Quantitative Enumeration of Macrophomina phaseolina in Soybean Tissues

G. E. Short, T. D. Wyllie, and V. D. Ammon

Respectively, former Postdoctoral Fellow and Professor, Department of Plant Pathology, University of Missouri-Columbia, Columbia, MO 65201; and Assistant Professor, Department of Plant Pathology and Weed Science, Mississippi State University, State College, MS 39762. Contribution of the Missouri Agricultural Experiment Station. Scientific Journal Series Paper No. 7848.

Accepted for publication 14 Oct 1977.

ABSTRACT

SHORT, G. E., T. D. WYLLIE, and V. D. AMMON. 1978. Quantitative enumeration of Macrophomina phaseolina in soybean tissues. Phytopathology 68: 736-741.

Macrophomina phaseolina penetrated roots of soybean plants that ranged in age from 7 to 42 days. The fungus invaded cortical tissue intercellularly; later, intracellular invasion occurred and that was followed by the formation of sclerotia. The large size of the sclerotia, the apparent disruption of host cell walls by sclerotia, and the appearance of sclerotia in stem tissues only after loss of color suggested that sclerotia in tissues are indicative of host cell death. A technique was developed for determining mycelial and sclerotial propagative units in infected soybean tissue. The method was used to measure disease development in field- and greenhouse-grown soybeans. Mycelial propagative units always exceeded sclerotia until moribundicity of the host was apparent. Sclerotia were not abundant in root or stem tissues until after the onset of host moribundicity. Following plant death, sclerotia were most numerous in the roots, less abundant in the lower one-half of the stem, and least numerous in the upper one-half of the stem. We suggest that differential propagule enumeration in diseased tissues quantitatively measures the degree of compatibility between selected soybean cultivars and *Macrophomina phaseolina*.

Additional key words: charcoal rot, *Glycine max*.

Charcoal rot, which is caused by *Macrophomina phaseolina* (Tassi) Goid, is a widespread root and stem disease of over 300 plant species (10), including soybean [*Glycine max* (L.) Merr.]. Disease development is favored by high temperature (30-35 C) and moisture stress (8, 14). The mycelium of the fungus penetrates and colonizes root tissues of young seedlings (1, 5), and subsequently invades stem tissues. Small, black, multicellular sclerotia are formed within infected host tissues following anthesis (21).

The conversion of mycelia into sclerotia (22) during the maturation and senescence of host tissues suggested that sclerotia might be indicative of host cell death. The objectives of this investigation were to enumerate populations of mycelia and sclerotia propagules in infected soybean plants with time, and to determine whether the appearance of sclerotia is indicative of moribundicity of host cells. A preliminary report has been published (18).

MATERIALS AND METHODS

Heat and chemical treatments of fungal propagules.—Stems of field-grown soybeans containing sclerotia of *M. phaseolina* were ground in a Wiley mill fitted with a 370 μm (40-mesh) screen. Sclerotia were extracted from the plant debris by dry-sieving using a series of screens (200-, 270-, and 325-mesh) with openings of 74, 53, and 44 μm, respectively; thus, sclerotia with diameters of 45-53 μm also were separated from sclerotia with diameters of 54-74 μm. Sclerotia were submerged in distilled water at 50 C for 15 or 30 min, or in 0.5% sodium hypochlorite for 5 or 10 min. Heat- or sodium hypochlorite-treated sclerotia were placed on Millipore filters in a Büchner funnel and rinsed with sterile distilled water. Nontreated sclerotia served as controls. One hundred sclerotia per treatment were individually transferred onto a solidified, cloroneb-mercuric chloride-rose bengal selective medium (CMRA) (13) as modified by Moustafa (16). Some sclerotia were heat-treated by immersion in CMRA at 50 C for 20 min, after which the agar containing the sclerotia was poured into petri dishes and allowed to solidify. The sclerotia in all treatments were incubated at 33 C on CMRA for 1 wk. Viability of sclerotia was determined by their ability to germinate and produce a colony.

