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A Modified Method to Screen for Partial Resistance to Phytophthora sojae in Soybean

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Abstract
Phytophthora root and stem rot, caused by Phytophthora sojae Kauf and Gerd, is one of the most damaging diseases of soybean [Glycine max (L.) Merr.] in the United States. Partial resistance (PR), which is defined as the relative ability of a soybean plant to survive root infection without showing severe symptoms, is an effective way to manage this disease. A modification of the layer test method used to screen for PR to P. sojae in soybean was evaluated. Instead of the P. sojae-colonized agar layer, which is used in the current greenhouse cup assay, P. sojaeinfested rice (Oryza sativa L.) grains were used. In addition, a dry root weight (Drw) measurement was compared to a disease severity root rot score, which uses a 1 to 10 visual scale. The rice method was not statistically different from the layer test for the variables evaluated. Advantages of the rice method include reduced cost and the ability to screen soybean germplasm with more than one pathotype of the pathogen in a single assay. A mixture of several pathotypes of P. sojae ensures compatible interactions between isolates used and all known Rps genes, thus avoiding Rps genes that could go undetected and mask PR during screening. Although collecting and handling of roots for Drw data may require more time, it is a more objective variable, which assures precise scoring, it is not rater dependent, and less training of personnel is required.

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A Modified Method to Screen for Partial Resistance to *Phytophthora sojae* in Soybean

Silvina Stewart, and Alison E. Robertson*

ABSTRACT

Phytophthora root and stem rot, caused by *Phytophthora sojae* Kauf and Gerd, is one of the most damaging diseases of soybean [*Glycine max* (L.) Merr.] in the United States. Partial resistance (PR), which is defined as the relative ability of a soybean plant to survive root infection without showing severe symptoms, is an effective way to manage this disease. A modification of the layer test method used to screen for PR to *P. sojae* in soybean was evaluated. Instead of the *P. sojae*-colonized agar layer, which is used in the current greenhouse cup assay, *P. sojae*-infested rice (*Oryza sativa* L.) grains were used. In addition, a dry root weight (Drw) measurement was compared to a disease severity root rot score, which uses a 1 to 10 visual scale. The rice method was not statistically different from the layer test for the variables evaluated. Advantages of the rice method include reduced cost and the ability to screen soybean germplasm with more than one pathotype of the pathogen in a single assay. A mixture of several pathotypes of *P. sojae* ensures compatible interactions between isolates used and all known *Rps* genes, thus avoiding *Rps* genes that could go undetected and mask PR during screening. Although collecting and handling of roots for Drw data may require more time, it is a more objective variable, which assures precise scoring, it is not rater dependent, and less training of personnel is required.

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Abbreviations: cDrw, corrected dry root weight; Drw, dry root weight; PR, partial resistance; QTL, quantitative trait loci.

Phytophthora root and stem rot caused by *Phytophthora sojae* Kauf and Gerd is an economically important disease of soybean [*Glycine max* (L.) Merr.] in Iowa and other states of the north central soybean production region of the United States and worldwide (Buzzel and Anderson, 1982; Hartman et al., 1999; Wrather et al., 2001; Wrather and Koenning, 2009). The most effective means of managing the disease is by use of genetic resistance in the soybean (Robertson et al., 2009). So far, Phytophthora root and stem rot has been effectively controlled through the use of single-gene, race-specific host resistance (Dorrance et al., 2003a). The pathogen, however, has the ability to rapidly adapt and evade this type of resistance (Schmitthenner et al., 1994).

More than 200 pathotypes of *P. sojae* have been identified (Dorrance et al., 2003a) on the basis of compatible (susceptible) and incompatible (resistant) reactions on inoculation on a set of soybean differential genotypes that each carry a single *Rps* gene conditioning resistance to one or more pathotypes of *P. sojae* (Dorrance et al., 2004, 2003b; Ferro et al., 2006). Since there are 15 known *Rps* genes, there are potentially 32,768 (2^{15}) possible virulence combinations or pathotypes (Dorrance and Schmitthenner, 2000; Gijzen and Qutob, 2009; Sun et al., 2010). New virulence gene combinations or pathotypes are likely to continue to emerge in the pathogen as resistant cultivars possessing *Rps* genes exert selection pressure on the pathogen population (Abney et al., 1997; Dorrance et al., 2003a; Jackson et al., 2004; Leitz et al., 2000; Malvick and Grunden, 2004; Ryley et al., 1998; Schmitthenner et al., 1994; Yang et al., 1996).

