Inoculum Density, Pathogenicity, and Interactions of Soybean Root-Infecting Fungi

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ABSTRACT

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In autoclaved and nonautoclaved soil (91 ppm P and pH 6.0), the percentages of infected roots and diseased plants increased with increasing levels of inoculum of *Macrophomina phaseolina* and *Rhizoctonia solani*. Fifty percent of the seedlings were infected (ED_{50}) by *M. phaseolina* at approximately 0.8 and 3.8×10^4 sclerotia per kilogram of autoclaved and nonautoclaved soil, respectively. For *R. solani* the ED_{50}s were 0.1 and 0.2×10^4 sclerotia per kilogram of autoclaved and nonautoclaved soil, respectively. *M. phaseolina* and *R. solani* or *Fusarium solani* at known inoculum densities in autoclaved soil were studied for their effect on soybean plants in a greenhouse at 28–35 C. Within 25 days after planting, *M. phaseolina* had significantly reduced root weight; *R. solani* and *F. solani* had significantly reduced root weight, shoot weight, and plant height compared to the uninoculated control plants. The combination of *M. phaseolina* at 40×10^4 sclerotia per kilogram of soil with either *R. solani* at 0.1×10^4 sclerotia or *F. solani* at 300×10^4 chlamydospores per kilogram of soil significantly reduced all three parameters when compared to the control or to treatments with *M. phaseolina* alone. The percentage of roots infected with *M. phaseolina* significantly decreased when combined with *R. solani*.

Additional key words: Glycine max.

Interactions among soilborne, plant-pathogenic fungi can influence disease incidence and severity on many crops resulting in synergism (5,19) or antagonism (4,8,17,19) among pathogens. *Macrophomina phaseolina* (Tassi) Goid., *Fusarium solani* (Mart.) Synd. & Han., and *Rhizoctonia solani* Kuhn cause root and stem rot on soybean (*Glycine max* (L.) Merr.) throughout the world (2,23,24). Although most reports deal primarily with the effects of individual pathogens, *M. phaseolina* has been reported to interact with *Fusarium* spp. (7,15,22), *R. solani* (22), and *Verticillium* sp. (27) in increasing the severity of root rot on several crops. No information is available on the effect of *F. solani* or *R. solani* on the infection of soybean by *M. phaseolina* or on the effect of inoculum densities of the latter two fungi on disease severity of soybean. In our preliminary research results and field observations, we observed apparent interactions of *M. phaseolina* with *R. solani* and

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F. solani on soybean. Therefore, the purpose of this study was to determine the effect of inoculum density on root infection by *M. phaseolina* and hypocotyl infection by *R. solani* and the effect of *F. solani* or *R. solani* on *M. phaseolina* root infection and soybean growth.

MATERIALS AND METHODS

The fungi in this study were recovered from naturally-infected soybean plants in Florida. The isolates selected were the most virulent of those isolates screened for their ability to produce the largest lesions on wounded, inoculated, soybean-seedling hypocotyls (*M. phaseolina*) or to infect the greatest number of plants when grown in autoclaved, artificially infested soil (*R. solani* and *F. solani*). Arredondo fine sand (91 ppm P, 52 ppm K, 325 ppm Ca, 84 ppm Mg, 38 ppm NO_3, 1.96 ppm Zn, and pH 6.0) was used throughout this study after it was autoclaved twice at 121 C for 4 hr at 24-hr intervals.

Inoculum of *M. phaseolina* was prepared using methods similar to those of Meyer et al (13). Sclerotia were added to approximately 500 ml of water and their numbers estimated by counting ten 1-ml samples. The viability of surface disinfested (0.5% NaClO for 1 min) sclerotia was determined after rinsing in sterile water by plating on potato-dextrose agar (PDA) supplemented with 30 mg of streptomycin sulfate per liter of medium.

Sclerotia of *R. solani* were obtained by placing four 4-mm-diameter disks from a 2-day-old PDA culture into each of several 250-ml Erlenmeyer flasks containing 50 g of a cornmeal and sand mixture (45 g of double washed sand plus 5 g of cornmeal and 15 ml of distilled water). After incubation for 30 days at 27 C, the contents of several flasks were blended in sterile tap water for 60 sec at maximum intensity in a blender, and the resulting suspension was passed through nested 125- and 250-μm sieves. The mycelial fragments were removed by exposure to a high-pressure water spray. After resuspending the sclerotia in 1 L of tap water, the sclerotial numbers were estimated by counting ten 1-ml samples.