Mycelia of *M. phaseolina* also were subjected to heat or sodium hypochlorite. Blocks of potato-dextrose agar (PDA) with mycelia but no sclerotia were removed from the periphery of a growing colony of *M. phaseolina* and submerged in distilled water at 50 C for 0, 5, 10, 15, or 20 min. For sodium hypochlorite treatments, Millipore filters were placed on PDA next to a growing colony of *M. phaseolina*; these filters were removed after mycelium had grown over the surface of the filter. Filters containing mycelia, but no sclerotia, were immersed on 0.5% sodium hypochlorite for 0, 15, 30, or 60 sec, and rinsed in large volumes of sterile distilled water. Following heat or sodium hypochlorite treatment, agar blocks and

00032-949X/78/000124$03.00/0

Millipore filters containing mycelia were placed on solidified CMRA and incubated at 33 C for 1 wk to determine the viability of mycelia.

Light microscopy of infected tissues.—Seed of soybean cultivars Amsoy and Adelphia was surface sterilized in 1% sodium hypochlorite for 5 min followed by three successive rinses in sterile distilled water and then germinated on water agar. The resulting plants were transferred aseptically to clay pots containing sterile vermiculite. The pots were covered with Parafilm and placed in the greenhouse. Sterile Hoagland's solution was added to each pot twice; once at the beginning of the experiment, and again 7 days later. Daily watering with sterile distilled water through the Parafilm was discontinued after 9 days. Subsequent daily irrigation was done with unsterilized tap water. Amsoy and Adelphia plants 1, 2, 3, 7, 10, 14, 28, and 42 days old were inoculated by seeding each pot with 75-100 ml of a 3-day-old shake culture of *M. phaseolina* in Czapek's Dox broth. Plants within each age group were incubated with *M. phaseolina* for 1, 2, 3, 4, 6, 8, 10, and 15 days. Plants of both cultivars were harvested along with noninoculated controls, inspected, photographed, and fixed in formalin-acetic acid-alcohol (FAA). Not all of the fixed root samples were prepared for microscopic examination. A representative sample was selected, dehydrated in a *t*-butyl alcohol series, and embedded in paraffin. Thick sections (10 μm) were cut with a rotary microtome and stained with safranin O and fast green.

Determination of propagule populations in host tissues.—Five soybean seeds (cultivars Amsoy 71 and A-100) were planted in 15-cm-diameter clay pots containing soil infested with 128 sclerotia/g of soil. The clay pots were buried in a moist sand bed maintained at 30 ± 2 C using a heating cable. Ambient air temperature in the greenhouse was maintained at 30 ± 5 C. Uniform physiological maturity of seedlings was achieved by roguing all but two seedlings per pot 2 wk after planting. All rogued seedlings were infected with *M. phaseolina* as determined by isolation of the pathogen.

Amsoy 71 seeds also were planted on 12 May 1976 in soil containing 42 sclerotia/g on the Bradford Experimental Research Farm, Columbia, MO. All soybean seedlings were infected with *M. phaseolina* 4 wk after planting, as determined by isolation of the pathogen.

In field and greenhouse studies four to eight plants of each cultivar were sampled at 3-wk intervals for 24 and 33 wk, respectively. Roots were washed in running tap water, surface sterilized in 0.5% sodium hypochlorite, and rinsed in distilled water; nodules containing nitrogen-fixing bacteria were removed. Each root system was air-dried for 1-2 days, ground in a Wiley mill fitted with a 370 μm (40-mesh) screen, and divided into three samples (Fig. 1). One sample was immersed in molten CMRA at 50 C for 20 min, poured into petri dishes, and incubated. As root tissues died and were invaded by other soil organisms, sodium hypochlorite (0.5% for 10 min) was substituted for the heat treatment (Fig. 1); this was necessary to eliminate secondary invaders and to facilitate counting colonies of *M. phaseolina* (17). A second sample was added to molten CMRA at 45 C, poured immediately into petri dishes, and incubated. A third sample was used for moisture determination so that propagules per gram of tissue could be expressed on an oven-dry weight basis.

On 8 October 1976, stems of Amsoy 71 plants in the field experiment were excised at ground level, cut in half to give upper and lower portions, and further subdivided into nodal and internodal tissues (9). Each tissue sample was processed as previously described (Fig. 1).

