Host Specialization in the Charcoal Rot Fungus, *Macrophomina phaseolina*

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ABSTRACT

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To investigate host specialization in *Macrophomina phaseolina*, the fungus was isolated from soybean, corn, sorghum, and cotton root tissue and soil from fields cropped continuously to these species for 15 years in St. Joseph, LA. Chlorate phenotype of each isolate was determined after growing on a minimal medium containing 120 mM potassium chlorate. Consistent differences in chlorate sensitivity were detected among isolates from different hosts and from soil versus root. To further explore genetic differentiation among fungal isolates from each host, these isolates were examined by restriction fragment length polymorphism and random amplified polymorphic DNA (RAPD) analysis. No variations were observed among isolates in restriction patterns of DNA fragments amplified by polymerase chain reaction covering the internal transcribed

spacer region, 5.8S rRNA and part of 25S rRNA, suggesting that *M. phaseolina* constitutes a single species. Ten random primers were used to amplify the total DNA of 45 isolates, and banding patterns resulting from RAPD analysis were compared with the neighbor-joining method. Isolates from a given host were genetically similar to each other but distinctly different from those from other hosts. Chlorate-sensitive isolates were distinct from chlorate-resistant isolates within a given host. In greenhouse tests, soybean, sorghum, corn, and cotton were grown separately in soil infested with individual isolates of *M. phaseolina* that were chosen based on their host of origin and chlorate phenotype. Root colonization and plant weight were measured after harvesting. More colonization of corn roots occurred when corn was grown in soil containing corn isolates compared with isolates from other hosts. However, there was no host specialization in isolates from soybean, sorghum, or cotton. More root colonization in soybean occurred with chlorate-sensitive than with chlorate-resistant isolates.

The fungus *Macrophomina phaseolina* (Tassi) Goid. causes charcoal rot disease on more than 500 plant species worldwide (35). Agronomically important hosts in the United States include soybean (*Glycine max* [L.] Merrill), corn (*Zea mays* L.), sorghum (*Sorghum bicolor* [L.] Moench), and cotton (*Gossypium hirsutum* L.). Estimates from Missouri during the 1980s showed that annual losses in soybean to charcoal rot averaged 5% statewide, but sometimes reached 30 to 50% (34). Estimated soybean yield reductions caused by charcoal rot for the 16 southern states averaged 8.54×10^5 metric tons, making charcoal rot the second most damaging disease in this region from 1990 to 1994 (33).

Despite its wide host range, the genus *Macrophomina* contains only one species, *M. phaseolina*. Variation in morphology and virulence among isolates of *M. phaseolina* was reported in soybean, common bean, and other crops (6,9). However, efforts to identify subspecies of *M. phaseolina* based on microsclerotia size, culture characteristics, changes in soil population in response to rotation, and differences in pathogenicity failed usually because of the extreme variability within the species or difficulties in quantifying characteristics (5-7,24).

Recent efforts to classify isolates of *M. phaseolina* have considered colony morphology on media amended with chlorate (5,24,25). Pearson et al. (24,25) showed that isolates from corn stalks were chlorate-resistant and had a dense phenotype, whereas isolates from soybean root tissue and field soil were chlorate-sensitive and had feathery or restricted phenotypes. In another report, isolates from sorghum had a dense phenotype, whereas soybean

isolates had a restricted phenotype (5). It was suggested that this apparent relationship between phenotype and host could serve as a marker for identifying host-specific isolates of *M. phaseolina* (24). However, it is possible that isolates with similar morphology may not be genetically identical. Restriction maps, which can identify a series of unique sites in the genome and separate them from one another by actual distance along the DNA strand, are independent of gene function. Therefore, a polymorphism at this level can be detected regardless of whether the sequence of the genome changes the phenotype (21).

Ribosomal DNA has been used as an important genetic marker to separate species and strains of many organisms (4,18,19,23). Restriction fragment length polymorphisms (RFLPs) of mitochondrial rDNA and nuclear rDNA have been useful in identifying fungal genera and species and in recognizing intraspecific variation in ectomycorrhizal fungi (15). Fungi can be differentiated based on both length mutations and alteration in base sequences in RFLPs of rDNA (1,2,11,12).

