Recovery and Pathogenicity of *Rhizoctonia solani* and Binucleate *Rhizoctonia*-like Fungi in Forest Nurseries

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ABSTRACT

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A baiting technique using slash pine stem segments provided a rapid, sensitive, and accurate means of assessing inoculum potential of populations of *Rhizoctonia solani* and binucleate *Rhizoctonia*-like fungi (BRLF) in forest nursery soils. Recovery of *R. solani* and BRLF from soils was best when stem segments were from 45- to 75-day-old seedlings and when soil plates with stem segments were incubated for 48 hr at 20–30 C. Percentage of stem segments colonized was highly correlated ($r \geq 0.95$) with population density detected by the multiple-pellet soil sampler method. However, the stem segment method was more sensitive for detecting small populations of *R. solani* and BRLF in the forest nursery soil. In an inoculum density experiment, the number of segments colonized in the stem segment assay was highly correlated ($r \geq 0.85$) with damping-off severity. However, not all isolates that colonized stem segments were pathogenic to slash pine seedlings.

Additional keywords: loblolly pine, pine bark mulch

Since the association of *Rhizoctonia solani* Kühn with damping-off diseases of conifers was reported in 1929 (17), pathologists have been studying host-pathogen relationships for this fungus (15). The anastomosis group (AG) concept has enabled mycologists to classify groups within this broad species and has enabled plant pathologists to better understand the relationships between the fungus and its numerous hosts (1,3,14). Isolates in AG-4 are important plant pathogens with a wide host range (1). Binucleate *Rhizoctonia*-like fungi (BRLF) have mycelium characteristic of the genus *Rhizoctonia*, binucleate vegetative mycelial cells, and a *Ceratobasidium* teleomorph (3). These binucleate *Rhizoctonia*-like fungi include several important fungi pathogenic to forest trees (5,14,15). Isolates in anastomosis group 3 (CAG-3) of BRLF were commonly obtained in the southeastern United States from longleaf pine (5) as well as from agronomic crops (3). Many methods, including plant stem segment colonization (4,12,13,16,18), immersion tube colonization (4,13), debris particle isolation (4,13), disk-plate method (9), and seed colonization (11,13), have been used to assay the inoculum potential of these fungi in agronomic and horticultural soils.

In preliminary trials, we found that stem segments of slash pine (*Pinus elliottii* Engelm. var. *elliottii*) seedlings were colonized by *R. solani* and BRLF soon after insertion into soil. Therefore, the objectives of the present research were to develop a standardized method for bioassay of *R. solani* and BRLF in forest nursery soils and to relate the bioassay to relative pathogenicity of isolates of *R. solani* and BRLF on slash pine seedlings.

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MATERIALS AND METHODS

Soil. The study soil, collected from a forest site in Clarke County, Georgia, had a pH of 5.7 and a composition of 95.8% sand, 2.3% clay, 0.9% silt, and 1.0% organic matter. The soil was sifted through a 10-mesh (2-mm) screen and stored at 6% (w/w) moisture content in polyethylene bags.

Inoculum. Isolate BB-08 (AG-4) of *R. solani* was recovered from pine bark mulch, and isolate WH-10 (CAG-3) of a binucleate *Rhizoctonia*-like fungus was recovered from a seedling of loblolly pine (*P. taeda* L.) with symptoms of damping-off. Their anastomosis groups were determined by pairing with tester isolates (Rhs-81 and BN-07) (5). All isolates of *R. solani* and BRLF were maintained on potato-dextrose agar (PDA). For soil inoculation, chopped potato-soil inoculum was prepared (10). Infested soils were prepared by mixing 1:1 and 1:4 ratios of inoculum and soil fumigated with methyl bromide.

Assay of inoculum level. The amount of inoculum in soil was estimated by a stem segment colonization method. A 24-g subsample from each infested soil sample was dispensed into a petri dish with four sections, about 6 g of soil per section. Soil moisture was brought to 12–15% (w/w) with sterile distilled water. Stem segments were prepared by placing 6-wk-old slash pine seedlings in 1% sodium hypochlorite solution for 3 min, then cutting the stems into segments 5–7 mm long. Twenty-four segments (six per section) were then inserted vertically into the soil such that 2–3 mm was below the surface for baiting *Rhizoctonia* spp. Four replications of each soil were used for each experiment. After incubation for 48 hr at 25 C, stem segments were recovered and washed for 3 min with 1% sodium hypochlorite solution, blotted with paper towels, and plated on 2% water agar containing 300 μg/ml of streptomycin sulfate. After incubation at 25 C for 20 hr, the number of stem segments with signs of *Rhizoctonia* spp. was determined microscopically. Four factors affecting the recovery of *R. solani*

