Techniques

A Method for Estimating Numbers of Viable Sclerotia of *Sclerotium rolfsii* in Soil

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ABSTRACT

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Sclerotia of *Sclerotium rolfsii* germinate when stimulated by certain volatile components of the host plant. Methanol was used as a stimulant to develop a method for determination of numbers of sclerotia in field soil. Air-dried soil was allowed to imbibe a 1% (v/v) aqueous methanol solution until it was evenly moist. After 4 days incubation in a closed chamber, the number of white colonies of *S. rolfsii* on the soil surface was counted. The method is at least 75% effective for detecting viable sclerotia. This method is as accurate and simpler than the soil-sieving and flotation methods currently used for determining numbers of viable sclerotia in soil.

Additional key words: southern blight, peanuts, *Arachis hypogaea*, volatile compounds.

Determination of numbers of sclerotia in soil is a prerequisite for developing predictions on the severity of diseases caused by *Sclerotium rolfsii* Sacc. (4,6). Published methods for determining numbers of sclerotia in soil are based on extraction of sclerotia either by washing on a sieve (4) or by flotation of the sclerotia in a blackstrap molasses solution containing a flocculating agent and then by sieving (6). Although these methods are accurate, they are time consuming in that they require observation of the debris collected in the sieves to count the number of sclerotia. In addition, these methods do not give a measure of the viability of the extracted sclerotia; this requires a separate operation (4,6). Recent findings on the effect of methanol as a stimulant for germination of dormant sclerotia of *S. rolfsii* (5) suggested that solutions of this alcohol could be added to soil and after incubation the number of colonies of *S. rolfsii* developing from stimulated sclerotia could be counted directly on the soil. This paper describes a method for determination of viable sclerotia in soil and presents field soil data obtained with the method.

MATERIALS AND METHODS

Sclerotia for these studies were produced by spreading autoclaved oats colonized by *S. rolfsii* (3) on the surface of moist (17% w/w, −1/3 bar) field soil contained in 35 × 50-cm trays to a depth of 8 cm. The soil was a sandy loam from a soybean field and the oats were added at the rate of 1/cm². The trays were covered with a layer of Saran® plastic wrap to reduce moisture loss. After 2 wk in the greenhouse, mature sclerotia were hand picked, air dried, and kept in a vial at room temperature (27 C) until used.

Germination of sclerotia. Germination experiments were performed in 15-cm-diameter desiccators. Five-centimeter-diameter petri dishes containing 5 g BaO₂ and 10 ml of water were placed in the bottom of each dessicator to supply O₂ and remove CO₂ (2). The soil to be tested was air dried, sieved (2 mm), and spread evenly over a Whatman No. 1 filter paper covering the bottom of a 13.5-cm-diameter sieve. The sieve had a 1-mm mesh fiberglass screen and a plastic (PVC) rim 3 cm high. The sieve with soil, was placed in a 14.5-cm-diameter petri dish cover with sufficient water (12.5 ml/50 g soil) or stimulating solution to moisten the soil to 60% field capacity. After the soil was moistened the sieve was placed inside the desiccator and incubated at 28 C. After 4 days the white colonies of *S. rolfsii* on the soil surface were

easily seen and counted.

The effects of the concentration of methanol and soil depth on germination of sclerotia were studied with the method described. The sensitivity of the method using optimal soil depth and methanol concentration also was evaluated with the method. Details of these studies will be presented in the Results section for ease of interpretation.

Assays of field samples. Soils from three peanut fields were collected in March 1978. Samples were collected using standard 2.5-cm-diameter soil probes to a depth of 15 cm; a total of 20 cores were obtained at random from each field. The cores for each field were composited and the soil was air dried and sieved (2 mm). Samples were assayed for viable sclerotia using 50 gm of soil and moistening with 12.5 ml of 1% (v/v) methanol. Fields A and B had a high incidence of southern blight in 1977; the third field, C, had a low incidence of the disease (7). The assay of samples was performed using seven replications for each field.

