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A Method for Combining Isolates of Phytophthora sojae to Screen for Novel Sources of Resistance to Phytophthora Stem and Root Rot in Soybean

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Soybean cultivars with specific single resistance genes (*Rps*) are grown to reduce yield loss due to Phytophthora stem and root rot caused by the oomycete pathogen *Phytophthora sojae*. To identify novel *Rps* loci, soybean lines are often screened several times, each time with an isolate of *P. sojae* that differs in virulence on various *Rps* genes. The goal of this study was to determine whether several isolates of *P. sojae* that differ in virulence on *Rps* genes could be combined into a single source of inoculum and used to screen soybean lines for novel *Rps* genes. A set of 14 soybean differential lines, each carrying a specific *Rps* gene, was inoculated with three isolates of *P. sojae*, which differed in virulence on 6 to 10 *Rps* genes, individually or in a 1:1:1 mixture. Inoculum containing the 1:1:1 mixture of isolates was virulent on 13 *Rps* genes. The mixed-inoculum method was used to screen 1,019 soybean accessions in a blind assay for novel sources of resistance. In all, 17% of *Glycine max* accessions and 11% of *G. soja* accessions were resistant (≤30% dead plants), suggesting that these accessions may carry a novel *Rps* gene or genes. Advantages of combining isolates into a single source of inoculum include reduced cost, ability to screen soybean germplasm with inoculum virulent on all known *Rps* genes, and ease of identifying novel sources of resistance. This study is a precursor to identifying novel sources of resistance to *P. sojae* in soybean using RXLR effectors.

Phytophthora stem and root rot (PRR), caused by the oomycete *Phytophthora sojae* Kaufm. & Gerd. (syn. *P. megasperma* f. sp. *glycinea*), is an economically important disease of soybean (*Glycine max* (L.) Merrill), significantly affecting soybean production worldwide (Tyler 2007). Soybean yield loss caused by PRR averaged 43 million bushels annually in the United States (2006 to 2009) (Koenning and Wrather 2010) and more than $1 billion per year worldwide (Tyler 2007). Yield loss can increase substantially in years with abundant rainfall and warm soil temperatures in the spring (Robertson et al. 2009).

P. sojae is a soilborne pathogen that survives from one season to the next as oospores in infested crop residue and soil. Oospores are formed as a result of sexual reproduction. In flooded conditions, oospores germinate and give rise to sporangia from which zoospores (asexual reproduction) are produced. Infection of soybean occurs primarily via these zoospores that are chemotactically attracted to soybean roots, where they encyst, germinate, and infect the plant (Dorrance et al. 2007).

PRR is primarily managed by incorporating single resistance genes (*Rps*) into commercial soybean cultivars (Grau et al. 2004; Stewart et al. 2014). Twenty genes have been found to confer resistance to PRR, with five of those *Rps* genes identified in the past 5 years (Demirbas et al. 2001; Diers et al. 1992; Fan et al. 2009; Gordon et al. 2006; Lin et al. 2013; Lohnes and Schmitthenner 1997; Sandhu et al. 2005; Sun et al. 2011; Weng et al. 2001; Wu et al. 2011; Zhang et al. 2013; Zhu et al. 2007). Only a few of these genes (*Rps* 1a, *Rps* 1b, *Rps* 1c, *Rps* 1k, *Rps* 3a, and *Rps* 6) have been incorporated separately into soybean germplasm for commercial use (Dorrance and Schmitthenner 2000; Dorrance et al. 2007). More recently, a few cultivars with two *Rps* genes have become commercially available for farmers to plant; for example, *Rps* 1c pyramided with *Rps* 1k, *Rps* 1k with *Rps* 6, or *Rps* 1k with *Rps* 7 (Slaminko et al. 2010).

For each *Rps* gene, there is a corresponding avirulence (*Avr*) gene (Tyler 2007). *Avr* genes encode proteins (i.e., effectors) that interact with proteins encoded by *Rps* genes, resulting in activation of defense responses in the plant and plant resistance (Jiang and Tyler 2012; Staskawicz et al. 1995). These effectors have an N-terminal amino acid sequence motif (known as RxLR) that is required for effector translocation into the plant cell (Song et al. 2013; Tyler 2009). Several of these *Avr* genes have been molecularly cloned and all have been shown to encode secreted *Avr* proteins in the RxLR superfamily (Dong et al. 2011; Jiang and Tyler 2012). Individual isolates of *P. sojae* might have one or more *Avr* genes corresponding to known *Rps* genes (Dong et al. 2011; May et al. 2002). Isolates of *P. sojae* are classified into pathotypes (previously referred to as races) based on their ability to cause disease (virulence) on a standard set of differentials composed of soybean lines, each of which contain a different *Rps* gene (Robertson et al. 2009). The pathotype of a *P. sojae* isolate is represented as a virulence formula that indicates which *Rps* gene or genes show a susceptible response, such as a brown lesion spreading from the inoculation site (i.e., virulence) to that isolate.

