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Genetic diversity and aggressiveness of *Fusarium* species isolated from soybean in Alberta, Canada

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ABSTRACT

Soybean (*Glycine max* (L.) Merr.) has excellent potential as an alternative crop to canola in southern Alberta farming systems. However, soybean is susceptible to Fusarium root rot, which usually results in the occurrence of dead or dying plants in mid-to-late summer and severe reductions in yield. A total of 102 isolates identified as *Fusarium* were recovered from diseased soybean root samples collected from central and southern Alberta from 2011 to 2013. Ten species of *Fusarium* were identified, with *F. acuminatum* as the predominant species (31 out of 102 isolates, 30.39%), followed by *F. equiseti* (16.67%), *F. culmorum* (13.73%), *F. avenaceum* (10.78%), *F. oxysporum* (8.82%), *F. redolens* (7.84%), *F. torulosum* (4.90%), *F. tricinctum* (2.94%), *F. commune* (1.96%) and *F. proliferatum* (0.98%). This is the first report of *F. commune*, *F. redolens* and *F. torulosum* causing root rot in soybean in Canada. Phylogenetic analyses based on sequence data from the translation elongation factor 1-α (EF-1α) and the internal transcribed spacer (ITS) region were used to evaluate genetic variation and, in conjunction with morphological characters, for species identification. All of the *Fusarium* isolates were able to infect soybean, although some exhibited varying levels of aggressiveness.

1. Introduction

Soybean [*Glycine* max (L.) Merr.], native to Asia, is a member of the Leguminosae family, and became a commercial oilseed crop in Canada in the late 1920s (Pesticide Risk Reduction Program, 2006; Shurtleff and Aoyagi, 2010). Increased demand for vegetable oil and protein meal during the early 1940s firmly established the crop and by 1950, soybeans had become a major cash crop in Ontario (Pesticide Risk Reduction Program, 2006; Shurtleff and Aoyagi, 2010). While overall Canadian soybean production doubled from 2004 to 2014, soybean production in Manitoba and Saskatchewan has increased six-fold, accounting for 20.6% of Canadian soybean production in 2014 (Pulse Beat, 2015). Since the introduction of shorter season varieties of soybean, production of this crop has become more profitable in Alberta, and soybean acreages also are on the increase in Alberta.

Fusarium root rot is a major disease of soybean in Canada and the United States (Pesticide Risk Reduction Program, 2006; Wrather et al., 2001), and can cause significant yield reductions as a consequence of decreased plant stands, stunted seedlings and weakened root systems (Wrather et al., 2001; Wrather and Koenning, 2006). Around 20 species of *Fusarium* have been associated with root rot of soybean (Broders et al., 2007; Díaz-Arias et al., 2011; Díaz-Arias, 2012; Ellis et al., 2014; Lanubile et al., 2016). *Fusarium solani* (Mart.) Sacc., *F. oxysporum* Schlecht., *F. acuminatum* Ellis & Everhart and *F. graminearum* Schwabe have been reported as major pathogens in the fusarium root rot complex of soybean in North America (Bienapfl et al., 2010; Broders et al., 2007; Díaz-Arias, 2012; Díaz-Arias et al., 2013; Ellis et al., 2014; Nelson, 1999; Zhang et al., 2010). In Canada, eight *Fusarium* species including *F. oxysporum*, *F. graminearum*, *F. solani*, *F. avenaceum* (Fries) Saccardo, *F. tricinctum* (Corda) Saccardo, *F. sporotrichioides* Sherb., *F. equiseti* (Corda) Saccardo and *F. poae* (Peck) Wollenw. were reported to be associated with soybean roots in eastern Ontario (Zhang et al., 2013). Four *Fusarium* species, *F. proliferatum* (Matsushima) Nirenberg, *F. avenaceum*, *F. culmorum* (Smith) Saccardo and *F. oxysporum* were

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identified from infected soybean roots in Alberta and Manitoba (Chang et al., 2013, 2015; McLaren et al., 2014; Nyandoro et al., 2014).

Identification of *Fusarium* to the species level is traditionally accomplished by microscopic examination and the comparison of morphological characters based on the criteria of Leslie and Summerell (2006). However, the concept of species within this genus has varied greatly between broader and narrower concepts. In addition, isolates within a particular species may vary considerably, both morphologically (Leslie and Summerell, 2006; Schneider, 1958) and genetically (Edel et al., 2001). As a result, species identification has been quite difficult. Molecular techniques are a more reliable means for species identification, as well as for the assessment of genetic diversity among *Fusarium* isolates. Studies of the population structure of *Fusarium* spp. using molecular techniques are also useful for clarifying the disease etiology and devising effective management strategies.

Genetic variation detected by molecular approaches at or within the species level have been reported for *Fusarium* spp. from various hosts (Barros et al., 2014; Chen et al., 2014; Kristensen et al., 2005; Stewart et al., 2006; Yli-Mattila et al., 2004). A better understanding of genetic variation within a species is important because this information can affect the strategies that are selected for the development of control methods (McDonald, 1997). Genetic variation and/or *Fusarium* species classification have been assessed by sequence analysis of the internal transcribed spacers (ITS), the translation elongation factor 1-α (EF-1α), the ribosomal intergenic spacer (IGS) region, the β-tubulin gene and some other molecular markers, such as random amplified polymorphic DNA (RAPD), amplification fragment length polymorphism (AFLP), etc. (Barros et al., 2014; Chandra et al., 2011; Schmidt et al., 2004). ITS regions are sequences located in rRNA genes between the 18S and 5.8S rRNA coding regions (ITS1) and between the 5.8S and 28S rRNA coding regions (ITS2) (Porter and Golding, 2011). These spacer sequences are present in high copy numbers in all eukaryotic genomes (Faggian et al., 1998). Therefore, the ITS region can provide valuable marker information in the investigation of phylogenetic relationships. ITS regions have been sequenced in many fungi and are used in phylogenetic studies or for the development of species-specific diagnostic probes (Guarro et al., 1999), and as a universal DNA barcode marker for fungi (Schoch et al., 2012). In the genus *Fusarium*, the ITS regions have been used to study both inter- (O'Donnell and Cigelnik, 1997) and intra-specific (Bateman et al., 1996) variation.