TABLE 1. Effect of heat and sodium hypochlorite on viability of sclerotia of *Macrophomina phaseolina* from field-grown soybeans

Diameter of sclerotia (μm)	Treatment		Viability of sclerotia (%)
	Type	Duration (min)	
45-53	Heat[b]	15	43
		30	38
	Hypochlorite[c]	5	38
		10	49
	Control	...	44
54-74	Heat	15	64
		30	53
	Hypochlorite	5	53
		10	55
	Control	...	60

[a]Determined by ability to germinate on a cloroneb-mercuric chloride-rose bengal selective medium. Treatment means for either size did not differ significantly [LSD (P = 0.10)].
[b]Deionized water at 50 C.
[c]Formulation: 0.5% sodium hypochlorite.

RESULTS

Cytological observations.—After 1 day of incubation, *M. phaseolina* had penetrated the roots of soybean plants ranging in age from 7-42 days. Cortical invasion proceeded intercellularly (Fig. 5); the entire depth of cortex of Amsoy 71 (most susceptible) and Adelphia (least susceptible) (9) roots was colonized intercellularly within 3 days following inoculation. Later, intracellular invasion occurred followed by formation of sclerotia (Fig. 6) within 1 week following inoculation. The large size of the sclerotia, the apparent disruption of host cell walls by sclerotia (Fig. 7), and the notable absence of sclerotia in green stem tissues suggested that formation of these propagules is probably indicative of host cell death.

Sensitivities of mycelia and sclerotia to heat and sodium hypochlorite.—About 50% of the mycelium colonies survived for 10 min in water at 50 C, but none survived for 15 min at 50 C. Sclerotium viability was unaffected by 15 or 30 min in either water or CMRA at 50 C (Table 1). Similarly, although mycelia were killed by 15 sec or more in 0.5% sodium hypochlorite, sclerotia withstood that treatment up to 10 min (Table 1). Furthermore, sensitivity of sclerotia to heat and sodium hypochlorite was not affected by propagule size.

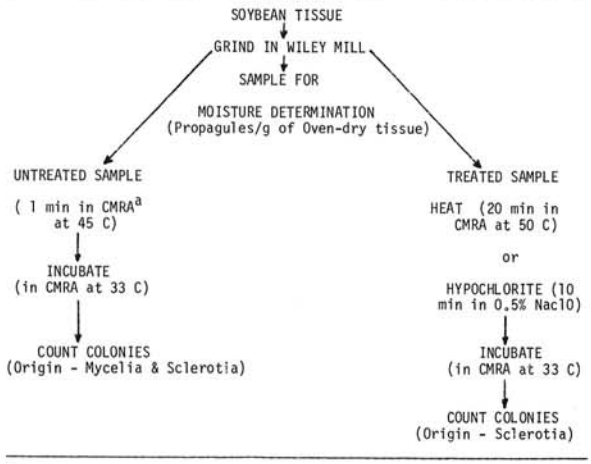
The differential sensitivity of propagules to heat and sodium hypochlorite treatment was exploited to enumerate mycelium and sclerotium propagules in soybean tissues during disease development. Prior to the late pod stage of plant development, root tissues were heat-treated in CMRA at 50 C for 20 min; thereafter,

tissues were treated by immersion in 0.5% sodium hypochlorite for 10 min to eliminate secondary invaders (primarily *Fusarium* spp.), rinsed for 30 min in sterile distilled water, and added to CMRA. Treated and nontreated samples were incubated in CMRA at 33 C for 1 wk. Colonies produced from heat- or sodium hypochlorite-treated samples arose from sclerotia; colonies produced from nontreated samples arose from both mycelium and sclerotium propagules (Fig. 1). By subtracting the number of colonies of the treated sample from the number of colonies of the nontreated sample, an estimate of the number of mycelial propagative units was obtained.

Disease development.—Surface-sterilized, intact root systems were plated on solidified CMRA 3 wk (Fig. 2, 3) or 4 wk (Fig. 4) after planting. All root systems were colonized by *M. phaseolina*, and usually mycelium growth was continuous along the length of the roots, indicating that infection was not localized. The rate of plant growth, development, and maturation appeared to be identical for plants of cultivars Amsoy 71 (maturity group II) and A-100 (maturity group I) in the greenhouse. However, Amsoy 71 plants required approximately 8 wk longer to mature in the greenhouse than in the field; for example, complete senescence of all plants was observed 22 wk after planting in the field (Fig. 4) compared to 30 wk in the greenhouse (Fig. 3).