Random amplified polymorphic DNA (RAPD) uses the polymerase chain reaction (PCR) to amplify DNA fragments from genomic DNA and examine differences in the size of amplified products via electrophoresis. This technique has been used to study population genetic variation in plants and fungi and has proven reliable in several taxonomic studies of higher plants (22,31). RAPD data generally agreed with those from more classical approaches that relied upon morphological, chemical, and molecular (isozyme, RFLP) methods (13). It has proven reliable for identifying individual isolates of ectomycorrhizal fungi as well (16).

Because of the wide host range of *M. phaseolina* and severe losses caused by this fungus worldwide, investigations were conducted concerning the relationship between *M. phaseolina* and its hosts. The objectives of our research were (i) to determine the relationship between host of origin and chlorate phenotype of *M.*

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phaseolina; (ii) to utilize rDNA RFLPs and RAPD to test for genetic differences among isolates of *M. phaseolina* from different hosts; and (iii) to determine if host specialization in *M. phaseolina* exists.

MATERIALS AND METHODS

Fungus isolation. In August 1996, soil and root samples were collected from soybean, corn, sorghum, and cotton plots in St. Joseph, LA. These plots were part of an ongoing crop rotation study that had been cropped to these species continuously for 15 years. We assumed that host specialization in *M. phaseolina*, if it exists, would be detectable after this time. Each crop was grown in four plots, and production practices typical for the area were implemented each year. Cover or rotation crops were not used in these plots. Twenty soil cores and 20 root systems were taken randomly from each plot. Each sample was bagged and labeled separately. Root systems were brought to the laboratory, rinsed thoroughly with water to remove soil, dried at 40°C in a forced air oven for 7 days, and ground in a Wiley mill to pass a 1-mm pore-size screen. Four soil subsamples (5 g) and four root subsamples (0.5 g) from each plot of each host were blended separately in 0.525% NaOCl for 3 min and collected on a 45-µm pore-size sieve. The triturate was washed well with water and added to 100 ml of potato dextrose agar (PDA) that had been autoclaved, cooled to 60°C, and amended with rifampicin (100 mg liter⁻¹), metalaxyl (224 mg liter⁻¹ a.i.), and tergitol NP-10 (1 ml liter⁻¹) (5). Plates were incubated at 30°C in darkness for 7 days, and 50 single microsclerotial isolates were collected from both soil and roots of each host. Cultures were incubated on PDA slants at 30°C in darkness for 7 days and stored in darkness at 6°C.

Chlorate phenotype. Isolates of *M. phaseolina* from roots (50 isolates from each host) and from soil (50 isolates from corn, sorghum, and soybean, 48 isolates from cotton) were examined for chlorate phenotype on PDA medium amended with 120 mM potassium chlorate (24). Three growth patterns generally were observed. A dense growth pattern similar to that obtained on medium without chlorate, indicated that the isolate was resistant to chlorate. Susceptibility to chlorate was evidenced either with a feathery spreading growth pattern or a restricted growth pattern (24). Cultures were incubated at 30°C in darkness, and colony phenotypes were recorded after 4 days by comparing with cultures of similar age that grew on the same medium without chlorate. The experiment was repeated once. The distributions of the chlorate phenotypes between hosts and between soil and root samples from each host were compared by Tukey's test (SAS Institute, Cary, NC).

DNA polymorphisms. Forty-five isolates were selected for molecular screening based on their chlorate phenotypes and hosts of origin (Table 1) (24). Cultures were grown in potato dextrose broth in 1.5-ml tubes for 3 days at 30°C in darkness. Total DNA was extracted from mycelia by grinding with a plastic pestle in 50 µl of lysis buffer (50 mM Tris-HCl at pH 7.2, 50 mM EDTA, 3% sodium dodecyl sulfate; autoclaved) combined with 3 µl of 2-mercaptoethanol and incubated in 300 µl of lysis buffer for 30 to 60 min at 55 to 65°C. The DNA was purified twice with 300 µl of phenol-chloroform and precipitated with 300 µl of isopropanol containing 1 drop of 3 M ammonium acetate. The DNA pellet was washed with 70% ethanol. Total DNA was diluted to a total volume of 200 µl, and 1 µl of RNase (10 mg/ml) was added to each sample. After incubation for 1 h at 37°C, the DNA was purified by extracting with phenol-chloroform, ammonium acetate and isopropanol, and finally with ethanol precipitation, as described above. The DNA was air-dried at room temperature (28°C), dissolved in sterile distilled water, and stored at -20°C for use (20).

