Relative Longevity of *Macrophomina phaseolina* and Associated Mycobiota on Residual Soybean Roots in Soil

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ABSTRACT

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Survival of the charcoal rot pathogen (*Macrophomina phaseolina*) in soybean (*Glycine max*) on residual root systems was studied over a 2-year period. Root segments colonized by *M. phaseolina* were placed into fiberglass-mesh bags and buried at depths of 0, 7.6, and 25.4 cm in a Marietta fine sandy loam soil in field microplots. Samples for year 1 and year 2 were buried in October 1999 and 2000, respectively, and sampled every 2 months over a 14-month period. Mean percent frequencies from both years for *M. phaseolina* showed a decreased linear trend over time from 91% before burial to 24.7% 2 months later, 10.3% at 6 months, and 0.3% after 14 months. Tissues were degraded in the June samplings of both years and this degradation was believed to be partially responsible for the reduced survival of *M. phaseolina*. Mean isolation frequencies were significantly ($P \leq 0.05$) greater at the 0-cm depth than at 7.6 and 25.4 cm across all sampling dates. Other fungi which were present in the preliminary assay, including *Fusarium* spp. and those in the *Phomopsis/Diaporthe* complex, also declined by the end of the study. *Trichoderma* spp. was isolated at significantly ($P \leq 0.05$) greater frequencies and a significant positive linear trend over time. It is possible that *Trichoderma* spp. were involved in direct tissue degradation and nutrient depletion of the root segments, or may have acted as a mycoparasite, reducing the survival of the pathogen *M. phaseolina* and other associated fungi. Results from this study indicate that farm practices which increase residue destruction immediately after harvest or those that enhance *Trichoderma* spp. populations may directly or indirectly lower the relative longevity of soilborne pathogens, including *M. phaseolina*.

Soil microflora consists of a diverse group of microorganisms, including bacteria, yeasts, and fungi (8). Pathogenic and saprophytic fungi are major components of the soil microflora and are known to overwinter on crop debris, including peanut (*Arachis hypogaea* L.), cotton (*Gossypium hirsutum* L.), canola (*Brassica napus* L. var. *oleifera* (Metzger) Sink.), and soybean (*Glycine max* (L.) Merr.) (1,3-5). Survival of pathogens on plant debris contributes to an increase in disease severity, especially when reduced tillage or continuous cropping systems are used by growers (1,13,17). Soybean seed and crop residues are recognized as sources of inoculum for numerous soybean pathogens, including *Phomopsis* spp. and *Diaporthe phaseolorum* var. *sojae* (Cooke & Ellis) Sacc. (9,14). One of the most important soybean pathogens is *Macrophomina phaseolina*

(Tassi) Goidanich, a root-inhabiting fungus, which does not commonly grow as a saprophyte in either soil or dead tissues of infected plants (12,16). The pathogen overwinters primarily as microsclerotia in soybean root and stem tissues and will continue to survive after the root and stem tissues decay (15). Cook et al. (6) reported that 23% of the microsclerotia, formed in invaded sorghum (*Sorghum bicolor* (L.) Moench) tissue, would germinate up to 16 months after initiation of the study. Furthermore, microsclerotia of *M. phaseolina* obtained from other hosts were reported to survive for several years (7). A study conducted in the midwestern United States reported that *M. phaseolina* could survive up to 2 years, but percent germination of the microsclerotia was lower after the first year. Information on the survival of *M. phaseolina* in soybean debris is limited for the southern portion of the United States. Inoculum density of *M. phaseolina* increases in soils planted continuously to susceptible soybean cultivars; therefore, understanding the importance of soybean root debris as an inoculum source for this pathogen to overwinter has implications in disease management strategies.

Tillage practices influence the distribution and size of plant debris left on the soil surface after harvest. Debris on or near the soil surface has been shown to influence

the diversity and density of pathogenic and saprophytic fungi (1,5). In previous studies, levels of *Rhizoctonia solani* Kühn AG-4 and *Leptosphaeria maculans* (Desmaz.) Ces. & De Not. were similar at depths of 0, 7.6, and 25.4 cm, but other fungi including *Trichoderma* spp., increased with soil depth. As isolation frequencies of the plant-pathogenic fungi decreased, *Trichoderma* spp. populations increased over time. It is uncertain, however, if *Trichoderma* spp. were important in degradation of soybean debris and in the biological control of *M. phaseolina*.