In previous reports, 54 and 56 different pathotypes of *P. sojae* were detected from two intensively sampled commercial soybean fields in Ohio (Dorrance et al., 2003a). In Iowa, 11 and

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18 pathotypes of the pathogen were recovered from two commercial soybean fields (Robertson et al., 2009). In this study, as many as four pathotypes of *P. sojae* were detected in some fields, indicating that a single soybean plant may be subjected to infection by more than one pathotype (Robertson et al., 2009). Consequently, complexity within the population of *P. sojae* in a single field and the possibility of multiple pathotypes co-infecting a single soybean plant (Stewart, 2011) make it difficult for a grower to choose a soybean cultivar with the appropriate *Rps* gene or genes required to resist pathogen infection in a particular field. Therefore, single gene resistance may not be the most appropriate genetic approach to use in managing the disease and preventing the appearance of new pathotypes in the oomycete population (Dorrance and McClure, 2001; Burnham et al., 2003).

Partial resistance (PR), also called tolerance or field, rate reducing, horizontal, or quantitative resistance, is an alternative to single gene mediated resistance (Burnham et al., 2003; Tucker et al., 2010). Partial resistance is polygenic and limits growth of the pathogen within the host tissue. It is expressed as a reduced level of root rot and consequently limits yield loss (Dorrance et al., 2003b; Ferro et al., 2006; Tooley and Grau, 1984). This type of resistance is effective against all physiological races or pathotypes of the pathogen. Partial resistance is durable, most likely due to its quantitative inheritance, which is more difficult for the pathogen to adapt to, irrespective of how many pathotypes may be present in an endemic population. In addition, PR may impose less selection pressure on the pathogen population, so the more virulent strains are less likely to become the majority in a population. Buzzel and Anderson (1982) proposed combining PR with specific *Rps* genes to provide long-term management of Phytophthora root and stem rot.

Currently, PR to *P. sojae* is identified in greenhouse assays by challenging soybean lines with a compatible pathotype to determine the extent to which plants roots are colonized (Dorrance et al., 2003b; Walker and Schmitthenner, 1984). This method, however, is cumbersome and laborious and the roots are difficult to rate, which may partially explain why few cultivars with high levels of PR are currently available (Dorrance et al., 2003b). The challenge to breeders and pathologists is to find easy, feasible, and effective ways to identify PR and incorporate it into soybean cultivars.

Since the early 1980s, researchers have evaluated numerous methods to screen for PR to *P. sojae* (McBlain et al., 1991a, b; Schmitthenner and Bhat, 1994; Thomison et al., 1991; Tooley and Grau, 1982; Wagner et al., 1992). Some methods include direct inoculation of wounded cotyledons or roots while others involve inoculation of nonwounded plants (McBlain et al., 1991b; Tooley and Grau, 1982; Wagner et al., 1992). The layer test has been the most widely accepted assay and has become the standardized method to screen soybeans for PR to *P. sojae* in greenhouse conditions (Dorrance et al., 2003b; Ferro et al., 2006; Jackson et al., 2004; Schmitthenner et al., 1994; Thomison et al., 1991). In the layer test, an agar culture of the pathogen is placed at a certain distance below the soybean seeds at planting time. Seedling roots grow through the *P. sojae*-colonized agar layer at approximately the same time that the seedling's unifoliate leaves expand, so root infection coincides with the VC stage of soybean development (Fehr et al., 1971), which is when PR is expressed in the plant (Dorrance and McClure, 2001; Dorrance et al., 2003a). Although the method is widely used in many soybean breeding programs to screen for PR, it is cumbersome because it requires handling one agar plate for each pathotype and for each soybean genotype that will be tested. Moreover, PR evaluation

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by the layer method is based on a visual rating scale (1 to 10) of root rot that requires trained personnel and can be subjective.

Our objective was to develop an easy and objective method that could be used by soybean breeders to screen for PR in large numbers of genotypes using more than one pathotype in a single test. The rice screen test that we propose is easier to use, more precise, and unbiased in its assessment of PR in soybean genotypes. Furthermore, in the standard agar layer test, a discrete variable (root rot) is assessed while in the rice screen test, a quantitative variable, root weight, is measured. Quantitative variables are preferred over discrete variables for identifying quantitative trait loci (QTL) for PR (Poland and Nelson, 2011).