PCR primers LR5 (5'-TCCTGAGGGAACTTCG-3') (27) and internal transcribed spacer (ITS) 5 (5'-GGAAGTAAAGTCG-TAACAAGG-3') (32) were used to amplify the ITS region and

part of the 25S rDNA. PCR was carried out in microcentrifuge tubes (0.5 ml) in a total volume of 50 µl, containing 1× reaction buffer; 0.2 µM each primer; 0.2 µM dATP, dGTP, dCTP, and dTTP; 1 µl of 0.5 to 1.0 µg/µl of DNA; and 1.25 units of *Taq* polymerase (Perkin-Elmer Corp., Norwalk, CT). Each PCR cycle consisted of 1 min of denaturation at 95°C, 1 min of annealing at 55°C, and 1.5 min of extension at 72°C. This cycle was repeated 35 times with 5 min of preheating at 95°C before the first cycle and 10 min of elongation at 72°C after the final cycle in a thermal cycler (Perkin-Elmer). A negative control with all reaction reagents except the DNA template was included with each set of reactions. The surface of the reaction mix was overlaid with 1 drop of mineral oil to prevent evaporation (32). The amplification was repeated once.

Restriction enzymes used for RFLP were chosen based on the *M. phaseolina* partial sequence of 18S ribosomal RNA gene, ITS1, the complete sequence of 5.8S ribosomal RNA gene, and ITS2 and partial sequence of 25S ribosomal RNA gene (GenBank Accession No. U97333). All digestions were repeated once. Isolates CRN R40, CRN S14, SORG R10, SOY R8, and COT

TABLE 1. *Macrophomina phaseolina* isolates used in laboratory and greenhouse studies

Source	Isolate ^a	Phenotype ^y	Study 1 ^z	Study 2 ^z
Soybean	S16	Feathery	—	—
Soybean	S17	Feathery	—	—
Soybean	R1	Feathery	+	+
Soybean	R2	Feathery	—	+
Soybean	R3	Feathery	—	—
Soybean	R17	Feathery	—	+
Soybean	S8	Restricted	+	+
Soybean	S41	Restricted	—	+
Soybean	S46	Restricted	—	—
Soybean	R8	Restricted	—	+
Soybean	R31	Dense	+	+
Sorghum	S1	Feathery	—	—
Sorghum	R1	Feathery	—	+
Sorghum	R10	Feathery	+	+
Sorghum	R20	Feathery	—	+
Sorghum	S5	Restricted	—	+
Sorghum	S8	Restricted	+	+
Sorghum	S10	Restricted	—	+
Sorghum	S2	Dense	—	—
Sorghum	S16	Dense	—	—
Sorghum	R27	Dense	—	+
Sorghum	R18	Dense	—	+
Sorghum	R30	Dense	+	+
Cotton	S3	Feathery	—	—
Cotton	S10	Feathery	—	—
Cotton	S27	Feathery	—	—
Cotton	R17	Feathery	—	+
Cotton	R33	Feathery	—	+
Cotton	R46	Feathery	+	+
Cotton	S22	Restricted	—	—
Cotton	S34	Restricted	—	+
Cotton	R31	Restricted	—	+
Cotton	R39	Restricted	+	+
Cotton	S8	Dense	—	—
Cotton	S30	Dense	—	—
Cotton	R2	Dense	—	+
Cotton	R20	Dense	—	+
Cotton	R49	Dense	+	+
Corn	S1	Restricted	—	+
Corn	S14	Restricted	+	+
Corn	S40	Restricted	—	+
Corn	S46	Dense	—	—
Corn	R1	Dense	+	+
Corn	R15	Dense	—	+
Corn	R40	Dense	—	+

^a S and R refer to soil and root, respectively.

^y Isolate phenotype on medium amended with 120 mM potassium chlorate (24).

^z + and — refer to isolates that were used or not used as inoculum in greenhouse studies, respectively.

