Molecular Assessment of Pathotype Diversity of *Phytophthora sojae* in Canada Highlights Declining Sources of Resistance in Soybean

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Abstract

The large-scale deployment of resistance to *Phytophthora sojae* (*Rps*) genes in soybean has led to the rapid evolution of the virulence profile (pathotype) of *P. sojae* populations. Determining the pathotypes of *P. sojae* isolates is important in selecting soybean germplasm carrying the proper *Rps*, but this process is fastidious and requires specific expertise. In this work, we used a molecular assay to assess the pathotypes of *P. sojae* isolates obtained throughout the provinces of Québec, Ontario, and Manitoba. In preliminary assays, the molecular tool showed equivalent prediction of the pathotypes as a phenotyping assay and proved to be much faster to apply while eliminating intermediate values. Upon analysis of nearly 300 isolates, 24 different pathotypes were detected in Québec and Ontario, compared with only eight in Manitoba, where soybean culture is more recent. Pathotypes 1a, 1c, and 1d was predominant

in Québec, while 1a, 1b, 1c, 1d, and 1k pathotypes were the most common in Manitoba. Overall, the results showed that 98 and 86% of the isolates carried pathotype 1a or 1c, respectively, suggesting that *Rps1a* and *Rps1c* were no longer effective in Canada. Based on the history of soybean varieties used in surveyed fields, it was found that 84% of them contained *Rps* genes that were no longer resistant against the pathotypes of the isolates found in the fields. While highlighting an easier and more precise option to assess pathotypes, this study presents the first pan-Canadian survey of *P. sojae* and stresses the importance of carefully managing the declining sources of resistance.

Keywords: avirulence genes, field survey, resistance genes, root rot, soybean

Phytophthora stem and root rot (PRR), caused by the oomycete *Phytophthora sojae* M.J. Kaufmann and J.W. Gerdemann, was first reported in Canada by Hildebrand (1954) and accounts for approximately \$50 M in annual losses in Canadian soybean (*Glycine max* [L.] Merr.) production (Sepiol et al. 2017). This soilborne pathogen is particularly devastating because it can attack soybean plants at all growth stages, causing pre- and postemergence damping-off and forcing farmers to replant (Dorrance et al. 2007; Tyler 2007). It also can cause stem and root rot of established plants, constraining the water-conducting capacity of the plant, which eventually causes wilting, foliar chlorosis, and plant death (Dorrance et al. 2007; Schmitthenner 2000). Factors that contribute to PRR include poor soil drainage that can be easily flooded because of compaction, poor leveling or high clay content of the soil, and the planting of susceptible cultivars.

An effective approach to control the disease is genetic resistance (Dorrance 2018). Vertical, or total, resistance leads to complete protection against specific *Phytophthora sojae* isolates, conferred by single resistance genes called *Rps* (resistance to *P. sojae*; Sugimoto et al. 2012). The effectiveness of *Rps* genes is dependent on the presence of corresponding avirulence genes (*Avr*) in the pathogen (Tyler 2007) based on the classical gene-for-gene model. Gene-for-gene resistance provides complete resistance to the disease if growers use the appropriate *Rps* gene(s) matching the *Avr* gene(s) present in

isolates that occur in a given field. To date, >33 *Rps* genes/alleles have been identified in soybean (Jiang et al. 2020). In North America, the most common resistance genes deployed in commercial soybean lines include *Rps1a*, *1c*, *1k*, *3a*, and *6* (Dorrance 2018). When a novel *Rps* gene is deployed into a soybean cultivar, it increases selection pressure on *P. sojae* against conserving *Avr* genes. The pathogen avoids recognition by the *Rps* gene products through various mutations within their *Avr* genes (Arsenault-Labrecque et al. 2018; Hartman et al. 2015; Tyler and Gijzen 2014). This rapid diversification of *Avr* genes has led to increased virulence profiles of *P. sojae* to >200 pathotypes (Dorrance et al. 2004) and limits the effectiveness of *Rps* genes over time (Grau et al. 2004; Schmitthenner 1985; Stewart et al. 2014). Because of the *Avr* diversification occurring within *P. sojae*, a great deal of effort has been invested in characterizing the pathotypes of *P. sojae* in soybean-producing areas to deploy the appropriate *Rps* genes. Plant pathologists in the U.S.A. have led the way in these endeavors with multiple race survey reports over the years, particularly in the Northern and Midwestern states including Iowa, Indiana, Illinois, Kansas, Michigan, Minnesota, Missouri, Nebraska, New York, Ohio, and North and South Dakota (Dorrance et al. 2003, 2016; Stewart et al. 2014; Yan and Nelson 2019). In Canada, where soybean production is more recent and more localized, only a few articles from the literature are available. The first survey was conducted in the 1980s in Ontario (Anderson and Buzzell 1992) and the second one, limited to Ontario again, highlighted the evolution of *P. sojae* pathotypes over two survey periods (Xue et al. 2015). Thereafter, a few reports, from the provinces of Manitoba or Alberta, provided the first description of *P. sojae* pathotypes in a limited number of fields (Chang et al. 2017; Henriquez et al. 2020). In all those surveys, the hypocotyl inoculation test, developed by Kaufmann and Gerdemann (1958), was used to phenotype *P. sojae* pathotypes. This method consists of wounding and inoculating young soybean hypocotyls of near-isogenic lines with different *Rps* genes with a mycelial slurry of an isolate of *P. sojae*. While this method is easy to apply, it has its shortcomings in that it is labor-intensive, can yield intermediate values, false-positives and

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false-negatives, and bypasses the natural method of zoospore infection of the root system (Lebreton et al. 2018). To alleviate these limitations, another assay was proposed in which inoculation took place in a hydroponic system to better recreate the natural course of infection (Lebreton et al. 2018). While providing a more accurate determination of the pathotypes (Arsenault-Labrecque et al. 2018), the assay remains laborious and time-consuming. In 2020, a molecular tool for predicting the pathotypes of *P. sojae* was developed by Dussault-Benoit et al. (2020). This molecular tool was designed based on genomic analyses in which haplotypes of *Avr* genes could be assigned to virulent or avirulent forms for seven *Avr* genes or alleles: *Avr1a*, *1b*, *1c*, *1d*, *1k*, *3a*, and *6* (Arsenault-Labrecque et al. 2018). This assay provides a relatively rapid and accurate identification of the virulence profile of many *P. sojae* isolates simultaneously.

