Development of a Medium for the Selective Isolation of Sclerotium rolfsii

P. A. Backman and R. Rodriguez-Kabana

Assistant Professor and Associate Professor, respectively, Department of Botany and Microbiology, Auburn University Agricultural Experiment Station, Auburn, Alabama 36830.

The technical assistance of Ms. Carol McLeod is gratefully acknowledged.

Accepted for publication 20 August 1975.

ABSTRACT

An agar medium for the selective isolation of *Sclerotium rolfsii* from contaminated field sclerotia and from organic matter is reported. Selectivity is based on tolerance and utilization of oxalate, with additional selection being contributed by the low pH of the medium, and by the addition of gallic acid.

Phytopathology 66:234-236.

Additional key words: crop loss.

Leach and Davey (4) showed that severity of disease caused by *Sclerotium rolfsii* Sacc. could be predicted by screening sclerotia from the soil, and determining their population. Sclerotial viability determinations in their study were hampered by internal contaminating organisms which ordinary laboratory techniques could not eliminate. Curl and Hansen (3) have reported that sclerotia of *S. rolfsii* are heavily infested with fungi and bacteria, and that some of these are inhibitory to *S. rolfsii*. A rapid flotation-sieving technique (7) for extraction of *S. rolfsii* sclerotia requires a viability check for accurate determination of live sclerotia. In addition, information is not available on the role that mycelium in organic debris may play in the perpetuation of the fungus.

Bateman and Beer (2), showed that *S. rolfsii* produces large amounts of oxalic acid during growth and pathogenicity. Maxwell and Bateman (5) indicated that the pH and buffer capacity of the medium are critical for maximal growth; the fungus produces a large amount of oxalic acid until the pH is reduced. Their literature review indicated that oxalate production is uncommon in the fungi, being restricted to *Aspergillus* spp. and *Botrytis* spp. These findings indicate a peculiar adaptation by *S. rolfsii* to growth in an oxalate-rich environment, and suggest that tolerance to, and/or metabolism of, oxalate could be used as the basis of a medium selective for *S. rolfsii*. This paper describes the development of a selective medium for *S. rolfsii* which is based on oxalate tolerance or metabolism.

MATERIALS AND METHODS.—During this study, mycelial dry weights were determined by filtering liquid cultures through a tared dacron cloth, followed by drying in a moving-air oven for 48 hours at 60 C. The hydrogen ion concentration in the filtrate from each flask was measured with a calomel electrode, and the oxalate concentration was determined by Baker's method (1). Growth on solid media was determined by measuring colony radii. Cultures of *S. rolfsii* on liquid and solid media were observed for growth characteristics during incubation.

Selectivity of prepared media was determined by the soil assay technique of Rodriguez-Kabana (6). One drop of an aqueous suspension of peanut field soil was placed in a 90-mm-diameter plastic petri dish. Twenty milliters of the melted agar medium (45 C) under study were added, and the plate was gently swirled. After 48 hours plates were read for the genera of fungi present. Additional plates containing the test medium were inoculated with oat seed pieces infested with *S. rolfsii*, and contaminated with peanut field soil.

A preliminary test to determine growth, oxalate production, and pH changes by *S. rolfsii* in a basal salts-glucose medium was developed. A peanut isolate of *S. rolfsii* was grown in a broth composed of 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 2.0 g KNO_3, 1.0 mg thiamine hydrochloride and 10 ml of a stock solution containing 1 g of $Fe_2SO_4 \cdot 7\ H_2O$, 1.0 g $ZnSO_4 \cdot 7\ H_2O$, and 0.6 g $MnSO_4 \cdot H_2O$ per liter. Thirty grams of glucose were added, and the solution was made to 1 liter with distilled water. Flasks containing 25 ml of the medium were inoculated with a 10-mm-diameter disk of *S. rolfsii* from the periphery of a 24-hour-old water agar-grown colony. Flasks were incubated at 27 C. At 0, 1, 3, 4, and 7 days after inoculation, four flasks were removed and sampled for pH, mycelial dry weight, and oxalate content.