In a second field experiment the same method was used to recover sclerotia in soil from microplots established on 20 December 1977 in a peanut field at the Agronomy farm at Auburn. Each microplot consisted of a 15-cm section of 10-cm diameter of PVC pipe buried to a depth of 13 cm. The soil in each microplot received 10 sclerotia. Twenty-one microplots received no amendment and another 21 received 4 g of dried decomposed peanut hay; a third group of 21 microplots received 4 g of the hay 2 mo after burial of the pipes with the 10 sclerotia. On 24 March 1978, soil from each microplot (approximately 500 g) was collected, air dried, sieved and spread evenly on a layer of absorbent paper towel in the bottom of a perforated (two 4-mm-diameter holes/10 cm²) 34 × 24 × 5-cm rectangular aluminum baking pan. Each pan was placed on a flat tray that contained 90 ml of 1% (v/v) methanol to moisten the soil. Pans with moistened soil were sealed inside clear polyethylene bags to reduce moisture loss. After 3 days of incubation at room temperature (27–30 C) the colonies of *S. rolfsii* on the surface were counted.

The effectiveness of the methanol method for determining numbers of viable sclerotia in soil was compared with that of the flotation-sieving method (6). A total of six peanut fields were randomly selected and sampled as described for fields A, B, and C. Each field was subdivided into 18 m² sections and soil samples were taken from each section. The samples were air dried, sieved (2 mm) and kept at room temperature in separate polyethylene bags until analyzed by the methanol and the flotation-sieving methods. The perforated pan procedure was followed for the methanol method and 250 g of soil was used per pan. Single determinations were performed for every sample with each method.

Statistical analysis. All data were analyzed following standard procedures for analysis of variance and differences between means were evaluated for significance by a modified Duncan's multiple range test (8). Unless otherwise specified, differences referred to in the text or tables were significant to the $P = 0.05$ or lower level of probability. Linear correlation coefficients were calculated following standard procedures (8).

RESULTS

Effect of methanol concentration and amount of soil. The effect of methanol concentration in the soil moistening solution was studied by using 50-g samples of dried soil each amended with 10 sclerotia. Solutions tested contained 0, 0.25, 0.50, 0.67, 1.00, 1.33, and 2.00% (v/v) methanol. A significant increase in germination over the control was observed with concentrations equal or higher than 0.5% (Table 1); differences in germination between solutions in the range of 0.5–1.0% were not significant. Highest percentage of germination was obtained with the 1.33% solution, but increasing methanol concentration to 2% reduced germination to less than that obtained with the 1.33% solution.

The effect of the amount of soil on the detectability of *S. rolfsii*

TABLE 1. Effect of methanol concentration of stimulating solutions on the germination of sclerotia of *Sclerotium rolfsii* on soil plates

Methanol (%, v/v)	Germination (%)
0.00	65* A[z]
0.25	77 B
0.50	85 C
0.67	80 C
1.00	85 C
1.33	100 D
2.00	85 C

[z] Figures represent the percent germination calculated from 10 sclerotia; figures within the same column followed by a common letter are not statistically different ($P = 0.05$).

TABLE 2. Relation between the amount of soil and colonies of *Sclerotium rolfsii* in soil from sclerotia stimulated to germinate with 1% (v/v) methanol

Amount of soil per sieve (g)	Rate (grams of soil per cm^2)	Thickness of soil in sieve (mm)	Germination (%)
25	0.17	1.12[y]	98 A[z]
50	0.34	2.24	93 A
75	0.50	3.36	83 B
100	0.68	4.48	85 B
150	1.00	6.42	73 C

[y] Thickness calculated on the basis on a soil density value of 1.52 for the dry soil used.
[z] Percent germination determined from 40 sclerotia; figures followed by the same letter were not statistically different ($P = 0.05$).

