Isolation and Quantitative Determination of Macrophomina phaseolina from Soil

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ABSTRACT

Selective media and a method were developed for the direct isolation of *Macrophomina phaseolina* from soil, and for the quantitative estimation of its inoculum density in soil. The selective basal agar contained commercial potato-dextrose agar and 25 and 100 mg/liter of chlortetracycline hydrochloride and streptomycin sulfate, respectively. The best combinations of other antimicrobial agents added to the basal medium were *p*-(dimethylamino) benzenediazo sodium sulfonate (DASS) + oxgall + rose bengal and DASS + oxgall + pentachloronitrobenzene. The isolation method involved wet-sieving of soil and exposure of selected sieve residues to NaClO solution for 8 minutes before aliquots were pipetted from the final dilutions onto the surface of the selective media. The method was used with naturally and artificially infested soils. Recovery of sclerotia from artificially infested soils was between 70 and 80 percent. Numbers of sclerotia in naturally infested soils ranged from none to more than 1,000/g soil. Most colonies from naturally infested soils originated from free sclerotia in soil.

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Macrophomina phaseolina (Tassi) Goid. [*Rhizoctonia bataticola* (Taub.) Butler] causes charcoal rot on corn, sorghum, soybean, and other economic crop plants (1, 4, 6). Apparently the pathogen survives in debris and soil as very small black sclerotia (2, 3, 6).

There are only a few reports on the quantitative isolation of *M. phaseolina* from soil. Watanabe et al. (6) developed a differential flotation technique for assaying populations of *M. phaseolina* sclerotia in pine nursery soils. Recently, Meyer et al. (4) described two selective media with rice agar as the basal medium to isolate *M. phaseolina* from soil and soybean debris. Selectivity in these two media was based on the addition to the basal medium of chloroneb (1,4-dichloro-2,5-dimethoxybenzene), rose bengal, and methoxymethylmercury chloride or mercuric chloride.

The present investigation describes a dilution-plate method and selective media for isolating *M. phaseolina* from soil and estimating its inoculum density.

MATERIALS AND METHODS.—Isolate Mp-1 was obtained from L. D. Dunkle, University of Nebraska; Mp-3 from T. D. Wyllie, University of Missouri; and Mp-7 from L. K. Edmunds, Kansas State University. Isolate Mp-10 was obtained with the dilution-plate method by the senior author from infested silty clay loam collected from a cornfield near Odelle, Nebraska.

Sclerotia were produced in the laboratory by a modification of a technique described by Bega and Smith

TABLE 1. Recovery of *Macrophomina phaseolina* from soil infested with sclerotia by the dilution-plate method as affected by various combinations of antimicrobial agents

Antimicrobial agent added to basal medium[a]	Concentration (mg/liter active)	Sclerotia/g oven-dry soil	
		Isolate Mp-1	Isolate Mp-7
None (control)		24 B[b]	14 B
Chloroneb + oxgall + rose bengal	300 + 1,500 + 100	250 A	130 A
Chloroneb + oxgall + chloramine-T[c]	300 + 1,000 + 500	240 A	140 A
Chloroneb + oxgall + DASS[d]	300 + 1,500 + 50	240 A	116 A
Chloroneb + chloramine-T + PCNB[e]	300 + 500 + 100	260 A	128 A
DASS + oxgall + PCNB	50 + 2,000 + 100	266 A	158 A
DASS + oxgall + rose bengal	50 + 1,500 + 150	246 A	134 A

[a]Basal medium consisted of potato dextrose agar containing per liter 25 mg chlortetracycline hydrochloride and 100 mg streptomycin sulfate.

[b]Numbers followed by the same letter are not significantly different ($P = 0.05$) by Duncan's multiple range test.

[c]Sodium *p*-toluenesulfonchloramide.

[d]*p*-(Dimethylamino) benzenediazo sodium sulfonate (DASS).

[e]Pentachloronitrobenzene.