The ability of *S. rolfsii* to grow in the presence of increasing levels of oxalate ions in a basal salts medium was tested. This medium was identical to the one previously described, but without glucose. Oxalate concentration in the medium was adjusted by addition of 0.54 M potassium oxalate buffer (pH 4.2) to achieve final oxalate concentrations of 0, 0.001, 0.003, 0.007, 0.013, 0.027, and 0.054 M. Volumes were adjusted by the addition of distilled water. Media thus prepared were sterilized through Coors sintered-glass filters into sterile receiving flasks. Aliquots (25 ml) of the sterile media were then dispensed into previously sterilized 125-ml flasks capped with Whatman No. 1 filter paper for uniform aeration. A 10-mm-diameter disk cut from the periphery of a 48-hour-old culture of *S. rolfsii* grown on water agar, was used as inoculum. Agar media were dispensed into 90-mm-diameter petri dishes at 20 ml per dish and inoculated with a 10-mm disk of *S. rolfsii*. Both tests were replicated eight times. After incubation at 27 C for 48

hours, radial growth was measured, and cultures were observed for growth characteristics for a week thereafter.

A test similar to the one above also was conducted, but each oxalate concentration contained 30 g glucose per liter. Growth in liquid and agar media was measured, and observed as before.

In order to increase selectivity, and to provide an additional carbon source, 130 mg of gallic acid per liter was added to the medium containing the highest oxalate level (no glucose) which allowed growth of *S. rolfsii*. Oxalate-gallate medium was compared to the corresponding medium without gallic acid, and to the basal salts medium for radial growth on solid medium. These media were again evaluated for selectivity and germinability of field sclerotia recovered by the flotation-sieving method.

RESULTS.—Growth of *S. rolfsii* on a basal salts medium containing glucose resulted in a rapid drop in pH with a concurrent increase in oxalic acid (Fig. 1). Four days after inoculation, oxalate concentration began to decrease, and pH began increasing.

No growth of *S. rolfsii* occurred in a liquid medium composed of basal salts and potassium oxalate. However, the fungus was very tolerant to levels of oxalate up to 0.054 M in agar media (Fig. 2). Growth on oxalate agar was sparse, however, and lysis occurred after 5 days. Addition of glucose to these media (both liquid and solid) resulted in excellent growth of *S. rolfsii* at all oxalate levels (Fig. 3). However, when solid media were checked for selectivity, there were many contaminants, particularly the penicillia and aspergilli.

Addition of gallic acid to 0.054 M oxalate, basal salts medium, and elimination of glucose, resulted in very little growth in the liquid medium. In the agar medium, however, *S. rolfsii* grew well (Fig. 4), did not lyse, and was moderately dense in growth habit. Selectivity was good, with only five to six contaminant colonies per plate being detected when a soil suspension was added to molten agar.

Plating of infested organic matter (sterilized, then inoculated oat seed pieces) contaminated with field soil revealed excellent selectivity, with *S. rolfsii* emerging from all seed pieces. Nonsterile sclerotia of *S. rolfsii*

