Applications of Next-Generation Sequencing for Large-Scale Pathogen Diagnoses in Soybean

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Abstract

Soybean (*Glycine max*) has become an important crop in Manitoba, Canada, with a 10-fold increase in dedicated acreage over the past decade. Given the rapid increase in production, scarce information about foliar diseases present in the province has been recorded. In order to describe the foliar pathogens affecting this legume, we harnessed next-generation sequencing (NGS) to carry out a comprehensive survey across Manitoba in 2016. Fields were sampled during the V2/3 (33 fields) and R6 (70 fields) growth stages, with at least three symptomatic leaves per field collected and subjected to RNA sequencing. We successfully detected several bacteria, fungi, and viruses known to infect soybean, including *Pseudomonas savastanoi* pv. *glycinea*, *Septoria glycines*, and *Peronospora manshurica*, as well as pathogens not previously identified

in the province (e.g., *Pseudomonas syringae* pv. *tabaci*, *Cercospora sojina*, and *Bean yellow mosaic virus*). For some microorganisms, we were able to disentangle the different pathovars present and/or assemble their genome sequence. Since NGS generates data on the entire flora and fauna occupying a leaf sample, we also identified residual pathogens (i.e., pathogens of crops other than soybean) and multiple species of arthropod pests. Finally, the sequence information produced by NGS allowed for the development of polymerase chain reaction-based diagnostics for some of the most widespread and important pathogens. Although there are many benefits of using NGS for large-scale plant pathogen diagnoses, we also discuss some of the limitations of this technology.

Over the course of millions of years of coevolution, microorganisms and plants have developed intricate and often highly specific biological interactions. Although many of these interplays are commensal or beneficial, some have a detrimental consequence on the host plant, inducing diseases and turning the microorganisms into “pathogens” (Ortiz-Castro et al. 2009; Turner et al. 2013). Under certain circumstances, pathogens can cause significant yield losses to economically important crops, and thus represent a significant threat to regional and even global food security. Each year, a myriad of infectious plant diseases are reported worldwide as a result of multiple factors, including environment, genetic recombination, and anthropogenic introduction (Anderson et al. 2004; Stukenbrock and McDonald 2008). This includes recent regional outbreaks that have resulted in significant regional losses to many economically important crops, including corn (De Groote et al. 2016; Mahuku et al. 2015), wheat (Islam et al. 2016), potato (Abdurahman et al. 2017), tomato (Timilsina et al. 2017), and coffee (Cristancho et al. 2012), among many others. Timely detection of these pathogens represents the first and most important step toward effective disease control and management. As such, multiple diagnostic techniques have been developed to aid in the fight against emerging and established plant pathogens in all crops worldwide (Schumann and D’Arcy 2012; Trigiano et al. 2004).

Over the years, technological and scientific advances have improved the accuracy, sensitivity, and efficiency of pathogen identification, providing a broader range of information and tools to lessen the impact of crop disease (Fang and Ramasamy 2015; Ray et al. 2017). Visual assessments of symptom development and traditional

microbiology techniques (e.g., morphology, motility, sporulation, staining, etc.) are commonly used for pathogen diagnosis. However, these approaches are limited by the expertise of the examiner, the time needed to carry out the tests, and the inability to culture some pathogens (McCartney et al. 2003). More recent serological testing (e.g., immunostrips or enzyme-linked immunosorbent assay [ELISA]) has allowed for the automatization of diagnosis to an extent but is still time-consuming, relies on the availability of specific antibodies, and may produce indecisive results (Lima et al. 2012; López et al. 2003; Narayanasamy 2011). The development of polymerase chain reaction (PCR) revolutionized the identification of microorganisms, providing a faster, more sensitive, and specific technique (Dickinson 2003; Henson and French 1993). Several PCR-based methods have been developed and successfully implemented (e.g., amplified fragment length polymorphism, restriction fragment length polymorphism, nested PCR, multiplex PCR, and quantitative PCR), but all require knowledge of the specific nucleotide sequences of the inferred pathogen (Garibyan and Nidhi 2013).

The relatively recent development of massive parallel next-generation sequencing (NGS) technologies has proven invaluable for a wide range of applications across multiple biological disciplines, including plant pathology (Behjati and Tarpey 2013; Studholme et al. 2011; Westermann et al. 2012). Although NGS requires a strong integration of bioinformatics (Conesa et al. 2016), many limitations of the aforementioned approaches for pathogen diagnosis can now be resolved using this technology. Not only does NGS provide superior resolution and sensitivity, but importantly, it also does not rely on previous knowledge of the nucleic acid to be sequenced or require specific antibodies. Several studies have harnessed NGS techniques to expand and enhance the diagnosis of pathogens even before symptoms appear. In virology, NGS has been particularly significant; it has accelerated the identification of viral pathogens in isolated samples of plant tissue and has become sufficiently cost-effective for use in larger-scale disease surveys (Eichmeier et al. 2016; Hadidi et al. 2016; Stewart et al. 2014). Furthermore, NGS has been successfully used to identify viruses infecting soybean plants (Han et al. 2016) and seeds (Groves et al. 2016), as well as viruses infecting their arthropod vectors (Feng et al. 2017). However, its application to other types of pathogenic microorganisms has been limited and has mostly focused on one particular pathogen per study (Chakraborty et al. 2016; Hayden et al. 2014).