(1). Cellophane disks (9-cm diameter) were boiled for 30 minutes in distilled water, washed with fresh distilled water, and autoclaved while floating in distilled water. Disks were laid over 15 ml of Difco potato-dextrose agar (PDA) in petri plates and inoculated at the center. The plates were incubated in the dark at 30 C for 6 days. To obtain sclerotia, the disks were lifted from the agar, the sclerotia were scraped from the cellophane with cotton swabs, the scrapings were comminuted in distilled water in a blender for 10 seconds, and the sclerotia were collected on filter paper. After the sclerotia were dried at room temperature, they were separated from the paper with a sterile spatula and passed through a 177-μm sieve (80-mesh). Clumps of sclerotia and mycelial fragments retained on the sieve were discarded, and individual sclerotia free of mycelia that passed the sieve were used in subsequent studies.

For germination studies, sclerotia were surface-disinfested with 0.25 or 0.5% NaClO solution, rinsed with sterile distilled water several times, and plated out on the surface of PDA or the selective media in petri plates. The plates were incubated at 30 C, and sclerotia were observed under a dissecting microscope for germination after 1, 2, 3, and 4 days.

Four replications were used throughout, and all experiments were repeated three times. For the soil isolation experiments, a complete extraction from a given soil constituted a replication.

RESULTS.—*The selective media.*—After several initial experiments on several kinds of basal media, pH values of media, and kinds and quantities of antimicrobial agents, six media were selected for isolation of *M. phaseolina* from soil (Table 1). The basal medium for all six media contained, per liter, PDA (29 g/liter, no pH adjustment); chlortetracycline hydrochloride, 25 mg; and streptomycin sulfate, 100 mg. The two antibiotics and the antimicrobial agents in Table 1 were added to autoclaved PDA when it cooled to 50-60 C. The media were poured into petri plates (15 ml/plate) 1 to 2 days before the assays, and the plates were kept at 25 C in the dark until used.

To compare the six media, natural soils were infested with individual isolates of *M. phaseolina* (ca. 300 sclerotia added/g soil), and soil dilutions of 1:10 were prepared after infestation. With two isolates, all media recovered high numbers of sclerotia from soil (Table 1). *M. phaseolina* colonies were small, gray-black to black, and easily identifiable; and isolations could easily be made directly from the dilution plates.

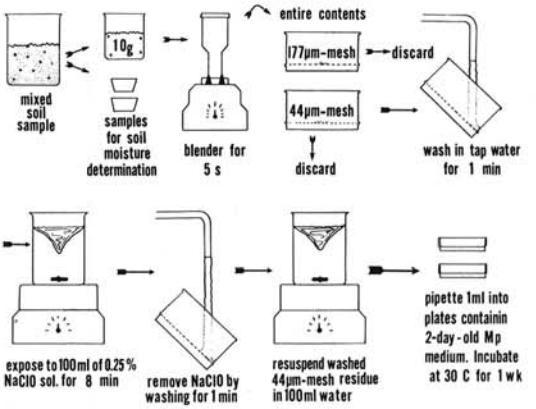

Fig. 1. Schematic diagram of the procedure used for the isolation of *Macrophomina phaseolina* from soil with the dilution-plate method.

From the six media, we finally selected the last two in Table 1. These were designated PDA-DOPCNB (basal medium containing *p*-(dimethylamino) benzenediazo sodium sulfonate [DASS, Dexon], oxgall, and pentachloronitrobenzene [PCNB] at 50, 2,000, and 100 mg/liter, respectively) and PDA-DORB (basal medium containing DASS, oxgall, and rose bengal at 50, 1,500, and 150 mg/liter, respectively). The other four media, containing chloroneb and chloramine-T (sodium *p*-toluenesulfonchloramide) were excellent for isolations, but undesirable for continuous laboratory use because their vapors irritated the eyes, nose, and throat.

The isolation method.—First, we experimented on sieve sizes and on pretreatment of sieve fractions with heat or antimicrobial agents to reduce competitive

TABLE 2. In vitro germination of sclerotia and recovery of *Macrophomina phaseolina* from soil infested with 200 sclerotia/g soil, as affected by length of exposure of final sieve fractions to 0.25% solution of NaClO

Length of exposure (minutes)	Germination of sclerotia on PDA-DORB[a] (%)				Recovery[b] from soil infested with specified isolates (%)		
	Mp-1	Mp-3	Mp-7	Mp-10	Mp-1	Mp-3	Mp-7
0	95	96	94	96	30	24	20
4	99	88	34	96	94	64	60
8	98	86	20	94	90	72	65
12	94	84	20	95	90	72	66
16	90	80	16	94	88	70	60

[a]PDA + *p*-(dimethylamino) benzenediazo sodium sulfonate + oxgall + rose bengal.