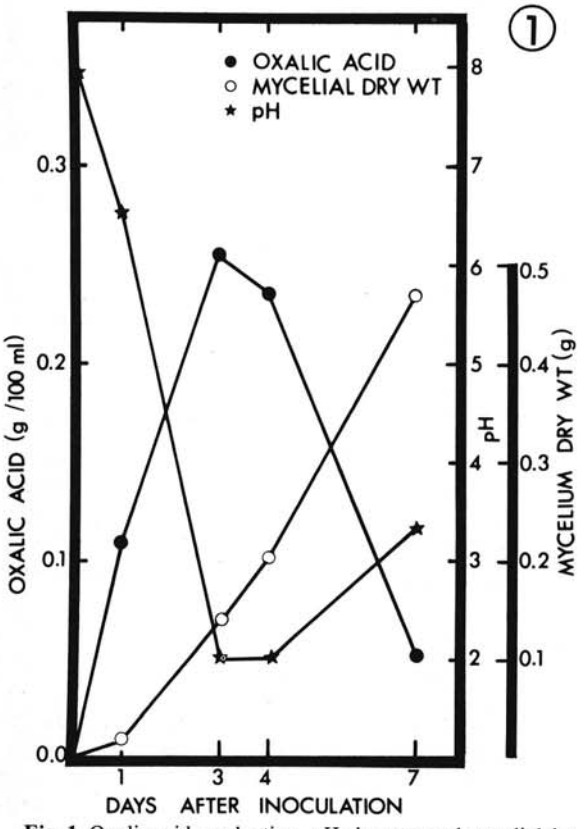

Fig. 1. Oxalic acid production, pH changes, and mycelial dry weight in a basal salts-glucose broth inoculated with *Sclerotium rolfsii*.

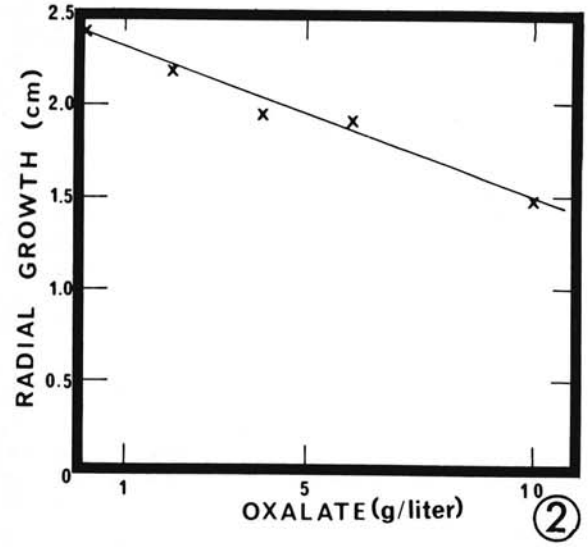

Fig. 2. Radial growth of *Sclerotium rolfsii* on basal salts agar (pH 4.2) containing increasing amounts of oxalate.

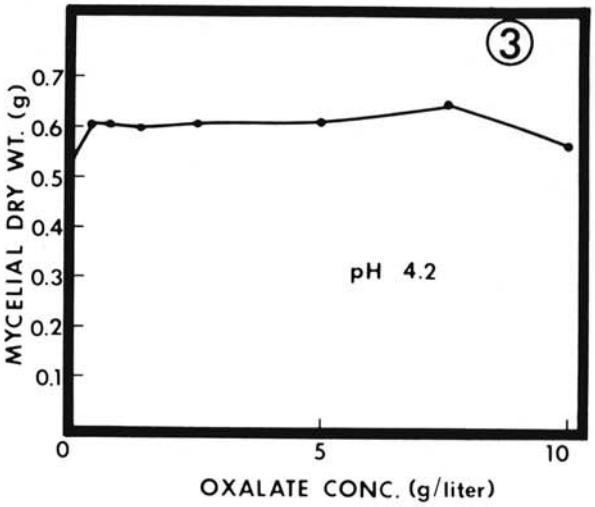

Fig. 3. *Sclerotium rolfsii* mycelial dry weight versus medium oxalate concentration in a basal salts-glucose (30 g/liter) broth with initial pH adjusted to 4.2.

recovered from peanut field soil showed >80% germination on oxalate-gallate medium, with no contaminating fungi radiating from the sclerotia. When *S. rolfsii* was plated on potato-dextrose agar, germination was less than 70%, and numerous sclerotia had mixed fungal populations and bacterial contamination.

DISCUSSION.—*Sclerotium rolfsii* grown on a basal salts-glucose medium produced oxalic acid initially followed by a reduction in oxalic acid levels. These data indicated that *S. rolfsii* can tolerate high concentrations of oxalate, and in later growth phases is able to metabolize it.

