Techniques

Rapid Isolation of Sclerotia of *Sclerotium cepivorum* from Muck Soil by Sucrose Centrifugation

B. Vimard, M. E. Leggett, and J. E. Rahe

Research assistant, former graduate student, and professor, respectively, Centre for Pest Management, Simon Fraser University, Burnaby, B.C., Canada V5A 1S6. Present address of the first author: Department of Plant Science, Macdonald Campus of McGill University, Ste. Anne de Bellevue, Quebec H9X 1C0. Present address of the second author: Department of Soil Science and Plant Nutrition, University of Western Australia, Nedlands, W.A. 6009 Australia.

We wish to thank E. R. Littley for advice throughout the project.

Accepted for publication 29 October 1985.

ABSTRACT

Vimard, B., Leggett, M. E., and Rahe, J. E. 1986. Rapid isolation of sclerotia of *Sclerotium cepivorum* from muck soil by sucrose centrifugation. Phytopathology 76:465-467.

A centrifugation procedure was developed to reduce the time needed to recover sclerotia of *Sclerotium cepivorum* from muck soil. Centrifugation of the soil fraction containing particles measuring 0.2–0.6 mm in diameter in 2.5 M sucrose speeded the density-dependent separation of sclerotia and reduced the number of soil particles retained with the sclerotia. Incubation of recovered sclerotia at 17 C selectively favored growth of *S. cepivorum* over contaminants and increased the sensitivity of the viability tests.

White rot, caused by *Sclerotium cepivorum* Berk., is an important disease of *Allium* spp. worldwide. The pathogen does not produce functional spores, but forms small (0.2–0.5 mm) spherical sclerotia that are long-lived in soil (11). The sclerotia are usually present in low numbers (7,9) and distributed unevenly throughout the field. Therefore, many soil samples must be analyzed to estimate numbers of sclerotia reliably.

Most of the techniques described for isolation of sclerotia of *S. cepivorum* from soil utilize wet-sieving, either alone or in combination with selective media (2,6,7). These methods are effective in most mineral soils, but are of limited use in muck soils, because many of the organic soil particles resemble sclerotia in both size and color. This problem was overcome by the sucrose flotation technique of Utkhede and Rahe (10). This method is reliable and accurate, but its usefulness is limited by the time required to process each sample. A second limitation of existing techniques is encountered in confirming the identity and viability of recovered sclerotia. In this paper, we describe the development and evaluation of a technique based on centrifugation in 2.5 M sucrose for density-dependent separation of sclerotia of *S. cepivorum* from muck soil and procedures for confirmation of their identity and viability.

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MATERIALS AND METHODS

Sclerotia of *S. cepivorum* were collected from naturally infected onions, *Allium cepa* L., in October 1983, added to freshly collected muck soil (200–300 sclerotia per gram), and stored at 17 C. In all recovery experiments, 25-g samples of muck soil were processed. This soil, to which known numbers of sclerotia were added, came from an uninfested field in Cloverdale, British Columbia. Each soil sample for analysis was kept in a coded polyethylene bag so the assayer would be unaware of the number of sclerotia added. These bags also were stored at 17 C prior to analysis.

After preliminary experiments to determine appropriate sieve sizes, sucrose concentration, size and shape of centrifuge tubes, and speed and period of centrifugation, the following technique for recovering sclerotia of *S. cepivorum* from soil was adopted. Soil samples were washed for at least 5 min with running tap water through two stacked brass sieves, the first with 0.6-mm mesh openings and the second with 0.212-mm mesh openings. The residue on the 0.6-mm mesh sieve was discarded. The soil fraction on the bottom sieve (0.212-mm mesh) was rinsed with tap water for an additional 2 min, then collected along one wall of the sieve. This was rinsed with water into a small sieve (50-mm-diameter, 0.177-mm mesh openings), and excess water was removed by pressing absorbent paper to the bottom of the sieve. The soil was then transferred quantitatively to a 50-ml plastic conical centrifuge tube (115 × 30 mm ID) containing about 5 ml of 2.5 M sucrose. The centrifuge tube was filled to approximately 50 ml with 2.5 M sucrose. The soil suspension was mixed thoroughly and centrifuged for 5 min at 2,125 *g*. During centrifugation, the soil particles

separated into a floating fraction and a pellet. The floating fraction, containing sclerotia and other particles, was decanted into the 50-mm-diameter, 0.177-mm mesh sieve. Any residual floating material adhering to the centrifuge tube was dislodged gently with water and poured into the sieve with the remaining sucrose. No sclerotia were found in the pellet in preliminary experiments, thus the pellet was discarded. The material collected on the 0.177-mm mesh sieve was rinsed with water and transferred to a 9-cm-diameter petri dish as a single dispersed layer barely covered with water. Sclerotia were located visually under a dissecting microscope at × 12 magnification. A grid drawn on the petri dish facilitated a systematic search. Sclerotia were removed with forceps and counted.

