Soybean Sudden Death Syndrome Causal Agent *Fusarium brasiliense* Present in Michigan

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Abstract

Sudden death syndrome (SDS), caused by members of *Fusarium solani* species complex (FSSC) clade 2, is a major and economically important disease in soybean worldwide. The primary causal agent of SDS isolated to date in North America has been *F. virguliforme*. In 2014 and 2016, SDS symptoms were found in two soybean fields located on the same farm in Michigan. Seventy *Fusarium* strains were isolated from roots of the SDS-symptomatic soybeans in two fields. Phylogenetic analysis of partial sequences of elongation factor-1 α , the nuclear ribosomal DNA intergenic spacer region, and the RNA polymerase II beta subunit revealed that the primary FSSC species isolated was *F. brasiliense* (58 and 36% in each field) and the remaining *Fusarium* strains were identified as *F. cuneirostrum*, *F. phaseoli*, an undescribed *Fusarium* sp. from

FSSC clade 2, and strains in FSSC clade 5 and FSSC clade 11. Molecular identification was supported with morphological analysis and a pathogenicity assay. The soybean seedling pathogenicity assay indicated that *F. brasiliense* was capable of causing typical foliar SDS symptoms. Both root rot and foliar disease severity were variable by strain, just as they are in *F. virguliforme*. Both FSSC 5 and FSSC 11 strains were also capable of causing root rot, but SDS foliar symptoms were not detected. To our knowledge, this is the first report of *F. brasiliense* causing SDS in soybean in the United States and the first report of *F. cuneirostrum*, *F. phaseoli*, an as-yet-unnamed *Fusarium* sp., and strains in FSSC clade 5 and FSSC clade 11 associated with or causing root rot of soybean in Michigan.

For the first time in recent U.S. history, planted soybean [*Glycine max* (L.) Merr.] acres surpassed planted corn [*Zea mays* L.] in 2018, at 89.6 million acres compared with 89.1 million acres (National Agricultural Statistics Service 2018). More than 80% of the soybean acreage is cultivated in the upper Midwest, and more than 2 million acres of soybean were planted and harvested in Michigan with an estimated yield of 2.83 metric tons in 2016. Although there is a high level of production, this can be affected by several factors. For example, one of the most destructive diseases in soybean is sudden death syndrome (SDS), which is caused by four *Fusarium* species (*F. virguliforme* O'Donnell & T. Aoki, *F. brasiliense* T. Aoki & O'Donnell, *F. crassispitatum* T. Aoki & O'Donnell, and *F. tucumaniae* T. Aoki, O'Donnell, & Y. Homma) that reside within clade 2 of the *F. solani* species complex (FSSC) (Aoki et al. 2003, 2005, 2012). Although SDS has been reported from all major soybean-producing states in the United States to date except North Dakota (Hartman et al. 2015; O'Donnell et al. 2010; Rupe et al. 2001; Wang and Chilvers 2016), *F. virguliforme* is the only SDS-causal pathogen reported in the United States. In contrast, all four *Fusarium* species causing SDS have been found in South America, specifically in Argentina and Brazil (Aoki et al. 2003, 2005), and both *F. virguliforme* and *F. brasiliense* were reported as causal agents

responsible for SDS outbreaks in South Africa (Tewoldemedhin et al. 2014, 2017).

The first symptoms of soybean SDS occur on the roots, with symptoms of necrosis and tap root decay (Roy et al. 1997). Foliar symptoms generally develop during mid- to late reproductive growth stages. Symptomatic leaves demonstrate interveinal chlorosis and necrosis, and premature defoliation can result (Hartman et al. 2015). All *Fusarium* species causing SDS are soilborne and are capable of producing macroconidia and chlamydospores as their major means of survival in soil or in plant debris (Westphal et al. 2008). In addition to soybean, *F. virguliforme* has been reported to infect a wide range of asymptomatic host plants (Kolander et al. 2012). Infested soil or plant debris serves as a primary inoculum for the next growing season, and the movement of infested soil or plant debris can contribute to pathogen dispersal (Nakajima et al. 1993; Roy et al. 1997).

The sickle- or boat-shaped macroconidium is one of the key morphological features to identify *Fusarium* species to the genus level (Leslie and Summerell 2006). Colony and microscopic morphology can resolve *Fusarium* strains into species or species complex levels, and additional colony and host pathogenicity characteristics can delineate the *Fusarium* species into further groupings such as formae specialis (Aoki et al. 2014). However, because of the high morphological similarity within some groups (e.g., within clades of the FSSC), molecular sequence markers are required for confident species discrimination (Aoki et al. 2003; Leslie and Summerell 2006; O'Donnell 2000). One such recommended region is parsimony informative characters within ribosomal DNA (rDNA) intergenic spacer (IGS) multiple sequence alignments, which can resolve species in clade 2 of the FSSC (Aoki et al. 2003, 2005, 2012; O'Donnell et al. 2010).

