A Quantifiable Type of Inoculum of *Rhizoctonia solani*

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ABSTRACT

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Production of sclerotia of *Rhizoctonia solani* was compared on six types of substrates in vitro. Autoclaved green bean pods were the most efficient substrate. About 1 million sclerotia measuring 300–710 μm in diameter were produced on 1 kg of frozen cut beans. The viability of sclerotia on potato-dextrose agar was greater with increasing size of sclerotia. Similarly, the ability of large sclerotia to grow on sand and infect a bean hypocotyl piece was better than that of small sclerotia. Sclerotia formed on bean pods were more vigorous than sclerotia of the same size produced on bean leaves. Only growth of small sclerotia from bean leaves was stimulated by the presence of a hypocotyl piece.

Rhizoctonia solani Kühn survives in soil in the form of thick-walled mycelium or sclerotia associated with organic debris (3). Formation of sclerotia in soil may be induced by nutritional exhaustion or possibly by water condensation on stones, organic matter, or other material in the soil (17). On culture media, sclerotial formation is determined by the nutritional status, notably the concentrations of carbon and nitrogen. Although the initial amount of carbohydrate is positively correlated with the numbers of sclerotia produced (12,14,19), excess carbohydrate inhibits sclerotial initiation (1) or maturation (19). Both inorganic nitrogen and amino acids promote sclerotial development (1,19), with the exception of sulfur-containing amino acids (15). Nutritional status also affects the virulence of sclerotia (6,12,22). Virulence of the infecting hyphae is dependent on the size and age of the parent propagules and on the quantity and quality of the food base as determined by the substrate (6,8,10,22). Propagules smaller than 150 μm produced on yeast-dextrose broth are noninfective (6) but become infective in the presence of yeast extract (10).

Inocula of *Rhizoctonia* used in inoculum density-disease relationship studies have been produced on autoclaved cornmeal-sand (11), infested lettuce leaves or chopped potato in soil (2), and oat or barley grains (16,21). Recently, McCoy and Kraft (12) advocated the use of sclerotia to screen peas (*Pisum sativum* L.) for resistance to *R. solani*, because sclerotia could be reproduced reliably and they were free from an extraneous nutrient source. However, individual sclerotia have been used rarely because of difficulties encountered in large-scale production and separation from mycelium and/or plant debris. Therefore, inoculum usually has not been quantified before being incorporated into soil. In inoculum density-disease studies, *Rhizoctonia* inoculum has been added on a volume-per-volume or weight-of-soil basis and then diluted with uninfested soil (11,18). Inoculum density has been checked with more or less quantitative isolation techniques, such as soil plating or pelleting on a selective medium (7,9) or with the use of baits (18).

We compared six methods of inoculum production to find a medium on which large numbers of sclerotia could be produced and from which the sclerotia could be removed as purely as possible. This study was conducted as part of a larger study on the effects of inoculum density of *R. solani* on infection of dry beans (*Phaseolus vulgaris*).

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MATERIALS AND METHODS

An isolate of *R. solani* (R-2, courtesy G. S. Abawi, N.Y. State Agricultural Experiment Station, Geneva) from table beet (*Beta vulgaris*) in New York was used. This isolate was highly pathogenic to beans and did not anastomose with tester isolates of AG 1–4 (5).

Inoculum production. The media tested either produced infested substrate particles (vermiculite, beet seed, and radish [*Raphanus sativus*] seed), or resulted in the formation of discrete sclerotia that had to be separated from their substrate (mycelial mats on soil or sand, potato-dextrose broth, bean tissues, and bean tissue extracts). The following substrates were tested:

1. *Vermiculite/V-8 juice.* Vermiculite was mixed with V-8 juice, water, and $CaCO_3$ as recommended for *Phytophthora* (13). About 70 ml of the mixture was added to each Erlenmeyer flask (100-ml).

2. *Radish or beet seeds.* Radish or beet seeds were moistened to full imbibition (3 hr) and autoclaved in 9-cm petri dishes (100 seeds per dish).

3. *Mycelial mats on clay or sand.* Mycelial mats of *R. solani* grown on potato-dextrose broth (Difco, Detroit, MI) for 4 days at 24 C were washed twice in sterile, distilled water and transferred onto a 2-mm layer of sterile clay soil (pH 5.8), dark sand (pH 7.5), or white quartz sand (pH 6.6) in 9-cm petri dishes (4).

