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SPECIES OF *FUSARIUM* CAUSING ROOT ROT OF SOYBEAN IN SOUTH DAKOTA: CHARACTERIZATION, PATHOGENICITY, AND INTERACTION WITH *HETERODERA GLYCINES*

BY

PAUL NYAWANDA OKELLO

A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Plant Science

South Dakota State University

2019

SPECIES OF *FUSARIUM* CAUSING ROOT ROT OF SOYBEAN IN SOUTH DAKOTA: CHARACTERIZATION, PATHOGENICITY, AND INTERACTION WITH *HETERODERA GLYCINES*

PAUL NYAWANDA OKELLO

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Plant Science degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this dissertation does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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DEDICATION

To my dear dad Meshack, and late loving mum Judith for their unending love for education, and giving me the wings to fly, roots to go back to, and reasons to remain hopeful...

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ABBREVIATIONS

^{0}C = degree celsius

ANOVA = analysis of variance

ATS = anova type statistics

DAP = days after planting

df = degree of freedom

EF1F = elongation factor 1 forward

EF1R = elongation factor 1 reverse

HG = *Heterodera glycines*

LSD = least significant difference

MG = maturity group

mm = millimeters

nparLD = non-parametric longitudinal data

PDA = potato dextrose agar

SCN = soybean cyst nematode

SRES = seed research equipment solution

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ABSTRACT

SPECIES OF *FUSARIUM* CAUSING ROOT ROT OF SOYBEAN IN SOUTH DAKOTA: CHARACTERIZATION, PATHOGENICITY, AND INTERACTION WITH *HETERODERA GYLCINES*

PAUL NYAWANDA OKELLO

2019

In South Dakota, *Fusarium*-associated diseases (e.g. seedling diseases, root rot and sudden death syndrome) are emerging threats to soybean production. Several species of *Fusarium* have been reported to cause these diseases in the soybean producing regions of the United States and in the world. However, little information is available on the species of *Fusarium* causing soybean root rot in South Dakota and their pathogenicity. Therefore, the objectives of this study were to (1) characterize the species of *Fusarium* causing soybean root rot in South Dakota; (2) evaluate the cross-pathogenicity of species of *Fusarium* causing root rot of soybean and corn; (3) screen soybean germplasm for resistance to *F.graminearum* under the greenhouse conditions; (4) determine the interaction of *F.graminearum* and *F.proliferatum* with *Heterodera glycines* (soybean cyst nematode, SCN) on soybean roots in the greenhouse; and (5) determine the effect of soil nutrients on the association of *F.virguliforme* and *F.proliferatum* with *H.glycines* under both greenhouse and field conditions.

In 2014, a survey of 200 commercial soybean fields across 22 counties in South Dakota was conducted during the reproductive growth stages of the soybean development. From the roots of the diseased plants sampled, 11 species of *Fusarium* were identified

using morphology and molecular techniques. Among the 11 species of *Fusarium*, *F.graminearum* (51%) followed by *F.acuminatum* (30%) were the most frequently recovered, while *F.virguliforme*, *F.solani*, *F.equiseti-incarnatum complex*, *F.commune,* and *F.subglutinans* were among the least frequently recovered (< 2%). Fifty-seven isolates were arbitrarily selected from a total of 1130 isolates that represented the 11 species of *Fusarium* to test for their pathogenicity on soybean. It was determined that isolates of *F.oxysporum*, *F.armeniacum*, and *F.commune* caused the greatest root rot severity on soybean plants in the greenhouse. In addition, there were significant differences in root rot severity caused among the 57 isolates on soybean when compared to the non-inoculated control plants and among the isolates within *F.acuminatum*, *F.oxysporum*, *F.proliferatum*, and *F.solani.* Results of the cross-pathogenicity among *Fusarium* isolates causing root rot of soybean and corn in South Dakota showed that there were significant differences among individual isolates and the non-inoculated control on both crops. Two *F.proliferatum* isolates and one *F.graminearum* isolate from corn caused significantly greater root rot severity compared to the others and the non-inoculated on soybean and corn.

Soybean germplasm screening for resistance to *F.graminearum*, revealed eight accessions that were significantly less susceptible to the fungus, and may be used as potential sources of resistance in breeding programs to develop soybean cultivars with resistance to root rot caused by *F.graminearum*. A greenhouse experiment on the association of *F.graminearum* and *F.proliferatum* with *H.glycines* detected no synergic interaction between the fungus and the nematode. The root rot severity caused by the *F.graminearum* and *F.proliferatum* isolates did not significantly increase in the presence of *H.glycines*. The presence of *F.graminearum* did not affect the nematode egg counts

when compared to *H.glycines* only treatment. However, the presence of *F.proliferatum* reduced the reproduction of *H.glycines* on soybean roots though not statistically significant.

To study the effect of soil nutrients on the association of *F.proliferatum* and *F.virguliforme* with *H.glycines* in the field, two rates of N-P-K fertilizers (15:15:15 and 50:80:110) were used on SCN susceptible and SCN resistant soybean varieties. The root rot severity caused by the isolates of *F.virguliforme* and *F.proliferatum* did not increase with either N-P-K rates application. At harvest, the number of SCN egg count per 100 cc of soil was higher (>9000 per 100 cc of soil) in SCN susceptible plots compared with SCN resistant variety irrespective of the N-P-K fertilizer rate application. The highest soybean yields were obtained from plots with SCN resistant soybean variety with application of starter N-P-K fertilizer rate.

Overall, the collective findings from this study indicate that the isolates representing the 11 species of *Fusarium* were pathogenic on soybean in South Dakota. In fields with a history of Fusarium root rot, soybean growers are recommended to adopt proactive management strategies to minimize the impact of the disease, which includes use of fungicide seed treatments, well-drained planting beds, tillage practices, and resistant cultivars (if available).

CHAPTER 1

Literature review

Soybean: An Overview

Soybean [*Glycine max* (L.) Merr.] is a leguminous field crop of the family Fabaceae (Phaseoleae) with origins in Asia. It was introduced in Georgia in the United States in 1765 (Hartman et al. 2011; Hartman et al 2015; Hymowitz and Harlan 1983), and currently accounts for about 90 percent of the U.S. oilseed production (USDA-ERS, https://www.ers.usda.gov/topics/crops/soybeans-oil-crops/). In the world, the United States (31%), Brazil (31%), Argentina (19%), China (5%) and India (4%) are the leading soybean producers (Hartman et al. 2015). In the United States, soybean is the second most cultivated field crop after corn, with 123.6 metric tons produced in 2018 (NASS. https://www.nass.usda.gov).

South Dakota ranks among the top 10 states for soybean production in the USA (Hartman et al. 2015). In the 2018 growing session, 5.58 million acres of soybean were harvested in the state (NASS. https://www.nass.usda.gov). The South Dakota soybean producers mostly plant the crop in rotation with corn (The South Dakota Cropping Systems Inventory, USDA-NRCS/SD, 2017). The crop is typically planted between May and early June and harvested from late September to October. A wide range of soybean maturity groups (MG = 0 to III) is recommended for South Dakota (Hall et al. 2012; Zhang et al. 2007).

Threats to soybean production

Several studies have shown that diseases limit soybean production in the United States (Allen et al. 2017; Diaz Arias et al. 2003; Koenning and Wrather 2010; Wrather et al. 1997; Wrather and Koenning 2009; 2006; 2003; Xing and Westphal 2006). The extent of economic plant damage as a result of disease is attributed to the pathogen or pest type, environmental conditions, susceptibility of the cultivar, other stress factors in the field, and plant growth stage (Hartman and Hill 2010; Hartman et al. 2011). Other factors limiting soybean production are caused by abiotic factors such as environmental conditions, soil nutrient availability, soil salinity, and response to photoperiod (Hartman et al. 2011). While, farming practices may be used to manage abiotic or biotic constraints, losses from few factors such as drought, flooding, and frost, may be difficult to manage.

Fusarium as plant pathogen

Species of *Fusarium* are important plant pathogens in the world that cause diseases in a wide range of host plants (Leslie and Summerell 2006; Leslie et al. 1990; Okello and Mathew 2019; Vicente 2014). These fungi are commonly recovered from the soil, roots and aerial plant tissues, crop residues and other organic substrates (Allen et al. 2017; Aoki et al. 2003; Bilgi et al. 2008; Broders et al. 2007; Diaz Arias 2013; Hartman et al. 2015; Kaur 2016; Mueller et al. 2016; Nelson et al. 1983; Newson and Martin 1953; Okello and Mathew 2019; Parikh et al. 2018). Species of *Fusarium* cause diseases such as crown rot, stalk rot, head blight, and scab on cereal and grains; vascular wilts on a wide range of horticultural crops such as tomato (*Solanum lycopersicum* Linnaeus); root rots in dry edible beans (*Phaseolus vulgaris* Linnaeus), peanuts (*Arachis hypogaea* Linnaeus), soybean, and asparagus (*Asparagus officinalis* Linnaeus); and also cankers, and other diseases (Diaz

Arias 2012; Leslie and Summerell 2006; Vicente 2014). Historically, some of the greatest social impacts of *Fusarium* has been the disease epidemic caused by *F.oxysporum* f. sp *cubense* on banana (Ploetz 2000) and by *F.graminearum* Schwabe on wheat (*Triticum aestivum* Linnaeus) and barley (*Hordeum vulgare* Linnaeus) (Goswami and Kistler 2004; McMullen et al. 1997; Stack 1999).

Fusarium-associated diseases in soybean

Multiple species of *Fusarium* cause diseases of soybean such as Fusarium blight/wilt (caused by *F.oxysporum* Schlechtend.), sudden death syndrome (SDS, caused by *F.virguliforme* O'Donnell and Aoki) and, Fusarium root rot and seedling disease (caused by several species of *Fusarium*) (Bienapfl 2011; Broders et al. 2007; Diaz Arias et al. 2013; Ellis et al. 2013; Ellis 2011; Hartman et al. 2015; Malvick 2018; Okello and Mathew 2019; Wrather 2006; Xue et al. 2007; Zhang et al. 2013; Zhang et al. 2010). In this document, we are focusing on Fusarium root rot of soybean.

Fusarium root rot of soybean

Fusarium root rot is a common disease that occurs in many soybean production areas in the U.S. At least 22 species of *Fusarium* have been isolated from roots of soybean plants (Hartman et al. 2015). In soybean production areas of the United States and Canada, *F.acuminatum* Ellis and Everhart, *F.graminearum*, *F.oxysporum* and *F.solani* (Martius) Appel and Wollenweber emend. Snyder and Hansen are among the most frequently recovered. The other species of *Fusarium* associated with root rot of soybean include *F.armeniacum* (Forbes, Windels and Burgess) Burgess and Summerell, *F.avenaceum* (Fries) Saccardo, *F.chlamydosporum* Wollenw. and Reinking, *F. equiseti* (Corda) Saccardo, *F.poae* (Peck) Wollenweber, *F.proliferatum* Matsushima,

F.pseudograminearum O'Donnell and Aoki, *F.redolens* Wollenweber, *F.semitectum* Berkeley and Ravenel, *F.sporotrichioides* Sherbakoff, *F.subglutinans* Wollenweber and Reinking, *F.tricinctum* (Corda) Saccardo, *F.verticillioides* (Sacc.) Nirenberg, and *F.virguliforme* (Broders et al. 2007; Díaz Arias et al. 2013; Malvick 2018; Nelson 1999).

Plants infected with Fusarium root rot exhibit poor or slow emergence. The symptoms are restricted to the roots and lower stems characterized by brown to dark discoloration. Under severe root infections, above ground symptoms such as stunting, leaf chlorosis, wilting and defoliation are developed.

Infection by species of *Fusarium* causing root rot is favored by cool temperatures and moist soil conditions early in the season, and dry conditions later in the season when soil moisture is limiting (Malvick 2018). Other factors that influence Fusarium root rot infection includes soybean cyst nematode (SCN), soil compaction, crop rotation history, soil pH and soil type (Malvick 2018; Nelson et al. 1997).

Disease cycle and epidemiology

Species of *Fusarium* can survive in the soil and plant debris as chlamydospores or mycelium for a long period of time (Gordon and Okamoto 1990; Smith and Snyder 1975). These pathogens may infect roots of soybean seedlings soon after planting and throughout the growing season. The fungus may enter the host plant by directly penetrating the host's epidermis, through natural openings or wounds and get into the root system to colonize the root cortex (Beckman and Roberts 1995; Nelson et al 1997). Once inside the root system, fungus spreads into the vascular bundle and occupy the xylem affecting host water and nutrient uptake.

Management of Fusarium root rot

Several management options are recommended to minimize the impact of Fusarium root rot.

Crop rotation: Crop rotation to non-host plants is recommended for fields with history of Fusarium root rot as this will help reduce the inoculum of species of *Fusarium*.

Seed treatment: Seed treatment is recommended for fields with a history of Fusarium root rot. Seeds treated with fludioxonil and *Bacillus subtilis* (Ehrenberg) Cohn in the greenhouse have been observed to reduce damage caused by Fusarium root rot (Broders et al. 2007; Zhang et al. 2009).

Cultural practices: These includes planting soybean seeds in well-drained soils, minimizing soil compaction, and plant stress caused by SCN, herbicides and iron deficiency (Hartman et al. 2015; Malvick 2018).

Host resistance: Ultimately, the use of resistant cultivars would be the most economical approach for managing Fusarium root rot. However, currently there are no commercial cultivars available with resistance to Fusarium root rot. Therefore, there is need to screen soybean germplasm to identify potential sources as parental materials for breeding commercial resistant cultivars.

Planting date: Early planting increases the risks of soybean infection. When planted in cool, wet soils, soybean seedlings are more susceptible to infection by species of *Fusarium*.

Interaction of species of *Fusarium* with biotic factors

Species of *Fusarium* are hypothesized to interact with biotic factors such as soybean cyst nematode. Among the species of *Fusarium* whose interaction with SCN have been studied extensively is *F.virguliforme*. Several studies have reported on the synergistic

interaction between *H.glycines* and *F.virguliforme* (Lawrence et al. 1988; McLean and Lawrence 1995, 1993; Melgar et al. 1994; Roy et al. 1989; Xing and Westphal 2006). For example, in the presence of SCN under greenhouse conditions, soybean seedlings inoculated with *F.virguliforme* have been observed to cause greater SDS foliar symptoms compared to seedlings inoculated only with the fungus (Lawrence et al. 1988; McLean and Lawrence 1995, 1993; Roy et al. 1989). Similarly, in the field, SDS foliar symptoms are observed to be more severe on soybean plants in plots infested with both *F.virguliforme* and SCN when compared to those plots inoculated with the fungus only (McLean and Lawrence 1993; Xing and Westphal 2006). In addition, the study by McLean and Lawrence (1993) observed that soybean yields were suppressed in plots where both *F.virguliforme* and SCN were present compared to where only the fungus was.

SCN has also been reported to interact with other plant pathogens and pests. For example, Tabor et al. (2003) observed that SCN can increase the incidence and severity of brown stem rot of soybean caused by *Cadophora gregata* (Allington and Chamberlain) Harrington and McNew [syn. *Phialophora gregata* (Allington and Chamberlain) Gams] on soybean cultivars with resistance to either of the pathogens under growth chamber conditions. In a greenhouse study on the interaction between *Phytophthora sojae* Kaufmann and Gerdemann (the causal agent of *Phytophthora* root and stem rot of soybean) and SCN on soybean, Adeniji et al. (1975) reported that seedlings of cv. Corsoy (susceptible to *P. sojae* and SCN) and cv. Dyer (susceptible to *P. sojae* and resistant to SCN) had a higher disease rating in the presence of the two pathogens as compared to when the seedlings were inoculated with only *P. sojae*. In the interaction between SCN and plant pests, McCarville et al. (2014), observed that the feeding by soybean aphid (*Aphis glycines*

Matsumura) improves the quality of soybean cultivar with resistance to SCN as a host for SCN and therefore increased SCN reproduction but decreased SCN reproduction on the susceptinle soybean cultivar at 30 days after infestation in a greenhouse experiment.

Interaction of species of *Fusarium* with abiotic factors (soil fertility)

Abiotic factors involving the soil components such as soil nutrients can play a significant role in the development of Fusarium root rot (Malvick 2018). Soil organic amendments can induce suppression of soil borne pathogens (Hoitink and Fahy 1986; Lazarovits et al. 2005). However, application of soil amendment can also increase disease incidence by creating within the soil an enabling environment for disease development. For example, Bonanomi et al. (2007) observed that organic matter amendments were suppressive in 45% of the studied cases; however, no significant changes in disease incidence were observed in 35% and in 20 % of the cases. Therefore, organic amendments incorporated to the soil can either suppress or create a conducive environment to plant pathogens by affecting soil biological, chemical and physical characteristics.

Sanogo and Yang (2001) reported that the sources of potassium (K) and phosphorus (P) fertilizer had an effect on sudden death syndrome development in soybean in a growth chamber study. The application of K and P fertilizer from potassium nitrate (KNO_3), potassium phosphate (K_2PO_4) and potassium sulfate (K_2SO_4) caused an increase in SDS severity by 45%, 32% and 43% respectively, while the addition of K from potassium chloride (KCL) decreased the severity of SDS by 36%.

In a comparable study to Sanogo and Yang (2001), Elmer 1989 examined the effect of nitrogen form on growth of asparagus (*Asparagus officinalis* Linnaeus) infected by *Fusarium oxysporum* Schlechtend and observed that potassium nitrate provided a more

conducive environment for disease and fungal root colonization than other nitrogen forms (e.g. calcium nitrate). Howard et al. (1992) reported that SDS incidence and severity reduced with application of potassium chlroide but increased with application of potassium sulfate.

Research justification

In South Dakota, a survey of 200 commercial soybean fields across 22 soybean-producing counties was conducted in 2014 and 11 species of *Fusarium* were recovered from diseased soybean roots. These surveyed commercial fields had a corn-soybean rotation history, and in a few of these fields, SCN and species of *Fusarium* were observed to coexist within the root rhizosphere, suggesting possibility of an interaction between the two pathogens. Therefore, it is important to advance our knowledge to gain a clearer understanding of the importance of species of *Fusarium* on soybean in South Dakota in order to provide growers with management practices that minimize the impact of root rot on soybean yield. The specific objectives of this study were to: (1) characterize the species of *Fusarium* causing soybean root rot in South Dakota; (2) evaluate the cross-pathogenicity of species of *Fusarium* causing root rot of soybean and corn; (3) screen soybean germplasm for resistance to *F.graminearum* in the greenhouse; (4) determine the interaction of *F.graminearum* and *F.proliferatum* with *H.glycines* on soybean roots in the greenhouse; and (5) determine the effect of soil nutrients on the association of *F.virguliforme* and *F.proliferatum* with *H.glycines* under both greenhouse and field conditions.

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CHAPTER 2

Characterize species of *Fusarium* causing root rot of soybean (*Glycine max* L.) in South Dakota

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Abstract

Fusarium root rot of soybean [*Glycine max* (L.) Merr.] has become a concern in the North Central United States. In 2014, soybean plants with diseased roots were sampled at the reproductive stages of soybean development from 200 commercial fields in South Dakota. In total, 1130 isolates of *Fusarium* were recovered and 11 species were identified. Fifty-seven isolates were arbitrarily selected and evaluated for their aggressiveness on 'Asgrow 1835' using the inoculum layer method in the greenhouse. At 14 days after inoculation, root rot severity caused by the isolates was assessed on a scale 1 to 5 and expressed as relative treatment effects (RTE). Isolates of *F.oxysporum* (nine isolates), *F.armeniacum* (one), and *F.commune* (one) caused significantly higher RTE compared to the control while one *F.acuminatum* isolate caused the least RTE when compared to the other isolates but significantly higher than the non-inoculated control. To identify sources of resistance, 21 accessions were screened using one isolate each of *F.graminearum*, *F.proliferatum*, *F.sporotrichioides*, and *F.subglutinans* and two checks ('Williams 82' and 'Asgrow 1835'). While PI361090 was significantly less susceptible to *F.graminearum*, *F.proliferatum* and *F.subglutinans* isolates compared to the checks, PI578386 was significantly less susceptible to *F.proliferatum* when compared to Williams 82. However, all accession were significantly susceptible to the *F.sporotrichioides* isolate. These

findings suggest that the 11 species of *Fusarium* are pathogenic on soybean in South Dakota, and the two accessions (PI361090 and PI578386) may be useful sources of resistance to the three pathogens for breeding programs.

Introduction

Root rot of soybean [*Glycine max* (L.) Merr.] is caused by several species of *Fusarium* in different production areas of the United States including Iowa, Ohio, Minnesota, Nebraska, and North Dakota (Aoki et al. 2003; Bienapfl et al. 2010; Broders et al. 2007; Diaz Arias et al. 2013a;2013b; Parikh et al. 2018). However, the species of *Fusarium* colonizing the root of soybean plants may either be the primary pathogen or one of the various fungi causing disease along with other pathogens such as species of *Phytophthora*, *Pythium*, and *Rhizoctonia*. Regardless, the symptoms caused by various root rotting pathogens including species of *Fusarium* on the root of soybean plants may be similar; for example, the infected tap and/or lateral roots are rotted and brown to black in color. Although the exact environmental conditions favoring Fusarium root rot of soybean are unclear, it is known that the disease is more severe under cool, wet weather conditions, particularly early in the growing season (Nelson et al. 1997). However, the pathogens also cause infection later in the growing season when the soil moisture becomes more limiting and soybean plants become stressed. Between 1994 and 2010, the average soybean yield losses from Fusarium root rot in the United States were estimated at 6.63 million bushels/year (Wrather and Koenning 2011).

Although root rot caused by species of *Fusarium* has been known as a common disease of soybean for a long time, management options for soybean producers are limited. For example, soybean producers are recommended to plant seeds in well-drained soils and

minimize soil compaction to reduce the favorable conditions for infection by species of *Fusarium*. Additionally, it is also recommended that stress and injury to plants caused by soybean cyst nematode, herbicides, iron deficiency, and other factors be minimized to reduce Fusarium root rot (Malvick 2018). Fungicidal seed treatments are recommended for fields with a history of root rot problems. The potential of biological control agents to manage Fusarium root rot have been investigated *in vitro*. For example, Zhang et al. (2010) evaluated the antagonistic activity of *Bacillus subtilis* Cohn strains on *F.oxysporum* Schlechtendal and *F.graminearum* Schwabe in the greenhouse and concluded that eight *B. subtilis* strains had the ability to protect soybean plants against the two pathogens when used as a seed treatment. In addition to the above management options, the use of resistant cultivars would be an economical approach for managing Fusarium root rot. However, there are limited or no commercial soybean cultivars available with resistance to any species of *Fusarium* [except for *F.virguliforme* O'Donnell and Aoki, the causal agent of sudden death syndrome (Zhang et al. 2015)] among the early maturity soybean varieties.

In the North Central United States, soybean researchers have indicated an increasing concern about Fusarium root rot causing poor root health based on field observations. Hence, in the U. S. state of Iowa, surveys were undertaken in 2007, 2008 and 2009 to characterize the prevalence and frequency of species of *Fusarium* associated with soybean root rot and 15 species were identified. While *F.acuminatum* Ellis and Everhart, *F.graminearum*, *F.oxysporum*, and *F.solani* (Martius) Appel and Wollenweber emend. Snyder and Hansen were the most commonly recovered fungi from the soybean roots, *F.virguliforme* was less frequently found (Díaz Arias et al. 2013a). In Minnesota, it was reported that there are species such as *F.graminearum*, *F. pseudograminearum* O'Donnell

and Aoki, *F. redolens* Wollenweber, *F.sporotrichioides* Sherbakoff and *F.proliferatum* (Matsushima) Nirenberg that are pathogenic (Bienapfl 2011). In South Dakota, soybean producers use corn-soybean or wheat-soybean rotation in combination with reduced-tillage or no-tillage practices, and while such practices provide numerous benefits, they can favor survival of species of *Fusarium* on crop residues (Ranzi et al. 2017). Although Fusarium root rot is a disease of wheat (Kaur 2016) and corn (Okello et al. 2019a) in South Dakota, there is no information about the species of *Fusarium* associated with root rot of soybean in the state. Therefore, the objectives of this study were to (1) identify the species of *Fusarium* causing soybean root rot in South Dakota; (2) determine the aggressiveness of isolates of *Fusarium* on soybean in the greenhouse; and (3) screen soybean accessions for resistance to *F.graminearum*, *F.proliferatum*, *F.sporotrichioides* and *F.subglutinans* under greenhouse conditions.

Materials and Methods

Survey, Isolation and Identification of *Fusarium* species

In 2014, soybean plants with diseased roots (e.g. no secondary roots, reduced roots) were arbitrarily sampled from a total of 200 commercial fields in 22 counties in eastern South Dakota, USA, where at least 70% of the soybean production takes place in the state. The plants were uprooted with care to maintain the entire root system and soil shaken off the roots before collection. These plants were arbitrarily sampled along five transects (50 m) and covering an area of 0.5 hectares in each field. The fields were arbitrarily selected and from each field, 10 plants were collected between the reproductive growth stages R1 (beginning flowering) and R5 (beginning seed) (Fehr et al. 1971) following rain in the months of August and September. The sampling was done at the soybean reproductive

growth stages to allow recovery of *F.virguliforme*, which is most prevalent during the reproductive growth stages of soybean (Cummings et al. 2018; Rupe and Gbur 1995; Ziems et al. 2006). The sampled fields had either corn, soybean or wheat as the previous crop (Table 2.1).

The sampled plant roots were brought to the laboratory and rinsed under running tap water for 2 to 5 min to remove soil particles and were observed to have light brown to black discoloration. Three long pieces (~1 cm) were cut from the diseased taproot and surface-disinfested in sodium hypochlorite (0.05%) and ethanol (70%) for 2 min each. The surface-disinfested root pieces were rinsed in autoclaved distilled water and blotted dry on sterile paper towels. These root pieces were plated on petri-dishes containing potato dextrose agar (PDA) amended with 0.02% streptomycin sulfate and incubated at 23 ± 2°C for seven days. Hyphal tips of putative *Fusarium* colonies were transferred to fresh PDA plates amended with 0.02% streptomycin sulfate to obtain pure cultures, which were later transferred to carnation leaf agar (CLA), incubated at 23 ± 2°C for seven days and identified based on morphological characteristics (Leslie and Summerell 2006).

In total, 1130 isolates were identified to species of *Fusarium* based on cultural (pigmentation) and morphological (microconidia and macroconidia production, shape, size, and septation) characteristics on PDA and CLA respectively (Leslie and Summerell 2006). To confirm the species identity, a subset of 57 *Fusarium* isolates representative of the morphological groups determined were selected arbitrarily for amplification and sequencing of the translation elongation factor 1-alpha (EF-1α) gene region. Briefly, DNA was extracted from the lyophilized mycelium of each of the 57 isolates using a FastDNA Spin Kit (MP Biomedicals, Solon, OH, USA). Following extraction, the translation

elongation factor 1-alpha (EF-1α) gene of the 58 isolates was amplified using the primers EF1F/EF1R (Geiser et al. 2004). Reactions for polymerase chain reaction (PCR) amplifications were performed in a 25-µl mixture containing 2.0 µl of fungal DNA (10 ng/µl), 0.75 µl forward primer (10.0 µM), 0.75 µl reverse primer (10.0 µM), 12.5 µl of 2x Taq PCR Master Mix containing Taq DNA Polymerase (Qiagen, Valencia, CA), and 9.0 µl of sterile nuclease-free water. The cycle parameters involved an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min (O'Donnell et al. 1998). A 5-µl aliquot of each PCR product was run electrophoretically on a 1% agarose gel stained with GelRed Nucleic Acid Gel Stain (Biotium, Inc., Hayward, CA, USA) at a final concentration of 0.25× to confirm amplification. DNA samples of the 58 isolates were sequenced (GenScript USA Inc., Piscataway, NJ) using the primers EF1F/EF1R (Geiser et al. 2004). The DNA sequences were subjected to BLASTN searches in the National Center for Biotechnology Information database (NCBI; http://www.ncbi.nlm.nih.gov/). The sequences of the 57 isolates generated in this study are deposited in NCBI under accession numbers MH822030 to MH822086 (Table 2.1).

To further confirm the identity of the 57 isolates, phylogenetic analysis of the EF-1α was performed. The EF-1α sequences of the isolates were adjusted manually and aligned with type sequences [generated by Chilvers and Brown-Rytlewski (2010), Obanor et al. (2010), Watanabe et al. (2011), Yli-Mattila et al. (2011), Funnell-Harris et al. (2015), Garibaldi et al. (2015, 2017), Stefanczyk et al. (2016), and Zhou et al. (2018)] and the outgroup sequences [*Neonectria ramulariae* CBS 151.29 (Accession number JF735791) and *N. ditissima* CBS 226.31 (Accession number JF735783)] using the default parameters

in ClustalW in Molecular Evolutionary Genetics Analysis (MEGA) software (v7; Kumar et al. 2016). Using the aligned EF-1α sequences, the maximum parsimony phylogeny was estimated using the heuristic search option and Tree-Bisection-Regrafting (TBR) algorithm in MEGA. In addition, tree length, consistency index (CI), retention index (RI), and rescaled consistency index (RC) were calculated for the EF-1α tree. Gaps were treated as missing data, and 1,000 replications were used to estimate bootstrap support in the maximum parsimony tree.

Aggressiveness of *Fusarium* isolates

For the aggressiveness study, the 57 isolates (Table 2.1) confirmed by morphology and phylogenetic analyses were used in a greenhouse experiment using Asgrow 1835, a cultivar with unknown resistance to *Fusarium* and the protocol of Bilgi et al. (2008). To prepare the inoculum, the 57 isolates were cultured on fresh PDA plates and incubated for 14 days at 23 ± 2°C. Five mycelial plugs (~15 mm square) of each isolate was transferred into 250 ml Erlenmeyer flask containing previously autoclaved sand-corn meal mixture (54 g of play sand, 6 g of cornmeal, and 10 ml distilled water) and incubated at 23 ± 2°C for 14 days. The conical flasks were kept at 23 ± 2°C for seven days and mixed every two days with an autoclaved spatula to allow the fungus to grow throughout the contents of the flask. At planting, 40 g of coarse dry vermiculite was added into 473 ml plastic drinking cups with three punched holes at the bottom, followed by 20 g of fungal inoculum, and then 20 g of vermiculite. In each cup, three seeds of 'Asgrow 1835' (Resistance to *Fusarium* is unknown; former Monsanto Co., St. Louis, MO, USA) were planted, and covered with a thin layer of vermiculite (approximately 20 g). The non-inoculated control plants were grown in vermiculite with a layer of autoclaved sand-cornmeal mix without

the fungus. For each isolate and the non-inoculated control, three cups (replicates) containing three plants (experimental units) were arranged in a complete randomized design and placed in trays that were kept on the greenhouse bench. A cycle of 12 h of light (light intensity of 450 $\mu Em^{-2}s^{-1}$) and 12 h of darkness was maintained in the greenhouse, with day and night temperatures of 22 ± 2°C. The cups were watered daily as need and no fertilizer was added. The experiment was performed twice.

At 14 days after inoculation, the plants were gently removed from cups and vermiculite was washed off the roots. Each of the plants in the cup was rated for root rot severity caused by the isolates. Disease severity was rated on a 1- to -5 scale (Acharya et al. 2015), where, 1 = germination and healthy seedlings with no visible root colonization, 2 = germination and 1 to 19% of the root having lesions, 3 = germination and 20 to 74% of the root having lesions, 4 = germination and 75% or more of the root having lesions; and 5 = no germination and complete colonization of seed. To confirm infection by the isolates and fulfill Koch's postulates, roots of inoculated soybean plants were selected at random to re-isolate the fungus.

The median root rot severity was calculated for the three plants in a cup. Since the median root rot severity was ordinal, the data were analyzed using the nonparametric method (Shah and Madden 2004). Prior to non-parametric analyses, homogeneity of variance was tested using the Fligner-Killen test (Conover et al. 1981) in R (R Core Team 2013) and satisfied. The data from the two experiments were combined for analysis (after no interaction between experiment run and disease severity was detected) and the analysis of variance type test statistics (ATS) was performed using the nparLD package (Noguchi et al. 2012) in R (R Core Team 2013). For each isolate, the rank was calculated as "$\overline{R}_i$ =

$\frac{1}{n_i}\sum_{k=1}^{n_i} R_{ik}$ (Akritas 1991), where $\overline{R}_i$ = the mean rank for the ith treatment, and R_{ik} = the rank of X_{ik} among all N observations" (Shah and Madden 2004).The root rot severity caused by each isolate was expressed as relative treatment effects (RTE), and RTE was calculated from mean ranks ($\overline{R}_i$) as $\widehat{p_i} = \frac{1}{N}(\overline{R}_i - \frac{1}{2})$ [where N = the total number of observations (Shah and Madden 2004)], and compared at 95% confidence intervals using the nparLD package.