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As with all other crops, soybean is affected by multiple abiotic and biotic disorders. Among the biotic issues, foliar diseases are considered a major problem in different parts of the world (Hartman et al. 2015) and cause roughly 52% of yield reductions in the top-eight producing countries (Wrather et al. 2010). Bacterial blight (*Pseudomonas savastanoi* pv. *glycinea*), brown spot (*Septoria glycines*), and *Cercospora* leaf blight (*C. kikuchii*) are among the most common foliar diseases of soybean. Other diseases, such as Asian rust (*Phakopsora pachyrhizi*) and frogeye leaf spot (*C. sojina*), have also caused important yield reductions in different regions around the world (Cruz et al. 2010; Soares et al. 2015; Yorinori et al. 2005).

Materials and Methods

In Manitoba, Canada, soybean has experienced a 10-fold increase of land dedicated to the crop during the last decade, increasing from 215,000 to 2,290,000 acres, and it is forecasted to continue its growth in the upcoming years (Statistics Canada 2018). Given the meteoric rise of soybean production, very scarce information about foliar pathogens is recorded for the province, and diagnoses have largely relied on visual observation and the culturing of microorganisms. Thus, the province represents an excellent study site to carry out an NGS-based comprehensive survey of the foliar pathogens infecting the crop. In 2016, we surveyed 81 soybean fields during the production season (V2/3 and R6 growth stages), uncovering a myriad of foliar disease-causing pathogens, including bacteria, fungi, and viruses. Importantly, some of the pathogens detected were not previously identified in the province. For some pathogens, we were even able to unravel the different pathovars present and/or assemble their genome sequence. Since NGS provides information on the entire flora and fauna inhabiting a given leaf sample, we also identified residual pathogens and arthropod pest and vector species. Finally, the sequence information generated by NGS enabled the development of rapid and cost-efficient PCR-based diagnostics for some of the most prevalent and important pathogens. Although the benefits and

Fig. 1. A representative sample of soybean leaves showing foliar disease symptoms that were collected for pathogen diagnoses by RNA sequencing using the Illumina platform: **A**, large, dark lesions without chlorosis; **B**, irregular and chlorotic lesions widely distributed on leaf; **C**, large, dark lesions with chlorotic edges, associated to main veins; **D**, large green to yellow lesions with necrotic tissue in the center; **E**, leaf showing crinkling and yellowing from edge to center; **F**, irregular and necrotic lesions of various sizes with chlorotic edges; **G**, small, light green spots widely distributed; and **H**, small, irregular, dark lesions with or without chlorotic edges.

Biosystems, Wilmington, MA). The cDNA libraries were then subjected to RNA sequencing in 100-bp paired-end fashion on two lanes of the Illumina HiSeq 2500 platform. The Illumina Analysis Package CASAVA 1.8.2 was used to perform bcl conversion and demultiplexing. Image deconvolution and quality value calculations were carried out using the Illumina GA pipeline (version 1.6). The raw sequence reads are available from the National Center for Biotechnology Information (NCBI) Short Sequence Read Archive under accessions SRR5480332 and SRR5480333 (study SRP105188).

Bioinformatics analysis. Raw reads were imported into CLC Genomics Workbench (version 10.0.1; Qiagen, Hilden, Germany), where adapter indexes and poly(A) tails were trimmed (ambiguous limit = 2, quality limit = 0.05). Reads were then mapped to the *G. max* Glyma2.0 (56,044 genes) reference cDNA database (retrieved using the BioMart tool in Phytozome) using the following parameters: similarity fraction = 0.95 and length fraction = 0.7 (default settings therein). The unmapped reads were assembled de novo using the RNA-SEQ function in CLC and the following parameters: bubble size = 500, similarity fraction = 0.9, and length fraction = 0.65 (default settings therein). Functional annotation of the resultant contigs was carried out using BLASTn against the NCBI nonredundant (nr) database. A minimum *E*-value $\leq 1 \times 10^{-100}$ with >90% sequence similarity and a contig match length >100 bp was required for

positive species diagnosis. In addition, a minimum of 100 reads for the sequences was used as the cut-off value for a confirmed pathogen.