4. *Bean tissues.* Layers (5–10 mm thick) of commercially available frozen green beans and dried and remoistened bean leaves and stems were added to petri dishes and autoclaved.

5. *Green bean pods on water agar.* Autoclaved slices of frozen green bean pods (5 mm thick) were placed into solidifying 3% Bactoagar (Difco) (five slices per petri dish).

6. *Liquid media.* Liquid media were included with the expectation that sclerotia could be separated easily from the substrate. Fifty grams of dry crushed bean leaves or green bean pods was autoclaved in 500 ml of water. The extracts were poured into petri dishes and autoclaved. Similarly, potato-dextrose broth (Difco) was autoclaved in petri dishes.

All media except the mycelial mats on sand/soil were infested with one 2-mm disk from a 3-day-old potato-dextrose agar (PDA) culture of *R. solani* and incubated at 18 C (17). There were three replicates for each medium. Sclerotia were separated from sand or soil by blending the contents of a petri dish with water, centrifuging the mixture in a clinical centrifuge (International Equipment Co., Needham, MA) for 1 min, stirring the pellet into a 75% (w/w) sugar solution, and floating off the sclerotia on sieves with openings of 710, 500, 300, 250, and 150 μm. Sclerotia were separated from plant material (bean pods, leaves, and stems) and broth and liquid extracts by blending the contents of a petri dish with water, adjusting the pH to 4 with 0.1 N HCl, adding 1 ml of pectinase per 5 g of material, then blending this mixture again the next day and separating the sclerotia according to size on sieves with openings of 710, 500, 300, 250 and 150 μm. All sclerotia were dried on filter paper in a Büchner funnel. The numbers of sclerotia per treatment were estimated by weight after determining the weight-to-number relationships under a dissecting microscope in subsamples of each treatment.

Assay of inoculum viability and virulence. Five sclerotia of four size classes were placed on each of three petri plates (9 cm in diameter) containing 12 ml of solidified PDA to test their viability. The plates were incubated at 24 C for 4 days.

Large (0.71–2 mm) and small (0.3–0.5 mm) sclerotia produced on bean leaves and green bean pods were placed on washed and autoclaved white quartz sand in the presence or absence of a 1-cm piece of fresh bean hypocotyl. The hypocotyl segments had been cut from 5-day-old seedlings and had been disinfested in 0.5% sodium hypochlorite for 2 min, rinsed three times in sterile water, and placed in the center of a petri dish with sterile sand (one piece per plate). Two similar sclerotia were placed 3 cm away on either side of a hypocotyl piece. There were four replicates per treatment. For comparison, infested radish seed and vermiculite were also used. Four plates with and without hypocotyls and without *R. solani* were kept as controls. All dishes were incubated at 24 C.

Large-scale inoculum production. Green bean pods were selected for large-scale inoculum production. Frozen green beans were defrosted, the liquid was drained off, and the beans were autoclaved in Pyrex storage dishes (100 g/dish) or widemouthed mason jars (200 g/jar) for 30 min. Each dish or jar was infested with three disks (2 mm in diameter) of 8- to 10-day-old cultures of *R. solani* on PDA. The infested beans were incubated at 27 C for 2 wk, then air-dried on paper towels. The dried bean material was ground in a Waring Blendor for about 1 min and sieved on 25- and 50-mesh sieves, so that sclerotia of 300–710 μm resulted.

RESULTS

Numerous sclerotia were formed on radish and beet seeds, but these were not counted separately from their substrates. No sclerotia were formed on vermiculite plus V-8 (Table 1). During the sieving procedures, the replicates were pooled, so statistical comparison of the media was not possible. Nevertheless, the numbers of sclerotia were clearly largest on autoclaved green beans. Almost all bean tissue turned into sclerotia, making separation of sclerotia from organic matter unnecessary. A chi-square test indicated that there were significantly more sclerotia in the larger size classes (>0.25 mm) on bean pod tissue than on all other media tested ($\chi^2 = 262$, $P < 0.005$).

In general, the viability of the smaller sclerotia was poorer than that of larger sclerotia (Table 2). Likewise, viability of sclerotia from bean stems and of infested vermiculite was less than that of other inoculum preparations (see 95% confidence intervals in Table 2).

Mycelial growth from sclerotia on sand was measured on the longest hyphae. No differences were noted between colony growth toward and away from the hypocotyl segments.

