Comparison of Techniques and Inoculum Sources in Evaluating Peas (*Pisum sativum*) for Resistance to Stem Rot Caused by *Rhizoctonia solani*

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ABSTRACT

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Differences in disease severity were compared in peas inoculated by planting seeds in soil infested with 20 sclerotia of *Rhizoctonia solani* per gram of soil or cornmeal-sand (cm-s) inoculum and by direct inoculation of seedling epicotyls. Similar disease severity ratings occurred in peas planted in soil infested with 20 sclerotia per gram or 3% cm-s inoculum. Increasing the cm-s concentration to 6% resulted in higher disease ratings. Direct inoculation of seedling epicotyls with mycelial disks of *R. solani* from dextrose-asparagine broth (DAT) caused more severe epicotyl rot than sclerotial inoculum did. Disks of *R. solani* grown on PDA or V-8 juice agar resulted in low disease ratings even on susceptible lines (plant introduction [PI] numbers 140165, 166159, and 223285). Although stem rot severity ratings for 20 pea lines differed among inoculation treatments, the overall relationship of these lines within an inoculum treatment (high to low) remained about the same. Incubation of *R. solani* on peptone-sucrose-yeast extract broth (PSY) containing 1 or 2% peptone and 2% sucrose resulted in abundant uniform sclerotial (250–425 μm) production within 9 days. Incubation of *R. solani* in potato-dextrose broth, V-8 juice broth, and DAT broth resulted in fewer sclerotia. Higher disease severity ratings occurred when pea seedlings were exposed to sclerotia produced in PSY medium containing 2% peptone and sucrose than in PSY medium containing 1% peptone or sucrose. One breeding line (B77-634-4), two PI accessions (189171, 197990), and Dark Skin Perfection were considered resistant.

Rhizoctonia solani Kühn is an important cause of seed rot, tip blight, and seedling stem rot of peas (*Pisum sativum* L.) (2,3). There are no reports of commercial pea cultivars immune to *R. solani*, although some germ plasm lines and cultivars have measurable levels of resistance (12).

Several methods have been reported (6,8,9,11,12) for evaluating prospective breeding lines of soybeans (*Glycine max* L.), beans (*Phaseolus vulgaris* L.), and peas for resistance to *R. solani*, but the initial inoculum concentration used on test plants was not quantified on a propagule basis. Inocula produced on cornmeal-sand (cm-s), host materials, or in liquid media were added to the soil on a weight or volume basis. Because of differences in mycelial growth rates, sclerotial production, and saprophytic ability of various *R. solani* AG-4 isolates, infestation of soil based on soil weight or volume can result in variable inoculum densities and potential.

Inoculum of *R. solani* exists in soil as active hyphae in plant refuse, as thick-walled resting hyphae and sclerotia (1). With the recent development of the pellet soil sampler (4) and a selective medium for *R. solani* (5), it is possible to more accurately determine population levels of *R. solani* in soil.

This research was conducted to develop a method of producing sclerotia of *R. solani* for use in a screening program that can be quantified in soil and to compare inoculum levels with reported methods of screening plants for resistance to *R. solani* stem rot. A portion of this work has been reported (7).

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MATERIALS AND METHODS

Sclerotial and mycelial production for soil infestation. Four liquid media were evaluated for ability to stimulate sclerotial production by five *R. solani* AG-4 isolates (10). The four media tested were 1) peptone-sucrose-yeast extract medium (PSY) with either 1 or 2% peptone or sucrose and 0.5% yeast extract, 2) dehydrated potato-flake-dextrose broth with 20 g potato flakes and 20 g dextrose per liter, 3) V-8 juice broth (200 ml cleared V-8 juice, and 2 g $CaCO_3$/L), and 4) dextrose-asparagine medium (DAT) (13) with the following amounts (g/L): dextrose, 20; asparagine, 2; KH_2PO_4, 1.8; $MgSO_4$, 0.8; $CaCl_2$, 0.05; $CuSO_4$, 0.0008; $FeCl_3$, 0.0010; NaMoO, 0.0005; $ZnSO_4$, 0.0009; and $MnSO_4$, 0.0003. Isolates of *R. solani* used to produce sclerotia were incubated in 250-ml wide-mouth Erlenmeyer flasks containing 35 ml of one of the four autoclaved media. A 9-mm plug from a 5-day-old *R. solani* culture on PDA was added to each flask and incubated as a still culture at 23 ± 2 C in the dark for 9 days. Mycelial mats were rinsed with tap water and triturated for 1 min at high speed in a blender. Suspensions were passed through three nested brass sieves of 425, 250, and 150 μm to separate sclerotia from the mycelium. Only sclerotia 250–425 μm in diameter produced on PSY medium were incorporated into soil as inoculum. Sclerotial inoculum levels in soil were determined with the multiple-pellet soil sampler (4) and the *Rhizoctonia*-selective medium of Ko and Hora (5). To obtain the desired inoculum density, sclerotia-infested soil was diluted with uninfested soil.