Comparison of recovery techniques. The sucrose-centrifugation technique and the wet-sieving flotation technique (10) were compared for accuracy and efficiency in recovering sclerotia from muck soil. Sixteen samples were processed with each technique, four replicates each of zero, one, two, and three sclerotia per 25 g of soil. In all cases, the assayer was unaware of the numbers of sclerotia present in the samples. For each sample, the percent recovery, the time to process, and the dry weight of the floating soil fraction were recorded.

To improve efficiency, an attempt also was made to identify points in the procedure where sclerotia may be lost. To this end, the soil particles in the 0.6-mm mesh sieve, the soil portion washed through the 0.212-mm mesh sieve, the pellets in the centrifuge tubes, and the glass flotation columns were examined for sclerotia under a dissecting microscope.

Comparison of suspending media. The centrifugation technique previously described was used to compare 4.0 M $CaCl_2$, 70% glycerol, and 2.5 M sucrose, each of which has a specific gravity of approximately 1.33. Seventeen samples, each containing 20 sclerotia per 25 g of muck soil were processed in each solution; percentage of recovery and the time required to recover sclerotia from the floating fraction were recorded.

Effects of temperature on culture of sclerotia. To study the effect of temperature on colony development from sclerotia of *S. cepivorum*, sclerotia recovered from soil in the various experiments were surface-sterilized in a 1.0% sodium hypochlorite solution for 3 min, rinsed in sterile distilled water for 1 min, and cut in half with fine forceps. Halves of sclerotia with firm mycelial contents were plated onto potato-dextrose agar (PDA) and 1.5% water agar (WA) at the rate of six to eight halves in each 9-cm-diameter petri dish. These were incubated at 10, 17, or 23 C, until confirmation of *S. cepivorum* was possible. The nature of contaminants growing around sclerotial halves from which colonies of *S. cepivorum* did not develop was recorded.

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

Method	Processing time (min)	Search time (min)	Residue dry wt (mg)	Recovery (%)
Centrifuge	20 a[z]	3 a	62 a	94 a
Wet-sieve	140 b	4 b	203 b	87 a

[y] Number of sclerotia per analysis ranged from zero to three per 25 g of soil.
[z] Values within a column followed by the same letter do not differ ($P \leq 0.05$) according to the two-sample *t* test.

TABLE 2. Effect of incubation temperature on viability of sclerotia of *Sclerotium cepivorum* on potato-dextrose agar

Temperature (C)	Time of incubation (days)	Viability of *S. cepivorum* confirmed (%)	Viability not confirmed major contaminants (%)		
			Bacteria	Fungi	None
10	17	92 a[z]	0 a	2 a	6 a
17	11	84 a	14 a	2 a	0 a
23	8	60 b	22 a	16 a	2 a

[z] Values within a column followed by the same letter do not differ ($P \leq 0.05$) according to the Student-Newman-Keuls *t* test.

RESULTS

Recovery techniques. There was no significant difference ($P \leq 0.05$) in sclerotia recovery between the wet-sieving flotation technique and the sucrose-centrifugation technique (Table 1). Both are accurate and sensitive for isolating low numbers of sclerotia (one to three per 25 g) in muck soil. The time required to process a 25-g sample of muck soil, the time required to examine the floating fraction and to count sclerotia, and the amount of material present in the floating fraction were each significantly less ($P \leq 0.05$) for the sucrose-centrifugation technique than for the wet-sieving flotation technique (Table 1).

No sclerotia were found in any of the normally discarded fractions when these were examined to identify points of possible loss of sclerotia in the procedure.

Comparison of suspending media. There were no significant differences ($P \leq 0.05$) in the times required for recovery or in the percentages of sclerotia recovered among the different suspending solutions. Mean search times were 3.3 min, 3.5 min, and 3.7 min, and mean recovery percentages were 82, 87, and 89% for samples centrifuged in 70% glycerol, 4.0 M $CaCl_2$, and 2.5 M sucrose, respectively.

Effects of temperature on culture of sclerotia. Surface-sterilized sclerotia recovered from soil following centrifugation in 2.5 M sucrose gave significantly ($P \leq 0.05$) higher confirmed viability on PDA at 10 and 17 C than at room temperature (23 C). There was no significant ($P \leq 0.05$) difference in the confirmed viability at 10 and 17 C, but a longer incubation time was required at 10 C than at 17 C (Table 2).