Chlamydospores of *F. solani* were obtained by placing two 4-mm-diameter disks from a 5-day-old PDA culture into each of several 250-ml Erlenmeyer flasks containing 50 ml of soil extract. Soil extract was prepared by autoclaving 500 g of field soil in 1 L of tap water for 30 min, filtering the extract through Whatman No. 10 filter paper, adjusting the pH to 6.5 with $CaCO_3$, and then autoclaving at 121 C for 15 min. After incubation for 7–10 days at 27 C the contents of several flasks were decanted onto a 44-μm (325-mesh) sieve and were gently rinsed with tap water. The rinsed mats were then ground in a Pyrex tissue grinder containing 10 ml of deionized water until no mycelia were evident. After grinding, the volume of the homogenate was increased with deionized water and 80 ml amounts were sonicated with a Braunsonic model 1510 (B. Braun Instruments, San Mateo, CA 94403) at 40% of maximum power for 40 sec. The number of chlamydospores per milliliter was determined with a hemacytometer.

Infested soil was plated on selective media for the quantitative population estimates of the pathogens. The number of propagules per gram of air-dried soil of *M. phaseolina* and *R. solani* were determined by the techniques described by Papavizas and Klag (18), and Ko and Hora (11), respectively. Colonies of *R. solani* and *M. phaseolina* were counted after incubation in the dark at 30 C for 36 hr and 12 days, respectively.

For studies on the relationships of inoculum densities to disease incidences, the following inoculum levels were established by mixing sclerotia into soil for 5 min with a model N-50 Hobart (Hobart Manufacturing Co., Troy, OH 43611) mixer: 0.0, 2.5, 5.0, 7.5, 10.0, 20.0, 40.0, and 80.0 sclerotia of *M. phaseolina* per gram of autoclaved soil; 0, 25, 50, 75, 100, 150, 250, and 500 sclerotia of *M. phaseolina* per gram of field soil; 0.0, 0.25, 0.50, 1.0, 2.0, 4.0, 6.0, and 8.0 sclerotia of *R. solani* per gram of either autoclaved or field soil. Water was added to the soil during the mixing process to give the final water content of 10% (w/w), a water potential of approximately −75 mbars. Inoculum densities per gram of autoclaved soil for *M. phaseolina* (40 sclerotia), *R. solani* (1 sclerotium), and *F. solani* (300 chlamydospores) used in interaction studies were based on propagule numbers frequently encountered in naturally infested soybean fields in Florida. Soil infestation with the three pathogens was achieved in the same manner as indicated above for inoculum density studies.

Experiments were conducted in a greenhouse in which ambient temperatures fluctuated from 28 to 35 C, day length varied from 13 hr, 20 min to 14 hr, and irradiance at noon at plant height was 800–1200 μEinstein$\cdot m^{-2}\cdot sec^{-1}$. There were thirty 10-cm, autoclaved clay pots per inoculum level containing 500 g of soil with two soybean plants per pot. Soybean seeds of cultivar Hood were used in all experiments. Seed were surface disinfested with 1.0% NaClO for 60 sec, rinsed three times with sterile water, and inoculated with a commercial strain of *Rhizobium japonicum*. Pots were watered on alternate days, or more frequently if necessary, to maintain active plant growth and to prevent plant wilting. Twenty-five days after seeding, soybean plants were harvested to evaluate percentage of root and stem infection, plant height, and root and shoot weights. For the evaluation of root infection, twenty 2.5-cm soybean-root pieces from treatments inoculated with *M. phaseolina* or *F. solani* were taken at random from each of 30 plants, and 30–40 soybean hypocotyls from treatments inoculated with *R. solani* were surface sterilized in a 0.5% solution of sodium hypochlorite, and plated on 2% water-agar plates. Twelve days after incubation at 27 C, the number of root pieces developing sclerotia of *M. phaseolina* and conidia of *F. solani* was counted to determine the percentage of root infection. Soybean hypocotyls with typical mycelia of *R. solani* were counted 36 hr after incubation. All experiments were repeated at least once.

