Morphological Instability on a Chlorate Medium of Isolates of *Macrophomina phaseolina* from Soybean and Sorghum

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ABSTRACT

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Soybean and sorghum were grown separately in soil infested with single, sclerotial isolates of *Macrophomina phaseolina* collected from either soybean or sorghum and together in soil infested with both isolates. Isolates were characterized by their growth on a chlorate-amended medium as having restricted or dense growth for the soybean or sorghum isolates, respectively. Roots from each treatment were assayed periodically for *M. phaseolina*, and the growth pattern on the chlorate-amended medium of the resultant isolates was determined. Significantly greater infection of soybean roots occurred when soybeans were grown in soil with the soybean isolate compared with either soil infested with both isolates or soil infested with the sorghum isolate. There were no differences in colonization of sorghum roots when sorghum was grown in soil infested with either or both isolates. The chlorate growth patterns of *M. phaseolina* obtained from infested soil in microplots or isolates in association with their respective hosts remained constant throughout the experiment. However, the chlorate growth patterns of isolates recovered from their alternative hosts shifted during reproductive development of the host plants.

Additional keywords: charcoal rot, *Glycine max, Sorghum bicolor.*

Macrophomina phaseolina (Tassi) Goid., the causal agent of charcoal rot, is a cosmopolitan soilborne pathogen with more than 400 known hosts (22). Although this wide host range suggests that *M. phaseolina* is not host specific, changes in soil populations in response to rotation (3), in vitro cultural differences (7,19), and differences in pathogenicity (3,19) indicate that host specialization may exist. Unfortunately, these characteristics have been either too variable (7,19) or too difficult to quantify (3,19) for adequate separation of this fungus into subgroups.

Recently, investigators have differentiated strains of fungi based on the ability of the fungi to utilize nitrate as a nitrogen source (2,5,6,11). Differentiation of strains is based on the reduction of chlorate, an analog of nitrate, to chlorite by the nitrate reductase pathway. Chlorite restricts growth when the nitrate reductase pathway is functional. In many fungi, sectors with unrestricted growth can develop from restricted colonies on media containing chlorate. Unrestricted growth in these sectors results from the inactivity of one or more of the five enzymes in the nitrate reductase pathway (15). Such isolates are designated *nit* mutants. These *nit* mutants have been used to identify vegetative compatibility groups within several species of plant pathogenic fungi. Compatibility groups designated from such tests have been correlated with host specialization (i.e., formae speciales) (6).

The generation of *nit* mutants to determine vegetative compatibility groups in *M. phaseolina* has been attempted but without success (C. A. S. Pearson, *personal communication*). However, media containing chlorate have been used to differentiate isolates on the basis of their growth morphology (16). Sclerotia free in the soil and isolates from soybean typically show restricted growth on media containing chlorate, because the isolates have an active nitrate reductase pathway. Isolates from sorghum and corn typically show dense growth on media containing chlorate, implying the absence or inactivity of the nitrate reductase pathway. Pearson et al (16,17) suggested that this difference in growth morphology on chlorate media was correlated with the host from which the isolate was recovered and could serve as a marker for the identification of host-specific isolates of *M. phaseolina*. Although this differentiation of *M. phaseolina* was based on a large number of isolates collected over an extensive geographical area, these researchers neither tested the relative ability of these isolates to colonize different hosts nor the stability of their chlorate phenotype when exposed to various hosts.

Our objectives were to determine if soybean and sorghum isolates of *M. phaseolina* colonize the roots of their respective hosts more frequently than their alternative host and to evaluate the effect of their respective and alternative hosts on the stability of the chlorate growth patterns of these isolates. Preliminary results have been reported (4).

MATERIALS AND METHODS

An experiment was conducted outdoors in 1988 and 1989 at the University of Arkansas Experimental Farm at Fayetteville in microplots constructed from plastic 208-L barrels (55 cm diameter, 121 cm high). Tops were removed, and eight 2.5-cm-diameter drainage holes were drilled in the bottom of each barrel. Barrels contained a 20-cm-deep layer of crushed limestone covered by a triple layer of cheesecloth and an 87-cm-deep layer of field soil (Zanesville silt loam) that had been disinfested with two applications of methyl bromide (Great Lakes Chemical Co., West Lafayette, IN) at 430 g m^{-3} per application. After the second application of methyl bromide, soil was assayed on nutrient agar to determine the extent of disinfestation. The soil was allowed to aerate for 2 wk before planting. Microplots were set above ground on a 15-cm-deep layer of limestone gravel. In the second year of the study, the soil in each microplot was removed and disinfested as described.