New pathotypes of *P. sojae* continue to evolve and the population of the pathogen in an individual field can be extremely diverse (Dorrance et al. 2003; Malvick and Grunden 2004; Robertson et al. 2009). Consequently, a single *Rps* gene that once was effective may be rendered ineffective against the same *P. sojae* isolates present in an individual field within a few years (Grau et al. 2004; Stewart et al. 2014). Because the pathotypic diversity of *P. sojae* is on the rise in the United States, soybean breeders continually screen germplasm for novel *Rps* genes.

The hypocotyl inoculation test is the traditional method of screening soybean lines or populations for resistance to *P. sojae* (Dorrance et al. 2004; Schmitthenner 1985). Kaufmann and Gerdemann (1958) compared several inoculation methods and demonstrated that hypocotyl

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inoculation clearly distinguished between resistant and susceptible plants. Moreover, this method had an added advantage, in that it reduced confusion due to poor germination of seed, a factor that often needs to be considered when screening germplasm accessions. Using the hypocotyl inoculation method, germplasm is typically screened several times using a different isolate of *P. sojae* with a known pathotype in each screen (Burnham et al. 2003; Dorrance and Schmitthenner 2000; Zhang et al. 2010). Thus far, no single *P. sojae* isolate is virulent on all known *Rps* genes; therefore, multiple isolates varying in pathotype need to be used to identify new sources of resistance to PRR (Gordon et al. 2007). In most cases, an average of two to eight isolates are used individually to inoculate plants (Burnham et al. 2003; Dorrance and Schmitthenner 2000; Gordon et al. 2007; Zhang et al. 2010), and soybean lines or populations are screened at least two times per isolate (Dorrance and Schmitthenner 2000; Zhang et al. 2010). Screening multiple isolates individually requires a large quantity of seed, additional laboratory materials, increased greenhouse space, and significantly more time.

Identifying novel sources of resistance to PRR is a key component of soybean breeding as a means of managing PRR. The possibility of combining *P. sojae* isolates with varying pathotypes into a single source of inoculum that could be used to identify novel *Rps* genes has not been evaluated. The objectives of this study were to determine whether (i) isolates of *P. sojae* with different pathotypes could be combined to produce a single source of inoculum that was virulent on 13 of the 14 standard differential lines and, thus, to almost all known *Rps* genes for which differential lines are available; and (ii) this source of inoculum could be used to screen soybean lines for novel sources of resistance to *P. sojae*.

Materials and Methods

***P. sojae* isolates.** Three isolates of *P. sojae* were used in this study: PT2004 C2.S1 (pathotype 1a, 1b, 1c, 1d, 1k, 2, 3c, 4, 6, 7) (Wang et al. 2012) was received from Dr. Brett Tyler (Virginia Tech), R7-2a (pathotype 1d, 2, 3a, 5, 6, 7) was received from Dr. Anne Dorrance (Ohio State University), and 1005-2.9 (pathotype 1a, 1b, 1c, 1k, 3b, 7) was recovered from a soil sample collected in 2009 from a soybean rotation study in Iowa (Stewart et al. 2014).

Inoculum preparation and inoculation method. *P. sojae* isolates were grown on separate plates containing half-strength V8 juice media amended with neomycin sulfate (50 μg/ml) and chloramphenicol (10 μg/ml) (DV8) for 5 to 7 days at 23°C in the dark. Inoculum was prepared from colonized DV8 plates using syringes to make an agar-based slurry of the pathogen, as outlined by Dorrance et al. (2004). Seven-day-old plants were inoculated with inoculum using the hypocotyl inoculation method described by Dorrance et al. (2004), in which approximately 0.2 to 0.5 ml of inoculum slurry was placed in a slit made in the hypocotyl of the plants being tested.