RESULTS

Inoculum density and disease incidence. After 25 days, 100% of the seedlings were infected in soils infested with 2×10^4 and 10×10^4 sclerotia of *M. phaseolina* per kilogram of autoclaved and nonautoclaved soil, respectively (Fig. 1). When the infection data were transformed to $\log_e 1/(1-x)$, in which x equals the proportion of the diseased seedlings, to adjust for multiple infections (25), and plotted against $\log_{10}$ of the number of sclerotia of *M. phaseolina* per kilogram of soil (Fig. 2), the slope of the line determined by linear regression analysis was 1.69 for autoclaved and 0.97 for nonautoclaved soil. The inoculum densities required for 50% infection of the plants (ED_{50}) were interpolated to be approximately 0.8 and 3.8×10^4 sclerotia per kilogram of autoclaved and nonautoclaved soil, respectively.

When soybean roots were plated on 2% water agar, 50% of the

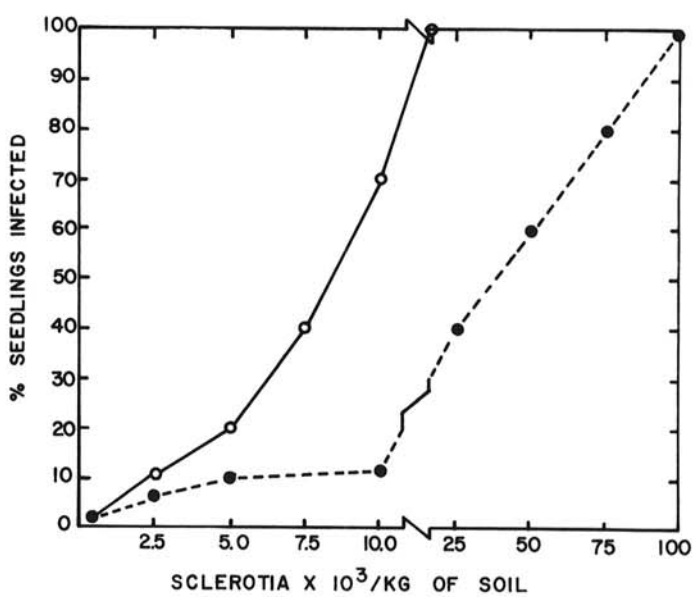

Fig. 1. The relationship of sclerotial density of *Macrophomina phaseolina* in autoclaved (o——o) and nonautoclaved (•------•) soil to percentage of soybean seedlings infected 25 days after planting.

root pieces were infected at approximately 2×10^4 and 15×10^4 sclerotia of *M. phaseolina* per kilogram of autoclaved or nonautoclaved soil, respectively (Fig. 3). The maximum proportion of infected roots obtained was 84% with approximately 8×10^4 sclerotia per kilogram in autoclaved soil, and 85% with 50×10^4 sclerotia per kilogram in nonautoclaved soil after 25 days.

For *R. solani*, 0.8×10^4 sclerotia per kilogram of soil, the highest level of inoculum used in the experiment, resulted in 100 and 92% of plants infected in autoclaved and nonautoclaved soil, respectively (Fig. 4). The slope of the line determined by linear regression analysis was 0.64 in autoclaved and 0.87 in nonautoclaved soil (Fig. 5). The inoculum level required for 50% infection of the plants was interpolated to be 0.1 and 0.2×10^4 sclerotia per kilogram of autoclaved and nonautoclaved soil, respectively.