[b]Dilutions of 1:20 were used on 20 petri plates of PDA-DORB.

microorganisms without affecting *M. phaseolina*. After experiments on these methods and on temperatures of incubation, an isolation procedure was adopted (Fig. 1). The final procedure is a modification of a method used for the isolation of *Sclerotium cepivorum* (5).

For artificially infested soils, 10 g of soil in 100 ml of sterile distilled water were comminuted for 5 seconds in a microblender at high speed (ca. 3,000 rpm). The entire contents were passed through a 177-μm pore size sieve (80-mesh) in tandem with a 44-μm pore size sieve (325-mesh). Residue on the 177-μm sieve was discarded, and the residue on the 44-μm sieve was washed with running tap water for 1 minute. The washed residue on the 44-μm sieve was concentrated in one side of the sieve and transferred by using a squeeze bottle to a 250-ml beaker containing 100 ml of 0.25 or 0.5% NaClO solution. After exposure to NaClO solution, the beaker contents were washed for 1 minute with running tap water on a 44-μm sieve to remove the NaClO. They were resuspended in 100 ml sterile distilled water to produce a 1:10 dilution.

Aliquots of 1.0 ml were removed from the final dilution, while the liquid was agitated, and pipetted onto the surface of the 1- to 2-day-old PDA-DORB or PDA-DOPCNB medium. Then the liquid was spread to cover the entire agar surface. The plates were incubated in the dark at 30 to 32 C and examined for *M. phaseolina* colonies after 6 to 7 days.

With naturally infested soils, the 177-μm sieve fractions were not discarded, but were treated the same as the 44-μm sieve fractions. With naturally infested soils, two sets of dilution plates were prepared, one from the 177-μm