MATERIALS AND METHODS

Phytophthora sojae Isolates and Pathotype Characterization

Two mono-zoosporic isolates of *P. sojae* recovered from fields in Iowa during 2008 were selected for testing on the basis of their pathotypes and on their interactions with soybean cultivars (Schmitthenner and Bhat, 1994). Isolate 1023-1c was compatible with *Rps*7 (race 1), and isolate 1019-1.11c was compatible with *Rps*1a, *Rps*1b, *Rps*1c, *Rps*1k, and *Rps*7 (race 25). The pathotypes were determined by the hypocotyl inoculation method on the following soybean differentials: L88-8470 (*Rps*1a from 'Mukden'), L77-1863 (*Rps*1b from 'Harrell'), Williams 79 (*Rps*1c from 'Lee68'), L99-3312 (*Rps*1d), Williams 82 (*Rps*1k from 'Kingwa'), L82-1449 (*Rps*2 from 'CNS'), L83-570 (*Rps*3a), L91-8347 (*Rps*3b), L92-7857 (*Rps*3c), L85-2352 (*Rps*4), L85-3059 (*Rps*5), L89-1581 (*Rps*6 from 'Altona'), L93-3258 (*Rps*7 from 'Harosoy'), PI 399073 (*Rps*8), and Williams (universal susceptible) (Buzzel et al., 1987; Dorrance et al., 2004; Wagner and Bernard, 1991). Differentials were considered susceptible when at least 7 out of 10 seedlings died or developed distinct symptoms of Phytophthora root and stem rot.

Inoculum Preparation

Phytophthora sojae-infested rice inoculum was prepared by autoclaving batches of 50 g of parboiled long grain rice (*Oryza sativa* L.) (Riceland Gold Perfected Rice) in 36 mL of distilled water in 250 mL Erlenmeyer flasks twice within a 24 h period. Cooled rice grains were separated under aseptic conditions in between each autoclaving. Erlenmeyer flasks were each inoculated with 10 pieces (0.5 cm^2) of 4- to 6-d-old mycelium of *P. sojae* isolates 1023-1c or 1019-1.11c grown on diluted V8 media (40 mL V8 juice [CSC Brands LP], 0.6 g $CaCO_3$, 0.2 g Bacto yeast extract [Becton, Dickinson and Company], 1 g sucrose, 0.01 g cholesterol, 20 g Bacto agar [Becton, Dickinson and Company], and 1.00 L distilled water). Inoculated flasks were kept at room temperature (20°C) for 10 to 14 d and shaken once per day to prevent clumping. Immediately before use, inoculated rice grains were removed from flasks and separated, and equal volumes of rice infested with each isolate (1:1 ratio) were then mixed together thoroughly. The amount of inoculated rice used in the screening test had been established in previous experiments in which no significant differences in root rot were found when 5, 10, or 15 cm^3 of inoculated rice per cup were used (data not shown). For this research, we used 10 cm^3 of inoculated rice per cup. For the standard layer test, inoculum consisted of the same two isolates, each grown for 10 d on plates of diluted V8 medium. One plate of each isolate was placed on top of the other to inoculate one cup. Noninoculated cups served as controls.

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Soybean Cultivars

Six cultivars with different sources of resistance to *P. sojae* were used in the research to compare the standard layer test to the rice method: the susceptible cultivar Sloan, with no *Rps* genes and low PR (Dorrance et al., 2008, 2009); the cultivar Stine 2402, with no *Rps* genes and moderate to high PR (Dorrance et al., 2009); 'Garst 2834 RR' with *Rps*1k and low PR (Dorrance et al., 2009); and three cultivars provided by Syngenta with high ('S37-F7'; relative maturity III), moderate ('S25-J5'; relative maturity II), and low ('S41-M5'; relative maturity IV) PR as described by the company (Table 1). The six cultivars lacked race-specific resistance to at least one of the isolates used, as determined earlier by hypocotyl inoculation assays (data not shown).

Partial Resistance Screening

Styrofoam cups (946 mL) with three holes punctured through the base were filled to a 5 cm depth with A4 coarse vermiculite. A layer of inoculum (*P. sojae*-infested rice or colonized agar) was placed on top of this basal layer of vermiculite, and the cups were filled with additional vermiculite to approximately 13 cm depth. Fifteen seeds of each soybean genotype were then placed in a small pile at the center of each cup, covered with vermiculite, and watered until runoff. Thereafter, cups were watered from overhead once daily and kept in a greenhouse at 20 ± 5°C with a 12-h supplemented light cycle.

