IDENTIFCATION, CHARACTERIZATION, AND MANAGEMENT OF FUSARIUM ROOT ROT PATHOGENS OF DRY BEANS IN MICHIGAN

By

Kirsten A. Oudman

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ABSTRACT

IDENTIFCATION, CHARACTERIZATION, AND MANAGEMENT OF FUSARIUM ROOT ROT PATHOGENS OF DRY BEANS IN MICHIGAN

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Michigan is the second-largest producer of dry beans in the United States. Among the yield-limiting diseases dry bean production faces is Fusarium root rot, which can lead to decreased nutrient and water uptake, plant stress, and even plant death. Cultural control can manage root rot, however tolerant cultivars or seed treatments may be more effective, especially if common casual agents are known. To understand which species of clade 2 of the *Fusarium solani* species complex (FSSC) were causing Fusarium root rot in Michigan, a survey was conducted revealing 50% of samples collected to be *F. cuneirostrum*, 37% *F. brasiliense*, and 13% as *F. phaseoli*. Isolates collected were moderately to highly pathogenic on two cultivars of dry beans. Isolates representative of both MAT idiomorphs were detected. An inoculated field trial was conducted to determine pathogenicity of *F. brasiliense* and *F. virguliforme* on two dry bean cultivars in the field. *F. brasiliense* decreased stand and dry plant mass while both pathogens increased root rot. Using two qPCR assays revealed the colonization potential of *F. brasiliense* in taproots to be greater than that of *F. virguliforme*. Two seed treatments were also examined as means of *F. brasiliense* management on dry beans. A seed treatment containing fluopyram reduced the quantity of *F. brasiliense* in the taproots of both cultivars though neither seed treatment influenced yield. Greater understanding of the Fusarium root rot pathogens present in Michigan and the efficacy of seed treatments can improve dry bean production .

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KEY TO ABBREVIATIONS

CB	Commercial base seed treatment
CB + F	Commercial base + Fluopyram seed treatment
CFU	Colony Forming Units
DIX	Disease Severity Index
DNA	Deoxyribonucleic acid
EF-1a	translation elongation factor – 1 alpha
FFSC	*Fusarium fujikuroi* species complex
FGSC	*Fusarium graminearum* species complex
FOSC	*Fusarium oxysporum* species complex
FRR	Fusarium root rot
FSSC	*Fusarium solani* species complex
IGS	Intergenic Spacer Region
MAS	Marker-assisted selection
MAT	Mating type locus
NIC	Non-inoculated control
NTC	Non-treated control
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
qPCR	quantitative Polymerase Chain Reaction
QTL	Quantitative trait loci

SDS	Sudden death syndrome
WMS	Water agar amended with metalaxyl and streptomycin

LITERATURE REVIEW

Dry Bean Origins and Production

Pulses

Pulses are grain legumes such as dry beans, peas, lentils, or chickpeas are important sources of food worldwide. Pulses are grown globally, with the largest share of pulses being produced in Asia, followed by Africa, and North and Latin America (Joshi and Rao 2017). Global trends reveal that since the 1980s, pulse production has experienced seasons of both increases and stagnations, though in recent years, pulse production has once more increased (Joshi and Rao 2017). Pulses are high in protein, fiber, and micronutrients rendering them valuable as an alternative to meat consumption (Kissinger 2016). Pulses account for 5% of total protein intake, with regions like Sub-Saharan Africa and Latin America incorporating a greater amount of pulses into their diets than other regions (Graham and Ranalli 1997, Joshi and Rao 2017, Rodriquez de Luque and Creamer 2014). In developed countries, 60-70% of pulses produced are used for animal feed while 75% of pulses in developing countries are consumed by humans (Joshi and Rao 2017).

In North America, pulses are predominately grown in the Great Plains region of the United States and Canada, though production still lags behind traditional cereal crops like wheat (Miller et al 2002, Urrea et al 2009). Production of pulses are encouraged in conventional cropping systems of developed countries as well as small-scale systems in developing nations due to their ability to fix atmospheric nitrogen through symbiosis with soilborne bacteria (Beckie and Brandt 1997, Joshi and Rao 2016, Miller et al 2002, Peoples et al 2009, Walley et al 2007). However, though pulses are recommended for crop systems worldwide, they vary widely in drought-tolerance, growth habits, and climate responses (Miller et al 2002).

Dry Bean Origins

Dry bean (*Phaseolus vulgaris*), also called common beans, are part of the *Phaseolus* genus that consists of 70 species endemic to the Americas, of which five species are domesticated (Kwak and Gepts 2009, Singh 2001). Crops other than the dry bean within the *Phaseolus* genus are the runner bean (*P. coccineus*), the Tepary bean (*P. acutifolius*), and the lima bean (*P. lunatus*) (Bitocchi et al 2017). Dry beans are the most widely grown pulse in the world, accounting for 37.5% of global production and grown on 29.5 million hectares with Latin America and Africa producing approximately 25 and 18% respectively of the world production and were one of the few pulse crops to experience yield increases without increased production area in the past decade (Joshi and Rao 2017, Rodriquez De Luque and Creamer 2014).