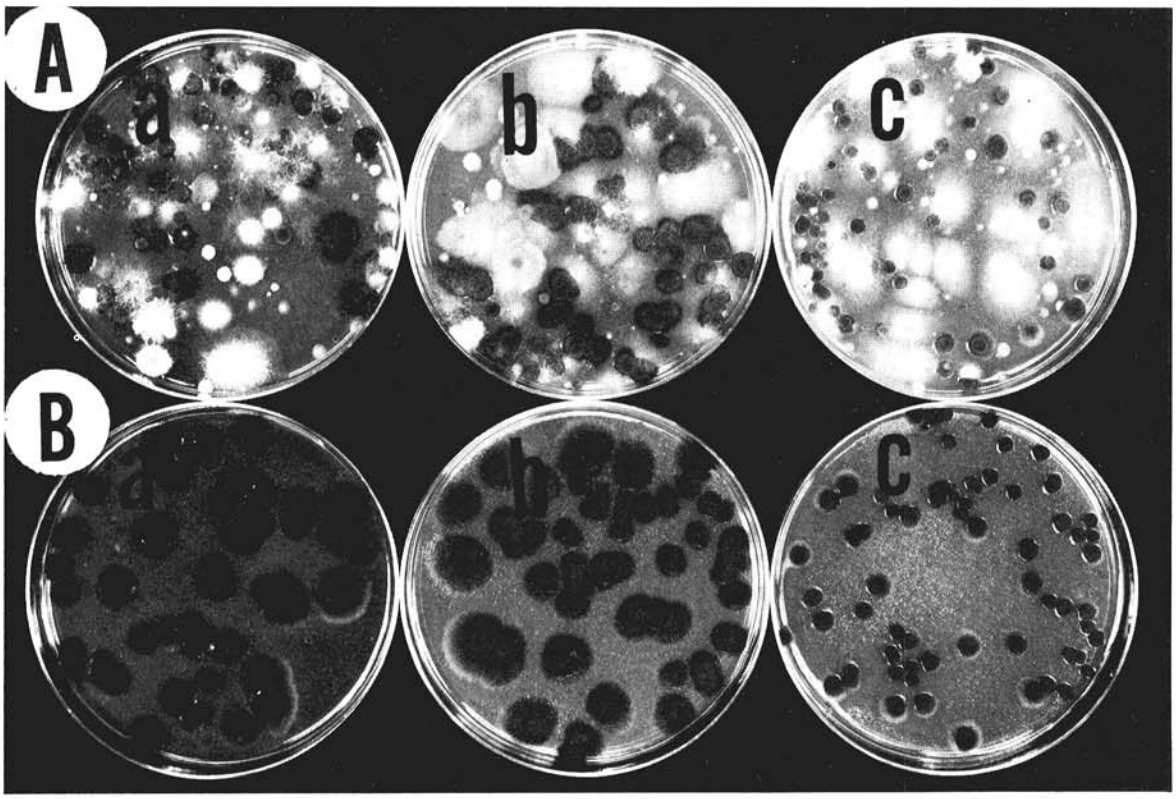

Fig. 2. Effect of NaClO on recovery of *Macrophomina phaseolina* from soil. (A) No NaClO; (B) 44-μm (325-mesh) sieve residue exposed to 0.25% NaClO solution for 8 minutes. A_a and B_a, PDA + *p*-(dimethylamino) benzenediazo sodium sulfonate (DASS) + oxgall + rose bengal; A_b and B_b, PDA + DASS + oxgall + pentachloronitrobenzene (PCNB); A_c and B_c, PDA + chloroneb + oxgall + chloramine-T.

TABLE 3. Recovery of sclerotia of *Macrophomina phaseolina* by the dilution-plate method on the PDA-DORB (PDA + DASS + oxgall + rose bengal) medium from soil to which various amounts of sclerotia of two isolates were added

Wt (mg) of sclerotia added/1.5 kg soil	Sclerotia/g soil: Estimated no. added	Sclerotia/g soil: No. reisolated[a] on PDA-DORB	Recovery (%)
Isolate Mp-1			
3.2	3	2	66
6.4	6	6	100
12.5	12	8	66
25.0	25	18	72
50.0	50	54	108
100.0	100	74	74
200.0	200	170	85
400.0	400	418	104
			Avg 84 ± 20[b]
Isolate Mp-7			
3.2	3	2	66
6.4	6	5	83
12.5	12	10	84
25.0	25	18	72
50.0	50	34	68
100.0	100	68	68
200.0	200	82	41
400.0	400	242	60
			Avg 68 ± 27[b]

[a]Dilutions of 1:10 were used for all concentrations of sclerotia. Average of three trials.
[b]Standard error of the mean, $P = 0.05$.

TABLE 4. Average number of viable sclerotia of *Macrophomina phaseolina*/g soil in naturally and artificially infested soils, as determined by the dilution-plate method on the PDA-DORB (PDA + DASS + oxgall + rose bengal) medium

Soil origin	Sclerotia/g oven-dry soil[a]: Debris inoculum (177-μm sieve residue)	Sclerotia/g oven-dry soil[a]: Soil inoculum (44-μm sieve residue)	Total[b] inoculum
Illinois No. 1	8	48	80
Illinois No. 2	4	24	32
Maryland No. 1[c]	530	890	1,300
Maryland No. 2	0	2	2
Missouri No. 1	8	36	54
Missouri No. 2	13	68	92
Nebraska No. 1	28	30	30
Nebraska No. 2	0	2	2
Nebraska No. 3	4	8	18
Nebraska No. 4[d]	250	780	1,030
Nebraska No. 5	0	0	2

[a]Dilutions of 1:10 were used on 20 petri plates for each sample except for Maryland No. 1 and Nebraska No. 4 soils.
[b]Total inoculum was determined by dilution of the 44-μm sieve residue, without the use of 177-μm sieve (Fig. 1).
[c]Soil had been artificially infested with sclerotia and planted to soybean several months before assay.
[d]Corn stalks with sclerotia of *M. phaseolina* were added to soil before assay (M. G. Boosalis, *personal communication).*

TABLE 5. Origin of colonies of *Macrophomina phaseolina* on dilution plates from two naturally infested soils prepared with the PDA-DORB (PDA + DASS + oxgall + rose bengal) medium

Soil and sieve fraction	Origin of colonies (%): From sclerotia	From organic debris	Undetermined
Illinois No. 1:			
177-μm sieve residue	62	38	0
44-μm sieve residue	75	21	4
Illinois No. 2:			
177-μm sieve residue	50	50	0
44-μm sieve residue	100	0	0