and BRLF in stem segment baits were evaluated: slash pine family, seedling age, temperature, and length of incubation period. Three families of slash pine (EMAN-5, 4555-5, and 3456-9), six ages of seedlings (25, 35, 45, 55, 65, and 75 days), seven incubation temperatures (5, 15, 20, 22, 25, 30, and 32 C), and six incubation periods (0, 12, 24, 36, 48, and 60 hr) were compared to establish a standard procedure for colonization of stem segments by *R. solani* (BB-08) and BRLF (WH-10).

Comparison with multiple-pellet soil sampler method. Inoculum density of infested soil was adjusted with fumigated soil to achieve seven ratios (1:1, 1:4, 1:16, 1:64, 1:256, 1:1,024, and 0:1) of infested to total soil, then the soil was moistened to about 12% (w/w) and placed on a medium selective for *Rhizoctonia* spp. (10) with a multiple-pellet soil sampler (8). For each inoculum concentration, 15 100- to 110-mg soil pellets were deposited on agar in each of 10 petri dishes. The petri dishes were incubated in the dark at 25 C for 24–48 hr, and colonies characteristic of *Rhizoctonia* spp. were counted. Four replicate experiments were conducted to determine recovery percentages of *Rhizoctonia* spp. in the seven inoculum levels. Each inoculum concentration was also sampled with the stem segment colonization method. Then, a correlation between the two methods was made.

Seedling disease assay. Seedling infection was determined by planting surface-sterilized germinating seeds (hypocotyls about 0.3 cm long) of slash pine (EMAN-5 family) in the infested soils in plastic flats (15 × 12 × 6.5 cm). Ten seeds were planted in each of four replicates, and the plants were maintained for 21 days in the greenhouse at 25–30 C. Disease severity was expressed as the percentages of seedlings with preemergence and postemergence damping-off. To consider multiple infections and compare the relationship of stem segment colonization to the severity of damping-off (combined preemergence and postemergence damping-off), we transformed the proportion of diseased plants (x) to $\ln [1/(1 - x)]$ (2,7,16). Statistical analyses were done with the SAS/STAT System for Personal Computers (SAS Institute Inc., Cary, NC). The identity of the pathogen was verified by reisolating the fungus from diseased plants on water agar and confirming its features microscopically.

Relation of stem segment colonization to pathogenicity of the isolates and field soil assay. Eleven isolates of *R. solani* and BRLF, originally obtained from a range of hosts or substrates, were tested for pathogenicity on seedlings of slash pine and for ability to colonize stem segments in soil. The effectiveness of stem segment colonization for monitoring naturally infested soils was evaluated in 10 soil samples from two nursery beds in Clarke County with loblolly and slash pine seedlings with and without damping-off; 96 stem segments were used for each soil sample.

RESULTS

Factors affecting stem segment colonization. Stem segments from half-sib progeny of three slash pine families (EMAN-5, 4555-5, and 3456-9) did not differ ($P = 0.05$) in recovery of *R. solani* AG-4 and BRLF CAG-3 from infested soil. *Rhizoctonia* spp. colonized 82–92% of the segments from all three families.

Seedling age. Stem segments from 45- to 75-day-old seedlings had the highest percentages of colonization (85–100%) and had small variations in colonization frequency (SD 0–3.5) by *R. solani* AG-4 and BRLF CAG-3 (Fig. 1). Segments from 25- and 35-day-old seedlings had lower percentages and larger variations (SD 6.0–19.0) in colonization than segments from older seedlings. Segments from seedlings less than 25 days old were not easily recovered from soil and broke down during washing.