Within dry beans, there is a large amount of genetic variation, seed type, growth habit, and pest and disease resistance diversity (Gepts et al 1986, Kwak and Gepts 2009, Singh et al 1991, Singh 2001). Dry beans are believed to have evolved in present-day Mexico from a wild common bean ancestor 4 to 6 million years ago (Bellucci et al 2014). The wild common bean is found dispersed from Northern Mexico to Argentina and both wild and cultivated beans share characteristics such as self-pollination abilities and diploid chromosomes (Beebe et al 2001, Kwak and Gepts 2009, Kelly 2010, Singh et al 1991). Dry beans were previously believed to have evolved from the wild common bean in present-day Ecuador and Peru prior to dispersing northwards into Central America and Mexico and southwards into the Andean mountain region, however recent genetic evidence instead favors a Mesoamerican origin with a southwards dispersal into the Andean region (Bitocchi et al 2012, Rossi et al 2009). The origin and subsequent dispersal of germplasm formed two gene pools, the Middle American and Andean that are characterized by genetic and morphological differences (Bitocchi et al 2012, Bellucchi et al 2014, Evans 1973,

Singh et al 1991). These two gene pools are characterized by differences in phaseolin seed protein types and vegetative and reproductive traits such as leaf shape, seed size, and leaf hairs (Singh et al 1991). Further within each of the diversity pools, there are six races, three in each pool that contain dry bean cultivars with specific characteristics unique to their respective race (Singh et al 1991).

Middle American pool originate from a region that extends from Mexico to Columbia. Middle American germplasms are generally categorized as small-seeded cultivars and have a larger amount of genetic diversity compared to the Andean germplasm (Cichy et al 2015, Singh 1989, Singh et al 1991). The three races within Middle American germplasm are Mesoamerican, Durango, and Jalisco (Singh et al 1991, Singh 1999). Mesoamerican beans are indeterminate and small-seeded and include market classes such as black and small white beans. Of all market classes, Mesoamerican beans, primarily black beans, are the most widely grown; predominately produced and consumed in North and South America (Mensack et al 2010, Singh 1999, 2001, Siddiq and Uebersax 2013). Mesoamerican beans are tolerant to low soil fertility, abiotic stresses, and several diseases (Singh et al 1991). Durango beans are also predominately indeterminate beans that are classified into pinto and small red market classes (Singh et al 2007, Singh 1999). Durango beans have the highest drought tolerance of all dry bean races and are consequently grown in semi-arid regions of the U.S. and Latin America (Singh et al 2007). While Mesoamerican and Durango races are small-seeded beans, the final Middle American race, Jalisco, has medium-sized seeds (Singh et al 1991, Singh 1999). Jalisco beans also boast high tolerance to a variety of diseases and low soil fertility (Singh 1989, Singh et al 1991, Singh 1999).

The Andean pool encompasses two centers, one in the northern Andes and another in the southern Andes, though the southern Andes region, which includes Peru and Argentina, is the

principal COD (Singh 1989). Beans in the Andean landrace are large-seeded cultivars and include races Nueva Granada, Chile, and Peru (Singh et al 1991). Nueva Granada is the major race of the Andean germplasm as it contains bean market classes such as cranberry and dark and light red kidney beans (Mensack et al 2010, Siddiq and Uebersax 2013, Singh 1999,2001). The Andean races have evolved to produce at high altitudes with intermittent precipitation, however have reduced tolerance to poor soil fertility and diseases as compared to Middle American races (Singh 1989).

Dry Bean Production

In the U.S., Middle American germplasm are most frequently planted, with pinto (race Durango), navy (race Mesoamerican), and black beans (race Mesoamerican) accounting for approximately 64% of planted dry bean acres in 2014 (Siddiq and Uebersax 2013, Wells et al 2014). While Andean beans make up a smaller share of the dry bean production in the U.S., dark red kidney beans are a large export class and beans of Andean germplasm account for the majority of production in both Europe and Africa (Bellucci et al 2014, Logozzo et al 2007, Wells et al 2014).

In 2010, 23.2 million metric tons of dry beans were produced globally, a 70% increase in production from 1980 (Wells et al 2014). While the area under cultivation for dry bean production has remained steady since 1980, the production has increased, due to improvements in dry bean cultivars and agricultural management techniques (Joshi and Rao 2017). The top five dry bean producing countries in 2010 were India, Brazil, Myanmar, China, and the U.S. In the U.S. two states make up approximately 50% of the total country's dry bean production, North Dakota and Michigan, with 36% and 13% of the share, respectively (Siddiq and Uebersax 2013). As the second largest producer of dry beans in the U.S., Michigan harvested around 88,200 hectares in 2017 for

a production value of $130 million (NASS 2017). In addition, Michigan is the largest U.S. producer of black beans and the second largest producer of navy beans (USDA 2016). Due to Michigan's contribution to dry bean production in the U.S., it is essential to address challenges facing dry bean production within the state.

Constraints to Dry Bean Production

Dry beans are grown throughout the world in a range of environmental conditions and cropping systems; from multiple-cropping systems on smallholder farms in the tropics to conventional monocultures in temperate regions. Due to the high diversity of conditions, dry beans face a wide array of pressures depending on the region of production and cultivars planted. Improvements to dry bean cultivars have mainly focused on disease and insect pressure (Graham and Ranalli 1997). With increasingly variable climates, drought has become a main constraint to dry bean production in all major dry bean production regions (Singh 2007). Dry bean races such as Durango and Mesoamerican have been explored extensively to identify mechanisms of drought tolerance that can be used to improve other cultivars (Acosta-Díaz et al 2009, Beebe et al 2008, Muñoz-Perea et al 2006, Singh 2007, Singh et al 2007). Indeed, dry bean growers in North Dakota and Minnesota reported drought was the primary problem facing their production in 2017 (Knodel et al 2018).

