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First report of *Fusarium proliferatum* causing root rot in soybean (*Glycine max* L.) in Canada

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ABSTRACT

Soybean plants showing symptoms of root rot were collected from fields in western Canada to determine the etiology of the disease. Four *Fusarium* spp., *Fusarium avenaceum*, *Fusarium culmorum*, *Fusarium oxysporum* and *Fusarium proliferatum*, were identified based on their cultural and morphological characteristics. All of the isolates of these species were pathogenic on soybean. Identification of *F. proliferatum* was confirmed by PCR analysis with the *F. proliferatum*-specific primer set CLPRO1/CLPRO2. Amplicons of the target fragments (partial calmodulin (cld) gene, 526 bp) were obtained only from DNA of isolates tentatively identified as *F. proliferatum*, and sequencing of the amplicon showed it shared 100% identity with the cld gene sequences of *F. proliferatum* in GenBank. *F. proliferatum* was the most aggressive of the four *Fusarium* species identified, causing the greatest root rot severity and reduction of seedling emergence. *F. avenaceum* was the second most aggressive and caused a greater reduction in seedling numbers than *F. culmorum* or *F. oxysporum*. Correlation analysis showed that seedling emergence, shoot dry weight, and seed yield decreased, and root rot severity increased, with the log of inoculum density of *F. proliferatum*. This is the first report of *F. proliferatum* causing soybean root rot in Canada.

1. Introduction

Root rot is a serious disease of soybean in Canada and effective root rot management is not yet available. *Fusarium* species were the predominant pathogens isolated from infected soybean roots collected in 2011 from one field in Manitoba, Canada and four fields in Alberta, Canada (Chang et al., 2013b; Nyandoro et al., 2014; McLaren et al., 2014). In eastern Ontario, Canada, eight *Fusarium* species including *Fusarium oxysporum* (Schlecht.) Snyder & Hansen, *Fusarium graminearum* Schwabe, *Fusarium solani* (Mart.) Sacc., *Fusarium avenaceum* (Corda ex Fr.) Sacc., *Fusarium tricinctum* (Corda) Sacc., *Fusarium sporotrichioides* Sherb., *Fusarium equiseti* (Corda) Sacc., and *Fusarium poae* (Peck) Wollenw. were reported to be associated with soybean roots (Zhang et al., 2013). Root rot disease is more common and severe when soybean is seeded into cold soils (Scherm and Yang, 1996). For this reason, Fusarium root rot may become more prevalent on the prairies than it is in Ontario. According to previous studies, *F. solani* and *F. oxysporum* are major causes of soybean root rot in North America (Nelson, 1999; Zhang et al., 2010). In Argentina, *F. graminearum* was also pathogenic on soybean, causing pre-emergence damping-off, reduced seedling height, and abnormal seedlings (Barros et al., 2014). The first report of *Fusarium proliferatum* (Matsushima) Nirenberg as a cause of soybean root rot was described in the USA by Díaz Arias et al. (2011). In Canada, *F. proliferatum* has been described as a pathogen of stalk rots and ear rots of maize (Bailey et al., 2003), but no previous reports have documented it as a cause of soybean root rot.

In southern Alberta, the area seeded to soybeans has grown from a few hectares to about 2400 ha in 2010 (Patrick Fabian, personal communication). Production is expected to increase as new cultivars with early maturity and cold resistance become available. In Manitoba, soybean acreage increased by 23% from 2011 to 2012 (Desjardins et al., 2012).

Fusarium root rot results in poor emergence, stunted seedlings, weak plant growth, and yield losses in soybean (Nelson, 1999). Losses of about 177,000 tons (1.8%) per year occurred in the United

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States during 2003–2005 (Wrather and Koenning, 2006). In Canada, yield losses due to Fusarium root rot of soybean were estimated to be over 7300 metric tons (2.5%) in 1998 (Wrather et al., 2001).

Species identification in the genus *Fusarium* has been based on morphological characters (Wollenweber and Reinking, 1935). Isolates within a species vary both morphologically (Leslie and Summerell, 2006; Schneider, 1958) and genetically (Edel et al., 2001; Feng et al., 2010a), which has caused difficulties in species identification (Leslie and Summerell, 2006). Therefore, molecular techniques have become the norm for species identification, assessment of genetic diversity among isolates, and even disease etiology. Some *Fusarium* species can be identified via polymerase chain reaction (PCR) analysis with specific primer sets (Demeke et al., 2005; Doohan et al., 1998; Turner et al., 1998).

The objectives of the current study were to identify and evaluate the pathogenicity of selected isolates of *Fusarium* spp. collected from roots of soybean with root rot symptoms from commercial fields in southern Alberta and Manitoba, and to examine the effect of inoculum density of *F. proliferatum* on seedling emergence, shoot weight, seed yield and root rot disease severity.