In this study, the general objective was to conduct the first comprehensive pan-Canadian analysis of *P. sojae* pathotypes in the major soybean-growing areas of the country. For this purpose, the molecular assay was first validated against the hydroponic phenotypic assay, and then exploited to analyze hundreds of *P. sojae* isolates and to relate the information in terms of pathotype distribution/evolution and management practices.

Materials and Methods

Field sampling. Soil samples were collected across Québec, Ontario, and Manitoba during the 2016 to 2019 growing seasons. These provinces account for 96% of the soybean production area in Canada, with 16% in Québec, 57% in Ontario, and 23% in Manitoba (Soy Canada 2020). The majority of soybean fields in Ontario and Québec were selected for sampling based on a history of PRR or a history of stand establishment problems, while in Manitoba the fields were selected randomly. Samples from Québec were collected from 71 commercial soybean fields, six experimental soybean fields, and four disease nurseries. Samples from Ontario were taken from 75 commercial soybean fields, five experimental soybean fields, and one disease nursery. Samples from Manitoba were taken from 84 commercial soybean fields. At least three soil cores were taken by sites where soybean plants with PRR symptoms were observed or in compaction zones. The soil samples (~1 kg) were taken at a depth of 15 cm from the rhizosphere using a shovel. The soil samples were air-dried and stored at 4°C until pathogen isolation. Whenever possible, in Québec and Manitoba, information on the cultivar planted in the sampled fields was collected for association studies between *P. sojae* pathotypes recovered and *Rps* genes used in the soybean cultivars.

Baiting procedures and isolation of *P. sojae*. The soybean seedling bioassay procedure (baiting method) described by Canaday and Schmitthenner (1982) was used to isolate *P. sojae* from soil. Each soil sample was placed in a 415-ml plastic cup, flooded for 24 h with distilled water, and then allowed to dry slowly in the dark at room temperature until the soil began to pull away from the sides of the glass. Cups were then closed with a lid for 2 weeks until planting. After incubation, five seeds of the soybean variety ‘Sloan’ or ‘Williams’ (susceptible to all known races of *P. sojae*) were planted and covered with a fine layer of vermiculite. The cups were placed inside a growth chamber set at 26°C day and 16°C night under a photoperiod of 13 h per day illuminated with incandescent and fluorescent (590 µMol light). After seed germination, the cups were flooded again for 24 h and then allowed to dry. Soybean plants were monitored for evidence of typical PPR symptoms, including root lesions or hypocotyl collapse, for 7 to 14 days. To isolate the pathogen, tissue sections (1 cm) cut from the margin of lesions on hypocotyls were surface-disinfested by submersion in 70% ethanol for 10 s, rinsed in sterilized distilled water and air-dried. The outer layer of the stem was removed very carefully, and the central tissues were sectioned into 0.5-cm pieces and placed on the semiselective V8 PBNIC agar medium containing Terrachlor (99% pentachloronitrobenzene; Sigma-Aldrich, St. Louis, MO) at 0.04 g liter⁻¹, Benlate (50% benomyl; Sigma-Aldrich) at 0.01 g liter⁻¹, neomycin sulfate at 0.05 g liter⁻¹, Rovral (50% iprodione; Bayer Cropscience, Montvale, NJ) at 0.04 g liter⁻¹, and chloramphenicol at 0.1 g liter⁻¹ in a V8 juice agar base (200 ml liter⁻¹ of clarified V8 juice; Campbell Soup

Co., Camden, NJ), and 15 g liter⁻¹ agar. To decrease bacterial growth, the medium was flipped to relocate the pieces between the bottom of the plate and the medium, and the Petri dishes were incubated at 28°C for 1 week. Petri dishes were monitored daily for presence of the pathogen. On the semiselective medium, *P. sojae* was easily identifiable by its creamy-white cottony appearance on the root tissues. If no growth of *P. sojae* was observed, the baiting steps were repeated twice. Hyphal tips of *P. sojae* growing from infected tissues were transferred onto regular V8 medium (200 ml liter⁻¹ of clarified V8 juice and 15 g liter⁻¹ agar).

Single-spore isolation. A single-spore isolate was recovered by oospore isolation. Briefly, a 7-to-10-day-old culture from a 5-ml Petri dish containing V8 Phytigel medium (rifampicin 0.1 g liter⁻¹; Sigma-Aldrich), Phytigel (4 g liter⁻¹), and clarified V8 (200 ml liter⁻¹) was blended with 30 ml of sterile water in a commercial blender for 10 s. The mixture was poured into a 50-ml sterile tube. The tube was placed on ice and kept at 4°C for 24 h to kill mycelium. The tube was centrifuged (1 m, 3,200 RCF) and decanted to separate the medium and the mycelium from the pellet containing the oospores. Thirty milliliters of water were added to the tube, vortexed, centrifuged, and decanted a second time. The pellet was diluted to a concentration of 10 oospores/ml and 200 µl of the oospore suspension was spread on regular V8 agar medium. The oospores were incubated at 28°C and began to germinate after 24 h. Germinated oospores were transferred onto regular V8 agar using a sterile needle under a stereomicroscope (60× magnification).

DNA extraction and molecular pathotype characterization. The single-spore isolates were subcultured on regular V8 agar medium covered with cellulose paper to facilitate the harvest of hyphae and spores. After 7 days, isolates were removed from the paper and placed in 1.5-ml tubes containing two porcelain beads (2.8 mm). The samples were homogenized (4 m s⁻¹ × 2 cycles of 30 s with a 40-s pause) with an Omni Bead Ruptor 24 (OMNI International, Tulsa, OK) and the DNA was then extracted using a cetyltrimethyl-ammonium bromide procedure (Werth et al. 2016).