Biodegradation of plant debris and relative longevity of an organism is dependent on several factors, such as available moisture and temperature in different geographical locations (5). Information on the overwintering or survival potential of *M. phaseolina* is limited to select host crops from the midwestern United States (15). Therefore, studies on the relative longevity of the soybean pathogen should be conducted within the southern region of the United States.

The objectives of this investigation were to determine the relative longevity of *M. phaseolina* in soybean debris buried at different soil depths and to document the occurrence of other potential pathogens, antagonists, and saprophytic fungi associated with *M. phaseolina* in decomposing soybean root tissues.

MATERIALS AND METHODS

This study was initiated on 19 October 1999 and 20 October 2000 in a field with no history of charcoal rot. Each year, microplots consisting of 91.4-cm-diameter open-ended fiberglass cylinders (surface area, 0.7 m²) placed 15.2 cm above and 3.0 m below the soil surface were established at the R. R. Foil Plant Science Research Center, Mississippi State University, Starkville, in a split-block design with four replications. Treatments consisted of burial depths and dates of sampling as discussed below. In all, 64 microplots were used during this 2-year study (32 microplots per year). The microplots were left fallow for at least 4 years prior to the study. The soil within each microplot was a Marietta fine sandy loam soil (fine-loamy, mixed, thermic siliceous, Aquic, Fluventic Eutrochrept, pH 6.0 to 6.2).

For the purposes of this article, a root segment refers to the tap, secondary, and

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feeder roots and the attached lower stem with a total length of 25 cm. A total of 500 root segments of the cultivar Bolivar were collected on 8 October 1999 and 5 October 2000 from two soybean fields with a history of charcoal rot, located near Starkville, MS. The root segments were placed into polypropylene bags, returned to the laboratory and stored at 20°C for 24 h.

Just prior to the establishment of the study, soybean root segments containing natural infections of *M. phaseolina* were separated from disease-free tissue. *M. phaseolina*-infected stems were identified based on visible discoloration and the presence of microsclerotia. Each root system was shortened to 25 cm in length just prior to initiation of the study and five of the pieces were placed into 30-cm² polypropylene (mesh size, 1 mm²) packets and sealed with nylon thread. A total of 96 packets per year were prepared and buried within the microplots. On 19 October 1999 and 20 October 2000, the packets (three per microplot), containing five root segments each, were placed within the microplots at each of the soil depths (0, 7.6, and 25.4 cm).

Laboratory procedures. Prior to the soybean root segments being placed into the packets for each year, 50 stem-root segments were randomly selected to determine the initial natural mycobiota, using methods previously described (5). From each root segment, four 1-cm-long pieces were removed, surface-sterilized, and cultured using the isolation procedures dis-

cussed below. Packets from each soil depth were collected every 2 months from December 1999 to December 2000 (year 1) and December 2000 through December 2001 (year 2). The packets of root segments were rinsed under running tap water for 1 h to remove excess soil and plant material. The five stem pieces were removed from the packet and 10 1-cm root sections were excised from tap and secondary roots. The 1-cm pieces were placed into 0.524% sodium hypochlorite solution (wt/vol) for 3 min. After the third sampling date (6 months after initiation of the study) in both tests, the soybean debris became progressively decomposed and surface disinfection time was reduced to 1 min. The 1-cm pieces, after sterilizing for 3 or 1 min., then were plated onto water agar (WA) (20 g of agar and 1 liter of distilled water) and potato-dextrose agar (PDA; 39 g Difco agar + 1 liter of distilled water; Difco Laboratories, Detroit) in petri plates (100 by 15 mm) amended with chlor-tetracycline at 50 mg/liter and streptomycin sulfate at 8 mg/liter (2). The 1-cm root pieces were incubated at room temperature up to 7 days and all mycelial colonies were transferred to PDA for initial identification. Macroscopic and microscopic cultural characters were used to identify the fungi. *M. phaseolina* was identified by the dark or black appressed mycelial growth and presence of the microsclerotia. The other fungi were identified using previously discussed methods (5). Data for each species were expressed as percent isolation frequency by dividing the number of recorded observations (isolations) in each by 50.