Dry bean production in tropic and sub tropic regions experience warmer, more humid environmental conditions that tend to favor pathogen diversity. The cropping systems of these regions also tend to increase the inoculum pressure as space pressures lead to continuous cropping and decreased crop rotation (Graham and Ranalli 1997). As the globe experiences warmer climates, fungi and bacteria have both been identified in increasing numbers at higher latitudes (Beebe et al 2013). Warming climates and increased pathogen pressure means diseases will be a

potent pressure to dry bean production, not only in tropical regions, but also in temperate regions. Growers in North Dakota and Minnesota named disease as a secondary pressure to drought in dry bean production (Knodel et al 2018). Among the most damaging dry bean diseases are anthracnose, angular leaf spot, Fusarium root rot, and bean common mosaic virus (Nene 1988). The four most serious diseases reported from the northern great plains region of the U.S. were rust, bacterial blight, white mold, and Fusarium root rot (Helm et al 1990, Knodel et al 2018).

Fusarium Root Rot

The *Fusarium* genus, in the *Ascomycota* phylum, is a vast genus comprising of over 300 species (Moretti 2009, Aoki et al 2014). *Fusarium* spp. can survive in diverse ecosystems and many are soilborne fungi (Aoki et al 2014). *Fusarium spp.* can be distinguished morphologically by curved asexual spores called macroconidia, however they also produce microconidia and chlamydospores (Leslie and Summerall 2006, Moretti 2009). Most economically important crops are affected by a *Fusarium*-induced disease, though some *Fusarium* spp. are also human pathogens (Aoki et al 2014, Leslie and Summerall 2006, O'Donnell et al 2006). In the genus of *Fusarium*, there are four species complexes from which the majority of phytopathogens are found: *Fusarium fujikuroi* species complex (FFSC), *F. graminearum* species complex (FGSC), *F. oxysporum* species complex (FOSC), and the *F. solani* species complex (FSSC) (Aoki et al 2014). Fusarium root rot (FRR) of dry beans can be caused by several *Fusarium* spp. such as *F. graminearum, F. oxypsorum, F. avenaceum, F. redolens*, and *F. solani*, however, *F. phaseoli* is the most commonly attributed causal agent of FRR on dry beans (Aoki et al 2005, Bilgi et al 2011, Hall and Phillips 1992, Gossen et al 2016, McFadden et al 1989, Lewis and Papavizas 1977, Sippell and Hall 1982).

Fusarium solani Species Complex Clade 2

F. phaseoli is located within clade 2 of the FSSC, a clade of eight phenotypically related species: *F. azukicola, F. brasiliense, F. cuneirostrum, F. crassistipitatum, F. phaseoli, F. tucumaniae, F. virguliforme,* and a yet-to-be characterized species related closely to *F. phaseoli* (Aoki et al 2003, 2005, 2014, O'Donnell et al 2010). Of the eight species, *F. brasiliense, F. cuneirostrum, F. crassistipitatum, F. tucumaniae,* and *F. virguliforme* are known soybean sudden death syndrome (SDS) causal agents, though *F. cuneirostrum* is also known to be a dry bean FRR pathogen. *F. phaseoli* is only known as a bean root rot pathogen and *F. azukicola* is a root rot pathogen of azuki bean (*Vigna angularis*) (Aoki et al 2005, 2012a, 2014, Sang et al in review). The eight species are characterized by unique morphological features as well as slow mycelial growth in culture (Aoki et al 2014, O'Donnell et al 2010). While the clade is considered endemic to South America, the actual distribution of the pathogens differs from species to species (O'Donnell et al 2000, 2013). Of the SDS pathogens, *F. tucumaniae* and *F. crassistipitatum* are only known to be present in South America, *F. virguliforme* is present in South America but is also the only known soybean SDS pathogen throughout the broad soybean production regions of the U.S. *F. brasiliense* was believed to be present only in South America until recent identification on soybeans in South Africa and on dry beans in Michigan (Aoki et al 2003, 2012b, 2014, Tewoldemedhin et al 2017, Jacobs et al 2018). *F. phaseoli* and *F. cuneirostrum* are distributed throughout North America, east Africa, central Asia, and South America (Aoki et al 2014, Bogale et al 2009, Hajieghrari 2009, Henriquez et al 2014, Mwang'ombe et al 2008, Naseri and Mousavi 2015, Sang et al 2018).

Mating in *Ascomycota* is regulated by the MAT locus that contains two mating-type alleles (Ni et al 2011). *Fusarium* spp. that sexually reproduce can either be homothallic, meaning the

species is self-fertile and have both mating-types present, or heterothallic, where species are self-sterile and require an individual with a contrasting, compatible mating type (Debuchy et al 2010, Ni et al 2011, Hughes et al 2014). Of the species within clade 2 of the FSSC, only F. *tucumaniae* is known to reproduce sexually (Covert et al 2007, Hughes et al 2014). Determining the mating types can allow for better understanding of genetic diversity and if species are reproducing sexually or clonally. Mating types have been determined for isolates from the clade 2 FSSC, revealing either MAT1-1 and MAT1-2 to be present in isolates of *F. azukicola*, *F. brasiliense*, and *F. phaseoli* (Covert et al 2007, Hughes et al 2014). The presence of both mating types in these species suggests sexual reproduction is possible but has yet to be observed (Hughes et al 2014). Only one mating type has been detected for the other four species, *F. virguliforme, F. cuneirostrum*, *F. crassistipitatum*, and undescribed *Fusarium* spp., suggesting clonal reproduction is ocurring (Hughes et al 2014).