The molecular assay allowed verification that the isolates recovered were *P. sojae*, because it is highly specific and also permitted the identification of the pathotype of each isolate using two PCR reactions following Dussault-Benoit et al. (2020). The first reaction was a multiplex PCR allowing simultaneous amplification of *Avr1a*, *Avr1b*, *Avr1d*, *Avr1k*, *Avr3a*, and *Avr6*; the second reaction amplified *Avr1c*. The multiplex PCR cocktail contained 1.5 µl of a mix of eight primer sets and the uniplex reaction contained only one primer set at a concentration described by Dussault-Benoit et al. (2020), 0.5 µl of 10 mM dNTPs, 0.1 µl of OneTaq DNA polymerase (5,000 units/ml; New England Biolabs, Ipswich, MA), and 4 µl of 5× OneTaq Standard Reaction Buffer to which was added 2 µl of 10 ng/µl DNA and sterile distilled water to a final volume of 20 µl. All reactions were performed in a T Professional Thermocycler (Biometra, Göttingen, Germany), using 40 cycles for the multiplex reaction and 30 cycles for the uniplex reaction. The thermal cycling settings consisted of an initial denaturation at 94°C for 5 min, cycles of denaturation at 94°C for 30 s, annealing for 30 s at 55°C for the multiplex PCR or 60°C for the uniplex PCR, extension at 68°C for 1 min, and a final extension at 68°C for 5 min.

Hydroponic pathotype characterization. To assess the validity and the precision of the molecular assay, a subsample of 18 isolates was arbitrarily chosen and also analyzed using a modified version of the hydroponic bioassay developed by Lebreton et al. (2018). The bioassay involved the inoculation of a panel of soybean differentials provided by Dr. Owen Wally (Harrow Research and Development Centre, Agriculture and Agri-Food Canada, Harrow, ON, Canada) possessing different *Rps* genes (‘Haro12’ [*Rps1a*], ‘Haro13’ [*Rps1b*], ‘Haro14’ [*Rps1c*], ‘Haro16’ [*Rps1d*], ‘Haro15’ [*Rps1k*], ‘Haro3272’ [*Rps3a*, 7], and ‘Haro6272’ [*Rps6*, 7]) with an isolate of *P. sojae*. Soybean seeds were sown in sterile vermiculite and the plantlets were transferred into the hydroponic system containing the nutritive solution 5 days’ postseeding. Twenty-four hours after the transfer of the plants, the hydroponic system was inoculated with the isolate of *P. sojae*. The inoculum was prepared by blending two 12-day-old cultures from 20-ml Petri dishes containing V8 Phytigel medium

with 60 ml of sterile water in a commercial blender. The resulting mycelial slurry was examined under a microscope (100×) to adjust the oospore concentration. Subsequently, the hydroponic system was inoculated with 1×10^6 oospores. Each isolate of *P. sojae* was characterized in two independent experiments ($n = 4$ plants per *Rps*). At 14 days postinoculation, plants were scored as resistant or susceptible to the isolate according to the method presented in Dussault-Benoit et al. (2020).

Results

Phytophthora sojae isolation. A total of 246 fields was sampled during the 2016 to 2019 seasons, including 81 in Québec, 81 in Ontario, and 84 in Manitoba. Altogether, 295 separate single-spore isolates of *P. sojae* were obtained originating as follows: 92 from Québec, 96 from Ontario, and 107 from Manitoba.

Molecular versus hydroponic assay. The DNA of the 295 single-spore isolates was extracted and then characterized by the molecular assay. This tool predicted the virulence profile of the isolates based on the amplification of seven avirulence genes for *Avr1a*, *1b*, *1c*, *1d*, *1k*, *3a*, and *6*. To assess the validity and the precision of this assay, a subsample of 18 isolates was also analyzed using the hydroponic bioassay made up of a panel of the different *Rps* genes that were challenged with a given isolate. The results obtained indicated a high level of consistency between the two methods (Table 1), wherein the genotyping approach matched the results of the phenotyping approach in 72 out of 76 alleles (95%). Interestingly, all four cases of disparity involved an additional virulence allele in the phenotyping approach, most of which (three out of four) were associated with *Avr3a*.

Pathotype diversity. After characterization of the 295 isolates with the molecular tool, 31 unique virulence formula or pathotype combinations based on the seven *Avr* genes were detected (Table 2). Some pathotype combinations were more common than others. For instance, pathotypes 1a, 1b, 1c, 1d, and 1k represented 34% of *P. sojae* isolates analyzed, and pathotypes 1a, 1c, and 1d represented 25%. Pathotypes 1a and 1c were found in 6% of isolates. All other combinations were found in percentages of <5%.

Pathotype distribution and complexity by province. In Québec, 24 different pathotype combinations were detected and pathotypes 1a, 1c, and 1d was predominant, representing 29% of the isolates characterized (Table 2), followed by pathotypes 1a, 1b, 1c, 1d, and 1k with a prevalence of 13%. In this province, the complex of pathotypes, expressed by the number of virulent alleles, predominantly involved three alleles (Fig. 1). Only a few isolates harbored more than five virulence alleles, while a complex of four alleles was found in approximately 10% of the isolates.

In Ontario, 23 pathotype combinations were detected. Pathotypes 1a, 1b, 1c, 1d, and 1k were predominant, comprising 23% of the isolates, followed by pathotypes 1a, 1c, and 1d, which accounted for 21% of the isolates (Fig. 1). The complex of pathotypes also mostly included three alleles with almost 40%, followed by approximately 20% of the isolates with five alleles. Thereafter, smaller percentages were associated with lower and higher virulence allele numbers.