The translation elongation factor 1-α (EF-1α) gene, which encodes an essential part of the protein translation machinery, provides highly useful phylogenetic information at the species level for *Fusarium*, and was used as a marker for resolving inter- and intra-species relationships within the *Fusarium* species complex (Geiser et al., 2004; Kristensen et al., 2005; Stewart et al., 2006).

In order to better understand the importance of soybean root-infecting *Fusarium* species in western Canada, it is critical to identify the *Fusarium* species and study their aggressiveness on soybean roots. In the present study, a total of 102 *Fusarium* isolates were obtained from plants in soybean fields in southern Alberta during the summers of 2011, 2012 and 2013 (Fig. 1). The objectives of this study were to identify these isolates to species level, measure the genetic variation within the fungal populations, and to assess the pathogenicity of the isolates.

2. Materials and methods

2.1. Fungal isolation and purification

Soybean roots showing symptoms of root rot were collected from 29 commercial fields in southern Alberta during the summers of 2011, 2012 and 2013 (Fig. 1, Table 1). Diseased tissue samples were cut into 2–5 mm pieces, and surface-sterilized in 1% NaOCl for 30 s and then washed under running tap water. All of the tissues were placed on fresh Difco™ potato-dextrose agar (PDA) plates (Becton, Dickinson and Company, Sparks, MD USA) for two days at about 22 °C under 12-h lighting, then transferred to peptone-pentachloronitrobenzene (PCNB) medium (Nash and Snyder, 1962) and incubated for 10–14 days under 12-h of lighting at about 22 °C. *Fusarium* cultures were transferred onto fresh PDA plates for further identification. Single-conidium isolates were established and cultured on PDA for 13–15 days at 22 °C under 12-h of lighting and stored at 4 °C for later use (Leslie and Summerell, 2006). The isolates were identified as *Fusarium* spp. based on their colony and morphological characteristics (Leslie and Summerell, 2006).

2.2. DNA extraction, PCR amplification, sequencing and identification of species

All of the isolates were sub-cultured on PDA for 10–14 days at 22 °C under 12-h lighting. Approximately 25 mg of mycelium was collected from a PDA plate of each isolate for fungal genomic DNA extraction using the modified rapid mini-preparation method of Feng et al. (2010b). The DNA concentration and quality was estimated using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer (Fisher Scientific, Nepean, ON, Canada) with the default set for DNA assays.

The ITS regions and the EF-1α gene of the 102 isolates of *Fusarium* were amplified with the primer sets ITS4/ITS5 and EF-1/EF-2 (O'Donnell et al., 1998; White et al., 1990), respectively. Reactions were conducted in a final volume of 25 μL, which contained EconoTaq® Plus 2× Master Mix (Lucigen, Middleton, WI), 0.5 μM of each primer and 10 ng of genomic DNA. The PCR amplification conditions in the thermocycler were set as follows: 5 min at 94 °C followed by 35 cycles of 50 s at 94 °C, 50 s at 55 °C (for EF-1α) or 58 °C (for ITS), and 60 s at 72 °C with a final extension of 10 min at 72 °C. The amplicons were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI). After purification, the amplicons were sequenced at the University of Alberta, Edmonton, AB, Canada. The ITS and EF-1α DNA sequences from all of the isolates were used to search for sequence similarity against the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) using the BLASTN program. Species were identified based using 100% sequence identity as the threshold.

2.3. Phylogenetic analysis

All of the sequences obtained and used for phylogenetic analyses were submitted to GenBank (KU891497-598 and KY659074-175). The sequence characters were weighted equally in all phylogenetic analyses.

Distance trees among these isolates were produced with PAUP 4.0b10 software (Swofford, 2002) using the neighbour-joining (NJ) approach (Saitou and Nei, 1987) after alignment with the Bioedit program v7.2.5 (Hall, 1999). Support for groups in the tree was assessed using a bootstrap analysis with 1000 replicates (Fig. 2 and Fig. 4). Prior to NJ analysis, the best-fit nucleotide substitution model was selected using the program ModelTest 3.7 (Posada and Buckley, 2004).

Bayesian inference (BI) was applied to estimate phylogenetic relationships among isolates using MrBayes 3.2 (Ronquist et al., 2012) with isolate F039 as an outgroup. Prior to Bayesian inference analysis, the same best-fit nucleotide substitution model selected by the program ModelTest 3.7 was used. Bayesian inference was performed with four chains (one cold and three heated under default heating values) in each of two parallel runs, with each chain starting with a random tree, and was subsequently run for 100,000 generations. Trees were sampled every 10 generations. Based on the trees (42,002 for EF-1α and 165,002 for ITS) sampled, a consensus tree of EF-1α and ITS (Fig. 3 and Fig. 5) was calculated after omitting the first 250 trees as burn in. All phylogenetic trees were graphically edited with TreeView v1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) to produce the figures.