Fusarium Root Rot Symptoms

Fusarium root rot (FRR) in dry beans manifests itself as reddish-brown lesions on the taproot that can initially be small in size but with time can discolor the entire taproot. Despite the rot on the taproot, the adventitious roots can still proliferate. If severe, the plant can experience chlorosis of the leaves and overall stunting (Hagedorn and Inglis 1986). Studies in Nebraska and North Dakota have both shown yield loss due FRR to be as high as 80%, though the exact yield loss from FRR is largely unknown (de Jensen et al 2002, Gossen et al 2016, Knodel et al 2007, Steadman et al 1975). The causal agents of FRR are not known to be transmitted through seeds and instead are distributed throughout a field or between fields due to contaminated equipment, crop debris, or soil water (Hagedorn and Inglis 1986). *Fusarium* can overwinter on crop debris in hardy structures known as chlamydospores and germinate in the spring to colonize plant tissue.

Environmental factors affect dry bean development and can exacerbate the interaction between host and pathogen. FRR can be exacerbated if the weather post-emergence is cool and wet or if soil conditions, such as compaction or flooding, restrict oxygen availability (Burke and Miller 1983). Cooler temperatures are commonly believed to increase incidence and severity of FRR. Tu (1994) conducted an experiment where peas were kept at temperatures between 10°C and 30°C, deteriming that the number of lesions on the root were maximized at 25°C, though the plants kept at 30°C were severely stunted or died. Other researchers found the optimal temperature for disease development of FRR to be at 21°C (Miller and Burke 1985, Sippell and Hall 1982). Cooler temperatures may hinder dry bean development, leaving them more susceptible to infection (Burke et al 1980). FRR may also be increased at cooler temperatures due to preferred chlamydospore germination conditions. At temperatures higher than 25°C, Mondal and Hyakumachi (1998) found that the carbon loss of *Fusarium phaseoli* chlamydospores was unsustainable and lead to a loss of viability. Soil moisture can also increase the incidence of FRR as an increase from 75% to 100% soil saturation increased severity of FRR by 20%, as did temporary flooding of the soil (Tu 1994). That cooler, wetter conditions encourage disease development can be a primary rationale for planting dry beans into warmer soils when soil moisture is under 50%.

Current Management Strategies

Host Resistance

The choice of dry bean cultivar is the first step in management of Fusarium root rot (Abawi et al 1985). Germplasm from Middle American and Andean gene pools differ in their resistance to FRR, specifically dry beans from the Andean gene pool have been shown to be more susceptible than Middle American beans (Beebe et al 1981, Miklas et al 2006, Singh 1989, Singh et al 1991,). However, breeding root rot-resistant dry beans has been challenging. Molecular techniques, such

as marker-assisted selection (MAS) or quantitative trait loci (QTL), have allowed for progress in dry bean improvement, though certain cultivars remain more disease-resistant (Miklas et al 2006). The use of QTL associated with resistance has led scientists to believe FRR resistance is quantitatively inherited (Dickson and Boettger 1977, Myers and Baggett 1999, Schneider and Kelly 2000, Schneider et al 2001). With quantitative resistance, susceptible and resistant phenotypes are continuous rather than discrete and depend on a larger number of genes to convey tolerance than qualitative resistance (Corwin and Kliebenstein 2017). Indeed, Schneider et al (2001) found that a single QTL only described approximately 15% of the phenotypic variation while a group of four QTL could explain 29%, a result similar to the search for dry bean resistance for other diseases (Miklas et al 1996).

In addition to the challenge of quantitative inheritance, because resistance is most commonly found in indeterminate Middle American beans, it is often difficult to transfer the resistance to Andean beans that are more commonly determinate (Burke and Miller 1983). Transferring resistance to Andean germplasm is often complicated to ensurethat market-desired traits, such as seed size and quality, are not lost in the process (Bilgi et al 2008, Roman-Aviles and Kelly 2005). Therefore, dry bean breeders have been considering root morphology characteristics as a way of improving germplasm against FRR (Cichy et al 2007, Roman-Aviles et al 2004, Snapp et al 2003). Characteristics such as increased root thickness appear related to increased resistance to FRR (Cichy et al 2007, Snapp et al 2003). In addition, beans that express more plant vigor characteristics such as drought-tolerance or thriving in compacted soils also tend to be more FRR tolerant (Cichy et al 2007, Miklas et al 2006). While the challenge of FRR resistance is not solved, host resistance remains the first line of defense and thus is most beneficial to combine tolerant cultivars with other agronomic techniques to reduce FRR incidence.

Cultural Management

Soil conditions can enhance plant stress that leads to a greater susceptibility to FRR, therefore it is crucial that proper management of soil is part of the suite of tools to alleviate FRR. As root vigor has been shown to be related with resistance of FRR, improving soil conditions that encourage extensive root growth, such as planting in warmer, non-compacted soil, could be helpful in decreasing the incidence and severity of FRR (Cichy et al 2007, Miller and Burke 1974, Roman-Aviles and Kelly 2005). Soil pH may also play a role in the ability of *Fusarium* spp. to interact with root tissue. In a hydroponic study, Schuerger and Mitchell (1992) found that pH was related to the number of macroconidia that at 25C, the number of *F. phaseoli* macroconidia attached to mung bean root tissue was significantly decreased at a pH of 7 compared to a pH of 4. Mondal and Hyakumachi (1998) also found a decreased viability of *F. phaseoli* chlamydospores at a pH of 8 instead of a pH of 5.