Soybean SDS has become increasingly problematic and well established across the soybean production region in Michigan. *F. virguliforme* was initially reported in six Michigan counties (Chilvers and Brown-Rytlewski 2010) and has since been detected in 28 counties as of 2016 (Chilvers et al. 2016; Wang and Chilvers 2016). As part of a population genetics project, more than 500 strains of *F. virguliforme* were collected from across the United States from 2012 to 2014, including from Arkansas, Illinois, Kansas, and Michigan

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(Wang and Chilvers, unpublished data). *F. virguliforme* isolate identity was confirmed with species-specific polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR) assays (Wang et al. 2015). In one Michigan location, diseased soybean plants with typical SDS symptoms were collected, and slow-growing *Fusarium* colonies, typical of FSSC clade 2 (Aoki et al. 2003), were isolated from roots. However, none of the isolates or root samples tested positive for *F. virguliforme*. Therefore, the objectives of this study were (i) to identify the *Fusarium* species causing SDS on soybean at this location and (ii) to confirm species identification using both morphological characteristics and molecular phylogenetics.

Materials and Methods

Isolation from soybean roots. SDS-symptomatic soybean plants were collected from two soybean fields located in Montcalm County, Michigan (43°18'36.00" N, -85°09'0.00" W). *Fusarium* strains were isolated from the root of SDS-symptomatic plants as described in Wang and Chilvers (2016). Briefly, taproots were vertically split into two or three pieces and then cut into 3- to 4-cm lengths. Root pieces were disinfested with 0.5% sodium hypochlorite for 3 min, rinsed in sterile deionized water for 1 min, and dried on sterilized paper towels. Three to four pieces of surface-disinfested root pieces were placed on 2% water agar amended with 300 mg per liter of streptomycin and 15 mg per liter of metalaxyl and incubated in the laboratory at room temperature (21°C) and in ambient light, with 12 h of light and 12 h of darkness per day for 5 days to induce formation of sporodochia. Conidia masses were picked from the sporodochia using a sterile microbiological loop, spread on 3% water agar, and incubated at room temperature for 8 to 10 h in ambient light. A single germinated macroconidium from each plate was transferred to potato dextrose agar (PDA) (39 g per liter; Neogen, Lansing, MI). For long-term storage, spore suspensions were stored in 20% glycerol at -80°C (Leslie and Summerell, 2006).

DNA extraction and PCR amplification. DNA from 70 *Fusarium* strains was extracted using the phenol-chloroform method according to Peever et al. (1999). PCR was performed in a DNA polymerase mixture (Phusion High-Fidelity; New England BioLabs, Ipswich, MA) containing ~50 to 250 ng of DNA from each strain to amplify partial sequences of translation elongation factor-1 α (EF-1 α) for all strains, the nuclear rDNA IGS region for strains identified as FSSC clade 2, and the RNA polymerase II beta subunit (RPB2) for strains identified as FSSC 5 and 11. Two primer sets, NL11/CNS1 and EF1/EF2, were used for the IGS region and EF-1 α amplification, respectively (Aoki et al. 2003; O'Donnell et al. 1998). Two previously published primer sets, 5F2/7cR and 7cF/11aR (O'Donnell et al. 2007; Reeb et al. 2004), were used for RPB2 amplification. In addition, four new primer sets were used. These included the following forward (F) and reverse (R) primers: F1-RPB2 (TACACCTTTGCATCCACTCTTTC) and R1-RPB2 (CATACCTGGTTGTGATCGGGG); F1-1-RPB2 (CTGCAAGCTCCA CTGCT) and R1-1-RPB2 (GGGGTGACTACATATTGTTAGCC); F2-RPB2 (CATTCCTTACTACCCGCAAAACC) and R2-RPB2 (AAGAACACCTGCGCTCGAA); and F2-1-RPB2 (GACAACTACT CTCGCCGTATG) and R2-1-RPB2 (CGACCATGTGACGGAGTC). These new primer sets were generated from consensus sequence by multiple sequence alignment of RPB2 of FSSC strains using MEGA version 6.06 (Tamura et al. 2013).

Sequence and phylogenetic analysis. PCR amplicons were purified with ExoSAP-IT (Affymetrix, Santa Clara, CA), and submitted to Macrogen Corporation (Rockville, MD) for sequencing using the aforementioned primer sets and internal IGS primers (Aoki et al. 2003). For phylogenetic analysis, EF-1 α , IGS, and RPB2 sequences of reference FSSC strains were obtained from the National Center for Biotechnology Information GenBank. Multiple sequence alignments were performed using MUSCLE (Edgar 2004). For each molecular marker, nucleotide substitution models were tested using the modelTest function in the phangorn R package (Schliep 2011) prior to building the phylogenetic trees. The best nucleotide substitution model was determined based on Bayesian information criteria.

Phylogenetic trees were built using MrBayes version 3 (Ronquist and Huelsenbeck 2003). The Bayesian consensus phylogenetic trees were visualized using the ggtree R package (Yu et al. 2017). EF-1 α , IGS, and RPB2 sequences of Michigan FSSC strains were deposited into GenBank (Table 1).

Examination of morphological characters. *Fusarium* strains were grown on PDA in 90-mm plates for 13 days at 25°C in the dark. The colony phenotypes were observed and images of colonies were taken. To characterize the morphology of macroconidia, the strains were grown on Spezieller Nährstoffarmer agar (SNA) for 13 days (Leslie and Summerell, 2006). Macroconidia stained with 1% safranin O solution were observed and photographed using a Leica DM5000 B microscope (Leica Microsystems, Buffalo Grove, IL) with a Leica DFC300FX digital camera. The size of 50 randomly selected macroconidia was measured using the Leica application suite (Leica Microsystems).