Sclerotia were not abundant in root tissue until after the onset of senescence (leaf-drop); this occurred 21 wk (Fig. 2, 3) and 16 wk (Fig. 4) after planting in the greenhouse and field, respectively. Sclerotia initially were most abundant toward the periphery of the root, and later also were plentiful toward the center of the root.

Mycelial propagative units always were more abundant than sclerotia until after the onset of senescence. The decline in mycelial propagules 24 wk (Fig. 3) and 27 wk (Fig. 2) after planting is partially an artifact. During the senescence of greenhouse-grown plants, colonies of *Fusarium* spp. (from nontreated root tissue cultured on CMRA) eventually outnumbered colonies of *M. phaseolina*, and at times masked the presence of *M. phaseolina*. However, *Fusarium* spp. were far less prevalent in nontreated root tissue from field-grown soybeans, where no decline in mycelial propagules was



[a]Chloroneb-mercuric chloride-rose bengal agar.

Fig. 1. Procedural scheme for determining mycelial and sclerotial propagative units of *Macrophomina phaseolina* in infected soybean root and stem tissue

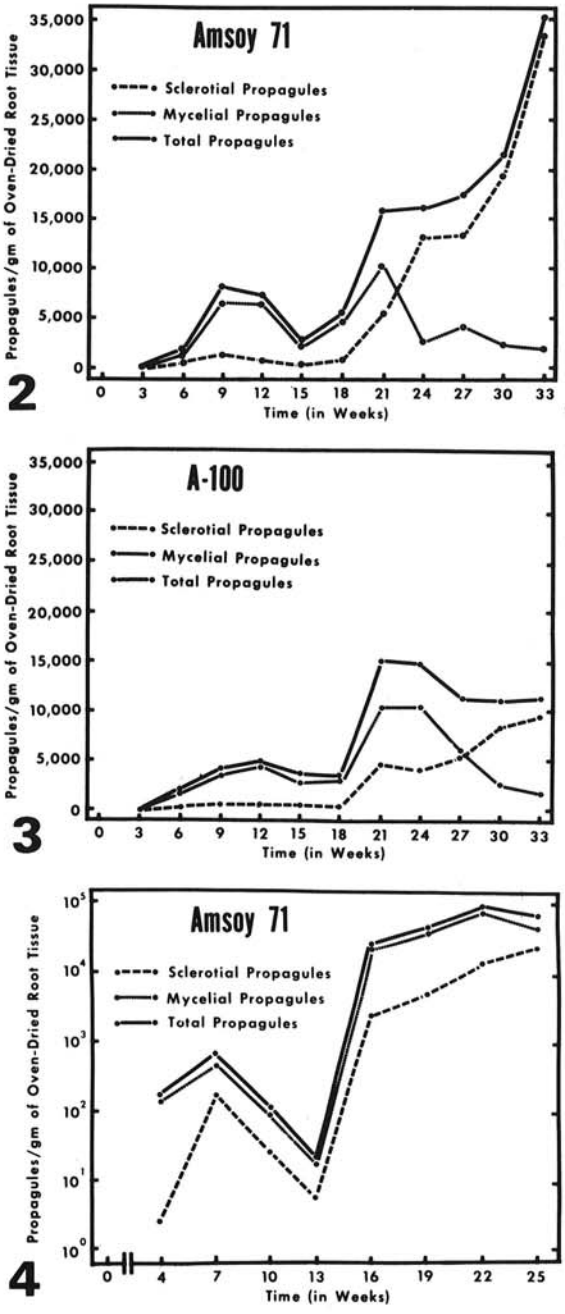


Fig. 2-4. Relation of time to charcoal rot disease development based on mycelial and sclerotial propagative units of *Macrophomina phaseolina* in roots of two soybean cultivars: **2)** cultivar Amsoy 71 grown in greenhouse, **3)** cultivar A-100 grown in greenhouse, and **4)** cultivar Amsoy 71 grown in field in 1976.

TABLE 2. Populations of mycelial and sclerotial propagative units of *Macrophomina phaseolina* in field-grown Amsoy 71 soybean plants differing in rate of senescence

Extent of plant senescence[y]		Propagules/g of root tissue[z]		
Stems	Leaves	Sclerotia (no.)	Mycelia (no.)	Total (no.)
Brown	Brown	2,295 ± 2,848	23,698 ± 6,748	25,993 ± 7,222
Green	Brown	112 ± 74	5,476 ± 4,263	5,588 ± 4,328
Green	Green	109 ± 60	511 ± 462	620 ± 501

[y]Determined by color of stems and leaves 16 wk after planting.
[z]Means and standard deviations of means.