Repeated production of dry beans or soybeans can lead to an increase in inoculum. In fields with 3-year dry bean monocultures, Burke and Kraft (1973) observed that the inoculum of *F. phaseoli* was increased and yield was significantly diminished by the third year. Maintaining a crop rotation that includes grains or alfalfa can lead to improved soil health as well as a decrease in soil inoculum (Burke and Miller 1983, Hall and Phillips 1992). Crops such as rye and corn could possibly also have an inhibitory effect on chlamydospore germination and lead to a decreased soil inoculum (Lewis and Papavizas 1977). Inoculum buildup is also a factor of compaction and soil layers. Burke et al (1972) found that though FRR was worse in compacted soil, and by utilizing subsoiling tillage practices, *Fusarium* inoculum could be distributed into subsoil instead of being concentrated in the surface soil and interacting with the rhizosphere.

Soil compaction and periods of excess soil water, even due to irrigation, can be detrimental to dry beans as they restrict the amount of oxygen available to the bean roots, causing the plants to be more susceptible to FRR pathogens (Burke and Miller 1983). Integrating cultural practices and host resistance can minimize plant stresses and reduce FRR incidence.

Biocontrol Management

Biocontrol, the use of living microorganisms to alleviate plant disease, is a specialized area of plant disease management. In 2001, biocontrol accounted for only 1% of chemical sales in agriculture compared to the share of 15% for fungicides (Lidert 2001). However, biocontrol methods remain desirable as it can be used in a crop protection chemical rotation due to differing modes of actions and can ameliorate public fears of pesticide residue (Fravel 2005). Currently, twenty-six bacteria and fungi are registered for use as biocontrol with common fungal genera being *Trichoderma* spp., *Gliocladium* spp., and *Aspergillus* spp., and common bacteria biocontrol genera being *Agrobacterium* spp., *Bacillus* spp., and *Pseudomonas* spp. (Dar et al 1996, de Jensen et al 2002, Fravel 2005, Kim et al 1997). The biggest constraints to biocontrol are reduced efficacy due to environmental conditions and inconsistency of efficacy between locations leading most producers to rely on chemical seed treatments rather than biocontrol (Keinath et al 2000, Pierson and Weller 1994).

Biocontrol agents have been tested on dry beans for control of such diseases as white mold common bacterial blight, anthracnose, charcoal rot, halo leaf blight, rust, and Fusarium wilt (Centurion and Kimati 1994, Huang et al 2000, Martin et al 2003, Mizubuti et al 1995, Zanatta et al 2007). Correa et al (2014) observed that combining two isolates of *Bacillus cereus* and *Pseudomonas fluorescens* was more effective than using a single biocontrol agent to control bacterial wilt, Fusarium wilt, charcoal rot, and angular leaf spot on dry beans. In a study

determining the efficacy of *B. subtilis* and seed treatments against root rot on dry beans, *B. subtilis* was observed to convey plant benefits such as increased plant biomass as compared to the non-treated control in inoculated soil (de Jensen et al 2002). Later, de Jensen et al (2004) observed less Fusarium root rot incidence and severity when dry beans were inoculated with *B. subtilis* and *Rhizobium*, results similar to Dar et al (1996) who found *Rhizobium*, in association with mycorrhiza *Glomus mosseae*, increased plant growth and reduced severity of root infection by *Fusarium solani* f. sp. *phaseoli* (Dar et al 1996, de Jensen et al 2002). Biocontrol of dry bean diseases has limitations in applications to field settings and limited commercial adoption means most dry bean producers stick with traditional chemical management.

Chemical Control

Chemical management of root rot is often accompanied with challenges due to root rot being caused by several species. Often, it is not clear which species are present and infecting dry beans making selection of a fungicide difficult as different fungicides have varying efficacy against the distinctive root rot pathogen species. Because root rot pathogens are soilborne organisms, fungicides must be present in the rhizosphere, meaning seed treatments or in-furrow applications are the most appropriate way of delivering fungicides (Natti and Crosier 1971). Seed treatments have been used on many different crops in order to protect them from seedling diseases or insects and are growing in popularity with growers (Taylor and Harman 1990). For example, only 8% of U.S. grown soybeans were treated with seed treatments in 1996 while the amount rose to 75% in 2015 (Gaspar et al 2017). Seed treatments can contain many active ingredients or biocontrol microorganisms (Gaspar et al 2017, Taylor and Harman 1990). The use of seed treatments allows for the utilization of systematic fungicides that aren't able to move basipetally throughout the plant

and for growers to gain protection for their crops without requiring in-furrow fungicide application equipment (Kandel et al 2016).

Many fungicides have been tested for efficacy against Fusarium root rot. Benzimidazoles, which target cellular mitosis and the B-tubulin assembly, were reported to be efficacious throughout the 1970's, though some researchers found benzamides to be too phytotoxic in seed treatments and favored application through soil drench and later, resistance to benzamides became a limitation to their use (Natti and Crosier 1971, Mussa and Russell 1977, Mussa 1986, Russell and Mussa 1977). In 1996, strobilurins were released as fungicides with efficacy against *Rhizoctonia*, *Fusarium*, *Phytophthora*, and *Pythium*, though Harveson et al (2005) found azoxystrobin to have minimal effect against *F. solani* (Uesugi 1998, Barlett et al 2002, Harveson and Stroup 2005, Bradley 2008). However, another strobilurin, trifloxystrobin, conveyed *in-vitro* control against *F. solani*, though did not reduce Fusarium root rot severity in the field (Hegde 2014). Metalaxyl have been used in various trials against root rot complex in field peas and dry beans, however most studies show that metalaxyl has little efficacy against Fusarium root rot, though it controls *Pythium* spp. (Kraft and Papvizas 1983, Hegde 2014). Other labeled seed treatments with action against *Fusarium* spp. are fludioxonil, fludioxonil + mefenoxam and Fluxapyroxad + pyraclostrobin + metalaxyl, though metalaxyl and mefenoxam have no efficacy specifically against *Fusarium* spp. (Barlett et al 2002, Bradley 2008, Harveson and Stroup 2005, McMullen and Markell 2011Uesugi 1998).