Pathogenicity tests. To determine the pathogenicity of *F. brasiliense* and other FSSC strains to soybeans, a seedling pathogenicity screen was performed using the soybean cultivar Sloan. The 17 strains used for pathogenicity tests were as follows: *F. brasiliense* (NRRL 22678, MIMtc-A8, MIMtc-C1, MIMtc-C9, and MIMtc-C12), *F. cuneirostrum* (NRRL 31157 and MIMtc-C11), *F. phaseoli* (NRRL 31156 and MIMtc-A12), an as-yet-unnamed *Fusarium* sp. (MIMtc-A4 and MIMtc-B8), *F. tucumaniae* (NRRL 31096), *F. virguliforme* (NRRL 22292), FSSC 5 (MIMtc-A16FS and MIMtc-C17FS), and FSSC 11 (MIMtc-B3FS and MIMtc-C13FS). NRRL strains were obtained from the Agricultural Research Service Culture Collection (Peoria, IL). Each strain was grown on Nash-Snyder medium (NSM) at room temperature for 14 days. An inoculum slurry of each strain was made by blending five colonized plates, five non-colonized NSM plates, and 500 ml of sterile deionized water in a Waring Pro blender. A 100-ml aliquot of the inoculum slurry was poured into a spawn bag containing 1.6 kg of autoclaved sorghum grain. The inoculum was allowed to colonize the sorghum grain for 4 weeks at room temperature under ambient light conditions. Inoculum (12 g) of each strain was mixed with 250 ml of medium vermiculite and placed into a 354-ml waxed paper cup (Solo, Lake Forest, IL) in which three 5-mm drainage holes had been made. An additional 70 ml of medium vermiculite was added to the cup and six soybean seeds (Sloan) were sown in each cup and covered with an additional layer of 70 ml of medium vermiculite. Cups containing no infested sorghum were used for the control. Foliar SDS disease severity (using a 1 to 9 scale, where 1 is a healthy plant, 2 to 5 indicate increasing severity of leaf interveinal chlorosis followed by necrosis, and 6 to 9 indicate increasing symptoms of defoliation and premature death) was rated 28 days after planting (growth stage: V4) in a growth chamber (20°C, 14 h of light) according to the modified method in Wang et al. (2019). The SDS disease severity index (DIX) score that accounted for both severity (DS) and disease incidence (DI) was calculated as follows: $DIX = DI \times (DS/\text{maximum disease score}) \times 100$. Roots were harvested, washed, and rated for root rot using a scale from 1 to 7 (where 1 is a plant with healthy roots and 7 indicates roots that are pithy or hollow, and having 80 to 100% reduction in root mass). The root rot DIX score was calculated as follows: $DIX = DI \times (DS/\text{maximum disease score}) \times 100$. Roots and shoots were dried for 48 h at 50°C and weighed for dry mass. The experiment was performed twice with five replicates. To assess differences among the results of SDS DIX scores (in percentages), values of the root rot DIX (in percentages), dry shoot mass (in grams), or dry root mass (in grams) caused by *Fusarium* strains, a mixed-model analysis of variance was performed using restricted maximum likelihood estimation. Species was treated as a fixed factor and strain nested within the interaction between species and replicate was used as a random factor. The Shapiro-Wilk test was conducted to test for normality (Shapiro and Wilk 1965). In addition, significant differences among the values of SDS DIX, values of root rot DIX, dry shoot mass, or dry root mass were separated using the Tukey honestly significant difference test for LSMeans ($\alpha = 0.05$). All statistical analyses were performed using JMP software (version 14.0; SAS Institute Inc., Cary, NC).

To complete Koch's postulates, reisolation of the pathogens was conducted from one of the five replicates, using the protocol stated above. Identification of *Fusarium* strains was performed using morphological and molecular identification techniques as described above.

Results

Species identification and abundance. In total, 70 *Fusarium* strains were isolated from SDS-symptomatic soybean roots based on sporodochia formation and colony morphological characteristics. After colony and microscopic identification, 50 strains were putatively identified to be members of the FSSC clade 2, whereas the other 20 *Fusarium* strains were placed into the FSSC but not clade 2, because the growth rate, colony color, and/or sporodochia formation were different.

Molecular phylogeny. Three molecular markers were used to identify *Fusarium* species recovered from soybean roots collected in Montcalm, Michigan. Phylogenetic analysis of EF-1 α sequences determined 50 strains to be within clade 2 of the FSSC and the other 20 strains belonged to clades 5 and 11 of the FSSC (Fig. 1). The EF-1 α sequence identified strains to the species complex level, but it did not resolve specific species, especially within clade 2 of the FSSC. Analysis of the rDNA IGS sequences identified most of the clade 2 FSSC strains to be *F. brasiliense* ($n = 35$), whereas the rest of strains were identified as *F. cuneirostrum* ($n = 9$), *F. phaseoli* ($n = 3$), and an as-yet-unnamed *Fusarium* sp. ($n = 3$) as shown in

Figure 2. In addition to strains belonging to clade 2 of the FSSC, *F. solani* species from clade 5 ($n = 6$) and clade 11 ($n = 14$) were identified and resolved using phylogenetic analysis of RPB2 sequences (Fig. 3).

