Comparison of Three Media for Enumeration of Sclerotia of *Macrophomina phaseolina*

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ABSTRACT

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The selective medium RB (39 g/L of potato-dextrose agar, 100 mg/L of rifampicin, 224 mg a.i./L of metalaxyl, and 1.0 ml/L of nonoxynol [Tergitol NP-10]) was compared with MP and MSK, two selective media commonly used to enumerate sclerotia of *Macrophomina phaseolina*. The comparison was made with 20 field soils collected from Arkansas that were naturally infested with *M. phaseolina* and with a sterilized soil artificially infested with various levels of sclerotia. The population of sclerotia enumerated with RB was as high or higher than that enumerated with MP or MSK. MP enumerated more sclerotia in artificially infested sterilized soil, but enumerations with all three media reflected the relative levels of *M. phaseolina* in the soil. RB does not contain the fungicide chloroneb (as do MP and MSK) or mercuric chloride (as does MSK). Sclerotia can be counted on RB 3–14 days after plating, compared with no more than 4 days on MP and no fewer than 7 days on MSK.

Additional keywords: charcoal rot

In plant pathology, selective media have long been recognized as vital to research on inoculum density, population dynamics, saprophytic behavior, taxonomy, and survival of fungi. For a medium to be considered selective, the target microorganism must be specifically isolated or enhanced from the vast number of soilborne inhabitants (9). Selectivity is accomplished directly by inhibiting the growth of undesirable microorganisms with antimicrobial chemical agents, allowing only the targeted microorganism to grow unimpeded. Antibiotics and fungicides, often used individually or in combination, can suppress contaminating microorganisms. Selectivity can also arise from chemical compounds that stimulate the growth of the targeted organism in culture. An indirect approach to selectively isolating targeted organisms is the use of nontoxic chemical compounds, such as surfactants, that suppress the growth of all microorganisms growing in culture, allowing the recognition of the targeted organism.

The effectiveness of a selective medium may vary greatly among locations because of differences in soil microbial populations. *Macrophomina phaseolina* (Tassi) Goidanich, the causal agent of charcoal rot, is a soilborne fungal plant pathogen that exists in the soil primarily as sclerotia. Several media are reported to be selective for *M. phaseolina* (5,6). Selectivity of each of these media relies on the fungicide chloroneb (Demosan), which suppresses growth of oomycetes. The medium described by Meyer et al (5) also incorporates rose bengal, which suppresses colony size to make the identification of the targeted microorganism easier. Suppressive agents, including rose bengal, nonoxynol (Tergitol NP, Igepal CO, and Triton N), and polysorbate 80 (Tween 80) have been used extensively to reduce colony size (2,3,7,8).

This paper compares a selective medium for *M. phaseolina* developed at the University of Arkansas with two other selective media widely used with this fungus (5,6). Soils from a number of locations in Arkansas and sterilized soil artificially infested with sclerotia produced on vermiculite were used.

MATERIALS AND METHODS

The three media compared for ability to enumerate populations of *M. phaseolina* from soil were MP (6), MSK (5), and a new one designated RB. MP consisted of Difco potato-dextrose agar (39 g), chloroneb (100 mg a.i.), streptomycin sulfate (250 mg a.i.), and 1 L of deionized water (6). MSK consisted of rice agar (10 g), chloroneb (300 mg a.i.), mercuric chloride (7 mg), rose bengal (90 mg), streptomycin sulfate (40 mg a.i.), penicillin G (60 mg a.i.), and 1 L of deionized water (5). RB consisted of Difco potato-dextrose agar (39 g), rifampicin (100 mg), metalaxyl (224 mg a.i.) (Ridomil 2E-G), Tergitol NP-10 (1 ml), and 1 L of deionized water. Before being added, rifampicin was dissolved in 1 ml of dimethyl sulfoxide. When used, rifampicin, streptomycin sulfate, penicillin G, metalaxyl, and Tergitol NP-10 were added after the media were autoclaved and had cooled to 55 C.

Twenty soil samples, each approximately 1 kg, were taken from naturally infested soils from seven counties in Arkansas on 11 October 1989 (Table 1). Sites were chosen on the basis of crops previously grown, soil type, and location. Samples were taken within crop rows to 15-cm depths and at random locations, within a diamond-shaped pattern, over the entire field. The samples were stored at 25 C for 1 wk until processed.

Field soil (Zanesville, silt loam) that had been sterilized with two applications of methyl bromide (430 g/m^2 per application) was artificially infested with *M. phaseolina*. Inoculum of the fungus was produced on a mixture of 300 g of vermiculite and 2 L of Difco potato-dextrose broth in autoclavable bags. After autoclaving, each bag was inoculated with an isolate of *M. phaseolina* and incubated at 30 C for 1 mo. Soil dilutions were prepared by mixing infested soil with sterilized field soil at ratios of 100:0, 75:25, 50:50, 25:75, and 0:100 g.