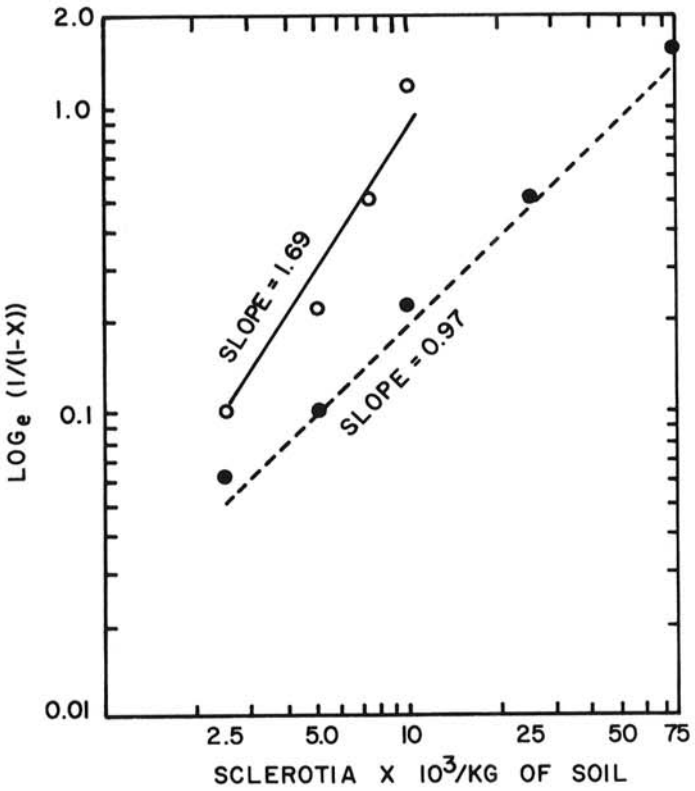

Fig. 2. The relationship of sclerotial density ($\log_{10}$) of *Macrophomina phaseolina* in autoclaved (o——o) and nonautoclaved (•----•) soil to percentage of infected soybean seedlings ($\log_{10}$ [$\log_e 1/(1-x)$]), where x = proportion of infected seedlings, 25 days after planting.

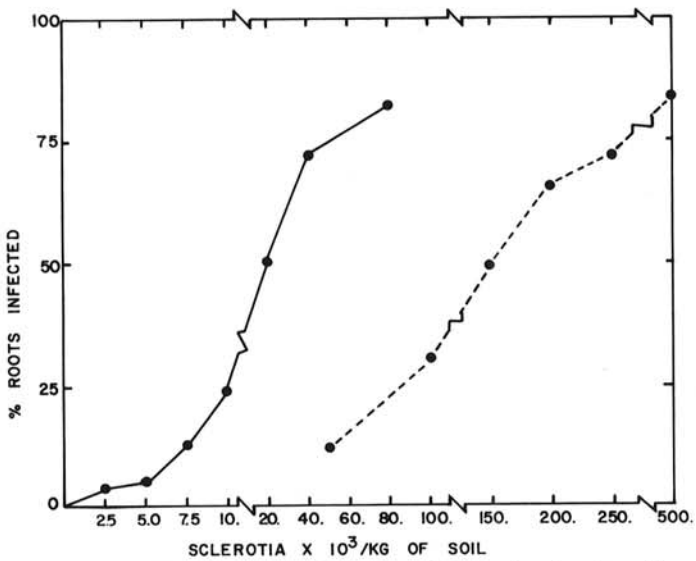

Fig. 3. The relationship of sclerotial density of *Macrophomina phaseolina* in autoclaved (•——•) and nonautoclaved (•------•) soil to percentage of root infection 25 days after planting.

Pathogen effects on soybean. Reduction in plant growth was the primary evidence for effect of the pathogens on soybean. All of the pathogens individually reduced either root weight, shoot weight, or plant height significantly, with *M. phaseolina* having the least effect in this regard (Table 1). Although *R. solani* resulted in moderate hypocotyl decay and *F. solani* resulted in small necrotic areas on the roots, no visible necrosis from *M. phaseolina* occurred at 25 days.

Plants in soil infested with *M. phaseolina* plus *R. solani* had significantly less shoot weight and plant height than plants with either pathogen alone. However, both the percentages of roots infected (Table 1) and the recovery of propagules from soil (data not shown) for *M. phaseolina* were reduced significantly when the pathogens were combined. When soil infested with *R. solani* alone was assayed, only *Trichoderma* spp. were recovered, a fungus not deliberately added to the soil. Root weight, shoot weight and plant height were reduced for plants in soil infested with both *M. phaseolina* and *F. solani* compared to plants inoculated with each pathogen alone.

