Inoculum Potential of Macrophomina phaseolina

G. E. Short and T. D. Wyllie

Former Postdoctoral Fellow and Professor, respectively, Department of Plant Pathology, University of Missouri-Columbia, Columbia, MO 65201.

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ABSTRACT

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Sodium hypochlorite was used to dissolve the melanin-like cementing agent that engulfs sclerotial cells of *Macrophomina phaseolina*. Then sclerotia were 'squashed' to enable enumeration of cells of the propagules. The number of cells per sclerotium was directly related to size of sclerotia; and sclerotium size appeared to depend on the available nutrients of the substrate on which the propagules were produced. Large sclerotia produced considerably more germ tubes than did small sclerotia when germinated on culture media. Sclerotia of *M. phaseolina* were sensitive to soil fungistasis; but in the spermosphere of soybean, sclerotia germinated within 2-3 mm of the seed surface and produced one to seven germ tubes per germinated sclerotium.

Additional key words: charcoal rot, decolorized sclerotia.

Macrophomina phaseolina (Tassi) Goid causes a root and stem rot of many agronomic crops, including soybean, corn, and sorghum (4, 13). Multicellular sclerotia (26) of variable size (11) enable the fungus to survive adverse environmental conditions (4) and later serve as sources of inoculum for infection (3, 21). Techniques have been described for determination of population densities of sclerotia in soil (11, 12, 14, 25); however, the number of cells per sclerotium have not been determined. Such information may more precisely elucidate the relationship between inoculum density and disease severity (2, 5).

The objectives of this investigation were: (i) to examine the relation between substrate, size of sclerotia, and number of cells per sclerotium; and (ii) to examine germination of sclerotia on culture media and in spermosphere (17, 23) soil.

MATERIALS AND METHODS

Production and treatment of sclerotia.—Sclerotia of *M. phaseolina* strain S (27) were produced in flasks containing 50 ml of potato-dextrose broth (PDB). After 2-3 wk of incubation at 33 C, sclerotial mats were rinsed three times with 100 ml of sterile water, air-dried, gently ground with a mortar and pestle, and placed on a 90-μm (170-mesh) sieve. Sclerotia were collected on a series of sieves (170, 200, 270, 325, and 500 mesh) according to size (>90 μm, 75-90 μm, 54-74 μm, 45-53 μm, and 25-44 μm diameter, respectively). Sclerotia also were produced in small quantities on 0.2% water agar, and size determinations were made microscopically using an ocular micrometer. Sclerotia from soybean stems [*Glycine max* (L.) Merr. 'Amsoy 71'] were collected by splitting the stem longitudinally, removing the pith containing the sclerotia, passing the pith and sclerotia through a Wiley mill using a 246-μm (60-mesh) sieve (27), and then sieving as with the PDB culture-produced sclerotia.

Sclerotia were decolorized for cell enumeration as follows: individual sclerotia were placed in a drop of 6.0% sodium hypochlorite on a microscope slide and covered with a coverslip. After 10-30 min, a slight amount of pressure was applied to the coverslip to 'squash' the sclerotium for cell counting.

Germination of sclerotia.—Sclerotia were incubated at 16, 24, or 32 C on 2.0% water agar (WA), potato-dextrose agar (PDA), or chloroneb-mercuric chloride-rose bengal agar (CMRA) (12). Sclerotia were examined microscopically every 24 hr to determine percentage germination and number of germ tubes per germinated sclerotium. Sclerotia were observed for 2 wk when incubated at 16 C. When both parameters ceased to increase, microscopic observations were discontinued.

Sclerotium germination also was observed in the spermosphere of soybean using a modification of a technique previously described (17). A sandy loam soil (58% sand, 27% silt, and 15% clay) was infested with sclerotia with diameters of 45-54 μm or 74-90 μm collected from stem tissue and from PDB culture, respectively. Amsoy 71 soybean seeds were planted in soil columns (17) 1 cm below the soil surface; soil moisture was established and maintained at 75% of moisture holding capacity. Sclerotia were incubated for 2 days at 32 C or for 4 days at 16 C following seed placement. Soil was embedded in agar, and then blocks of agar-embedded soil containing sclerotia were removed at defined millimeter increments from the seed (17) and placed on a microscope slide in a drop of 6.0% sodium hypochlorite