To gain some insights into the efficacy of NGS in resolving bacterial pathogens to their pathovar, the de novo contigs matching to *P. savastanoi* pv. *phaseolicola* were further analyzed using local BLASTn (*E*-value $\leq 1 \times 10^{-100}$), comparing the contigs against a customized *Pseudomonas* database, including deposited genomes of *P. savastanoi* pv. *glycinea* (GenBank accessions AB106910, AB112554, AB491987, AB683862, AF037443, AF061506, AF093246, AF135163, and AF169828; project accessions AEGG00000001, AEGH00000000, LGLL00000000, LGLM00000000, LGLO00000000, LGLP00000000, and LJQL00000000) and *P. savastanoi* pv. *phaseolicola* (GenBank accessions AB237164, AF461559, AJ870974, AY603426, CP000058, CP000059, CP000060, JN655687, JQ365663, KF937496, KT905434, KT905436, KT907498, and NR_076360). These represent all of the genomes available in GenBank for *P. savastanoi* pv. *glycinea* and *P. savastanoi* pv. *phaseolicola* to date and were included regardless of the assembly level (e.g., complete assembly, scaffolds, contigs). The local BLAST was set to present the top-three results of each contig. The analyses were carried out for each survey independently.

Development of molecular diagnostics. NGS provided a wealth of sequence information for pathogens, epiphytes, and endophytes

Fig. 2. Regional distribution of soybean fields surveyed in Manitoba in 2016. Surveys were carried out during the V2/3 (diamond) and R6 (square) growth stages. Fields surveyed during both growth stages are denoted with a diamond overlaying a square.

found in Manitoba, which can be used to design and validate PCR diagnostics in silico for some of the common pathogens. Contig sequences of selected pathogens were manually analyzed for specificity using BLASTn against the nr database. Sequences showing >96% within-species identity and low similarity (<5%) to other species in their genus were used for primer design using Primer3 (Untergasser et al. 2012). The primer sequences are listed in Supplementary Table S1.

For validation of PCR diagnostics, cDNA was synthesized from each field pool of the second survey using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA), following the manufacturer's instructions. PCR was performed on the cDNA using each set of primers. The PCR program used included one step of denaturation at 95°C for 5 min, followed by 35 cycles composed of 1 min at 95°C (denaturation), 30 s at 55°C (annealing), and 30 s at 72°C (extension). A final 5-min extension step was also included at the end of the program. PCR products were visualized on a 1% agarose gel stained with ethidium bromide, using an electrophoresis run of 55 min at 110 V.

PCR products from all different sets of primers were purified using the PureLin Quick PCR Purification Kit (Invitrogen, Lithuania) following the manufacturer's instructions and were later sent to the Centre for Applied Genomics (Toronto, ON, Canada) for Sanger sequencing. The sequences were visualized using FinchTV (version 1.4.0) and then compared in the NCBI nr database using BLASTn to validate the identified pathogens. The specificity of each primer set was further validated on soybean samples positive for the other common pathogens that were subjected to PCR diagnostic development.

Results

NGS as a tool for pathogen identification. Foliar diseases can be very destructive to soybean plants, and control is exceedingly difficult when there is little to no information on the pathogens established in the production areas. To explore the efficacy of NGS in identifying different types of pathogens, 25 cDNA libraries representing >650 symptomatic leaf samples (e.g., necrotic and chlorotic spots, crinkling, regular and irregular lesions, leaf deformation, streaking, specks, discoloration) from 81 Manitoba soybean fields surveyed twice in 2016 (V2/3 and R6 growth stages) were subjected to RNA sequencing. More than 700 million paired-end reads of 100 bp were generated, of which ~11% on average was not of soybean origin. The nonsoybean read subset of each cDNA library was assembled de novo into contigs (see Supplementary Table S2). Each contig was functionally annotated using BLASTn and then classified to the species level (where possible) based on the most significant match to the nr database. Our results show an array of different types of microorganisms associated with symptom development on soybean across Manitoba in both the early and later stages of development.

Pathogens previously reported in Manitoba. Table 1 summarizes the Canadian Plant Disease Surveys (CPDSs) of the last 15 years specific to Manitoba, using visual examination, culturing on culture medium, ELISA, and Agdia ImmunoStrips (Desjardins 2010; Desjardins and Bisht 2011; Desjardins et al. 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2012, 2013, 2014, 2015; Platford and Desjardins 1999, 2000; Platford and Kurtz 1997, 1998; Pradhan et al. 2016, 2017). These surveys were used as baseline data to determine which pathogens were previously reported in Manitoba. Of the 10 foliar diseases reported previously, nine of the causative agents were identified in our survey. This included pathogens of all five diseases found in each of the past 4 years (brown spot, downy mildew, anthracnose, bacterial blight, and *Alternaria* leaf spot), two others that have been sporadically reported (*Cercospora* blight and leaf spot), and two found in only one survey (bacterial pustule, 2003; gray mold, 2010). The only previously reported disease where the causative agent was not detected in our survey was *Soybean mosaic virus* (SMV), which was only found in 2010.