Statistical analyses. All data were analyzed employing Proc GLM (Statistical Analysis System, SAS Institute Inc., Cary, NC) as a split-block design with burial depths as factor A and sampling dates as factor B. Means were separated using Fisher's protected least significant difference (LSD), and orthogonal polynomials were employed (*F* test) to test for linear and quadratic trends for sampling date and depth.

RESULTS

In both years, the preliminary assay yielded mean isolation frequencies of 91, 5, and 14% for *M. phaseolina*, *Trichoderma* spp., and *Fusarium* spp., respectively (*data not shown*). Species within the *Diaporthe/Phomopsis* complex were isolated at the frequency of 6.2% and mean frequencies of other important fungi, including *Fusarium*, *Pythium*, and *Rhizoctonia* spp., were less than 5% prior to burial. All other fungi identified occurred in less than 1% of the root segments tested.

The most common genera isolated during the investigation was *Trichoderma* (42.1%, averaged across both years). The second most frequently identified taxa was *Fusarium* (16.9%), followed by *Macro-*

phomina (12.9%), *Basidiomycetes* (6.9%), the *Phomopsis/Diaporthe* complex (4.5%), and *Melanospora* (3.8%) (Table 1). A total of 20 genera of fungi also were identified from less than 1% of the root pieces (*data not shown*), including the pathogenic fungi *Cercospora*, *Colletotrichum*, *Rhizoctonia*, *Sclerotium* at less than 1%, and *Pythium* at 1.3%.

Isolation frequency was greater for *M. phaseolina* during year 2 of the study (12.8%) than year 1 (9.0%) (Table 1). The percent frequencies of *M. phaseolina* decreased linearly following burial, but was isolated from the debris throughout the study period (Table 2). Mean frequencies across years for *M. phaseolina* were 24.7% during the first sampling date in December, decreased to 10.3% in June at the 6-month sampling date, and further declined to 0.3% by the last sampling date 14 months after burial. When frequencies were compared by soil depth, the mean percentage at 0 cm of depth was significantly greater at 14.0% compared with 8.8% at 7.6 cm, but at the 25.4-cm depth the frequency was similar at 9.8% (*data not shown*).

Four commonly occurring *Fusarium* spp. identified during this investigation were isolated from 14% of the debris during the preliminary study. They included *Fusarium oxysporum* Schlechtend.:Fr., *F. pallidoroseum* (Cooke) Sacc., *F. sambucinum* Fuckel, and *F. solani* (Mart.) Sacc., and the frequencies declined linearly over time (Table 2). Mean frequencies varied from 25.2% after the first sampling period to 16.3% in June and 6.5% during the last sampling date in December. Significant linear responses were observed for the four *Fusarium* spp., but a significant quadratic trend also was noted for *F. solani* (Table 2). The quadratic response occurred due to the steady decline in isolation frequencies up to 10 months after sampling, followed by increased frequencies at 12 and 14 months. When isolation frequencies were compared among years, greater levels were observed during year 1 for *F. oxysporum* and *F. solani* and in year 2 for *F. pallidoroseum* and *F. sambucinum*. Isolation frequencies did not differ with soil depth for the four *Fusarium* spp. For total *Fusarium* spp., isolation frequencies between years were similar at the three soil depths and among both years (*data not shown*).

Mean isolation frequencies for the *Phomopsis/Diaporthe* complex were 6.2% during the preliminary study and increased to 9.7% 2 months after burial (Table 2). Following the first sampling date, percent frequencies were significantly lower for the remaining dates and levels for these taxa had a decreasing linear trend over time, with increases at 10 and 12 months after burial resulting in a significant quadratic response. No differences in mean isolation frequencies occurred for the *Pho-*

Table 1. Survival of *Macrophomina phaseolina* and isolates of other fungi from soybean root segments buried in soil during a 2-year study