Hyphal growth of *R. solani* on sand was significantly related to size of sclerotia and bean tissues on which they were produced (Table 3). An analysis of variance for these data indicated that after 2 and 3 days, hyphae from large sclerotia were significantly ($P = 0.0001$) longer than those from small sclerotia, and those from sclerotia produced on green bean pods were significantly ($P = 0.002$) longer than those produced on

Table 1. Mean production of sclerotia of *Rhizoctonia solani* per petri dish

Medium	No. of sclerotia produced in six size classes (mm)					
	>2.0	0.71–2.0	0.5–0.71	0.3–0.5	0.25–0.3	0.15–0.25
Vermiculite/V-8 juice	0	0	0	0	0	0
Mycelial mats on						
Clay	0	10	20	50	20	30
Dark sand	0	10	20	50	70	170
White sand	0	6	13	80	30	0
Bean tissues						
Green bean pods	13	670	1,170	2,670	1,000	2,000
Bean leaves	1	130	330	170	150	130
Bean stems	0	30	50	70	70	60
Green bean pods on water agar	1	15	50	50	30	25
Liquid media						
Potato-dextrose broth	0	3	20	70	100	0
Bean leaf extract	0	1	2	3	0	0
Bean pod extract	0	1	60	500	330	200

Table 2. Mean percent viability (with a 95% confidence interval) of 15 sclerotia of *Rhizoctonia solani* and seeds or vermiculite infested with *R. solani* on potato-dextrose agar

Source	Percent viability of sclerotia in five size classes (mm)					
	>2.0	0.71–2.0	0.5–0.71	0.3–0.5	0.25–0.3	Mean
Seeds						
Radish	80 (55–95)[a]	...	...	...	...	...
Beet	100 (80–100)	...	...	...	...	...
Vermiculite/V-8 juice	...	67 (36–86)	20 (5–45)	13 (2–37)	20 (5–45)	30 (19–43)
Mycelial mats on						
Clay	...	87 (63–98)	80 (55–95)	60 (33–81)	33 (14–64)	65 (53–78)
Dark sand	...	100 (80–100)	60 (55–95)	13 (2–37)	33 (14–64)	58 (38–65)
White sand	...	100 (80–100)	67 (36–86)	80 (55–95)	40 (19–67)	72 (58–83)
Bean tissues						
Green bean pods	...	80 (55–95)	87 (63–98)	93 (69–100)	60 (29–81)	80 (67–89)
Bean leaves	...	87 (63–98)	93 (69–100)	60 (33–81)	33 (14–64)	68 (55–79)
Bean stems	...	60 (33–81)	27 (9–56)	80 (55–59)	13 (2–37)	45 (33–62)
Green bean pods on water agar	...	100 (80–100)	87 (63–98)	93 (69–100)	27 (9–56)	77 (63–86)
Liquid media						
Bean pod extract	...	80 –[b]	87 (63–98)	87 (63–98)	67 (36–86)	80 (67–89)
Mean		90 (25–98)	85 (76–91)	67 (58–75)	64 (55–72)	36 (28–45)

[a] 95% Confidence interval.
[b] Only five sclerotia available.

bean leaves. There was no interaction between the size and source of sclerotia. The presence of hypocotyl pieces did not affect hyphal growth, and there were no interactions between the presence of hypocotyl and sclerotial size or origin. After 12 days, *R. solani* from large sclerotia produced on bean pods had covered the whole plate, so the data of this treatment were excluded from statistical analysis. Again, the differences in hyphal growth between large and small sclerotia were significant ($P = 0.0015$) but not between those from leaves and pods. There was also no significant interaction between size of sclerotia and origin and the presence of a hypocotyl segment, despite the apparent stimulation of mycelial growth by the hypocotyl in the case of small sclerotia produced on bean leaves.

After 12 days, all colonies growing from large sclerotia produced on bean pods had invaded the hypocotyls (Table 3), resulting in collapse of the hypocotyl tissue and formation of new sclerotia on one hypocotyl segment. Four of the colonies from large sclerotia produced on bean leaves had reached and invaded two hypocotyl pieces. Only two colonies from small sclerotia produced on bean leaves had reached and invaded one hypocotyl piece, and two colonies from small sclerotia produced on green bean pods had reached one hypocotyl but had not yet invaded it. Growth of *R. solani* from infested radish seed was comparable to that of large sclerotia from bean leaves; mycelial growth seemed to be stimulated by a hypocotyl piece. Only one hypha was observed growing out of a vermiculite particle, thus vermiculite was not included in Table 3. The control dishes with sand or sand plus a hypocotyl piece did not have any fungal growth, except in one dish in which the hypocotyl piece was degraded by *Fusarium* sp.