Soil temperature. Recovery of *R.*

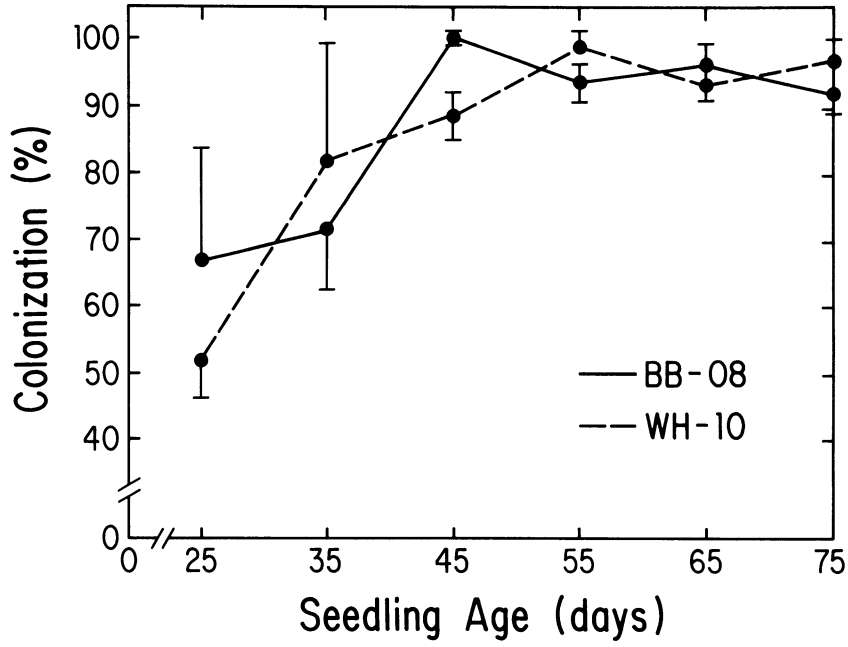

Fig. 1. Colonization of stem segments of slash pine seedlings of different ages in infested nursery soil at 25 C. BB-08 is isolate of *Rhizoctonia solani* AG-4, and WH-10 is isolate of binucleate *Rhizoctonia*-like fungus CAG-3. Vertical bars represent standard deviation ($n = 4$).

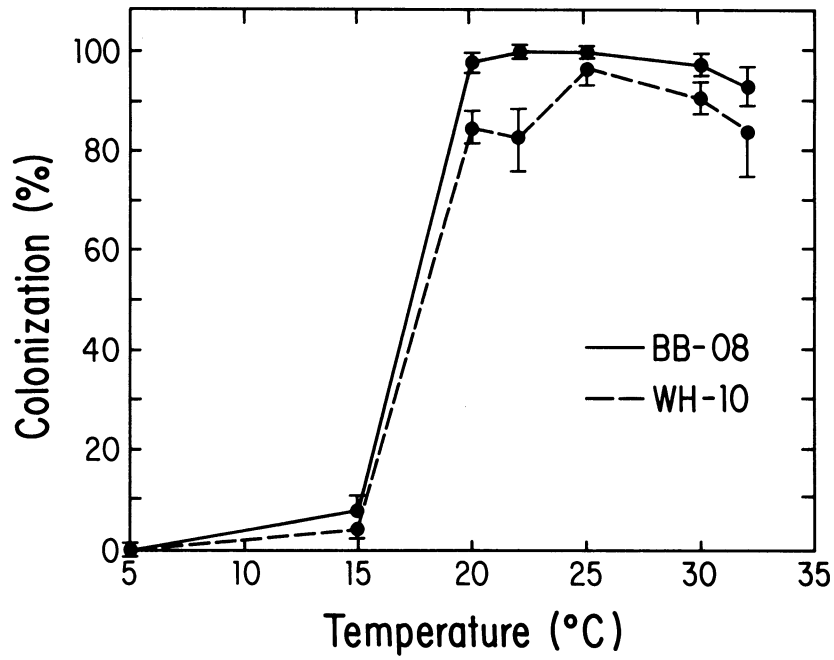

Fig. 2. Effect of temperature on colonization of stem segments of slash pine seedlings by isolate BB-08 of *Rhizoctonia solani* AG-4 and isolate WH-10 of *Rhizoctonia*-like fungus CAG-3. Vertical bars represent standard deviation ($n = 4$).