Experimental Design, Assessment, and Analysis

A completely randomized factorial design with two factors (six cultivars and three treatments) and five replications was used (Table 1). Treatments were (i) 10 cm^3 of a 1:1 volume of rice infested with *P. sojae* isolates 1023-1c and 1019-1.11, (ii) a double agar layer in which each layer corresponded to a fully grown culture of *P. sojae* isolates 1023-1c and 1019-1.11, and (iii) a noninoculated control. The experiment was repeated twice, on 11 Nov. 2010 and 18 June 2011. Four weeks after planting, the number of surviving plants in each cup was recorded. Plants were removed from the cups, and the roots washed gently to remove all traces of vermiculite. Partial resistance was evaluated by a trained rater using a visual 1 to 10 scale in which 1 represented no root rot, 3 indicated high levels of PR with one-third of the roots rotted, and 10 indicated all seedlings had been killed before emergence (Dorrance et al., 2009; Schmitthenner and Bhat, 1994). After rating, roots from individual cups were cut at the soil line, placed in a paper bag, and oven dried in a Precision Thelco oven (Precision Scientific Company) at 50°C for 24 h. Total dry root weight (Drw) for each cup was determined, and the mean weight for each root was obtained by dividing total Drw by the number of surviving plants present in each cup. Corrected dry root weight (cDrw) was calculated as percent root weight of a cultivar relative to the average root weight of its noninoculated control.

Analysis of variance and contrasts were done using PROC GLM in SAS version 9.2 (SAS Institute, Cary, NC). Contrasts were used to test predetermined categories of PR (low, moderate, or high) as well as treatments and methods. Each cultivar in the study was assigned to a *P. sojae* PR category on the basis of published information or information supplied by the source company. Experiment repetitions were considered random effects while cultivars and treatments were considered fixed. No transformation of the data was needed because residuals for the variables evaluated were distributed randomly. Correlations between the visual scale versus Drw and cDrw were computed with PROC CORR using SAS (SAS Institute, Cary, NC).

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RESULTS

A significant experiment × treatment × cultivar interaction for the variables evaluated was not observed, so data from both experiments were pooled for analysis. The level of PR differed among cultivars as expected (Table 1). Cultivar rankings for PR were similar to published data (Dorrance et al., 2009) and company information. Cultivars reported as having moderate to high levels of PR had the lowest root rot severity ratings using the scale, the heaviest root weight using Drw, and the highest cDrw. Of the three variables, cDrw best separated the cultivars (Table 1). Stine 2402 ranked the highest for PR, based on cDrw, followed by Syngenta S27-F7 and S25-J5. When contrast statements were used to differentiate high, moderate, and low PR cultivars, all variables (root rot severity rating, Drw, and cDrw) significantly ($p < 0.001$) differentiated low PR from high PR and also low PR from moderate PR. Corrected Drw, however, was the only variable that significantly ($p < 0.001$) differentiated moderate from high PR cultivars. Root rot severity ratings were negatively and significantly ($p < 0.0001$) correlated with Drw ($r = -0.779$). The relationship between the two variables, however, was improved when root weights for each cultivar in the inoculated treatments were corrected for the mean root weights of the corresponding noninoculated control (cDrw) ($r = -0.833$, $p < 0.0001$).

Single degree of freedom comparisons of noninoculated control versus inoculated treatments using contrast statements revealed highly significant differences ($p < 0.0001$) for root rot severity ratings and Drw measurements (Fig. 1 and 2, respectively). When contrasts were used to compare the standard layer method and the rice method, no significant differences between the two methods were detected for the root rot severity rating, Drw, and cDrw ($p = 0.193$, 0.489, and 0.136, respectively) (Fig. 1, 2, and 3, respectively). There was an overall reduction of 68.2% in Drw when inoculated roots were compared to noninoculated roots across cultivars.

DISCUSSION

Our results showed no significant differences between the standard agar layer test and the rice method, indicating that screening for PR is effective using either of the two methods. The rice method, however, facilitates screening of large number of genotypes, which in itself may be advantageous to soybean breeders. It also it has several other advantages: (i) it is less than one-tenth as expensive to set up as the agar layer test, considering the price of rice versus the price of agar, V8 juice, and petri plates, (ii) during preparation, hundreds of plates can be replaced with two or three flasks of inoculated rice in the lab, and (iii) the ability to screen for PR using more than one pathotype is practical, since mixing equal volumes of inoculated rice of each desired pathotype is easily done.

A major constraint in screening a large number of genotypes is the choice of pathogen isolates, since this is critical to accurately evaluate PR (Dorrance et al., 2008). The chosen isolate(s) of *P. sojae* should have a compatible interaction (susceptible response) on all of the soybean genotypes to be tested. The presence of an *Rps* gene in the genotypes to be screened will interfere with PR assessment; therefore, a hypocotyl test needs to be done on each genotype before screening for PR to ensure the isolate's compatibility. This step could be avoided when using the rice method as long as the mixture of pathotypes of *P. sojae* used is compatible with all known *Rps* genes.