R46 (Table 1) were chosen to represent the most diverse situation among all isolates for screening of restriction enzymes. PCR products from each isolate that were amplified by primers ITS5 and LR5 were digested with each of the restriction enzymes *MspI* (Gibco BRL, Life Technologies, Rockville, MD), *HaeIII* (Boehringer GmbH, Mannheim, Germany), *MboI* (Proem, Madison, WI), and *RsaI* (Gibco BRL) at 37°C for 2 h, and with enzyme *TaqI* (Gibco BRL) at 65°C for 1 h with 1 drop of mineral oil overlaid on the reaction mix under buffer conditions suggested by the manufacturer. Ten micrometers of reaction solution was prepared by combining 2 µg of PCR products, 1× reaction buffer, 2 units of enzyme, and sterile distilled water. One microliter of 10× dye was added to the reaction solutions to stop the reaction. After digestion, 10 µl of each reaction mix was exposed to electrophoresis through 2% agarose gel in 1× Tris-acetate-EDTA (TAE) buffer at 70 V for 40 min followed by staining in ethidium bromide for 30 min. The gel was visualized and photographed under UV light (30).

Ten random primers (OPA-02, OPA-08, OPA-09, OPA-10, OPA-11, OPA-12, OPA-14, OPA-15, OPA-16, and OPA-18) (Operon Kit A; Operon Technologies, Alameda, CA) were chosen to perform random amplification of genomic DNA for all selected isolates (Table 1). Sample DNA was purified again by RNase treatment as described previously. DNA purity was verified by electrophoresis through a 0.7% agarose gel, and DNA concentration was determined by spectrophotometry before the sample DNA was diluted in 50 µl of sterile distilled water to give similar DNA concentrations for all isolates. Amplification was performed in 50 µl of solution containing 1× reaction buffer; 0.8 µM primer; 25 ng of DNA; 0.2 µM each of dATP, dGTP, dCTP, and dTTP; 0.5 mM MgCl₂; and 1 unit of *Taq* polymerase. The surface of the reaction mix was overlaid with a drop of mineral oil. Each cycle of PCR consisted of 15 s of denaturation at 95°C, 45 s of annealing at 36°C, and 90 s of elongation at 72°C. This cycle was repeated 45 times with 5 min of preheating at 95°C before the first cycle and 10 min of extension at 72°C after the final cycle. The PCR reaction with each primer was repeated twice, and the products were stored at 4°C. Ten micrometers of the PCR product generated with each primer was separated by electrophoresis

through a 2% agarose gel prepared with 1× TAE buffer, stained with ethidium bromide, and photographed under UV light.

To characterize the variations in DNA banding patterns, a data matrix was constructed with the computer program MacClade (version 3.01; Sinauer Associates, Sunderland, MA) by scoring the presence or absence of a band as 1 or 0, respectively (26). The computer program PAUP (test version 4.0d64) (17) was used to analyze the relationships between *M. phaseolina* isolates. A dendrogram was generated by the neighbor-joining method (29). Statistical support for the internal branches within the resulting trees was obtained by bootstrap analysis (10) from 1,000 replications.

Greenhouse studies. Root isolates ($n = 11$ and 31 for studies 1 and 2, respectively) that showed consistent chlorate phenotypes in preliminary tests were selected to serve as inoculum for all greenhouse experiments (Table 1). For each isolate, 50 g of corncob grits (60 mesh, Bio-Serv, Frenchtown, NJ) were moistened with 200 ml of potato dextrose broth in a 400-ml glass beaker, covered with aluminum foil, and autoclaved for 40 min. The moistened mixture in each beaker was infested with six plugs (5-mm diameter) of one *M. phaseolina* isolate from PDA plates. After 60 days of incubation at 30°C in darkness, the mixture of mycelium, microsclerotia, and corncob grits was dried in a forced air oven at 35 to 40°C for 7 days and powdered with a food processor. The inoculum density (CFU per gram of inoculum mixture) was determined by dilution plating (28). Soil was infested with inoculum to yield 10 CFU/g of soil prior to filling pots. This level was chosen because it represented an average level for *M. phaseolina* infestation in soil at the study site (J. S. Russin, unpublished data).