DISCUSSION

The slope of the log-log transformation of number of *M. phaseolina* sclerotia to the proportion of infected plants approached 1.0 in nonautoclaved soil, which indicates direct proportionality; whereas for autoclaved soil the slope was 1.69. The high slope of *M. phaseolina* obtained in autoclaved soil might be explained by a reduction in inhibition of *M. phaseolina* resulting from the reduction of antagonistic organisms in the soil (16,17), or by the reduction in competition for nutrients at the onset of sclerotium germination. Lower inoculum densities of *M. phaseolina* and *R. solani* were required for 50% infection of soybean seedlings in autoclaved than in nonautoclaved soil; this was expected because of the apparent lack of competition of other microorganisms with *M. phaseolina* and *R. solani* for nutrients in

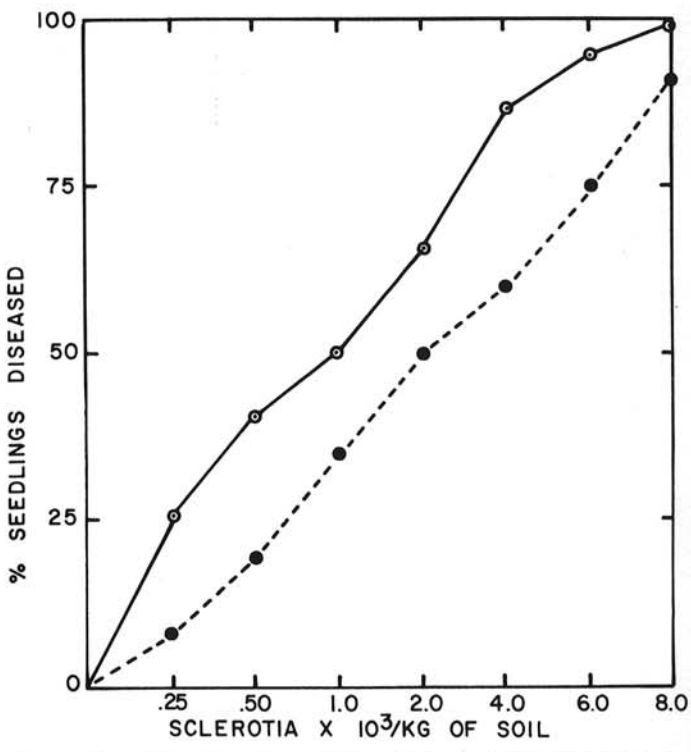

Fig. 4. The relationship of sclerotial density of *Rhizoctonia solani* in autoclaved (o——o) and nonautoclaved (•------•) soil to percentage of diseased soybean seedlings 25 days after planting.

TABLE 1. Plant growth response and soybean stem and root rot 25 days after infesting autoclaved soil with known quantities[x] of *Macrophomina phaseolina* plus *Rhizoctonia solani* or *Fusarium solani*

Treatments	Root weight (g)	Shoot weight (g)	Plant height (cm)	Roots with *M. phaseolina* (%)	Disease index *R. solani*[y]	Roots with *F. solani* (%)
Uninoculated (control)	3.5 a[z]	4.2 a	30.2 a	0.0	0.0	···
M. phaseolina	2.5 b	2.6 b	28.7 a	35.3 a	0.0	···
R. solani	2.0 c	2.6 b	26.1 b	0.0	2.3 a	···
M. phaseolina + *R. solani*	2.1 c	2.4 c	24.4 c	10.6 b	3.1 a	···
Uninoculated (control)	2.7 a[z]	3.1 a	29.9 a	0.0	···	0.0
M. phaseolina	2.0 b	2.8 a	26.8 ab	32.6 a	···	0.0
F. solani	1.6 b	1.8 b	25.0 b	0.0	···	33.0 a
M. phaseolina + *F. solani*	0.8 c	1.0 c	21.9 c	26.3 a	···	43.6 a

[x] Inoculum densities were 40×10^4 sclerotia of *M. phaseolina* per kilogram of soil; 1,000 sclerotia of *R. solani* per kilogram of soil and 300×10^4 chlamydospores of *F. solani* per kilogram of soil.

[y] Disease index rating was based on the hypocotyl decay on a scale of 0 to 4 with 0 = no symptoms and 4 = most severe symptoms (dead plant).