Virulence assessment. The pathotype of each isolate was determined by assessing its virulence on a set of 14 soybean differential lines that each have a specific *Rps* gene (Table 1). Simultaneously, a 1:1:1 mixture of the three isolates was pathotyped on the same set of differential lines. 'Sloan' soybean (background 'Williams'), which has no *Rps* genes, was included with each differential set to confirm the pathogenicity of each isolate. Ten seeds of each soybean line were grown for 7 days in 8-oz polystyrene cups filled with coarse vermiculite at 25°C in the greenhouse. All seedlings were inoculated using the hypocotyl inoculation method (Dorrance et al. 2004). Immediately after inoculation, plants were kept in a dew chamber at 25°C for 24 h in the dark before being placed in the greenhouse. Plants were scored 7 days postinoculation. The isolate was considered virulent on an *Rps* differential line when >70% of seedlings were killed and avirulent when ≤30% of the seedlings died (Table 2). The experiment was replicated three times.

Plant material. Soybean germplasm used in this study to screen for *P. sojae* resistance consisted of 490 accessions of *Glycine max* and 529 accessions of *G. soja.* The Maroof lab chose lines based on background information of each line according to the Germplasm Resources Information Network (GRIN) database (http://www.ars-grin.gov), Dorrance and Schmitthenner (2000), and Pazdernik et al. (1997). In the GRIN database, over 14,000 *G. max* accessions have been tested for reaction to *P. sojae*. Of the 490 selected *G. max* accessions used in our study, 356 had been screened previously and showed a resistance reaction to at least 3 pathotypes, and up to 11 pathotypes for some accessions. The parents of the soybean Nested Association Mapping populations, 40 *G. max* accessions, were also chosen for this study. The remaining 94 *G. max* accessions we screened were chosen arbitrarily, with no regard for resistance to the pathogen. Of the 1,179 *G. soja* accessions in the GRIN database, *P. sojae* screening data for 448 accessions were available from previous reports (Pazdernik et al. 1997). From this set, we selected 42 accessions which had shown resistance to pathotype 1a, 7 (race 3) of *P. sojae*. In addition, we arbitrarily chose 487 accessions with no prior *P. sojae* screening data to bring our *G. soja* sample size to a total of 529 accessions. Seed for all *G. max* and *G. soja* accessions was obtained from the United States Department of Agriculture (USDA) Soybean Germplasm Collection, increased at Virginia Tech, and screened at the Robertson Lab at Iowa State University (ISU). All *G. max* and *G. soja* accessions were screened blindly at ISU, as described below.

Identification of potential lines for resistance to *P. sojae*. Ten seeds of each *G. max* accession were grown for 7 days in 8-oz polystyrene cups filled with coarse vermiculite at 25°C in the greenhouse. As described above, inoculated plants in polystyrene cups were kept in a dew chamber at 25°C for 24 h in the dark and then placed in the greenhouse. Because the hypocotyls of *G. soja* were small and difficult to inoculate using the cup assay, 10 seeds of each accession were grown on germination paper for 7 days at 25°C in a growth chamber in the dark. The hypocotyls of the plants were inoculated as described above; then, the inoculated plants in rolled germination paper were placed in a growth chamber for 7 days at 25°C in the dark. During each replication of the germplasm screening, a differential set that included Sloan was inoculated with the same source of inoculum as a control. Plants were assessed 7 days postinoculation, at which time the number of dead plants in each accession was recorded.

Table 1. Soybean differentials and corresponding *Rps* genes used in this study[a]

Differential	*Rps* gene	Background	Source of *Rps* gene
L88-8470	1a	Williams	Mukden
L77-1863	1b	Williams	Harrell
Williams 79	1c	Williams	Lee 68
L93-3312	1d	Williams	PI 103091
Williams 82	1k	Williams	Kingwa
L82-1449	2	Williams	CNS
L83-570	3a	Williams	PI 86972-1
L91-8347	3b	Williams	PI 172901
L92-7857	3c	Williams	PI 340046
L85-2352	4	Williams	PI 86050
L85-3059	5	Williams	PI 91160
L89-1581	6	Williams	Altona
L93-3258	7	Williams	Harosoy
PI 399073	8	…	…

[a] Genetic background and the source of each *Rps* gene were obtained from Dorrance et al. (2004).

Table 2. Assessment scales used in this study to evaluate the virulence of the combined mixture of *Phytophthora sojae* isolates used as inoculum and screen *Glycine max and Glycine soja* accessions for resistance to *P. sojae*

Assessment	Scale (%)[a]	Virulence
Virulence assay	≤30	Avirulent
	>70	Virulent
Resistance screening assay	0	100% resistant
	≤30	Strongly resistant
	>30 to ≤70	Moderately resistant
	>70	Susceptible

[a] Percentage of dead seedlings.