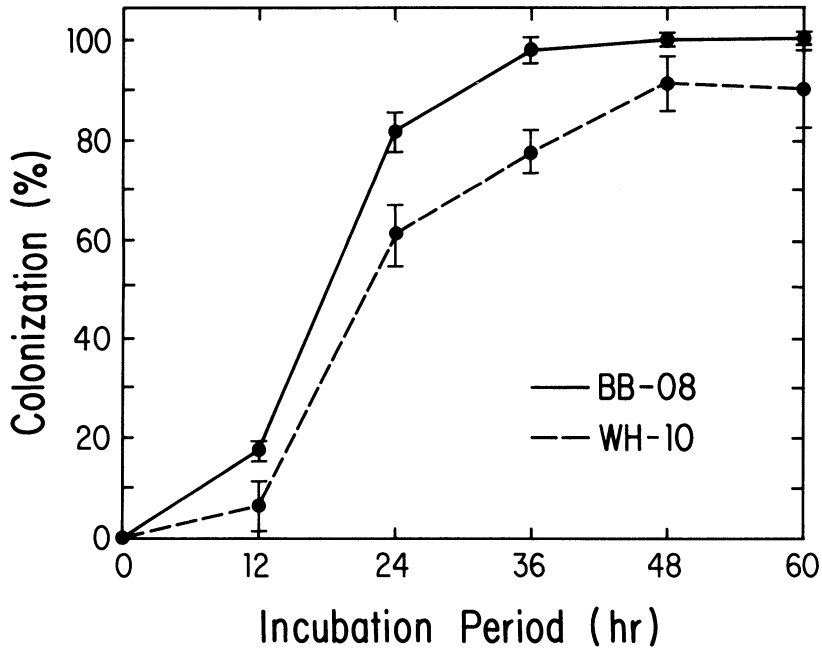

Fig. 3. Colonization of *Rhizoctonia* spp. on stem segments of slash pine seedlings after different incubation periods at 25 C. BB-08 is isolate of *Rhizoctonia solani* AG-4, and WH-10 is isolate of binucleate *Rhizoctonia*-like fungus CAG-3. Vertical bars represent standard deviation ($n = 4$).

Table 1. Sensitivity of stem segment colonization and multiple-pellet soil sampler methods for recovery of *Rhizoctonia solani* AG-4 and binucleate *Rhizoctonia*-like fungus from soils infested with different inoculum density levels of each fungus at 25 C

	Rhizoctonia recovered (% of segments or samples)[y]			
	Isolate BB-08		Isolate WH-10	
Inoculum level[x]	Stem segment colonization method	Multiple-pellet soil method	Stem segment colonization method	Multiple-pellet soil method
1:1	99 ± 1.8[z]	72 ± 4.3[z]	98 ± 2.1[z]	79 ± 5.8[z]
1:4	70 ± 3.4	33 ± 4.2	65 ± 9.5	31 ± 1.8
1:16	27 ± 4.7	9 ± 2.9	34 ± 6.2	11 ± 1.1
1:64	17 ± 2.9	3 ± 1.1	9 ± 1.8	2 ± 0.9
1:256	3 ± 1.8	1 ± 0.4	3 ± 3.4	1 ± 0.7
1:1,024	1 ± 1.8	0 ± 0	1 ± 1.8	0 ± 0
0:1	0 ± 0	0 ± 0	0 ± 0	0 ± 0

[x] Ratio of potato-soil inoculum to soil fumigated with methyl bromide.
[y] Relationship between recovery percentage of *Rhizoctonia* spp. (x) detected by multiple-pellet soil sampler method and stem segment percentage ($\hat{Y}$) was $\hat{Y}_{BB\text{-}08} = 7.75 + 1.37X$ ($r = 0.96$) for *Rhizoctonia solani* isolate BB-08 and $\hat{Y}_{WH\text{-}10} = 8.21 + 1.24X$ ($r = 0.95$) for binucleate *Rhizoctonia*-like fungus isolate WH-10.
[z] Standard deviation.

solani AG-4 and BRLF CAG-3 was best at 20–30 C (Fig. 2). At 5–15 C, *R. solani* AG-4 and BRLF CAG-3 apparently grew very slowly and colonized stem segments less often during the 48-hr incubation period.

Incubation period. A high frequency of colonization by BB-08 and WH-10, with relatively little competition from other organisms, was obtained at 36–60 hr (Fig. 3). The highest frequency of colonization occurred with a 48-hr incubation period.

Comparison between stem segment colonization and multiple-pellet soil sampler methods. There was a high correlation ($r \geq 0.95$) between percentage of stem segments colonized by *R. solani* and BRLF and population density detected by the multiple-pellet soil sampler method (Table 1). The stem segment colonization method, however, was the most sensitive for detecting small populations of *Rhizoctonia* spp. in the forest nursery soil.