R. solani was incubated on autoclaved cm-s mixture (3%, w/w) for 10 days at room temperature before incorporation into soil as mycelium. The mycelial inoculum was mixed into uninfested soil at 3 or 6% (w/w) and planted immediately with test lines.

Screening for resistance. Four breeding lines, 10 plant introduction (PI) accessions, and six cultivars were used to compare screening techniques (Table 1). Seeds of each pea line were placed on the soil surface of each pot oriented with the hilum down in plastic pots (10 × 10 × 9 cm) containing 400 g of uninfested soil.

Two hundred grams of infested soil containing either sclerotia or cm-s inoculum was layered 2.5 cm deep over the seeds. Uninfested soil was placed over seeds to be used for epicotyl inoculation. Three days after emergence, seedlings were inoculated in pots by removing soil from one side of the pea epicotyl midway between the cotyledonary attachment area and the soil surface, then covering with soil. Primary inoculum for epicotyl inoculations consisted of either a 4-mm agar plug from a 5-day-old culture on V-8 juice agar or PDA or a 4-mm mycelial

disk from a 5-day-old culture on DAT broth.

Tests were conducted twice with 15 seeds of each line per test. Disease severity was determined 14 days after planting for the soil-infestation technique and 8 days after epicotyl inoculation. A disease severity scale (0 = no symptoms and 5 = 100% epicotyl girdling) was used in all tests.

Tests were conducted in controlled-environment chambers with soil temperature at 19 ± 1 C and a 14-hr photoperiod of 400–500 μEinsteins/m^{-2}/sec^{-1} at plant height. Soil in pots was maintained at 60–70% water-holding capacity for the duration of each test.

Table 1. Comparison of inoculum source and screening technique on stem rot severity of peas caused by *Rhizoctonia solani*

	Disease severity rating[w]			
	Inoculation by soil infestation[x]			Epicotyl inoculation[y] (DAT)
Line	6% cm-s	3% cm-s	Sclerotia	
PI 223285	5.00 a[z]	4.60 a	4.62 a	4.46 a
PI 166159	5.00 a	4.75 a	4.60 a	4.37 ab
PI 140165	4.80 a	4.22 b	4.13 b	4.55 a
Frimas	4.13 b	3.23 cde	3.35 d	3.98 cde
PI 257593	4.08 bc	3.10 def	3.10 defg	4.25 abc
PI 378159	4.07 bc	3.47 c	3.15 def	3.75 def
G19169	4.05 bcd	3.10 def	3.33 d	3.13 hi
Mini	3.88 bcde	2.92 cdefg	3.72 c	4.08 bcd
M410	3.88 bcde	3.18 cde	3.07 defg	3.45 fgh
74SN5	3.83 bcdef	3.25 cde	3.05 defg	3.73 ef
Minn 108	3.78 bcdefg	3.30 cd	3.27 d	3.68 ef
PI 244128	3.75 cdefg	3.25 cde	2.77 gh	3.23 gh
PI 194006	3.70 defg	3.08 def	2.90 efg	3.93 cde
74SN3	3.70 defg	2.82 fg	2.77 gh	3.33 gh
Horal	3.65 efg	3.32 cd	2.87 fg	3.48 fg
Finale	3.50 fgh	2.68 g	3.23 de	2.88 i
PI 189171	3.45 gh	2.92 efg	2.50 hi	2.90 i
B-77-634-4	3.21 hi	2.33 h	2.23 i	2.45 j
PI 197990	3.02 i	2.82 fg	2.20 i	2.85 i
D.S. Perfection	2.95 i	2.58 gh	2.30 i	2.83 i
Treatment mean	3.87 a	3.25 c	3.16 c	3.57 b

[w]Disease severity rating based on a scale of increasing disease severity for 0–5 where 0 = healthy epicotyl and 5 = a completely girdled epicotyl. Averages represent two tests with a total of 30 seedlings evaluated for each line.