TABLE 3. Populations of sclerotia of *Macrophomina phaseolina* in root and stem tissues of Amsoy 71 soybeans 22 wk after planting in the field

Tissue	Sclerotia/g of tissue[a]	
	Mean (no.)	Range (no.)
Nodes (upper half of stem)	73	(22-103)
Internodes (upper half of stem)	356	(21-948)
Nodes (lower half of stem)	1,706	(33-6,079)
Internodes (lower half of stem)	1,330	(305-2,253)
Root	12,416	(3,478-46,339)

[a]Mean and range.

observed until late senescence (Fig. 4). Apparently, the gradual decline in mycelial propagules in moribund roots from the greenhouse (Fig. 2 & 3) was caused by a high *Fusarium* population that masked *M. phaseolina* colony development on CMRA. That mycleium was present in the tissues is proven by the continued increase in the pathogen population as measured by numbers of sclerotia (Fig. 3).

The relation of moribundicity to disease development was also examined in the field. On 1 September, 16 wk after planting, Amsoy 71 plants were observed to be senescing at different rates. Populations of *M. phaseolina* were determined in roots of plants which apparently were healthy (green stems and leaves), dying (green stems, but brown leaves), or apparently dead (brown stems and leaves) (Table 2). Both mycelial and sclerotial propagative units were least in plants that appeared healthy, and greatest in plants that appeared dead. Populations in root tissues obtained in subsequent sampling periods (Fig. 4) indicated that colonization by mycelia and formation of sclerotia continued even after apparent death of the plant.

Stem segments from greenhouse-grown plants were plated periodically on CMRA to determine upward progression of *M. phaseolina*. Upward movement was extremely limited until after the onset of moribundicity. For example, the percentage of colonized stem pieces located between the cotyledonary nodes and the primary leaf nodes increased with each 3-wk interval as follows: 8, 17, 25, 38, 56, 66, 83, 92, 100%. Thus, more than 24 wk were required for *M. phaseolina* to reach the stem of all plants. A similar phenomenon occurred in the field. Populations of sclerotia in senescent root and stem tissues were greatest in the root and least near the stem apex (Table 3).

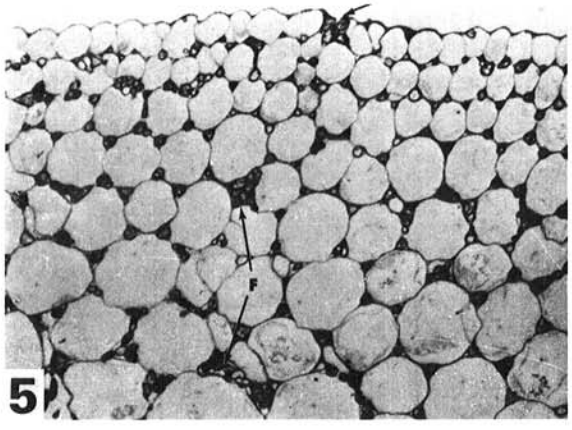
DISCUSSION

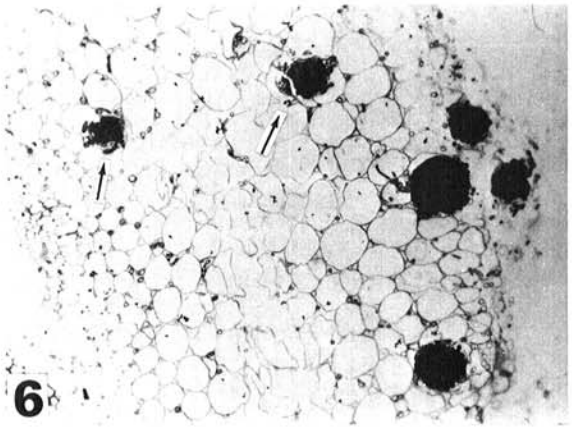
The extent of fungal colonization of living plant tissue frequently has been determined by sectioning the tissues into segments and placing them on selective media (7, 20). This technique later was refined (15) by grinding infected tissue segments in a Wiley mill before incorporation into a selective medium for propagule enumeration. Our procedure permitted the differentiation of propagule types in ground tissue. Unfortunately, size of tissue fragments was variable; thus, mycelial propagative units were variable in size, and presumably some fragments contained more than a single sclerotium. Despite these limitations, the method provided an indication of the extent of tissue colonized as well as quantitative estimates of mycelia and sclerotia in the tissue. Sclerotia of *M. phaseolina* has been shown to be fairly resistant to heat (3) and sodium hypochlorite (17), but this is the first report of the sensitivity of the vegetative mycelium to these treatments.