Screening of soybean accessions for resistance

Four isolates - *F.graminearum* (FUS052), *F.proliferatum* (FUS026), *F.sporotrichioides* (FUS002) and *F.subglutinans* (FUS035) - were used to evaluate 21 accessions for resistance to these fungi in separate greenhouse experiments. These species were selected because they were either frequently isolated from diseased soybean roots in South Dakota (*F.graminearum*) or under-studied pathogens of soybean anywhere the crop is grown (*F.proliferatum*, *F.sporotrichioides*, and *F.subglutinans*). For the aggressiveness study, single isolates of *F.sporotrichioides* and *F.subglutinans* confirmed by molecular identification were used. As for *F.graminearum* and *F.proliferatum*, significant differences in aggressiveness were not observed among isolates within these species and their isolates were arbitrarily selected after confirmation of their identity by sequencing the EF-1α gene. As for the accessions, considering soybean varieties belonging to maturity group I (MG-I) are popular in the upper Midwest, seeds of MG-I accessions were obtained from Iowa State University, Ames, Iowa, USA. These accessions originated from a total of seven countries (Austria, China, Georgia, Japan, Oman, Russian Federation, and Ukraine). Two soybean cultivars 'Asgrow 1835' and 'Williams 82' were used as control checks. The inoculum of

the four isolates was prepared, and the greenhouse experiments were established using the protocol of Bilgi et al. (2008) as described previously.

For each variety-isolate combination, there were two cups (replications) with three plants (experimental units) each and these cups were arranged in a completely randomized design. The greenhouse temperature was maintained at 22 ± 2°C with 12 h photoperiod. The plants were lightly watered once daily, and no fertilizer was added during the experiments. At 14 days after inoculation, disease severity caused by the isolates on the roots of the soybean plants was evaluated using a 1- to -5 rating scale (Acharya et al. 2015). The experiments were performed twice for each variety-isolate combination. The median disease rating for the three plants in a cup was calculated, and ATS was performed separately for each isolate using the nparLD package in R as described previously.

Results

Survey, Isolation and Identification of *Fusarium* species

In total, 1130 isolates were recovered from a total of 2000 plants sampled from commercial fields in South Dakota. Eleven species of *Fusarium* (*F.acuminatum* =30.0%, *F.armeniacum* =3.4%, *F.commune* = 0.1%, *F.equiseti-incarnatum complex* = 0.4%, *F.graminearum* = 51.0%, *F.oxysporum* = 8.0%, *F.proliferatum* =2.0%, *F.solani* =1.2%, *F.sporotrichioides* =2.4%, *F.subglutinans* = 0.1%, and *F.virguliforme* =1.6%) were identified using morphology.

Fusarium acuminatum: Three hundred and thirty-nine of the 1130 isolates were identified as *F.acuminatum* based on the characteristics of producing blood-red pigmentation on PDA and on CLA, long tapering apical cell, curved and slender macroconidia in abundance with three to five septate (*n*=100; 8.0 to 11.0 x 0.7 to 1.2 μm); and no observed microconidia

but with chlamydospores formed in chains. Of the 339 isolates, the EF-1α gene region of 13 isolates was sequenced. A BLASTN search of GenBank performed for the EF-1α sequence of 13 isolates showed the best match was *F.acuminatum* strain D22-2 (Accession # KY365595) with identity of 100% (FUS009, FUS010, FUS022, FUS023, FUS038, FUS041, FUS042, FUS043, FUS045, FUS046, FUS047 and FUS051) and 99% (FUS037).

Fusarium armeniacum: Thirty-eight of the 1130 isolates were identified as *F.armeniacum* Forbes, Windels and Burgess. The colonies on PDA produced white aerial mycelium and reddish-orange sporodochia in the center of the culture. On CLA, macroconidia (*n*=100; 1.4 to 2.0 x 0.6 to 0.8 μm) in orange sporodochia on carnation leaves and chlamydospores formed abundantly, but microconidia were absent. Of the 38 isolates, the EF-1α gene region of three isolates was sequenced. A BLASTN search of GenBank performed for the EF-1α sequence of FUS018, FUS021 and FUS049 showed the best match was *F.armeniacum* strain NRRL 6227 (Accession # HM744692) with an identity of 100%.

Fusarium commune: One isolate was identified as *F.commune* Skovg., O'Donnell and Nirenberg (Skovgaard et al. 2003). On PDA, the colonies had fluffy aerial mycelium with magenta to violet pigmentation. The macroconidia (*n*=100; 2.6 to 7.0 x 0.6 to 0.8 μm) were three septate with slightly curved apical cell and the chlamydospores formed singly. The microconidia (*n*=10; 1.2 to 2.2 x 0.5 to 0.7 μm) were produced in abundance in false heads on monophialides or polyphialides, and were oval in shape with no septate. The EF-1α gene region of the *F.commune* isolate identified was sequenced. A BLASTN search of GenBank performed for the EF-1α sequence showed the best match was *F.commune* strain F022 (Accession # KY659116) with an identity of 100%.

Fusarium equiseti-incarnatum complex: Four isolates were identified as *F. equiseti* (Corda) Saccardo. The PDA colonies were abundant with whitish aerial mycelium that turned brownish with age. The macroconidia (*n*=100; 7.0 to 21.4 x 0.3 to 0.6 µm) had 5 to 6 septa and were long, slender and whip-like. Microconidia were absent but chlamydospores were produced in hyphae and appeared either singly or in chains. Of the four isolates, the EF-1α gene region of the two isolates was sequenced. A BLASTN search of GenBank performed for the EF-1α sequence of FUS030 and FUS048 showed the best match was *F. incarnatum* strain kw58 (Accession # KY509036) with identity of 100% and was considered to belong to the *Fusarium equiseti-incarnatum* complex.

Fusarium graminearum: Five hundred and seventy-six of the 1130 isolates were identified as *F.graminearum* characterized by rapid colony growth on PDA, which was pale orange to yellow in color. The macroconidia (*n* = 100; 5.8 to 11.1 x 0.6 to 1.1 µm) were observed to have five to six septate, slightly curved and generally slender. There were no observed microconidia and the chlamydospores were produced singly. Of the 576 isolates, the EF-1α gene region of the 15 isolates was sequenced. A BLASTN search of GenBank performed for the EF-1α sequence of 15 isolates showed the best match was strains of *F.graminearum*: (1) strain clone spt002 (Accession # JF270169) with identity of 100% (FUS001, FUS003, FUS004, FUS006, FUS007, FUS044, FUS050, and FUS056) and 99% (FUS008, FUS052, FUS054 and FUS057); and (2) strain ATCC 60309 (Accession # GU370498) with identity of 100% (FUS039, FUS040 and FUS055).

Fusarium oxysporum: Ninety isolates were identified as *F.oxysporum* observed to produce a white to pale violet pigmentation on PDA while on CLA the macroconidia (*n*=100; 3.3 to 7.4 x 0.7 to 1.2 µm) were sparse, of medium length and had three septate. The

microconidia (*n*=100; 1.3 to 2.1 x 0.5 to 0.7 µm) were observed in abundance formed on false heads on short monophialides. Of the 90 isolates, the EF-1α gene region of 11 isolates was sequenced. A BLASTN search of GenBank performed for the EF-1α sequence of 11 isolates showed the best match was strains of *F.oxysporum*: (1) strain F67 (Accession # EU091065) with identity of 100% (FUS005, FUS015, FUS016, FUS017, FUS019, FUS027, FUS029, and FUS033) and 99% (FUS013); (2) strain IT22 (Accession # KY563701) with identity of 99% (FUS024); and (3) strain DB14DIC06M1 (Accession # KT149291) with identity of 99% (FUS036).

Fusarium proliferatum: Twenty-three of the 1130 isolates were identified as *F.proliferatum* which produced abundant aerial white mycelium that turned violet to dark purple in pigmentation on PDA. On CLA, the microconidia (*n* = 100; 1.6 to 2.5 x 0.6 to 0.7 µm) appeared in abundance, single-celled, oval in shape with no septate and in chains on both monophialides and polyphialides. The macroconidia (*n* = 100; 6.2 to 7.7 x 0.8 to 1.2 µm) had three-to-five septate and were slender with curved apical. Of the 23 isolates, the EF-1α gene region of the four isolates was sequenced. A BLASTN search of GenBank performed for the EF-1α sequence of FUS014, FUS025, FUS026 and FUS058 showed the best match was *F.proliferatum* strain M05-1891S-1_DCPA (Accession # KM462975) with identity of 100%.

Fusarium solani species complex: Thirteen of the 1130 isolates recovered from the soybean roots were identified as *F.solani*. These isolates produced green sporodochia and abundant macroconidia (*n*=100; 4.5 to 6.2 x 0.8 to 1.2 µm) with five to seven septate. The microconidia (*n*=100; 1.4 to 3.0 x 0.6 to 2.6 µm) were oval in shape with zero to one septate. Of the 13 isolates, the EF-1α gene region of the five isolates was sequenced. A

BLASTN search of GenBank performed for the EF1-α sequence of five isolates showed the best match was strains of *F.solani* species complex: (1) strain N305 (Accession # KP400707) with identity of 100% (FUS011, FUS028, FUS031 and FUS053); and (2) strain CH-3P (Accession # KY486699) with identity of 99% (FUS012).

Fusarium sporotrichioides: Twenty-seven of the 1130 isolates matched descriptive characters of *F.sporotrichioides* with fast growing colonies, orange sporodochia and, between three to five septate macroconidia (*n*=100; 4.3 to 6.5 x 0.5 to 1.0 µm) that were moderately curved. The microconidia (*n*=100; 0.7 to 2.2 x 0.4 to 0.6 µm) were oval-shaped and abundantly produced. Of the 27 isolates, the EF-1α gene region of one isolate was sequenced. A BLASTN search of GenBank performed for the EF1-α sequence of FUS002 showed the best match was *F.sporotrichioides* strain NRRL 53434/2616/11 (Accession # FJ768703) with identity of 100%.

Fusarium subglutinans: One isolate was identified as *F.subglutinans* Wollenweber and Reinking observed to produce violet mycelial pigmentation that initially were white. The macroconidia (*n*=100; 6.2 to 10.8 x 0.5 to 0.7 µm) were produced in abundance and were generally slender and thin-walled with curved apical cell. The microconidia (*n*=100; 0.6 to 2.3 x 0.3 to 0.5 µm) were oval shaped, non-septate and produced in abundance. The EF-1α gene region of the one isolate identified as *F.subglutinans* was sequenced. A BLASTN search of GenBank performed for the EF-1α sequence of FUS035 showed the best match was *F.subglutinans* strain OS15 (Accession # JX867945) with identity of 100%.

Fusarium virguliforme: Eighteen isolates were identified as *F.virguliforme* that produced green sporodochia and abundant macroconidia (*n*=100; 4.2 to 6.2 x 0.6 to 1.2 µm) with three to seven septate. The microconidia (*n*=100; 1.4 to 2.0 x 0.6 to 0.8 µm) were oval in

shape with zero to one septate. Of the 18 isolates, the EF-1α gene region of the one isolate was sequenced. A BLASTN search of GenBank performed for the EF-1α sequence of isolate FUS020 showed the best match was *F.virguliforme* strain DAOM23740 (Accession # EF512023) with identity of 100%.

For the EF-1α tree, out of 1030 aligned characters, 336 were parsimony-informative characters, which was included in the maximum parsimony analyses and resulted in 10 most parsimonious trees. The parsimony-informative sites had a consistency index of 0.68, retention index of 0.96, and a composite index of 0.64. The EF-1α based-phylogeny grouped the 57 isolates in 11 well-supported clades (bootstrap value = 95 to 99%) that included type sequences of the 11 species previously identified by BLASTN (Figure 2.1).

Aggressiveness of *Fusarium* species

The homogeneity of variance between the two experimental runs was satisfied using the Fligner-Killen test (P = 0.34).

A significant effect of RTEs (ATS = 14.3; df = 4.2; $P = 2.7 \times 10^{-12}$) caused by the treatments was observed on soybean seedlings at 14 days after inoculation. All the isolates caused root rot of soybean seedlings at 14 days' post-inoculation and there were no visual differences in the symptoms caused by the isolates. Significant differences in RTE caused by the 57 isolates on soybean roots were observed (Table 2.2). No discoloration was observed on the roots of the control plants.

Among the isolates, *F.oxysporum* isolates FUS016, FUS017, FUS024, FUS029, FUS033, and FUS036, and *F.armeniacum* isolate FUS018 caused the highest RTEs, which was significantly different from 42 other isolates and the non-inoculated control. *Fusarium*

acuminatum isolate FUS045 caused the least RTE, which was significantly different from the non-inoculated control (Table 2.2).

Significant differences in aggressiveness were observed among isolates within *F.acuminatum*, *F.oxysporum*, *F.proliferatum*, and *F.solani*. For example, *F.acuminatum* isolate FUS010 caused RTE that was significantly higher than that caused by ten other *F.acuminatum* isolates (FUS038, FUS09, FUS041, FUS042, FUS022, FUS037, FUS047, FUS043, FUS046, and FUS045). The RTEs caused by *F.oxysporum* isolates FUS016, FUS017, FUS024, FUS029, FUS033, and FUS036 were significantly higher than that caused by FUS027, FUS013, and FUS005. *Fusarium proliferatum* isolate FUS058 caused RTE that was significantly higher than FUS025. Similarly, *F.solani* isolate FUS012 caused RTE that was significantly higher than FUS028, FUS011, and FUS053 (Table 2.2).

Screening of soybean accessions for resistance

The assumptions of homogeneity of variance between the two experimental repeats was satisfied using the Fligner-Killen test (*F.graminearum* isolate FUS052 - P = 0.47; *F.proliferatum* isolate FUS026 -P = 0.34; *F.sporotrichioides* isolate FUS002 - P = 1.00, and *F.subglutinans* isolate FUS035 - P = 0.30).

For *F.graminearum* isolate FUS052, a significant effect of RTEs (ATS = 3.3; df = 2.0; P = 0.04) caused by the fungi was observed on seedlings of the 23 accessions at 14 days after inoculation. Based on 95% confidence intervals, PI361090 was significantly less susceptible when compared with Williams 82 and Asgrow 1835 (Table 2.3).

For *F.proliferatum* isolate FUS026, a significant effect of RTEs (ATS = 3.2; df = 2.6; P = 0.03) caused by the fungi was observed on the 23 cultivars at 14 days after inoculation. Two accessions (PI361090 and PI578386) were significantly less susceptible

when compared to Williams 82 and one accession (PI361090) was significantly less susceptible than Asgrow 1835 (Table 2.3).

For *F.sporotrichioides* isolate FUS002, no significant effect of RTEs (ATS = 1.0; df= 1.8; *P* = 0.37) caused by the fungi was observed on the 21 accessions and the checks at 14 days after inoculation (Table 2.3).

For *F.subglutinans* isolate FUS035, a significant effect of RTEs (ATS = 3.1; df = 4.2; *P* = 0.01) caused by the fungi was observed on seedlings of the 22 accessions and Asgrow 1835 at 14 days after inoculation. One accession (PI361090) was significantly less susceptible than the two checks (Table 2.3).

Among the 21 accessions, PI361090 was observed to be significantly less susceptible to isolates of *F.graminearum*, *F.proliferatum* and *F.subglutinans* when compared to Williams 82 and Asgrow 1835 (Table 2.3).

Discussion

Eleven species of *Fusarium*, *F.acuminatum*, *F.armeniacum*, *F.commune*, *F.equiseti-incarnatum* complex, *F.graminearum, F.oxysporum, F.proliferatum*, *F.sporotrichioides*, *F.solani*, *F.subglutinans*, and *F.virguliforme* were identified causing soybean root rot in South Dakota. Six *F.oxysporum* isolates (FUS033, FUS017, FUS024, FUS029, FUS036, and FUS016), and one *F.armeniacum* isolate FUS018 caused significantly higher RTE when compared to the non-inoculated control, while *F.acuminatum* isolate FUS045 caused the lowest RTE when compared to the other *Fusarium* isolates, but was significantly higher than the non-inoculated control. One soybean accession, PI361090 was significantly less susceptible to the isolates of *F.graminearum* and *F.subglutinans* compared to the checks Asgrow 1835 and Williams

82. Two accessions, PI361090 and PI578386 were significantly less susceptible to *F.proliferatum* isolate compared to the two checks, and Williams 82 respectively. However, for *F.sporotrichioides*, no accession was observed to be significantly less susceptible compared to the checks. Our results also indicate that PI361090 and PI578386 may be promising parental materials for development of soybean cultivar with resistance to one or multiple species of *Fusarium*.

In this study, *F.graminearum* (51%) was the most frequently recovered fungus followed by *F.acuminatum* (30%), while *F.virguliforme*, *F.solani*, *F.equiseti-incarnatum complex, F.commune,* and *F.subglutinans* were among the less frequently recovered (< 2%). Our findings of *F.graminearum* as the most frequently recovered fungus is not surprising and is consistent with the studies from the states neighboring South Dakota, which includes Iowa (Diaz Arias et al. 2013a), Minnesota (Bienapfl 2011; French and Kennedy 1963), Nebraska (Parikh et al. 2018) and North Dakota (Nelson and Windels 1992). First, *F.graminearum* is frequently isolated from plants sampled in the reproductive stage of soybean development based on previous studies (Diaz Arias et al. 2013a; Zhang et al. 2013). For our study, sampling of diseased soybean plants was conducted between the reproductive growth stages R1 and R5 (Fehr et al. 1971) during the survey. Second, *F.graminearum* is an important pathogen of corn (*Zea mays* L.) (Carter et al. 2002; Leslie et al. 1990) and wheat (*Triticum aestivum* L.) (Jones 1999). Moreover, these crops are commonly rotated with soybean, corn and wheat crop residues are important sources of inoculum for species of *Fusarium* including *F.graminearum* (Dill-Macky and Jones 2000; Manstretta and Rossi 2015; Pereyra and Dill-Macky 2008). In our study, the soybean fields sampled, had mostly corn and in few instances, had wheat as the previous crop. Besides

F.graminearum, *F.acuminatum* was also prevalent in this study, which is consistent with the previous studies (Bienapfl 2011; Diaz Arias et al. 2013a; French and Kennedy 1963; Nelson and Windels 1992; Parikh et al. 2018; Zhou et al. 2018). *Fusarium acuminatum* is commonly found in the cold environments (e.g. South Dakota) and can be isolated from the diseased roots of soybean (Diaz Arias et al. 2013a), corn (Okello et al. 2019a) or wheat (Gonzalez and Trevathan 2006; Tinline 1977). However, *F.acuminatum* is considered to be a saprophyte and a secondary invader of agricultural crops (Altomare et al. 1997). Other species of *Fusarium* such as *F.equiseti-incarnatum complex, F.subglutinans and F.virguliforme* were among those recovered less frequently in this study. The fungi less frequently recovered were found in less than 10% of the soybean fields sampled and their low numbers may either be because the plants were randomly sampled or because their recovery affected by the plant growth stage at time of sampling. In Iowa, Diaz Arias et al. (2013a) reported *F.armeniacum*, *F. equiseti, F.subglutinans*, and *F.virguliforme* among nine *Fusarium* isolates that were recovered the least.

For the aggressiveness study, our results showed that isolates of *F.oxysporum*, *F.armeniacum*, and *F.commune* caused the highest RTE and this may be because these isolates caused damping off of soybean seedlings. Our results of isolates causing severe damping off is consistent with previous studies on these pathogens (Diaz Arias et al. 2013b, Ellis et al. 2012a; 2012b). For example, Diaz Arias et al. (2013b) observed *F.oxysporum* isolate recovered from soybean roots in Iowa to cause severe damping-off, while Ellis et al. (2012a; 2012b) reported that the soybean seedling emergence in soil infested with *F.armeniacum* and *F.commune* were less than 50%. In this study, significant differences in aggressiveness were observed among isolates within *F.acuminatum*, *F.oxysporum*,

F.proliferatum, and *F.solani*; however not in the case of *F.armeniacum*, *F.commune*, *F.equiseti-incarnatum*, *F.graminearum*, *F.sporotrichioides*, *F.subglutinans*, and *F.virguliforme*. In contrast, besides *F.acuminatum*, *F.oxysporum*, and *F.solani*, the Iowa study by Diaz Arias et al. (2013b) reported significant variation among isolates within *F.equiseti*, but not *F.proliferatum*. We suspect the difference between Diaz Arias et al. (2013b) and our study to be due to soybean cultivars used, inoculation method, the virulence of the isolates, and the greenhouse conditions that may have influenced the disease development caused by the *F. equiseti-incarnatum* and *F.proliferatum* isolates. In the Iowa study, soybean Asgrow 2403 was used in the pathogenicity test set up in a water bath maintained at 18 ± 1^{0}C in a greenhouse at 23 ± 5°C; while in our study, Asgrow 1835 seeds were planted on plastic cups arranged on the bench at the greenhouse maintained at 22 ± 2°C. Further, the isolates in the Iowa study were recovered from soybean roots sampled at both vegetative (V2 to V5) and reproductive growth stages (R2 to R4) (Diaz Arias et al. 2013a), while in our study, the isolates were recovered from plant roots sampled at the reproductive growth stages R1 to R5.

In this study, while only 21 accessions were screened for resistance to *F.graminearum*, *F.proliferatum*, *F.sporotrichioides*, and *F.subglutinans*, these accessions varied in their level of susceptibility to the four species. For example, while PI578386 was significantly less susceptible to *F.proliferatum*, it was susceptible to the other three species. PI361090 was observed to be significantly less susceptible to the South Dakota isolates of *F.graminearum*, *F.proliferatum* and *F.subglutinans* when compared to Williams 82 and Asgrow 1835. However, we used only single isolates of the three species in this study and the germplasm screening experiments for these pathogens were performed at the same

greenhouse temperature (22°C). Therefore, additional studies are required involving multiple isolates of *F.graminearum*, *F.proliferatum* and *F.subglutinans* and genotyping to confirm the resistance in PI361090 to these fungi as reported in this study. As for *F.sporotrichioides*, none of the 21 accessions were observed to be significantly less susceptible to when compared to either Asgrow 1835 or Williams 82 and this may be because of several reasons. First, we evaluated only MG-I accessions in this study since we were identifying parental resistant materials that can be incorporated by breeding programs in the North Central Region of the U.S. Second, despite that we evaluated accessions from seven countries, the genetic bases of these accessions may be narrow. For example, the genetic base of Chinese soybean cultivars has 339 ancestors, which is considered the largest compared to other countries such as Japan with 74 ancestors, is yet classified as narrow (Cui et al. 2000; Zhou et al. 2000). Third, although Asgrow 1835 and Williams 82 were observed to be susceptible to multiple species of *Fusarium* in this study and studies by Okello et al. (2019b), these two cultivars are not universal susceptible checks to screen germplasm for resistance to *F.sporotrichioides*. Finally, the greenhouse conditions provided for screening soybean accessions for resistance to *F.sporotrichioides* was not ideal. Our greenhouse aggressiveness study was performed at 22°C, while few other studies (e.g. Nazari et al. (2014)), the pathogen infected durum wheat spikes at temperatures >25°C.

In summary, our study has demonstrated that 11 species of *Fusarium* are involved in soybean root rot in South Dakota and these fungi were observed to cause lesions on the roots (>1% when compared to the non-inoculated control) to complete seed colonization of soybean plants in the greenhouse. While only 21 accessions were screened for resistance

to *F.graminearum*, *F.proliferatum*, *F.sporotrichioides*, and *F.subglutinans*, PI361090 and PI578386 were identified as possible sources of resistance to one or multiple species of *Fusarium*. However, it has to be noted that soybean cultivar resistance may vary with isolates within a given species of *Fusarium*, greenhouse conditions and the screening methods used for inoculations. In general, the level of resistance among soybean accessions are known to be affected by environmental conditions within a particular species and among species. Hence, to determine resistance, comparisons among accessions will have to be made in the field under natural disease pressure from species of *Fusarium*. While identifying and incorporating traits associated with resistance to Fusarium root rot in soybean may take time, growers in soybean producing areas should consider an integrated management approach to manage the disease, which includes use of soybean cultivars with resistance (if available), tillage practices, removal of crop residues, proper field drainage, and fungicide seed treatments.

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Table 2.1 Information of 57 isolates of *Fusarium* that were recovered from soybean roots sampled in South Dakota in 2014 and used in the greenhouse study.

Isolate ID	Species of *Fusarium*	County	Growth stage at time of collection[a]	Previous Crop	Accession Numbers
FUS001	*F.graminearum*	Union	R3-R4	Corn	MH822047
FUS002	*F.sporotrichioides*	Clay	R3-R4	Corn	MH822032
FUS003	*F.graminearum*	Yankton	R3-R4	Corn	MH822046
FUS004	*F.graminearum*	Kingsbury	R4-R5	Corn	MH822054
FUS005	*F.oxysporum*	Sanborn	R4-R5	Corn	MH822078
FUS006	*F.graminearum*	Sanborn	R1-R2	Corn	MH822045
FUS007	*F.graminearum*	Hanson	R4-R5	Corn	MH822053
FUS008	*F.graminearum*	Minnehaha	R2-R3	Corn	MH822073
FUS009	*F.acuminatum*	Deuel	R3-R4	Corn	MH822067
FUS010	*F.acuminatum*	Brookings	R3-R4	Corn	MH822065
FUS011	*F.solani* species complex	Union	R3-R5	Corn	MH822035
FUS012	*F.solani* species complex	Yankton	R3-R4	Corn	MH822033
FUS013	*F.oxysporum*	Sanborn	R4-R5	Corn	MH822074
FUS014	*F.oxysporum*	Davison	R4-R5	Corn	MH822086
FUS015	*F.oxysporum*	Codington	R3-R4	Corn	MH822080
FUS016	*F.oxysporum*	Spink	R3-R4	Corn	MH822076
FUS017	*F.oxysporum*	Brown	R3-R4	Corn	MH822079
FUS018	*F.armeniacum*	Sanborn	R4-R5	Corn	MH822071
FUS019	*F.oxysporum*	McCook	R3-R5	Corn	MH822075
FUS020	*F.virguliforme*	Clay	R3-R4	Corn	MH822030
FUS021	*F.armeniacum*	Brookings	R3-R4	Corn	MH822070
FUS022	*F.acuminatum*	Sanborn	R4-R5	Corn	MH822057
FUS023	*F.acuminatum*	McCook	R4-R5	Corn	MH822063
FUS024	*F.oxysporum*	Brown	R3-R4	Corn	MH822038
FUS025	*F.proliferatum*	Clay	R3-R4	Corn	MH822084
FUS026	*F.proliferatum*	McCook	R4-R5	Corn	MH822083
FUS027	*F.oxysporum*	Moody	R3-R4	Corn	MH822081
FUS028	*F.solani* species complex	Clay	R3-R4	Corn	MH822037
FUS029	*F.oxysporum*	Brown	R3-R4	Corn	MH822082
FUS030	*F.equiseti-incarnatum*	McCook	R4-R5	Corn	MH822040
FUS031	*F.solani species complex*	Brookings	R3-R4	Corn	MH822036
FUS033	*F.oxysporum*	Brookings	R3-R4	Corn	MH822077

FUS034	*F.commune*	Brookings	R3-R4	Corn	MH822055
FUS035	*F.subglutinans*	Sanborn	R4-R5	Corn	MH822031
FUS036	*F.oxysporum*	Clark	R3-R4	Corn	MH822039
FUS037	*F.acuminatum*	McCook	R4-R5	Corn	MH822061
FUS038	*F.acuminatum*	McCook	R4-R5	Corn	MH822062
FUS039	*F.graminearum*	Minnehaha	R2-R3	Corn	MH822043
FUS040	*F.graminearum*	Minnehaha	R3-R4	Corn	MH822044
FUS041	*F.acuminatum*	Kingsbury	R3-R4	Corn	MH822059
FUS042	*F.acuminatum*	Kingsbury	R3-R4	Corn	MH822060
FUS043	*F.acuminatum*	Hanson	R4-R5	Corn	MH822058
FUS044	*F.graminearum*	Miner	R3-R4	Corn	MH822049
FUS045	*F.acuminatum*	Miner	R3-R4	Corn	MH822068
FUS046	*F.acuminatum*	Miner	R3-R4	Corn	MH822064
FUS047	*F.acuminatum*	Miner	R3-R4	Corn	MH822056
FUS048	*F.equiseti-incarnatum*	Miner	R3-R4	Corn	MH822041
FUS049	*F.graminearum*	Union	R3-R4	Corn	MH822069
FUS050	*F.graminearum*	Clay	R3-R4	Corn	MH822051
FUS051	*F.solani* species complex	Hamlin	R3-R4	Corn	MH822066
FUS052	*F.graminearum*	McCook	R4-R5	Corn	MH822072
FUS053	*F.solani* species complex	Hamlin	R3-R4	Corn	MH822034
FUS054	*F.graminearum*	Grant	R3-R4	Corn	MH822050
FUS055	*F.graminearum*	Grant	R3-R4	Corn	MH822048
FUS056	*F.graminearum*	Brookings	R3-R4	Wheat	MH822042
FUS057	*F.graminearum*	Brookings	R4-R5	Corn	MH822052
FUS058	*F.proliferatum*	McCook	R4-R5	Corn	MH822085

Soybean plants were sampled from commercial fields at reproductive growth stages R1= beginning bloom; R2 = full flowering; R3 = beginning pod development; R4 = full pod and R5 = beginning seed (Fehr et al. 1971).

Table 2.2. Median, mean rank, and relative treatment effects (RTE) for root rot severity caused by 57 isolates of *Fusarium* on Asgrow 1835 in a greenhouse study.