Only eight pathotype combinations were observed in Manitoba. Pathotypes 1a, 1b, 1c, 1d, and 1k were predominant, representing 62% of the isolates, followed by pathotypes 1a, 1c, and 1d representing 25% of the isolates (Fig. 1). As in Québec and Ontario, the

Table 2. Molecular assessment of pathotypes of 295 isolates of *Phytophthora sojae* recovered from soybean fields in Québec, Ontario, and Manitoba between 2016 and 2019

Pathotypes	Québec	Ontario	Manitoba	Total	%
1a	2	9	0	11	4
1a, 1b, 1c	1	5	0	6	2
1a, 1b, 1c, 1d	1	1	0	2	1
1a, 1b, 1c, 1d, 1k	12	22	66	100	34
1a, 1b, 1c, 1d, 1k, 3a, 6	1	1	0	2	1
1a, 1b, 1c, 1d, 1k, 6	2	0	0	2	1
1a, 1b, 1c, 1k	2	3	5	10	3
1a, 1b, 1c, 3a, 6	0	1	0	1	0
1a, 1b, 1d, 1k	1	0	0	1	0
1a, 1b, 1k	1	4	0	5	2
1a, 1b, 3a, 6	0	1	0	1	0
1a, 1c	8	8	1	17	6
1a, 1c, 1d	27	20	27	74	25
1a, 1c, 1d, 1k	1	0	1	2	1
1a, 1c, 1d, 1k, 3a, 6	0	1	0	1	0
1a, 1c, 1d, 3a	1	0	4	5	2
1a, 1c, 1d, 3a, 6	4	1	2	7	2
1a, 1c, 1d, 6	1	0	1	2	1
1a, 1c, 3a	0	1	0	1	0
1a, 1c, 3a, 6	3	1	0	4	1
1a, 1c, 6	5	1	0	6	2
1a, 1d	1	2	0	3	1
1a, 1d, 6	0	1	0	1	0
1a, 3a	2	0	0	2	1
1a, 3a, 6	11	2	0	13	4
1a, 6	2	5	0	7	2
1b, 1c, 1d, 1k	1	0	0	1	0
1b, 1c, 1d, 3a	0	1	0	1	0
1b, 1c, 1k	1	2	0	3	1
1c	1	0	0	1	0
1c, 1d	0	3	0	3	1
Total	92	96	107	295	100

Table 1. Characterization of the pathotypes of 18 isolates of *Phytophthora sojae* from Québec, Ontario, and Manitoba with the hydroponic bioassay (phenotype) and with the molecular tool (genotype)

Province	Pathotypes ^a		
	Identification	Phenotype	Genotype
Québec	Ps_0762	1a, 1b, 1c, 1d, 1k, 6	1a, 1b, 1c, 1d, 1k, 6
	Ps_0802	1a, 1b, 1c, 1k	1a, 1b, 1c, 1k
	Ps_0768	1a, 1c, 1d	1a, 1c, 1d
	Ps_0584	1a, 1c, 1d, 1k	1a, 1c, 1d, 1k
	Ps_0767	1a, 1c, 1d, 3a, 6	1a, 1c, 1d, 3a, 6
	Ps_0781	1a, 1c, 3a ^b , 6	1a, 1c, 6
Ontario	Ps_0604	1a, 1b, 1c, 1d	1a, 1b, 1c, 1d
	Ps_0627	1a, 1b, 1c, 1d, 1k	1a, 1b, 1c, 1d, 1k
	Ps_0639	1a, 1c ^b	1a
	Ps_0685	1a, 1c, 1d	1a, 1c, 1d
	Ps_0703	1a, 1c, 1d, 3a, 6	1a, 1c, 1d, 3a, 6
	Ps_0722	1a, 1c, 3a ^b , 6	1a, 1c, 6
Manitoba	Ps_0476	1a, 1b, 1c, 1d, 1k	1a, 1b, 1c, 1d, 1k
	Ps_0492	1a, 1b, 1c, 1d, 1k	1a, 1b, 1c, 1d, 1k
	Ps_0539	1a, 1b, 1c, 1d, 1k, 3a ^b	1a, 1b, 1c, 1d, 1k
	Ps_0591	1a, 1c, 1d	1a, 1c, 1d
	Ps_0444	1a, 1c, 1d, 3a	1a, 1c, 1d, 3a
	Ps_0515	1a, 1c, 1d, 6	1a, 1c, 1d, 6

^a Although *Avr7* was not included in the genotype test, pathotype 7 was recorded in all isolates subjected to the hydroponic assay.

^b Indicates presence of a virulence allele with the phenotyping approach that was absent with the genotyping approach.

dominance of three and five virulence alleles was caused by the fact that 1b and 1k tended to be present or absent in tandem in all isolates analyzed.

Virulence allele distribution. Virulence on *Rps1a* was detected in 96, 94, and 100% of *P. sojae* tested from Québec, Ontario, and Manitoba, respectively (Fig. 2). This was followed closely by pathotypes that were virulent on the resistance allele *Rps1c*, representing 77, 75, and 100% of the isolates, respectively.

Virulence on *Rps1k* was present only in 16 and 33% of the isolates from Québec and Ontario, respectively. This percentage rose to 67% in isolates from Manitoba. Virulence on *Rps1b* followed the same pattern as *Rps1k* in all provinces. Virulence on *Rps3a* and 6 was observed occasionally, particularly in Manitoba, with the exception of virulence on *Rps6*, which occurred in 30% of the isolates in Québec. Finally, considering that *Rps1d* is not commercially deployed, it was rather surprising to find isolates virulent on this genotype in 50% of the collections from Québec and Ontario and 90% in those from Manitoba.

Pathotypes versus *Rps* genes in field. During the course of these surveys, whenever possible, the identity of the soybean variety in the sampled fields was recorded. This information was available for 64 fields in Québec, where 87 isolates of *P. sojae* were recovered, and for 30 fields in Manitoba, where 49 isolates were collected. No such information was obtained in Ontario. As shown in Figure 3, *Rps1c* was the most common resistance gene deployed, followed by *Rps1k*, *Rps1a*, and *Rps3a*, while nearly one-third of the fields were planted with cultivars carrying no *Rps* genes.