Taxa	Isolation frequency (%) ^z	
	1999–2000	2000–2001
Ascomycetes		
<i>Melanospora</i>	0.2	6.5
Basidiomycetes		
Unknown spp.	1.3*	12.5*
Fungi imperfecti		
<i>Alternaria</i>	4.4	<1
<i>Fusarium</i>		
<i>oxysporum</i>	9.3*	4.3*
<i>F. sambucinum</i>	1.0*	5.9*
<i>F. solani</i>	8.2*	4.7*
<i>F. pallidoroseum</i>	0*	2.3*
<i>Macrophomina</i>	10.7*	15.0*
<i>Phomopsis</i>	5.3*	3.6*
<i>Trichoderma</i>	48.5*	35.8*
<i>Verticillium</i>	2.1	0
Oomycetes		
<i>Pythium</i>	0*	2.6*
Zygomycetes		
<i>Mucor</i>	1.3	<1
Unknown fungi	<1	<1

^z Burial for each year of the study was in October and samples were taken bi-monthly up to 14 months after burial. Percentage of soybean stems (600/sampling date) yielding cultures; * = significantly (*P* ≤ 0.05) different frequencies per year.

mopsis/Diaporthe complex when compared by soil depths.

Trichoderma spp. were isolated from 1.6% of the debris tissue just prior to burial in both years of the study. Greater mean frequencies occurred with time for *Trichoderma* spp., with levels ranging from 18.2% 2 months after burial to 29.2% in June and 60.8% on the last sampling date (Table 2). A significantly greater mean frequency of *Trichoderma* spp. occurred during year 1 (48.5%) than year 2 (35.8%) of the investigation. Mean isolation frequencies for *Trichoderma* spp. showed significant linear and quadratic responses across sampling dates in both years (Table 2). Depth did not influence recovery of *Trichoderma* spp.

Common fungi associated with plant tissue degradation isolated during the study including *Melanospora* sp. and basidiomycete isolates (Table 1). A *Melanospora* sp. was isolated throughout the study, but the greatest frequency occurred on the fourth sampling date (June), which was responsible for the significant quadratic response observed (Table 2). Furthermore, the *Melanospora* sp. was isolated at a greater frequency during year 2 (6.5%) than year 1 (0.2%), but percentages were similar when compared among the three soil depths. Basidiomycetes were identified based on cultural morphology, presence of clamp connections, or dolipore septum, but their frequencies were always <1%, except on the last sampling dates (1.8%) (*data not shown*).

DISCUSSION

During both years, mean *M. phaseolina* isolation frequencies declined linearly over time, which may have been affected by the root segment degradation or interactions with other microorganisms. The 25-cm depth segments were in fragments 6

months after burial and were completely shredded at 14 months. Even though the isolation frequencies of *M. phaseolina* differed among depths, debris degradation was consistent, regardless of depth. In a previous study, survival of the pathogen *R. solani* AG-4 on peanut shell debris was significantly lower at 18 and 24 months than at 6 and 12 months following burial (1). Formation of microsclerotia is an important survival mechanism for *M. phaseolina*. Unless destroyed by environmental factors or other microorganisms, these structures will continue to germinate and infect host tissues during subsequent growing seasons. Microsclerotia generally form below the epidermal tissues of the lower stems, hypocotyl, or roots near the soil surface of most host crops (6), but little or no growth occurs in soil or dead host tissue (12). The microsclerotia which form during invasion of soybean roots remain protected within the dead tissues until degradation. In this study, it could not be determined if the reduction in isolation frequencies was directly associated with destruction of the pathogen during the plant tissue degradation process or release of the microsclerotia into the soil. In one previous study, exposed microsclerotia of *M. phaseolina* were reported to have a 23% germination rate 16 months after burial (6), but microsclerotia were reported to survive up to several years in a later investigation (7). Therefore, rotation with nonhost crops for 2 to 3 years is necessary to lower *M. phaseolina* infection levels in severely infested fields (18).