Accessions were scored as either susceptible (>70% of plants were dead), moderately resistant (>30 to ≤70% of plants were dead), or strongly resistant (≤30% of plants were dead) (Table 2). For each accession of *G. max* and *G. soja*, 40 seedlings were screened for resistance. For accessions that showed strong resistance, additional screenings of up to 300 seedlings of 39 *G. max* accessions and up to 100 seedlings of 52 *G. soja* accessions were performed on two to four separate occasions.

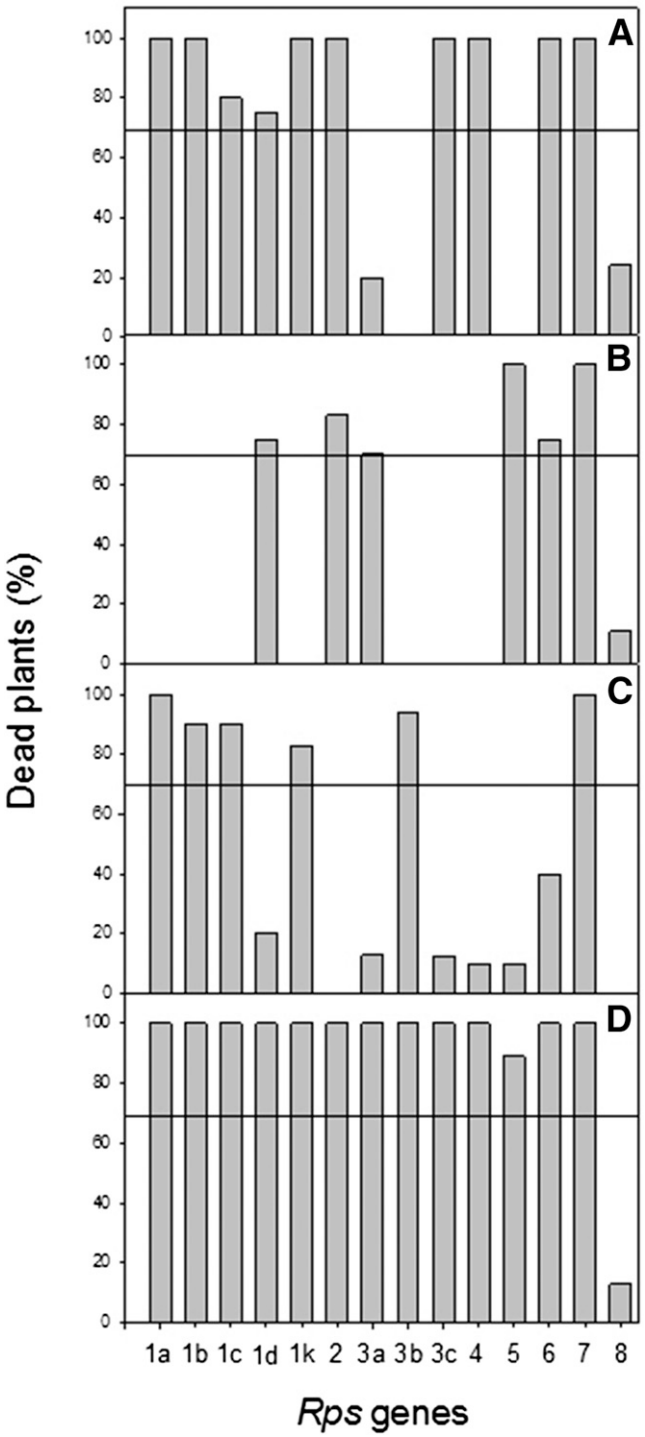

Fig. 1. Mean percentage of dead plants of soybean differentials, each containing a specific *Rps* gene, used to pathotype *Phytophthora sojae* isolates **A,** PT2004 C2.S1; **B,** R7-2a; **C,** 1005-2.9; and **D,** a 1:1:1 mixture of all three isolates. Thirty plants of each differential were inoculated and assessed. Solid line signifies >70% of dead plants indicating virulence of the isolate or mixture on that differential.