New pathogens to Manitoba. We previously reported two viral pathogens detected in our surveys that were not previously known to infect soybean: *Brome mosaic virus* (BMV; genus *Bromovirus*) (Díaz-Cruz et al. 2018) and *Tobacco necrosis virus* strain D (TNV-D; genus *Betanecrovirus*) (Díaz-Cruz et al. 2017). A group of soybean pathogens that have been reported in other regions but not in the province of Manitoba were also identified (Table 2), including *Bean yellow mosaic virus* (BYMV; genus *Potyvirus*) (Tu 1986), *C. sojina* (frog-eye leaf spot) (Anderson and Tenuta 2001), *Pleospora herbarum* (leaf blight), and *Boeremia exigua* var. *exigua* (leaf spot) (Hartman et al. 2015).

Disentangling the disease-causing species. Previous surveys in Manitoba were based on visual assessment and culturing. Consequently, the putative diseases are reported but the causative agent is rarely identified to the species level. NGS allows us to elucidate this by obtaining multiple sequences and reads that match to specific organisms, provided the genomic resources are available. Among the diseases that have not been reported to the species level is anthracnose, caused by the genus *Colletotrichum*; in the current study, two species previously identified in other regions but not in Manitoba were detected. Similarly, the bacterial genus *Pseudomonas* has not yet been fully identified to the species level in some soybean isolates but in this case, *P. cichorii* (a well-known pathogen) was detected.

In many cases, the sensitive and powerful nature of NGS also allowed for identification of pathogens to the pathovar level. We used *P. savastanoi* as an example of this. Contigs matching *P. savastanoi* pv. *phaseolicola* (syn. *P. syringae* pv. *phaseolicola*) were compared using desktop-downloaded BLASTn against a customized database of nucleotide sequences and genomes of varying completeness for *P. savastanoi* pv. *glycinea* (syn. *P. syringae* pv.

Table 1. Foliar pathogens previously reported in Manitoba soybean, based on Canadian Plant Disease Surveys (2001 to 2015)^a

Disease	Pathogen	Year of report														
		2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015
Brown spot	<i>Septoria glycines</i>		X	X			X		X	X		X	X	X	X	X
Downy mildew	<i>Peronospora manshurica</i>		X	X	X	X		X	X	X	X	X	X	X	X	X
Bacterial pustule	<i>Xanthomonas axonopodis</i> pv. <i>glycines</i>				X											
<i>Cercospora</i> blight	<i>Cercospora kikuchii</i>									X					X	
Leaf spot	<i>Phyllosticta</i> spp.									X	X	X	X			X
Anthracnose	<i>Colletotrichum</i> spp.						X				X	X	X	X	X	X
Gray mold	<i>Botrytis cinerea</i>										X					
Bacterial blight	<i>Pseudomonas</i> sp.	X	X	X	X		X		X	X	X	X	X	X	X	X
Seed discoloration	<i>Soybean mosaic virus</i>													X		
<i>Alternaria</i> leaf spot	<i>A. alternata</i>														X	X

^a Positive years for each pathogen are denoted with an "X" and the disease caused by each pathogen is also shown. With the exception of *Soybean mosaic virus*, all pathogens were found in our 2016 surveys. Sources are as follows: Desjardins (2010), Desjardins and Bisht (2011), Desjardins et al. (2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2012, 2013, 2014, 2015), Platford and Desjardins (1999, 2000), Platford and Kurtz (1997, 1998), and Pradhan et al. (2016, 2017).

glycines) and *P. savastanoi* pv. *phaseolicola*. Focusing on the top-three BLAST hits of contigs across the V2/3 and R6 surveys, 55% had one *P. savastanoi* pv. *glycinea* and two *P. savastanoi* pv. *phaseolicola* matches, whereas 44% had all three matches to *P. savastanoi* pv. *glycinea* (Supplementary Table S3). Other combinations of matches did not exceed 1%.

Nonfoliar soybean pathogens. Most pathogens preferentially infect and spread to specific tissues of the host plant, and they are generally classified as pathogens of the leaf, stem, or root. In several regions of our survey, NGS identified soybean pathogens that are known to predominantly infect tissues other than leaves. Two species within the *Diaporthe* genus, *D. eres* and *D. longicolla* (causal agents for seed and stem diseases), appeared as part of the survey in multiple regions. Interestingly, *Phytophthora sojae* was detected on leaves across all of the regions of study, despite being a root pathogen (Supplementary Table S4).

Residual pathogens. In many cases, foliar pathogens overwinter in the soil and can reinfect next year's crop. Crop rotation helps protect against this, as many pathogens are host specific and cannot successfully invade the foliar tissues of the newly planted crop. Therefore, we explored our contig lists for pathogens known to infect other crops planted in the province but not soybean; this group is referred to hereafter as "residual pathogens" (Supplementary Table S5). The pathogens detected are parasitic predominantly to cereals (e.g., wheat, barley, maize, oat, rye) and brassicas (e.g., canola) but also to other groups such as legumes and trees. Some genera with several species detected included *Bipolaris* (maize) (Manamgoda et al. 2014), *Colletotrichum* (multiple crops) (Cannon et al. 2012), *Leptosphaeria* (canola) (Fernando et al. 2016), *Phaeosphaeria* and *Pyrenophora* (wheat) (Gilbert and Woods 2001), and *Xanthomonas* (canola) (Vicente and Holub 2013).