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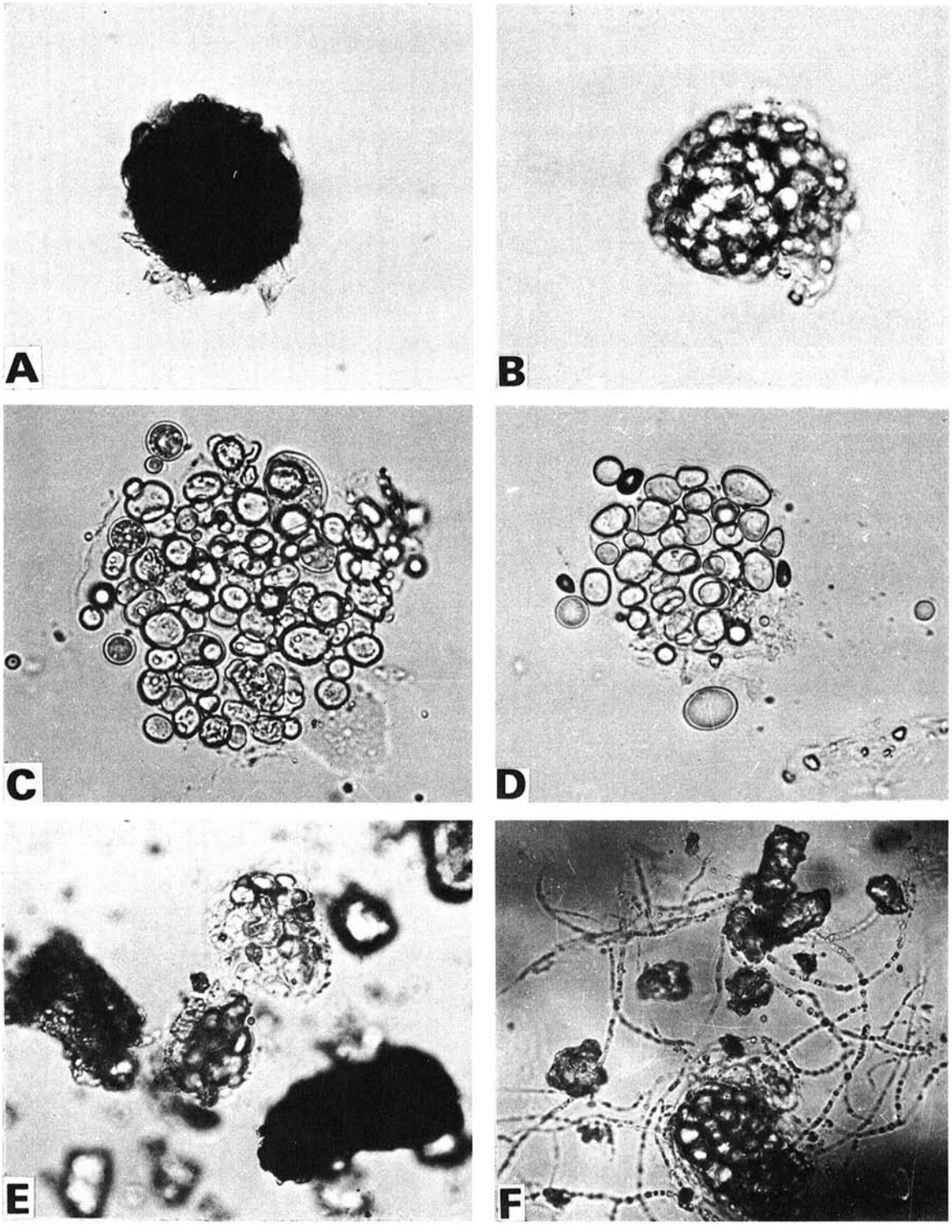

Fig. 1-(A to F). Effect of sodium hypochlorite on sclerotia of *Macrophomina phaseolina*. **A)** Nontreated sclerotium (×1,100). **B)** Sodium hypochlorite-treated sclerotium (×1,100). **C-D)** Sclerotia decolored in sodium hypochlorite and 'squashed' (×1,100). **E)** Decolored, nongerminated sclerotia on soil smear (×1,100). **F)** Decolored, germinated sclerotium on soil smear (×1,100).

to decolor the sclerotia (16). The slide was warmed slightly to melt the agar, and a soil smear was made for microscopic examination of sclerotium germination.