Two isolates of *M. phaseolina* were collected in the fall of 1987, one from a naturally infected soybean plant and one from a naturally infected sorghum plant. Isolates were stored on potato-dextrose agar (PDA; Difco Laboratories, Detroit, MI) at 15 C. The chlorate phenotype of the isolates was determined on the medium described by Pearson et al (16). On this medium, the soybean isolate had a colony diameter of 2 cm after 7 days, whereas

the sorghum isolate grew 26 cm in 7 days at 30 C; they were designated restricted and dense, respectively.

Inoculum was grown on a mixture of 300 g of vermiculite and 2 L of potato-dextrose broth (Difco) in autoclavable bags. After autoclaving, each bag was infested with 10 0.5-cm-diameter agar plugs of either the dense or restricted isolate grown on PDA and incubated at 30 C for 1 mo.

The entire disinfested soil layer (87 cm) in each microplot was infested thoroughly with 200 g of inoculum of *M. phaseolina* and with *Bradyrhizobium japonicum* (Kirchner) Jordan (The Nitragin Co., Milwaukee, WI) at a rate of 10^7 viable bacterial cells per microplot. In the treatment in which both isolates were added to the disinfested soil, 100 g of inoculum per isolate was added to keep the inoculum density consistent with the treatments incorporating one isolate. In addition to inoculum, NH_4NO_3 (34-0-0), P_2O_5 (0-46-0), KCl (0-0-60), and $CaCO_3$ were added to each microplot at recommended rates for soybean and sorghum growth (1).

The experiment was conducted in a split-plot randomized complete block design with five replications. Main plots were the various isolate × host treatments, and the split-plots were sample times. There were six isolate × host treatments: soybean (*Glycine max* (L.) Merrill 'Douglas') grown in soil infested with the soybean isolate; soybean grown in soil with the sorghum isolate; soybean grown in uninfested soil (which served as a control); sorghum (*Sorghum bicolor* (L.) Moench 'Funks 522 DR') grown in soil infested with the sorghum isolate; sorghum grown in soil infested with the soybean isolate; and soybean and sorghum grown in soil infested with both isolates. A total of 20 seeds (soybean and/or sorghum) were surface-disinfested for 3 min in a 0.5% NaOCl solution, rinsed in sterile distilled water for 3 min, and planted in each microplot.

The density of sclerotia in the microplots was determined at planting and harvest. Sclerotial density determinations were made by a modification of the method of McCain and Smith (12). Soil cores, consisting of approximately 250 g, were taken randomly from each microplot to a depth of 15 cm and mixed thoroughly in a plastic bag. One 5-g subsample was taken from the composite 250-g sample. The subsample was blended for three 30-s intervals alternated with three 30-s idle periods in 250 ml of 0.5% NaOCl. The resultant soil slurry was passed through a 0.045-mm sieve, the debris then was washed into a beaker with 25 ml of water, and 90 ml of molten medium (55 C) was added. The medium consisted of PDA (39 g L^{-1}) amended with rifampicin (100 mg L^{-1}), metalaxyl (224 mg L^{-1} a.i.), and tergitol NP-10 (1 ml L^{-1}). The contents of the beaker were mixed, poured into six petri dishes (100 × 15 mm), and incubated at 30 C for 4 days. Sclerotial numbers were estimated from the number of colony-forming units (cfu) on the amended PDA medium, then colonies were transferred to the chlorate medium to determine their growth patterns. Fifty colonies were transferred from microplots infested with either the dense or restricted isolate, and 100 colonies were transferred from microplots infested with both isolates.

Soybean and sorghum were sampled at six different growth stages for determination of the degree of colonization. Soybean plants were sampled at V3, V8, R2 (full bloom), R3 (pod-set), R6 (bean fill), and R7 (senescence) by removing the entire plant from the soil and keeping the roots intact (8). Sorghum plants were sampled in the same way at growth stages 2, 3, 4 (reproductive initiation), 5 (boot stage), 7 (soft dough), and 8 (hard dough) (21). At each growth stage, two plants were removed randomly from each microplot. Roots were washed to remove soil, and 10 1.0-cm-long root segments per intact root system were selected at random. Root segments were surface-disinfested in 0.5% NaOCl for 5 min, rinsed in distilled water for 2 min, and plated on PDA amended with rifampicin (100 mg of L^{-1}) and metalaxyl (224 mg of L^{-1} a.i.). After 2 days at 30 C, the number of root segments colonized by *M. phaseolina* was determined, and each isolate was transferred to the chlorate medium to determine its chlorate growth pattern.