Isolate ID[a]	Species of *Fusarium*	Median disease rating	Mean rank[b]	Relative treatment effects[c]
FUS016	*F.oxysporum*	5	306.50	0.88 a
FUS017	*F.oxysporum*	5	306.50	0.88 a
FUS018	*F.armeniacum*	5	306.50	0.88 a
FUS024	*F.oxysporum*	5	306.50	0.88 a
FUS029	*F.oxysporum*	5	306.50	0.88 a
FUS033	*F.oxysporum*	5	306.50	0.88 a
FUS036	*F.oxysporum*	5	306.50	0.88 a
FUS010	*F.acuminatum*	5	286.50	0.82 ab
FUS021	*F.armeniacum*	5	286.50	0.82 ab
FUS034	*F.commune*	5	286.50	0.82 ab
FUS015	*F.oxysporum*	5	277.42	0.80 ab
FUS058	*F.proliferatum*	4	266.50	0.76 ab
FUS023	*F.acuminatum*	4.5	257.42	0.74 abc
FUS014	*F.oxysporum*	5	248.33	0.71 abcd
FUS019	*F.oxysporum*	5	248.33	0.71 abcd
FUS031	*F.solani species complex*	4.5	238.33	0.68 bcd
FUS012	*F.solani* species complex	4	228.33	0.65 bcd
FUS027	*F.oxysporum*	4	228.33	0.65 bcd
FUS026	*F.proliferatum*	4	227.42	0.65 bc
FUS035	*F.subglutinans*	4	227.42	0.65 bc
FUS025	*F.proliferatum*	3.5	189.25	0.54 cd
FUS013	*F.oxysporum*	3	161.08	0.46 cde
FUS038	*F.acuminatum*	3	161.08	0.46 cde
FUS055	*F.graminearum*	3	161.08	0.46 cde
FUS 002	*F.sporotrichioides*	3	151.08	0.43 de
FUS052	*F.graminearum*	3	151.08	0.43 de
FUS056	*F.graminearum*	3	151.08	0.43 de
FUS008	*F.graminearum*	3	142.58	0.41 de
FUS001	*F.graminearum*	3	132.00	0.38 e
FUS 003	*F.graminearum*	3	132.00	0.38 e
FUS006	*F.graminearum*	3	132.00	0.38 e
FUS009	*F.acuminatum*	3	132.00	0.38 e
FUS020	*F.virguliforme*	3	132.00	0.38 e
FUS022	*F.acuminatum*	3	132.00	0.38 e

FUS028	*F.solani* species complex	3	132.00	0.38 e
FUS039	*F.graminearum*	3	132.00	0.38 e
FUS040	*F.graminearum*	3	132.00	0.38 e
FUS041	*F.acuminatum*	3	132.00	0.38 e
FUS042	*F.acuminatum*	3	132.00	0.38 e
FUS054	*F.graminearum*	3	132.00	0.38 e
FUS057	*F.graminearum*	3	132.00	0.38 e
FUS050	*F.graminearum*	3	132.58	0.38 de
FUS037	*F.acuminatum*	3	124.08	0.36 cdef
FUS005	*F.oxysporum*	3	114.08	0.33 def
FUS047	*F.acuminatum*	3	114.08	0.33 def
FUS004	*F.graminearum*	3	113.50	0.32 ef
FUS007	*F.graminearum*	3	113.50	0.32 ef
FUS030	*F.equiseti-incarnatum*	3	113.50	0.32 ef
FUS043	*F.acuminatum*	3	113.50	0.32 ef
FUS044	*F.graminearum*	3	113.50	0.32 ef
FUS049	*F.graminearum*	3	113.50	0.32 ef
FUS051	*F.solani* species complex	3	113.50	0.32 ef
FUS011	*F.solani* species complex	3	95.00	0.27 ef
FUS053	*F.solani* species complex	3	95.00	0.27 ef
FUS046	*F.acuminatum*	2.5	76.50	0.22 ef
FUS048	*F.equiseti-incarnatum*	2.5	76.50	0.22 ef
FUS045	*F.acuminatum*	2	58.00	0.17 f
Non-inoculated control	Non-inoculated control	1	3.50	0.01 g

[a] The root rot caused by the *Fusarium* isolates was evaluated 14 days after inoculation on a 1 to 5 scale (Acharya et al. 2015), where, 1 = germination and healthy seedlings with no visible root colonization, 2 = germination and 1 to 19% of the root having lesions, 3 = germination and 20 to 74% of the root having lesions, 4 = germination and 75% or more of the root having lesions; and 5 = no germination and complete colonization of seed.

[b] The mean rank corresponding to each isolate was calculated using the nparLD package (Noguchi et al 2012) in R version 2.1 (R Core Team 2013).

[c] The root rot caused by each isolate was expressed as relative treatment effects (RTE) calculated from mean ranks using the nparLD package in R and compared at 95% confidence intervals. RTEs with different alphabetical letters indicate significant differences in root rot severity caused by the *Fusarium* isolates.

Table 2.3. Soybean accessions screened for resistance to *F.graminearum* FUS052, *F.proliferatum* FUS026, *F.sporotrichioides* FUS002 and *F.subglutinans* FUS035 in the greenhouse.

Soybean accession[a]	Country of origin	Relative Treatment Effects (RTE)[a,b]			
		FUS052	FUS026	FUS002	FUS035
Asgrow 1835 (Check)	United States	0.55 ab	0.48 ab	0.36 a	0.57 a
Williams 82 (PI518671; Check)	United States	0.55 ab	0.59 a	0.47 a	0.57 a
PI361090	Austria	0.09 c	0.15 c	0.70 a	0.14 b
PI437343	Russian Federation	0.55 ab	0.59 a	0.58 a	0.38 a
PI461509	China	0.65 a	0.68 a	0.47 a	0.38 a
PI467307	China	0.55 ab	0.30 abc	0.47 a	0.51 a
PI506678	Japan	0.65 a	0.77 a	0.47 a	0.44 a
PI532229	Oman	0.32 ab	0.33 abc	0.48 a	0.57 a
PI561333	China	0.65 a	0.48 ab	0.47 a	0.57 a
PI578374	China	0.65 a	0.59 a	0.47 a	0.56 a
PI578384	China	0.43 ab	0.86 a	0.47 a	0.44 a
PI578385	China	0.43 ab	0.59 a	0.70 a	0.57 a
PI578386	China	0.55 ab	0.26 bc	0.47 a	0.44 a
PI578474	China	0.65 a	0.68 a	0.70 a	0.51 a
PI467311A	China	0.32 ab	0.37 abc	0.47 a	0.56 a
PI512322C	Georgia	0.65 a	0.48 ab	0.47 a	0.51 a
PI578380A	China	0.55 ab	0.59 a	0.58 a	0.57 a
PI593982	Japan	0.20 b	0.26 abc	0.37 a	0.51 a
PI592907C	Russian Federation	0.55 ab	0.48 ab	0.47 a	0.57 a
PI597397A	Russian Federation	0.43 ab	0.37 abc	0.48 a	0.51 a
PI597405B	Ukraine	0.65 a	0.68 a	0.36 a	0.57 a
PI612754	China	0.32 ab	0.48 ab	0.47 a	0.51 a
PI603339A	China	0.55 ab	0.48 ab	0.58 a	0.57 a

[a]Soybean accessions were evaluated for resistance to *F.graminearum* FUS052, *F.proliferatum* FUS026, *F.sporotrichioides* FUS002 and *F.subglutinans* FUS035 on a 1 to 5 rating scale (Acharya et al. 2015).

[b]The root rot severity caused by the *Fusarium* isolates on the soybean accessions was expressed as relative treatment effect (RTE). RTE with different alphabetic lettering

indicate significant differences among soybean accessions and the susceptible checks in response to screening against the *Fusarium* isolates.

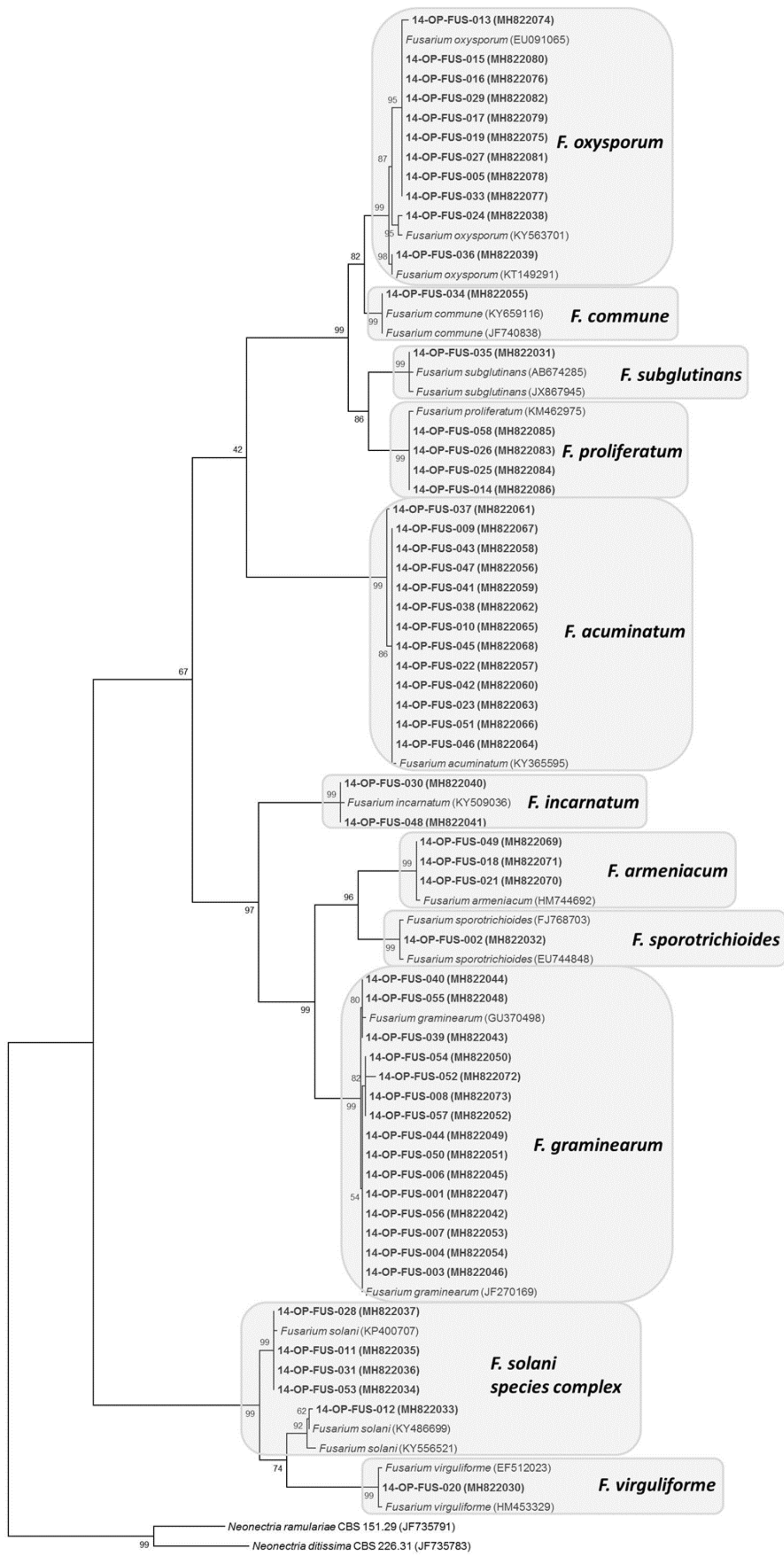
14-OP-FUS-013 (MH822074)
Fusarium oxysporum (EU091065)
14-OP-FUS-015 (MH822080)
14-OP-FUS-016 (MH822076)
14-OP-FUS-029 (MH822082)
14-OP-FUS-017 (MH822079)
14-OP-FUS-019 (MH822075)
14-OP-FUS-027 (MH822081)
14-OP-FUS-005 (MH822078)
14-OP-FUS-033 (MH822077)
14-OP-FUS-024 (MH822038)
Fusarium oxysporum (KY563701)
14-OP-FUS-036 (MH822039)
Fusarium oxysporum (KT149291)
F. oxysporum
14-OP-FUS-034 (MH822055)
Fusarium commune (KY659116)
Fusarium commune (JF740838)
F. commune
14-OP-FUS-035 (MH822031)
Fusarium subglutinans (AB674285)
Fusarium subglutinans (JX867945)
F. subglutinans
Fusarium proliferatum (KM462975)
14-OP-FUS-058 (MH822085)
14-OP-FUS-026 (MH822083)
14-OP-FUS-025 (MH822084)
14-OP-FUS-014 (MH822086)
F. proliferatum
14-OP-FUS-037 (MH822061)
14-OP-FUS-009 (MH822067)
14-OP-FUS-043 (MH822058)
14-OP-FUS-047 (MH822056)
14-OP-FUS-041 (MH822059)
14-OP-FUS-038 (MH822062)
14-OP-FUS-010 (MH822065)
14-OP-FUS-045 (MH822068)
14-OP-FUS-022 (MH822057)
14-OP-FUS-042 (MH822060)
14-OP-FUS-023 (MH822063)
14-OP-FUS-051 (MH822066)
14-OP-FUS-046 (MH822064)
Fusarium acuminatum (KY365595)
F. acuminatum
14-OP-FUS-030 (MH822040)
Fusarium incarnatum (KY509036)
14-OP-FUS-048 (MH822041)
F. incarnatum
14-OP-FUS-049 (MH822069)
14-OP-FUS-018 (MH822071)
14-OP-FUS-021 (MH822070)
Fusarium armeniacum (HM744692)
F. armeniacum
Fusarium sporotrichioides (FJ768703)
14-OP-FUS-002 (MH822032)
Fusarium sporotrichioides (EU744848)
F. sporotrichioides
14-OP-FUS-040 (MH822044)
14-OP-FUS-055 (MH822048)
Fusarium graminearum (GU370498)
14-OP-FUS-039 (MH822043)
14-OP-FUS-054 (MH822050)
14-OP-FUS-052 (MH822072)
14-OP-FUS-008 (MH822073)
14-OP-FUS-057 (MH822052)
14-OP-FUS-044 (MH822049)
14-OP-FUS-050 (MH822051)
14-OP-FUS-006 (MH822045)
14-OP-FUS-001 (MH822047)
14-OP-FUS-056 (MH822042)
14-OP-FUS-007 (MH822053)
14-OP-FUS-004 (MH822054)
14-OP-FUS-003 (MH822046)
Fusarium graminearum (JF270169)
F. graminearum
14-OP-FUS-028 (MH822037)
Fusarium solani (KP400707)
14-OP-FUS-011 (MH822035)
14-OP-FUS-031 (MH822036)
14-OP-FUS-053 (MH822034)
14-OP-FUS-012 (MH822033)
Fusarium solani (KY486699)
Fusarium solani (KY556521)
F. solani
species complex
Fusarium virguliforme (EF512023)
14-OP-FUS-020 (MH822030)
Fusarium virguliforme (HM453329)
F. virguliforme
Neonectria ramulariae CBS 151.29 (JF735791)
Neonectria ditissima CBS 226.31 (JF735783)
20

Figure 2.1. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 10 most parsimonious trees (length = 550) is shown.

The consistency index is (0.67), the retention index is (0.95), and the composite index is 0.65 (0.64) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 79 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 499 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

CHAPTER 3

Seven species of *Fusarium* from soybean and corn are pathogenic on the two crops in South Dakota

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Abstract

In South Dakota, despite that IPM options are available, Fusarium root rot is an emerging disease on soybean (*Glycine max* L.) and corn (*Zea mays* L.). Surveys were conducted across South Dakota on soybean and corn fields in 2014 and 2015 respectively to assess the prevalence of species of *Fusarium* causing root rot. *Fusarium acuminatum*, *F. equiseti*, *F.graminearum*, *F.oxysporum*, *F.proliferatum*, *F.solani*, and *F.subglutinans* were identified common to soybean and corn. A total of 21 isolates, representing these seven species, were evaluated for their pathogenicity on soybean (Williams 82) and corn (B73) using the inoculum layer inoculation method in the greenhouse. At 14 days' post-inoculation, the seedlings were evaluated for root rot severity (1-to-5 rating scale), and relative treatment effects (RTE) were estimated. A significant effect of RTE caused by the treatments was observed on the seedlings of soybean ($P = 1.1 \times 10^{-07}$) and corn ($P = 3.0 \times 10^{-14}$). Two *F.proliferatum* isolates and one *F.graminearum* isolate from corn caused significantly greater RTE than the other treatments (including non-inoculated control) on soybean and corn. Results indicate that soybean and corn can serve as inoculum sources of the seven species of *Fusarium* that are pathogenic to both crops.

Introduction

Root rots and seedling diseases, together, are among the ten most destructive diseases that limit corn (*Zea mays* L.) (Mueller et al. 2016) and soybean (*Glycine max* L.) (Allen et al. 2017) production in the United States and Canada (Ontario). For example, on corn, the total yield losses due to root rots and seedling diseases were estimated to be 1.5 million metric tons in 2015 (Mueller et al. 2016). On soybean, the total yield losses due to seedling diseases were estimated to be 6.6 million metric tons in 2014 (Allen et al. 2017). Among the pathogens that cause root rot and seedling diseases of soybean and corn, species of *Fusarium* are found in all corn and soybean growing areas of the United States. Currently, there are no commercial cultivars of the two crops available that have complete resistance to Fusarium root rot. In South Dakota, most producers plant corn and soybean early into cool and wet soils. They also use a corn-soybean rotation in combination with tillage practices that leave more than 30% crop residue cover on the soil surface (The South Dakota Cropping Systems Inventory, USDA-NRCS/SD, 2017). These cropping practices can increase the risk of root rot and seedling diseases for the two crops in the production areas.

In 2014 and 2015, root rots and seedling diseases were observed in commercial corn and soybean fields in South Dakota with symptoms such as yellowing, loss of stand, wilting, and stunting of plants (F. Mathew, *unpublished*). Root rot surveys were conducted across South Dakota on soybean and corn fields respectively to assess the presence of pathogens causing root rot. Species of *Fusarium* were identified to be most prevalent among the fungal pathogens causing root rot of soybean and corn. Of the total 11 and eight species of *Fusarium* identified on soybean and corn (P. Okello and F. Mathew,

unpublished; Okello et al. 2019), seven species of *Fusarium*, *F.acuminatum* Ellis and Everhart, *F. equiseti* (Corda) Saccardo (syn. *F.equiseti-incarnatum complex*), *F.graminearum* Schwabe, *F.oxysporum* Schlecht, *F.proliferatum* Matsushima, *F.solani* (Martius) Saccardo, and *F.subglutinans* Wollenweber and Reinking, were common to the two crops. While *F.acuminatum*, *F. equiseti*, *F.graminearum*, *F.oxysporum*, and *F.subglutinans* were demonstrated to be pathogenic on both corn and soybean in cross-pathogenicity studies (Broders et al. 2007; Parikh et al. 2018), there is no information available on the cross-pathogenicity of *F.proliferatum* and *F.solani* causing root rot of the two crops. For this study, a total of 21 isolates representing *F.acuminatum*, *F. equiseti*, *F.graminearum*, *F.oxysporum*, *F.proliferatum*, *F.solani*, and *F.subglutinans* were selected from the isolates recovered from corn and soybean surveys in South Dakota based on their aggressiveness levels on a susceptible variety of corn (Adhikari et al. 2016) or soybean (Okello et al. 2016). The objective of this study was to evaluate the cross-pathogenicity of the 21 isolates from soybean and corn on the two crops in the greenhouse.

Isolation and Identification of *Fusarium* Species

For soybean, ten plants with diseased roots (e.g. reduced tap root, no secondary roots, brown discoloration) were arbitrarily sampled along five transects (50 m) and covering an area of 0.5 hectares from a total of 200 commercial fields in 22 counties (nine fields per county) in 2014. The soybean plants were sampled between R1 (beginning flowering) to R5 (beginning seed) growth stages (Fehr et al. 1971), where excess moisture was observed in soils following rain and previous crop was corn. For corn, five plants were arbitrarily sampled along two transects (50 m) and covering an area of 0.5 hectares from a total of 50 commercial fields in 24 counties (two field per county) in 2015. The corn plants

were sampled between V1 (first leaf) to V3 (third leaf) growth stages (Ritchie et al. 1992), where moisture was observed in soils from rain and previous crop was soybean. The commercial fields were randomly selected for the two surveys and located in eastern South Dakota, where over 50% of soybean and corn production takes place in the state. Distance between the commercial fields ranged from approximately 1 to 2 km.

For fungal isolation, the main root of all plant samples was first rinsed under running tap water for 2 to 5 min and then cut into small pieces (~15 mm long). The root pieces were surface-disinfested in 0.05% sodium hypochlorite and 70% ethanol for 1 min each, washed with sterile distilled water, and then dried on autoclaved filter papers. Three root pieces of each plant were placed on petri-dishes containing potato dextrose agar [PDA; prepared as per the protocol by Leslie and Summerell (2006)] amended with 0.02% streptomycin sulfate and incubated at 23 ± 2°C for 7 days under 16 h fluorescent light/dark conditions. Seven days after incubation, cultures were examined for the presence of *Fusarium* using the Olympus CX31 Binocular Microscope (Olympus Corporation, Center Valley, PA). Putative *Fusarium* colonies were transferred to fresh PDA plates by removing a mycelial plug (~3 mm square) from the edge of a growing colony with a sterile scalpel. A total of 1130 and 198 isolates (=1328) were recovered from soybean and corn respectively, which were grouped into a total of 12 species (11 species from soybean and eight from corn) based on morphology on PDA (colony appearance) and carnation leaf agar (sporodochia production and color) (Leslie and Summerell 2006). Of the 12 species of *Fusarium*, seven species [(*F.acuminatum*, 27.6%), (*F. equiseti*, 2.7%), (*F.graminearum*, 47.4%), (*F.oxysporum*, 10.1%), (*F.proliferatum*, 3.4%), (*F.solani*, 1.6%), and (*F.subglutinans*, 0.9%)] were identified common to corn and soybean. The other five

species of *Fusarium* were identified only to soybean [(*F.armeniacum*, 2.9%*, F.commune*, 0.1%*, F.sporotrichioides*, 2.0% and *F.virguliforme*, 1.4%)] or to corn (*F. boothi*, 0.1%).

From the 1328 isolates, 84 isolates (57 isolates from soybean and 27 from corn) were selected from different South Dakota counties to represent the 12 species and single-conidial isolates were established. DNA was extracted from the single-conidial cultures of the 84 isolates using the FastDNA Spin Kit (MP Biomedicals, Solon, OH, USA), and the translational elongation factor 1-alpha (EF1-α) gene region was sequenced using the primers EF1F/EF1R (EF1F: 'ATGGGTAAGGARGACAAGAC' and EF1R: 'GGARGTACCAGTSATCATGTT') (Geiser et al. 2004). The polymerase chain reaction (PCR) amplifications were performed in a 25 µl mixture containing 2.0 µl of fungal DNA (10 ng/µl), 0.75 µl forward primer (10.0 µM), 0.75 µl reverse primer (10.0 µM), 12.5 µl of 2x Taq PCR Master Mix containing Taq DNA Polymerase (Qiagen, Valencia, CA), and 9.0 µl of sterile nuclease-free water. The PCR parameters included an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, elongation at 72°C for 1 min, and a final elongation at 72°C for 10 min (O'Donnell et al. 1998). To confirm amplification, a 5 µl aliquot of both PCR products was run on an agarose gel (2%). The PCR products were sequenced by GenScript USA Inc., Piscataway, NJ. The isolates were identified to 12 species of *Fusarium* using the Basic Local Alignment Search Tool Nucleotide (BLASTN) searches at the *Fusarium*-ID database (http://isolate.fusariumdb.org/index.php). For this study, the EF1-α sequences of the 10 soybean isolates are deposited in GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) under the accession numbers MH822031, MH822037, MH822040, MH822048, MH822059, MH822068, MH822072, MH822081,

MH822083 and MH822084, and those of the 11 corn isolates are under the accession numbers MH595503, MH595499, MH595503, MH595505, MH595507, MH595510, MH595513, MH595516, MH595517, MH595519, and MH595522 (Table 3.1).

Pathogenicity of *Fusarium* Isolates from Soybean and Corn

For the cross-pathogenicity experiment, a total of 21 isolates from soybean and corn were used to determine their pathogenicity on 'Williams 82' and 'B73' in the greenhouse in two experiments (Table 3.1). These isolates were selected based on the level of aggressiveness of 57 soybean isolates on a soybean susceptible variety (Okello et al. 2016) or 27 corn isolates on a corn susceptible hybrid (Adhikari et al. 2016) in greenhouse experiments. It was determined in the studies by Okello et al. (2016) and Adhikari et al. (2016) that while individual isolates varied significantly in their aggressiveness on soybean or corn respectively, significant differences in aggressiveness were not observed among isolates within a particular species. In the total 21 isolates, there are four isolates each of *F.acuminatum*, *F.graminearum* and *F.proliferatum*; three isolates of *F.oxysporum*, and two isolates each of *F. equiseti*, *F.solani*, and *F.subglutinans* (Table 3.1). To prepare inoculum, each isolate was initially grown on potato dextrose agar and five mycelial plugs (~15 mm square) were transferred into a 250-ml Erlenmeyer flask containing previously autoclaved sand-corn meal mixture (54 g of play sand, 6 g of cornmeal, and 10 ml distilled water) based on the protocol of Bilgi et al. (2008). The inoculum for each of the 21 isolates was incubated for 14 days at 22 ± 2°C and mixed every other day by manually shaking the flask.

For the two experiments, each isolate was used to inoculate soybean or corn seedlings using the inoculum layer method modified from Bilgi et al. (2008). Briefly,

plastic cups (473 ml) were first filled with 40 g coarse vermiculite, followed by 20 g of fungal inoculum and then 20 g of coarse vermiculite to avoid direct contact of the seedlings with the inoculum. Two pre-germinated seeds of either ‘Williams 82’ or ‘B73’ were planted into the plastic cups containing vermiculite- inoculum mixture and then 20 g of coarse vermiculite was used to cover the seedlings. To germinate the soybean or corn seeds, the seeds were spread on a moist filter paper placed in petri plates and germinated at 23 ± 2°C for three days. After the seeds sprouted roots, these were planted into plastic cups containing vermiculite- inoculum mixture. For the non-inoculated control treatments, previously autoclaved sand-cornmeal mixture containing no fungus was used as inoculum for the two experiments.

Following the experimental setup, soybean or corn plants were placed in a completely randomized design (CRD) with five replications (each replication is a cup containing two plants) on a greenhouse bench at 22 ± 2°C with 16 h photoperiod. The plants were watered once daily, and no fertilizer was added during the experiment. At 14 days after inoculation, root rot severity caused by the isolates on soybean and corn plants was evaluated using a 1- to -5 rating scale (Acharya et al. 2015); where, 1 = germination and healthy seedlings with no visible root colonization, 2 = germination and 1 to 19% of the root having lesions, 3 = germination and 20 to 74% of the root having lesions, 4 = germination and 75% or more of the root having lesions; and 5 = no germination and complete colonization of seed. The experiments were performed twice.

Each plant in the cup was rated for root rot severity and the mean root rot severity was calculated for the two plants in a cup. The root rot severity data is ordinal and hence, the data were analyzed using nonparametric statistics (Shah and Madden 2004). Prior to

performing the nonparametric analyses, the Fligner-Killen test for homogeneity of variance was satisfied for the two experimental runs for soybean (P = 0.85) and corn (P = 0.08). Therefore, the data from the two experimental runs were combined and the analysis of variance (ANOVA) type test statistics (ATS) of ranked data was determined using the nparLD package (Noguchi et al. 2012) in R v 2.1 (R Core Team 2013). For either soybean or corn, the rank of each isolate was calculated as " $\overline{R}_i = \frac{1}{n_i}\sum_{k=1}^{n_i} R_{ik}$ (Akritas 1991), where $\overline{R}_i$ = the mean rank for the ith treatment, and R_{ik} = the rank of X_{ik} among all N observations" (Shah and Madden 2004). The root rot severity caused by each isolate was expressed as relative treatment effects (RTE), and calculated from mean ranks ($\overline{R}_i$) as $\widehat{p_i} = \frac{1}{N}(\overline{R}_i - \frac{1}{2})$ [where N = the total number of observations (Shah and Madden 2004)] and compared at 95% confidence intervals using the nparLD package in R.

At 14 days after inoculation, a significant effect of treatments was observed on root rot severity for soybean (ATS = 7.2, df = 6.0, $P = 1.1 \times 10^{-7}$) and corn (ATS = 11.5; df = 6.6, $P = 3.0 \times 10^{-14}$). All the 21 isolates caused root rot on both soybean and corn. No damping-off was observed on any of the plants. On the non-inoculated control plants, no root discoloration (disease rating =1) was observed on either soybean or corn plants.

On soybean, significant differences in RTE were observed among individual isolates and non-inoculated control. *Fusarium proliferatum* isolates C1FP and C14FP from corn caused the greatest RTE, which was not significantly different from that of the *F.graminearum* isolate C12FG. Significant differences in RTE were observed among soybean and corn isolates within *F.graminearum*, and *F.proliferatum* (Fig. 3.1).

On corn, significant differences in root rot severity were observed among individual isolates and non-inoculated control. *Fusarium proliferatum* isolate C1FP from corn caused

the greatest RTE, which was not significantly different from that of the *F.proliferatum* isolate C14FP. Significant differences in RTE were observed among soybean and corn isolates within *F.graminearum*, *F.subglutinans*, and *F.proliferatum* (Fig. 3.2).

To fulfill Koch's postulates, isolates from soybean and corn were re-isolated from the roots of inoculated soybean and corn plants. The diseased plants were randomly selected, and discolored root pieces were plated on PDA as described previously. The identity of the isolates was confirmed by sequencing the EF1-α gene. No species of *Fusarium* was recovered from the roots of the non-inoculated soybean and corn plants.

Discussion

In this study, the severity of root rot on soybean and corn plants was observed to differ among the 21 isolates, with two *F.proliferatum* isolates (F1 and F14FP-PB) and one *F.graminearum* isolate (F12) from corn causing significantly greater root rot severity on both the hosts. Our findings of *F.graminearum* isolates from corn and soybean being virulent on the two hosts are consistent with previous cross-pathogenicity studies (Broders et al. 2007; Parikh et al. 2018). However, in contrast to the study by Broders et al. (2007) who observed that isolates of *F.acuminatum*, *F. equiseti*, *F.oxysporum*, and *F.subglutinans* caused no lesion on the roots of soybean or corn (a mean pathogenicity score of <1.0), we observed that isolates of these fungi caused brown to black discoloration of the primary root (20 to 74% of the root had lesions) of the seedlings in this study. Between Broders et al. (2007) and our study, we suspect differences in the soybean or corn cultivars used, inoculation method, type of inoculum, virulence of isolates, and greenhouse conditions may have contributed to the differences in disease development by *F.acuminatum*, *F.equiseti*, *F.oxysporum*, and *F.subglutinans* on the two hosts. As for *F.proliferatum* and

F.solani, we have not found published work on the cross-pathogenicity of isolates of these fungi on soybean and corn despite that these pathogens are known to cause root rot of soybean and corn. Our study was able to demonstrate that the isolates of *F.proliferatum* and *F.solani* from soybean and corn are virulent (at least 10% of the root had lesions) on the two hosts.

Overall, our cross pathogenicity experiments showed that South Dakota isolates of *Fusarium acuminatum*, *F.equiseti*, *F.graminearum*, *F.oxysporum*, *F.proliferatum*, *F.solani*, and *F.subglutinans* from either soybean or corn are pathogenic to both crops, despite that only a small number of isolates was used for the study. This suggests that rotating soybean and corn in South Dakota will not help reduce inoculum of species of *Fusarium* that are pathogenic to the two crops. Unfortunately, these pathogens can cause root rot on other crops grown in South Dakota [e.g. wheat (*Triticum aestivum* L.), Kaur 2016], which suggests exposure of soybean and corn to virulent isolates of *Fusarium* can be expected even if crop rotations are diversified. Thus, it is critical to adopt an integrated approach to manage Fusarium root rot in South Dakota, which includes combining tillage practices, weed management, use of fungicide seed treatments, adjusting planting dates, and using resistant cultivars/hybrids. Additionally, future studies are needed to explain how much corn and soybean can individually contribute to maintaining inocula of these fungi in fields with a known rotation of the two hosts.

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Table 3.1. Information on the 21 isolates of *Fusarium* evaluated on soybean and corn in the greenhouse.

Isolate ID[a]	Pathogen	Year of isolation	Host of origin	South Dakota County	GenBank Accession Number
FUS041	*F.acuminatum*	2014	Soybean	Kingsbury	MH822059
FUS045	*F.acuminatum*	2014	Soybean	Miner	MH822068
FUS052	*F.graminearum*	2014	Soybean	McCook	MH822072
FUS055	*F.graminearum*	2014	Soybean	Grant	MH822048
FUS027	*F.oxysporum*	2014	Soybean	Moody	MH822081
FUS026	*F.proliferatum*	2014	Soybean	McCook	MH822083
FUS025	*F.proliferatum*	2014	Soybean	Clay	MH822084
FUS030	*F. equiseti*	2014	Soybean	McCook	MH822040
FUS028	*F.solani*	2014	Soybean	Clay	MH822037
FUS035	*F.subglutinans*	2014	Soybean	Sanborn	MH822031
F8	*F.acuminatum*	2015	Corn	Charles Mix	MH595499
F6	*F.acuminatum*	2015	Corn	Browns	MH595497
F13	*F.graminearum*	2015	Corn	Codington	MH595507
F12	*F.graminearum*	2015	Corn	Codington	MH595505
F4	*F.oxysporum*	2015	Corn	Brookings	MH595513
F3	*F.oxysporum*	2015	Corn	Brookings	MH595510
F14FP-PB	*F.proliferatum*	2015	Corn	Codington	MH595517
F1	*F.proliferatum*	2015	Corn	Brookings	MH595516
F16	*F. equiseti*	2015	Corn	Days	MH595503
F11	*F.solani*	2015	Corn	Clay	MH595519
P5	*F.subglutinans*	2015	Corn	Browns	MH595522

[a] Species identity of the 21 isolates (10 from soybean and 11 from corn) was established by morphology and sequencing the EF1-α gene region using the primers EF1F/EF1R (Geiser et al. 2004). The EF1-α sequences of the 10 soybean and 11 corn isolates are deposited in the GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/).