RESULTS

Sclerotia of *M. phaseolina* were thoroughly decolored by 6.0% sodium hypochlorite in less than 0.5 hr (Fig. 1-A, B). The apparent dissolution of the black, melanin-like cementing agent (6) also permitted the sclerotia to be 'squashed' to such an extent that individual cells of the propagules could be counted easily (Fig. 1-C, D). Decoloring sclerotia on soil smears facilitated a positive identification of nongerminated (Fig. 1-E) and germinated (Fig. 1-F) sclerotia. Cells of sclerotia decolored in 1.2% to 6.0% sodium hypochlorite were no longer viable.

Effect of substrate on inoculum.—The mean diameter and standard deviation of 100 sclerotia produced on PDB (130 ± 30 μm) was significantly greater than when sclerotia were produced on 0.2% WA (63 ± 8 μm) or in stems of naturally-infected field-grown soybeans (70 ± 18 μm). Indeed, 74% of the sclerotia produced on PDB had diameters greater than 90 μm; whereas, all of the sclerotia produced on 0.2% WA or naturally-infected stems of soybeans had diameters of less than 75 μm (Table 1). Finally, the number of cells per sclerotium increased directly with size of sclerotia (Table 1).

Relation of inocula characteristics to sclerotium germination.—Large sclerotia (74-90 μm diameter) produced twice as many germ tubes when germinated on 2.0% WA as did smaller sclerotia (45-53 μm diameter) (Table 2). Since it was difficult to count all of the germ tubes produced by some sclerotia, a maximum of 10 germ tubes per germinated sclerotium was counted; therefore, the total number of germ tubes per germinated sclerotium probably was underestimated, particularly among the large (74-90 μm diameter) sclerotia, and among sclerotia germinating on PDA, where rapid growth obscured later-emerging germ tubes. Temperature affected the rapidity of sclerotium germination, but the final number of germ tubes was not affected. Particulate matter and color of CMRA, and microbial contamination on PDA, resulted in selecting WA as the most suitable medium to assay for germinability.

Germination of sclerotia (74-90 μm diameter) in soil at 32 C was confined primarily to a zone within 2 mm of the soybean seed (Table 3), with one to seven germ tubes produced by each germinated sclerotium. When smaller sclerotia (45-53 μm diameter), obtained from stems of naturally-infected soybeans, were incubated in soil at 16 C, sclerotium germination was not observed beyond 2 mm of the seed surface.

DISCUSSION

Severity of diseases caused by soilborne pathogens is dependent upon many factors, including the inoculum potential of the infective propagules in the soil (2, 5). Inoculum potential measures the ability of the pathogen to infect its host (5). Garrett (8) and Baker (2) expressed this ability in terms of biological energy available for infection. To measure inoculum potential in fundamental units of biological energy would be ideal, but in this instance, energy is difficult to measure in an absolute sense (2). Consequently, biological energy has been expressed as a function of the genetic capacity of the pathogen, environmental factors, available nutriment (both internal and external to the propagule), and inoculum density (2). Incidence of disease caused by *M. phaseolina* has been directly related to the population of sclerotia in soil (13, 24), but the sclerotium population is an extremely crude measure of inocula such as with sclerotia of *M. phaseolina*, which are multicellular (Fig. 1-A, B, C, D) and capable of producing multiple germ tubes (Table 2, Fig. 1-F) and capable of repeated germination. Our method of decolorizing and 'squashing' sclerotia has made it possible to estimate the inoculum potential of this pathogen on a numerical cellular basis.

Sodium hypochlorite previously has been used to

TABLE 1. In vivo and in vitro production of sclerotia by *Macrophomina phaseolina*: relation of propagule size to number of cells per propagule

Substrate	Characteristics of sclerotia		
	Diameter range (μm)	Proportion[a] (%)	Cells per sclerotium (no.)
Potato dextrose broth	25-44	1	27 (14-44)[b]
	45-53	1	59 (28-98)
	54-74	6	79 (53-102)
	75-90	18	109 (76-195)
	>90	74	214 (128-320)
Soybean tissue	25-44	27	27 (11-43)
	45-53	22	47 (35-75)
	54-74	51	69 (43-104)
0.2% water agar	25-44	11	24 (14-43)
	45-53	47	
	54-74	42	