Analysis of variance was performed on the data, based on the split plot design (20). Where there were significant effects, treatments were separated by the least significant difference test at $P = 0.05$. Because analysis of variance indicated no significant effect of year or year × treatment interaction, data from both years were combined.

TABLE 1. Estimation of sclerotial population densities of *Macrophomina phaseolina* in microplots at planting and harvest

Treatment (Host/isolate)[a]	Sclerotial population (cfu g^{-1})	
	Planting	Harvest
Control	0.0[b]	0.0
Soy/Soy	91.9	128.9
Sorg/Sorg	73.0	74.2
Soy Sorg/Soy Sorg	97.6	131.8
Soy/Sorg	79.3	91.7
Sorg/Soy	120.9	81.1

[a] Treatments consisted of soybeans (Soy), sorghum (Sorg), or both (Soy Sorg) grown in soil with either the soybean isolate (Soy), the sorghum isolate (Sorg), or both (Soy Sorg) in a split-plot randomized complete block design with five replicates.

[b] Differences in sclerotial population densities were significantly different ($P = 0.05$) between planting and harvest within a treatment at LSD = 9.8. Differences between treatments within a sampling time were significantly different ($P = 0.05$) at LSD = 22.1.

RESULTS

Soil in control microplots remained free of *M. phaseolina* (Table 1). Population densities of the pathogen in the infested soil ranged from 73 to 120 cfu g^{-1} of soil at planting and from 74 to 131 cfu g^{-1} at harvest. Most treatments with infested soil had similar soil population densities of *M. phaseolina* at planting, although

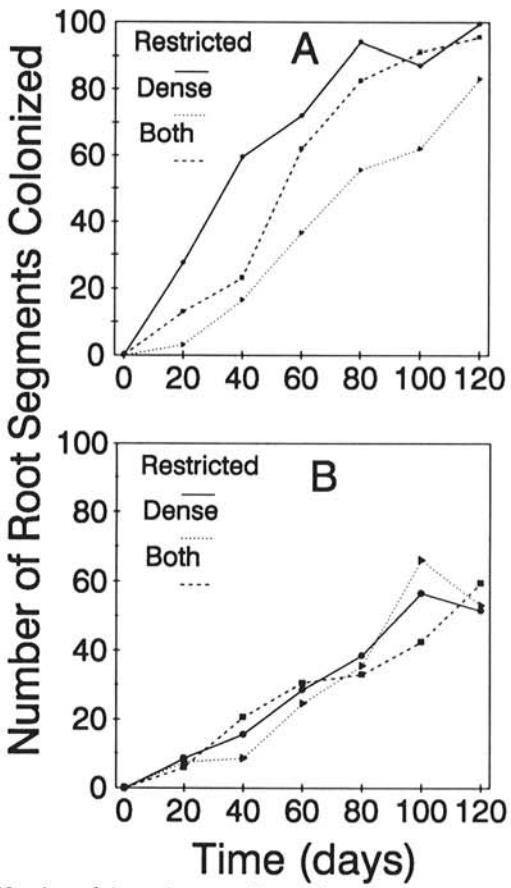

Fig. 1. Number of **A,** soybean, or **B,** sorghum, root segments colonized at various times from plants grown in soil infested with an isolate of *Macrophomina phaseolina* collected from soybean (restricted), sorghum (dense), or both isolates.

there were changes in some of these populations from planting to harvest.

In both years, soybean roots in control microplots remained free of colonization by *M. phaseolina* (data not shown). A greater ($P < 0.01$) number of soybean root segments were colonized by the soybean isolate 20 and 40 days after planting in soil infested with only the soybean isolate than in soil with both isolates (Fig. 1A). Likewise, a greater ($P < 0.01$) number of root segments were colonized by the soybean isolate in soil infested with the soybean isolate than in the soil infested with only the sorghum isolate up to 100 days after planting. There were no significant differences among treatments in colonization of sorghum roots, except at 100 days when the treatment with both isolates had significantly less ($P = 0.02$) colonization than the treatment with the sorghum isolate (Fig. 1B).