All experiments were conducted in a randomized complete block design with four replications. Plastic pots (15-cm diameter) containing 3 kg of Convent silt loam soil (pH 7.0; organic matter ≤ 0.1%; 80.3% sand, 5.8% silt, and 13.9% clay) were used in all experiments. Soil was fertilized at planting and once every week after planting with Miracle-Gro (Stern's Miracle-Gro Products, Port Washington, NY) to provide 25 mg/kg of N as (NH₄)₃PO₄ and urea, 25 mg/kg of P₂O₅, and 25 mg/kg of K₂O. Four seeds per pot were planted. One week after emergence, plants were thinned to one per pot, and seedlings of similar size were kept.

The objective of greenhouse study 1 was to determine whether isolates from different host species colonized the roots of their hosts more extensively than roots of other host species. Treatments were established in a 4 by 11 factorial arrangement consisting of four host species (soybean, grain sorghum, corn, and cotton) and 11 isolates of *M. phaseolina*, each replicated four times. Isolates of *M. phaseolina* were chosen to represent all three chlorate phenotypic classes from each of the four hosts (Table 1). Because none of the corn isolates showed a consistent feathery phenotype in the presence of chlorate, only corn isolates with restricted and dense phenotypes were included. The experiment was repeated once. Seeds of 'Buckshot 66' soybean, 'DPL G-4666' corn, 'DK-50' sorghum, and 'Suregrow' cotton were planted 21 March (test 1) and 29 April 1998 (test 2). Plants were harvested after 8 weeks by cutting shoots 1 cm above the soil line. Root systems were removed from pots and washed gently in water to remove soil. Dry weights of shoots and roots were recorded after drying for 1 week at 40°C. Procedures for determining root colonization rate were modified from those described by Cloud and Rupe (5). Dry roots were ground in a Wiley mill fitted with a 1-mm pore-size screen. A 1-g sample of ground root taken from each host × isolate combination was subjected to dilution plating as described previously. Colonies of *M. phaseolina* were enumerated after 4 days at 30°C in darkness, and root colonization was expressed as CFU gram⁻¹ dry root tissue.

The objective of greenhouse study 2 was to confirm whether isolates from different host species colonize soybean roots differentially. This study differed from study 1 by including more isolates ($n = 31$) from each host × phenotype combination. Treatments were established in a 4 by 3 by 3 factorial design and

TABLE 2. Chlorate phenotypes of *Macrophomina phaseolina* isolates collected from soybean, corn, sorghum, and cotton roots and associated field soil in St. Joseph, Louisiana in 1996

Host	Sensitivity	Phenotype	Isolate ^x	
			Soil ^y	Roots ^z
Soybean	Sensitive	Feathery	39.0 a A	35.0 a A
Soybean	Sensitive	Restricted	11.0 b A	4.5 b A
Soybean	Resistant	Dense	0 b A	8.5 b A
Sorghum	Sensitive	Feathery	11.0 b A	39.0 a A
Sorghum	Sensitive	Restricted	36.0 a A	0 b B
Sorghum	Resistant	Dense	2.0 b A	11.0 b A
Cotton	Sensitive	Feathery	17.0 a A	13.0 ab A
Cotton	Sensitive	Restricted	14.0 a A	3.0 b A
Cotton	Resistant	Dense	17.0 a A	34.0 a A
Corn	Sensitive	Feathery	4.5 b A	0 b A
Corn	Sensitive	Restricted	44.5 a A	0.5 b B
Corn	Resistant	Dense	0.5 b A	49.5 a B

^x Within source (soil or root) and host, means followed by the same lower-case letter did not differ ($P > 0.05$) according to Tukey's honestly significant difference test. Between soil and root isolates within host, means followed by the same uppercase letter did not differ ($P > 0.05$) according to Tukey's honestly significant difference test.

^y Mean number of isolates from soil (50 total for soybean, sorghum, and corn; 48 total for cotton) in each chlorate phenotype class from two experiments. Due to death of some isolates during incubation, mean number may be <50.