TABLE 3. Relation between the methanol-pan method and the flotation-sieving method for determination of sclerotia of *Sclerotium rolfsii* in soil from six peanut fields in Alabama

Peanut field	Samples per field (no.)	Sclerotia/50 cm^3 soil[y]		Correlation coefficient
		Range	Average	
I	34	0–11	2.70	0.51**[z]
II	47	1–21	2.95	0.54**
III	55	1– 9	2.05	0.55**
IV	68	0–11	2.42	0.55**
V	54	1–10	1.89	0.91**
VI	38	0– 9	2.52	0.50**

[y] Based on extraction by flotation sieving.
[z] Figures followed by ** represent highly significant ($P = 0.01$) linear correlation coefficients.

colonies from sclerotia was studied by using 1% (v/v) methanol and 25-, 75-, 100-, and 150-g amounts of silt loam. The amount of soil in the sieves influenced the number of colonies of *S. rolfsii* developing from stimulated sclerotia (Table 2). At concentrations of 0.17 and 0.34 g of soil per cm, equivalent to soil thickness of 1.12 and 2.24 mm, the number of colonies detected corresponded to 98 and 93% of the sclerotia added, respectively; however, higher soil concentrations resulted in significantly fewer colonies being detected.

Sensitivity of the method. A total of 19 colonies of the possible 22 were detected in sieves with soil (1% methanol, 50 gm soil) containing a single sclerotium (86% recovery). In an experiment with 10 sclerotia per sieve, 152 colonies were detected of a possible 200 (76% recovery).

Field samples. Samples of fields A and B yielded 1.28 and 1.14 colonies per 50 g of soil, respectively; these figures were not statistically different but were significantly higher than the corresponding figure for field C (0.28 colonies per 50 g of soil).

Samples from the microplots yielded an average of 7.86 colonies per plot for the treatment with no amendment, 11.62 colonies for the treatment with sclerotia and hay added simultaneously, and 11.52 colonies for that in which the hay was added 2 mo after addition of the sclerotia. Differences between amended plots were not significant but both of these treatments resulted in detection of significantly more colonies than the unamended control.

Comparisons of the flotation-sieving and the methanol-pan methods for extraction fo sclerotia from field soil (Table 3) indicated a close and highly significant degree of correlation between the two methods.

DISCUSSION

Results obtained with the method described here indicate that it is as effective for estimation of sclerotia in soil as are the published methods based on direct extraction of sclerotia from soil (4,6). Our method succeeds because of the stimulatory effect of methanol on sclerotial germination. This alcohol is one of the components of peanut (authors' *unpublished*) and alfalfa hay volatiles (5). It is probable that methanol serves as a carbon source for the germinating sclerotia and perhaps inhibits competing microorganisms in and around the sclerotia of *S. rolfsii* thus permitting germination on the soil surface, in a manner shown by Linderman (5) with weak NaClO solutions and earlier by Leach (4) with HgCl$_2$ treatments of sclerotia.

Another critical variable of the method is the thickness of the layer of soil spread on the sieves or pans. *S. rolfsii* has a high O$_2$ requirement and occurs typically in nature at or near the soil surface where it competes favorably with other organisms (1). Our results indicate that the layer of soil should not be thicker than 2mm. Thicker layers apparently reduce the competitive ability of the fungus and make visualization of colonies from sclerotia deep in the soil difficult.

We described two systems for determination of sclerotia. In one system, 50 g of soil as placed in 13.5-cm-diameter sieves enclosed in desiccators with an O$_2$ source. This system is recommended for laboratory tests in which a small amount of soil with high numbers of sclerotia are to be analyzed. The use of pans which permit processing of larger samples while maintaining the thickness of the soil layer within the 2-mm limit is more practical for field samples containing lower numbers of sclerotia. The pan system also has the advantage of not requiring BaO$_2$ since sufficient O$_2$ and CO$_2$ exchange occurs through the polyethylene bag to permit good growth of the pathogen. The pans are available at any local market and are those commonly used for roasting or cooking. They are relatively inexpensive and can be perforated readily for the requirements of the method

Our results from field samples suggest a probable correlation between the number of sclerotia in the soil and the severity of disease on peanuts in the preceding season. A greater number of samples would be needed to establish correlations to develop equations for accurate predictions of peanut yield losses to southern blight. Accumulation of these data is currently in

progress.

Results from the samples from microplots indicated that it is possible to follow changes in numbers of sclerotia with this method in response to organic amendments and underline the usefulness of the method for ecological studies.

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