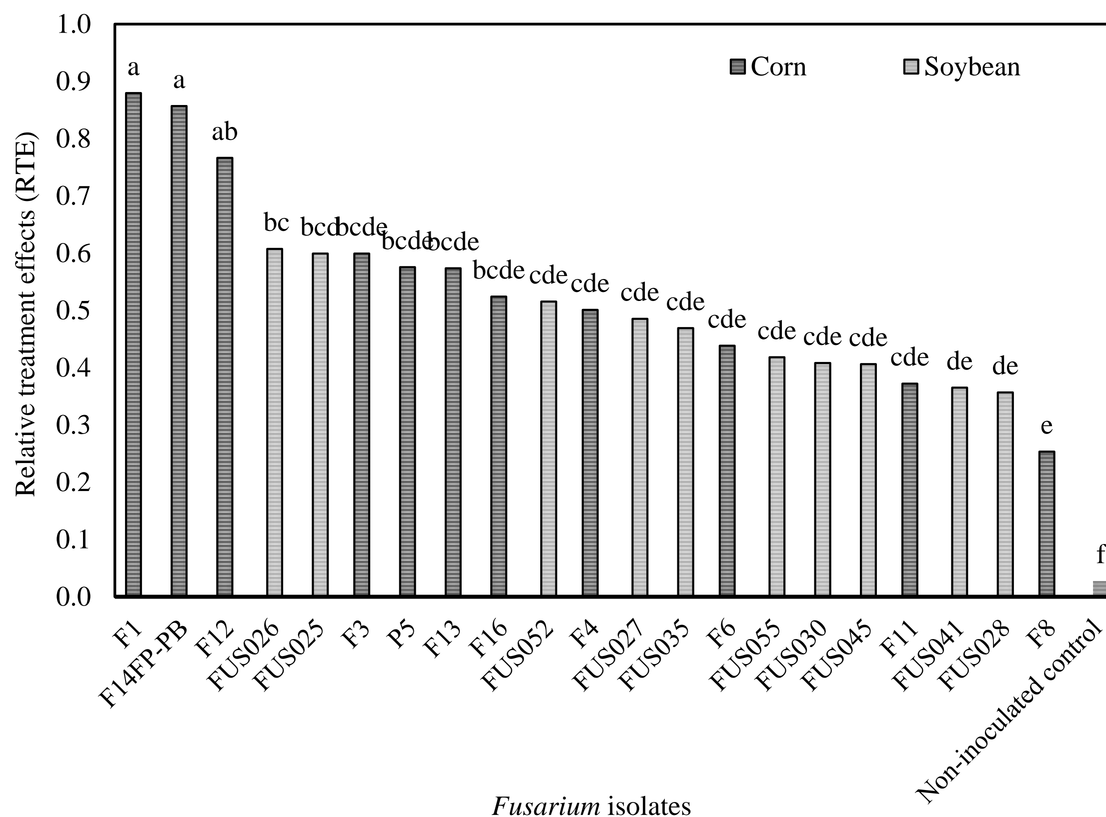

Figure 3.1. Root rot severity (expressed as RTE) caused by *Fusarium* isolates from corn (dark grey) and soybean (light grey) on the soybean cv. Williams 82 at 14 days post-inoculation in the greenhouse. RTE with the same letter are not significantly different based on 95% confidence intervals. *Fusarium* isolates: *F.acuminatum* = F6, F8, FUS041 and FUS045A; *F. equiseti* = F16 and FUS030; *F.graminearum* = F12, F13, FUS052 and FUS055; *F.oxysporum* = F3, F4 and FUSO27; *F.proliferatum* = F1, F14FP-PB, FUS025, FUS026; *F.solani*= F11 and FUS028; *F.subglutinans* = P5 and FUS035.

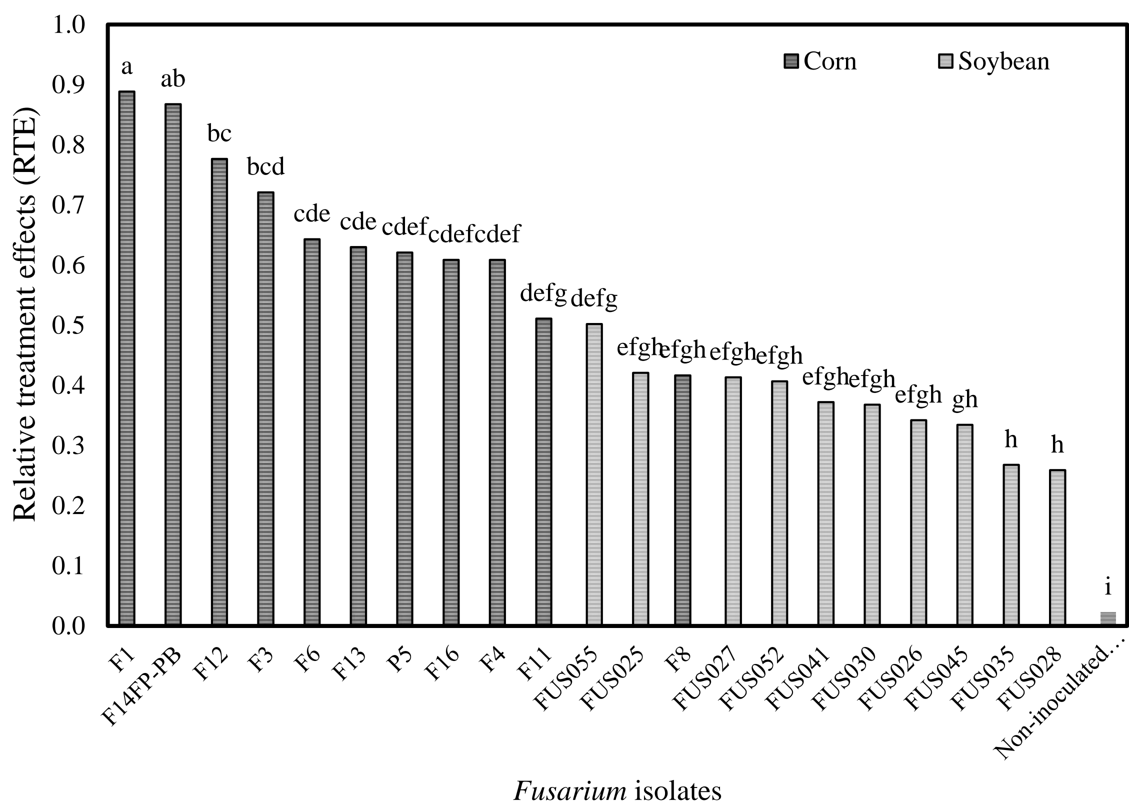

Figure 3.2. Root rot severity (expressed as RTE) caused by Fusarium isolates from corn (dark grey) and soybean (light grey) on the corn inbred line 'B73' at 14 days post-inoculation in the greenhouse. RTE with the same letter are not significantly different based on 95% confidence intervals. Fusarium isolates: F.acuminatum = F6, F8, FUS041 and FUS045A; F. equiseti = F16 and FUS-030; F.graminearum = F12, F13, FUS052 and FUS055; F.oxysporum = F3, F4 and FUSO27; F.proliferatum = F1, F14FP-PB, FUS025, FUS026; F.solani= F11 and FUS028; F.subglutinans = P5 and FUS035.

CHAPTER 4

Identification of sources of resistance to *Fusarium graminearum* in soybean

A paper to be submitted to the journal *Plant Disease*

Abstract

Fusarium graminearum Schwabe is now recognized as a primary pathogen on soybean causing root rot, seed rot, and seedling damping-off in the United States including South Dakota. The development of host resistance is considered the best long-term management option in reducing the impacts of *F.graminearum* on soybean plants. Therefore, the objective of this study was to identify accessions from the USDA soybean germplasm collection in Maturity Groups (MG) 00 to V with resistance to *F.graminearum* in the greenhouse. Two hundred and forty-seven accessions were screened for their resistance to a single isolate of *F.graminearum* using the inoculum layer method with two susceptible checks, Williams 82 and Asgrow 1835. Disease severity caused by the *F.graminearum* isolate was evaluated 21 days post-inoculation on a 1-to-5 rating scale and expressed as relative treatment effects (RTE). Eight soybean accessions (PI437949, PI438292, PI612761A, PI438094B, PI567301B, PI408309, PI361090 and P188788) were observed to be significantly less susceptible to *F.graminearum* when compared to Williams 82 and Asgrow 1835. The eight accessions may be used in breeding programs as sources of resistance to *F.graminearum* for advance screening and development of resistant soybean cultivars. Future studies will focus on identifying single nucleotide polymorphism markers associated with *F.graminearum* resistance using mapping strategies.

Introduction

Fusarium graminearum Schwabe is known to cause seedling and root rot, damping-off and pod blight on soybean (*Glycine max* L.) (Broders et al. 2007; Diaz Arias et al. 2013a; Ellis et al. 2011; Martinelli et al. 2004; Pioli et al. 2004). Symptoms caused by the fungus on seedlings of soybean include brown lesions on the roots and shoot, which become necrotic and cause the plant to die. Occasionally, lesions that are irregularly shaped can develop on the cotyledons and leaves. Pod may be infected during the reproductive growth stages, which will inhibit development of seed (Martinelli et al. 2004). Besides the visual symptoms caused by *F.graminearum* on the plants, the fungus as a root rot pathogen can also cause reduction in soybean yield (Allen et al. 2017; Diaz Arias et al. 2013a; Koenning and Wrather 2010). The development of the disease caused by *F.graminearum* is favored by warm and humid soil conditions (optimum soil temperature range of 20 to 25°C), which influences the levels of inoculum present in the soil (Beyer et al. 2004; Broders et al. 2007; Doohan et al. 2003; Ellis et al. 2011; Leslie and Summerell 2006). The fungus can survive on infested plant residues of host crops such as corn (*Zea mays* L.), soybean and wheat (*Triticum aestivum* L.) on the soil surface (Anderson et al. 1988; Harrington et al. 2000; Khonga and Sutton 1988; Leslie et al. 1990; Sutton 1982; Windels et al. 1988).

Management of root rot caused by *F.graminearum* and other species of *Fusarium* is limited to seed treatment, and cultural practices such as planting in well-drained soils, reduced tillage, and minimizing the effect of soil compaction (Broders et al. 2007; Ellis et al. 2011; Malvick 2018). For example, Broders et al. (2007) observed fludioxonil to be effective in amended agar plate assays but mutants insensitive to fludioxonil was easily

generated in the amended agar plate assays. Hence, the development of cultivars with resistance to *F.graminearum* remains the key component in reducing the impact of *F.graminearum* on soybean roots (Tu, 1992; Abawi et al. 2006).

Several studies have focused on identification of soybean accessions with resistance to *F.graminearum* (Ellis et al. 2012; Acharya 2014; Acharya et al. 2015; Zhang et al. 2010). For example, Ellis et al. (2012) identified from a preliminary screen of 24 soybean genotypes, five quantitative traits loci (QTL) for resistance to *F.graminearum* isolate recovered from soybean in Ohio using a rolled towel assay. The soybean genotypes were inoculated using macroconidia suspension of the *F.graminearum* isolate and evaluated 7 days after inoculation by determining disease severity index using lesion length caused by the fungal isolate and plant length for each seedling. From the five QTLs, four resistance alleles were from Conrad and one from Sloan. Acharya (2014) evaluated soybean germplasm, parents and recombinant inbred lines (RILs) of two soybean populations: ‘Wyandot’ x PI 567301B and ‘Conrad x Sloan’ using the rolled towel assay and 100 µl suspension of *F.graminearum* macroconidia as inoculum. From the 200 soybean genotypes evaluated for resistance, Acharya (2014) identified 30 soybean genotypes with moderate to high levels of resistance to *F.graminearum*. Soybean genotypes PI567301B and ‘Conrad’ were identified with high and moderate levels of resistance to *F.graminearum* respectively. Zhang et al. (2010) on the other hand evaluated in the greenhouse 57 commercial soybean cultivars (MG 00 to 0) for resistance to a single isolate of *F.graminearum* recovered from soybean roots from a crop rotation experiment in Ontario, Canada. Fourteen days after inoculation using wheat kernel colonized by the

F.graminearum isolate mixed with soil, nine cultivars were observed as most resistant to *F.graminearum* based on root rot severity caused by the isolate.

In all these studies, a local isolate of *F.graminearum* was used for screening soybean accessions for resistance to the fungus. Since isolates of *F.graminearum* from a local population and from different geographical regions are known to vary in their virulence (Diaz Arias et al. 2013a), there is a need to evaluate soybean accessions for resistance to *F.graminearum* using a local isolate. In addition, most of the previous screening evaluations for resistance to *F.graminearum* were focused mostly on soybean accessions belonging to maturity groups (MG) 00 and IV and less of early maturity groups (0 to III) adapted for South Dakota. These evaluations were performed in the greenhouse as disease phenotyping can be efficient in a controlled environment where the soybean cultivars are challenged with a single pathogen. The objective of this study was to identify soybean accessions resistant to *F.graminearum* in MG 00 to V from the United States Development of Agriculture (USDA) germplasm collection (Urbana, IL) in the greenhouse.

Materials and Methods

Screening of soybean accessions for resistance

In total, 247 soybean accessions (excluding two control checks) obtained from Iowa State University (ISU) in Ames, Iowa and the USDA soybean germplasm collection were evaluated for their resistance to root rot caused by *F.graminearum*. The 247 accessions ranged in maturity groups 00 to V and originated from 24 countries (Table 4.1). The accessions were selected to represent a diverse maturity group and included accessions previous evaluated for their resistance to root rot caused by *F.graminearum* (Ellis et al.

2012; Zhang et al. 2010) and *F.virguliforme* (Mueller et al. 2002). Two cultivars 'Asgrow 1835' and 'Williams 82' (Resistance to *F.graminearum* is unknown) were used as the checks.

For inoculum, the *F.graminearum* isolate (FUS052) recovered from diseased soybean roots sampled from McCook County, SD was used to inoculate the roots of the soybean accessions and determine their resistant levels. The isolate was arbitrarily selected from 15 isolates used to study aggressiveness in a greenhouse experiment (Okello et al. *unpublished*). In the aggressiveness experiment, all the 15 isolates of *F.graminearum* had a median root rot severity of 3 (= germination and 20 to 74% of the root having lesions; Okello and Mathew, *unpublished*) on Asgrow 1835.

For screening, the inoculum was prepared by growing the *F.graminearum* isolate on a sand-cornmeal mixture (9:1) that was twice autoclaved in aluminium foil steam table pans (25.5 in W x 13 in D x 3 in H). The inoculum was incubated for 2 weeks at 22±2°C in the lab and mixed every other day with sterilized spatula until the sand-cornmeal mixture was fully colonized. At planting, the inoculum layer method (Okello et al. 2019) was used in which 40 g (~60 mm level) of coarse dry vermiculite was added into 473 ml plastic drinking cups, followed by 20 g (~10 mm level) of fungal colonized sand-cornmeal inoculum and another 20 g layer of vermiculite. In each cup, five seeds of each of the accessions were planted and covered with approximately 20 g layer of vermiculite. The number of seedlings was thinned to three per cup after the seedling emergence. Non-inoculated control plants were included in which seeds were grown in vermiculite with no layer of the fungal inoculum. The cups were arranged on the greenhouse bench in a randomized complete block design, watered to saturation at planting and thereafter every

other day as per the volumetric water holding capacity of ~61%. Each plastic cup was considered as an experimental unit, and consisted of three replicate blocks per accession. A cycle of 12 h of light (light intensity of 450 $\mu Em^{-2}s^{-1}$) and 12 h of darkness was maintained in the greenhouse, with day and night temperatures of 23 ±2°C. No fertilizer was added and the experiment was performed twice.

At 21 days post-inoculation, the seedlings were gently removed from the cups and the vermiculite was washed off the roots. Root rot caused by *F.graminearum* was evaluated for each seedling on a 1 - to -5 scale (Acharya et al. 2015), where, 1 = germination and healthy seedlings with no visible root colonization, 2 = germination and 1 to 19% of the root having lesions, 3 = germination and 20 to 74% of the root having lesions, 4 = germination and 75% or more of the root having lesions; and 5 = no germination and complete colonization of seed. To confirm root infection due to *F.graminearum* and fulfill Koch's postulates, diseased roots of soybean plants were selected at random to re-isolate the fungus.

Data analysis

To analyze root rot severity caused by *F.graminearum* on roots of each soybean accession line, the median rating of the three plants in a cup was calculated. Fligner-Killen test of homogeneity of variance (Conover et al. 1981) in R (R Core Team 2013) was satisfied among the replicate blocks ($P = 0.43$). Since the root rot severity data was ordinal, the data were analyzed using the nonparametric procedure described by Shah and Madden (2004). The analysis of variance type test statistics (ATS) of ranked data was conducted using the nparLD package (Noguchi et al. 2012) in R (R Core Team 2013). For each accession line, the rank was calculated as "$\overline{R}_i = \frac{1}{n_i}\sum_{k=1}^{n_i} R_{ik}$ (Akritas 1991), where $\overline{R}_i$ = the

mean rank for the ith treatment, and R_{ik} = the rank of X_{ik} among all N observations" (Shah and Madden 2004).The root rot severity caused by *F.graminearum* on each accession line was expressed as relative treatment effects (RTE), and was calculated from mean ranks ($\overline{R}_i$) as $\widehat{p_i} = \frac{1}{N}(\overline{R}_i - \frac{1}{2})$ [where N = the total number of observations (Shah and Madden 2004)], and compared at 95% confidence intervals using the nparLD package.

Results

Screening of soybean accessions for resistance

The *F.graminearum* isolate FUS-052 caused root rot on the seedlings of 247 accessions; however, the disease severity varied among the accessions. Out of the 247 accessions, *F.graminearum* was observed to kill the seedlings before emergence or after emergence in 85 accessions. In the remaining accessions, the fungus produced dark brown lesions that were either small (~ 5 mm in length) or covered the entire seedling.

A significant effect of RTE (ATS = 23.50; df = 1.96; $P = 8.88 \times 10^{-11}$) caused by *F.graminearum* isolate was observed on seedlings of 249 accessions (including the two controls) at 21 days after inoculation. Compared to Williams 82 and Asgrow 1835, eight accession lines were observed to be significantly less susceptible to the *F.graminearum* isolate (Table 4.1). The eight accessions originated from Austria (PI361090-MG I), China (PI88788 -MG III; PI437949 -MG I; PI612761A - MG 0; PI438094B -MG 1; and PI567301B –MG IV), Japan (PI438292-MG I), and South Korea (PI408309 - MG IV).

Discussion

In this study, 247 soybean accessions were screened for their resistance to a single isolate of *F.graminearum* recovered from soybean roots in McCook County, South Dakota.

Eight accessions (PI437949, PI438292, PI612761A, PI438094B, PI567301B, PI408309, PI361090, and PI88788) from four countries (Austria, China, South Korea, and Japan) were observed to be significantly less susceptible to *F.graminearum* when compared to the controls, Williams 82 and Asgrow 1835. These accessions have been reported to have resistance to other soybean pathogens such as *Phytophthora sojae* Kaufmann and Gerdemann, the causal pathogen of Phytophthora root and stem rot, and soybean cyst nematode (SCN). For example, PI408309 has resistance against root rot caused by races of *Phytophthora* (Dorrance and Schmitthenner 2000) while PI88788 have resistant genes against SCN race 14, and race 3 and moderate resistance to race 5 (Anand and Gallo 1984; Glover et al. 2004). As for PI408309, it has been reported to be resistant to Frogeye race 2; Phytophthora rot race 17; and Phytophthora rot race 25 and is mostly resistant to SDS (Dorrance and Schmitthenner 2000; Mueller et al. 2002; Nelson et al. 1987). This may indicate that the resistance to *F.graminearum* in the accessions is linked to alleles contributing to *Phytophthora* and SCN resistance. Regardless, these eight accessions can be used as potential sources of resistance to *F.graminearum* in breeding programs.

Among the soybean accessions selected to screen for resistance to *F.graminearum*, PI567301B is a key accession for greenhouse resistance as it was identified to be significantly less susceptible to the *F.graminearum* isolate used in this study and in the study by Acharya (2014). PI567301B was used in the study by Acharya et al. (2015) to identify quantitative trait loci (QTL) associated with resistance to *F.graminearum* and they concluded that in the cross of PI567301B with Wyandor, that PI567301B contributed the resistance alleles of two QTL (one major on chromosome 8 and one minor on chromosome 6) identified conferring resistance to *F.graminearum*. Among the other accessions,

PI361090 was previously screened for resistance against a single isolate of *F.proliferatum*, *F.sporotrichioides,* and *F.subglutinans,* as well as the *F.graminearum* isolate used in this study (Okello et al. *unpublished*). PI361090 was observed to be significantly less susceptible to *F.graminearum* and *F.subglutinans* when compared to the controls, Asgrow 1835 and Williams 82, and to *F.proliferatum* when compared to the control Asgrow 1835; however, the study by Okello et al. (*unpublished*) did not perform mapping analyses to confirm the presence of resistance genes in PI361090. To the best of our knowledge, the other accessions identified in this study that are significantly less susceptible to *F.graminearum* when compared to the controls, PI437949, PI438292, PI612761A and PI438094B have not been previously screened for resistance to *F. graminearum*.

In this study, among the 247 soybean accessions (excluding the two susceptible checks) screened with a single isolate of *F.graminearum*, eight accessions were identified as potential parental materials in breeding programs to develop resistant cultivars to root rot caused by *F.graminearum*. However, it has to be noted that the resistance in soybean cultivar may vary with isolates within *F.graminearum*, greenhouse conditions and the screening methods used for inoculations. For this study, the inoculum layer method was used to screen soybean accessions for resistance to *F.graminearum* in the greenhouse maintained at day and night temperatures of 23 ±2°C. The disease severity caused by the *F. graminearum* was then evaluated at 21 days after inoculation. Therefore, further screening is required to determine resistance to *F.graminearum* among accessions under field conditions and at multiple locations. Future studies will focus on association mapping analysis to identify resistance genes regions in these accessions associated with *F.graminearum*. The findings from this study, therefore, provide information on accessions

that may be used to develop breeding lines that resist infection by *F.graminearum* and ensure sustainable yields for soybean farmers.

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Table 4.1. Information on the soybean accessions evaluated for resistance to *Fusarium graminearum* isolate FUS052.

Soybean accession[a]	Country of Origin	Maturity group	Relative treatment effect (RTE)[b]
FC30684	China	0	0.78
PI131531	Poland	I	0.78
PI153229	France	I	0.78
PI153250	Belgium	I	0.78
PI153251	Unknown	0	0.78
PI153265	France	I	0.78
PI153306	France	0	0.78
PI154194	Netherlands	0	0.78
PI154196	Netherlands	0	0.78
PI189857	France	0	0.78
PI189870	France	0	0.78
PI189873	France	0	0.78
PI189876	France	0	0.78
PI189919	France	I	0.78
PI189950	France	0	0.78
PI189951	France	0	0.78
PI205085	Japan	I	0.78
PI227325	Japan	I	0.78
PI227565	Japan	0	0.78
PI232996	Germany	0	0.78
PI248509A	China	I	0.78
PI253652C	China	I	0.78
PI253658A	China	I	0.78
PI257436	Germany	00	0.78
PI290149	Hungary	I	0.78
PI291331	China	0	0.78
PI297513	Russia	I	0.78
PI297523	China	0	0.78
PI297538	Hungary	I	0.78
PI 347549	Kazakhstan	0	0.78
PI358316C	Japan	0	0.78
PI378658	Ukraine	0	0.78
PI 378674A	Bulgaria	0	0.78
PI417139	Japan	I	0.78
PI424148	South Korea	0	0.78

PI437090	Russian Federation	0	0.78
PI437098	Russian Federation	I	0.78
PI437141	Russian Federation	0	0.78
PI437174B	Russian Federation	I	0.78
PI437202	Moldova	0	0.78
PI437207	Moldova	0	0.78
PI437230	Moldova	0	0.78
PI437238	Moldova	0	0.78
PI437255	Moldova	0	0.78
PI437263	Moldova	0	0.78
PI437295	Moldova	0	0.78
PI437306A	Russian Federation	0	0.78
PI437425	Russian Federation	I	0.78
PI437519	Russian Federation	I	0.78
PI437558	China	I	0.78
PI437570	China	0	0.78
PI437594A	China	I	0.78
PI437682A	China	I	0.78
PI437712	China	0	0.78
PI437716A	China	I	0.78
PI437738B	China	I	0.78
PI437757	China	I	0.78
PI437982	China	0	0.78
PI437995A	China	0	0.78
PI438031	China	I	0.78
PI438148	China	0	0.78
PI438218	China	I	0.78
PI438239A	China	0	0.78
PI467311A	China	I	0.78
PI475821	China	0	0.78
PI483459	China	I	0.78
PI507201	Japan	0	0.78
PI507685B	Ukraine	0	0.78
PI507688	Moldova	0	0.78
PI512322C	Georgia	I	0.78
PI518706A	China	I	0.78
PI522188A	Russian Federation	I	0.78
PI538403	Japan	I	0.78
PI538410B	Japan	I	0.78
PI548329	Japan	I	0.78
PI567223	Russian Federation	I	0.78

PI578374	China	I	0.78
PI578375B	China	I	0.78
PI578380A	China	I	0.78
PI578384	China	I	0.78
PI578474	China	I	0.78
PI578485A	China	0	0.78
PI592907C	Russian Federation	I	0.78
PI 593970	Japan	I	0.78
PI594170B	Japan	I	0.78
PI594296	Japan	I	0.78
PI594898	China	I	0.78
PI594902	China	I	0.78
PI603339A	China	I	0.78
PI603371	China	I	0.78
PI603426F	China	I	0.78
PI603546A	China	I	0.78
PI603587A	China	I	0.78
PI603704A	China	I	0.78
PI612752	China	I	0.78
PI612759C	China	I	0.78
PI612760	China	I	0.78
PI70241	China	I	0.78
PI81765	China	I	0.78
PI88295	China	I	0.78
PI88497	China	I	0.78
PI89060	China	I	0.78
PI91733	China	I	0.78
PI189903	France	0	0.70
PI378663	Russian	0	0.70
PI378679	France	I	0.70
PI538393	China	I	0.70
PI561346	China	I	0.70
PI437561	China	0	0.63
PI437812	China	0	0.63
PI437846	China	I	0.63
PI445827B	Romania	0	0.63
PI71161	China	I	0.63
PI437156C	Russian Federation	I	0.48
PI437553	China	0	0.48
PI445819	Germany	I	0.48
PI79648	China	I	0.48

PI468907	China	I	0.40
PI520733	South Korea	IV	0.40
PI647087	USA	III	0.39
PI562387	Japan	I	0.38
PI132207	Netherlands	0	0.32
PI181536	Japan	I	0.32
PI189866	France	0	0.32
PI189916	China	I	0.32
PI189961	France	0	0.32
PI238921	Germany	0	0.32
PI243547	Japan	0	0.32
PI250844	Iran	I	0.32
PI290116A	Hungary	0	0.32
PI291276	China	I	0.32
PI291277	China	I	0.32
PI291278	China	I	0.32
PI291309C	China	I	0.32
PI297532	China	0	0.32
PI326579	Romania	I	0.32
PI342619A	Russian Federation	0	0.32
PI347565B	China	0	0.32
PI358323	China	0	0.32
PI408235	South Korea	IV	0.32
PI417458	Japan	0	0.32
PI417517	Former Serbia and Montenegro	I	0.32
PI424216	South Korea	IV	0.32
PI424221B	South Korea	IV	0.32
PI424354	South Korea	IV	0.32
PI437091	Russian Federation	I	0.32
PI437100	Russian Federation	0	0.32
PI437116	Russian Federation	I	0.32
PI437165A	Russian Federation	I	0.32
PI437267	Moldova	0	0.32
PI437343	Russian Federation	I	0.32
PI437509	Russian Federation	I	0.32
PI437563	China	II	0.32
PI437786	China	I	0.32
PI437886B	China	II	0.32
PI445833	Romania	0	0.32
PI458095	South Korea	IV	0.32

PI458144	South Korea	IV	0.32
PI461509	China	I	0.32
PI467313	China	0	0.32
PI467324	China	I	0.32
PI504485	Japan	I	0.32
PI506595A	Japan	II	0.32
Williams 82 (PI518671; Check)	USA	III	0.32
PI518711	China	II	0.32
PI525453	USA	II	0.32
PI548398	Canada	00	0.32
PI548504	Canada	00	0.32
PI548592	Canada	00	0.32
PI548655	USA	V	0.32
PI561232	China	I	0.32
PI561242	China	0	0.32
PI561315	China	I	0.32
PI567163	China	I	0.32
PI567173	China	00	0.32
PI567229B	Russian Federation	I	0.32
PI567417B	China	I	0.32
PI578362	China	I	0.32
PI578385	China	I	0.32
PI578386	China	I	0.32
PI578505	China	II	0.32
PI592912A	Russian Federation	I	0.32
PI593654	USA	IV	0.32
PI595843	USA	II	0.32
PI602497A	China	I	0.32
PI603151A	North Korea	I	0.32
PI603334	China	I	0.32
PI603690	China	IV	0.32
PI603706A	China	IV	0.32
PI603753B	China	III	0.32
PI642768	USA	III	0.32
PI643146	USA	IV	0.32
PI676304	Unknown	II	0.32
PI79694	China	I	0.32
PI92706	China	I	0.32
Asgrow 1835 (Check)	USA	III	0.32
PI153282	Belgium	0	0.23

PI248403	Serbia	0	0.23
PI291274B	China	I	0.23
PI291275	China	I	0.23
PI291319B	China	0	0.23
PI326580	Germany	I	0.23
PI401418	Russian Federation	I	0.23
PI407655B	China	II	0.23
PI408211B	South Korea	0	0.23
PI417513B	Eastern Europe	I	0.23
PI423936	Japan	II	0.23
PI437477A	Russian Federation	I	0.23
PI437523	Turkmenistan	0	0.23
PI458825B	China	I	0.23
PI467307	China	I	0.23
PI468906	China	0	0.23
PI468910	China	0	0.23
PI476345	Moldova	I	0.23
PI506678	Japan	I	0.23
PI540556	USA	II	0.23
PI548354	China	0	0.23
PI548607	Canada	00	0.23
PI561333	China	I	0.23
PI567159A	China	I	0.23
PI567229A	Russian Federation	I	0.23
PI567516C	China	IV	0.23
PI578382	China	I	0.23
PI584469	USA	III	0.23
PI592534	USA	00	0.23
PI597397A	Russian Federation	I	0.23
PI597405B	Ukraine	I	0.23
PI603337A	China	I	0.23
PI603424C	China	I	0.23
PI612711B	China	I	0.23
PI612754	China	I	0.23
PI257432	Germany	0	0.14
PI438376	France	I	0.14
PI468904	China	0	0.14
PI548311	Canada	0	0.14
PI54854	China	I	0.14
PI548544	Canada	00	0.14
PI548595	Canada	00	0.14

PI593982	Japan	I	0.14
PI79727	China	I	0.14
PI88310	China	III	0.14
PI361090	Austria	I	0.05
PI408309	South Korea	IV	0.05
PI437949	China	I	0.05
PI438094B	China	0	0.05
PI438292	Japan	I	0.05
PI567301B	China	IV	0.05
PI612761A	China	0	0.05
PI88788	China	II	0.05

[a]Soybean accessions that were evaluated for resistance to *F.graminearum* isolate FUS052 on a 1 to 5 rating scale (Acharya et al. 2015).

[b]The root rot severity caused by the *F.graminearum* isolate FUS052 on the soybean accessions were expressed as relative treatment effect (RTE). The RTE in bold indicate significant differences among soybean accessions and the susceptible checks in response to screening against *F.graminearum* isolate FUS052.

CHAPTER 5

Interaction of *Fusarium graminearum* and *F.proliferatum* with *Heterodera glycines* on soybean (*Glycine max* L.)