Relation of stem segment colonization to severity of damping-off of slash pine seedlings. It was important to determine at what level of stem segment colonization a given amount of damping-off of slash pine seedlings was produced. An increase in *Rhizoctonia* spp. infestation in soil enhanced both the colonization activity of the fungi and their pathogenic effect on slash pine seedlings (Table 2). There was a high correlation ($r \geq 0.85$) between damping-off severity (percentage) or disease index ($\ln [1/(1 - x)]$ transformation) and percentage of stem segments colonized.

Relation of stem segment colonization to pathogenicity of *Rhizoctonia* spp. isolates. Isolates from a variety of hosts and substrates not only caused severe preemergence and postemergence damping-off of slash pine but also resulted in 88–94% colonization on stem segments (Table 3). Isolate Rhs-81, obtained from cotton hypocotyl, colonized only 44% of the stem segments but caused 63% damping-off. Isolates BN-01 and BN-14a

Table 2. Colonization of pine stem segments by *Rhizoctonia* spp. compared with damping-off of slash pine seedlings and a disease index in soil with various concentrations of inoculum of *R. solani* isolate BB-08 and binucleate *Rhizoctonia*-like fungus isolate WH-10

	Isolate BB-08[w]			Isolate WH-10[w]		
Inoculum level[v]	Stem segment colonization (%)	Seedling damping-off[x] (%)	Disease index[y]	Stem segment colonization (%)	Seedling damping-off[x] (%)	Disease index[y]
1:1	89 ± 3.7[z]	83 ± 4.3[z]	1.78 ± 0.30[z]	90 ± 2.0[z]	88 ± 4.3[z]	2.13 ± 0.03[z]
1:4	61 ± 3.5	88 ± 4.3	2.12 ± 0.30	62 ± 11.9	88 ± 4.3	2.13 ± 0.30
1:16	26 ± 3.5	75 ± 5.0	1.40 ± 0.20	29 ± 2.8	78 ± 8.3	1.58 ± 0.45
1:64	16 ± 3.4	45 ± 5.0	0.60 ± 0.09	12 ± 3.6	40 ± 7.1	0.52 ± 0.12
1:256	3 ± 1.8	18 ± 4.3	0.19 ± 0.05	2 ± 2.1	23 ± 4.3	0.26 ± 0.06
1:1,024	0 ± 0	5 ± 5.0	0.05 ± 0.05	0 ± 0	3 ± 4.3	0.03 ± 0.04
0:1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

[v] Ratio of potato-soil inoculum to soil fumigated with methyl bromide.
[w] Relationship of stem segment colonization (X) with seeding damping-off ($\hat{Y}$) was $\hat{Y}_{BB\text{-}08} = 18.54 + 0.93x$ ($r = 0.85$) for isolate BB-08 and $\hat{Y}_{WH\text{-}10} = 19.19 + 0.94x$ ($r = 0.86$) for isolate WH-10; with disease index, ($\hat{Y}$) was ($\hat{Y}_{BB\text{-}08} = 0.244 + 0.023x$ (r = 0.87) and $\hat{Y}_{WH\text{-}10} = 0.241 + 0.024x$ ($r = 0.88$).
[x] Includes preemergence and postemergence damping off.
[y] Disease index was determined by $\ln [1/(1 - x)]$ instead of x (proportion of seedlings with damping-off).
[z] Standard deviation.

(from cucumber root) and Rhs-01 (from spruce seedling) colonized 100, 100, and 50% of stem segments, respectively, but did not cause damping-off of slash pine.

Application of stem segment colonization technique to naturally infested soil. Soil from two nursery beds in Clarke County had been fumigated with methyl bromide. In spite of this, *Rhizoctonia* spp. were present in several small areas and loblolly pine seedlings were killed by BRLF CAG-3 in May 1987. The stem segment colonization technique was used to monitor the survival of *Rhizoctonia* spp. in these two beds. About 15% of stem segments of slash pine seedlings were colonized by BRLF CAG-3 in soil where damping-off of loblolly pine seedlings occurred. However, *R. solani* and BRLF were not recovered in soil from beds without symptoms of damping-off.

DISCUSSION

Using infection of pine seedlings to monitor biocontrol or chemical control generally requires considerable space in a greenhouse. Furthermore, seedling infection requires a longer exposure period than does stem segment baiting. Therefore, pine stem segment colonization was exploited to solve these two problems before research was extended to the greenhouse and field. The bioassay with pine stem segments is convenient and efficient for detecting the inoculum potential of *R. solani* and BRLF in forest nursery soils.