Tables 3 and 4 present a comprehensive comparison between the *P. sojae* pathotypes found in a given field and the *Rps* genes used in that field. As expected, for all fields where *Rps1a* was used, all *P. sojae* isolates recovered had the corresponding pathotype 1a. A very similar association was observed for *Rps1c*, where in 96% of the cases, the isolates were pathotype 1c, resulting in a compatible

Fig. 1. Percentage of *Phytophthora sojae* isolates carrying a given pathotype complexity based on virulence genes *avr 1a*, *1b*, *1c*, *1d*, *1k*, *3a*, and *6*. The percentage is based on 295 isolates of *P. sojae* recovered in Québec, Ontario, and Manitoba fields in 2018 and 2019.

Fig. 2. Percentage of *Phytophthora sojae* isolates carrying a given pathotype. The percentage is based on 295 isolates of *P. sojae* recovered in Québec, Ontario, and Manitoba fields in 2018 and 2019.

interaction. These predicted compatible reactions were somewhat less common in fields where *Rps1k*, *Rps3a*, or *Rps6* were used as sources of resistance. Nevertheless, in both Québec and Manitoba, >80% of the sampled fields used soybean varieties that were not resistant to the *P. sojae* pathotypes present in the fields.

Discussion

With the rapid expansion of soybean production in Canada, *P. sojae* arguably has become the most an important limiting factor in its production. For this reason, a greater awareness of the pathotype diversity of this pathogen must be taken into consideration when applying genetic approaches to manage the disease. This study reports findings of the first pan-Canadian survey of the genetic complexity of *P. sojae* isolates in soybean fields across the provinces of Québec, Ontario, and Manitoba, where 96% of soybean production occurs. To carry out these analyses, *P. sojae* pathotypes were assessed based on a molecular test by Dussault-Benoit et al. (2020), which allowed greater ease, speed, and accuracy in determining the virulence formula of the tested isolates. This exhaustive characterization of *P. sojae* should help soybean growers, breeders, and seed suppliers in making educated decisions when deploying *Rps* genes for the purpose of reducing losses to PRR.

Historically, all studies that have analyzed the pathotype diversity of *P. sojae* isolates have relied on the hypocotyl assay, which involves testing single isolates on a set of soybean differentials carrying different *Rps* genes and alleles. This method is both time- and labor-intensive, and also is prone to yielding false positives or negatives when intermediate responses occur. While the hydroponic assay developed by Lebreton et al. (2018) may be more accurate, it remains as fastidious as the hypocotyl assay. For this reason, the molecular assay used in this study represents an important breakthrough in pathotype studies, because it is much less demanding in terms of time, space, and manpower. It is also theoretically more accurate, as amplification of the virulence genes is less open to interpretation. When comparing the molecular assay with the hydroponic method, this study demonstrated that a high level of consistency among 95% of the alleles studied. Interestingly, the most common, albeit infrequent, discrepancy was related to *Avr3a*. In their description of the molecular assay, Dussault-Benoit et al. (2020) also observed that results for *Avr3a* were sometimes at odds with the

Fig. 3. Relative field distribution of different *Rps* genes present in soybean cultivars in 64 and 30 fields in the provinces of Québec and Manitoba, respectively, sampled for the presence of *Phytophthora sojae* in 2018 to 2019. Pyramiding of *Rps* genes in some cultivars explain total exceeding 100%.

hydroponic assay, while Arsenault-Labrecque et al. (2018) reported a perfect match between the molecular assay and the hypocotyl assay for detecting *Avr3a*. This may simply indicate that the hydroponic assay is sometimes too stringent for allowing the timely expression of *Rps3a*. Nevertheless, the results of this study suggest that the molecular assay is, on many levels, a significant advance for the easy and rapid assessment of *P. sojae* pathotypes.

Overall, the pathotype combination 1a, 1c, and 1d was the most common across all three provinces. Regardless of the diversity, nearly all isolates analyzed were virulent against *Rps1a* genotypes, and the vast majority were also virulent on *Rps1c* genotypes. These results might be explained by the fact that *Rps1a* and *Rps1c* were the first resistance genes deployed commercially in soybean in Canada, and that selection pressure has led to an increase in the prevalence of virulence for these genes (Jackson et al. 2004; Schmitthenner et al. 1994). This study clearly indicates that *P. sojae* has overcome those resistance genes and that good resistance stewardship practices are necessary to preserve the other ones.

An unexpected finding in the course of our surveys was the frequent detection of some pathotypes with virulence on *Rps1a*, *Rps1b*, *Rps1c*, *Rps1d*, and *Rps1k*, especially in Manitoba. Incidentally, there was a much higher percentage of isolates with pathotypes 1b and 1k in that province than in Ontario and Québec. This was surprising because expansion of the cultivation of short-season soybeans

capable of growing in Manitoba has occurred predominantly within the past 10 years. A possible explanation is that *Rps1k* has been heavily deployed in Manitoba in adapted varieties, while being more gradually integrated in the Québec or Ontario production areas. The deployment of newer *Rps* genes such as *Rps1k* in commercial soybean cultivars has contributed to the emergence of new pathotypes with increased complexity (Xue et al. 2015). This situation also was reported in the USA (Dorrance et al. 2016; Jackson et al. 2004; Yan and Nelson 2019) and Argentina (Grijalba et al. 2020). It is also interesting to observe how the presence of the virulence or avirulence alleles of 1b and 1k were nearly always synchronized and also reported in other studies (Whisson et al. 1995). This suggests that a more thorough characterization of *Rps1b* and *Rps1k* should be conducted to explain this phenomenon.