Arthropods. Many arthropods cause damage to host plants through their feeding behavior, by serving as vectors that spread pathogens plant to plant. Since NGS provides information on the leaf and all of the organisms associated with a given leaf sample, we also identified sequencing matching arthropod pest and vector species in both surveys (Supplementary Table S6). Members of the Lepidoptera order (*Bombyx mori*, *Helicoverpa zea*, *Heliothis virescens*, *Spodoptera exigua*, *S. frugiperda*, *Trichoplusia ni*) were found only during the early survey (presumably coinciding with their wingless larval stages), whereas the member of Homoptera (*Acyrtosiphon pisum*) was present only in the second survey. Species within the orders Thysanoptera (*Frankliniella occidentalis*, *F. tritici*) and Trombidiformes (*Tetranychus pueraricola*, *Tetranychus urticae*) were present in both surveys. At least 13 of the identified arthropod species are known pests of soybean (Abd El-Wahab 2016; Gotoh et al. 2004; Hartman et al. 2015; Keough et al. 2016; Nikolova et al. 2014; Pobozniak 2011; Suekane et al. 2012; Viteri et al. 2010). Overall, the arthropods were found in several locations throughout the province but were predominately found in the southwestern and central regions.

Whole genome sequences. The baseline sequence data generated using NGS are assembled into larger sequences that reflect the mRNA sequences expressed by the pathogens. If the pathogen is a virus with a relatively small RNA genome, it is often possible to generate its complete or near-complete genome. As such, we previously reported the whole genome sequence of TNV-D, which was isolated from a leaf sample collected during our V2/3 survey (Díaz-Cruz et al. 2017). Similarly, the contig sequences for BYMV covered ~92% of the 9.5 Mb genome available in NCBI (GenBank accession KF632713), with ~97% identity. For BMV, the coverage was lower at 41%, but the contigs analyzed included all three RNA segments of the virus with no less than 99% similarity (GenBank accession DQ530423).

Development of molecular diagnostics. Although NGS has proven to be an exceptional tool, it is impractical for use as a routine diagnostic. A major benefit of our approach is that it generated DNA sequence data for strains of pathogens found in Manitoba. We used this information to develop PCR-based diagnostics for seven of the most prevalent and important soybean foliar pathogens present in the province. Each diagnostic was tested and validated on 70 fields from our 2016 survey (Supplementary Table S7). These new tools will allow for fast, sensitive, reliable, and cost-efficient pathogen identification in future provincial surveys and in isolated diagnoses.

Discussion

Similar to other crops, soybean is susceptible to multiple types of pathogens (e.g., bacteria, fungi, viruses, oomycetes), some of which threaten the normal development of plants and could severely impact yield. Many of the traditional approaches for pathogen detection (e.g., visual observation of symptoms, culturing) are not sensitive and/or are prone to misdiagnosis. Newer diagnostic tools (e.g., PCR, ELISA) are more accurate and sensitive but require a priori knowledge of the pathogens present. In this study, we assessed the capabilities of NGS (RNA) in identifying diverse microorganisms inhabiting soybean leaves. More than 650 samples exhibiting foliar symptoms of disease were collected across Manitoba, Canada in June (V2/3) and August (R6) of 2016. As discussed below, we identified bacterial, oomycete, fungal, and viral pathogens, including some microorganisms that were not previously reported in the province. NGS was sensitive enough to uncover the pathovars present for some of our pathogens, assemble complete or near-complete genome sequences of the identified RNA viruses, and provide information on the residual pathogens and arthropod pest and vector species found in the fields. Although many nonpathogenic microorganisms were also detected, it was not the intent of this study to describe them; thus, they were omitted from the data set.

Annual CPDSs identify soybean pathogens in Manitoba based mostly on visual assessment of the disease symptoms and culturing techniques. Consequently, several of the reported causal disease

Table 2. Foliar pathogens identified in Manitoba soybean in 2016 by next-generation sequencing, which were not previously reported in the province^a