2. Materials and methods

2.1. Fungal isolation and purification

Approximately 300 samples of plants with root rot symptoms were collected from four soybean fields in southern Alberta, Canada during the summer of 2011, and 600 samples were collected from 40 fields in Manitoba, Canada in 2012. The diseased samples were surface-sterilized in 1% NaOCl for 30 s and washed under running tap water. All of the tissues were placed on fresh potato-dextrose agar (PDA) dishes (Difco™, Becton, Dickinson and Company, Sparks, MD) for 2 days at room temperature with a 12-h photoperiod, then transferred to peptone-pentachloronitrobenzene (PCNB) medium (Nash and Snyder, 1962) and incubated for 10–14 days. Based on colony characteristics, 334 putative *Fusarium* isolates were identified. They were transferred onto fresh PDA, then single-spore sub-cultures were made, cultured on PDA for 13–15 days at room temperature and identified based on colony and morphological characteristics (Leslie and Summerell, 2006). The single-spore isolates were stored at 4 °C for later use.

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

Prior to DNA extraction, each isolate was subcultured on PDA for 10–14 days at room temperature. Approximately 25 mg of mycelium of each isolate was collected for genomic DNA extraction using the modified rapid mini-preparation method of Feng et al. (2010b). DNA concentration and quality were estimated using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer (Fisher Scientific, Nepean, ON, Canada).

PCR analysis using the *F. proliferatum*-specific primer pair CLPRO1/CLPRO2 was used to assess the identity of 16 isolates with colony morphology similar to *F. proliferatum* (Mulè et al., 2004). The primer set consisted of a forward primer CLPRO1 (5′- TGCATCAGA CCACTCAAATCCT -3′), and a reverse primer CLPRO2 (5′- TGTCAGTA ACTCGACGTTGTTGTT -3′). Reactions were conducted in a final volume of 25 μL, which contained EconoTaq® Plus 2× Master Mix (Lucigen, Middleton, WI), 0.5 mM 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 at 94 °C for 50 s, 50 s at 55 °C, and 60 s at 72 °C with a final extension of 7 min at 72 °C. The amplicons were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI). The amplicons were mixed with a Safe-Green loading dye (Applied Biological Materials, Richmond BC) and separated on a 1.2% agarose gel in 1 × Tris-acetate-EDTA (TAE) buffer at 100 V for 1.2 h. The gels were visualized under UV light with a Gel DocTM XR System (BIO-RAD, Columbus OH). After purification, the amplicons were sequenced at the University of Alberta, Edmonton, AB, Canada. The DNA sequences from each 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 batch BLAST program hosted at Greengene (http://greengene.uml.edu/programs/NCBI_Blast.html).

2.3. Symptoms on soybean seedlings

Eight isolates, including three isolates each of *F. avenaceum* and *F. oxysporum*, and one isolate each of *Fusarium culmorum and F. proliferatum*, were tested for their pathogenicity on soybean cultivar 'TH29002RR' (Pioneer Hi-Bred, Chatham, ON), a widely grown cultivar in Alberta, which is susceptible to *Fusarium* spp. For each single-spore-derived isolate, two 8-mm-diameter agar plugs of live mycelium were cut and transferred onto two separate PDA dishes and incubated at room temperature under 12-h ambient room lighting for 13–15 days. The two PDA dishes, when fully covered with mycelium (about 30 mL of propagule solution in total for each isolate), were cut into pieces of approximately 1–2 mm^2 and suspended in about 90 mL deionized water. A 20-mL aliquot of the suspension was added evenly to the surface of 9-cm-diameter cups filled with 300 mL of sterilized Promix PGX soil (Evergro Canada Inc., Delta, BC). Seven soybean seeds were sown on the top of each cup, covered with 40 mL sterilized Promix PGX potting mix, and grown at about 25 °C and 12-h photoperiod in a greenhouse. Two dishes of PDA without fungal colonization were used for the negative controls. For each isolate, six replications (cups) were inoculated. The cups were arranged in a randomized complete block (RCB) design. The experiment was repeated once under the same greenhouse conditions.

Data on seedling emergence and plant height was recorded 12 days after seeding. At 20 days after seeding, root rot lesions were observed on the basal stem. The root of each soybean plant was collected, washed under running tap water, and assessed for the presence and severity of root rot symptoms on a 0–4 scale according to Zhou et al. (2014), where: 0 = no symptoms, 1 = mild symptoms (discoloration but no visible lesions), 2 = obvious lesions, 3 = severe lesions on the stem and diminished plant vigor, and 4 = stem rotten, plant dead (Fig. 1). The shoots growing in each cup were bulked, dried in an oven at 37 °C for 2 days, and then weighed.

2.4. Inoculum density

Inoculum of *F. proliferatum* was produced on a wheat grain medium following the procedure described by Hwang et al. (2014). After soaking in tap water overnight, 1L of whole wheat was put in an autoclavable bag and autoclaved for 90 min. The wheat grains were inoculated with agar plugs from a 14- to 15-day-old potato dextrose agar culture of *F. proliferatum*. The inoculated wheat was incubated in darkness at room temperature for 5 weeks to allow colonization of the kernels. The inoculum was then air dried at 25 °C for 3 days, ground, and sieved to obtain particle sizes between 250 and 1000 μm. Polar® 415- mL cups (Polar Pak., Montreal, QC) were filled with a steam-sterilized (121°C for 2 h) mixture (2:1, v:v) of soil-less mix (Promix PGX) and natural loam soil collected from a field near the Crop Diversification Centre–North research station in Edmonton, Alberta. This mixture was selected to optimize the growth of soybean seedlings. The inoculum was mixed with the soil mixture to produce the desired inoculum density. The