Three subsamples, 10 g of field soil or 1 g of artificially infested soil, were taken from each soil sample to estimate the sclerotia populations of *M. phaseolina*. Three additional 10-g subsamples were placed in an oven (105 C) to determine percentage soil moisture. The procedure used to estimate the number of sclerotia of *M. phaseolina* was a modification of the procedure described by McCain and Smith (4). Each subsample was blended for three 30-sec intervals alternated with three 30-sec idle periods in 250 ml of 0.5% NaOCl. The soil slurry was passed through a 0.045-mm sieve, and the debris on the sieve was rinsed for 2 min under distilled water, then washed into a beaker with 25 ml of water. The contents of each beaker were mixed with 100 ml of MSK (5), 100 ml of MP (6), or 90 ml of RB, then dispensed into six 100-mm-diameter plastic petri dishes. The MP and RB plates were incubated at 30 C for 4 days and the MSK plates for 7 days before colonies of *M. phaseolina* were counted.

Data were analyzed by analysis of

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Table 1. Comparison of three selective media for enumeration of sclerotia of *Macrophomina phaseolina* in 20 naturally infested Arkansas field soils

Previous crop	Soil type[x]	County	Medium[y] (sclerotia/g dry soil) RB	MP	MSK
Soybean	Grenada SiL	Jefferson	24.3 a[z]	21.9 a	10.9 b
	Bowie FSiL	Hempstead	74.7 a	43.3 b	16.5 c
	Pickwick SiL	Washington	115.5 a	90.1 b	73.9 c
	Calhoun SiL	St. Francis	62.1 a	35.2 b	41.5 b
Sorghum	Pheba SiL	Jefferson	27.7 a	10.6 b	17.5 a
	Bowie FSiL	Hempstead	60.5 a	12.1 b	22.2 b
	Zanesville SiL	Washington	119.3 a	55.8 c	88.0 b
	Calhoun SiL	St. Francis	67.4 a	41.5 a	33.8 a
Corn	Crowley SiL	Lonoke	28.0 a	11.6 a	13.9 a
	Loring SiL	Lee	32.9 a	19.8 a	19.9 a
	Bowie FSiL	Hempstead	50.1 a	15.8 b	25.7 b
	Crowley SiL	Lonoke	73.0 a	34.3 a	43.2 a
Rice	Portland SiL	Jefferson	24.6 a	8.2 a	7.5 a
	Hebert SiL	Lonoke	3.8 a	1.8 a	3.4 a
	Hebert SiL	Lonoke	9.6 a	12.5 a	11.2 a
Wheat	Sawyer L	Hempstead	33.5 a	9.3 b	11.2 b
Cotton	Roxana SiL	Jefferson	28.0 a	12.5 b	16.6 ab
	Loring SiL	Lee	3.3 a	1.4 a	1.8 a
	Smithdale FSiL	Lincoln	4.4 b	1.3 b	7.9 a
	Loring SiL	Lee	26.6 a	17.4 a	10.5 a

[x]F = fine, L = loam, S = sand, Si = silt.
[y]RB = 39 g of potato-dextrose agar, 100 mg of rifampicin, 224 mg a.i. of metalaxyl, 1 ml of Tergitol NP-10, and 1 L of deionized water; MP = as described by Mihail and Alcorn (6); MSK = as described by Meyer et al (5).
[z]Means of three replications followed by the same letter within a soil are not significantly different ($P = 0.05$) according to ANOVA and LSD.

variance of the number of sclerotia per soil sample for each of the three media. Mean separations were obtained at the $P = 0.05$ level by the least significant difference (LSD) (1).

RESULTS

The efficacy of the three media compared across the 20 field soils by analysis of variance showed significant differences among soils ($P < 0.01$), among media ($P < 0.01$), and in interactions of soil and medium ($P < 0.01$). In all 20 soils, population estimates of *M. phaseolina* were either highest with RB or not significantly different from the highest estimate, with the exception of cotton soil collected in Lincoln County (Table 1). Population estimates were generally lower with MP and MSK than with RB. In six soils, MP and MSK showed significant differences, but population estimates were significantly higher with MP in only three of the six. With RB and MSK, colony diameters of all fungi rarely exceeded 1.5–2.0 cm and did not interfere with enumerating sclerotia of *M. phaseolina*. Contaminating fungi were suppressed up to 4 days with MP. After 4 days, however, the overgrowth of contaminating fungi obscured colonies of *M. phaseolina*, making enumeration impossible. Colonies could not be enumerated on MSK until 7 days after plating but could be counted as late as 14 days after plating. Colonies on RB could be enumerated as early as 3 days and as late as 14 days after plating.