A fairly large number of *P. sojae* isolates were shown to possess virulence on *Rps1d*, although the corresponding resistance gene has not been deployed commercially in soybean varieties in Canada. This observation has been reported in other soybean-growing regions in the USA, where isolates were virulent on *Rps* genes that were not known to be deployed in the region (Dorrance et al. 2016; Jackson et al. 2004; Schmitthenner et al. 1994; Stewart et al. 2014).

The only information regarding the occurrence of *P. sojae* pathotypes, dating as far back to the 1980s, is from Ontario, which was the first province to invest in soybean research. Two large-scale

Table 3. Association between the pathotypes of *Phytophthora sojae* isolates in a soybean field and the *Rps* genes present in the cultivar used in the field for 87 isolates from Québec

Québec							
ID	Pathotype	Rps	Expected response	ID	Pathotype	Rps	Expected response
Ps_0545	1a, 1d	—	Disease	Ps_0727	1a, 1c	1c	Disease
Ps_0573	1a, 6	—	Disease	Ps_0744	1c	1c	Disease
Ps_0547	1a, 1c, 1d, 1k	—	Disease	Ps_0745	1a, 1c, 1d	1c	Disease
Ps_0728	1a, 3a	—	Disease	Ps_0730	1a, 1c	1c	Disease
Ps_0549	1a, 1c, 3a, 6	—	Disease	Ps_0544	1a, 1c, 1d	1c	Disease
Ps_0550	1a, 1c	—	Disease	Ps_0732	1a, 1b, 1c, 1d, 1k	1c	Disease
Ps_0731	1a, 3a	—	Disease	Ps_0733	1a, 1b, 1c, 1k	1c	Disease
Ps_0552	1a	—	Disease	Ps_0734	1b, 1c, 1k	1c	Disease
Ps_0544	1a, 3a, 6	—	Disease	Ps_0735	1a, 1c, 1d	1c	Disease
Ps_0554	1a, 1c, 1d	—	Disease	Ps_0746	1a, 1c, 1d	1c	Disease
Ps_0555	1a, 1c, 1d	—	Disease	Ps_0737	1a, 1b, 1c, 1d, 1k	1c	Disease
Ps_0739	1a, 1c, 1d	—	Disease	Ps_0738	1a, 1c, 6	1c	Disease
Ps_0570	1a, 1c, 1d, 3a, 6	—	Disease	Ps_0546	1a, 1c, 1d	1c	Disease
Ps_0558	1a, 1c, 6	—	Disease	Ps_0551	1a, 1b, 1c, 1d, 1k	1c	Disease
Ps_0559	1a, 1b, 1c, 1d, 1k	—	Disease	Ps_0560	1a, 1c, 1d, 3a, 6	1c	Disease
Ps_0729	1a, 1b, 1c, 1d, 1k	—	Disease	Ps_0747	1a, 1c, 1d	1c	Disease
Ps_0561	1a, 1c, 1d	—	Disease	Ps_0548	1a, 1c, 6	1c	Disease
Ps_0562	1a, 1c, 1d	—	Disease	Ps_0748	1a, 1c, 1d	1c	Disease
Ps_0736	1a, 1c, 1d, 3a	—	Disease	Ps_0749	1a, 1c, 1d, 3a, 6	1c	Disease
Ps_0564	1a, 1c	—	Disease	Ps_0750	1a, 1c	1c	Disease
Ps_0565	1a, 1c, 6	—	Disease	Ps_0751	1a, 1c, 1d	1k	Disease
Ps_0739	1a, 3a, 6	—	Disease	Ps_0563	1a, 1b, 1c, 1d, 1k	1k	Disease
Ps_0740	1a, 1b, 1c, 1k	—	Disease	Ps_0557	1a, 1b, 1c, 1d, 1k	1k	Disease
Ps_0568	1a, 1c, 1d	—	Disease	Ps_0752	1b, 1c, 1d, 1k	1k	Disease
Ps_0569	1a, 1c, 1d	—	Disease	Ps_0774	1a, 3a, 6	1k	Resistance
Ps_0741	1a, 1c, 1d	1a	Disease	Ps_0775	1a, 1c, 1d	1k	Resistance
Ps_0571	1a, 3a, 6	1a	Disease	Ps_0753	1a, 1c, 6	1k	Resistance
Ps_0572	1a, 1c, 1d	1a	Disease	Ps_0553	1a, 6	1k	Resistance
Ps_0742	1a, 3a, 6	1a	Disease	Ps_0556	1a, 1b, 1c	1k	Resistance
Ps_0574	1a, 1c, 3a, 6	1c	Disease	Ps_0754	1a, 1b, 1c, 1d	1k	Resistance
Ps_0755	1a, 1c, 1d	1c	Disease	Ps_0762	1a, 1b, 1c, 1d, 1k, 6	1k	Disease
Ps_0756	1a, 1c, 1d	1c	Disease	Ps_0763	1a, 1b, 1c, 1d, 1k	1k	Disease
Ps_0771	1a, 3a, 6	1c	Resistance	Ps_0764	1a, 3a, 6	1k	Resistance
Ps_0772	1a, 1c, 1d	1c	Disease	Ps_0566	1a, 1c	1k + 3a	Resistance
Ps_0757	1a, 1b, 1c, 1d, 1k	1c	Disease	Ps_0567	1a, 1b, 1k	1k + 3a	Resistance
Ps_0758	1a, 1b, 1c, 1d, 1k	1c	Disease	Ps_0765	1a, 3a, 6	3a	Disease
Ps_0759	1a, 1c, 1d	1c	Disease	Ps_0766	1a, 3a, 6	3a	Disease
Ps_0760	1a, 1c, 1d, 6	1c	Disease	Ps_0767	1a, 1c, 1d, 3a, 6	3a	Disease
Ps_0761	1a, 1c, 1d	1c	Disease	Ps_0776	1a, 1c, 3a, 6	3a	Disease
Ps_0773	1a, 1b, 1c, 1d, 1k	1c	Disease	Ps_0777	1a	3a	Resistance
Ps_0778	1a, 1b, 1c, 1d, 1k, 6	1c	Disease	Ps_0768	1a, 1c, 1d	3a	Resistance
Ps_0779	1a, 1b, 1c, 1d, 1k	1c	Disease	Ps_0769	1a, 3a, 6	3a	Disease
Ps_0780	1a, 1c	1c	Disease	Ps_0770	1a, 1b, 1c, 1d, 1k, 3a, 6	3a	Disease
Total of isolates = 87		Disease = 86%		Resistance = 14%			