Incorporation of oxalate into agar medium showed that *S. rolfsii* could tolerate oxalate and grow, but hyphal extension was sparse and soon lysed, indicating that oxalate would not serve as the sole carbon source. Addition of glucose to the medium improved growth of *S. rolfsii*, but decreased selectivity to the point that it was not a usable medium. Addition of gallic acid to the oxalate medium (pH 4.2) provided excellent selectivity and growth of *S. rolfsii*, indicating that the following formula was best for selective isolation of *S. rolfsii* from contaminated sources:

1. KH_2PO_4	1.0 g
$Mg\ SO_4 \cdot 7H_2O$	0.5 g
KNO_3	2.0 g
Thiamine · HCl	1.0 mg
Minor element solution	10.0 ml
Gallic acid	160.0 mg
Potassium oxalate	10.0 g
Distilled water to 250 ml	
Filter-sterilize, adjust pH to 4.2 with HCl	
2. Agar	20.0 g
Distilled water to 750 ml	
Steam sterilize 15 minutes at 121 C, cool to 60 C	
3. Combine 1 with 2, pour plates immediately	

The major problem encountered in using this medium occurred when attempts were made to isolate *S. rolfsii* from large pieces of organic debris. The larger particles apparently provided a sufficient food source for contaminating fungi, particularly *Rhizopus* spp., which quickly overgrew the agar surface. When smaller debris particles were used, this problem did not occur.

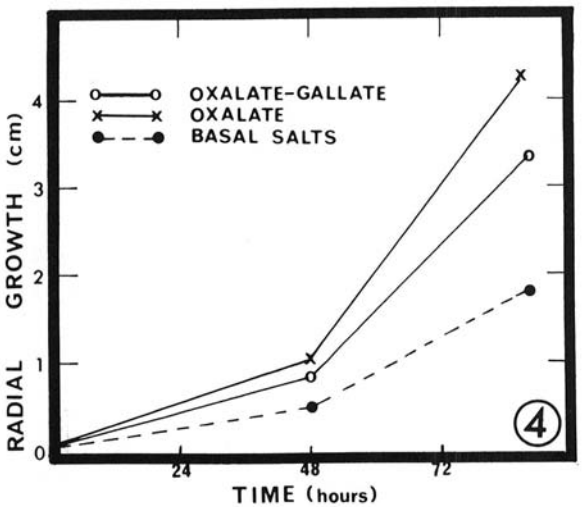

Fig. 4. Comparison of *Sclerotium rolfsii* radial growth on basal salts, oxalate, and oxalate-gallate agar media.

This medium allows for easy determination of viability of field-collected sclerotia of *S. rolfsii*. In addition, it may provide a means for determining the role played by mycelium in infested organic matter, both in perpetuation of the fungus, and serving as a primary inoculum source.

LITERATURE CITED

1. BAKER, C. J. L. 1952. The determination of oxalates in fresh plant material. Analyst 77:340-344.
2. BATEMAN, D. F., and S. V. BEER. 1965. Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by Sclerotium rolfsii. Phytopathology 55:204-211.
3. CURL, E. A., and J. D. HANSEN. 1964. The microflora of natural sclerotia of Sclerotium rolfsii and some effects upon the pathogen. Plant Dis. Rep. 48:446-450.
4. LEACH, L. D., and A. E. DAVEY. 1938. Determining the sclerotial population of Sclerotium rolfsii by soil analysis and predicting losses of sugar beet on the basis of these analyses. J. Agric. Res. 56:619-631.
5. MAXWELL, D. P., and D. F. BATEMAN. 1968. Oxalic acid biosynthesis by Sclerotium rolfsii. Phytopathology 58:1635-1642.
6. RODRIGUEZ-KABANA, R. 1967. An improved method for assessing soil-fungus population density. Plant Soil 26:393-396.
7. RODRIGUEZ-KABANA, R., P. A. BACKMAN, and E. A. WIGGINS. 1974. Determination of sclerotial populations of Sclerotium rolfsii in soil by a rapid flotation-sieving technique. Phytopathology 64:610-615.