Morphological characterization. Colony morphology of FSSC clade 2 strains (MIMtc-A1, MIMtc-C11, MIMtc-A12, and MIMtc-B8) grown on PDA for 13 days displayed undulating margins with variations in color of white to tints of yellow with grayish-blue and/or pink on the aerial hyphae. Orange exudates were observed as droplets on the surface of the colony of *F. cuneirostrum* strain MIMtc-C11 (Fig. 4, A to D). Colonies of fast-growing FSSC 5 (MIMtc-A16FS) and FSSC 11 (MIMtc-C13FS) on PDA for 7 days displayed entire margins and were white (Fig. 4, E and F). Sporodochia of FSSC strains on SNA were yellowish-white to pale yellow, and macroconidia were falcate, mostly 3-5 septate, apex rounded, or a distinct sickle-shape with a notched foot cell (Fig. 4, G to L). The sizes of macroconidia were as follows: *F. brasiliense* strain MIMtc-A1, 41.76 to 47.0 $\times$ 5.4 to 6.5 μ m on average; *F. cuneirostrum* strain MIMtc-C11, 46.6 to 51.6 $\times$ 5.3 to 6.8 μ m on average; *F. phaseoli* strain MIMtc-A12, 40.4 to 46.7 $\times$ 5.5 to 6.8 μ m on average; *Fusarium* sp. strain MIMtc-B8, 48.6 to 53.2 $\times$ 5.4 to 7.0 μ m on average; FSSC 5 strain MIMtc-A16FS, 42.0 to 46.5 $\times$ 5.3 to 6.4 μ m on average; and FSSC 11 strain MIMtc-C13FS, 40.0 to 47.0 $\times$ 5.4 to 6.5 μ m on average.

Pathogenicity tests. Soybean inoculated with stains *F. brasiliense* NRRL 22678, MIMtc-C1, or *F. tucumaniae* NRRL 31096 showed

Table 1. *Fusarium* spp. strains isolated from sudden death syndrome-symptomatic soybean roots in Michigan from two different years with location and GenBank accession numbers of elongation factor 1 α (EF-1 α), intergenic spacer (IGS), and RNA polymerase II beta subunit (RPB2) sequences^y

Strain	Species	Isolation year	Location	GenBank accession number ^z		
				EF-1 α	IGS	RPB2
MIMtc-A1	<i>F. brasiliense</i>	2014	Montcalm A	MH541870	MH539737	NA
MIMtc-A13	<i>F. brasiliense</i>	2014	Montcalm A	MH541882	MH539734	NA
MIMtc-A17	<i>F. brasiliense</i>	2014	Montcalm A	MH541857	MH539733	NA
MIMtc-A3	<i>F. brasiliense</i>	2014	Montcalm A	MH541858	MH539732	NA
MIMtc-A7	<i>F. brasiliense</i>	2014	Montcalm A	MH541886	MH539729	NA
MIMtc-A8	<i>F. brasiliense</i>	2014	Montcalm A	MH541856	MH539728	NA
MIMtc-A9Brn	<i>F. brasiliense</i>	2014	Montcalm A	MH541914	MH539727	NA
MIMtc-A9Wht	<i>F. brasiliense</i>	2014	Montcalm A	MH541916	MH539726	NA
MIMtc-B1	<i>F. brasiliense</i>	2014	Montcalm A	MH541868	MH539725	NA
MIMtc-B11	<i>F. brasiliense</i>	2014	Montcalm A	MH541871	MH539724	NA
MIMtc-B13	<i>F. brasiliense</i>	2014	Montcalm A	MH541913	MH539723	NA
MIMtc-B14Blu	<i>F. brasiliense</i>	2014	Montcalm A	MH541891	MH539721	NA
MIMtc-B15	<i>F. brasiliense</i>	2014	Montcalm A	MH541873	MH539720	NA
MIMtc-B5	<i>F. brasiliense</i>	2014	Montcalm A	MH541899	MH539718	NA
MIMtc-B6	<i>F. brasiliense</i>	2014	Montcalm A	MH541854	MH539717	NA
MIMtc-B7	<i>F. brasiliense</i>	2014	Montcalm A	MH541915	MH539716	NA
MIMtc-B9Blu	<i>F. brasiliense</i>	2014	Montcalm A	MH541894	MH539714	NA
MIMtc-B9Brn	<i>F. brasiliense</i>	2014	Montcalm A	MH541866	MH539713	NA
MIMtc-C1	<i>F. brasiliense</i>	2014	Montcalm A	MH541906	MH539712	NA
MIMtc-C10	<i>F. brasiliense</i>	2014	Montcalm A	MH541904	MH539711	NA
MIMtc-C12	<i>F. brasiliense</i>	2014	Montcalm A	MH541872	MH539709	NA
MIMtc-C15	<i>F. brasiliense</i>	2014	Montcalm A	MH541877	MH539708	NA
MIMtc-C2	<i>F. brasiliense</i>	2014	Montcalm A	MH541905	MH539707	NA
MIMtc-C3Brn	<i>F. brasiliense</i>	2014	Montcalm A	MH541889	MH539706	NA
MIMtc-C6	<i>F. brasiliense</i>	2014	Montcalm A	MH541901	MH539705	NA
MIMtc-C9	<i>F. brasiliense</i>	2014	Montcalm A	MH541885	MH539704	NA
MIMtc-A6	<i>F. cuneirostrum</i>	2014	Montcalm A	MH541853	MH539730	NA
MIMtc-A11	<i>F. cuneirostrum</i>	2014	Montcalm A	MH541897	MH539736	NA
MIMtc-C11	<i>F. cuneirostrum</i>	2014	Montcalm A	MH541850	MH539710	NA
MIMtc-A12	<i>F. phaseoli</i>	2014	Montcalm A	MH541881	MH539735	NA
MIMtc-B4	<i>F. phaseoli</i>	2014	Montcalm A	MH541910	MH539719	NA
MIMtc-A4	<i>Fusarium</i> sp.	2014	Montcalm A	MH541883	MH539731	NA
MIMtc-B14	<i>Fusarium</i> sp.	2014	Montcalm A	MH541912	MH539722	NA

