot was not found on onions were virulent. Some other Fraser Valley muck soils on which white rot occurred at economic levels in 1976 (Provincial regulations prohibit further onion production on infested fields in the Fraser Valley) and mineral soils with long-standing infestations (based on observed disease in *Allium* crops) at Olympia, WA, and Kelowna, B.C. also were analyzed and recoveries of viable sclerotia of *S. cepivorum* ranged from 0.03 to 0.43/g of air-dry soil. For comparison, the numbers of viable sclerotia recovered in 1977 from an experimental plot on muck soil which was established with introduced inoculum in 1976 (7) were 0.42 and 2.2/g of air-dry soil from portions of the plot used for chemical and varietal trials, respectively.

Confirmation of the identity of recovered sclerotia as *S. cepivorum* on PDA after surface sterilization and splitting varied markedly for different soil samples, but averaged 82% (range 37% to 94%) for sclerotia recovered from fields in which white rot occurred on *Allium* spp. in 1976 or 1977. In contrast, only 20% of sclerotia recovered from fields in which white rot was not observed were confirmed to be *S. cepivorum*; the reamining sclerotia either failed to germinate (68%) or were other species of *Sclerotium* or possibly *Sclerotinia* (12%).

DISCUSSION

Papavizas (6) reported that competitive saprophytes precluded isolation of *S. cepivorum* from a naturally infested muck soil by a wet-sieving and dilution-plate technique, and that the presence of

TABLE 1. Efficiency of recovery of sclerotia of *Sclerotium cepivorum* from muck soil

Analyses (no.)	Sclerotia added[y]	Time of floatation (hr)	Sieve range (mm)	Sclerotia on[y]		Recovery[z] (%)
				Top sieve	Bottom sieve	
8	28.12	3	0.210–0.500	5.75	22.25	78.75 a
6	26.33	2	0.210–0.595	0.00	25.83	98.17 b
					S.E. of mean	1.58

[y]Mean number of sclerotia per analysis.
[z]Figures followed by the same letters do not differ significantly, $P = 0.01$.

large numbers of organic particles precluded direct recoveries with McCain's method (5). Both of these difficulties are overcome to a large extent by the method reported here, as are the other problems Papavizas (6) associated with a dilution-plate method for isolation of *S. cepivorum*.

Comparision of the specific gravities of natural and laboratory-reared sclerotia revealed that a sucrose solution with $\geqslant$1.330 sp gr is essential for quantitative recovery of sclerotia by the wet-sieving floatation technique. Germination of sclerotia was not affected by exposure to the 1.330 sp gr sucrose solution for up to 24 hr. The sieve sizes finally adopted (0.210 mm and 0.595 mm) provided quantitative recovery of sclerotia of the isolate occurring in commercial fields in the lower Fraser Valley of British Columbia.

We found surface sterilization of recovered sclerotia in hypochlorite followed by rinsing in water, splitting, and plating of the halves on PDA to be superior to the use of selective media (5,6) for confirmation of the viability and identity of sclerotia. Contaminants were not entirely eliminated by this procedure but growth of *S. cepivorum* began sooner from the split halves than from intact sclerotia. Early growth from split sclerotia was more competitive with contaminants than was delayed growth from intact sclerotia. Splitting of sclerotia provided an additional advantage which became apparent during analyses of field soil samples collected during the spring (March-April). These samples contained numerous cleistothecia and/or non-ostiolate perithecia which were similar in size and appearance to sclerotia of *S. cepivorum* but these were readily distinguishable after being split.

The technique described here is simple, economical, and rapid. One person routinely can process 18 to 20 samples per day (more for mineral soils in which the soil residues are minimal). We have found that relatively untrained personnel quickly become proficient in this technique, and it has been successfully utilized for large scale survey of the distribution of *S. cepivorum* in muck soils of the commercial onion growing areas of the Fraser Valley of British Columbia. These levels of natural infestation are considerably below those reported for four mineral soils by Papavizas (6). Quarantine restrictions prohibiting onion production on infested fields in the Fraser Valley presumably contribute to this difference

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