Select environmental conditions such as temperatures and rainfall are known to affect the survival and growth of fungi. In this study, soil temperatures for each 14-month period averaged 20°C in year 1 and 21°C in year 2. Prior to burial of the root segments in October through February of

the following year for both years, temperatures continued to decrease until March and then increased through September. Rainfall accumulation during years 1 and 2 was 195 and 239 cm, respectively, and isolation frequencies for the fungi varied between years. Isolation frequencies for *M. phaseolina* were greater in year 2 than year 1, but the same decreasing trends in survival of the pathogen occurred both years during this study. Furthermore, the pathogen is known to grow actively and compete for substrate and predominate in host tissues over other fungi during dry conditions more than when moisture is readily available (18). Four common genera of fungi were isolated at greater frequencies in year 2, but seven had greater percentages in year 1 (Table 1). No trends in isolation frequencies could be shown based on the differences in precipitation amounts between years 1 and 2.

Trichoderma spp. were the most commonly occurring fungi identified during the investigation (Table 1). Isolation frequencies which were low prior to burial of the root segments rapidly increased over time and were greatest by the end of the study. The increased frequencies corresponded with reductions in population levels of *M. phaseolina* and *Fusarium* spp. *Trichoderma* spp. are known to rapidly colonize plant debris of different crop tissues that are in contact with the soil (5). These fungi degrade hemicellulose of the plant cell walls by the production of the enzyme cellulase (10). The degradation of this food reserve by *Trichoderma* spp. may be important for limiting populations of selected fungal species and may reduce levels of *M. phaseolina* and *Fusarium* spp. Furthermore, *Trichoderma* spp. are mycoparasites and have been reported to attack the pathogens *Fusarium*, *Pythium*, and *Rhizoctonia* spp. (10,11). *M. phaseolina*

Table 2. Mean isolation frequencies (%) of *Macrophomina phaseolina* and other fungi from soybean root segments during seven sampling dates averaged over burial depths of 0, 7.6, and 25.4 cm^x

Taxa ^y	Months after burial (sampling dates)							L ^z	Q ^z
	2 (Dec)	4 (Feb)	6 (Apr)	8 (Jun)	10 (Aug)	12 (Oct)	14 (Dec)		
<i>Macrophomina phaseolina</i>	24.7 a	15.5 b	13.6 b	10.3 bc	6.8 c	4.9 cd	0.3 d	**	NS
Total <i>Fusarium</i> spp.	25.2 a	26.8 a	19.6 ab	16.3 b	7.5 c	7.0 c	6.5 c	**	NS
<i>F. oxysporum</i>	6.9 ab	6.8 ab	9.7 a	6.9 ab	4.4 bc	3.1 bc	2.3 c	**	NS
<i>F. pallidoroseum</i>	0 b	8.2 a	0 b	0 b	0 b	0 b	0 b	**	NS
<i>F. sambucinum</i>	3.6 ab	4.5 a	4.1 ab	4.0 ab	1.6 ab	1.1 b	1.8 ab	**	NS
<i>F. solani</i>	14.3 a	6.3 b	5.5 b	4.2 bc	0.9 c	2.8 bc	4.5 bc	**	**
<i>Melanospora</i>	2.3 b	0.8 b	2.7 b	10.3 a	0 b	3.6 b	1.5 b	NS	**
<i>Phomopsis/Diaporthe</i>	9.7 a	3.9 b	0.3 b	0.3 b	2.3 b	4.6 b	0.2 b	**	**
<i>Pythium</i>	0 b	0 b	5.3 a	0 b	0 b	0.9 b	1.4 b	NS	NS
<i>Rhizoctonia solani</i>	0 b	0 b	0 b	0 b	0.2 a	0 b	0 b	NS	NS
<i>Trichoderma</i>	18.2 c	26.8 bc	22.3 c	29.2 bc	38.8 b	53.5 a	60.8 a	**	**

^x Mean percent isolation frequencies averaged across 2 years and three depths; mean isolation frequencies were derived from the number of isolation from 150 debris pieces from each of four depths for each sampling date; and frequencies within a row followed by the same letter are not significantly ($P \leq 0.05$) different according to least significant difference.

^y Taxa: *Fusarium* spp. include *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. pallidoroseum*, *F. sambucinum*, *F. solani*, and *F. verticillioides*; *Phomopsis/Diaporthe* spp. included *D. phaseolorum* var. *caulivora*, *D. phaseolorum* var. *sojae*, and *P. longicolla*; *Rhizoctonia solani* includes AG-4, AG2-2, and AG-5.