^z Mean number of isolates from roots (50 total) in each chlorate phenotype class from two experiments. Due to death of some isolates during incubation, mean number may be <50.

included four host species (soybean, sorghum, corn, and cotton), three chlorate phenotypes (feathery, resistant, and dense), and three isolates in each host $\times$ phenotype combination. Each treatment was replicated four times. The experiment had unbalanced data because only one isolate from soybean exhibited a dense chlorate phenotype and no isolates from corn exhibited a feathery chlorate phenotype. The experiment was repeated once. 'Buckshot 66' soybean seeds were planted 1 April (test 1) and 18 April 1998 (test 2). Plants were harvested after 8 weeks by cutting plant shoots 1 cm above the soil line. Procedures for measuring plant growth and determining root colonization by *M. phaseolina* isolates were as described previously.

Main treatment effects and interactions among treatments in greenhouse experiments were tested using general linear methods procedure (SAS). When necessary, treatments were separated with

Tukey's test (SAS Institute) at $P = 0.05$. In greenhouse experiment 2, only one dense isolate from soybean and no feathery isolates from corn were included. Therefore, data from this experiment were analyzed as an 11 by 3 factorial treatment arrangement by combining the host and phenotype factors. Data were transformed according to LOG (root colonization + 1) prior to analysis because root colonization data followed a geometric distribution.

RESULTS

Chlorate phenotype. Mycelial growth of *M. phaseolina* on chlorate medium was classified into three categories (restricted, feathery, and dense) as described by Pearson et al. (24). Restricted and feathery isolates were sensitive to chlorate, whereas dense isolates were resistant to chlorate. Isolates belonging to each phe-

Fig. 1. Dendrogram of isolates of *Macrophomina phaseolina* from soybean (SOY), sorghum (SORG), cotton (COT), and corn (CRN). For each isolate, R and S after the host designation represents root or soil as the source; R, F, or D at the end of the isolate designation refers to restricted, feathery, or dense chlorate phenotype. Numbers on the branches indicate the percentage of bootstrap samplings, derived from 1,000 samples, supporting the internal branches of 50% or higher.

notypic class were recovered from soil and from roots of all hosts. Analysis of variance showed no test by treatment interactions, therefore, data from repeated experiments were combined prior to analysis. Consistent differences in chlorate sensitivity were detected among isolates from different hosts and between soil and root (Table 2).

Host and source (root or soil) had significant effects on chlorate phenotype of *M. phaseolina* isolates. The dominant phenotype differed between soil and root isolates for each host and among different hosts (Table 2). Among soybean isolates, feathery isolates were much more abundant than either restricted or dense isolates, and the frequency of phenotypes did not differ between soil and root. Feathery isolates predominated on sorghum roots, but restricted isolates predominated in soil cropped to sorghum. Paired comparisons showed that there were significantly more restricted isolates in soil than in roots. Isolates from cotton soil showed no predominant phenotype, although root isolates exhibited primarily a dense phenotype. Restricted isolates predominated in soil cropped to corn, whereas dense isolates predominated in corn roots.

DNA polymorphisms. DNA amplification using primers ITS5 and LR5 yielded a fragment of approximately 1,600 bp, identical in size in all isolates tested. The amplified region then was digested with restriction enzymes *MspI*, *HaeIII*, *MboI*, *RsaI*, and *TaqI*. More than four fragments (≈ 800 and 340 bp, and more than two fragments smaller than 200 bp) resulted from digestion with *MspI*; two major fragments ($\approx 1,000$ and 500 bp) and some small fragments (<100 bp) resulted from digestion with *HaeIII*; more than five fragments that were less than 400 bp resulted from digestion with *MboI*; and more than three fragments, including two major fragments (510 and 380 bp), and some bands smaller than 200 bp resulted from digestion with *RsaI*. Digestion with enzyme *TaqI* resulted in three major fragments (≈ 400 , 380, and 280 bp) that were shared by all five isolates. No site or length variations were observed among these isolates based on the banding patterns resulting from digestion of PCR products by these five restriction enzymes.

Fig. 2. Effects of host of origin (cotton, corn, sorghum, and soybean) on root colonization by *Macrophomina phaseolina*. Each isolate was used to inoculate all hosts. Letters above bars indicate significance ($P < 0.05$) according to Tukey's honestly significant difference based on geometric transformed means. Means presented in this figure are actual values. Within each inoculated host, means with the same letter do not differ.