During the initial stages of pathogenesis, the mycelium penetrated the root epidermis, but was restricted primarily to the intercellular spaces of the cortex (Fig. 5). Later, the fungus invaded host cells intracellularly. Recognizable host organelles disappeared (1) and sclerotia were formed within the host cells, frequently disrupting host cell walls (Fig. 7). Fully-formed sclerotia are metabolically quiescent propagules, which no longer live at the expense of the host (2, 6, 19). Thus, similar to *Sclerotium rolfsii* (4), *M. phaseolina* appears to have two ecologically distinct phases–a mycelial, or parasitic phase, and a sclerotial, or nonparasitic phase. The gradual shift from a parasitic (mycelial) to an increasingly nonparasitic (sclerotial) existence of *M. phaseolina* in soybean (Fig. 2, 3, 4) may be indicative of moribundicity of the host.

The population of sclerotia in roots and stems of a moribund host might be indicative of the degree of compatibility between host and pathogen. Indeed, sclerotial populations of *M. phaseolina* in Amsoy 71 (highly susceptible) roots were two to three times greater

than in A-100 (less susceptible) roots (Fig. 2, 3). Perhaps the greater amount of disease in Amsoy 71 (maturity group II) soybeans than in A-100 (maturity group I) soybeans caused Amsoy 71 plants to senesce simultaneously with A-100 plants. The populations of sclerotia in individual roots were extremely variable; a maximum range of 582 to 17,074 sclerotia/g of root tissue was observed 19 wk after planting in the field. This variability might be due to genetic, physiological, and environmental factors influencing the longevity of the life of the plant, or possibly to differences in multiple host genes in individual plants that result in different levels of compatibility to *M. phaseolina*. It will not be possible to determine which of these possibilities may be the true explanation of these data until much more information is gained concerning the biological, physiological, and genetic parameters that influence the ultimate phenotypic expression of this particular host-pathogen interaction. A new approach to plant breeding (11, 12) that enumerated mycelial and sclerotial propagative units in soybean tissues might prove useful in screening for general resistance to *M. phaseolina* on a single-plant basis (12) as a beginning in solving these problems.





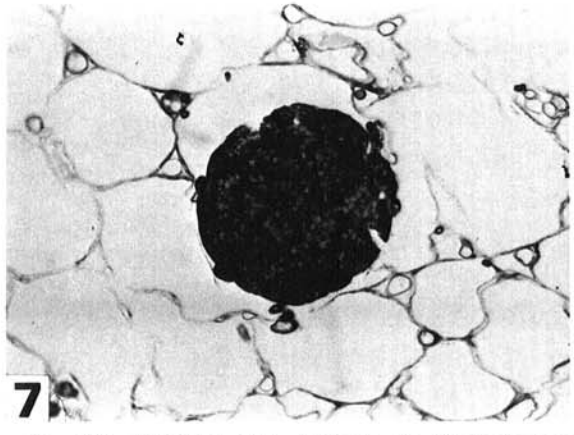


Fig. 5-7. Mycelium and sclerotia of *Macrophomina* phaseolina in soybean tissues. 5) Cross section through infected tissue. Mycelium initially restricted to intercellular space. Note arrows. ×130. 6) Cross section through 10-day-old soybean root tissue which was incubated with *Macrophomina phaseolina* for 7 days. Note intracellular invasion of tissue in the form of sclerotia. Note arrows ×150. 7) Cross section through soybean tissue. Note mature sclerotium causing cellular disruption. ×600.