For large-scale inoculum production, 1 kg of frozen green beans produced about 1 million ($0.96 \pm 0.18 \times 10^6$) sclerotia 300–710 μm in diameter. The numbers of sclerotia per 10 mg sclerotial mass, as counted under a dissecting microscope, were 333 ± 21. The weight per milliliter of sclerotia was 192 ± 39 mg.

DISCUSSION

Of the six groups of substrates tested, the vermiculite/V-8 mixture appeared to be the least effective substrate for sclerotial production of *R. solani*. Hyphal growth from the vermiculite particles was poor, both on PDA and on sand. The medium, as recommended for *Phytophthora* (13), was probably too wet for *R. solani*. Besides, V-8 juice itself may not be conducive for sclerotial production (12).

Of the other substrates used, green bean pods were most effective in sclerotial production. The efficient turnover of green bean pods into sclerotia of *R. solani* may be attributed to a relatively low C/N ratio in pods compared with that in older leaves or stems. The importance of the C/N ratio of a substrate for sclerotial production has been pointed out earlier (14,19).

Of the liquid media, bean pod extract gave rise to the most sclerotia, and potato-dextrose broth produced only moderate numbers. This agrees with results obtained by McCoy and Kraft (12), who obtained more sclerotia from peptone-sucrose-yeast extract than from potato-dextrose broth.

Transfer of mycelial mats from potato-dextrose broth onto sand or soil, as suggested by Christias and Lockwood (4), enhanced sclerotial production only moderately compared with that on potato-dextrose broth alone.

Sclerotial production on radish and beet seed was abundant, but sclerotia were not counted individually. However, each seed could be considered as a unit of inoculum as was done for infested barley grains (16).

Virulence of *R. solani* is known to be dependent on size and nutritional status of the propagules (6,12,22). This difference in virulence could at least partly be explained by a difference in saprophytic growth (6).

Our results confirmed that a nutritionally rich substrate (green bean pods) produced sclerotia with vigorous hyphal growth on PDA and sand. Besides, green hypocotyl segments were more readily invaded by hyphae from large sclerotia produced on bean pods than from those produced on bean leaves or radish seed. Whether sclerotia produced on bean pods would be more virulent on actively growing bean hypocotyls remains to be shown. Hyphal growth was stimulated in the presence of a hypocotyl piece only in the case of small sclerotia produced on bean leaves. This indicated that only weak sclerotia need an extraneous nutrient source to stimulate hyphal growth.

Large-scale production of sclerotia on green bean pods proved to be efficient and convenient. Because of the particulate nature of this type of inoculum, it could be mixed into soil easily and homogeneously and could be quantified before incorporation into soil. Field plots were infested homogeneously with this type of inoculum, and consistent relationships were obtained between inoculum densities and infections of bean seedlings by *R. solani* (20).

Table 3. Mean hyphal length of *Rhizoctonia solani* after 2, 3, and 12 days of growth on sand in the presence or absence of a bean hypocotyl piece and percentage of hypocotyls infected

Sclerotia		Presence of	Mean hyphal length (mm)			Percent infected
Size (mm)	Origin	hypocotyl	2 Days	3 Days	12 Days	hypocotyls
0.7–2.0	Leaves[a]	+	3.6 (1.9)[b]	7.4 (3.1)[b]	22.4 (6.8)[b]	50
		–	7.0 (2.6)	10.4 (2.9)	30.0 (6.0)	···
0.7–2.0	Pods[a]	+	8.6 (1.7)	17.6 (2.9)	>30 (–)	100
		–	10.3 (0.8)	19.8 (2.8)	>30 (–)	···
0.3–0.5	Leaves[a]	+	1.1 (0.5)	3.0 (1.8)	13.1 (6.4)	25
		–	0.5 (0.2)	0.5 (0.2)	3.3 (2.4)	···
0.3–0.5	Pods[a]	+	3.3 (1.1)	5.6 (1.3)	15.0 (3.0)	0
		–	3.5 (1.2)	6.4 (1.7)	12.1 (3.2)	···
2.0–2.5	Radish[c]	+	4.0 (1.4)	8.5 (7.8)	22.0 (0.0)	50
		–	0.0 (0.0)	0.0 (0.0)	14.5 (7.8)	···

[a] Mean of eight replicates.
[b] Standard error of the mean.
[c] Mean of two replicates.

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