All RAPD primers tested yielded amplification products. Twenty three and twenty four polymorphic bands were obtained using primers OPA-02 and OPA-09, respectively, whereas only seven bands were obtained using primer OPA-11. All of the primers resulted in amplification patterns characterized by group-specific and individual-specific bands. For example, primer OPA-02 produced one monomorphic band that was shared by all isolates tested, three bands specific to corn isolates, and one band for restricted corn isolates; primer OPA-09 produced one band exclusive to all soybean isolates. A total of 142 polymorphic bands was scored, and polymorphism was found in RAPD profiles of the 45 isolates examined. Based on analysis using the neighbor-joining method, isolates from soybean separated distinctly from isolates from sorghum, cotton, and corn; sorghum isolates separated distinctly from isolates from cotton and corn; cotton isolates separated distinctly from corn isolates (Fig. 1). Furthermore, chlorate-sensitive isolates separated distinctly from chlorate-resistant isolates within a given host. Within chlorate-sensitive isolates for each host, only isolates from sorghum showed distinct differences between the feathery and restricted phenotypes.

Greenhouse studies. In both greenhouse studies, analysis of variance indicated no test by treatment interactions for any of the dependent variables measured in these studies; therefore, data from duplicate tests for each of these two cross-inoculation experiments were combined prior to analysis.

In study 1, isolates of *M. phaseolina* from cotton, corn, sorghum, and soybean did not differ in their colonization of cotton, sorghum, and soybean roots. However, isolates from corn exhibited higher colonization rates in corn roots than did isolates from cotton, sorghum, or soybean (Fig. 2). None of the isolates affected dry shoot or dry root weights of any of the host species (data not shown).

In study 2, corn isolates colonized soybean roots more extensively than did sorghum isolates, otherwise colonization of soybean roots did not differ among isolates from other hosts (Fig. 3). There was more soybean root colonization by chlorate-sensitive isolates than by chlorate-resistant isolates, especially feathery isolates (Table 3), across all hosts of origin. Isolates from cotton, corn, sorghum, and soybean did not affect dry shoot weight of soybean (data not shown). There was a greater reduction of dry root weight of soybean by soybean isolates than by sorghum isolates ($P = 0.0327$).

Fig. 3. Effects of host of origin on soybean root colonization by isolates of *Macrophomina phaseolina* in a cross-inoculation experiment. Letters above bars indicate significance ($P < 0.05$) according to Tukey's honestly significant difference based on geometric transformed means. Means presented in this figure are actual values. Means with the same letter do not differ.

DISCUSSION

DNA polymorphism indicates that *M. phaseolina* isolates from the four host species used in this study are closely related but that detectable genetic variation exists among isolates based on their hosts. This is revealed through analysis of the RFLPs of ITS regions, 5.8S rDNA, and part of the 25S rDNA as well as RAPDs of genomic DNA. The variation that does exist among the isolates is confined to populations with boundaries defined by host species.

Restriction and length polymorphisms in the ITS region, the 5.8S rDNA, and part of the 25S rDNA gene were not detected between and within *M. phaseolina* isolates from four hosts. A comparison of restriction patterns derived from this region was not useful to separate *M. phaseolina* isolates based on host of origin. This study suggests that four groups of *M. phaseolina* isolates, based on host, are very closely related and are not distinguishable from each other by RFLP analysis. Hence, there is no basis for further subdividing *M. phaseolina* taxonomically.

Results from RAPD analysis showed that *M. phaseolina* isolates were clearly differentiated from each other based on their host of origin, and isolates within each host which have different sensitivity to chlorate also separated distinctly. Isolates of *M. phaseolina* in this study were recovered from a field in which soybean, sorghum, corn, and cotton were cropped without rotation for 15 years. This unique sampling site, combined with our molecular analysis, suggest that host specialization in this fungus has developed to some extent. The dendrogram pattern, which clearly delineates subpopulations based on host of origin, can be explained in terms of adaptation of isolates to their hosts in that populations recovered from specific hosts may be subjected to similar selection pressures. Because no sexual stage of *M. phaseolina* was observed, it is assumed that *M. phaseolina* is an asexual fungus and that microsclerotia and pycnidiospores were the only reproductive propagules produced (8). However, microsclerotia are formed by swelling of a single cell or fusion of two cells from two neighboring hyphae (8). Fusion between vegetative cells may form heterokaryons, and parasexual recombination between nuclear genes may occur (3).