[x]Source of inoculum: cm-s = cornmeal-sand added to uninfested soil on a 3 or 6% (w/w) air-dry basis; sclerotia = 20 sclerotia (250–425 μm diameter) per gram of soil.

[y]DAT = dextrose-asparagine mycelial disks (4 mm).

[z]Means within vertical columns followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

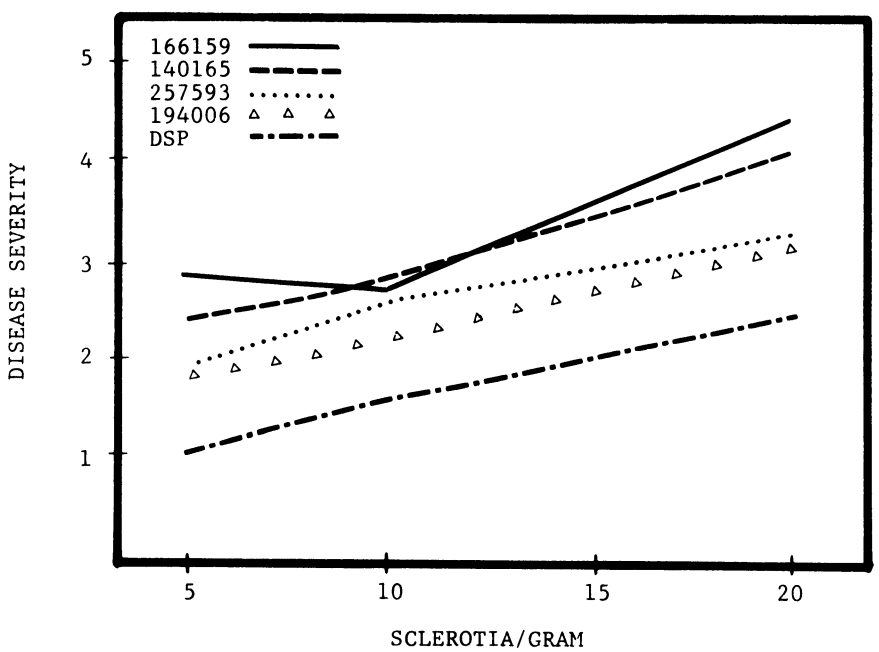

Fig. 1. Effect of sclerotial numbers in soil on severity of epicotyl rot of 14-day-old pea seedlings. Disease severity based on a 0–5 scale, where 0 = healthy epicotyl and 5 = a completely girdled epicotyl. Numbers represent plant introduction accession numbers and DSP = Dark Skin Perfection.

RESULTS

Epicotyl rot severity and inoculum concentration were associated almost linearly up to 20 sclerotia per gram of soil (Fig. 1). Sclerotial concentrations greater than 20/g resulted in a greater incidence of preemergence death with all lines tested. Consequently, 20 sclerotia per gram was chosen for future screening tests.

Five isolates of *R. solani* produced substantially more sclerotia (250–425 μm) on PSY medium after 9 days than on potato-dextrose broth, V-8 juice broth, or DAT broth. When varying levels of peptone and sucrose in PSY medium were tested, lower numbers of sclerotia were produced in the 1% peptone and sucrose medium (Table 2).

Virulence of sclerotia was directly affected by peptone and sucrose concentrations. Three lines (Dark Skin Perfection and PIs 223285 and 257593), when exposed to 20 sclerotia per gram of soil produced in PSY medium containing 2% peptone and sucrose, had higher disease ratings than peas grown in soil infested with 20 sclerotia per gram produced on the other two levels of peptone and sucrose (Table 2).