The fungicide fluopyram (ILeVO; Bayer CropScience, Research Triangle Park, NC) was labeled in 2014 for soybean as a seed treatment against *F. virguliforme*, the causal agent of soybean SDS (Labourdette et al 2010). Zaworski (2014) observed reduced root rot severity in soybeans under greenhouse conditions when using a fluopyram seed treatment. In a five-location field trial

conducted throughout the United States and Ontario, Canada, fluopyram was effective at reducing foliar symptoms of SDS, though not root rot symptoms or concentrations of *F. virguliforme* in root DNA (Kandel et al 2016). Further multi-location field trials of fluopyram on soybeans found fluopyram increased stand and yield in all locations (Gaspar et al 2016). *In-vitro* baseline sensitivity trials show *F. virguliforme* to be quite sensitive to fluopyram (Wang et al 2017). As *F. virguliforme* is in the same clade as the causal agents of dry bean Fusarium root rot, the dry bean pathogens may also be sensitive to and be controlled by fluopyram. While seed treatments exist to control Fusarium root rot on dry beans, there is still much unknown. Because Fusarium root rot is a chronic disease that affects the plant throughout the growing season, seed treatments may have limited efficacy due to their narrow protection window. Fungicides, as well as cultural management and host resistance must be integrated to provide the best control against this fungal disease distributed globally that can lead to decreased yield or plant death.

CHAPTER 2. IDENTIFICATION AND CHARACTERIZATION OF *FUSARIUM SOLANI* SPECIES COMPLEX CLADE 2 ISOLATES FROM DRY BEAN IN MICHIGAN

ABSTRACT

Fusarium root rot of dry beans presents a challenge for producers in Michigan and for dry bean breeders as they work to incorporate germplasm resistance into future varieties. Fusarium root rot can be caused by many species within the *Fusarium* genus such as *Fusarium oxysporum*, *F. avenaceum*, *F. incarnatum-equiseti*, and *F. phaseoli*, however species in clade 2 of the *Fusarium solani* species complex (FSSC), like *F. phaseoli* and *F. cuneirostrum*, have been most commonly attributed to dry bean root rot in Michigan. To determine which FSSC clade 2 species are present and infecting dry beans in Michigan, a survey was conducted using a two-step isolation process from symptomatic dry bean roots throughout the dry bean production region in Michigan. Species were identified as belonging within FSSC clade 2 through the translation elongation factor 1-alpha (EF-1α) gene and were further identified to the species level using the rDNA Intergenic Spacer region (IGS). Ninety-eight FSSC clade 2 isolates were collected from eight Michigan counties from the years 2014 to 2016, with 50% of the isolates identified as *F. cuneirostrum*, 37% as *F. brasiliense*, and 13% as *F. phaseoli*. Mating type idiomorphs were then determined for ninety isolates (S.1). Seedling pathogenicity assays on two dry bean genotypes revealed isolates of the three species to be pathogenic on both a dark red kidney and black bean cultivar with *Fusarium* species and isolates expressing a range of virulence. Both mating types were identified in isolates of all three species, confirming prior knowledge of putative heterothallism of *F. brasiliense* and *F. phaseoli*, and detecting MAT1-2 in *F. cuneirostrum* for the first time, though no sexual reproduction has been observed. This study finds that *F. brasiliense* was present in all eight counties surveyed, *F. cuneirostrum* was present in seven counties, and *F. phaseoli* was present in

five counties. Based on the long history of dry bean production in Michigan, the established presence of three pathogenic species with a potential for sexual reproduction due to the presence of both mating types is a cause for continued breeding efforts to increase resistance to Fusarium root rot.

Keywords: Fusarium root rot, dry beans, *Fusarium brasiliense, F. cuneirostrum, F. phaseoli,* mating type idiomorphs

INTRODUCTION

Pulses are significant crops, especially in developing countries where they are critical sources of proteins. Dry bean (*Phaseolus vulgaris*) is the most important pulse crop, being produced globally on 29.5 million hectares (Joshi and Rao 2016). Dry beans are a short-season crop, typically reaching maturity in approximately 100 days. Due to insect and pathogen pressures and limited time allotted for dry beans to recover following a stress, dry beans face many production constraints. One such constraint to dry bean production is root rot. Root rot is a caused by a complex of *Fusarium* spp., *Pythium* spp., and *Rhizoctonia solani* (Schwartz and Pastor Corrales 1989). These species can act in concert or singularly. Common symptoms of root rot are damping off, reduced root mass, decreased stand, foliar chlorosis, and reduced yield (Gossen et al 2016). Root rot caused by *Fusarium* spp. is prevalent throughout dry bean production areas in Michigan. Symptomatic plants can be stunted and display reddish-brown lesions along the taproot, though adventitious roots can still proliferate despite loss of the taproot to rot.