Because isolates from the four hosts generally showed three kinds of chlorate phenotypes, it is impossible to separate isolates from different hosts based only on chlorate phenotype. Other workers classified subspecies of *M. phaseolina* based on differences in microsclerotia size, culture characteristics, and pathogenicity (5–7,24). However, no conclusive results were obtained in those studies. It may now be possible to determine whether an isolate obtained from the field is derived from any of these four hosts, although additional studies are needed to confirm these findings. According to the dendrogram obtained using the neighbor-joining method from RAPD banding patterns, it appears that sorghum and soybean isolates are less diverse than cotton and corn isolates. Also, chlorate-sensitive isolates showed fewer differences from each other when compared with chlorate-resistant isolates within each host.

Host specialization in *M. phaseolina* appears to occur with corn, but not with sorghum, cotton, or soybean, as indicated by results of cross-inoculation experiments. Colonization of corn roots was significantly greater with corn isolates than with isolates from other hosts. Although Cloud and Rupe (5) reported host specialization with *M. phaseolina* from soybean, they used only one

soybean isolate and one sorghum isolate to inoculate soybean and sorghum. Our results may be more representative because of the number of isolates used as well as the fact that isolates were recovered from their respective hosts after 15 years of continuous cropping. The influence of host of origin on pathogenicity of *M. phaseolina* is not an isolated example of host specialization in a nonspecific pathogen. For example, host specialization in *Heterobasidion annosum* (Fr.) Bref. on pine was documented (14).

The specific mechanisms contributing to this special relationship between host and *M. phaseolina* were not determined. Association with one host species for a long period of time may cause an adaptation to the nitrogenous compounds available from the respective hosts, and individuals within this population may be more suited to colonize their respective hosts (24). Further studies are required to elucidate mechanisms of host specialization in *M. phaseolina* with corn.

Results from the present study showed that chlorate-sensitive isolates were better able than chlorate-resistant isolates to colonize soybean roots. Chlorate-sensitive isolates of *M. phaseolina* do not utilize the same nitrogenous compound as chlorate-resistant isolates (25). It is possible that soybean has an effect on the nitrogen metabolism of *M. phaseolina*. This prospect warrants further investigation. Also, it was reported that sorghum isolates of *M. phaseolina* with a dense phenotype, when inoculated onto soybean, began to change to the restricted growth pattern (5). Further work needs to be conducted with mixtures of the three phenotypes to determine if soybean selects one phenotype over the others and if this selection is related to nitrogen nutrition.

Based on the results of the chlorate tests, the differences in dominant chlorate phenotypes among hosts and sites (soil and root) strongly suggest that host preference or selection functions in the *M. phaseolina*–host pathosystem. Otherwise, the frequency of each phenotype should be similar in soil and root populations (25). However, except for soybean, results from the current study show that the composition of the soil population is not the same as the root population. It should be noted that no chlorate-sensitive (feathery or restricted) isolates were recovered from corn root tissue, whereas most of corn soil isolates were restricted, and no restricted isolates were recovered from sorghum root tissue, whereas 36 of 50 sorghum soil isolates were restricted. This suggests that corn and sorghum imposed a functional preference on the population of *M. phaseolina* so that chlorate-resistant isolates were more likely to infect corn roots than were chlorate-sensitive isolates, and restricted isolates were less likely to infect sorghum roots than were dense and feathery isolates. Reasons for the phenotype shifts are not clear. They may be associated with the heterogeneous, possibly polygenic nature of microsclerotia (8). But the fact that no sectors were observed on media amended with chlorate argues against this explanation (5). More work needs to be done to determine the mechanisms of chlorate assimilation in *M. phaseolina*.

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TABLE 3. Effects of chlorate sensitivity and chlorate phenotype of *Macrophomina phaseolina* on root colonization in soybean

Contrast	<i>P</i> > <i>F</i>
Chlorate-sensitive vs. chlorate-resistant	0.0001
Feathery vs. dense	0.0001
Restricted vs. dense	0.0012
Feathery vs. restricted	0.2322

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