[z] Values (mean of 40 plants) in vertical columns for each separated experiment followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

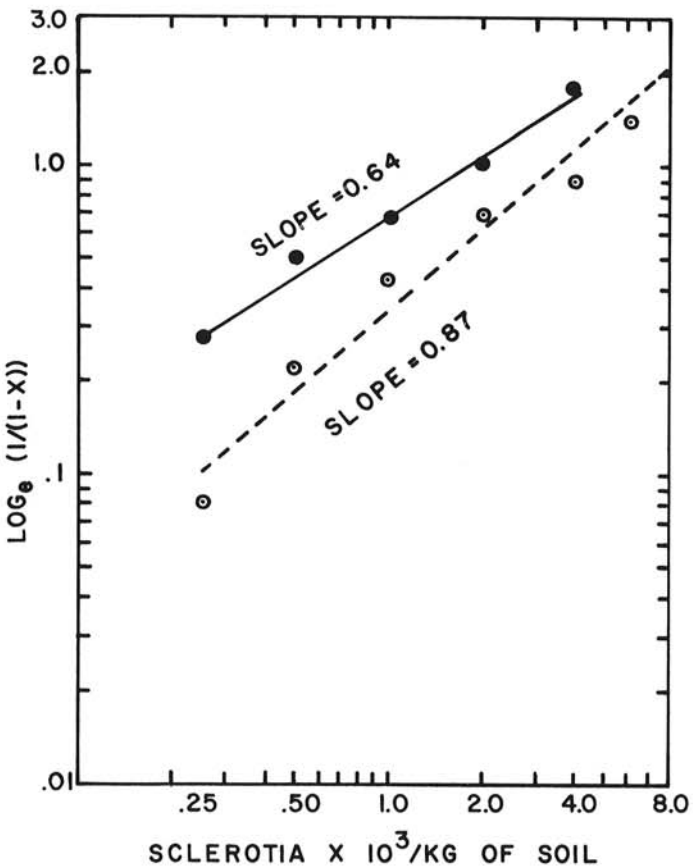

Fig. 5. The relationship of sclerotial density ($\log_{10}$) of *Rhizoctonia solani* in autoclaved (•——•) and nonautoclaved (o- - - -o) soil to percentage of diseased soybean seedlings ($\log_{10}[\log_e 1/(1-x)]$), in which x = proportion of diseased seedlings, 25 days after planting.

autoclaved soil.

Weinhold et al (26) reported that *R. solani* can utilize effectively exogenous nutrients to increase virulence. Sclerotia of *M. phaseolina* have been reported to germinate in the spermosphere of soybean seeds (21), which contains sucrose and fructose (10), compounds known to stimulate germination of sclerotia of *M. phaseolina* (1). Furthermore, the reduction of root infection by *M. phaseolina* by antagonistic fungi has been reported on several crops (8,9). Luttrell and Garren (12) reported that infection of snap bean by *M. phaseolina* was less in nonautoclaved field soil than in autoclaved soil.

Approximately 9 and 45 times fewer sclerotia per gram of nonautoclaved and autoclaved soil, respectively, were required in our study for 50% infection of soybean seedlings by *M. phaseolina* than that reported by Meyer et al (14). They found that 50% infection of soybean plants by *M. phaseolina* required approximately $35–37 \times 10^4$ propagules per kilogram of autoclaved soil mixed with autoclaved silica sand (1:1, v/v) after it had been recolonized by microorganisms for 2 mo. Several factors might have contributed to the discrepancy of the ED_{50}s found in the two experiments, such as different cultivars, environmental conditions, isolates, amount of infested soil to which plants were exposed, and length of exposure of the plants to infested soil.

For *R. solani*, the slopes of the log-log transformation of the numbers of sclerotia to the proportions of infected plants were less than 1.0 in both autoclaved and nonautoclaved soil. Less than direct proportionality of inoculum density to disease incidence may have occurred because other soilborne microorganisms residing in nonautoclaved soils or introduced into either of the soils as airborne contaminants may have acted as antagonists to or competitors with the pathogen. At the end of the experiment, *R. solani* could not be recovered from autoclaved soil, but *Trichoderma* spp. that were not included in the artificial infestation were recovered. This failure to recover *R. solani* may have resulted from the presence of *Trichoderma* spp. in soil since *Trichoderma* spp. strongly influence the recovery of fungi from peanut pods (3) and soil (6).

Schenck and Kinloch (20) presented evidence that *M. phaseolina, R. solani*, and *F. solani* occur in association in the field, indicating they may form a complex on soybean. The consistent increased reduction of plant growth parameters by *R. solani* and *F. solani* when combined with *M. phaseolina* would support this disease complex hypothesis. Garcia and Mitchell (4) also reported high frequencies of isolation of *F. solani* and *R. solani* in interactions with *M. phaseolina* on peanut pods. Increased disease has been reported for other soilborne, root-infecting fungi when combined with *M. phaseolina* (7,15,23).

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