A paper to be submitted to the journal *Plant Disease*

Abstract

In this study, the interaction of *F.graminearum* and *F.proliferatum* with *Heterodera glycines* was investigated on a SCN susceptible cv. Williams 82 in the greenhouse. The experiments were set up in a randomized complete block design using an inoculum-layer method with four treatments (fungus only, SCN only, fungus + SCN and non-inoculated control) and four replications per treatment. At 40 days post-inoculation, no synergism was detected between the fungi and SCN. No significant differences in stem and root lengths and root dry weight were observed among fungus only, SCN only and fungus + SCN treatments for *F.graminearum* and *F.proliferatum*. The shoot dry weight was not significantly different among *F.graminearum*, SCN, and *F.graminearum* + SCN treatments. However, the shoot dry weight was significantly different among *F.proliferatum*, SCN and *F.proliferatum* + SCN treatments. Root rot severity caused by the two fungi was not significantly increased in the presence of SCN. The nematode egg counts were not affected by *F.graminearum* when compared to the SCN only treatment. However, the presence of *F.proliferatum* inhibited SCN reproduction. Future studies involving seed treatments or host resistance may be necessary to study the interaction between the two pathogens.

Introduction

Soybean cyst nematode (*Heterodera glycines* Ichinohe, SCN) is one of the major production constraints of soybean, *Glycine max* (L) Merrill in the United States and Canada (Ontario) (Allen et al. 2017). The nematode was first discovered in the U. S. state of North Carolina in 1954 (Winstead et al. 1955). Since 1954, SCN has spread to 30 states in the United States and into Canada (Poromarto et al. 2010; Tylka et al. 2017). In 2014, yield losses from SCN in the United States and Canada (Ontario) were estimated to be 3.5 million metric tons ($1.3 billion of revenue according to the 2014 market values for soybean) (Allen et al. 2017). The nematode reduces yield by feeding on nutrients from the plants, reducing root and plant growth, disrupting uptake of water and nutrients from roots, and inhibiting root nodulation of soybean (Poromarto et al. 2010; Williamson and Hussey 1996). However, the challenge with SCN is that there can be a 30% yield loss without any obvious aboveground symptoms such as stunting, yellowing, and wilting of soybean plants (Niblack et al. 1992; Noel and Edwards 1996; Riggs and Niblack 1999; Wang et al. 2003; Young 1996).

This hidden yield loss can be a problem for soybean growers who may not realize that they have SCN in their fields. In subsequent years, the symptoms caused by SCN become visible on soybean plants, which may be influenced by the nematode population density, soil texture, soil fertility, plant age and vigor, soil moisture, and planting of susceptible varieties (Chen et al. 2001; Davis and Tylka 2000; Donald et al. 2006; Koenning 2004; Koenning and Barker 1995; Niblack et al. 1992; Wang et al. 2003; Wheeler et al. 1997). Regardless of whether symptoms appear in a field, once SCN becomes established, the nematode cannot be eradicated. However, SCN can be managed

by planting non-hosts (crop rotation for two years) or nematode-resistant soybean varieties. Despite that management options are available to soybean farmers, SCN continues to be a problem because the nematode is adapting to PI88788, which is the single source of resistance used in more than 95% of commercial SCN-resistant soybean varieties (Niblack et al. 2008).

Another possible challenge with SCN is that it is known to interact with other plant pathogens on soybean (Adeniji et al. 1975; Back et al. 2002; Brzostowski et al. 2014; Díaz Arias 2012; Gao et al. 2006; Lawrence et al. 1988; McLean and Lawrence 1993, 1995; Meyer et al. 1998; Pacumbaba 1992; Roy et al. 1989; Sanogo and Yang 2001; Tabor et al. 2003; Xing and Westphal 2006). For instance, Tabor et al. (2003) observed that SCN can increase the incidence and severity of brown stem rot caused by *Cadophora gregata* (Allington and Chamberlain) Harrington and McNew [syn. *Phialophora gregata* (Allington and Chamberlain) Gams] on soybean cultivars with resistance to either of the pathogens under growth chamber conditions. Adeniji et al. (1975) observed an interaction between *Phytophthora sojae* Kaufmann and Gerdemann (the causal agent of Phytophthora root and stem rot of soybean) and SCN in the greenhouse. They observed that seedlings of soybean cultivars ‘Corsoy’ (susceptible to *P. sojae* and SCN) and ‘Dyer’ (susceptible to *P. sojae* and resistant to SCN) had a higher disease rating when the two pathogens were present as compared to when the seedlings were inoculated with only *P. sojae*.(Adeniji et al. 1975).

Among the species of *Fusarium* whose association with SCN have been investigated, the interaction between *F.virguliforme* O’Donnell and Aoki and *H.glycines* is the most studied. Hirrel (1983) was the first to observe the association of SCN with

sudden death syndrome (SDS) caused by *F.virguliforme* in 30 soybean fields across four U. S. states. Since the study by Hirrel (1983), there have been several studies on the synergistic interaction between SCN and *F.virguliforme* (Lawrence et al. 1988; McLean and Lawrence 1995, 1993a, b; Melgar et al. 1994; Roy et al. 1989; Xing and Westphal 2006). For example, in the greenhouse, concomitant inoculation of soybean seedlings with *F.virguliforme* and SCN resulted in greater severity of SDS foliar symptoms than when the seedlings were inoculated with the fungus only (Lawrence et al. 1988; McLean and Lawrence 1995, 1993a; Roy et al. 1989). In the field, McLean and Lawrence (1993a) and Xing and Westphal (2006) observed that SDS foliar symptoms were more severe on soybean plants in plots infested with both *F.virguliforme* and SCN when compared to those plots inoculated with the fungus only. The McLean and Lawrence (1993a) study also observed that soybean yields were suppressed in plots containing both *F.virguliforme* and SCN compared to *F.virguliforme* only. In contrast to these studies, Gao et al. (2006) observed that SCN did not increase the severity of SDS foliar symptoms on soybean plants in the greenhouse.

While *F.virguliforme* is an economically important pathogen, there are other species of *Fusarium* causing root rot of soybean in the United States and Canada (Bienapfl et al. 2010; Broders et al. 2007; Chang et al. 2015; Carter et al. 2002; Díaz Arias et al. 2011; Díaz Arias et al. 2013; Ellis et al. 2012; French and Kennedy 1963; Hartman et al. 1999; Pioli et al. 2004; Xue 2007; Zhang et al. 2013; Zhou et al. 2018). For example, Diaz Arias et al. (2013) identified nine species of *Fusarium* causing root rot of soybean, among which the greatest root rot severity was caused by *F.graminearum* Schwabe on

soybean plants, followed by *F.virguliforme*, *F.proliferatum* Matsushima, *F.sporotrichioides* Sherbakoff, and *F.solani* (Martius) Saccardo.

In South Dakota, seedling and root diseases of soybean were observed in the lower areas of the commercial fields with symptoms such as yellowing, loss of stand, wilting, and stunting of plants in 2014 and 2015 (F. Mathew, *unpublished*). Surveys were conducted across the soybean producing counties of South Dakota to assess the presence of fungal pathogens causing root rot and species of *Fusarium* were identified to be most prevalent. Of the total 11 species of *Fusarium* identified on soybean, *F.acuminatum* Ellis and Everhart, *F.graminearum* Schwabe, *F.oxysporum* Schlecht, *F.proliferatum* Matsushima, and *F.sporotrichioides* Sherbakoff, were associated with soybean plants having foliar symptoms (wilting and yellowing) and SCN cysts on the roots. While SCN is known to predispose soybean plants to invasion by *F.virguliforme* based on greenhouse and field experiments (Lawrence et al. 1988; McLean and Lawrence 1995, 1993a, b; Melgar et al. 1994; Roy et al. 1989; Xing and Westphal 2006), there is no information available on whether other species of *Fusarium* can interact with the nematode. Among these five pathogens, *F.graminearum* and *F.proliferatum* are known to survive in warm and humid soil conditions (Doohan et al. 2003; Elmer 2001, 2000; Moretti et al. 1997; Vigier et al. 1997), which may coincide with the optimal temperature for SCN hatching and development (24 and 30°C; Alston and Schmitt 1988). Thus, the objective of this study was to get a basic understanding of the influence that SCN has on root rot of soybean caused by *F.graminearum* and *F.proliferatum*; and the effect of the two fungal pathogens on the reproduction of the nematode on a SCN-susceptible soybean cultivar ('Williams 82') in the greenhouse.

Materials and methods

Source of *F.graminearum* and *F.proliferatum* inoculum

For this study, a single isolate of *F.graminearum* (FUS052) and *F.proliferatum* (FUS026) was used, which were selected based on the level of aggressiveness of 57 soybean isolates of *Fusarium* on a soybean susceptible variety in the greenhouse (Okello et al. 2016). It was determined in the study by Okello et al. (2016) that significant differences in aggressiveness were not observed among isolates within *F.graminearum*. As for *F.proliferatum*, the isolate which caused the greatest root rot severity rating (i.e. 4 = germination and 75% or more of the root having lesions) was selected for this study. These isolates were recovered from diseased soybean roots sampled from McCook County, in South Dakota (Okello and Mathew 2019).

To prepare inoculum, the *F.graminearum* isolate and *F.proliferatum* isolate were cultured on potato dextrose agar (PDA) and the PDA plates were incubated for seven days at 22±2°C under 12-h fluorescent light. Ten plugs (15 mm squared) taken from the edge of 7-day-old fungal colonies were transferred to 1-liter flasks containing 400 g of Japanese millet (*Echinochloa esculenta* (Braun) Scholz) grains that were previously autoclaved twice at 121°C for 60 min. The flasks were incubated (22±2°C) for 14 days in the lab under 12-h fluorescent light. The inocula were mixed every two days by manually shaking the flasks to ensure full colonization of the millet grains by the fungus.

Source of SCN inoculum

An HG type 0 isolate of SCN from Brookings County, SD was provided by Dr. E. Byamukama (South Dakota State University, Brookings, SD). An HG type 0 population of SCN is defined as "having less than 10% reproduction on all published sources of

H.glycines resistance” (McCarville et al. 2014). The HG type 0 isolate was maintained on cv. ‘Williams 82’ in the greenhouse at 22±2°C for 35 days prior to this experiment.

To obtain inoculum, the nematode cysts were extracted from the soil by decanting and sieving method. The soil containing SCN (in subsamples of 100 cc) was placed into a 2-gallon bucket that was subsequently half filled with water. The soil and water mixture was mixed by stirring with hand and then allowed to stand until water almost stops swirling. The resulting suspension was poured through the USA standard testing sieves 850-µm-pore sieve (No. 20), which was nested over a 250-µm-pore sieve (No. 60). The soil contents that were collected on the two sieves were rinsed with a spray of tap water, and the soil debris on the 850-µm-pore sieve was discarded. The SCN cysts on the 250-µm-pore sieve were washed into a beaker with a spray of water and the water level was brought to 50 ml. The cysts were ruptured with a rotating rubber stopper to release eggs (Faghihi and Ferris 2000), which were then collected under USA standard testing sieves 75-µm-pore sieve (No. 200) nested over a 25-µm-pore sieve (No. 500). SCN eggs were suspended in 100 ml of distilled water. A 1 ml sample of the suspension was collected. The number of eggs present were counted under a dissecting microscope at 40X magnification (Olympus BX41, Leeds Precision Instruments, Inc., Minneapolis, MN) using a nematode counting slide (Chalex Cooperation, Portland, OR). The total number of SCN eggs was adjusted to approximately 2000 eggs (2000 ± 100) per ml by diluting the suspension of eggs with distilled water.

Experimental design

The experiments were arranged separately for *F.graminearum* and *F.proliferatum* in a randomized complete block design (RCBD). The treatments were fungus only, SCN

only, fungus + SCN, and a non-inoculated control. Each treatment had four replications represented by 7.5-liter plastic buckets. The buckets were filled with sand, and each had five 164 ml plastic cones (Ray Leach Cone-tainers, Stuewe and Sons, Inc., Tangent, OR) of the same treatment. The buckets were placed in a greenhouse water bath maintained at a temperature of 25±3°C and under 16-h fluorescent light (intensity of 1000 $\mu Em^{-2}s^{-1}$). The water bath was maintained at an average temperature of 25°C for SCN development based on previous studies (Lauritis et al. 1983; Skotland 1957). For *F.graminearum* and *F.proliferatum*, the pathogenicity experiments on different hosts have been established in several studies at any temperature between 16 and 26°C (Bilgi et al. 2011; Chang et al. 2015; Cong et al. 2016; Díaz Arias et al. 2013; Ellis et al. 2012; Elshahawy et al. 2017; Hudec and Muchova 2010; Marin et al. 1995; Scruggs and Quesada-Ocampo 2016; Sutton 1982).

To set up the interaction experiment, the inoculum layer method modified from Bilgi et al. (2008) was used. Seeds of cv. Williams 82 were germinated for three days on a damp 1-ply brown paper towel before planting. For the fungus only treatments, the cones were filled with 60 g of steam pasteurized sand: soil (3-parts construction sand: 1-part silty clay loam soil) mixture, which was followed by 20 g of fungal inoculum and then 20 g of the sand: soil mixture. One pre-germinated seed of Williams 82 was planted into the sand: soil mixture and an additional 20 g of the sand: soil mixture was used to cover the soybean seedling. For the fungus + SCN treatments, after the pre-germinated seed was covered with 20 g of the sand: soil mixture, a hole (20 mm deep) was made close to the seedling using a glass rod (0.5 mm diameter) and a suspension of approximately 2000 eggs per ml was added into the hole using a disposable pipette (Frohning 2013). After adding the nematode,

the hole was covered with sand: soil mixture. For the SCN only treatments, the cones were filled as described previously for the fungus + SCN treatments but with twice autoclaved non-infested millet grains. For all SCN treatments, the suspension containing nematode eggs was mixed uniformly before pipetting into the hole. Additionally, care was taken to ensure that the primary root (radicle) of the seedling was in contact with the suspension containing the SCN eggs. The non-inoculated treatment was set up by first filling the cone with 100 g sand: soil mixture, and then placing the seedling into the mixture and covering the seedling with 20 g of the sand: soil mixture. For all treatments, one square piece (127 mm by 127 mm) of a weed barrier fabric (The Master Gardner Company, Spartanburg, SC) was placed at the outer end of each cone and held by a rubber band to reduce drainage and contain the soybean roots within the cones. The soybean plants in each cone were watered once every other day with approximately 17 ml of tap water, which was approximately 50% of the water holding capacity of sand: soil mixture. No fertilizer was added to any of the cones during the experiment. The experiments were performed a total of four times.

Data collection

At 40 days after SCN inoculation, when the nematode would have completed one life cycle, the experiments were terminated. The soybean plants were removed from cones and the roots were gently washed with tap water. The stem length and root length (tap root) of plants were manually measured with a ruler. Shoot and root dry weights of each soybean plant were weighed with the help of an electronic analytical balance after the plants were air-dried in the greenhouse for five days.

To determine the pathogenicity of *F.graminearum and F.proliferatum isolates*, root rot severity was assessed on plants using a visual scale of 1 to 5 (Acharya et al. 2015);

where, 1 = germination and healthy seedlings with no visible root colonization, 2 = germination and 1 to 19% of the root having lesions, 3 = germination and 20 to 74% of the root having lesions, 4 = germination and 75% or more of the root having lesions; and 5 = no germination and complete colonization of seed. To confirm pathogenicity and fulfill Koch's postulates, the two fungi were re-isolated from the roots of randomly selected plants inoculated with either of the fungus. Briefly, the taproot of the soybean plants from these treatments was cut into three pieces (~15 mm each), surface-sterilized in 0.05% sodium hypochlorite (NaOCl) and 70% ethanol for 1 min each, rinsed in sterile distilled water and then blotted dry between sterile filter papers. The sterile root pieces were incubated on freshly prepared PDA plates for a week. After seven days of incubation at 22°C under 12 h fluorescent light, single-spore cultures of *F.graminearum* and *F.proliferatum* were prepared on fresh PDA and then transferred onto CLA for morphological identification (Leslie and Summerell 2006). DNA was extracted from the pure colonies on CLA and the pathogen identity was confirmed by sequencing the EF1-α gene region using EF1F and EF1R primers (Geiser et al. 2004).

To obtain SCN cysts, the cones containing nematode treatments were soaked in a bucket of water for 10 to 15 min to loosen soil and the soybean plants were gently removed from the cones. The root of the soybean plants was placed on a No. 20 sieve stacked over a No. 60 sieve and sprayed with a strong stream of water to collect the SCN cysts on the No. 60 sieve (Tabor et al. 2003). In addition to washing the roots, the soil mixture in the bucket was poured into the nested sieves (No. 20 on top and No. 60 on the bottom) to collect SCN cysts. The inside of the No. 60 sieve was gently washed to collect the SCN cysts and the water containing cysts was poured into a sterile 100 ml glass beaker. The

SCN cysts were ruptured with a rotating rubber stopper to release eggs (Faghihi et al. 1986). The SCN eggs were collected into a 100 ml beaker into which one eye-dropper (~0.05 ml) of acid fuchsin stain (Daykin and Hussey 1985) was added. The beaker containing SCN eggs and fuchsin stain was heated using an Isotemp Basic Stirring Hotplate (Fisher Scientific, Hampton, NH) to near boiling (approximately 90 s for six samples). The beakers were cooled and then placed in the refrigerator. Prior to counting, the suspension containing SCN eggs was thoroughly mixed. Using a sterile glass Pasteur pipette, 1 ml sample was drawn from the suspension and the number of SCN eggs was counted under a dissecting microscope with a 40X magnification. The total number of SCN eggs per gram of dry root weight was calculated. The non-inoculated control and the fungus only treatments were also examined for SCN eggs.

Data analysis

Data were analyzed in R (R core team 2013; https://www.rstudio.com/) at $P = 0.05$. Prior to the data analyses, homogeneity of variance tests using Bartlett's (for stem length, root length, dry shoot weight, dry root weight and number of SCN eggs) or Fligner-Killer's (for root rot severity) were performed using the stats package in R (Conover et al. 1981) to compare the variances of the four experimental runs.

The data for stem length, root length, dry shoot weight, dry root weight and number of SCN eggs reproduced per gram of dry root weight did not satisfy the normality test (Shapiro-Wilk test), and therefore were analyzed as the ordinal root rot severity data using the nonparametric method (Shah and Madden 2004). The overall effect of each treatment was determined by the ANOVA type test statistic (ATS) of ranked data using the nparLD package (Noguchi et al. 2012) in R. The mean ranks for each treatment were calculated as

"$\overline{R}_i = \frac{1}{n_i}\sum_{k=1}^{n_i} R_{ik}$, where $\overline{R}_i$ is the mean rank for the ith treatment and R_{ik} is the rank of X_{ik} among all N observations" (Akritas 1991; Shah and Madden 2004). From the mean ranks ($\overline{R}_i$), the relative treatment effects (RTE) for each treatment was calculated as "$RTE = \frac{1}{N}(\overline{R}_i - \frac{1}{2})$ where N is the total number of observations (Shah and Madden 2004)". The relative treatment effects were compared at 95% confidence intervals using the nparLD package in R.

To determine the type of interaction between SCN and the two fungi (*F.graminearum* and *F.proliferatum*), RTE values were used to calculate reduction in stem length, root length, shoot dry weight and root dry weight of soybean plants caused by the fungus and nematode, either singly or in combination, when compared to the non-inoculated control plants. A synergy was considered to occur when the reduction due to fungus + SCN treatment was greater than the total reduction due to the fungus only and SCN only treatments.

Results

Effects of *F.graminearum* isolate on soybean plant growth, root rot severity, and SCN reproduction

At 40 days after inoculation, brown lesions were observed on the roots of all soybean plants inoculated with the *F.graminearum* isolate and a combination of *F.graminearum* and SCN. From the discolored roots of the soybean plants, the *F.graminearum* isolate was recovered and identity confirmed by sequencing of EF1-α gene region. No damping off was observed on soybean plants caused by the *F.graminearum* isolate. In addition, no lesions were observed on the roots of plants

inoculated with SCN only or from the control treatment and neither was *F.graminearum* isolated from these plants.

The *F.graminearum* isolate was observed to affect all the host response variables measured in the presence of SCN at 40 days post inoculation when compared to the other treatments (Table. 5.1). A significant effect of RTE caused by *F.graminearum* isolate was observed for stem length (ATS = 16.17; df = 2.02; $P = 8.40 \times 10^{-8}$), root length (ATS = 15.58; df = 2.16; $P = 6.31 \times 10^{-8}$), dry root weight (ATS = 13.41; df = 2.44; $P = 1.62 \times 10^{-7}$), dry shoot weight (ATS = 14.79; df = 2.00; $P = 3.75 \times 10^{-7}$), root rot severity (ATS = 160.09; df = 1.00; $P = 1.08 \times 10^{-36}$) and SCN egg counts (ATS = 69.97; df = 1.67; $P = 1.72 \times 10^{-26}$) at 40 days after inoculation.

Root length was significantly reduced by fungus only, and fungus + SCN when compared to the control. In addition, root length of plants inoculated with SCN only was significantly lower than that of control plants (Table. 5.1).

The dry shoot and dry root weights were significantly lower when the soybean plants were inoculated with fungus only, SCN only and fungus + SCN as compared to the control plants.

SCN reproduction was not significantly reduced by the *F.graminearum* isolate when compared to SCN only treatment (Table. 5.1). In addition, the presence of SCN did not significantly increase root rot severity caused by *F.graminearum* isolate compared to the fungus only treatment (Fig. 5.1).

Stem length of soybean plants (expressed as RTE) was significantly reduced by 46.5% and 68.6% when plants were inoculated with fungus only and fungus + SCN respectively compared to the control (Fig. 5.2).

The interaction between SCN and *F.graminearum* was observed to be not synergistic for all variables measured based on reduction in stem length, shoot dry weight, root length and root dry weight in soybean plants inoculated with *F.graminearum* only, SCN only and, *F.graminearum* + SCN compared to non-inoculated control (Fig 5.2).

Effects of *F.proliferatum* isolate on soybean plant growth, root rot severity, and SCN reproduction

For the interaction between the *F.proliferatum* isolate and SCN, brown lesions were observed on the roots of all soybean plants inoculated with the *F.proliferatum* isolate and the combination of *F.proliferatum* and SCN. To confirm pathogenicity and fulfill Koch's postulates, *F.proliferatum* was re-isolated from the roots of randomly selected plants inoculated with the fungus. The identity of *F.proliferatum* was confirmed by sequencing of EF1-α gene region. Forty days post-inoculation, no damping off was observed on soybean plants, and neither were lesions observed on the roots of plants inoculated with SCN only or from the control treatment. *Fusarium proliferatum* was not isolated from SCN only or the control treatments.

Similar to the *F.graminearum* isolate, *F.proliferatum* was observed to affect all the response variables measured in the presence of SCN when compared to the other treatments (Table. 5.2). A significant effect of RTE caused by *F.proliferatum* isolate was observed for stem length (ATS = 15.07; df = 2.42; $P = 2.50 \times 10^{-8}$), root length (ATS = 16.97; df = 1.81; $P = 1.54 \times 10^{-7}$), dry root weight (ATS = 13.44; df = 2.31; $P = 2.94 \times 10^{-7}$), dry shoot weight (ATS = 23.83; df = 2.91; $P = 5.15 \times 10^{-15}$), root rot severity (ATS = 199.63; df = 1.0; $P = 2.52 \times 10^{-45}$) and SCN egg counts (ATS = 65.68; df = 1.68; $P = 5.68 \times 10^{-25}$) at 21 days after inoculation.

Root length was significantly reduced by the fungus only, SCN only, and fungus + SCN when compared to the control (Table. 5.2). Inoculation with the *F.proliferatum* isolate did not significantly reduce SCN reproduction when compared to SCN only treatment (Table. 5.2). The presence of SCN did not significantly increase the root rot disease severity caused by *F.proliferatum* (Fig. 5.3).

Stem length of soybean plants (expressed as RTE) was reduced by 55.2% and 67.8% when plants were inoculated with fungus only and fungus + SCN respectively compared to the control (Fig. 5.4).

The shoot dry weight and root dry weight were significantly lower when the soybean plants were inoculated with fungus only, SCN only and fungus + SCN as compared to the control plants. In addition, the shoot dry weight was significantly lower when the plants were inoculated with the fungus only and fungus + SCN compared to the SCN only.

The interaction between SCN and *F.proliferatum* was observed to be not synergistic for all host variables measured based on reduction in stem length, shoot dry weight, root length and root dry weight in soybean plants inoculated with *F.graminearum* only, SCN only and, *F.proliferatum* + SCN compared to non-inoculated control (Fig 5.4).

Discussion

In this study, single isolates of *F.graminearum* and *F.proliferatum* were used to examine the interaction of these fungi with SCN on soybean. We hypothesized that although the two fungi can negatively impact soybean plants by damaging roots, they may not affect plant growth in the presence of SCN. The results from this study show that the presence of SCN did not significantly increase the root rot severity caused by the isolates

of *F.graminearum* and *F.proliferatum.* For neither of the fungi, there was no observed significant differences in stem length, root length, shoot dry weight, and root dry weight between the fungus only, and fungus + SCN treatments. In addition, the presence of *F.graminearum* or *F.proliferatum* did not significantly reduce the reproduction of SCN eggs on 'Williams 82'.

For *F.graminearum*, the reduction in stem length, shoot dry weight, root length, and root dry weight caused by the fungus only, SCN only and fungus + SCN compared to the non-inoculated control were not synergistic at 40 days post-inoculation (Fig 5.2). The reduction of stem length caused by the fungus + SCN was greater than that caused by either the fungus or SCN. The reduction in shoot dry weight caused by the fungus was greater than that caused by the SCN and fungus + SCN. The root length reduction caused by the fungus only, SCN only and fungus + SCN were approximately the same at 60% while the reduction in root dry weight caused by SCN was greater than that by the fungus and fungus +SCN. For *F.proliferatum*, the reduction in stem length, shoot dry weight, root length, and root dry weight caused by the fungus only, SCN only and fungus + SCN compared to the non-inoculated control were not synergistic at 40 days post-inoculation (Fig 5.4). The reduction of stem length caused by the fungus + SCN was greater than that caused by either the fungus or SCN. The shoot dry weight reduction caused by the fungus was greater than that caused by the SCN and fungus + SCN. The reduction in root length caused by the fungus only was greater than that caused by either SCN only or fungus + SCN and the reduction in root dry weight caused by SCN was greater than that caused by the fungus and fungus +SCN. A possible explanation to the effect of the fungi (*F.graminearum* and *F.proliferatum*) on shoot dry weight and root length of the soybean plant is that the SCN

may be involved in modifying the physiology of the host, and thereby increasing or decreasing the susceptibility of the host plant to the fungus. The greater reduction in stem length caused by the presence of both the pathogens (either *F. graminearum* or *F. proliferatum* and SCN) may be due to the host plant response to the impact of the two pathogens. As for the greater reduction in root dry weight caused by SCN, the nematode causes wounds as it enters the roots and then creates a feeding site depleting the roots of essential nutrients. This action by the SCN likely contributes to greater root reduction.

Previous studies have observed a significant reduction in the number of SCN eggs on plants co-inoculated with *Fusarium* and SCN (Gao et al. 2006; McLean and Lawrence 1993a). In contrast, in this study, the presence of the fungus did not significantly reduce SCN egg numbers on cv. Williams 82 compared to the SCN only treatment. The lack of reduced SCN eggs numbers may have been due to cortical root decay that would limit the space for SCN development and reproduction. This speculation may hold for the effect of both the *F. graminearum* and *F.proliferatum* on soybean roots. However, it is also possible that the SCN may have infested the roots before the colonization by the fungus, and thereby outcompeted the fungus for nutrients to occupy the space for its development and reproduction. The other possibilities to explain the effect of the fungus on SCN eggs are the differences in inoculum concentration of the fungus, initial SCN population density, viability of SCN eggs and second stage juveniles, level of toxic metabolites released by the fungus and or different watering regimes. For, *F. graminearum*, while the inoculum levels were maintained consistently during each experimental run with no observable variability, the SCN egg count observed from the two cones may have been affected by the amount of inoculum from the two pathogens, and variability between the experiment replications.

However, this hypothesis has to be verified by evaluating different inoculum levels of SCN and *F.graminearum*.

In summary, this study has shown that the association of *F.graminearum* and *F.proliferatum* with SCN appeared to be not synergistic for the host response variables measured. A study by Gao et al. 2006 indicated no synergistic interaction between *F.virguliforme* (formerly *F.solani* f.sp. *glycines*) and SCN due to the use of different levels of nematode and fungi, use of different soybean cultivars and experimental conditions; and these may apply to *F.graminearum* and *F.proliferatum* used in this study. However, there is need to examine further this possibility by use of seed treatment in addition to different greenhouse conditions.

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Table 5.1. Effects of *F.graminearum* on soybean plants and SCN reproduction.

Treatment	Relative Treatment Effects (RTE)[a,b]				
	Stem length	Root length	Shoot dry weight	Root dry weight	SCN reproduction (Eggs/g of root dry weight)
F.graminearum	0.46 (0.37,0.56)	0.38 (0.26,0.55)	0.26 (0.17,0.42)	0.39 (0.28, 0.52)	0.25 (0.25,0.25)*
F.graminearum +SCN	0.27 (0.19,0.41)	0.37 (0.27,0.51)	0.39 (032, 0.46)	0.46 (0.37, 0.57)	0.76 (0.68, 0.81)
SCN	0.40 (0.28,0.56)	0.36 (0.26,0.50)	0.49 (0.35,0.63)	0.28 (0.18, 0.46)	0.74 (0.67, 0.79)
Control	0.86 (0.79,0.87)*	0.88 (0.88,0.88)*	0.86 (0.74,0.87)*	0.86 (0.82,0.87)*	0.25 (0.25,0.25)*

[a]The stem and root lengths, shoot and root dry weights, and number of SCN reproduced per root dry weight were analyzed as non-parametric data and expressed as relative treatment effects (RTE). The RTE for each treatment was determined from mean ranks ($\overline{R}_i$) as $\widehat{p_i} = \frac{1}{N}(\overline{R}_i - \frac{1}{2})$ [where N is the total number of observations; Shah and Madden 2004] and compared at 95% confidence intervals using the nparLD package in R.

[b] Asterisk indicates significant differences between treatments in response to inoculation with either fungus only, SCN only or fungus + SCN.

Table 5.2. Effects of *F.proliferatum* on soybean plants and SCN reproduction.

Treatment	Relative Treatment Effects (RTE)[a,b]				
	Stem length	Root length	Shoot dry weight	Root dry weight	SCN reproduction (Eggs/g of root dry weight)
F.proliferatum	0.39 (0.27,0.53)	0.27 (0.17,0.46)	0.26 (0.19, 0.37)*	0.43 (0.34,0.53)	0.25 (0.25, 0.25)*
F.proliferatum +SCN	0.28 (0.20, .41)	0.39 (0.31,0.50)	0.33 (0.26, 0.43)*	0.41 (0.31,0.53)	0.70 (0.62, 0.75)
SCN	0.46 (0.35, .58)	0.46 (0.37,0.55)	0.55 (0.46,0.63)**	0.29 (0.19,0.46)	0.80 (0.70, 0.85)
Control	0.87 (0.80, 87)*	0.88 (0.88, 88)*	0.86 (0.73, 0.87)*	0.86 (0.80,0.87)*	0.25 (0.25, 0.25)*

[a]The stem and root lengths, shoot and root dry weights, and number of SCN reproduced per root dry weight were analyzed as non-parametric data and expressed as relative treatment effects (RTE). The RTE for each treatment was determined from mean ranks ($\overline{R}_i$) as $\widehat{p_i} = \frac{1}{N}(\overline{R}_i - \frac{1}{2})$ [where N is the total number of observations; Shah and Madden 2004] and compared at 95% confidence intervals using the nparLD package in R.

[b] Asterisk indicates significant differences between treatments in response to inoculation with either fungus only, SCN only or fungus + SCN.