studies were previously conducted, the first by Anderson and Buzzell (1992), which reported on the different “races” of *P. sojae* in the 80s and the second by Xue et al. (2015), which reported similar results from the early 2010s. Those studies provide an important window into the evolution of *P. sojae* pathotypes over time for a given region (Table 5). The first striking feature is that the first study described 10 races, while the second study reported 23 races, which represented a considerable increase. According to Xue et al. (2015), pathotype complexity in Ontario has increased, involving one to five alleles in the 1980s versus two to eight in 2010. Because the complexity of the pathotypes in this study was determined on the basis of seven *Avr* genes with the molecular tool, a direct comparison was limited as *Avr7* is not part of the molecular assay. It can be inferred from the two previous studies, however, that all the isolates in this study carried virulence for *Rps7* because it was the first *Rps* gene to be deployed (Xue et al. 2015). On the other hand, an increase in the complexity of the pathotypes obtained in this study was found in comparison with earlier investigations. Indeed, over time, pathotypes 1a and 7 have become rare, while more complex pathotypes such as 1a, 1c, 7 and 1a, 1c, 1d, 7 have appeared. Pathotypes 1a and 7 decreased from 25.1% in the report of Anderson and Buzzell (1992) to 12% in the report of Xue et al. (2015), to just 9.4% in this study. Pathotype 1k was quite prevalent in both our study and that of Xue et al. (2015), but was conspicuously absent in the survey by Anderson and Buzzell (1992). These results demonstrate that pathotype diversification is driven by selection pressure from *Rps* genes, because *Rps1k* was only deployed in Ontario after the initial survey.

Based on this last statement, it seems contradictory to observe the increasing presence over time of virulence for *Rps1d*, reaching 23% in this study. This may indicate that *Rps1d* has been deployed unknowingly in certain commercial soybean varieties, as has been reported for *Rps6* (Anderson et al. 2007; Athow and Lavolette 1982). Additionally, some studies have found a significant percentage of isolates with virulence on *Rps1d* (Dorrance et al. 2016; Stewart et al. 2014), thus supporting the unknown presence of *Rps1d* in some soybean varieties.

Henriquez et al. (2020) characterized the pathotypes of some *P. sojae* isolates from Manitoba collected in 2014, 2016, and 2017 using the hypocotyl test. Their results were fairly similar to this

study, with the notable exception that >90% of our isolates carried virulence for *Rps1d*, while nearly none did in the former study. Based on the results of this study, this may indicate that issues existed with the *Rps1d* differential used in that study, a problem previously reported among differentials of a similar gene in other studies (Dorrance et al. 2004). In as much as the presence of the avirulence gene will lead to an incompatible reaction, as shown in an overwhelming percentage in previous papers (Arsenault-Labrecque et al. 2018; Dussault-Benoit et al. 2020), the molecular assay can thus eliminate the bias owing to the differential source.

To our knowledge, this is the first study in Canada reporting on *P. sojae* pathotypes that has a direct association with the *Rps* genes present in soybean varieties in sampled fields. Admittedly, although sampling in Québec in this study was biased toward fields with a suspected presence of PRR, sampling in Manitoba was arbitrary and the results indicated, in both provinces, a high percentage of fields using soybean varieties with an inappropriate *Rps* gene for the pathotypes found in the field. *Rps1a* was the first resistance gene to be widely deployed and, according to the results in this study, it is, for all practical purposes, no longer effective, because most isolates have acquired virulence against *Rps1a*; but 5% of the fields surveyed still relied on that gene to manage *Phytophthora* stem and root rot. More troublesome is the fact that *Rps1c* has become almost as obsolete, but was still used in 30% of the fields. This information should serve as a warning to farmers and seed suppliers to alter their selection and introgression of *Rps* genes. In this context, some soybean growers did opt for varieties with other *Rps* such as *Rps1k*, 3a, and 6, which appeared to fare better. It is worrisome to note, though, that almost 70% of the isolates from Manitoba carry virulence for *Rps1k*, and this leaves *Rps3a* and *Rps6* – which are often less frequently available in suppliers’ catalogs – as the sole effective options.

Whereas the effectiveness of *Rps1a* appeared to last nearly 40 years (Schmitthenner 1972; Schwenk and Slim 1974), *Rps* genes deployed in more recent years seem to have had a much shorter lifespan (Dorrance et al. 2016). This reality was highlighted in this study, along with the need to better manage the available sources of resistance. In addition, the development of new molecular tools, such as the assay employed here, allows for much faster, easier, and more precise analysis of evolving pathotypes, providing information that can be exploited in disease management and associated decision-

Table 4. Association between the pathotypes of *Phytophthora sojae* isolates in a soybean field and the *Rps* genes present in the cultivar used in the field for 49 isolates from Manitoba