(Continued on next page)

^y The rDNA IGS and/or the RPB2 gene was not sequenced for all strains because of their ability to resolve species identification in clade 2 and other clades of the *F. solani* species complex (FSSC).

^z NA indicates that sequences were not used in molecular phylogenetics and thus were not obtained.

severe interveinal chlorosis and necrosis on leaves, typical of SDS. *F. phaseoli* strain MIMtc-A12 and *Fusarium* sp. strain MIMtc-A4 also caused foliar chlorosis but not typical SDS symptoms. All strains of FSSC clade 2 (*F. brasiliense*, *F. cuneirostrum*, *F. phaseoli*, an as-yet-unnamed *Fusarium* sp., *F. tucumaniae*, and *F. virguliforme*) caused more root rot symptoms compared with the FSSC 5 strains and the noninoculated control. Strains of FSSC 5 and FSSC 11 (MIMtc-A16FS, MIMtc-C17FS, and MIMtc-C13FS) caused moderate root rot (~50 to 60% root rot), except for strain MIMtc-B3FS (FSSC 11), which was more aggressive (~88% root rot). All strains significantly reduced soybean dry shoot and root mass compared with the noninoculated control. Strains MIMtc-A12 (*F. phaseoli*), MIMtc-C1 (*F. brasiliense*), MIMtc-C11 (*F. cuneirostrum*), NRRL 22678 (*F. brasiliense*), and MIMtc-B8 (an as-yet-unnamed *Fusarium* sp.) caused more than 83% shoot mass reduction compared with the noninoculated control. Strains MIMtc-A12 (*F. phaseoli*), NRRL 22678 (*F. brasiliense*), MIMtc-C11 (*F. cuneirostrum*), and MIMtc-C1 (*F. brasiliense*) reduced root mass by 79% compared with the noninoculated control (Table 2). Koch's postulates were completed by reisolating *Fusarium* strains from the diseased roots and confirming their identity.

Discussion

In North America, *F. virguliforme* has been known as the only causal pathogen of soybean SDS (Aoki et al. 2003; Hartman et al. 2015; Wang and Chilvers 2016). This study confirms *F. brasiliense*

as an additional SDS-causal pathogen in the United States. *F. brasiliense* is known to cause SDS in South America and Africa (Aoki et al. 2005). This finding is significant because it will affect disease management strategies and soybean breeding efforts, as *F. virguliforme* is no longer the sole SDS-causal pathogen in the United States.

F. brasiliense causing soybean SDS was first reported in Brazil (Aoki et al. 2005) and recently in South Africa (Tewoldemedhin et al. 2017). Tewoldemedhin et al. (2017) speculated that the origin of *F. brasiliense* in South Africa was from infested soybeans possibly imported from South America. O'Donnell et al. (2010) also speculated that SDS-causing *Fusarium* species may have originated in South America. Although *F. brasiliense* has been primarily reported in South America until recently, the first isolation of *F. brasiliense* (strain NRRL 22678) in the United States was from an unknown host in California in 1993 (Aoki et al. 2005; O'Donnell et al. 2010; Rupe et al. 2001), which is 1 year after the first isolation of *F. brasiliense* in Brazil (Aoki et al. 2005). The presence of the species in California and Michigan may indicate that *F. brasiliense* was present prior to modern agriculture. Alternatively, although these *Fusarium* species have never been isolated from seed, seed lots have the potential to be contaminated by infested soil, potentially facilitating movement between continents.