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Partial resistance has been typically evaluated by visual scoring of disease severity. Since disease severity is a quantitative trait, accuracy and precision of the visual estimate is critical and particularly impacts identification of disease resistance QTL when molecular information is gathered (Poland and Nelson, 2011). Poland and Nelson (2011) reported that variability existed between individual raters using direct percentage and a 0-to-9 rating scale, which resulted in variation in the identification of QTL that was dependent on the subset of raters. This rater-dependent scoring variation is avoided when objective measurements, such as cDrw, which was shown to be highly correlated to root rot severity ratings, are used as a measure of PR. Other objective measurements, such as lesion length, have been used to assess PR to *P. sojae* in recombinant soybean inbred lines to map QTL (Tucker et al., 2010). Although objective measurements such as Drw or lesion length may be more time consuming than assessing root rot severity, final choice of the assessment method to measure PR could be decided by the breeder depending on their breeding objectives and allocation of resources.

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Figure 1. Overall mean root rot rating across cultivars and experiments for three treatments (noninoculated control, layer method, and rice method) used to screen soybean cultivars for partial resistance to *Phytophthora sojae*. Letters above columns indicate differences among treatments. Noninoculated controls differed ($p < 0.0001$) from the other two treatments for root rot severity rating according to GLM contrast. The layer method did not differ from the rice method for root rot severity rating according to GLM contrast ($p = 0.193$). †Root rot severity rating on 1 to 10 scale in which 1 represents no root rot and 10 represents all seedlings killed before emergence (Schmitthenner and Bhat, 1994).

Figure 2. Overall mean dry root weight across cultivars and experiments for three treatments (noninoculated control, layer method, and rice method) used to screen soybean cultivars for partial resistance to *Phytophthora sojae*. Letters above columns indicate differences among treatments. Noninoculated controls differed ($p < 0.0001$) from the other two treatments for dry root weight according to GLM contrast. The layer method did not differ from the rice method for dry root weight according to GLM contrast ($p = 0.489$).

Figure 3. Overall mean corrected dry root weight (cDrw) across cultivars and experiments for two treatments (layer method and rice method) used to screen soybean cultivars for partial resistance to *Phytophthora sojae*. The layer method did not differ from the rice method for cDrw according to GLM contrast ($p = 0.136$). ‡Corrected dry root weight (cDrw) was calculated as percent root weight of an inoculated cultivar relative to the mean root weight of its noninoculated control: cDrw = (Treatment dry root weight [Drw]/Mean Drw of noninoculated controls) × 100. Noninoculated controls were not considered in the analysis of cDrw.

Table 1. Overall mean disease rating, dry root weight (Drw) (g), and corrected dry root weight (cDrw) of six soybean cultivars evaluated for partial resistance to *Phytophthora sojae* in the greenhouse using a mixture of two isolates of *P. sojae* as either an infested rice layer or a double agar layer.

Cultivar name	Phytophthora root and stem rot resistance†	Rating‡	Drw§ (g)	cDrw¶
Stine 2402	HPR, *rps*	4.2 a#	0.089 a	0.563 a
Syngenta S25-J5	MPR (C)	4.2 a	0.077 b	0.362 bc
Syngenta S37-F7	HPR (C)	4.4 a	0.071 b	0.443 b
2834 RR	LRP, *Rps*1k	4.5 a	0.074 b	0.323 c
Syngenta S41-M5	LPR (C)	5.1 b	0.054 c	0.307 c
Sloan	LPR, *rps*	6.0 c	0.055 c	0.116 d

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[†]Level of *P. sojae* partial resistance based on published information and information supplied by company: HPR, high partial resistance, indicates that all seedlings survived and 10 to 39% of the roots showed root rot symptoms associated with infection by *P. sojae*; MPR, moderate level of PR, indicates that all seedlings survived and 40 to 70% of the roots showed root rot symptoms; LPR, low level of PR, indicates all roots were rotted and 50 to 89% of the seedlings were killed according to Dorrance et al. (2009). Names followed by C indicate the seed companies rating for this trait.

[‡]Root rot severity rating on 1 to 10 scale in which 1 represents no root rot and 10 represents all seedlings killed before emergence, according to Schmitthenner and Bhat (1994).

[§]Dry root weight per root in grams.

[¶]Corrected dry root weight = Treatment Drw/(Mean Drw of noninoculated controls).

[#]Values within a column followed by the same letters were not significantly different according to LSD test ($p \leq 0.05$).