				Manitoba			
ID	Pathotype	Rps	Expected response	ID	Pathotype	Rps	Expected response
Ps_0585	1a, 1d	–	Disease	Ps_0516	1a, 1c, 1d	1c	Disease
Ps_0586	1a, 6	–	Disease	Ps_0517	1a, 1b, 1c, 1d, 1k	1c	Disease
Ps_0587	1a, 1c, 1d, 1k	–	Disease	Ps_0522	1a, 1b, 1c, 1d, 1k, 6	1c	Disease
Ps_0588	1a, 3a	–	Disease	Ps_0523	1a, 1b, 1c, 1d, 1k	1c	Disease
Ps_0589	1a, 1c, 3a, 6	–	Disease	Ps_0528	1a, 1c	1c	Disease
Ps_0598	1a, 1c	–	Disease	Ps_0529	1a, 1c	1c	Disease
Ps_0599	1a, 3a	–	Disease	Ps_0593	1a, 1c, 1d	1k	Disease
Ps_0600	1a	–	Disease	Ps_0594	1a, 1b, 1c, 1d, 1k	1k	Disease
Ps_0504	1a, 3a, 6	–	Disease	Ps_0518	1a, 1b, 1c, 1d, 1k	1k	Disease
Ps_0505	1a, 1c, 1d	–	Disease	Ps_0519	1b, 1c, 1d, 1k	1k	Disease
Ps_0506	1a, 1c, 1d	–	Disease	Ps_0520	1a, 3a, 6	1k	Resistance
Ps_0510	1a, 1c, 1d	–	Disease	Ps_0524	1a, 1c, 1d	1k	Resistance
Ps_0511	1a, 1c, 1d, 3a, 6	–	Disease	Ps_0525	1a, 1c, 6	1k	Resistance
Ps_0512	1a, 1c, 6	–	Disease	Ps_0526	1a, 6	1k	Resistance
Ps_0513	1a, 1b, 1c, 1d, 1k	–	Disease	Ps_0527	1a, 1b, 1c	1k	Resistance
Ps_0514	1a, 1b, 1c, 1d, 1k	–	Disease	Ps_0530	1a, 1b, 1c, 1d	1k	Resistance
Ps_0590	1a, 1c, 3a, 6	1c	Disease	Ps_0531	1a, 1b, 1c, 1d, 1k, 6	1k	Disease
Ps_0591	1a, 1c, 1d	1c	Disease	Ps_0532	1a, 1b, 1c, 1d, 1k	1k	Disease
Ps_0592	1a, 1c, 1d	1c	Disease	Ps_0595	1a, 1c	1k + 3a	Resistance
Ps_0601	1a, 3a, 6	1c	Resistance	Ps_0521	1a, 1b, 1k	1k + 3a	Resistance
Ps_0602	1a, 1c, 1d	1c	Disease	Ps_0596	1a, 3a, 6	3a	Disease
Ps_0603	1a, 1b, 1c, 1d, 1k	1c	Disease	Ps_0597	1a, 3a, 6	3a	Disease
Ps_0604	1a, 1b, 1c, 1d, 1k	1c	Disease	Ps_0507	1a, 1c, 1d, 3a, 6	3a	Disease
Ps_0605	1a, 1c, 1d	1c	Disease	Ps_0508	1a, 1c, 3a, 6	3a	Disease
Ps_0515	1a, 1c, 1d, 6	1c	Disease				
Total of isolates = 49		Disease = 82%		Resistance = 18%			

Table 5. Comparison of the evolution of relative pathotype diversity of isolates of *Phytophthora sojae* collected in Ontario over three survey periods

Pathotype ^a	% of Isolates		
	2018 to 2019	2010 to 2012 ^b	1980 to 1989 ^c
1a, 7	9.4	12	25.1
1a 1c, 6, 7	1.0	9.0	3.9
1a, 1b, 1c, 7	5.2	0.0	0.0
1a, 1b, 1c, 1d, 7	1.0	0.0	0.0
1a, 1b, 1c, 1d, 1k, 7	22.9	0.0	0.0
1a, 1b, 1c, 1d, 1k, 3a, 6, 7	0.0	0.8	0.0
1a, 1b, 1c, 1k, 7	3.1	14.3	0.0
1a, 1b, 1c, 1k, 3a, 7	0.0	0.6	0.0
1a, 1b, 1c, 1k, 3a, 6, 7	0.0	0.6	0.0
1a, 1b, 1c, 1k, 6, 7	0.0	5.0	0.0
1a, 1b, 1c, 3a, 6, 7	1.0	0.0	0.0
1a, 1b, 1k, 7	4.2	6.7	0.0
1a, 1b, 1k, 3a, 6, 7	0.0	2.2	0.0
1a, 1b, 1k, 6, 7	0.0	1.1	0.0
1a, 1b, 3a, 6, 7	1.0	0.0	0.0
1a, 1b, 6, 7	0.0	0.3	0.0
1a, 1c, 7	8.3	8.7	13.9
1a, 1c, 1d, 7	20.8	0.8	0.0
1a, 1c, 1d, 1k, 3a, 6, 7	1.0	0.0	0.0
1a, 1c, 1d, 3a, 6, 7	1.0	0.0	0.0
1a, 1c, 3a, 7	1.0	0.0	0.0
1a, 1c, 3a, 6, 7	1.0	2.0	0.3
1a, 1d, 7	2.1	0.0	0.0
1a, 1d, 3a, 6, 7	0.0	5.3	0.2
1a, 1d, 6, 7	1.0	5.0	0.2
1a, 1k, 7	0.0	1.1	0.0
1a, 3a, 7	0.0	0.0	0.3
1a, 3a, 6, 7	2.1	6.2	33.6
1a, 6, 7	5.2	9.5	14.4
1b, 1c, 1d, 1k, 3a, 6, 7	1.0	3.1	0.0
1b, 1c, 1d, 3a, 7	1.0	0.0	0.0
1b, 1c, 1k, 7	2.1	0.0	0.0
1b, 1d, 3a, 6, 7	0.0	0.6	0.0
1c, 7	0.0	2.0	0.0
1c, 1d, 7	3.1	0.0	0.0
1c, 6, 7	0.0	0.3	0.0
6, 7	0.0	0.3	1.0
7	0.0	0.8	0.0

^a Data for 2018 to 2019; pathotype 7 was deemed present on all isolates through phenotyping.

^b Data from Xue et al. (2015).

^c Data from Anderson and Buzzell (1992).

making. Thus, soybean growers, breeders, and seed suppliers will be better positioned to make science-based choices in their efforts to curb losses caused by *P. sojae*.

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