Interestingly, recent surveys of dry bean root rots in Michigan have revealed a high proportion of *F. brasiliense* (Jacobs et al. 2018). The source of *F. brasiliense*, *F. cuneirostrum*, and *F. phaseoli* in this study might be attributable to rotation with dry beans

Table 1. (Continued from previous page)

Strain	Species	Isolation year	Location	GenBank accession number ^z		
				EF-1 α	IGS	RPB2
MIMtc-B8	<i>Fusarium</i> sp.	2014	Montcalm A	MH541890	MH539715	NA
MIMtc-A16FS	FSSC 5	2014	Montcalm A	MH541859	NA	MH684734
MIMtc-B10	FSSC 5	2014	Montcalm A	MH541903	NA	MH684731
MIMtc-B16FS	FSSC 5	2014	Montcalm A	MH541862	NA	MH684730
MIMtc-C17FS	FSSC 5	2014	Montcalm A	MH541884	NA	MH684729
MIMtc-A2FS	FSSC 11	2014	Montcalm A	MH541867	NA	MH684723
MIMtc-B12FS	FSSC 11	2014	Montcalm A	MH541863	NA	MH684732
MIMtc-B13FS	FSSC 11	2014	Montcalm A	MH541898	NA	MH684728
MIMtc-B2FS	FSSC 11	2014	Montcalm A	MH541864	NA	MH684726
MIMtc-B3FS	FSSC 11	2014	Montcalm A	MH541865	NA	MH684733
MIMtc-C13FS	FSSC 11	2014	Montcalm A	MH541887	NA	MH684727
MIMtc-C3FSwht	FSSC 11	2014	Montcalm A	MH541860	NA	MH684717
F-16-11	<i>F. brasiliense</i>	2016	Montcalm B	MH541911	MH539751	NA
F-16-20	<i>F. brasiliense</i>	2016	Montcalm B	MH541875	MH539754	NA
F-16-38	<i>F. brasiliense</i>	2016	Montcalm B	MH541848	MH539747	NA
F-16-39	<i>F. brasiliense</i>	2016	Montcalm B	MH541902	MH539752	NA
F-16-43	<i>F. brasiliense</i>	2016	Montcalm B	MH541907	MH539744	NA
F-16-51	<i>F. brasiliense</i>	2016	Montcalm B	MH541878	MH539743	NA
F-16-54	<i>F. brasiliense</i>	2016	Montcalm B	MH541851	MH539741	NA
F-16-58	<i>F. brasiliense</i>	2016	Montcalm B	MH541849	MH539739	NA
F-16-59	<i>F. brasiliense</i>	2016	Montcalm B	MH541892	MH539738	NA
F-16-22	<i>F. cuneirostrum</i>	2016	Montcalm B	MH541855	MH539753	NA
F-16-30	<i>F. cuneirostrum</i>	2016	Montcalm B	MH541895	MH539749	NA
F-16-31	<i>F. cuneirostrum</i>	2016	Montcalm B	MH541909	MH539748	NA
F-16-40	<i>F. cuneirostrum</i>	2016	Montcalm B	MH541908	MH539746	NA
F-16-42	<i>F. cuneirostrum</i>	2016	Montcalm B	MH541893	MH539745	NA
F-16-52	<i>F. cuneirostrum</i>	2016	Montcalm B	MH541880	MH539742	NA
F-16-16	<i>F. phaseoli</i>	2016	Montcalm B	MH541874	MH539750	NA
F-16-21	FSSC 5	2016	Montcalm B	MH541900	NA	MH684736
F-16-33	FSSC 5	2016	Montcalm B	MH541876	NA	MH684735
F-16-8	FSSC 11	2016	Montcalm B	MH541869	NA	MH684719
F-16-48	FSSC 11	2016	Montcalm B	MH541896	NA	MH684721
F-16-49	FSSC 11	2016	Montcalm B	MH541917	NA	MH684725
F-16-61	FSSC 11	2016	Montcalm B	MH541888	NA	MH684718
F-16-67	FSSC 11	2016	Montcalm B	MH541861	NA	MH684720
F-16-69	FSSC 11	2016	Montcalm B	MH541852	NA	MH684722
F-16-72	FSSC 11	2016	Montcalm B	MH541879	NA	MH684724

Fig. 1. Bayesian phylogenetic tree of elongation factor-1 α (EF-1 α) sequences showing the phylogenetic relationship of *Fusarium brasiliense* with other species within the *F. solani* species complex (FSSC). Posterior probabilities are above the nodes. This tree is rooted on the sequences of *F. staphyleae*, *F. illudens*, and *F. plagianthi* from the National Center for Biotechnology Information GenBank. Strains labeled "MIMtc" and "F" were collected from Montcalm, Michigan, in 2014 and 2016, respectively. Strains labeled "NRRL" are from the Agricultural Research Service Culture Collection.

(*Phaseolus vulgaris*) in these fields, which is not common for the bulk of soybean production fields in the United States. Both *F. cuneirostrum* and *F. phaseoli* are known to cause root rot of dry bean (Aoki et al. 2003; Henriquez et al. 2014; Jacobs et al. 2018) and strains of these two pathogens (*F. cuneirostrum* NRRL 31157 and *F. phaseoli* NRRL 31156) used in this study were isolated in previous studies from dry bean in Michigan (Aoki et al. 2005; O'Donnell

et al. 2010). In addition, three strains of an unnamed *Fusarium* sp. grouped with NRRL 22411 isolated from soybean in South Africa (Tewoldemedhin et al. 2017). Tewoldemedhin et al. (2017) proposed that this *Fusarium* sp. is a fifth species capable of causing soybean SDS. Our results agreed with this suggestion, because the strain *Fusarium* sp. MIMtc_A4 caused SDS-like symptoms in our growth chamber assay. Members of the same FSSC 5 and FSSC 11 from