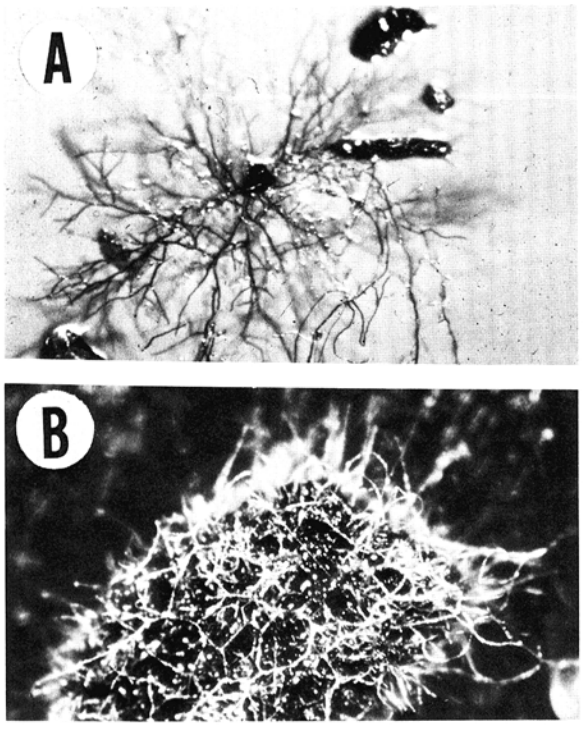

Fig. 3-(A, B). Origin of colonies on dilution plates from a soil naturally infested with *Macrophomina phaseolina*. **A)** Colony originating from a single sclerotium (36 hours after dilution, × 21.6). **B)** Colony originating from a debris particle (48 hours after dilution, × 13).

sieve fraction, and the second from the 44-μm sieve fraction. The number of colonies/g of the two fractions were combined to obtain the total number of propagules/g soil (oven-dry basis).

In vitro germination of sclerotia and recovery of M. phaseolina from soil as affected by length of exposure to NaClO.—Sclerotia of three isolates could tolerate up to 16 min of exposure to 0.25% NaClO solution without losing their germinability (Table 2). Even exposure to 0.5% NaClO solution for up to 8 minutes did not reduce germinability of the sclerotia of isolates Mp-1, Mp-3, and Mp-10. Germinability of isolate Mp-7 was considerably reduced by the NaClO treatment.

Although wet-sieving, washing with tap water, and using antimicrobial agents in PDA-DORB or PDA-DOPCNB reduced competition from soil microbes to *M. phaseolina* on the dilution plates, fungal colonies which developed on the surface of the plates obscured the pathogen. To improve the isolation technique, the final sieve fractions were exposed to 0.25% NaClO for 0, 4, 8, 12, and 16 minutes before the final dilutions.

Exposure for up to 16 minutes did not reduce recovery of two isolates of *M. phaseolina* on the PDA-DORB medium (Table 2); and the exposure to NaClO reduced competition from other fungi. Even recovery of the sensitive isolate Mp-7 was improved with the use of NaClO solution. *M. phaseolina* colonies appeared discrete and the plates were almost free of other organisms when NaClO solution was used (Fig. 2). An 8-minute exposure to 0.25% NaClO solution was adopted for subsequent experiments.

Effectiveness of the dilution-plate method.—Tests were performed to evaluate the effectiveness of the isolation technique (Fig. 1) as a quantitative dilution-plate method. Batches of known weights of dry sclerotia of several isolates were counted to determine the number of sclerotia/mg. Increasing weights of sclerotia were then added to 1.5-kg soil samples to produce increasing concns of sclerotia ranging from 3 to 400 sclerotia/g soil (Table 3).

The average percentage of sclerotia recovered was 84% with isolate Mp-1, with a range of 104-64 at $P = 0.05$. With isolate Mp-7, more sensitive to NaClO than Mp-1, the average percentage recovery was 68%, with a range of 95-41.