Results

Evaluating virulence of the combined mixture of *P. sojae* isolates used as inoculum. No difference in experimental replicates was detected for PT2004 C2.S1, R7-2a, 1005-2.9, or the combined mixture ($P < 0.6177$, 0.9975, 0.2156, and 0.3806, respectively); therefore, disease assessment of all replicates per isolate was combined. Using the 14 differentials described in Table 1, the virulence and pathotype of each of the isolates used in this study were confirmed. Moreover, Sloan soybean, which has no *Rps* genes, was 100% susceptible to all three isolates (Fig. 1A to C). None of the isolates were virulent on *Rps* 8 under our laboratory conditions. When the differentials were inoculated with a 1:1:1 mixture of the three isolates, the mixture was virulent on all differential lines (>70% dead plants), except PI399073, which contains the *Rps* 8 gene, on which it was avirulent (≤30% dead plants) (Fig. 1D). The susceptible check, Sloan, was 100% susceptible to the isolate. Thus, the pathotype of the 1:1:1 inoculum mixture was 1a, 1b, 1c, 1d, 1k, 2, 3a, 3b, 3c, 4, 5, 6, 7 (Fig. 1D).

Screening *G. max and G. soja* accessions for resistance to *P. sojae*. The mixed-inoculum method was used to screen lines of *G. max* and *G. soja* for potential novel sources of resistance to *P. sojae*. No difference in replicates was detected for either *G. max* or *G. sojae* ($P < 0.1768$ and 0.2603, respectively); therefore, disease assessment of all replicates per species was combined. Of the 490 *G. max* accessions screened, 325 lines were susceptible (>70% dead plants), 82 lines displayed moderate resistance (>30 to ≤70% dead plants), and 83 lines exhibited strong resistance (≤30% dead plants). Moreover, 1 of these 83 lines was 100% resistant; in other words, no plants died as a result of being inoculated with the 1:1:1 mixture of the *P. sojae* isolates (Fig. 2A). Of the 529 *G. soja* accessions screened, 384 lines were susceptible (>70% dead plants) to infection, 85 lines were moderately resistant (>30 to ≤70% dead plants), and 60 lines were strongly resistant (≤30% dead plants), with 13 of those 60 lines being 100% resistant (Fig. 2B).

Discussion

In this study, we combined agar-based cultures of three *P. sojae* isolates in a 1:1:1 single agar-based slurry and identified 83 *G. max* and 60 *G. soja* resistant accessions based on the criterion of ≤30% dead plants (Supplementary Table S1). It is expected that the most resistant accessions among this set have either *Rps* 8 or a novel *Rps* gene or genes. Although the number of identified resistant accessions may seem more than what would be expected, the majority of accessions we screened had been previously identified as having resistance to *P. sojae* (http://www.ars-grin.gov). Moreover, it is possible that some of the accessions screened were duplicates, because these are known to exist within the USDA Soybean Germplasm Collection. For example, Song et al. (2012) reported that 23 and 30% of the USDA accessions are redundant for *G. max* and *G. soja*, respectively, based on analysis with more than 42,000 single-nucleotide polymorphisms. All accessions identified as being resistant in our study were screened at least twice and sometimes up to four times, and consistently showed strong resistance (≤30% plants dead after inoculation with *P. sojae*). In addition, 1 *G. max* and 13 *G. soja* lines were 100% resistant (no plants dead), providing strong evidence of the presence of a novel *Rps* resistance gene or genes.

The distribution of *P. sojae* resistance in the *G. max* and *G. soja* accessions we screened was similar. *G. soja* is a wild relative of soybean and it may be argued that more diversity in resistance might have been expected, particularly because the genetic basis of current soybean cultivars is somewhat limited. Because we did not assay all available *G. soja* accessions and the *G. max* accessions we screened were not arbitrarily chosen but selected based on prior studies, it is possible that we were not able to detect this greater diversity in *G. soja*.

Combining three isolates of *P. sojae* that were virulent on many of the known *Rps* genes was an effective method of quickly screening soybean accessions for novel sources of resistance of the pathogen. Including a set of soybean differentials and a susceptible control (i.e., Sloan) in each screening experiment was important to ensure that the mixture of inoculum was virulent on all *Rps* genes. Using this

method, we consistently identified accessions that have potential novel sources of resistance to *P. sojae.* It is important to note that the Robertson Lab screened all accessions blindly; data were sent to the Maroof Lab, where accessions were chosen for retesting and sent back to ISU with a new identifier.