Fig. 2. Bayesian phylogenetic tree of intergenic spacer (IGS) sequences showing the phylogenetic relationship of *Fusarium brasiliense* with other species within the *F. solani* species complex (FSSC). Posterior probabilities are above the nodes. This tree is rooted on a sequence of *F. virguliforme* from the National Center for Biotechnology Information GenBank. Strains labeled "MIMtc" and "F" were collected from Montcalm, Michigan, in 2014 and 2016, respectively. Strains labeled "NRRL" are from the Agricultural Research Service Culture Collection.

soybean roots as in the current study were also found in soybean fields in North Dakota and Minnesota (Chitrampalam and Nelson 2016). Members of FSSC 11 have been associated with root rot of pea and soybean (Chitrampalam and Nelson 2016; Snyder and Hansen 1941), and FSSC 5 includes clinically important species (O'Donnell et al. 2008; Zhang et al. 2006). A more thorough evaluation of these species and their possible contribution to soybean root rot and SDS appears to be necessary.

Identifying *Fusarium* strains to the species level often requires specific molecular markers. EF-1 α sequences can resolve some *Fusarium* to the species level or at least the species complex level (Aoki et al. 2014; Geiser et al. 2004). However, species within the FSSC clades require additional markers such as the rDNA IGS or RPB2 (O'Donnell et al. 2010). *Fusarium* species in FSSC clade 2 were able to be classified based on the phylogeny of the rDNA IGS marker (O'Donnell et al. 2010). However, the rDNA IGS sequence works poorly to identify *F. solani* species in other clades of the FSSC. The RPB2 marker has primarily been used for FSSC besides clade 2, particularly for the study of clinical isolates (O'Donnell et al. 2013). Thus, a combination of appropriate markers to conform

to the genealogical concordance phylogenetic species recognition concept (Taylor et al. 2000) was used in this study.

Strains within the same *Fusarium* species caused variable soybean foliar and root disease symptoms. For example, *F. brasiliense* strain MIMtc-C1 caused typical SDS foliar chlorosis but strains MIMtc-A8 and MIMtc-C12 did not, and *Fusarium* sp. strain MIMtc-A4 caused typical SDS foliar symptoms but strain MIMtc-B8 did not. Intriguingly, the *F. brasiliense* strain causing more root rot induced more foliar symptoms, whereas the *Fusarium* sp. strain causing more root rot induced fewer SDS foliar symptoms. Within *F. virguliforme*, these SDS foliar symptoms are attributed to fungal phytotoxins such as FvTox1 and FvNIS1 (Chang et al. 2016; Hartman et al. 2015; Pudake et al. 2013). The variation among SDS foliar symptoms in this study might be dependent on the ability of each strain to colonize soybean roots or possibly because of different amounts or types of toxins produced by each strain. In addition to the known SDS-inducing species, *F. phaseoli* strain MIMtc-A12 caused SDS-like foliar symptoms on soybeans under our assay conditions. This agrees with the work of Aoki et al. (2005), who demonstrated that soybean infected by some strains of *F. phaseoli* also exhibit moderate SDS

Fig. 3. Bayesian phylogenetic tree of RNA polymerase II beta subunit (RPB2) sequences showing the phylogenetic relationship of *Fusarium* species isolated from soybean roots with other species within the *F. solani* species complex (FSSC). Posterior probabilities are above the nodes. This tree is rooted on sequences of *F. staphyleae*, *F. illudens*, and *F. plagianthi* from the National Center for Biotechnology Information GenBank. Strains labeled "MIMtc" and "F" were collected from Montcalm, Michigan, in 2014 and 2016, respectively. Strains labeled "NRRL" are from the Agricultural Research Service Culture Collection.

foliar symptoms. Further studies of phytotoxin production and other virulence factors within the FSSC are required to elucidate the complete mechanisms of foliar and root disease in soybean.

Members of FSSC clade 2 caused more root rot than those of FSSC clade 5; however, members of FSSC clade 5 still produced root rot severity scores greater than 50% that resulted in root and shoot mass reduction. This is in contrast with the findings of Chitrampalam and

Nelson (2016), who suggested that members of FSSC clade 5 could be facultative pathogens because isolates caused limited lesions or were nonpathogenic on legume species (soybean, pea, dry bean, and lentil). Additional work is needed to explore the contribution of the various members of the FSSC to soybean disease. For example, coinoculation experiments and field trials with different species of FSSC may provide additional insights.

Fig. 4. The colonies and macroconidia of representative *Fusarium solani* species complex (FSSC) strains isolated from soybean in Michigan. Thirteen-day-old colony of *F. brasiliense* strain MIMtc-A1 (A), *F. cuneirostrum* strain MIMtc-C11 (B), *F. phaseoli* strain MIMtc-A12 (C), and *Fusarium* sp. strain MIMtc-B8 (D) on potato dextrose agar (PDA). Seven-day-old colony of FSSC 5 strain MIMtc-A16FS (E) and FSSC 11 strain MIMtc-C13FS (F) on PDA. Macroconidia of *F. brasiliense* strain MIMtc-A1 (G), *F. cuneirostrum* strain MIMtc-C11 (H), *F. phaseoli* strain MIMtc-A12 (I), *Fusarium* sp. strain MIMtc-B8 (J), FSSC 5 strain MIMtc-A16FS (K), and FSSC 11 strain MIMtc-C13FS (L) on Spezieller Nährstoffarmer agar and stained with safranin O.