			Region										
			NW		SW		N ^b	CN		CS		NE ^b	SE ^b
Type	Pathogen	Disease	V2/3 (n = 2)	R6 (n = 2)	V2/3 (n = 11)	R6 (n = 12)	R6 (n = 2)	V2/3 (n = 3)	V2 (n = 5)	V2/3 (n = 16)	R6 (n = 33)	R6 (n = 11)	R6 (n = 5)
Fungi	<i>Alternaria tenuissima</i>	Leaf and stem spot	–	+	+	+	+	–	+	+	+	+	+
	<i>Cercospora sojae</i>	Frogeye	–	+	+	+	+	–	+	+	+	+	+
	<i>Colletotrichum gloeosporioides</i>	Anthrachnose	–	–	–	–	–	–	–	–	+	–	–
	<i>Colletotrichum graminicola</i>	Anthrachnose	–	–	–	–	–	–	–	–	+	–	–
	<i>Diaporthe eres</i>	Seed decay	–	–	–	–	–	–	–	–	+	+	–
	<i>Pleospora herbarum</i>	Leaf blight	–	–	–	–	–	–	–	+	–	–	–
Bacteria	<i>Pseudomonas cichorii</i>	Leaf spot	–	–	+	–	–	–	–	+	+	+	+
	<i>Pseudomonas syringae</i> pv. <i>tabaci</i>	Wildfire	–	+	+	–	–	+	–	+	+	+	–
Virus	<i>Bean yellow mosaic virus</i>	Mosaic of legumes	–	–	–	+	–	–	–	–	–	–	–

^a The distribution of pathogens is categorized by region (NW = Northwest, SW = Southwest; N = North, CN = Northcentral, CS = Southcentral, NE = Northeast, and SE = Southeast), where a plus sign indicates a positive diagnosis (i.e., pathogen is present) and a minus sign indicates a negative diagnosis. The number of fields surveyed per region is also displayed. V2/3 and R6 represent the growth stage of soybean surveyed.

^b Samples from these regions were taken only during the R6 survey.

agents have only been identified to their genus: bacterial blight (*Pseudomonas* spp.), leaf spot (*Phyllosticta* spp.), and anthracnose (*Colletotrichum* spp.). Application of NGS allowed for unparalleled resolution of the species present in Manitoba that are responsible for these diseases. Indeed, two *Colletotrichum* species (*Colletotrichum gloeosporioides* and *Colletotrichum graminicola*) and two *Pseudomonas* species (*P. savastanoi* and *P. syringae*) appear to contribute to anthracnose and bacterial blight in the province, respectively. Although the CPDS attributed leaf spot to *Phyllosticta* and we did not identify any species in this genus, other soybean pathogens conferring similar disease symptoms were present (e.g., *A. alternata*, *Boeremia exigua*). In particular, the *Boeremia* and *Phyllosticta* genera comprise closely related, morphologically similar *Phoma*-like species (Chen et al. 2015; de Gruyter et al. 2013), which can easily lead to misdiagnosis.

Of the seven pathogens reported by CPDSs to the species level, six were corroborated by NGS; the exception was SMV, which has not been reported in the province since 2013. Plants developing from infected seed serve as the primary inoculum for insect vectors that disseminate the pathogen to healthy plants (Domier et al. 2007; Porto and Hagedorn 1975). Given the relatively high titers of SMV RNA in infected leaves (Díaz-Cruz and Cassone 2018) and the absence of observable disease symptoms corresponding to SMV in our samples, it is probable that SMV was absent from the fields used during our surveys. The most likely explanation for the absence of SMV in this study is perhaps some combination of the use of clean seed in the fields surveyed, low incidence of the disease, and insufficient sampling power. A more targeted study is needed to better infer this.

Although the CPDS makes no formal attempts in identifying bacterial pathogens to their pathovar, we demonstrated with *Pseudomonas* that pathogens can even be delineated to their pathovar if appropriate genomic resources are available and there are regions of the genome showing sufficient genetic differentiation. This information is valuable because different pathovars could induce identical symptoms in infected plants but have a different genetic composition, as a result of recombination and development of different strains (Nowell et al. 2016).

The primary goal of this study was to assess the ability of NGS to detect pathogens not previously reported in Manitoba. We hypothesized that because soybean is a relatively new crop in the province and has dramatically expanded in acres planted, there could be several pathogens that are in the province but have not yet been reported. With sufficient sequencing coverage, NGS provides a snapshot of all the microorganisms inhabiting a given leaf sample. As expected, we found a myriad of pathogens new to Manitoba; these may have already been established in the region but not identified by traditional surveys or may represent new invasions in 2016. Among these were two bacteria of the pseudomonads: *P. cichorii*, reported as a pathogen of soybean only in South Korea (Yu and Lee 2012), and *P. syringae* pv. *tabaci*, causal agent of tobacco wildfire, previously detected in regions of Canada (Anderson and Tenuta 2001). New fungal pathogens include *A. tenuissima*, a pathogen that has more impact on seed germination but can also live on and damage foliar tissue (Jasnić et al. 2011; Larran et al. 2002; Shortt et al. 1982; Thomma 2003). *C. sojae*, causal agent of frogeye leaf spot, was also detected. Previously reported as a minor pathogen in Canada (Wrather and Koenning 2006), *C. sojae* has shown more importance in the United States as a result of fungicide resistance (Zhang et al. 2012). Stemphylium leaf blight, a minor disease caused by *Pleospora herbarum* (Hartman et al. 2015), was identified as well. A report on fungal communities found that *Pleospora herbarum* is part of healthy soybean leaves (Larran et al. 2002), but pathogenic reports are limited (Caudillo-Ruiz et al. 2017). Finally, BYMV (genus *Potyvirus*) was the only newly detected viral pathogen. In soybean, it causes yellow mottling and typical mosaic symptoms (Campos et al. 2014; Golnaraghi et al. 2004). Although not previously reported to infect soybean in Manitoba, BYMV has been found to infect faba bean in the province (Frowd and Bernier 1977).