Recovery from infested soils.—Eleven soils naturally or artificially infested with *M. phaseolina* were assayed with the dilution-plate method on the PDA-DORB medium. The 177-μm sieve fractions from these soils were exposed to 0.25% NaClO solution for 8 minutes and dilutions were prepared as for the 44-μm sieve fractions. Besides the inoculum determined separately in the 177-μm sieve and 44-μm sieve fractions, total inoculum for the soils was also estimated by use of only a 44-μm sieve to collect the residue that would normally be fractionated into two portions (177-μm and 44-μm sieve portions). Except for Nebraska No. 2 soil, high numbers of sclerotia were recovered from the 177-μm sieve fractions. Because this fraction consisted mainly of debris particles, the inoculum in that fraction was referred to as "debris inoculum." The inoculum in the 44-μm sieve fraction was referred to as "soil inoculum."

With all soils tested, the soil inoculum was higher than the debris inoculum (Table 4). The populations of *M. phaseolina* estimated as debris, soil, or total inoculum were very low in some soils and very high in others, ranging from 2 to more than 1,000 sclerotia/g soil. Maryland No. 1 and Nebraska No. 4 soils had very high populations. Both soils had been artificially infested with sclerotia and cropped to soybeans several times before the dilution-plate assays.

Origin of colonies in naturally infested soils.—The identity of the surviving propagules of *M. phaseolina* in naturally infested soils was determined by direct observation of developing colonies on dilution plates of the PDA-DORB medium. With a dissecting microscope of low magnification (× 10), small colonies were spotted within 24 to 48 hours from the start of each experiment. Colony centers were then examined carefully with high magnification (× 40) to determine the origin of colonies developing on the agar.

Most colonies on dilution plates from two naturally infested Illinois soils came from single sclerotia of *M. phaseolina* (Table 5). It could not be ascertained by the microscope whether colonies developing around debris particles originated from single sclerotia, mycelia, or both (Fig. 3). A higher percentage of colonies originated from single sclerotia in the 44-μm sieve fraction than in the 177-μm sieve fraction. Similar results were obtained with other naturally infested soils.

DISCUSSION.—It appeared from limited

observations in the literature that *M. phaseolina* was a poor competitor on agar plates (2). Even a few colonies of saprophytic fungi growing on agar next to *M. phaseolina* colonies prevented or reduced growth of the latter and masked its colonies or inhibited production of sclerotia needed for identification. These difficulties were resolved with the development and standardization of selective media and a rapid technique for isolation and enumeration. The success of soil bioassays for *M. phaseolina* depended upon: (i) mechanical concentration of sclerotia and debris infested with sclerotia by wet-sieving, (ii) reduction of saprophytic fungi by washing the sieve residues, (iii) elimination of bacteria by antibiotics, and (iv) exposure of the final sieve fractions to NaClO to eliminate or reduce saprophytes without damaging *M. phaseolina* sclerotia. NaClO was used also by McCain and Smith (3). This assay technique may be used for survival studies in soil, and for determining inoculum density of *M. phaseolina* and correlating this with disease development.

The data obtained with naturally infested soils showed that field soils may vary in inoculum density from very few to more than 10 propagules/g soil. Most of the soils averaged between 10 and 100 propagules/g soil. These data substantiated observations by others (2, 6) who reported small numbers of sclerotia/g soil. These observations on the low inoculum density of *M. phaseolina* in field soils are not new with sclerotial soil-borne plant pathogens. Papavizas (5) found fewer than 10 viable sclerotia of *S. cepivorum* in naturally infested soils from various parts of the United States.

More than 60% of developing colonies of *M. phaseolina* on dilution plates from field soils originated from sclerotia; therefore, it seems likely that the pathogen survives in soil as sclerotia. A smaller percentage of colonies originated from debris particles that remained on the 177-μm sieve. This observation would suggest at least two things. First, the 177-μm sieve fractions should not be discarded in wet-sieving assays, if accurate determinations of inoculum density are to be made in field soils. Second, the number of free sclerotia in soil may be related to the stage of crop-residue decomposition. The smaller the degree of residue decomposition, the lower the number of sclerotia to be found in soil. This observation agrees with that of Cook et al. (2), who observed that sclerotia of *M. phaseolina* could survive in corn and sorghum stalk residues in soil for 16 to 18 months. More studies are needed to determine the length of time of survival of sclerotia in soil and crop residues.

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