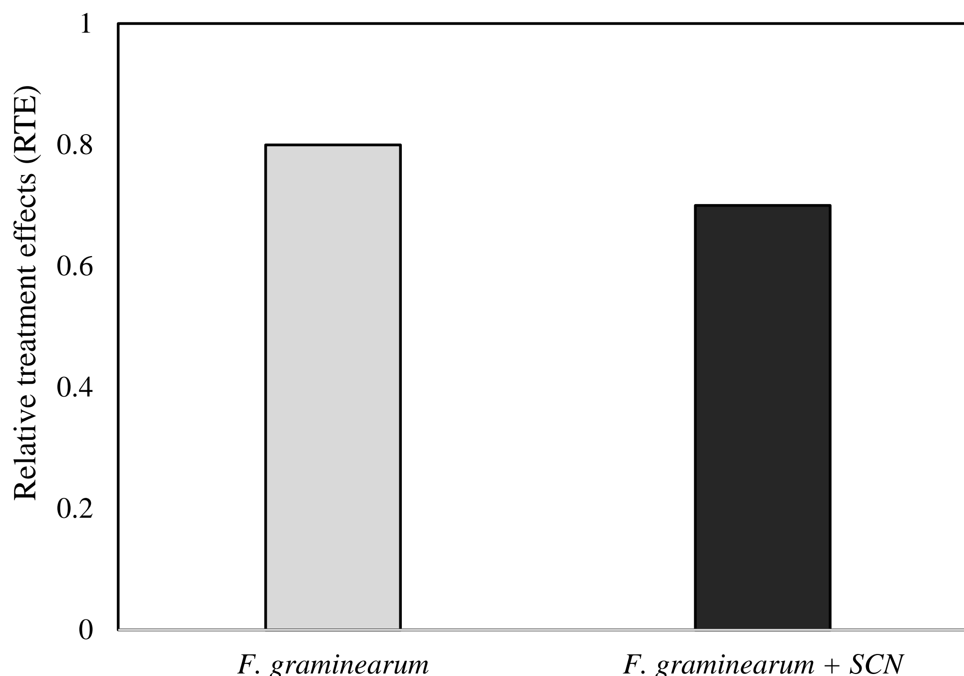

Figure 5.1. Root rot severity (expressed as RTE) caused by the *F.graminearum* only and *F.graminearum* + SCN at 40 days after inoculation on a 1-to-5 rating scale (Acharya et al. 2015), where 1 = germination and healthy seedlings with no visible root colonization, 2 = germination and 1 to 19% of the root having lesions, 3 = germination and 20 to 74% of the root having lesions, 4 = germination and 75% or more of the root having lesions; and 5 = no germination and complete colonization of seed. The relative treatment effects (RTE) for each treatment was determined from mean ranks ($\overline{R}_i$) as $\widehat{p_1} = \frac{1}{N}(\overline{R}_i - \frac{1}{2})$ [where N is the total number of observations; Shah and Madden 2004] and compared at 95% confidence intervals using the nparLD package in R.

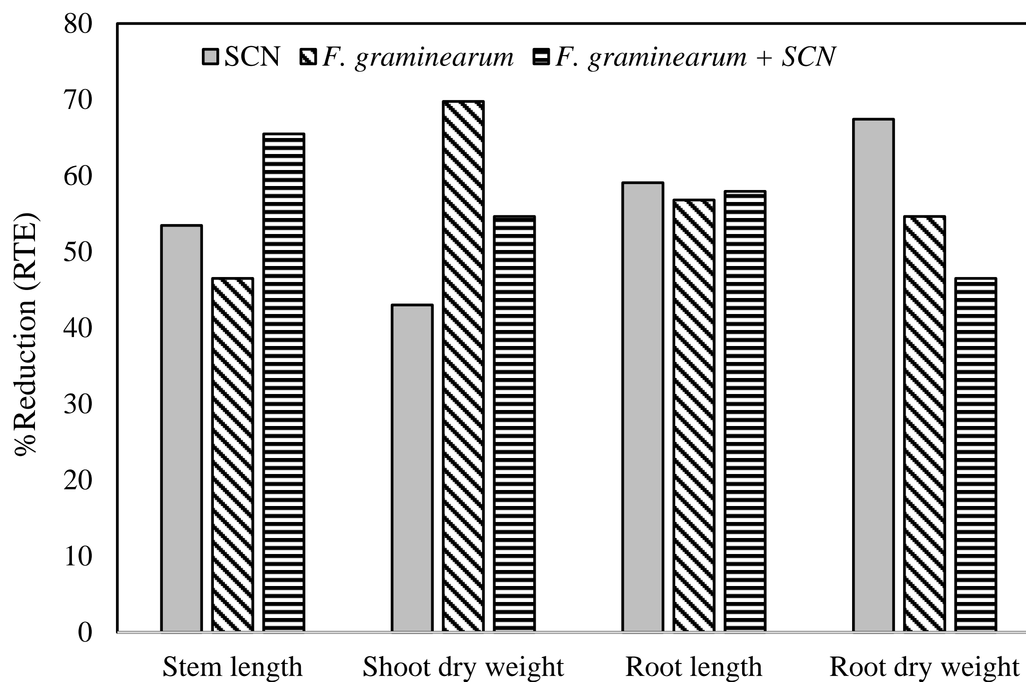

Figure 5.2. Reduction in stem length, shoot dry weight, root length and root dry weight in soybean plants inoculated with *F.graminearum* only, SCN only and, *F.graminearum* + SCN compared to non-inoculated control.

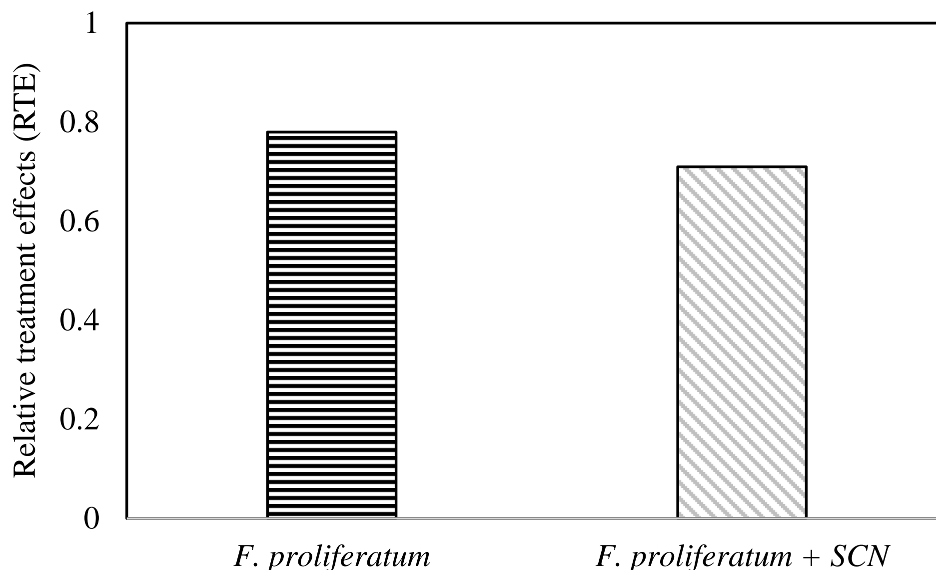

Figure 5.3. Root rot severity (expressed as RTE) caused by the fungus only and fungus + SCN at 40 days after inoculation on a 1-to-5 rating scale (Acharya et al. 2015), where 1 = germination and healthy seedlings with no visible root colonization, 2 = germination and 1 to 19% of the root having lesions, 3 = germination and 20 to 74% of the root having lesions, 4 = germination and 75% or more of the root having lesions; and 5 = no germination and complete colonization of seed. The relative treatment effects (RTE) for each treatment was determined from mean ranks ($\overline{R}_i$) as $\widehat{p_i} = \frac{1}{N}(\overline{R}_i - \frac{1}{2})$ [where N is the total number of observations; Shah and Madden 2004] and compared at 95% confidence intervals using the nparLD package in R.

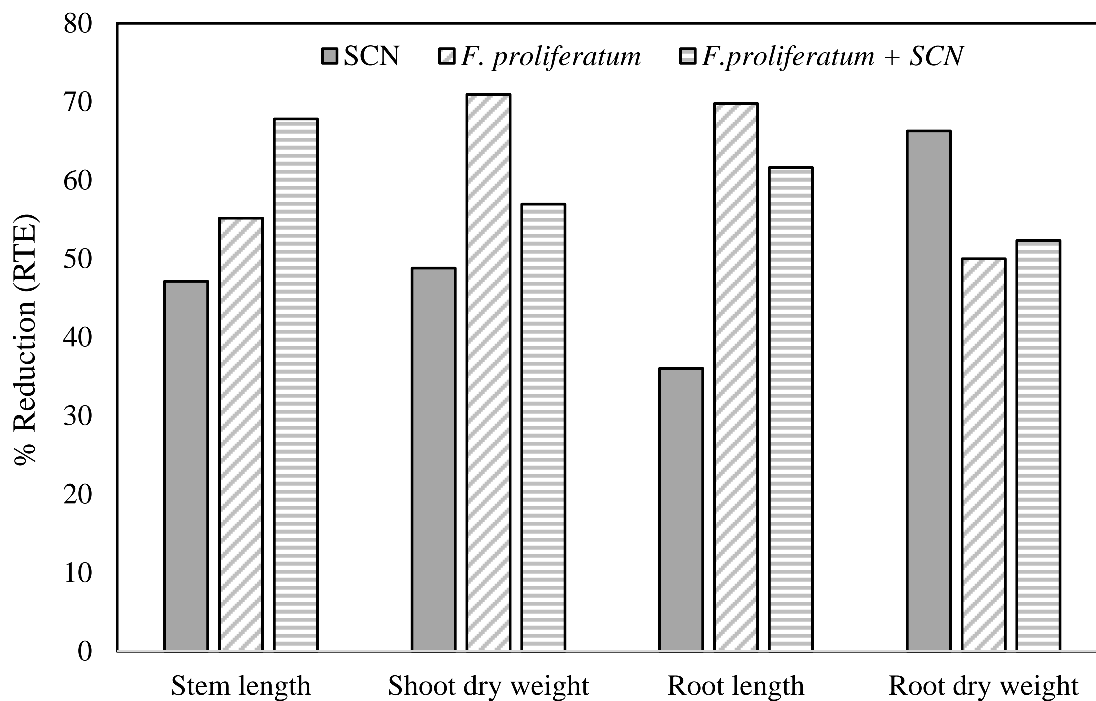

Figure 5.4. Reduction in stem length, shoot dry weight, root length and root dry weight in soybean plants inoculated with *F.proliferatum* only, SCN only and, *F.proliferatum* + SCN compared to non-inoculated control.

CHAPTER 6

Effect of soil nutrients (N-P-K) on the interaction between *Fusarium proliferatum* and *Fusarium virguliforme* with *Heterodera glycines* on soybean roots

A paper to be submitted to the journal *Plant Disease*

Abstract

In South Dakota, soybean cyst nematode, (SCN), *Heterodera glycines* and species of *Fusarium* are yield-limiting pathogens that co-exist in soybean fields. In this study, we hypothesize that root rot caused by *F.proliferatum* and *F.virguliforme* may be high in the presence of SCN and excess nutrients. Field studies were conducted in 2016 and 2017 to test the hypothesis using SCN susceptible and SCN resistant soybean varieties and two N-P-K fertilizer at the rate 15:15:15 for starter and 50:80:110 for high levels treatment. It was observed that the root rot severity caused by *F.proliferatum* and *F.virguliforme* did not increase with either of the N-P-K rates. The initial SCN population densities per 100 cc of soil ranged from 325 to 1175 depending on the inoculation with either *F.proliferatum*, *F.virguliforme* or non-inoculated control. At harvest, the SCN susceptible plots had higher SCN population densities (> 9000 eggs per 100 cc of soil) compared to plots with SCN resistant soybean variety. Soybean yield was highest in plots with SCN resistant soybean with starter N-P-K rate. Our results suggest that soybean farmers should continue to use SCN- resistant varieties to manage the two pathogens for better yields.

Introduction

Soybean, [*Glycine max* (L) Merrill], is a major field crop grown in the United States including South Dakota. It is a host to economically important plant pathogens, soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe and species of *Fusarium*. These

pathogens cause yield reduction in most soybean producing U. S. states (Allen et al. 2017; Hartman et al. 2015; Tylka and Marett 2014). In 2014, the estimated soybean yield reduction caused by SCN and *Fusarium* associated diseases (Sudden Death Syndrome, Fusarium wilt, and Fusarium root rot) in the United States and Canada (Ontario) was 3.5 million metric tons and 7.3 million metric tons respectively (Allen et al. 2017).

In South Dakota, SCN is found in 30 soybean-producing counties and is continuing to spread to other counties where soybean is grown (Acharya 2015; Basnet 2018). Species of *Fusarium* have been commonly found in many of the same fields (P. Okello and F. Mathew, *unpublished*) where SCN has been reported, suggesting the possibility of an interaction between the two pathogens. Interestingly, plant-parasitic nematodes and fungal pathogens may be affected not only by the host but also by abiotic factors such as soil pH and soil nutrients. For example, urea and ammonia- releasing fertilizers are considered effective in the management of plant-parasitic nematodes (Eno et al. 1955; Mojtahedi and Lownsberry 1976; Walker 1971). Devi and Gupta (1999) reported that the population of pigeon pea cyst nematode, *Heterodera cajani* Koshi was significantly reduced in treatments with potash application either alone or in combination with phosphorus or nitrogen compared to control with no fertilizer. Melakeberhan (2007) observed that nematode-infected plants grow better in soils rich in nutrients and nitrogen application decreased nodulation in soybean roots as much as SCN infection. However, Melakeberhan (2007) found no evidence if starter nitrogen application has benefits under the field conditions.

A high concentration of ammonia (N) has been reported to stimulate the development of *Fusarium*-associated diseases (Kato et al. 1981; McClellan and Stuart

1947; Woltz and Engelhard 1973; Woltz and Jones 1973). Species of *Fusarium* are capable of surviving in the soil for long periods as chlamydospores (Guerra and Anderson 1985). The germination of chlamydospore is stimulated by carbon and nitrogen sources such as ammonium (Cook and Schroth 1965; Loffler et al. 1986; Hendrix and Toussoun 1964). The reverse is true- nitrogen deficiencies may inhibit chlamydospore maturation and stimulate spore lysis (Griffin 1970; 1976).

In all, soil nutrients are important for the growth and development of plants and microorganisms and may play an important role in the interactions between the host plant and the pathogen causing the disease. However, the effect of each nutrient and response of the plant to disease infection may vary depending on the plant-disease complex. Generally, the complex involving soil nutrient-plant-pathogen interactions are not understood. Therefore, the objectives of this study were to evaluate the effect of N-P-K fertilizer on the association of *Fusarium proliferatum* (Matsushima) Nirenberg and *F.virguliforme* O'Donnell and Aoki with SCN on plant population densities, Fusarium root rot, SCN reproduction and yield of soybean under field conditions.

Materials and methods

Field trials were established at the South Dakota State University Southeast Research Farm in Beresford, South Dakota in 2016 and 2017.

Source of *Fusarium* inoculum

In this study, one isolate each of *F.proliferatum* (FUS026) and *F.virguliforme* (FUS020) were used, which were recovered from diseased soybean roots in South Dakota during a 2014 survey of commercial fields. The isolates were grown on potato dextrose agar (PDA) for 14 days at 22±2°C under 12-h fluorescent light. The inoculum was then

prepared by growing the *F.proliferatum* isolate on twice autoclaved Japanese millet (*Echinochloa esculenta* (Braun) Scholz) grains and the *F.virguliforme* isolate on sorghum (*Sorghum bicolor* (L.) Moench) seeds. The inoculum for the field study was prepared in aluminum foil steam table pans (25.5 in W x 13 in D x 3 in H) and incubated for three weeks at 22 ± 2ºC in the lab and mixed occasionally with sterilized spatula until the millet grains and sorghum seeds were fully colonized. After incubation, the colonized millet grains and sorghum seeds were air dried and stored within room temperature until use.

Field experiments

In 2016, the experimental design was a randomized complete block with four replications in a factorial arrangement using two soybean cultivars (Monsanto, St. Louis, MO - SCN susceptible ‘AG2531 (RM 2.4)’ and SCN resistance ‘AG2336 (RM 2.3)), two levels of fertilizer treatments [‘starter’ (15:15:15) and high rate (50:80:110)] and two fungal treatments (*F.proliferatum* and *F.virguliforme*). In 2017, the experimental design was a randomized complete block with four replications in a factorial arrangement using two soybean cultivars (Monsanto, St. Louis, MO - SCN susceptible ‘AG2531 (RM 2.4)’ and SCN resistance ‘AG2336 (RM 2.3)), two levels of fertilizer treatments [‘starter’ (15:15:15) and high rate (50:80:110)] and one fungal treatment (*F.proliferatum*). The location had a history of SCN. The experimental plots were 4 rows with a 76 cm spacing and were 6 m. long. The plots were planted using a four-row SRES Precision Planter at a seeding rate of 66,773 seeds/ha.

To study the effect of soil nutrients (N-P-K) on their interaction between *Fusarium* spp. and SCN, both the fungal inoculum (*F.proliferatum*-colonized Japanese millet seeds and *F.virguliforme*-colonized sorghum seeds) and the fertilizer rates (starter fertilizer -

15:15:15 and high levels -50:80:110) were surface broadcast using a fertilizer cart on the applicable plots within 7 days after planting to coincide with the rain.

Prior to planting, initial surface (0-15 cm) and subsurface (15-30 cm) soil test characteristics of soil pH, extractable phosphorus and exchangeable potassium were determined by a soil testing laboratory (Lindquist 2010). Extractable P (Olsen P) was determined using the $NaHCO_3$ method (Olsen 1954) while the exchangeable K was determined using the NH_4Ac method (Brown and Warncke 1988). The initial and final SCN egg population density for each plot was also determined by collecting soil samples and extracting SCN eggs.

To determine stand emergence of soybean plants, stand counts were conducted for the middle two rows of each plot at 14 (June 2) and 28 days (June 16) after planting when the soybeans were in the vegetative growth stage V2 (second trifoliate) and V4 (fourth trifoliate) (Fehr et al. 1971). During stand count, plants in each plot were examined for symptoms of damping-off caused by the two species of *Fusarium*.

To determine SCN counts, soil samples were collected from each plot first at planting and later at harvest. SCN eggs were then extracted from the soil samples collected and counted using the methodology described by Tabor et al. (2003).

Ten soybean plants were sampled at each stand count and soil sampling date from the outer two rows to rate for root rot severity. The root rot severity caused by the two species of *Fusarium* from each plant sampled were rated as a percentage of the lesion produced on the soybean tap roots. At R4 growth stage (full pod development), the plants in each plot were assessed for visible foliar symptoms of SDS. To complete Koch's postulates, the roots of diseased plants sampled were randomly selected and plated on to

PDA plates to recover the inoculant fungal pathogens. Soybean yield was estimated by harvesting the middle two rows of each plot.

Cumulative monthly precipitation and average temperatures during the growing seasons in 2016 and 2017 were obtained from the weather data collected by Southeast Farm personnel in cooperation with South Dakota State Climatologist, South Dakota Office of Climatology and SDSU Extension, and the National Weather Service, Sious Falls, SD, and also from the South Dakota State University – South Dakota Climate and Weather site: http://climate.sdstate.edu/climate_site/climate.htm.

The data collected of the root rot disease severity and SCN eggs count per plot were analyzed by analysis of variance (ANOVA) for a completely randomized block design using R (R Core Team 2013) (v3.2.2;https://www.r-project.org/). Fisher's least significant differences at $P \leq 0.05$ was used to compare treatment means. The analysis of all main effects and interactions were conducted using all treatment combinations. Data from the two years were analyzed separately.

Results

For 2016, in the *F.proliferatum* inoculated plots, at 14 days after planting (DAP), there was no significant difference observed among treatments for the plant stand counts ($P = 0.44$). However, there were numerical differences between treatments of either of the two fertilizer rates and where no fertilizer was applied (Table 6.1). At the vegetative (VE-V1) growth stage, the disease severity caused by *F.proliferatum* was not significantly different in either of the soybean cultivars with either fertilizer rate application. However, higher disease severity (>40%) were observed at reproductive stages compared to vegetative growth stages (Table 6.1). In *F.proliferatum* inoculated plots, the initial SCN

egg count (100 cc of soil) ranged from 575 to 625 eggs/ 100 cc of soil on plots planted with soybean cultivar susceptible to SCN, and from 675 to 950 eggs /100 cc of soil on plots planted with SCN resistant cultivar. At harvest, the SCN egg counts were higher (> 9000 SCN eggs /100cc of soil) in plots planted with soybean susceptible to SCN compared to plots with SCN resistant soybean varieties (< 2800 SCN eggs /100 cc of soil). Soybean yields were highest in plots with SCN resistant soybean varieties in combination with starter fertilizer application (Table 6.1).

For plots inoculated with *F.virguliforme*, there were no observed significant differences ($P = 0.49$) in plant stand counts at 14 days after planting (DAP). However, there were observed numerical differences between treatments of either of the two fertilizer rates and the plots where fertilizer was not applied (Table 6.2). At the vegetative (VE-V1) growth stage, the disease severity caused by *F.virguliforme* was not significantly different ($P = 0.15$). However, similar to plots inoculated with *F.proliferatum*, higher disease severity (>40%) were observed at reproductive stages compared to vegetative growth stages (Table 6.2). The *F.virguliforme* inoculated plots had initial SCN egg count (100 cc of soil) that ranged from 325 to 425 eggs /100cc of soil on plots planted with soybean cultivar susceptible to SCN, and from 500 to 825 eggs /100cc of soil on plots planted with SCN resistant cultivar. At harvest, the SCN egg counts were higher (> 9000 SCN eggs /100cc of soil) in plots planted with soybean susceptible to SCN compared to plots with SCN resistant soybean variety (< 2700 SCN eggs /100cc of soil). The soybean yields were highest in plots with SCN resistant soybean variety planted in combination with starter fertilizer application (Table 6.2).

In 2016, the average monthly temperatures were higher during the months of June (19.1ºC) and July (22.2ºC) and lower in the months of May (13.8ºC) and September (15.0 ºC) (Figure. 6.1). The monthly accumulate precipitation were higher in June (86.1 mm) and July (90.9 mm) and lower in August (67.0 mm) and September (60.9 mm) (Figure. 6.1).

For 2017 field trial, the effect of N-P-K was evaluated on the interaction between *F.proliferatum* and SCN. At 14 days after planting, the plant stand count was not significantly different ($P > 0.05$) among treatments. However, numerical differences were observed between either of the two N-P-K fertilizer applications and plots where no fertilizers were applied (Table 6.3). At vegetative growth stage (VC-V1~ cotyledon and first trifoliate) lesion length caused by *F.proliferatum* on soybean roots was not significantly different ($P > 0.05$) in both SCN-susceptible and resistant variety plots. However, at the reproductive stage (R8 ~full maturity), there was observed significant difference in lesion length ($P = 0.02$). SCN resistant variety plants had shorter lesions compared to SCN susceptible plants. In all, at reproductive growth stage R8, plots inoculated with *F.proliferatum* had longer lesion length in plots where both the SCN susceptible and resistant varieties were planted compared to those at vegetative growth stages VC-V1.

In plots inoculated with *F.proliferatum*, the initial SCN population ranged from approximately 113 to 1238 eggs /100 cc of soil compared to a range of 225 to 700 eggs /100 cc of soil in the non-inoculated plots. At harvest, the SCN population was higher (>1500 SCN eggs/100 cc of soil) in SCN susceptible plots than in plots with SCN resistant soybean variety (< 700 SCN eggs/100 cc of soil). The soybean yield harvested from the

two middle rows were observed to be significantly different among the treatments ($P < 0.05$). The SCN resistant plots with starter fertilizer application recorded the highest yields of 4455.3 kg/ha (Table 6.3).

In 2017, the average monthly temperatures were higher during the months of June (21.6ºC) and July (24.1ºC) and lower in the months of May (13.8ºC) and September (17.9ºC) (Figure. 6.1). The monthly accumulate precipitation were higher in May (141.4 mm) and August (202.4 mm) and lower in June (53.8 mm) and July (34.2 mm) (Figure. 6.1).

Discussion

In this study, field experiments were conducted to determine the effect of N-P-K on the interaction between the fungi (*F.proliferatum* and *F.virguliforme*) and SCN. The application of either rate of N-P-K fertilizer did not have a significant effect on the plant stand count or contribute to an increase in root rot severity caused by the two fungal pathogens on either soybean variety. However, the root rot severity caused by the two fungal pathogens was observed to be numerically higher at the reproductive growth stage compared to the vegetative growth stage regardless of whether fertilizer was applied or not. At harvest, high SCN population densities (> 9000 eggs per 100 cc of soil) were observed more in SCN susceptible plots compared to SCN resistant plots regardless of N-P-K fertilizer rate applied. In addition, soybean yield was higher in plots with SCN resistant soybean variety where starter N-P-K fertilizer was applied compared to the SCN susceptible variety where no fertilizer was applied and either or both pathogen present. This suggests that the use of resistant variety and appropriate fertilizer rates may help manage SCN in infested fields and ensure higher yields (Howard et al., 1998;

Melekeberhan, 1999, 2007; Mitchum, 2016). For example, Howard et al. (1998) observed that high rates of PK fertilizer increased yield of two soybean cultivars with different levels of SCN resistance and reduced the SCN population under field conditions in Tennessee while Melakeberhan (2007) reported that starter N fertilizer increased soybean yield under conditions of high SCN population densities for soybean cultivar Jack in Michigan.

Under field conditions, a major factor influencing the cause-and-effect association between pathogens and the host plant is the contribution of environment driven factors. Species of *Fusarium* that affect soybean roots thrive early in the growing season under cool temperatures and moist soil conditions and also later in the season when soil moisture is limiting (Malvick 2018). There was variation in the pattern of monthly accumulate precipitation and average monthly temperatures between the two years of the experiment. In 2016, higher precipitation was recorded during the months of June and July, and higher average monthly temperatures in the months of July and August. In the 2017 growing season, higher precipitation was received in the months of May and August and the average monthly temperature was higher during the months of June and July. Therefore, the disease severity observed at both the vegetative and reproductive growth stages may be attributed to the cool and wet soil conditions as well as the limiting soil moisture conducive for soil-borne fungal pathogens to cause root infection. Whereas targeted plots were broadcast with the inocula of *F.proliferatum* and *F. virguliforme* isolates in 2016 and with *F.proliferatum* in 2017, other root rot fungal pathogens were recovered from the soybean plants sampled from the experimental plots. The root rot fungal pathogens included *F.graminearum*, *F.oxysporum*, *F.sporotrichioides*, *F.acuminatum*, *Rhizoctonia* species and *Diaporthe* species among others. In 2016, no sudden death syndrome (SDS) foliar symptoms were

observed on plots inoculated with *F. virguliforme* and the fungus was less frequently recovered from plants sampled on plots inoculated with the pathogen. This may be attributed to the application of K fertilizer from potassium chloride (KCL), which has been reported to decrease the severity of SDS by 36% (Sanogo and Yang 2001). However, the *F. proliferatum* was frequently recovered from plots inoculated with the fungus. Therefore, in 2017 the experiment was repeated with plots inoculated only with *F. proliferatum*.

Moisture levels may have an effect on SCN hatching (Tefft et al. 1982). Tefft et al. (1982) reported the optimum moisture for SCN hatching at 25%, and an increase above that would lead to a decline. Therefore, in this study the SCN reproduction may have been reduced during the months when higher rainfall was recorded minimizing the chances of interaction between the fungal pathogens, nematode and fertilizer rates. In addition, the field site where the experiment was set up has been observed to have low SCN population based upon the SCN count per 100 cc of soil. Regardless, the findings from this study that plots with no SCN resistance cultivars resulted in lower yield and higher SCN population density supports the past study by McLean and Lawrence, (1993) that yield reduction is higher in the presence of SCN. The higher yields observed are therefore due to the use of SCN resistance cultivar rather than the application of fertilizer regimes.

The lack of a consistent pattern on the effect of N-P-K fertilizer rates on plant count, disease severity, SCN counts and yield may suggest that the interaction between N-P-K fertilizer rates, isolates of *F. proliferatum* and *F. virguliforme* and SCN is complex. This interaction may be affected by multiple factors such as population density of SCN at planting, environmental conditions such as temperature and precipitation (soil moisture), soil pH, soil fertility, inoculum concentration of the fungal pathogen (i.e *F.proliferatum* or

F.vrguliforme) in the field, and susceptibility of the cultivar planted. In 2016, most of the N-P-K fertilizer applied was washed off the plots after heavy rainfall early in the growing season. For *F.proliferatum* inoculated plots with SCN susceptible cultivar, application of starter N-P-K fertilizer contributed to higher yield (460 kg/ha more) that application of high N-P-K fertilizer rate. In plots with SCN resistant cultivar, starter N-P-K fertilizer rate increased the soybean yield by 110 kg/ha. For *F.virguliforme* inoculated plots with SCN susceptible cultivar, the application of N-P-K fertilizer did not improve soybean yield. However, the application of high N-P-K fertilizer increased the yield of SCN resistant cultivar by 1183 kg/ha. In 2017, under plots inoculated with *F.proliferatum* and with SCN susceptible cultivar, application of high rate N-P-K fertilizer rate increased soybean yield by 145 kg/ha and by 173 kg/ha in plots with SCN resistant cultivar.

Generally, balanced nitrogen, phosphorus, and potassium nutrients in the soil result in less *Fusarium*-associated disease (Walker and Foster 1946). However, the application of both starter N-P-K (15:15:15) and high N-P-K (50:80:110) fertilizer rates did not reduce the root rot severity or the SCN population, and therefore may not be an effective management option. Despite that, regular soil tests are necessary to maintain appropriate soil nutrient levels, which are essential for plant growth and development. Residual fertility from previous crops and manure applications should be taken into consideration when determining application amounts. Findings from this study suggest that soybean growers will need to monitor diseases caused by the two soil-borne pathogens, continue the use of SCN resistant varieties and treated seeds and formulate fertilizer regimes only after conducting soil nutrient tests.

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Table 6.1. Field evaluation of the effect of N-P-K fertilizer on the interaction between *F.proliferatum* and SCN on soybean plants in Beresford, SD in 2016.

Variety	Treatment [a]	Fertilizer rates (N:P:K)	Stand count (14 DAP)	Disease severity (VE-V1)	Disease severity (R4-R5)	Initial SCN count	Final SCN count	Yield (Kg/ha)
SCN susceptible	FP+SCN	0:0:0	118483.20	28.30	48.90	625.00	29300.00	2423.33
	SCN		108682.20	40.05	48.40	325.00	9375.00	2323.33
	FP+SCN	15:15:15	103455.00	31.75	49.15	575.00	26425.00	2750.00
	SCN		105633.00	32.25	49.45	325.00	10825.00	3066.67
	FP+SCN	50:80:110	111513.60	24.50	49.40	575.00	29350.00	2290.00
	SCN		75358.80	22.50	47.75	500.00	14775.00	3090.00
SCN resistant	FP+SCN	0:0:0	103890.60	30.25	48.75	850.00	2725.00	4303.33
	SCN		108682.20	34.75	47.50	550.00	1350.00	4013.33
	FP+SCN	15:15:15	108028.80	33.75	45.70	675.00	2775.00	4473.33
	SCN		102583.80	20.25	45.65	1175.00	2250.00	4250.00
	FP+SCN	50:80:110	91911.60	29.00	47.35	950.00	2000.00	4363.33
	SCN		80586.00	38.90	46.35	425.00	2675.00	3560.00
P- Value			0.44	0.08	1.00	0.42	0.11	0.93
LSD			37352.68	12.27	10.65	748.18	23802.75	1910.67

[a]Abbreviations: FP = *F.proliferatum*; SCN = soybean cyst nematode

Table 6.2. Field evaluation of the effect of N-P-K fertilizer on the interaction between *F.virguliforme* and SCN on soybean plants in Beresford, SD in 2016.

Variety	Treatment [a]	Fertilizer rates (N:P:K)	Stand count (14 DAP)	Disease severity (VE-V1)	Disease severity (R4-R5)	Initial SCN count	Final SCN count [b]	Yield (Kg/ha)
SCN susceptible	FV+SCN	0:0:0	62944.20	29.50	45.70	425.00	10350.00	2500.00
	SCN		108682.20	40.05	48.40	325.00	9375.00	2323.33
	FV+SCN	15:15:15	77754.60	34.50	47.70	425.00	10975.00	2906.67
	SCN		105633.00	32.25	49.60	325.00	14775.00	3066.67
	FV+SCN	50:80:110	107593.20	32.25	45.60	325.00	44125.00	3133.33
	SCN		75358.80	22.50	47.75	500.00	14775.00	3090.00
SCN resistant	FV+SCN	0:0:0	109771.20	37.75	49.05	600.00	1850.00	4256.67
	SCN		108682.20	34.75	47.50	550.00	1350.00	4013.33
	FV+SCN	15:15:15	93218.40	35.00	47.35	825.00	2125.00	4706.67
	SCN		102583.80	20.25	45.65	1175.00	2250.00	4250.00
	FV+SCN	50:80:110	100623.60	34.25	44.75	500.00	1975.00	3523.33
	SCN		80586.00	38.90	46.35	425.00	2675.00	3560.00
P- Value			0.49	0.15	0.98	0.10	0.04*	0.67
LSD			49456.80	13.67	8.84	516.75	21149.87	1564.67

[a]Abbreviations: FV = *F.virguliforme*; SCN = soybean cyst nematode.