Colonization of a substrate is not necessarily correlated with pathogenicity (11). Papavizas and Davey (12) reported that isolates of *Rhizoctonia* spp. obtained from various stem segment baits ranged from nonpathogenic to strongly pathogenic. Our data (Table 3) indicated that pathogenicity of 11 isolates on slash pine was divided into three groups: virulent, intermediately virulent, and avirulent. The colonization capacity of the isolates was also divided into three levels: high capacity (80–100%), intermediate capacity (40–50%), and no capacity (0%). Although virulence was not consistently correlated with colonization, five pathogenic isolates (BB-08, WH-10, Rhs-103, BN-07, and Rhs-81) were able to colonize stem segments, and three nonpathogenic isolates (BN-12, Rhs-36, and Rhs-45) did not colonize stem segments. When stem segments of slash pine are used to detect the population density of *Rhizoctonia* spp. in naturally infested soils, the capacity of the population of isolates obtained to cause damping-off of seedlings must also be tested; that requirement has been demonstrated with *R. solani* and BRLF on agronomic crops (12).

The percentage of diseased plants observed in an experiment does not necessarily reflect the number of successful infections. An individual plant may be invaded by a pathogen many times but would be recorded only once as being diseased (2). The reason for making the $\ln[1/(1-x)]$ transformation is that one would expect to find the number of infections related to inoculum density (7,16). However, the correlation coefficients between colonization and the nontransformed data (damping-off severity) were equal to those between colonization and the transformed data (disease index, or number of infections). This result indicates that it is not necessary to consider transformation of multiple infections when monitoring damping-off severity with the stem segment colonization method.

The multiple-pellet soil sampler method was reported to be both accurate and convenient for estimating the quantity of *R. solani* in soil (8). The sensitivity of the stem segment colonization method for recovery of *R. solani* and BRLF was compared with that of the multiple-pellet soil sampler method. Results were highly correlated (Table 1), but lower levels of *Rhizoctonia* spp. were detected by stem segment baiting. With the pellet soil sampler, the detectable level of propagules of *Rhizoctonia* spp. in the soil is limited by the number of replicates, by the size of the pellets (8), and by the selectivity of the growth medium. Many soil microorganisms are competitive saprophytes (6), and prolonging the incubation period allows them to physically obscure colonies of *Rhizoctonia* spp. on the assay plates (11). Growth of microorganisms other than *R. solani* on the medium of Ko and Hora (10) cannot be eliminated completely, and delaying the recording of *R. solani* growth beyond 24 hr allows such microorganisms as *Trichoderma* and *Fusarium* to overgrow *R. solani*, making quantitative assessment difficult (4). The pine stem segment baiting technique, on the other hand, limits competitive saprophytic pressure of other fungi and thus has an advantage over the multiple-pellet soil sampler method.

Table 3. Assay of *Rhizoctonia solani* and binucleate *Rhizoctonia*-like fungi in soil by colonization of slash pine seedling stem segments and pathogenicity of isolates obtained to slash pine seedlings

Isolates[x]	Anastomosis group	Original host or substrate	Stem segment colonization (%)	Seedling damping-off[y] (%)
BB-08	AG-4	Pine bark mulch	94 b[z]	100 a[z]
Rhs-103	AG-5	Turfgrass	94 b	95 a
WH-10	CAG-3	Pine seedling	88 c	90 a
BN-07	CAG-3	Peanut pod	94 b	73 b
Rhs-81	AG-4	Cotton hypocotyl	44 e	63 b
BN-01	CAG-2	Cucumber root	100 a	10 c
BN-14a	CAG-5	Cucumber root	100 a	10 c
Rhs-01	AG-1	Spruce seedling	50 d	10 c
BN-12	CAG-4	Soybean root	0 f	10 c
Rhs-36	AG-2-2	Corn brace root	0 f	8 c
Rhs-45	AG-2-1	Peanut root	0 f	5 c
Check	...	...	0 f	8 c

[x] All isolates except BB-08 and WH-10 were provided by D. R. Sumner, University of Georgia.
[y] Includes preemergence and postemergence damping-off; data were recorded 21 days after germinating seeds were planted.
[z] Means (four replicates) in the same column followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

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