LITERATURE CITED

1. AMMON, V., T. D. WYLLIE, and M. F. BROWN, JR. 1974. An ultrastructural investigation of pathological alterations induced by Macrophomina phaseolina (Tassi) Goid in seedlings of soybean, Glycine max (L.) Merrill. Physiol. Plant Pathol. 4:1-4.
2. AYANRU, D. K. G., and R. J. GREEN, JR. 1974. Alteration of germination patterns of sclerotia of Macrophomina phaseolina on soil surfaces. Phytopathology 64:595-601.
3. BEGA, R. V., and R. S. SMITH. 1962. Time-temperature relationships in thermal inactivation of sclerotia of Macrophomina phaseoli. Phytopathology 52:632-635.
4. BOYLE, L. W. 1961. The ecology of Sclerotium rolfsii with emphasis on the role of saprophytic media. Phytopathology 51:117-119.
5. BRISTOW, P. R., and T. D. WYLLIE. 1975. The effect of crop rotation and date of planting on charcoal rot of soybean. Proc. Am. Phytopathol. Soc. 2:83 (Abstr.).
6. CHET, I., and Y. HENIS. 1975. Sclerotial morphogenesis in fungi. Annu. Rev. Phytopathol. 13:169-192.
7. DHINGRA, O. D., and J. B. SINCLAIR. 1974. Effect of soil moisture and carbon:nitrogen ratio on survival of Macrophomina phaseolina in soybean stems in soil. Plant Dis. Rep. 58:1034-1037.
8. EDMUNDS, L. K. 1964. Combined relation of plant maturity, temperature, and soil moisture to charcoal stalk rot development in grain sorghum. Phytopathology 54:514-517.
9. GANGOPADHYAY, S., and T. D. WYLLIE. 1973. Biochemical comparison of nine soybean varieties in relation to their susceptibility to Macrophomina phaseolina. Indian J. Mycol. Plant Pathol. 3:131-140.
10. GHAFFAR, A., and G. A. ZENTMYER. 1968. Macrophomina phaseolina on some new weed hosts in California. Plant Dis. Rep. 52:223.
11. GOODE, M. J., and J. L. BOWERS. 1973. Breeding for anthracnose resistance in cucumber. Phytopathology 63:442 (Abstr.).
12. LOEGERING, W. Q., D. A. SLEPER, J. M. JOHNSON, and K. H. ASAY. 1976. Rating general resistance on a single-plant basis. Phytopathology 66:1445-1448.
13. MEYER, W. A., J. B. SINCLAIR, and M. N. KHARE. 1973. Biology of Macrophomina phaseoli in soil studied with selective media. Phytopathology 63:613-620.
14. MEYER, W. A., J. B. SINCLAIR, and M. N. KHARE.

1974. Factors affecting charcoal rot of soybean seedlings. Phytopathology 64:845-849.

15. MOUSTAFA, A. M., and T. D. WYLLIE. 1976. Evaluation of soybean cultivars for response to infection by M. phaseolina. Proc. Am. Phytopathol. Soc. 3:290 (Abstr).
16. MOUSTAFA, A. M. 1976. Efficacy of systemic fungicides for the control of charcoal rot disease of soybean. Ph.D. Thesis. University of Missouri-Columbia, Columbia, MO. 119 p.
17. PAPAVIZAS, G. C., and N. G. KLAG. 1975. Isolation and quantitative determination of Macrophomina phaseolina from soil. Phytopathology 65:182-187.
18. SHORT, G. E., and T. D. WYLLIE. 1976. A quantitative technique for measuring vegetative growth and sclerotia production by Macrophomina phaseolina in soybean tissues. Proc. Am. Phytopathol. Soc. 3:222 (Abstr.).
19. SMITH, W. H. 1969. Germination of Macrophomina phaseoli sclerotia as effected by Pinus lambertiana root exudate. Can. J. Microbiol. 15:1387-1391.
20. STOVER, R. H., and B. H. WAITE. 1953. An improved method of isolating Fusarium spp. from plant tissue. Phytopathology 43:700-701.
21. WYLLIE, T. D., and O. H. CALVERT. 1969. Effect of flower removal and pod set on formation of sclerotia and infection of glycine max by Macrophomina phaseoli. Phytopathology 59:1243-1245.
22. WYLLIE, T. D., and M. F. BROWN. 1970 Ultrastructural formation of sclerotia of Macrophomina phaseoli. Phytopathology 60:524-528.