In artificially infested soil, MP had the highest population estimates, but these were significantly higher than estimates with RB only at the 50:50 dilution (Table 2). Population estimates with all three media reflected the dilution series.

DISCUSSION

The RB medium was as effective, or more so, in enumerating populations of *M. phaseolina* in field soil as the currently used selective media MP and MSK (Table 1). In only one of the 20 soils, which had a very low population of *M. phaseolina*, did RB give a lower population estimate than MSK (Table 1). This difference may have been due to sampling variability caused by the low population level of the fungus. The media showed no significant differences, however, in two other soils with low population levels—one of the rice soils from Lonoke County and the cotton soil from Lee County. More variability in population estimates was observed between MP and MSK than with RB. MP and MSK differed significantly with each other in six of the 20 soils, but differences were not consistent (Table 1). MP gave higher estimates than MSK in one-half of the soils, and MSK gave higher estimates than MP in the other half. Differences were not associated with cropping history, soil type, location, or population levels of *M. phaseolina*.

In artificially infested soil, the three media reflected the dilution series equally well, although MP generally had the highest sclerotia counts (Table 2).

Besides giving higher sclerotia counts in field soils and being more consistent over a range of soils, the RB medium has other advantages over the MP and MSK media. Accurate counts can be made over a longer period of time (3–14 days), making RB more flexible in scheduling research activities than the other media. Unless counts are made 3–4 days after plating with the MP medium, the colonies become obscured by contaminating fungi. With the MSK medium, counts cannot be made earlier than 7 days after plating. In addition, MSK contains mercuric chloride, which is highly toxic and presents disposal problems. RB does not contain mercuric chloride. One disadvantage of the RB medium is the cost—45% greater than that of MP or MSK.

This study shows that the RB medium is as effective, or more so, in enumerating sclerotia of *M. phaseolina* in the soil as the other commonly used selective media. Flexibility in use and the lack of toxic chemicals are other advantages of this medium.

Table 2. Comparison of three selective media for enumeration of sclerotia of *Macrophomina phaseolina* from dilutions of artificially infested field soil

Soil dilution[x]	Medium[y] (sclerotia/g dry soil) RB	MP	MSK
100:0	128.96 ab[z]	191.66 a	114.30 b
75:25	99.90 ab	145.00 a	90.13 b
50:50	61.80 b	124.33 a	57.70 b
25:75	45.80 a	52.00 a	29.56 b
0:100	0.00 a	0.00 a	0.00 a

[x]Infested:noninfested. Inoculum was produced on a mixture of 300 g of vermiculite and 2 L of potato-dextrose broth in autoclavable bags and incubated at 30 C. After 1 mo, inoculum was added to sterilized soil (Zanesville silt loam).
[y]RB = 39 g of potato-dextrose agar, 100 mg of rifampicin, 224 mg a.i. of metalaxyl, 1 ml of Tergitol NP-10; and 1 L of deionized water; MP = as described by Mihail and Alcorn (6); MSK = as described by Meyer et al (5).
[z]Means of three replications followed by the same letter within a dilution are not significantly different ($P = 0.05$) according to ANOVA and LSD.

LITERATURE CITED

1. Anonymous. 1988. SAS User's Guide: Statistics. Version 6.03 ed. SAS Institute, Cary, NC. 1,028 pp.
2. Honour, R. C., and Tsao, P. H. 1974. Production of oospores by *Phytophthora parasitica* in liquid medium. Mycologia 66:1030-1038.
3. Martin, J. P. 1949. Use of acid, rose bengal, and streptomycin in the plate method for estimating soil fungi. Soil Sci. 69:213-232.
4. McCain, A. H., and Smith, R. S., Jr. 1972. Quantitative assay of *Macrophomina phaseoli* from soil. Phytopathology 62:1098.
5. Meyer, W. A., Sinclair, J. B., and Khare, M. N. 1973. Biology of *Macrophomina phaseoli* in soil studied with selective media. Phytopathology 63:613-620.
6. Mihail, J. D., and Alcorn, S. M. 1982. Quantitative recovery of *Macrophomina phaseolina* sclerotia from soil. Plant Dis. 66:662-663.
7. Ritchie, D. F., and Klos, E. J. 1978. Differential medium for isolation of *Erwinia amylovora*. Plant Dis. Rep. 62:167-169.
8. Sneh, B. 1972. An agar medium for the isolation and macroscopic recognition of *Phytophthora* spp. from soil on dilution plates. Can. J. Microbiol. 18:1389-1392.
9. Tsao, P. H. 1970. Selective media for isolation of pathogenic fungi. Annu. Rev. Phytopathol. 8:157-186.