[a] Based on 100 sclerotia per substrate.
[b] Mean and range of 25 sclerotia per treatment.

decolorize microsclerotia of *Verticillium albo-atrum* (16), which then were stained and cleared to reveal the origin of germ tubes. However, the method has not been used widely, even though our experience with *M. phaseolina* and other sclerotia-producing fungi suggests a wide applicability. For example, the difficulty in differentiating between microsclerotia and certain soil particles on modified soil smears (15) was resolved easily with hypochlorite–each sclerotium was seen as a distinct clump of cells unlike anything else in the soil (Fig. 1-E, F). Unfortunately, the hypochlorite treatment was lethal to the cells; thus, the ability of each cell to produce a germ tube could not be tested directly. However, larger sclerotia produced more germ tubes than smaller sclerotia (Table 2), and sclerotia of *M. phaseolina* are capable of repeated germination (Short and Wyllie, *unpublished*). It is not known if single cells are capable of either multiple or repeated germination. Regardless, it should be possible to determine indirectly the relationship between cell number and potential number of germ tubes per sclerotium.

Sclerotia of *M. phaseolina* buried in nonamended soil failed to germinate unless suitable nutrients were added (3, 22). Smith (22) found the amino acid fraction of root exudate from sugar pine seedlings to be particularly stimulatory to germination, and *M. phaseolina* is known to use nearly all amino acids for growth (7). Soybean seed exudates contain sucrose and fructose (9) compounds also known to stimulate germination of sclerotia of *M. phaseolina* (1). Sclerotia of *M. phaseolina* have been reported to germinate in the rhizosphere of pine seedlings (21) and we have observed sclerotium germination in the spermosphere of soybean seeds (Table 3). The maximum distance from the soybean seed at which sclerotia germinated (2-3 mm) was considerably less than with chlamydospore germination of *Fusarium solani* (17, 23) in the spermosphere of pea (7-8 mm) and bean (10-12 mm). Sclerotia of *M. phaseolina* require nearly 24 hr to germinate under optimum conditions, in contrast to the 4-5 hr required for chlamydospore germination of *F. solani* f. sp. *phaseoli* (23). Competition for nutrients (20) at the onset of sclerotium germination (24 hr) was

TABLE 2. Effect of source and size of sclerotia, substrate, and temperature on germination of sclerotia of *Macrophomina phaseolina*

Characteristics of sclerotia		Germination substrate[w]	Temp (C)	Germination of sclerotia (%)	Germ tubes per germinated sclerotium (apparent) (no.)
Source	Diameter (μm)				
Strain S[x]	45-53	PDA	16	31 a[z]	3.0 a
		PDA	32	33 a	3.2 a
Field[y]	45-53	WA	16	37 ab	6.4 b
		WA	32	37 ab	4.8 ab
Strain S	45-53	CMRA	24	49 ab	6.3 b
		WA	24	52 b	5.0 ab
Strain S	45-53	WA	16	52 b	5.0 ab
		WA	32	57 b	4.4 ab
Strain S	75-90	WA	16	90 c	9.8 c
		WA	32	91 c	8.7 c

[w]Potato-dextrose agar (PDA), 2.0% water agar (WA), or chloroneb-mercuric chloride-rose bengal agar (CMRA).
[x]Sclerotia produced in potato-dextrose broth.
[y]Sclerotia collected from stems of field-grown, naturally-infected soybeans.
[z]Means in each column followed by the same letter do not differ significantly ($P = 0.05$) according to Tukey's test.

TABLE 3. Germination of *Macrophomina phaseolina* sclerotia[z] at 32 C in the spermosphere of Amsoy 71 soybean

Distance from seed (mm)	Germination of sclerotia (%)	Germ tubes per germinated sclerotium (apparent) (no.)
0-1	42	2.2
1-2	16	2.2
2-3	3	2.5
3-4	0	0.0

[z]Sclerotia (74-90 μm diameter) were obtained from PDB cultures of *M. phaseolina* strain S.

probably much more intense than at the onset of *F. solani* f. sp. *phaseoli* chlamydospore germination (5 hr). Other factors of possible importance include soil moisture and temperature (17, 23), seed quality (18, 19), and fungistatic level of the propagule (10).

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