Chlorate growth patterns of sclerotia recovered from soil remained the same from planting to harvest regardless of treatment. Isolates collected from roots of soybeans grown in soil infested with the soybean isolate had predominantly restricted growth on the chlorate medium, whereas isolates collected from sorghum grown in soil with the sorghum isolate had predominantly a dense growth pattern (Fig. 2). When grown in soil with both isolates, isolates collected from soybean had predominantly restricted growth, with only 5–12 isolates having a dense growth pattern (Fig. 3A). The dense isolate was the predominant isolate recovered from sorghum roots, with only 3–16 isolates having a restricted growth pattern (Fig. 3B). Initially, the predominant isolate recovered from roots of soybeans grown in soil with the sorghum isolate was the dense isolate, but the restricted isolate was recovered with increasing frequency beginning at the V3 growth stage and became the predominant isolate at R3 (Fig. 4A). The predominant isolate recovered from roots of sorghum grown in soil with the soybean isolate was restricted (Fig. 4B). The dense isolate first was recovered at growth stage 4, was greatest at growth stage 5 (24 colonized root segments), and declined at growth stages 7 and 8.

DISCUSSION

Host specialization in *M. phaseolina* appears to occur with soybean, but not with sorghum. Colonization of soybean roots was significantly greater with the soybean isolate than with the sorghum isolate throughout the season, but there were no significant differences in sorghum root colonization with either isolate (Fig. 1).

Although host specialization appears to occur in *M. phaseolina,* the growth pattern on chlorate-amended medium does not appear to be a reliable marker in characterizing isolates. Shifts in the chlorate growth pattern of the isolates occurred in response to the host; the dense (sorghum) isolate, when exposed to only soybean, began to change to the restricted growth pattern (Fig. 4A). A similar change occurred when the restricted (soybean) isolate was exposed only to sorghum (Fig. 4B). These shifts were manifested in both hosts primarily during reproductive development of the plant and may be due to the types and concentrations of nitrogenous compounds in the plant. Although nitrogenous compounds of the host plants were not measured in this study, it is known that the amino acid composition can differ widely between types of crops (13,14) and that the chlorate-sensitive isolates of *M. phaseolina* do not utilize all the same nitrogenous compounds as the chlorate-resistant isolates (18). Further, nitrate reductase may function as an allosteric enzyme, based on fluctuations of its activity in the presence of certain amino acids (9). If the nitrate reductase enzyme is allosteric, then isolates of *M. phaseolina,* in association with their respective hosts, should exert minimal fluctuations in enzyme activity due to the

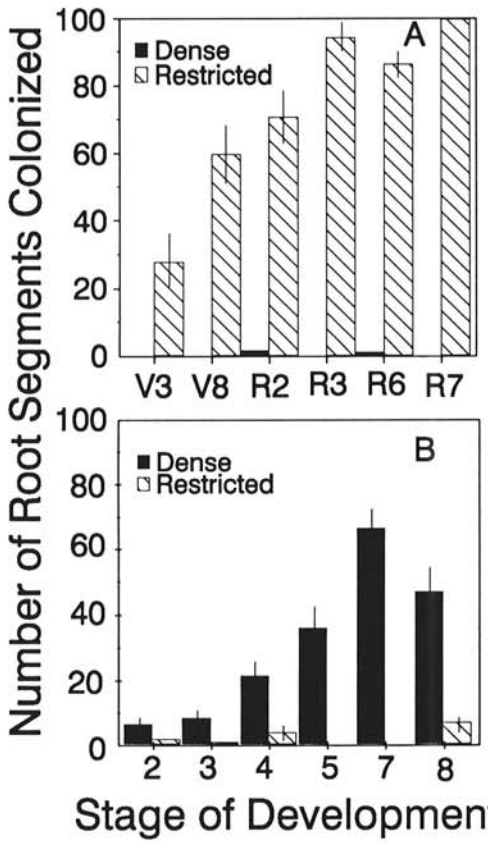

Fig. 2. Number of root segments colonized at various growth stages by the restricted or dense isolates of *Macrophomina phaseolina* from **A,** soybeans grown in soil with the soybean (restricted) isolate, or **B,** sorghum grown in soil with the sorghum (dense) isolate. Histograms represent the mean of five replicates in two experiments. Vertical bars represent the standard error of the mean.