[b]Asterisk: indicates significant differences at $P = 0.5$ between plots with different rates of N-P-K fertilizer applications.

Table 6.3. Field evaluation of the effect of N-P-K fertilizer on the interaction between *F.proliferatum* and SCN on soybean plants in Beresford, SD in 2017.

Variety	Treatment[a]	Fertilizer rates (N:P:K)	Stand count (14 DAP)	Lesion length (VC-V1)	Lesion length (R8)[b]	Initial SCN count	Final SCN count[b]	Yield (bu/A)[b]
SCN susceptible	FP+SCN	0:0:0	88535.70	34.75	116.00	362.50	3337.50	3650.00
	SCN		78190.20	38.50	104.25	337.50	2450.00	3183.33
	FP+SCN	15:15:15	83417.40	33.63	128.88	250.00	2962.50	3728.67
	SCN		64904.40	29.63	118.50	700.00	2725.00	3472.00
	FP+SCN	50:80:110	73943.10	30.63	109.88	1237.50	3187.50	3873.33
	SCN		67082.40	31.88	120.75	225.00	1700.00	3720.00
SCN resistant	FP+SCN	0:0:0	89515.80	35.63	115.75	250.00	662.50	4222.00
	SCN		78625.80	35.88	90.50	700.00	312.50	4023.33
	FP+SCN	15:15:15	67953.60	31.63	93.13	437.50	575.00	4133.33
	SCN		83526.30	34.13	89.25	387.50	400.00	4455.33
	FP+SCN	50:80:110	75578.60	31.88	94.75	112.50	375.00	4306.67
	SCN		85486.50	35.38	89.75	287.50	375.00	4388.67
P- Value			> 0.05	> 0.05	0.02*	> 0.05	0.00*	0.01*
LSD			43534.59	10.53	26.22	1979.77	1603.61	656.67

[a]Abbreviations: FP = *F.proliferatum*; SCN = soybean cyst nematode.

[b]Asterisk: indicates significant differences at $P = 0.5$ between plots with different rates of N-P-K fertilizer applications.

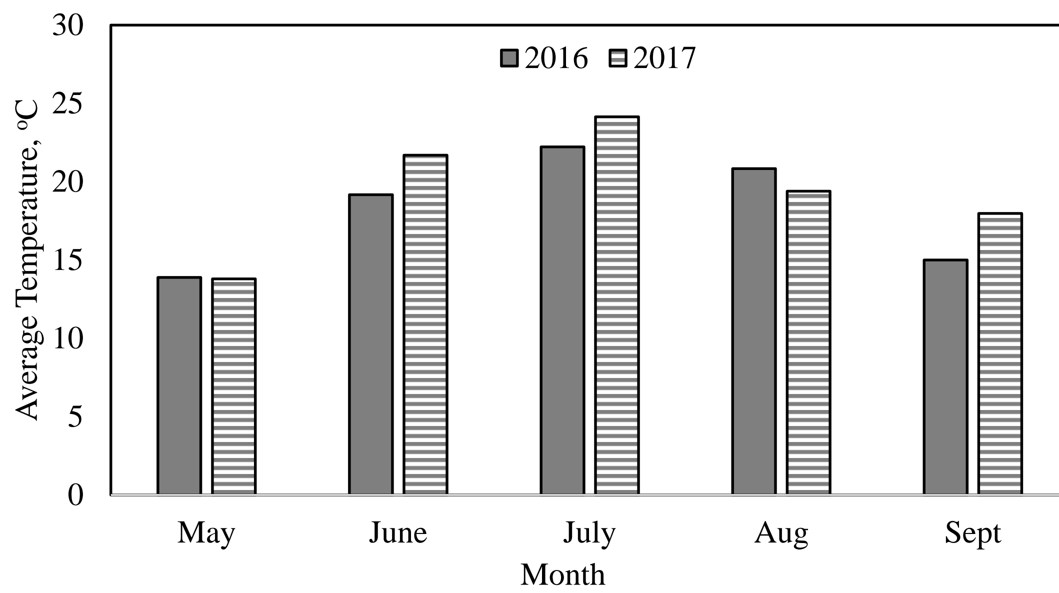

Figure 6.1. Monthly average temperature (°C) during the 2016 and 2017 growing season at the Southeast Research Farm in Beresford, SD.

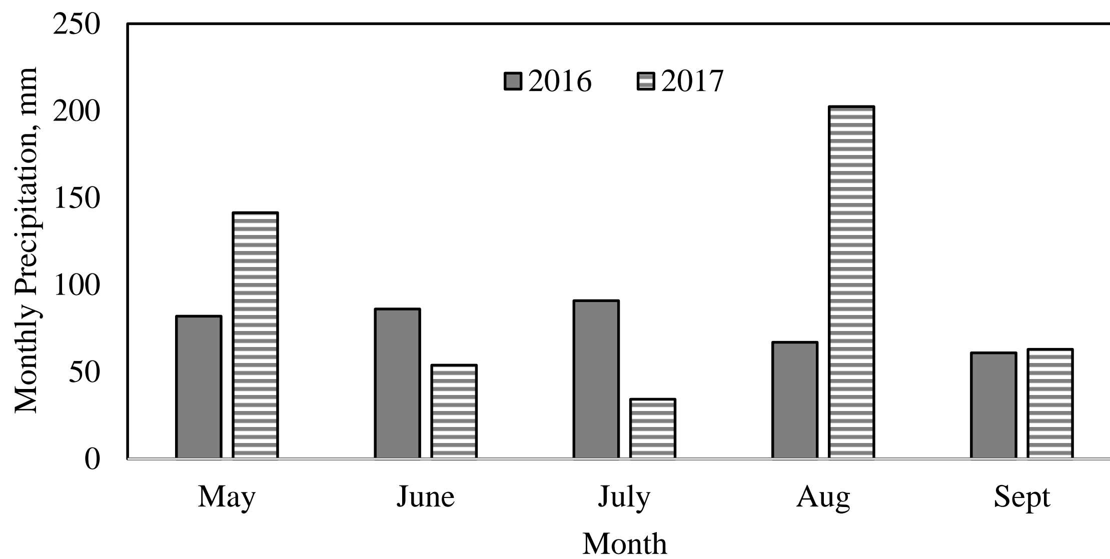

Figure 6.2. Monthly accumulate precipitation (mm) during the 2016 and 2017 growing season at the Southeast Research Farm in Beresford, SD.

Chapter 7

General conclusions and recommendations

This dissertation presents research findings from both greenhouse and field studies relating to the pathogenicity of species of *Fusarium* causing root rot on soybean in South Dakota. The specific objectives of the studies were to (1) characterize the species of *Fusarium* causing soybean root rot in South Dakota; (2) evaluate the cross-pathogenicity of species of *Fusarium* causing root rot of soybean and corn; (3) screen soybean germplasm for resistance to *Fusarium graminearum* in the greenhouse; (4) determine the association of *F.graminearum* and *F.proliferatum* with *Heterodera glycines* (soybean cyst nematode, SCN) on soybean roots in the greenhouse; and (5) determine the effect of soil nutrients on the association of *F.virguliforme* and *F.proliferatum* with *H.glycines* under field conditions.

To realize these objectives, a survey was conducted on commercial soybean and corn fields during the 2014 and 2015 growing seasons respectively to isolate the species of Fusarium causing root rot. In all, 200 commercial soybean fields (~10 plants collected per field) across 22 counties and 50 corn fields (~5 plants collected per field) across 24 counties in South Dakota were sampled for diseased plants. On soybean, 11 species of *Fusarium* were identified causing root rot based on morphology and confirmed through molecular technique. The species included *F.acuminatum*, *F.armeniacum*, *F.commune*, *F.equiseti-incarnatum* complex, *F.graminearum*, *F.oxysporum*, *F.proliferatum*, *F.solani*, *F.sporotrichioides*, *F. subglutinans* and *F.virguliforme*. *F.graminearum*, *F.acuminatum*, and *F.oxysporum* were among the most frequently recovered while *F.commune*,

F.subglutinans, and *F.sporotrichioides* were recovered the least. There were significant differences in aggressiveness among the isolates of *Fusarium* and also among isolates within particular species of *Fusarium* such as *F.acuminatum*, *F.oxysporum*, *F.proliferatum* and *F.solani*. In general, *F.oxysporum*, *F.armeniacum* and *F.commune* caused the highest root rot severity (expressed as relative treatment effects).

On corn, eight species of *Fusarium* were identified of which seven were common to soybean fields. The species were *F.acuminatum*, *F.equiseti-incarnatum complex*, *F.graminearum*, *F.oxysporum*, *F.proliferatum*, *F.solani*, and *F.subglutinans*. A greenhouse cross-pathogenicity study on the seven species of *Fusarium* common to soybean and corn observed that the isolates recovered from either crop were pathogenic to both soybean and corn. The *Fusarium* isolates recovered from corn were generally more aggressive on both crops. For example, two *F.proliferatum* isolates and one *F.graminearum* isolate from corn were observed to be more aggressive on both crops than the others. This suggests that soybean and corn can serve as primary sources of inoculum for species of *Fusarium* that affects both crops and therefore crop rotation between the two crops may not be effective in the management of Fusarium root rot. There are at least 22 species of *Fusarium* that have been reported on soybean. It is possible that there may be other species of *Fusarium* not reported here causing root rot on soybean and corn in South Dakota as more land is put into soybean production and the soybean-corn rotation with reduced tillage practice continues. Therefore, annual survey, identification and pathogenicity tests of species of *Fusarium* on commercial soybean and corn fields is recommended.

The use of host resistance cultivars has been recommended as the best long-term management option to reduce the impacts of species of *Fusarium*. Presently there are no known commercial cultivars with resistance to species of *Fusarium* (except *F.virguliforme*). Our greenhouse screening of the soybean germplasm determined eight plant introduction lines which were significantly less susceptible to *F.graminearum.* The eight accessions, PI 437949, PI 438292, PI 612761A, PI 438094B, PI 567301B, PI 408309, PI 361090 and PI 88788 may be used as parental materials in breeding programs to develop commercial cultivars with resistance to *F.graminearum*. Future research should focus on genotyping to locate genes in the eight soybean accessions associated with resistance to *F.graminearum*.

During the 2014 survey of commercial soybean fields in South Dakota, cysts of the soybean cyst nematode were observed present on roots to which species of *Fusarium* were later isolated. This suggested the possibility of an interaction between the two soil-borne pathogens. Our greenhouse study on the interaction between two species of *Fusarium*, (*F.graminearum* and *F.proliferatum*) and soybean cyst nematode (*Heterodera glycine*) on an SCN susceptible cultivar Williams 82 showed no synergism or antagonism between either of the fungus and SCN. The presence of SCN did not significantly increase root rot severity caused by either species of *Fusarium*, and while the number of SCN egg counts were not affected by *F.graminearum* when compared to SCN only treatment, *F.proliferatum* in the presence of SCN inhibited the reproduction of SCN. However, further research under field conditions is necessary to evaluate the nature of the interaction between the fungal pathogens and SCN.

Plant infection by species of *Fusarium* and SCN may be influenced by abiotic factors such as soil nutrients. We conducted field studies to determine the effect of soil nutrients on the interaction between two fungi (*F.virguliforme* and *F.proliferatum*) and *Heterodera glycines*. The effect of two rates of N-P-K fertilizer (starter 15:15:15 and high rate 50:80:110) were used on two soybean varieties (Monsanto, St. Louis, MO- 'AG2531 (RM 2.4)' SCN susceptible and 'AG2336 (RM 2.3)' SCN resistance). In the field, it was observed that the root rot severity caused by the *Fusarium* isolates did not increase when either N-P-K rates was applied. At harvest, the nematode eggs count per 100 cc of soil was higher compared to the initial counts. The highest soybean yield was obtained from plots planted with SCN resistant soybean variety and application of starter N-P-K rate. This may suggest that use of SCN resistant varieties and seed treatment are better management options for the two pathogens and that fertilizer regimes should be formulated that do not initiate or aggravate existing disease problems.

In all, the research presented in this thesis has advanced the emerging threat of Fusarium root rot in soybean and corn production in South Dakota but also presented soybean accessions with potential for development of resistant cultivar. In particular, this research has enriched our understanding of species of *Fusarium* causing soybean root rot in South Dakota, their aggressiveness, cross-pathogenicity of *Fusarium* isolates from soybean and corn, interaction of *F.graminearum* and *F.proliferatum* with SCN and also the effect of N-P-K on the interaction between two species of *Fusarium* (*F.proliferatum* and *F.virguliforme*) and SCN. Our findings therefore provide useful information for soybean producers to develop integrated pest management programs in fields with history of Fusarium root rot and presence of SCN.

Appendix 1.

Root rot caused by species of *Fusarium* on *Brassica carinata* in South Dakota

A paper accepted by the journal *Plant Health Progress*

Abstract

Brassica carinata is an emerging oilseed crop in the United States and a root disease that has the potential to cause yield losses in production is those caused by *Fusarium*. In this study, *B. carinata* plants were randomly sampled at vegetative and seed development plant stages from South Dakota State University experimental plots. Reddish-brown lesions were observed on roots of sampled plants from which *F.acuminatum, F.oxysporum, F.solani* and *F.sporotrichioides* were recovered. The *Fusarium* species were identified based on morphology and phylogenetic analyses of the translation elongation factor 1-α gene region. Pathogenicity of the four *Fusarium* species was evaluated on five *B. carinata* accessions using a modified inoculum layer method in the greenhouse. At 21 days after inoculation, root rot severity caused by *Fusarium* on the *B. carinata* accessions was assessed on a 0-to-4 rating scale and evaluated using relative treatment effects (RTE). The *F.oxysporum* isolate caused significant differences in RTE (P = 0.01) among the *B. carinata* accessions. However, there was no significant differences in RTE among the *B. carinata* accessions in response to *F.acuminatum* (P = 0.82), *F.solani* (P = 0.76) and *F.sporotrichioides* isolates (P = 0.47).

Brassica carinata Braun, also known as Ethiopian mustard, is an oilseed crop in the Brassicaceae family. In North America, there is an increased interest in the commercial production of *B. carinata* because of continued search for alternative biofuel sources

(Seepaul et al. 2016; Warwick et al 2006). In South Dakota, where it has been difficult to achieve the productivity potential of canola (commonly called *Brassica napus* L.; rapeseed), *B. carinata* is currently being considered as an alternative crop. However, because South Dakota temperatures can be 100°F or higher in the summer and 0°F freezing in the winter, the environment is highly conducive to maintaining organisms capable of causing plant diseases throughout the year (Elad and Pertot 2014). Under such conditions, newly introduced crops such as *B. carinata* may be susceptible to plant pathogens already prevalent in the production regions of South Dakota and it is important to monitor for diseases.

In May and September of 2017, plants of *B. carinata* at the South Dakota State University experimental plots in Brookings County, South Dakota (44°18′37″ N, 96°40′25″ W) were randomly sampled twice during the growing season. During both months, excess moisture was observed following rain. Ten random plants were sampled at each of the two plant growth stages, vegetative (May) and seed development (September). On the roots of the sampled *B. carinata* plants, reddish-brown lesions were observed and from these roots, species of *Fusarium* were isolated. The objectives of this study were to (i) identify the species of *Fusarium* causing root rot of *B. carinata*; and (ii) evaluate the pathogenicity of species of *Fusarium* on five *B. carinata* accessions in the greenhouse.

Fusarium isolation and identification

To isolate the causal pathogen, the diseased roots were washed under running tap water for 2 min to dislodge soil particles and the tap root was cut into small pieces (~ 5 mm). Three root pieces were surface-sterilized in sodium hypochlorite (0.05%) and ethanol (70%) for 1 min each, and then rinsed in sterile distilled water. The sterilized root pieces

were plated on Komada's medium (Davet and Rouxel 2000) amended with 0.02% streptomycin sulfate, and incubated at 23 ± 2°C for seven days. A total of 41 isolates were tentatively identified as *Fusarium* (12 isolates at vegetative stage and 29 isolates at seed development stage, Fig.1) and 33 isolates as species of *Alternaria* (10 isolates at vegetative stage and 23 isolates at seed development stage). The leading edge of the colony of the *Fusarium* isolates was transferred to carnation leaf agar (CLA) for morphology based identification. The cultures on the CLA plates were incubated for seven days at 23 ± 2°C under a 12 h dark and light cycle. Following the key of Leslie and Summerall (2006), the 41 *Fusarium* isolates were identified to species based on morphological characteristics. Among the 41 isolates, seven isolates (two isolates at vegetative and five isolates at seed development growth stages) were tentatively identified as *F.acuminatum* Ellis and Everhart based on the characteristics of producing red pigmentation; slender, curved macroconidia with three to five septate (n=10; 3.0 to 4.0 x 0.7 to 1.0 µm); and no microconidia but with chlamydospores present in chain formation. Twenty isolates (10 isolates at vegetative and 10 isolates at seed development growth stages) were tentatively identified as *F.oxysporum* Schlechtend because they produced a white to pale violet pigment, false heads of microconidia on short monophialides, and sparse macroconidia that were medium in length and had three septate (n=10; 3.1 to 4.9 x 0.6 to 1.0 µm). Two isolates (5%) recovered from the *B. carinata* roots at the seed development growth stages were tentatively identified as *F.solani* (Martius) Appel and Wollenweber as they produced green sporodochia, macroconidia with five to seven septate (n=10; 4.6 to 7.2 x 0.8 to 1.0 µm), oval shaped microconidia with one to two septate (n=10; 1.2 to 5.6 x 0.8 to 1.5 µm). Twelve isolates (29%) recovered from the *B. carinata* roots at the seed development

growth stages had fast growing colonies that were pink-white with dense mycelia and orange sporodochia, three to five septate macroconidia that were moderately curved (*n*=10; 4.4 to 8.5 x 0.7 µm), and oval-shaped microconidia (*n*=10; 4.7 to 8.2 x 0.8 to 3.0 µm) matched descriptive characters of *F.sporotrichioides* Sherbakoff.

For molecular confirmation, one isolate representing *F.oxysporum* (FUS-CAR002), *F.acuminatum* (FUS-CAR003), *F.solani* (FUS-CAR004) and *F.sporotrichioides* (FUS-CAR008) were arbitrarily selected for DNA extraction and sequencing of the translational elongation factor 1-α (TEF) gene region (Geiser et al. 2004). The TEF phylogenetic tree was inferred using the Maximum Parsimony analyses in MEGA6 (Tamura et al, 2013) with bootstrap analyses of 1000 replications. The TEF phylogeny grouped isolates in four well-supported clades that contained type sequences of *F.oxysporum, F.acuminatum*, *F.sporotrichioides*, and *F.solani* obtained from *Fusarium*-ID, with a bootstrap value of 99% or 100% (Fig 2). The TEF sequences of the four *Fusarium* isolates generated in this study have been indicated on the tree as well as deposited in the NCBI GenBank under the accession numbers MH142728 and MH175701 to MH175703.

Greenhouse pathogenicity tests

The virulence of the four *Fusarium* isolates (FUS-CAR003, FUS-CAR002, FUS-CAR008 and FUS-CAR004) was evaluated on five *B. carinata* accessions (PI273640; PI274283; PI360887; PI195552; PI199950) in separate greenhouse experiments.

For inoculations, a modified inoculum layer technique (Bilgi et al. 2008) was used. The *Fusarium* isolates were initially grown on potato dextrose agar (PDA) media in petri-dish for seven days and five mycelial plugs (~5 mm) were transferred into 1000 ml conical flasks containing autoclaved millet (for isolates FUS-CAR003, FUS-CAR002 and

FUS-CAR008) or sorghum grains (for isolate FUS-CAR004) that were wetted before autoclaving. The conical flasks were incubated at 23 ± 2°C for 14 days.

For each *Fusarium* isolate, the experiments were conducted as a completely randomized design with six plants (experimental unit) evaluated for each of the five *B. carinata* accessions and performed twice. To grow the five *B. carinata* accessions, two seeds of each accession were planted into 164 ml plastic cone-tainers (Stuewe and Sons, Tangent, OR) wrapped with a piece of weed barrier fabric (The Master Gardner Company, Spartanburg, SC) to contain the *B. carinata* roots within the cone-tainer. Each cone-tainer was one third filled with potting mix (Sunshine Mix no. 1; Sun Gro Horticulture, MA, USA), followed by 3 g of *Fusarium* inoculum. The inoculum of the *Fusarium* isolates was covered with approximately 3 g of potting mix to avoid the direct contact of the seed and inoculum and then the *B carinata* seeds were added. The seeds were covered with 3 g of potting mix. All cone-tainers were incubated on a greenhouse bench at 22 to 25°C under 16 h photoperiod with a light intensity of 450 $\mu Em^{-2}s^{-1}$. The plants were watered once every day. Seven days after planting, one *B. carinata* seedling plant was left after thinning out of the cone-tainer. At 21 days after inoculation, the experiments were terminated and the *B. carinata* roots were assessed for root rot severity on a 0-to-4 rating scale modified from Hwang et al. (1994), where 0 = no symptoms, 1 = discoloration but no visible lesions, 2 = obvious lesions, 3 = severe lesions and 4 = plant dead.

The ordinal data from the root rot severity rating scale were not normally distributed, and therefore analyzed using the nonparametric procedure (Shah and Madden 2004). Prior to performing the non-parametric analyses, the homogeneity of variance between the two experimental repeats was satisfied using the Fligner-Killen test for

F.acuminatum (P = 0.82), *F.solani* (P = 0.76), *F.oxysporum* (P = 0.60), and *F.sporotrichioides* (P = 0.47). The overall effect of the treatment (*Fusarium* isolate) on the root rot severity of the *B. carinata* accessions was determined by the ANOVA type test statistics (ATS) of ranked data. The ranks corresponding to each accession were calculated using the nparLD package (Noguchi et al. 2012) in R v 2.1 (R Core Team 2013) as "$\overline{R}_i = \frac{1}{n_i}\sum_{k=1}^{n_i} R_{ik}$ (Akritas 1991), where $\overline{R}_i$ is the mean rank for the ith treatment and R_{ik} is the rank of X_{ik} among all N observations" (Shah and Madden 2004). The relative treatment effects (RTE) for each treatment was calculated from mean ranks ($\overline{R}_i$) as $\widehat{p_i} = \frac{1}{N}(\overline{R}_i - \frac{1}{2})$ [where N is the total number of observations; Shah and Madden 2004] and compared by calculating their 95% confidence intervals using the nparLD package in R.

All the four *Fusarium* isolates caused root discoloration of the five *B. carinata* accessions at 21 days after inoculation (an example is provided in Fig 3) and no any visual differences were observed in the symptoms caused by the four *Fusarium* species on *B. carinata* roots. Although non-inoculated control plants were included for each of the *B. carinata* accessions, no discoloration (disease rating = '0') was observed on the roots of the control plants (Fig. 3) and hence they were not included in the statistical analyses.

For *F.oxysporum* isolate FUS-CAR002, significant differences in root rot severity (expressed in terms of RTE) were observed among the five *B. carinata* accessions (P = 0.01). Based on 95% confidence intervals, PI 360887 had a significantly higher RTE from the pathogen than PI195552, PI273640 and PI274283, while PI199950 had a significantly higher RTE compared to PI 195552 (Table 1). For *F.acuminatum* isolate FUS-CAR003 (Table 2; P = 0.82), *F.solani* isolate FUS-CAR004 (Table 3; P = 0.76) and

F.sporotrichioides isolate FUS-CAR008 (Table 4; *P* = 0.47), no significant differences in RTE was observed among the five *B. carinata* accessions.

To fulfill Koch's postulates, *Fusarium* were isolated from the diseased roots of the five *B. carinata* accessions using the protocol as described previously. After isolation, the identities of the four species of *Fusarium* were confirmed by morphology after growing the pathogen on carnation leaf agar. From the non-inoculated control plants, *Fusarium* was not recovered.

Significance of this study

In South Dakota, the common practices of no-till will provide a better opportunity for species of *Fusarium* to survive longer on crop residues and therefore, pose a greater risk for infecting possible crops rotated with *B. carinata* such as wheat (*Triticum* sp.). In this study, only five *B. carinata* accessions were screened for their response to *F.acuminatum*, *F.oxysporum*, *F.solani* and *F.sporotrichioides* and the pathogens were virulent on all the accessions despite using a randomly selected isolate of the fungus. Hence, larger number of *B. carinata* accessions will have to be screened to identify accessions with resistance to the four species of *Fusarium*. Future management of Fusarium root rot of *B. carinata* will depend on use of resistant varieties, in combination with other Integrated Pest Management strategies, such as seed treatments (fungicide or biological) and tillage practices. To the best of our knowledge, this is the first report of *F.acuminatum*, *F.oxysporum*, *F.solani* and *F.sporotrichioides* causing root rot of *B.carinata*.

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Table 1. Median, mean rank and relative treatment effects for root rot severity caused by *Fusarium oxysporum* isolate FUS-CAR002 on five *B. carinata* accessions in the greenhouse.

Brassica carinata accessions	Origin	Median disease ratings [a]	Mean rank [b]	Relative Treatment Effects (RTE) [c, d]
PI 195552	Ethiopia	1.50	20.75	0.34 (0.24, 0.46)*
PI 274283	Ethiopia	2.00	26.88	0.44 (0.32, 0.57)*
PI 273640	Ethiopia	2.00	27.13	0.44 (0.33, 0.57)*
PI 199950	Ethiopia	2.00	36.75	0.60 (0.47, 0.72)
PI 360887	Sweden	2.00	41.00	0.68 (0.59, 0.75)**

[a] *Brassica carinata* roots were assessed for root rot severity at 21 days after inoculation on a 0-to-4 rating scale modified from Hwang et al. (1994), where 0 = no symptoms, 1 = light symptoms (discoloration but no visible lesions), 2 = obvious lesions, 3 = severe lesions and 4 = plant dead.

[b] The ranks corresponding to each treatment was calculated using the nparLD package (Noguchi et al. 2012) in R v 2.1 (R Core Team 2013) as " $\overline{R}_i = \frac{1}{n_i}\sum_{k=1}^{n_i} R_{ik}$ (Akritas 1991), where $\overline{R}_i$ is the mean rank for the ith treatment and R_{ik} is the rank of X_{ik} among all N observations" (Shah and Madden 2004).

[c] The relative treatment effects (RTE) for each treatment was calculated from mean ranks ($\overline{R}_i$) as $\widehat{p_i} = \frac{1}{N}(\overline{R}_i - \frac{1}{2})$ [where N is the total number of observations; Shah and Madden 2004] and compared by calculating their 95% confidence intervals (lower and upper limits in parentheses) using the nparLD package in R.

[d] Asterisk indicates significant differences among *B. carinata* accessions in response to *Fusarium oxysporum* isolate FUS-CAR002.

Table 2. Median, mean rank and relative treatment effects for root rot severity caused by *Fusarium acuminatum* isolate FUS-CAR003 on five *B. carinata* accessions in the greenhouse.

Brassica carinata accessions	Origin	Median disease ratings [a]	Mean rank [b]	Relative Treatment Effects (RTE) [c, d]
PI 274283	Ethiopia	2.0	27.17	0.44 (0.33, 0.57)
PI 195552	Ethiopia	2.0	29.50	0.48 (0.37, 0.60)
PI 360887	Sweden	2.0	31.33	0.51 (0.39, 0.64)
PI 273640	Ethiopia	2.0	31.33	0.51 (0.41, 0,61)
PI 199950	Ethiopia	2.0	33.17	0.54 (0.43, 0.65)

[a] *Brassica carinata* roots were assessed for root rot severity at 21 days after inoculation on a 0-to-4 rating scale modified from Hwang et al. (1994), where 0 = no symptoms, 1 = light symptoms (discoloration but no visible lesions), 2 = obvious lesions, 3 = severe lesions and 4 = plant dead.

[b] The ranks corresponding to each treatment was calculated using the nparLD package (Noguchi et al. 2012) in R v 2.1 (R Core Team 2013) as " $\overline{R}_i = \frac{1}{n_i}\sum_{k=1}^{n_i} R_{ik}$ (Akritas 1991), where $\overline{R}_i$ is the mean rank for the ith treatment and R_{ik} is the rank of X_{ik} among all N observations" (Shah and Madden 2004).

[c] The relative treatment effects (RTE) for each treatment was calculated from mean ranks ($\overline{R}_i$) as $\widehat{p_i} = \frac{1}{N}(\overline{R}_i - \frac{1}{2})$ [where N is the total number of observations; Shah and Madden 2004] and compared by calculating their 95% confidence intervals (lower and upper limits in parentheses) using the nparLD package in R.

[d] No significant differences among *B. carinata* accessions in response to *Fusarium acuminatum* isolate FUS-CAR003

Table 3. Median, mean rank and relative treatment effects for root rot severity caused by *Fusarium solani* isolate FUS-CAR004 on five *B. carinata* accessions in the greenhouse.

Brassica carinata accessions	Origin	Median disease ratings [a]	Mean rank [b]	Relative Treatment Effects (RTE) [c, d]
PI 199950	Ethiopia	2.0	27.50	0.45 (0.32,0.59)
PI 360887	Sweden	2.0	29.13	0.48 (0.35, 0.61)
PI 273640	Ethiopia	2.0	30.75	0.50 (0.38, 0.63)
PI 195552	Ethiopia	2.0	31.75	0.52 (0.43, 0.61)
PI 274283	Ethiopia	2.0	33.38	0.55 (0.45, 0.64)

[a] *Brassica carinata* roots were assessed for root rot severity at 21 days after inoculation on a 0-to-4 rating scale modified from Hwang et al. (1994), where 0 = no symptoms, 1 = light symptoms (discoloration but no visible lesions), 2 = obvious lesions, 3 = severe lesions and 4 = plant dead.

[b]The ranks corresponding to each treatment was calculated using the nparLD package (Noguchi et al. 2012) in R v 2.1 (R Core Team 2013) as “ $\overline{R}_i = \frac{1}{n_i}\sum_{k=1}^{n_i} R_{ik}$ (Akritas 1991), where $\overline{R}_i$ is the mean rank for the ith treatment and R_{ik} is the rank of X_{ik} among all N observations” (Shah and Madden 2004).

[c] The relative treatment effects (RTE) for each treatment was calculated from mean ranks ($\overline{R}_i$) as $\widehat{p_i} = \frac{1}{N}(\overline{R}_i - \frac{1}{2})$ [where N is the total number of observations; Shah and Madden 2004] and compared by calculating their 95% confidence intervals (lower and upper limits in parentheses) using the nparLD package in R.

[d] No significant differences among *B. carinata* accessions in response to *Fusarium solani* isolate FUS-CAR004

Table 4. Median, mean rank and relative treatment effects for root rot severity caused by *Fusarium sporotrichioides* isolate FUS-CAR008 on five *B. carinata* accessions in the greenhouse.

Brassica carinata accessions	Origin	Median disease ratings [a]	Mean rank [b]	Relative Treatment Effects (RTE) [c, d]
PI 273640	Ethiopia	1.5	25.50	0.42 (0.28, 0.58)
PI 199950	Ethiopia	2.0	29.08	0.48 (0.35, 0.61)
PI 274283	Ethiopia	2.0	30.88	0.51 (0.39, 0.63)
PI 360887	Sweden	2.0	30.88	0.51 (0.40, 0.61)
PI 195552	Ethiopia	2.0	36.17	0.59 (0.47, 0.70)

[a] *Brassica carinata* roots were assessed for root rot severity at 21 days after inoculation on a 0-to-4 rating scale modified from Hwang et al. (1994), where 0 = no symptoms, 1 = light symptoms (discoloration but no visible lesions), 2 = obvious lesions, 3 = severe lesions and 4 = plant dead.