There was a highly significant difference in response of various pea lines to the sources of inoculum used (Table 3). In addition, there was a significant effect of media × variety interaction. Stem rot severity ratings for peas grown in soil infested with 3% cm-s inoculum or with 20 sclerotia per gram were similar (treatment means of 3.25 and 3.16, respectively) (Table 1). An increase in the cm-s concentration from 3 to 6% resulted in an increase in the disease ratings (Table 1). Of the three media used to produce inoculum for epicotyl inoculations, only *R. solani* incubated in DAT broth caused severe disease. Although not given in Table 1, mean disease severity ratings for test lines exposed to inoculum produced on V-8 juice and PDA were 2.8 and 2.4, respectively. Of the 20 pea lines evaluated, one breeding line (B77-634-4), two PI accessions (189171, 197990), and one cultivar (Dark Skin Perfection) consistently had the lowest disease ratings with any medium or inoculation procedure tested.

DISCUSSION

One problem in screening peas for resistance to *R. solani* is that the medium used to increase primary inoculum can affect the virulence of the isolate used (Table 1). All inoculum treatments resulted in severe disease symptoms on

the susceptible lines (PIs 223285, 166159, and 140165) except the stem-inoculation procedure using PDA or V-8 juice agar inoculum. Shehata et al (12) stated that PDA inoculum did not separate pea lines for resistance or susceptibility to Rhizoctonia stem rot within 1 wk, but after 2 wk, there were clearcut differences. In our trials, pea lines exposed to PDA or V-8 juice agar inoculum after 8 days showed differences in severity of epicotyl rot between susceptible (Mini and PIs 140165, 223285, and 166159) and resistant lines (Dark Skin Perfection, PIs 189171, and 197990, and B77-634-4) that were not readily distinguishable. Stem inoculations with disks of inoculum incubated on DAT broth gave quick, reliable differentiation of peas for resistance to Rhizoctonia stem rot because resistant and susceptible lines were easily identified. Unfortunately, epicotyl inoculation with inoculum produced on DAT medium is time consuming when large populations are screened. Either sclerotial or cm-s inoculum gave similar resistance and susceptibility ratings among the 20 pea lines tested; however, only the inoculum concentration of sclerotia-infested soil could be readily determined and be reproduced reliably. Preliminary unreported results indicated that significant differences in concentrations of inoculum in soil resulted when five isolates of *R. solani* (AG-4) were incubated on cm-s. Another advantage of using sclerotial inoculum is that no extraneous nutrient source is added to soil along with the primary inoculum.

Significant differences in disease severity occurred within the same pea line when exposed to a similar inoculum density, resulting from sclerotia produced under varying peptone and sucrose concentrations (Table 2). This work is in agreement with that of Weinhold et al (13), who also found that virulence of mycelial inoculum of *R. solani* was directly affected by the carbon and nitrogen concentration.

Even though inoculum produced on other growth media resulted in clearcut differences between resistant and susceptible pea lines for Rhizoctonia stem rot, only sclerotial inoculum could be quantified on a propagule level. For this reason, we recommend use of sclerotial inoculum at a concentration of about 20 propagules per gram of soil when screening peas for resistance to Rhizoctonia stem rot.

Table 2. Effects of varying concentrations of peptone and sucrose on sclerotium production and on disease severity

Growth medium[w]	Sclerotia per flask[x]	Disease severity[y]		
		DSP	PI 223285	PI 257593
2% Peptone + 2% sucrose	10,200 a[z]	3.0 a	4.5 a	3.6 a
1% Peptone + 2% sucrose	9,200 a	2.2 b	3.6 b	2.0 b
1% Peptone + 1% sucrose	2,300 b	1.8 c	3.1 c	1.8 b

[w] PSY = peptone-sucrose-yeast extract broth containing 5.0 g/L yeast extract.
[x] Number of viable sclerotia (250–425 μm) produced by *R. solani* isolate R-1, from each of three flasks estimated by addition to air-dry soil and measured by the multiple soil pellet technique.
[y] Disease severity ratings were based on a 0–5 scale, where 0 = clean, uninfected epicotyl and 5 = completely girdled stem. Data is an average of three tests with a total of 42 seedlings per line evaluated.
[z] Data in vertical columns followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

Table 3. Analysis of variance of disease severity among 20 pea lines exposed to three soil infestations and one stem-inoculation procedure using a split-block analysis

Source	df	Mean square	*F*
Total	2,399	...	...
Main plots (replicate × media)	119	...	...
Replications	29	11.75	0.41
Media	3	63.82	28.04*[a]
Error A	87	2.28	...
Pea Lines	19	41.53	183.02*
Media × variety interaction	57	1.68	7.39*
Error B	2,204	0.23	...

[a] * = Significant at $P = 0.01$.

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