A particularly interesting finding was that soybean fungal pathogens known to infect nonfoliar tissues were also detected in foliar

tissues. *Phytophthora sojae* is a severe root and stem pathogen (Tyler 2007) and was recovered from most regions. However, under certain conditions, the pathogen may move up to the foliar tissue (Cabrera et al. 2009). *D. eres* and *D. longicolla* are part of a genus containing multiple species infecting soybean (Gomes et al. 2013; Santos et al. 2011; Udayanga et al. 2015) and have been reported to cause seed decay (Petrović et al. 2015) and stem disease, respectively. Future studies are needed to discern whether they are truly invading the tissues or simply attaching onto the surfaces of leaves or are the result of soil contamination, or less likely, misdiagnosis caused by the database used as reference.

Prior to the survey, we had no expectations on the ability of NGS to generate information on residual pathogens or arthropod species. A total of 61 bacterial, fungal, and oomycete microorganisms known to infect crops other than soybean were identified. This included pathogens of other economically important crops in Manitoba, such as canola, wheat, and maize. The presence of residual pathogens in soybean fields is likely attributable to the proximity of other crops, spontaneous plants from previous crop cycles in the same field, pathogens with overwintering capacities (Duczek et al. 1999), as well as weeds, which often represent the main reservoir of pathogens (Wisler and Norris 2005). Pathogenicity tests are required to determine the risk these microorganisms pose to both soybean and other crops, as well as the potential of soybean as an inoculum source. Nonetheless, the activity of certain pathogens in soybean fields may advise growers of crops that may or may not be desirable to plant in the subsequent year. Consequently, there may be benefit to incorporating NGS into crop rotation analysis, to determine the presence of specific pathogens, in similar fashion to the work carried out in this study.

The detection of arthropods in our samples shows that NGS can also extend to pests that cause damage by feeding or spreading pathogens among plants. NGS has been shown to be more accurate, sensitive, and effective than Sanger sequencing for barcoding of arthropods (Shokralla et al. 2015). In addition, the analysis of potential vectors has aided in the detection of plant pathogens (Nouri et al. 2016). We were able to detect sequences belonging to members of four different orders: Homoptera, Lepidoptera, Thysanoptera, and Trombidiformes. The identified organisms that are problematic for soybean include the fall armyworm (*S. frugiperda*) (da Silva et al. 2017; Peruca et al. 2018), five species of genus *Frankliniella* (Pobozniak 2011), and three species in the *Tetranychus* genus (Gotoh et al. 2004; Nikolova et al. 2014; Suekane et al. 2012). Compared with the report of insects affecting soybean in 2016 for the province of Manitoba, there is consistency with the presence of spider mites (*Tetranychus* spp.), whereas our survey did not detect soybean aphid (*Aphis glycines*) or green cloverworm (*Hyena scabra*) (Gavloski 2016).

The amount of short-read sequence data generated by NGS has increased dramatically (Muir et al. 2016; Zhang et al. 2011). It is now possible to produce hundreds of millions of reads in a single sequencing run and even generate complete or near-complete genomes of microorganisms inhabiting different host tissues (Cassone et al. 2015). Previously, we reported the assembly of the viral pathogen TNV-D (Díaz-Cruz et al. 2017), facilitated by the high amount of reads and full coverage compared with other genomes available. In this study, a similar situation was determined for BYMV and BMV, although we could not achieve complete coverage. This technology has been useful to detect and describe novel mycoviruses associated with soybean leaves (Marzano and Domier 2016). In order to improve genome assembly from plant material, a minimum coverage of 30× and different RNA extraction methods have been proposed (Kehoe et al. 2014). Although our study used RNA sequencing, DNA isolation and sequencing would likely result in draft assemblies of bacterial and fungal pathogens.

In addition to genome assemblies, the large volume of sequence information produced by NGS can be filtered to identify genes of specific pathogens that are highly expressed during the infection process. These genes, if conserved, can be targeted for primer development and robust PCR-based diagnostics that are specific to the strains found in a given geographic region. Based on our surveys, our primers were designed and optimized for seven relevant pathogens in

Manitoba that infect soybean. However, implementing the primers designed through NGS in diagnostic clinics would require a more thorough validation of their sensitivity and specificity to the targeted pathogen across its range distribution. Furthermore, primers developed for genomic DNA would likely be more ideal, as this would omit the reverse transcription step.