Analysis of variance showed that the three suspending media had no significant ($P \leq 0.05$) effect on confirmed viability of recovered sclerotia. Thus, the confirmed viability data for sclerotia recovered from 70% glycerol, 4.0 M $CaCl_2$, and 2.5 M sucrose were pooled for analysis of the effects of incubation temperature and incubation medium on confirmed viability (Table 3). In contrast to the results obtained on PDA (Tables 2 and 3), temperature did not affect the proportion of sclerotial halves giving confirmed viability on WA. Moreover, confirmed viability did not differ significantly ($P \leq 0.05$) when incubation on PDA at 17 C and on WA at 23 C was compared in a separate experiment. Sclerotia did not form on colonies developing from sclerotial halves plated on WA at any of the incubation temperatures tested, but formed within 11 days on colonies developing on PDA at 17 C.

DISCUSSION

The wet-sieving flotation technique reported by Utkhede and Rahe (10) is an improvement over previous methods (2,6,7) for recovery of sclerotia from muck soil, but it is time-consuming. Centrifugation greatly reduces the time required for separation of sclerotia from more dense soil particles compared with flotation. The reduced quantity of soil particles present in the floating fraction obtained with the centrifugation technique compared with

TABLE 3. Effect of incubation temperature on viability of sclerotia of *Sclerotium cepivorum* on potato-dextrose and water agars

Medium and temperature (C)	Time of incubation (days)	Viability of *S. cepivorum* confirmed (%)	Viability not confirmed major contaminants (%)		
			Bacteria	Fungi	None
Potato-dextrose agar					
10	17	93 a[z]	2 a	1 a	5 a
17	11	95 a	4 a	1 a	0 b
23	9	63 b	29 b	4 a	3 ab
Water agar (1.5%)					
10	17	95 a[z]	0 a	1 a	4 a
17	12	95 a	0 a	1 a	5 a
23	9	88 a	1 a	3 a	8 a

[z] Values within a column for each individual medium separately followed by the same letter do not differ ($P \leq 0.05$) according to the Student-Newman-Keuls *t* test.

flotation in a glass column (10) reduced both the time and tedium associated with visual search for and removal of sclerotia from the floating fraction. Moreover, the centrifugation technique is as sensitive as the wet-sieving flotation technique for recovery of low numbers of sclerotia (one to three per 25 g) in muck soil. This is important because the natural populations of *S. cepivorum* in field soils can be low (7,9).

Because no sclerotia were lost in any discarded fraction examined, we conclude that any loss likely occurs during transfer of material. The column flotation technique requires the draining of a loosely compacted bottom fraction from the column without loss of sclerotia from the floating fraction; this step is eliminated in the centrifugation technique, making this technique more reliable and easier for an untrained person.

A centrifugation technique using 70% glycerol was reported for recovery of sclerotia of *Sclerotinia minor* (1). The use of solutions other than 2.5 M sucrose (70% glycerol and 4.0 M $CaCl_2$) for the flotation of sclerotia of *S. cepivorum* does not affect sclerotia recovery as long as the specific gravity of that solution is approximately 1.33. No striking effects on viability were demonstrated, but 4.0 M $CaCl_2$ gave a slightly lower germination than did the other two solutions.

Propagules of *S. cepivorum* compete poorly during germination and early stages of development with saprophytes in the soil and on agar (7,8). A few colonies of saprophytic fungi and/or bacteria on agar can prevent or reduce the germination or growth of sclerotia or developing colonies of *S. cepivorum*, making viability tests unreliable. Coley-Smith and Javed (4) used combinations of antibiotics to control bacterial contamination, but 20% of the sclerotia were still contaminated by other fungi. Utkhede and Rahe (10) showed that surface sterilization of sclerotia with 0.25% sodium hypochlorite solution, accompanied by splitting the sclerotia, increases their germination but does not eliminate the growth of contaminants. We found that incubation at 17 C or lower, combined with surface sterilization, favored *S. cepivorum*, thereby permitting its identification. The increase in germination of *S. cepivorum* is probably due to a suppressive effect of the low temperature on the growth of fungal and bacterial contaminants.

Results of viability tests done on WA at room temperature and on PDA at 17 C were similar, but the identification of *S. cepivorum* is more laborious on WA. Easily recognized sclerotia of *S. cepivorum* do not develop on WA. Instead, there is formation of sporodochia which produce microconidia (5). The microconidia may be misidentified as water droplets, if not examined under a microscope. Even then, many other fungi form microconidia similar in size and shape.

Low-temperature incubation of surface-sterilized sclerotial halves is preferable to other viability tests (3–5) because it does not require antibiotics, and only 11 days are required for formation of sclerotia. Moreover, identification of sclerotia is easily done with the unaided eye.

With combination of sucrose centrifugation and the low-temperature viability test, a technician can process 16 or more samples per day. This procedure can be an important tool for research studies or for disease management programs.

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