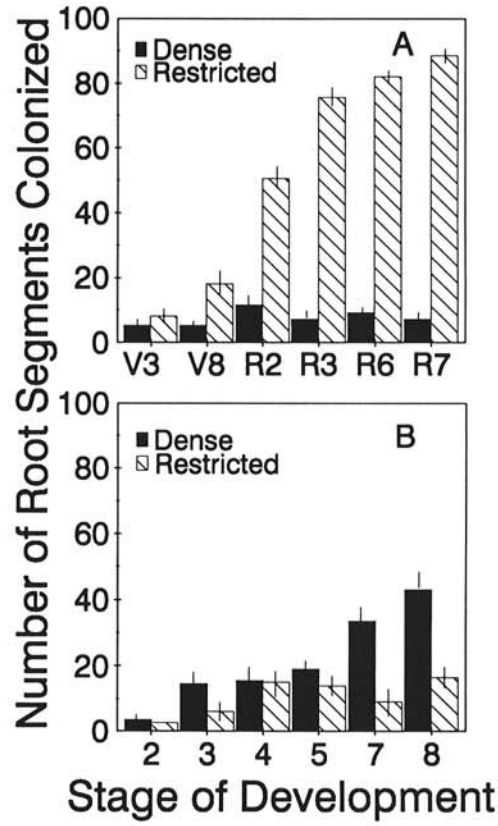

Fig. 3. Number of root segments colonized at various growth stages by the restricted or dense isolates of *Macrophomina phaseolina* from **A,** soybeans, and **B,** sorghum, grown together in soil infested with both the soybean (restricted) isolate and the sorghum (dense) isolate. Histograms represent the mean of five replicates in two experiments. Vertical bars represent the standard error of the mean.

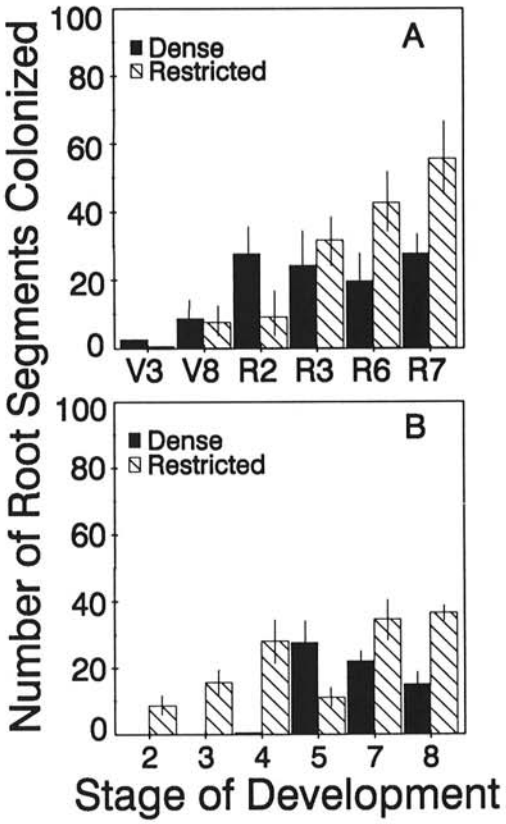

Fig. 4. Number of root segments colonized at various growth stages by the restricted or dense isolates of *Macrophomina phaseolina* from **A,** soybeans grown in soil with the sorghum (dense) isolate, or **B,** sorghum grown in soil with the soybean (restricted) isolate. Histograms represent the mean of five replicates in two experiments. Vertical bars represent the standard error of the mean.

consistency of the nitrogenous compounds present in the host plant. Under these conditions, the nitrate reductase enzyme in an isolate of *M. phaseolina* would remain relatively constant, resulting in a stable chlorate growth pattern (Fig. 2). Likewise, when soybeans and sorghum were grown in soil with both isolates, the predominant isolate recovered from soybean was the restricted isolate, whereas the dense isolate was predominantly recovered from sorghum (Fig. 3). It is not possible at this time to determine whether host specialization or a shift in the chlorate phenotype was responsible for the predominance of one isolate over the other in these hosts.

The shift in the chlorate phenotype may reflect heterogeneity in the inoculum because multicelled sclerotia were used in this experiment. Even hyphal cells of *M. phaseolina* are multinucleate, so heterogeneity could exist within single hyphal cells (10). However, it is unlikely that heterogeneity in the inoculum was responsible for this shift in chlorate growth pattern, because sectoring of the restricted isolate on chlorate-amended medium does not occur (17,18). Other possible explanations for the shift in the chlorate growth patterns of these isolates are contamination of the soil, which was not apparent in the soil assays (Table 1), or seed contamination, which is possible but unlikely because the seeds were surface-disinfested.

Further work with additional isolates and hosts is necessary, but from our results, we conclude that host specialization does occur, at least in soybeans. Although host specialization appears to occur, growth on chlorate-amended medium is not a reliable marker for characterizing isolates. From our results, we also suggest that the host has a strong effect on the nitrogen metabolism of *M. phaseolina.*

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