The genome of *P. sojae* is very plastic (Dong et al. 2011; Tyler et al. 1995) and the pathogen often evolves to overcome deployed *Rps* genes (Dorrance et al. 2003; Stewart et al. 2014). The extent of virulence diversity of *P. sojae* varies from state to state. For example, pathotype diversity is low in Iowa (Robertson et al. 2009) when compared with other states such as Ohio (Dorrance et al. 2003), Minnesota (Nelson et al. 1996), or Illinois (Malvick and Grunden 2004). Therefore, the importance of finding and implementing novel sources of resistance to *P. sojae* to manage PRR is economically important to soybean production in the United States. Because no one isolate of *P. sojae* has been recovered from the field that can overcome all *Rps* genes (Gordon et al. 2007), combining *P. sojae* isolates to make a source of inoculum covering all known *Rps* genes should be useful for screening soybean lines that may have multiple or novel *Rps* genes.

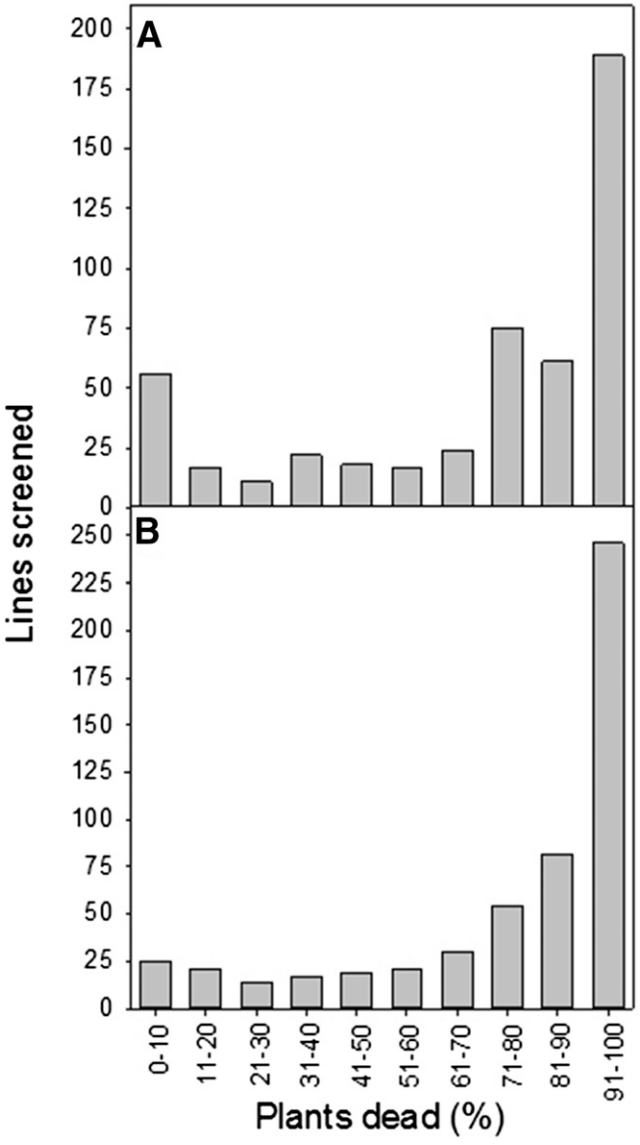

Fig. 2. Number of **A,** *Glycine max* and **B**. *G. soja* lines screened for resistance using a 1:1:1 mixture of *Phytophthora sojae* isolates PT2004 C2.S1, R7-2a, and 1005-2.9. Forty seedlings of each *G. max* and *G. sojae* line were screened initially. Lines showing strong resistance (≤30% dead plants) were screened two to four additional times (up to 300 and 100 seedlings for *G. max* and *G. sojae*, respectively).

Extensive surveys of *G. max* accessions for reaction to *P. sojae* have been conducted by many investigators in the past. However, screening of *G. soja* germplasm has been very limited (Pazdernik et al. 1997). Our work included the majority of the *G. soja* accessions available at GRIN and identified 60 resistant accessions, 13 of which showed 100% resistance to *P. sojae*. These accessions could be used in breeding programs to develop germplasm with resistance to PRR. In our study, we identified *G. max* and *G. soja* lines with potentially novel *Rps* genes with resistance to *P. sojae* that are potentially valuable sources of resistance in field isolates that are virulent on the most commonly deployed *Rps* genes. Research is currently underway to determine which of these lines recognize core RXLR effectors that are broadly conserved and essential for virulence.

In conclusion, this study is novel in that we demonstrated that isolates of *P. sojae* varying in pathotype could be combined and used as a single source of inoculum to effectively screen soybean germplasm for novel sources of resistance to the pathogen using the hypocotyl method. This method significantly reduces time, labor, space, and materials normally associated with screening germplasm and, therefore, is extremely cost effective.

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