[b] The ranks corresponding to each treatment was calculated using the nparLD package (Noguchi et al. 2012) in R v 2.1 (R Core Team 2013) as “ $\overline{R}_i = \frac{1}{n_i}\sum_{k=1}^{n_i} R_{ik}$ (Akritas 1991), where $\overline{R}_i$ is the mean rank for the ith treatment and R_{ik} is the rank of X_{ik} among all N observations” (Shah and Madden 2004).

[c] The relative treatment effects (RTE) for each treatment was calculated from mean ranks ($\overline{R}_i$) as $\widehat{p_i} = \frac{1}{N}(\overline{R}_i - \frac{1}{2})$ [where N is the total number of observations; Shah and Madden 2004] and compared by calculating their 95% confidence intervals (lower and upper limits in parentheses) using the nparLD package in R.

[d] No significant differences among *B. carinata* accessions in response to *Fusarium sporotrichioides* isolate FUS-CAR008

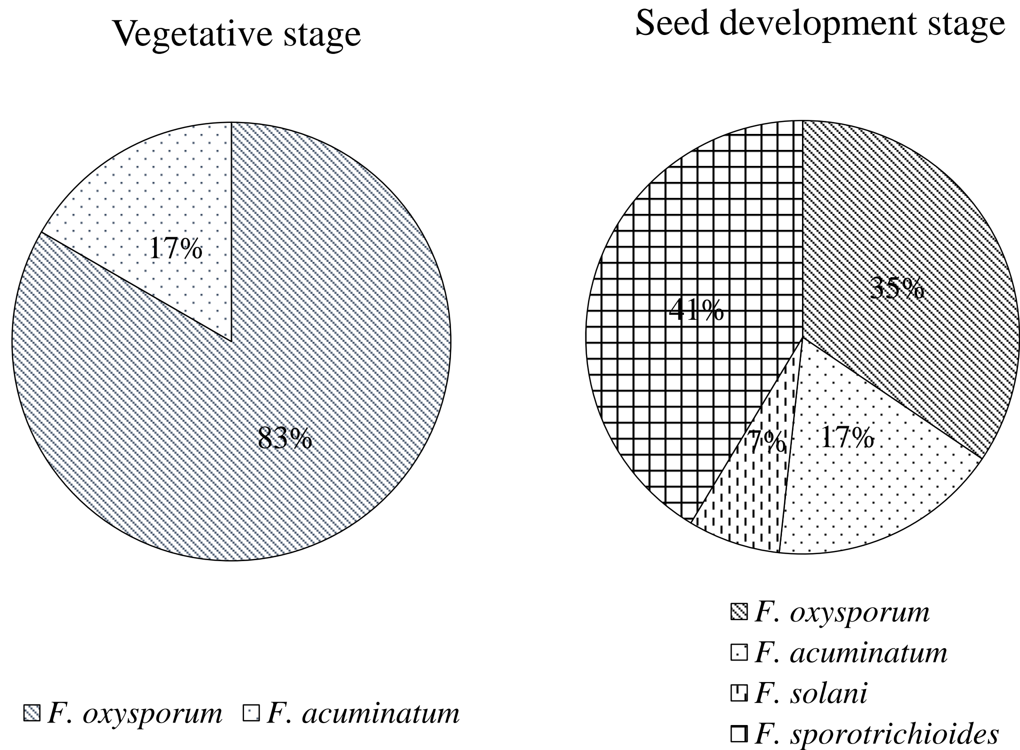

Fig. 1. Frequency (%) of isolation of species of *Fusarium* from diseased roots of *B. carinata* plants sampled at the vegetative and seed development growth stages of the crop from experimental plots at the South Dakota State University Research Farm in Brookings County, South Dakota in 2017.

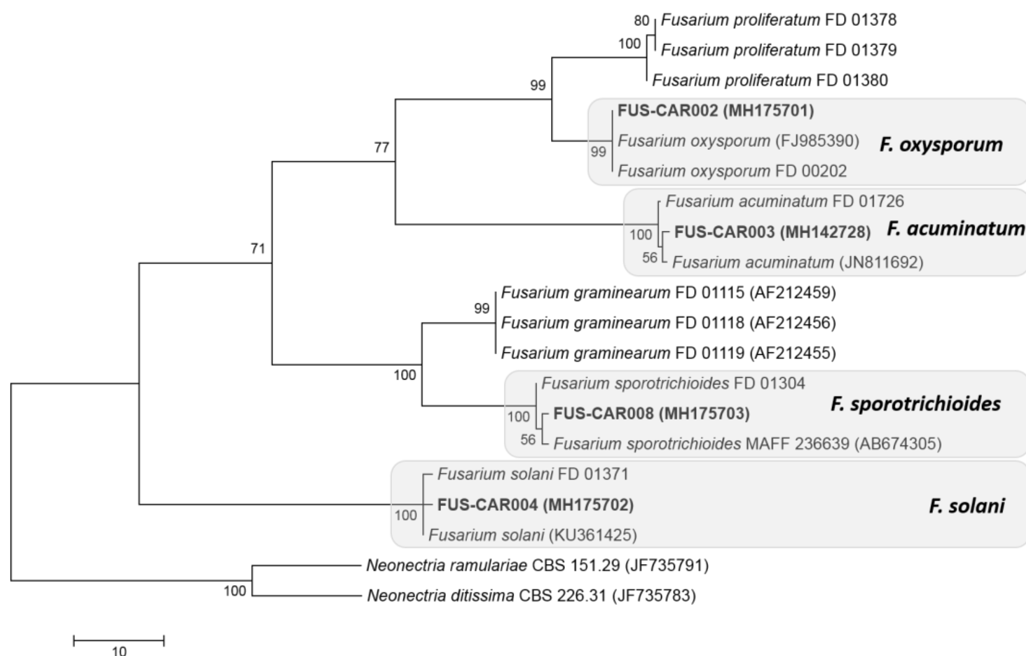

Fig. 2. Phylogenetic tree of the TEF gene of *Fusarium* isolates recovered from diseased roots of *B. carinata* plants in South Dakota. The TEF phylogenetic tree was inferred using the Maximum Parsimony analyses in MEGA6 (Tamura et al. 2013) with bootstrap analyses of 1000 replications. The TEF phylogeny grouped isolates in four well-supported clades that contained type sequences of *F.oxysporum, F.acuminatum*, *F.sporotrichioides*, and *F.solani* obtained from *Fusarium*-ID, with a bootstrap value of 99% or 100%. Species of *Neonectria* (Nectriaceae, Hypocreales) were used as the outgroup (JF735791 and JF735783).

Appendix 2.

Eight species of *Fusarium* cause root rot of corn (*Zea mays* L.) in South Dakota.

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Abstract

Fusarium root rot of corn (*Zea mays* L.) is yield-limiting in the United States, but there is no information available on the disease in South Dakota. In 2015, corn seedlings with discolored roots were arbitrarily sampled from 50 South Dakota fields and 198 isolates were recovered. Eight species (*F.acuminatum*, *F.boothii*, *F.equiseti-incarnatum complex*, *F.graminearum*, *F.oxysporum*, *F.proliferatum*, *F.solani*, and *F.subglutinans*) were identified by morphology and TEF1-α gene sequencing. *Fusarium graminearum* (26.8%) was the most common fungus, and *F.boothii* (0.5%) was the least recovered. Fourteen isolates, representing the eight species, were evaluated for their pathogenicity on two-week old seedlings of inbred 'B73' using the inoculum layer method in the greenhouse. Fourteen days' post-inoculation, root rot severity was evaluated on a 1 to 5 rating scale and expressed as relative treatment effects (RTE). *Fusarium proliferatum* isolate P2 caused significantly greater RTE (based on 95% CI) on seedlings than the other isolates and the non-inoculated control except *F.graminearum* isolate FG23. This study indicates that the eight species of *Fusarium* are aggressive root rot pathogens of corn in South Dakota, and this information will help evaluate strategies for producers to manage the disease in their fields.

Diseases of corn (*Zea mays* L.) caused by species of *Fusarium* (e.g. Fusarium ear rot, root rot, and stalk rot) are yield-limiting in the United States and Ontario, Canada (Mueller et al. 2016). In 2015, the total yield losses due to *Fusarium*-associated diseases of corn in the United States and Ontario, Canada were estimated at 6.3 million metric tons

(Mueller et al. 2016). For all *Fusarium*-associated diseases, the causal pathogens are either soil-borne or seed-borne (Dodd and White 1999; Ocamb and Komedahl 1994).

Among the *Fusarium*-associated diseases, root rot of corn may be understudied (Smit 1998). This maybe because diagnosis of Fusarium root rot is complicated since multiple organisms can be isolated from a single diseased corn plant and these include species of *Fusarium* (Dodd and White 1999; Ocamb and Komedahl 1994), *Rhizoctonia* (Sumner and Bell 1982), and *Pythium* (Matthiesen et al. 2016). Among the species of *Fusarium* reported to colonize corn roots, *F.acuminatum* Ellis and Everhart, *F. chlamydosporum* Wollenweber and Reinking, *F. culmorum* (Smith) Saccardo, *F. equiseti* (Corda) Saccardo (syn. *F.equiseti-incarnatum complex*), *F.graminearum* Schwabe, *F.oxysporum* Schlechtendal, *F. poae* (Peck) Wollenweber, *F.proliferatum* (Matsushima) Nirenberg, *F.redolens* Wollenweber, *F.semitectum* Berkeley and Ravenel, *F.solani* (Martius) Saccardo, *F.subglutinans* (Wollenweber and Reinking) Nelson, Toussoun and Marasas, and *F.verticillioides* (Saccardo) Nirenberg (syn. *F.moniliforme* Sheldon), are important (Kuhnem et al. 2015; Leslie et al. 1990; Munkvold and O'Mara 2002; Munkvold and Desjardins 1997; Ocamb and Kommedahl 1994; Parikh et al. 2018; Ranzi et al. 2017; Soonthornpoct et al. 2001). In general, corn seedlings affected by Fusarium root rot have brown to dark discoloration and decayed roots (Gilbertson et al. 1985; Soonthornpoct et al. 2001; Wise et al. 2016).

In South Dakota, most producers rotate corn with soybean (*Glycine max* L.), wheat (*Triticum aestivum* L.), or sunflower (*Helianthus annuus* L.) and combine the rotation with no-tillage systems. Such cropping practices can favor the survival of species of *Fusarium* as substantial amount of crop residue may be left on the soil surface, which can increase

the amount of inoculum for the crop in the subsequent season. Despite that fungicides are used to treat corn seeds, seedling, and root diseases of corn are becoming a concern in the United States. At this time, there is no information available on the pathogens causing root rot of corn in South Dakota. However, several species of *Fusarium* were recently isolated from diseased corn seedlings and therefore, the objectives of this study were to characterize species of *Fusarium* associated with root rot of corn and determine their aggressiveness in the greenhouse.

Isolation and Identification of *Fusarium*

In 2015, corn plants with discolored roots were arbitrarily sampled from a total of 50 commercial fields (five samples per field) across 24 counties in eastern South Dakota, where over 50% of corn production takes place. The corn plants were sampled early in the season (following rain) between V1 (first leaf) and V3 (third leaf) vegetative growth stages (Ritchie et al. 1992), along five transects (50 m) that covered an area of two hectares of the field where excess moisture was observed in soils. The distance between the corn fields ranged from approximately 1 to 2 km.

To isolate fungi, the corn roots were washed under running tap water for 2 to 5 min to remove soil particles and any debris from the field. The infected root tissues from each plant were cut into small pieces of approximately 15 mm long and surface-disinfested in sodium hypochlorite (0.05%) and ethanol (70%) for 1 min each, rinsed with sterile distilled water, and blotted dry with sterile paper towels. Three root pieces (~15 mm) were plated on potato dextrose agar (PDA) amended with streptomycin sulfate (0.02%) and incubated at 23 ± 2°C for 7 days.

From five plants sampled per field, one to four putative *Fusarium* isolates were recovered on PDA. In total, 198 isolates were collected and identified to species level by transferring hyphal tips of the colonies onto fresh PDA plates to obtain pure cultures. From the growing edge of the colony of the *Fusarium* isolates, one mycelial plug (~3 mm square) was removed with a sterile scalpel and transferred to carnation leaf agar (CLA) to examine morphological characteristics (Leslie and Summerell 2006). The isolates were identified to eight species of *Fusarium* (*F.acuminatum*, *F.boothii* O'Donnell, Aoki, Kistler and Geiser, *F.equiseti-incarnatum* complex, *F.graminearum*, *F.oxysporum*, *F.proliferatum*, *F.solani*, and *F.subglutinans*). Among the 198 isolates, *F.graminearum* (26.8%) was the most commonly recovered, followed by *F.oxysporum* (22.2%), *F.equiseti-incarnatum complex* (16.2%), *F.acuminatum* (13.6%), *F.proliferatum* (11.1%), *F.subglutinans* (5.6%), *F.solani* (4.0%), and *F.boothii* (0.5%).

Twenty-seven isolates identified as *F.acuminatum* produced red pigment, curved macroconidia with three to five septate (*n* =100; 7.0 to 10.0 x 0.7 to 1.0 μm), and no microconidia but with chain formation of chlamydospores. One isolate was tentatively identified as *F.boothii* as it produced thick-walled macroconidia with five to seven septate (*n* =100; 5.9 to 10.0 x 0.8 to 1.1 μm). Thirty-two isolates were tentatively identified as *F.equiseti-incarnatum complex*, given the macroconidia on CLA were relatively long and narrow (*n* =100; 12.6 × 20.4 μm), with an average of five septa and whip-like bent apical cells. Chlamydospores and microconidia were not observed on CLA. Fifty-three isolates were tentatively identified as *F.graminearum* given the macroconidia were slender and slightly curved with five to six septate (*n* = 100; 4.5 to 11.5 x 0.8 to 1.1 μm). No microconidia were observed but chlamydospores were present in singular form. Forty-four

isolates were tentatively identified as *F.oxysporum* given the macroconidia were less abundant, and were three septate (*n* =100; 5.6 to 7.1 x 0.5 to 0.9 µm). The microconidia were in abundance and formed on false heads (*n* =100; 0.6 to 2.0 x 0.3 to 0.6 µm). Twenty-two isolates were tentatively identified as *F.proliferatum* given the macroconidia were relatively straight with curved apical cell. The septate were two to five in number (*n* = 100; 4.8 to 8.1 x 0.7 to 1.2 µm). The microconidia were produced in abundance, oval in shape without septate. They were formed in chains on both monophialides and polyphialides (*n* = 100; 0.6 to 1.3 x 0.5 to 0.7 µm). Eight isolates were tentatively identified as *F.solani* as they produced green sporodochia. The macroconidia had five to seven septate (*n*=100; 4.6 to 6.6 x 0.6 to 1.1 µm). The microconidia were oval shaped with one to two septate (*n*=100; 1.2 to 2.5 x 0.4 to 1.0 µm). Eleven isolates were tentatively identified as *F.subglutinans*, given the macroconidia were abundant, slender, thin-walled and with curved apical cell (*n*=100; 5.2 to 7.3 x 0.4 to 1.1 µm). The microconidia were produced in abundance, oval shaped and non-septate (*n*=100; 1.2 to 2.6 x 0.4 to 0.8 µm).

For molecular identification, a total of 25 isolates were selected by South Dakota County to represent the eight species (Table 1). DNA was extracted from the isolates and the translational elongation factor 1-α (TEF1-α) gene region was sequenced using the primers EF1F and EF1R (Geiser et al. 2004). The TEF1-α sequences were used for performing maximum parsimony phylogenetic analysis in Molecular Evolutionary Genetics Analysis (MEGA) software (v7; Kumar et al. 2016). Out of 761 aligned characters, 343 were parsimony-informative characters, which was included in the maximum parsimony analyses and resulted in seven most parsimonious trees. The consistency index was (0.72), the retention index was (0.93), and the composite index is

0.67 (parsimony-informative sites = 0.66) for all sites. The TEF1-α based-phylogeny grouped the isolates in eight well-supported clades (bootstrap value from 94 to 100%) that included type sequences of the eight species identified by BLASTN searches in the National Center for Biotechnology Information database (NCBI; http://www.ncbi.nlm.nih.gov/) (Fig. 1).

Greenhouse experiment to evaluate aggressiveness of *Fusarium* isolates

For the aggressiveness study, 14 isolates representing the eight species of *Fusarium* were selected arbitrarily based on morphological grouping from the 25 isolates and evaluated on the corn inbred 'B73' (PI 550473) in the greenhouse. To prepare the inoculum, each of the 14 isolates was initially grown on PDA and then five mycelial plugs (~15 mm square) were transferred into a 250-ml Erlenmeyer flask containing autoclaved sand-corn meal mixture (54 g of play sand, 6 g of cornmeal, and 10 ml distilled water). The flasks containing the inoculum were mixed every other day by manually shaking the flask to ensure uniform colonization of the sand-corn meal mixture. For the non-inoculated control, sand-cornmeal mixture without any fungus was used for inoculum. These were incubated at 23 ± 2°C for 14 days.

At planting, the inoculum layer method from Bilgi et al. (2008) was used. The plastic cups (473 ml) were first filled with 40 g coarse vermiculite, followed by 20 g of inoculum and then 20 g of coarse vermiculite before planting the pre-germinated seeds of 'B73'. The seeds were pre-germinated in petri plates for 5 days on a wet filter paper and after the seeds sprout roots, they were transplanted into plastic cups. In each cup, two sprouting seeds were planted and covered with additional 20 g of coarse vermiculite. The experiment was set up as a completely randomized design (CRD) with five replications

(cups) and performed twice. The temperature in the greenhouse was maintained at 22 ± 2°C under 16 h photoperiod. The plants were watered once daily and no fertilizer was added during the experiment.

The experiment was terminated at 14 days after inoculation and root rot severity caused by the isolates on corn seedlings was evaluated on a 1 to 5 rating scale (Acharya et al. 2015), where, 1 = germination and healthy seedlings with no visible root colonization, 2 = germination and 1 to 19% of the root having lesions, 3 = germination and 20 to 74% of the root having lesions, 4 = germination and 75% or more of the root having lesions; and 5 = no germination and complete colonization of seed.

The root rot severity data was not normally distributed and therefore, analyzed using nonparametric statistics (Shah and Madden 2004). The Fligner-Killen test for homogeneity of variance between the two experimental repeats was tested and satisfied (P = 0.09) prior to data analysis. The nparLD package (Noguchi et al. 2012) in R v2.1 (R Core Team 2013) was used to determine the analysis of variance (ANOVA) type test statistics (ATS) of ranked data, which indicated the overall effect of the treatments. The nparLD package calculated the rank of each isolate (treatment) as "$\overline{R}_i = \frac{1}{n_i}\sum_{k=1}^{n_i} R_{ik}$ (Akritas 1991), in which $\overline{R}_i$ = the mean rank for the ith treatment, and R_{ik} = the rank of X_{ik} among all N observations" (Shah and Madden 2004). The root rot severity was expressed as relative treatment effects (RTE) [calculated as $\widehat{p_i} = \frac{1}{N}(\overline{R}_i - \frac{1}{2})$, where, $\overline{R}_i$ = mean ranks and N = the total number of observations (Shah and Madden 2004)] and compared at 95% confidence intervals in R using the nparLD package (Noguchi et al. 2012).

A significant effect of RTEs (ATS = 30.11; df = 4.94; P = 2.20 x 10^{-30}) caused by the treatments was observed on corn seedlings at 14 days after inoculation. All the 14

isolates caused discoloration on the roots of the corn seedlings. No discoloration was observed on the roots of the control seedlings. Among the treatments, *F.proliferatum* isolate P2 (median disease rating = 4.0) caused significantly higher RTE (based on 95% confidence intervals) than the other treatments except *F.graminearum* isolate F23 (median disease rating = 3.5). The RTE caused by *F.oxysporum* isolate P1 (median disease rating = 1.5) was significantly lower compared to that of all the other isolates (Fig. 2).

Significant differences in RTE were observed among isolates within *F.graminearum*, *F.oxysporum* and *F.proliferatum*. The RTE caused by *F.graminearum* isolate F23 (median disease rating = 3.5) was significantly higher than that caused by F10 (median disease rating = 2.0). The RTE caused by *F.oxysporum* isolate P7 (median disease rating = 2.0) was observed to be significantly higher than that caused by PI (median disease rating =1.5), and the RTE caused by *F.proliferatum* isolate P2 (median disease rating = 4.0) was significantly higher compared to that of either F14-PB (median disease rating = 3.0) or F1 (median disease rating = 3.0) (Fig. 2).

To fulfill Koch's postulates, roots of inoculated seedlings were randomly selected and the fungi were re-isolated by plating diseased root pieces on PDA as previously described. Colonies of the isolates were transferred to CLA for morphology based identification. From the roots of the non-inoculated corn seedlings, species of *Fusarium* were not recovered.

Summary and Importance

Our study confirms that eight species of *Fusarium* are capable of causing root rot of corn in South Dakota. Among the eight species, *F.graminearum* was the most commonly recovered from the roots of the diseased corn seedlings, followed by

F.oxysporum and *F.equiseti-incarnatum complex*. In the greenhouse, isolates of *F.acuminatum*, *F.boothii*, *and F.equiseti-incarnatum complex, F.graminearum, F.oxysporum*, *F.proliferatum*, *F.solani*, and *F.subglutinans* were determined to be aggressive on 'B73'. In addition, significant differences in aggressiveness were observed among isolates within *F.graminearum*, *F.oxysporum*, and *F.proliferatum*.

In the aggressiveness study, *F.oxysporum* caused little discoloration from the two isolates on the corn roots when compared to the non-inoculated plant roots. This is possibly because our experiment was performed at a greenhouse temperature of 22°C and the roots were not wounded. Based on the study by Warren and Kommedahl (1973), *F.oxysporum* can cause root rot of corn only under high temperatures (e.g. 29°C), in the presence of another species of *Fusarium* or other fungi, and if the roots are wounded. In contrast to *F.oxysporum*, isolates of *F.acuminatum*, *F.boothii*, *F.graminearum*, and *F.proliferatum* caused severe discoloration of corn roots. The severity of root rot observed in seedlings inoculated with *F.graminearum* isolates is consistent with that of previous research (Broders et al. 2007). However, in the case of *F.acuminatum* and *F.proliferatum* isolates, our study is not consistent with the previous studies that these two fungi contributed little or none to root rot development on corn (Mao et al. 1998; Ocamb and Kommedahl 1994; C. M. Ocamb and T. Kommedahl, *unpublished data*). In our study, isolates of *F.acuminatum* and *F.proliferatum* caused necrotic lesions on the root of corn plants and we suspect that lower temperature in the greenhouse played a role in disease development. As for *F.boothii*, the fungus was reportedly caused Gibberella ear rot in China (Duan et al. 2016), Mexico (Cerón-Bustamante et al. 2018), and South Africa (Boutigny et al. 2011). *Fusarium boothii* has been reported on corn in the United States, but the pathogenicity of

the fungus was not examined (Aoki et al. 2012). Although one isolate of *F.boothii* was used for this study, it caused severe root rot which was not significantly different from that caused by *F.graminearum* (F23) and *F.proliferatum* (F14-PB) isolates. This suggests that *F.boothii* is pathogenic on corn, and to our knowledge, this is the first report of the fungus causing Fusarium root rot of corn.

This study suggests that species diversity of *Fusarium* associated with corn roots may have changed since the research by Warren and Kommedahl (1973), who found six species of *Fusarium* (*F. episphaeria* (Tode) Snyder and Hansen, *F. moniliforme* Sheldon, *F.oxysporum*, *F. roseum* Link, *F.solani*, and *F. tricinctum* (Corda) Saccardo) colonizing corn roots, rhizosphere, residues and soil. However, additional research is required to study the environmental factors affecting root rot development of corn (e.g. temperature) caused by the eight species of *Fusarium* and to evaluate integrated pest management tools (e.g. genetic resistance, fungicide seed treatments) to manage Fusarium root rot in South Dakota corn fields.

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Table 1. Information on the representative *Fusarium* isolates used for molecular identification and greenhouse aggressiveness study.

Isolate[a]	Fungal names	County in South Dakota	Growth stage at time of collection	Previous Crop	GenBank Accession Number
F7	*Fusarium acuminatum*	Sanborn	V2	Soybean	MH595496
F25	*F.acuminatum*	Clark	V2- V3	Wheat	MH595498
F6	*F.acuminatum*	Miner	V2	Corn	MH595499
F8	*F.acuminatum*	Spink	V1	Corn	MH595497
F9	*F.boothii*	Clark	V2- V3	Wheat	MH595500
F16	*F. equiseti-incarnatum*	Minnehaha	V3	Corn	MH595503
F27	*F. equiseti-incarnatum*	Hamlin	V3	Soybean	MH595501
F28	*F. equiseti-incarnatum*	Hamlin	V3	Soybean	MH595502
F10	*F.graminearum*	Codington	V1	Corn	MH595504
E22	*F.graminearum*	Hutchinson	V1-V2	Corn	MH595506
F12	*F.graminearum*	Clark	V2- V3	Wheat	MH595505
F13	*F.graminearum*	Lincoln	V2	Soybean	MH595507
F23	*F.graminearum*	Douglas	V2	Corn	MH595508
F2	*F.oxysporum*	Brookings	V1	Corn	MH595509
F3	*F.oxysporum*	Brookings	V1	Corn	MH595510
E15	*F.oxysporum*	Davison	V1-V2	Corn	MH595511
P1	*F.oxysporum*	Brown	V2-V3	Corn	MH595514
P4	*F.oxysporum*	Lake	V2	Corn	MH595515
P7	*F.oxysporum*	Minnehaha	V3	Soybean	MH595512
F1	*F.proliferatum*	Brookings	V1	Corn	MH595516
F14-PB	*F.proliferatum*	Lincoln	V3	Soybean	MH595517
P2	*F.proliferatum*	Brookings	V1	Corn	MH595518
F11	*F.solani*	Clay	V2	Corn	MH595519
P6	*F.solani*	Browns	V1-V2	Wheat	MH595520
P5	*F.subglutinans*	Miner	V2	Corn	MH595522

[a]These isolates were recovered from diseased corn roots sampled from 50 commercial fields (across 24 counties) in South Dakota during the 2015 growing season.

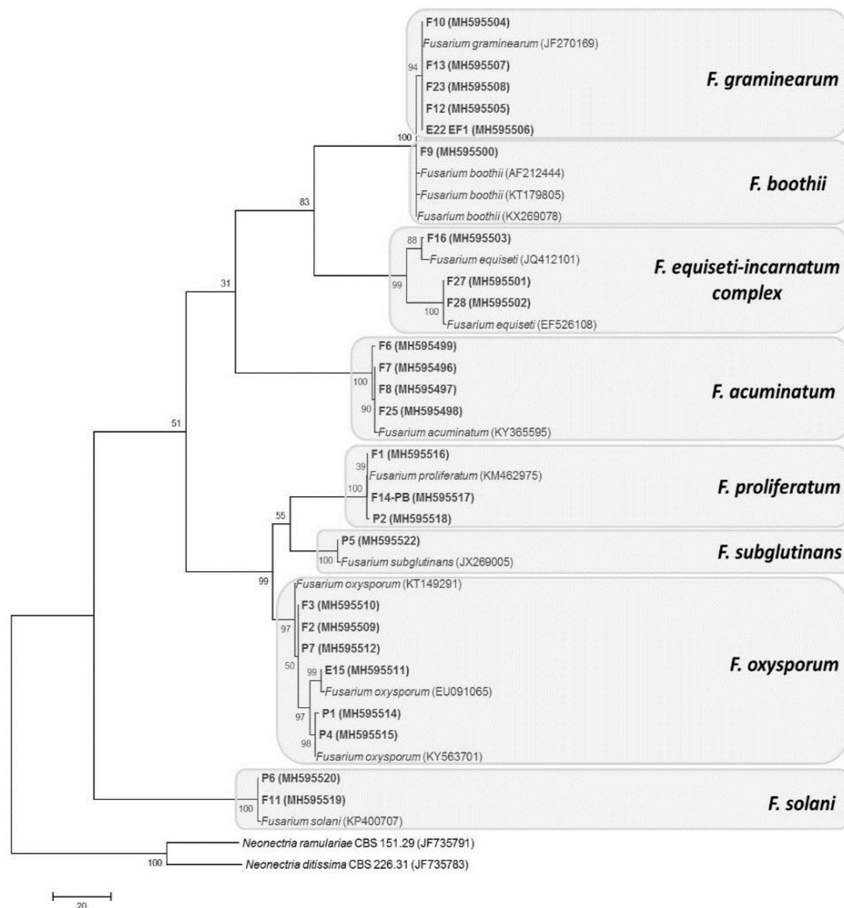

Fig. 1. The evolutionary history was inferred using the Maximum Parsimony method (MP) in Molecular Evolutionary Genetics Analysis (MEGA) software (v7; Kumar et al. 2016). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 40 nucleotide sequences included the outgroups *Neonectria ramulariae* and *N. ditissima*. The new generated sequences are in bold.

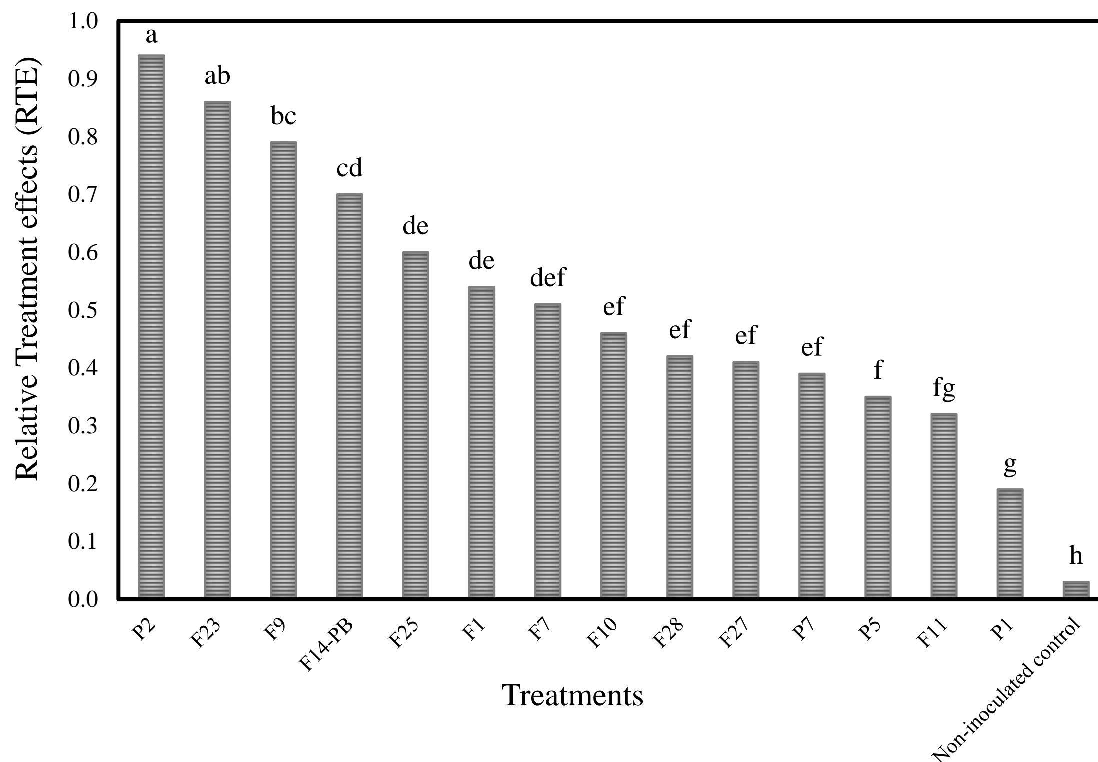

Fig. 2. Root rot severity (expressed as RTE) caused by 14 isolates on the seedlings of the corn inbred 'B73' in the greenhouse. RTE with the same letter are not significantly different among treatments based on 95% confidence intervals. Abbreviations: P2= *F.oxysporum*, F23= *F.graminearum*, F9= *F. boothi*, F14-PB= *F.proliferatum*, F25= *F.acuminatum*, F1= *F.proliferatum*, F7= *F.acuminatum*, F10= *F.graminearum*, F28= *F.equiseti-incarnatum*, F27=*F.equiseti-incarnatum*, P7= *F.oxysporum*, P5= *F.subglutinans*, F11= *F.solani*, P1= *F.oxysporum.*