Table 2. Pathogenicity of strains of *Fusarium* species originally isolated from soybean roots in Michigan (MIMtc) or obtained from the Agricultural Research Service Culture Collection (NRRL) on soybean cultivar Sloan in the growth chamber^v

<i>Fusarium</i> species ^w	Strain	SDS DIX (%) ^x	Root rot DIX (%) ^y	Dry shoot mass (g)	Dry root mass (g)
<i>F. brasiliense</i>	NRRL 22678	43.8 a	92.1 ab	0.25 ef	0.16 cd
<i>F. tucumaniae</i>	NRRL 31096	43.8 a	89.3 ab	0.53 c–f	0.29 b–d
<i>F. brasiliense</i>	MIMtc-C1	33.8 ab	90.0 ab	0.14 f	0.20 b–d
<i>F. phaseoli</i>	MIMtc-A12	29.4 a–c	96.4 a	0.13 f	0.06 d
<i>F. virguliforme</i>	NRRL 22292	28.8 a–c	86.4 ab	0.92 b–d	0.55 bc
<i>Fusarium</i> sp.	MIMtc-A4	27.5 a–d	80.7 a–c	0.67 b–f	0.27 b–d
<i>F. brasiliense</i>	MIMtc-C9	21.9 b–e	83.6 ab	0.45 c–f	0.33 b–d
<i>Fusarium</i> sp.	MIMtc-B8	18.8 b–e	92.1 ab	0.36 d–f	0.28 b–d
<i>F. brasiliense</i>	MIMtc-C12	18.1 b–e	83.6 ab	0.74 b–f	0.35 b–d
<i>F. cuneirostrum</i>	MIMtc-C11	18.1 b–e	93.6 ab	0.24 ef	0.20 cd
<i>F. cuneirostrum</i>	NRRL 31157	14.4 c–e	76.4 bc	1.06 bc	0.56 bc
FSSC 11	MIMtc-B3FS	11.9 c–e	87.9 ab	0.85 b–e	0.47 b–d
Noninoculated control		11.9 c–e	21.4 e	2.18 a	1.01 a
<i>F. brasiliense</i>	MIMtc-A8	11.3 c–e	82.9 ab	0.67 b–f	0.42 b–d
FSSC 5	MIMtc-C17FS	11.3 c–e	50.0 d	1.25 b	0.64 ab
<i>F. phaseoli</i>	NRRL 31156	9.4 de	81.4 ab	0.69 b–f	0.41 b–d
FSSC 11	MIMtc-C13FS	8.8 de	48.6 d	1.16 b	0.47 b–d
FSSC 5	MIMtc-A16FS	6.9 e	62.1 cd	0.97 b–d	0.43 b–d
<i>P</i> value					
Species		<0.0001	<0.0001	<0.0001	<0.0001
Strain (species × replicate) ^z		<0.0001	<0.0001	<0.0001	<0.0001

^v Plants were examined for both foliar symptoms typical of sudden death syndrome (SDS) and root rot. Means followed by the same letter are not significant ($P < 0.05$) according to Fisher's protected least significant difference test.

^w *Fusarium* sp. indicates that the species has not yet been named. FSSC = *F. solani* species complex.

^x The SDS disease severity index (DIX) that accounted for both disease severity (DS) and disease incidence (DI) was calculated as follows: $DIX = DI \times (DS / \text{maximum disease score}) \times 100$.

^y The root rot DIX was calculated as follows: $DIX = DI \times (DS / \text{maximum disease score}) \times 100$.

^z Strain nested within the interaction between species and replicate was the random effect.

To date, the management of SDS in the United States has focused on *F. virguliforme*. These newly reported *Fusarium* species may need to be included for phenotyping of disease severity to improve soybean resistance to both foliar SDS and root rot. In addition, the efficacy of the recently labeled fungicide seed treatment, fluopyram (succinate dehydrogenase inhibitor), for SDS management (Kandel et al. 2016, 2018) should be determined for these additional species. The effect of crop rotation should also be determined, because some of these species are reported to colonize or be pathogenic on various crops (Kolander et al. 2012). It has been demonstrated that single-year corn rotations offer little value to reducing subsequent SDS pressure when *F. virguliforme* is the primary pathogen present; however, extended rotations with diversified crop systems coupled with the use of organic matter amendments have demonstrated promise (Leandro et al. 2018). Our study indicates that rotating to dry bean may favor other members of the FSSC. We hypothesize that this may be a reason why *F. brasiliense* is more common in South America, where dry bean is a major crop (Broughton et al. 2003). In addition, current qPCR assays specifically designed for the detection of *F. virguliforme* are specific only to *F. virguliforme* and other SDS-causing pathogens cannot be detected. Therefore, diagnostic assays that are specific to or can distinguish between *Fusarium* SDS- and root rot-causing pathogens are needed.

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