Some of the Fusarium root rot causal agents include: *Fusarium graminearum*, *F. incarnatum-equiseti*, *F. acuminitatum*, *F. avenaceum*, *F. oxysporum*, and *F. solani* f. sp. *phaseoli* (Bilgi et al 2011, Bogale et al 2009, Hajieghrari 2009, Mwang'ombe et al 2008, Nino-Sanchez et al 2015, Zhou et al 2018). With improved molecular tools, *F. solani* f. sp. *phaseoli* was differentiated to include distinct species, *F. phaseoli* and *F. cuneirostrum* (Aoki et al 2005). These species are grouped within clade 2 of the *Fusarium solani* species complex (FSSC), a complex comprised of eight species which are known causal agents of dry bean root rot or soybean sudden death syndrome (SDS) (Aoki et al 2003, 2005, 2012). *F. phaseoli* and *F. cuneirostrum* are prevalent worldwide, being reported in eastern Africa, central Asia, South

America, and throughout North America (Aoki et al 2014, Bogale et al 2009, Hajieghrari 2009, Henriquez et al 2014, Mwang'ombe et al 2008, Sang et al 2018a).

In addition to *F. cuneirostrum* and *F. phaseoli*, two other FSSC clade 2 species have been recently identified in Michigan. *F. virguliforme*, the primary soybean SDS pathogen in the United States, was first detected in 1971 in Arkansas before spreading northwards and being reported in Michigan in 2009 (Chilvers and Brown-Rytlewski 2010, Hirrel 1983). Since its initial report in Michigan, *F. virguliforme* has spread rapidly through soybean production areas in the state, which often overlap with dry bean production regions. *F. brasiliense*, a known soybean SDS pathogen in South America and South Africa, was identified from both root rot symptomatic dry beans and from SDS-symptomatic soybeans in Michigan (Jacobs et al 2018, Sang et al 2018b). *F. brasiliense* was described as a SDS pathogen first by Aoki et al (2005). NRRL strains of *F. brasiliense* have been collected primarily from SDS-symptomatic soybeans in Brazil or Argentina, with the exception of one strain from an unknown host in California (Aoki et al 2005). *F. brasiliense* has been shown to produce typical SDS foliar symptoms as well as root rot symptoms on soybeans and ranges from highly to weakly pathogenic, depending on the strain (Aoki et al 2014). In 2017, *F. brasiliense* was identified with molecular and phylogenetic tools as a causal agent of SDS in South Africa, having previously been identified as *F. virguliforme* (Tewoldemedhin et al 2017).

Knowledge of reproductive ability of such pathogens is crucial in order to improve management techniques. If a species reproduces sexually, they may be able to adapt more rapidly and overcome host resistance or sensitivity to a fungicide. Mating is regulated in the FSSC clade 2 species by the mating-type (MAT) locus. Mating types are determined by the presence of one or two alleles at the MAT locus, MAT1-1, or MAT1-2 (Nelson 1996). The presence of one or both alleles, or mating types, determines whether a species is heterothallic or homothallic.

Homothallic species contain both mating types, MAT1-1 and MAT1-2, are self-fertile while heterothallic species require an individual with the opposite mating type to sexually reproduce (Ni et al 2011). Within clade 2 of the FSSC, only *F. tucumaniae* has been confirmed to be heterothallic and capable of sexually reproducing (Covert et al 2007, Scandiani et al 2010). Hughes et al (2014) identified the mating type of strains of each species in clade 2 of FSSC, finding both mating types were present in strains of *F. azukicola*, *F. brasiliense*, *F. phaseoli,* and *F. tucumaniae*. The presence of both mating types allows for the possibility of sexual reproduction to occur but it has not been observed as of yet in any species other than *F. tucumaniae*. Other strains of clade 2 FSSC species, *F. crassistipitatum*, *F. cuneirostrum*, and *F. virguliforme*, were observed to only possess one of the mating types, suggesting clonal reproduction (Hughes et al 2014). Determining the distribution of mating types in Michigan FSSC clade 2 species can lead to further understanding of the pathogens as well as have practical implications for management of root rot.

With the recent knowledge of *F. brasiliense* in South Africa and Michigan, as well as the importance of dry bean production worldwide, a study was conducted on a collection of FSSC isolates recovered from a survey of dry bean in Michigan from 2014-2016 (Jacobs and Chilvers, unpublished). The objectives of this study were to (i) identify which species are present and causing root rot on dry beans, (ii) to determine the mating type of the isolates collected, (iii) to determine the phylogenetic relationships between the species, and (iiii) evaluate the pathogenicity of a panel of FSSC clade 2 isolates collected. Putative *Fusarium* spp. isolates were collected from dry bean roots and identified using the translation elongation factor 1-a (EF-1α) and ribosomal Intergenic Spacer region (IGS). Surveyed isolates were randomly selected and used to conduct seedling pathogenicity assays to evaluate the virulence on two dry bean cultivars. In addition, MAT idiomorphs were determined in all collected FSSC clade 2 isolates.

This study aims to improve understanding of which species from the FSSC clade 2 are present and causal agents of Fusarium root rot in Michigan in order to benefit dry bean breeding efforts and root rot management.

MATERIALS AND METHODS

Species-level Identification, Distribution, and MAT characterization.

Sample Collection

Isolates of *Fusarium* were obtained from dry bean root tissue from plants exhibiting chlorotic foliage, wilt, and stunting symptoms within MSU research farms and commercial fields during the growing season in 2014 through 2016 (Jacobs and Chilvers, unpublished). Roots were washed under tap water for 20 minutes to remove soil debris then patted dry with paper towels. The roots were sliced down the middle with a sterile razor blade and one side of each root was plated on water agar amended with metalaxyl (300 µg/mL) and streptomycin (15 µg/mL) (WMS). The roots were incubated at room temperature and fast growing putative *Fusarium* spp. were hypal tip transferred to potato dextrose agar (PDA) to acquire a pure culture. To obtain isolates of the slower growing FSSC clade 2 organisms, culture plates were allowed to colonize for 7 to 14 d to allow for sporodochium development. If sporodochium was observed on the root tissue, the sporodochium was touched with a sterile pin, plated on WMS, and incubated for 24 h. After 24 h, if macrocondium germination was observed under a dissecting scope, a single spore was transferred to PDA to obtain a pure culture.