^z L refers to linear responses and Q quadratic responses of the mean isolation frequencies; ** indicates significant linear or quadratic responses; NS is nonsignificant at $P \leq 0.05$.

also was shown to be parasitized by two *Trichoderma* spp. in vitro (R. E. Baird, unpublished data), but these results were based on observed outgrowth and subsequent death of the *M. phaseolina* colonies.

The combined role of *Trichoderma* spp. as mycoparasites and strong cellulolytic degraders may have resulted in reduced survival of *M. phaseolina*. It is uncertain, however, if mycoparasitism by *Trichoderma* spp. results in destruction of the microsclerotia during the debris degradation process and, if so, what percentage of microsclerotia are viable in the soil following release from these plant tissues. In two previous studies, increases in *Trichoderma* spp. population levels corresponded with decreases in the plant pathogens *R. solani* AG-4 and *L. maculans* (1,5). As *Trichoderma* spp. levels increased over time, percent frequencies of the two pathogens declined in those two studies. A similar trend was observed for *M. phaseolina* and *Trichoderma* spp. in the current investigation.

Soil depth had a significant effect on the survival of *M. phaseolina* during this study, with a greater frequency at the 0-cm depth (14.0%) than at 7.6 cm (8.8%), but 25.4-cm depth (9.8%) was numerically the same. In a previous study, the pathogen *R. solani* AG-4 was isolated from peanut shells with greater frequencies in debris collected from the soil surface than buried (1). Contrary to those results, *L. maculans* was isolated from canola debris at similar frequencies from different soil depths (5). As discussed previously, cellulose-degrading fungi such as *Trichoderma* spp. and basidiomycetes may be more active within the soil due to available moisture and more stable temperatures resulting in greater activity. Plant tissue degradation by microorganisms could be partially responsible for the reduced survival of *M. phaseolina* at the 7.6- and 25.4-cm depths in this study. *Trichoderma* spp. occurred at greater frequencies with increased burial depths of 7.6 and 25.4 cm compared with the surface debris. Actual causes of the reduced survival of the two pathogens were not determined (1,5). In this study, however, *Trichoderma* spp. levels were similar for all soil depths and could not be correlated with changes in mean frequencies of *M. phaseolina* (Table 1).

Mean isolation frequencies for total *Fusarium* spp. and the four most common species showed a decreasing response over each 14-month period during each year of the investigation (Table 2). A similar trend

occurred on canola debris, where isolation frequencies of *Fusarium* spp. decreased over a 12-month period (5). Contrary to those results, *Fusarium* spp. significantly increased over a 24-month period on peanut shell debris (1). When mean frequencies were compared by depth and years for total *Fusarium* spp. and individual species, data were similar. Similar results occurred in two previous studies comparing survival of saprophytic and parasitic fungi on other crop debris (1,5).

The *Phomopsis/Diaporthe* complex, an important group of soybean-pathogenic fungi, followed the same general pattern as *M. phaseolina* of reduced frequencies over time. A significant quadratic response occurred among the sampling dates for *Phomopsis/Diaporthe*. The difference was that there were decreasing mean percent isolation frequencies up to 8 months after burial, with slight increases at 10 and 12 months. At 14 months, however, the frequency decreased and was lower than for any other sampling date. The overall reduced frequencies over time also may have occurred as with *M. phaseolina* due to increased colonization, nutrient utilization by different fungi, or mycoparasitism by *Trichoderma* spp.

Based on the isolation frequency data, significant quadratic and linear responses could not be related to changes in soil temperature or moisture (data not shown). The significant responses, however, may reflect degradation of the root segments and subsequent changes in nutrient requirements for the different fungi.

The results from this study indicate that farming practices which enhance shredding of the debris and burial through tillage practices may increase the rate of wood degradation or colonization by antagonistic fungi, which reduces the relative longevity of *M. phaseolina* in debris. Many soybean fields in Mississippi, however, are left as no-tillage or minimum tillage, or the debris is not disturbed until just prior to planting the new crop almost 1 year later. Strategies to improve management techniques which increase soybean tissue fragmentation and may enhance colonization, nutrient depletion, and mycoparasitism by fungi such as *Trichoderma* spp. resulted in elimination or decreased viability of microsclerotia of *M. phaseolina* and should be further investigated.

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