Although the benefits of using NGS have been discussed, there are several limitations of implementing the technology for large-scale diagnoses. In terms of bacterial or fungal pathogens, NGS can only be used to detect their presence in a given sample. Additional assays are required to isolate the causative organisms and determine whether they are truly pathogenic. The technology also relies on the availability of genomic resources. Although soybean pathology sequence data have been rapidly expanding, many important pathogens do not have complete genome sequences currently available and some have extremely limited resources. In these cases, the diagnoses must be inferred based on the sequences of closely related organisms that have more accessible nucleic acid resources. In our study, the causal agent of downy mildew (*Peronospora manshurica*) had only five nucleotide sequences reported in the NCBI database at the moment of the analysis, but four genomes in the genus *Peronospora* were available. Other examples in our results where genomic resources may play a significant role for the proper identification of pathogens include the *Septoria/Mycosphaerella* group (Crous 2009; Soares et al. 2015; Verkley et al. 2013; Videira et al. 2017), the *Xanthomonas axonopodis* group (Constantin et al. 2016), the *Phomopsis/Diaporthe* complex (Gomes et al. 2013; Mengistu et al. 2014; Nevena et al. 1997; Udayanga et al. 2014, 2015), and *Phoma*-like species (Chen et al. 2015; de Gruyter et al. 2013).

The same limitation applies for pathovar identification. A relatively large number of reads and contigs were assigned to the bacterial pathogen *P. savastanoi* pv. *phaseolicola*, which has minor significance to soybean (Hartman et al. 2015). In contrast, the number of reads and contigs assigned to the more important *P. savastanoi* pv. *glycinea* was much lower. When the BLAST analysis was done for this study, the NCBI nr database had a *P. savastanoi* pv. *phaseolicola* complete genome available, whereas *P. savastanoi* pv. *glycinea* presented only independent genes. A previous study on the *P. savastanoi* pv. *glycinea* genomes determined that *P. savastanoi* pv. *phaseolicola* strain 1448A is the most closely related pseudomonads genome to two *P. savastanoi* pv. *glycinea* genomes, sharing more than 4,500 genes (Qi et al. 2011). In this particular case, the similarity of sequences seems to play a significant role in the outcome of the analysis. Since the group *P. syringae*/P. *savastanoi* is divided into pathovars based on the biological interaction with host plants rather than genomic diversity, the use of NGS is incomplete for identification, requiring other methods of confirmation before a final report is made for each microorganism.

Although NGS generates data on the entire biota inhabiting a host, in most cases the majority of reads are host derived. Our study was no exception, as nearly 90% of reads were of soybean origin. This means that the high-throughput nature of NGS is effectively reduced. This must be taken into consideration in the experimental design, as it may require multiple sequencing runs to achieve the desired resolution. A potential solution is to use prokaryote enrichment kits to facilitate the detection of bacterial pathogens; this was not applicable in our study, given the goal of identifying diverse organisms. In terms of analysis, although the application for plant pathogen detection does not necessarily require the use of a plant reference genome, its availability expedites the de novo assembly and functional annotation processes. Additional issues are related to the database (in our case, NCBI nr) used for diagnosis and include error rates and sequences matching to multiple organisms. The latter is particularly problematic for NGS studies based on internal transcribed spacer (ITS)/16S sequencing. For global RNA sequencing approaches, the bulk of generated sequences are considerably more conserved and assembled into larger contigs, thus providing better resolution at the species level.

Other issues that should be considered for larger-scale application of NGS are the associated costs and expertise required for analysis.

Although NGS costs have declined dramatically and NGS now represents a relatively cost-effective tool, it is still considerably more expensive than more traditional diagnostic approaches and would therefore be difficult to justify implementing NGS into long-term annual surveys. A cost-effective alternative could be to carry out 16S or ITS amplicon sequencing for surveys focused on only fungal or bacteria species. In terms of time, NGS requires multiple molecular approaches, sequencing, and high-throughput analysis, which justifiably take much longer than a PCR or serological test and thus would not be recommended if immediate diagnosis is needed. Finally, most of the freely accessible software used for data analysis requires some skill at computational programming. There is some graphical interface-based software available as used in this study; however, licensing and support fees can be expensive.

In our study, we successfully used NGS to identify multiple foliar microorganisms with pathogenic potential present in soybean across the province of Manitoba, Canada. No prior knowledge of these microorganisms was required for identification, but the accuracy and precision of the method relied on the availability of genomic resources accessed in NCBI. This situation highlights the importance of further research in genomes and transcriptomes of soybean pathogens, to increase the chances of proper identification. In addition, we were able to detect pathogenic microorganisms of crops other than soybean, suggesting that NGS could be used in the analysis of residual pathogens, a major concern for crop rotation strategies. We were also able to detect arthropod sequences, meaning that further studies in the epidemiology of viral diseases could include NGS to confirm the identity of potential vectors. Taken collectively, although NGS is not perfect, it represents a promising option for large-scale disease surveys.

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