DNA Extraction

Isolates were grown in potato dextrose broth (PDB) for 1- 2 weeks. Mycelium was harvested from the PDB, transferred to a 2.0 mL microcentrifuge tube, and lyophilized for 24 h

hours. DNA was extracted from the lyophilized mycelium using a Mag-Bind Plant DNA Plus (Omega Bio-Tek) kit and a KingFisher Flex Purification System (Thermo Fisher Scientific) following manufacturer's instructions.

Isolate Identification

A portion of the translation elongation factor- 1 alpha (EF-1α) gene was amplified and sequenced using primers and reagents described by (O'Donnell et al 1998). EF-1α PCR reactions were conducted using DreamTaq Buffer (ThermoFisher Scientific, Waltham, MA, USA) under thermocycler conditions: 94°C for 3 m, 35 cycles of 94°C for 30 s, 54° C for 30 s, and 72°C for 30 s, followed by one cycle of 72°C for 5 m and a 4°C wash. Ninety-Eight *Fusarium* spp. isolates identified belonging to clade 2 of the FSSC through EF-1α sequences were further classified to the species-level using the IGS region, amplified through PCR using primers NL11 and CNS1 (Aoki et al 2003). PhusionTaq (New England BioLabs, Ipswich, MA, USA) was used in IGS PCR reactions under conditions: one cycle of 98°C for 30 s, 35 cycles of 98°C for 10 s, 55°C for 20 s, and 72°C for 60 s, followed by one cycle of 72°C for 5 m, and a 4°C wash. Four internal primers, S3, S5, S7, and S8, were used in sequencing the IGS region (Aoki et al 2003). All primers used in this study are listed in Table 1.1.

Gel electrophoresis was used to confirm amplification of DNA using 0.5X Tris-borate buffer with EDTA (TBE buffer) and 1.5% agarose gel. PCR products were cleaned using an ExoSAP-IT PCR Product Cleanup Reagent kit (Thermo Fisher Scientific). Three µL of PCR purified product was loaded into 96-well plates with 3µL of 10µM primer, and sent to Macrogen Inc (Macrogen Inc., Rockville, MD, USA) for sequencing. Consensus sequences were assembled from forward, reverse, and internal sequences using CodonCode Aligner version 4.2.7 (www.codoncode.com). Aligned sequences were compared to fungal DNA sequences using the

Basic Local Alignment Search Tool (BLAST) program from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/).

Mating Type Identification

The mating idiomorphs of 90 isolates identified by IGS as belonging within clade 2 of the FSSC were characterized using the primers and reaction described by Hughes et al (2014) (Table S.1.). PCRs reactions used PlatinumTAQ (ThermoFisher Scientific, Waltham, MA, USA) using the parameters: 95°C for 30s, 40 cycles of 95°C for 30 s, 57°C for 30 s, and 68°C for 50 s, followed by 68°C, for 5 m, and a 4°C wash. Isolate mating type was confirmed and visualized through a 1.5% agarose gel and ethidium bromide straining. A PCR product band of 496 bp confirmed a MAT1-1 idiomorph while a band of 260 bp confirmed a MAT1-2 idiomorph.

Table 1.1. List of primers used in this study.

Reference	Primer Name	Sequence (5'-3')	Length (bp)	Tm (°C)
EF-1α	EF-1	ATGGGTAGGAAGACAAGAC	19	55
(O'Donnell et al 1998)	EF-2	GGAAGTACCAGTGATCATGTT	21	57
IGS	NL11	CTGAACGCCTCTAAGTCAG	19	56
(Aoki et al 2003)	CNS1	GAGACAAGCATATGACTAC	19	51
	S3	GGTCTGAAAGATCAGGTACG	20	57
	S5	TACCCTATACCTCCGCCAAC	20	60
	S7	TACCCTATACCACCTAGTAGC	21	57
	S8	TTTCGCTTTTACCTACCCTG	20	57
MAT Locus	MAT1-1F	ATCACTCCGGAGGAACTGAAG	21	61
(Hughes et al 2014)	MAT1-1R	TGTCTGTACAACACCCACGCG	21	65
	MAT1-2F	GATTCCACGACCCCCYAACGC	21	65
	MAT1-2R	CGGTACTGGTAGTCGGGAT	19	60

Phylogenetic Analysis.

IGS sequences of reference *Fusarium* spp. strains were obtained from GenBank (NCBI). Multiple sequence alignments were performed using Multiple Alignment using Fast Fourier Transform (MAFFT) (Katoh et al 2002). Sequences of 17 isolates from this study were selected to be used in the phylogeny, 7 *F. brasiliense* isolates, 7 *F. cuneirostrum*, and 3 *F. phaseoli*. Nucleotide substitution models were tested using the model selection tool in MEGA7 prior to

building phylogenetic trees. The Hasegawa-Kishino-Yano (HKY) model was determined to be the best substitution model as determined by Bayesian information criteria (BIC). A maximum likelihood tree using IGS sequences was created by using the HKY substitution model with 1000 bootstrap replications in MEGA7. IGS sequences used in the study were deposited into GenBank (Table S.1).

Seedling Pathogenicity Assays.

Experimental Setup

Seedling pathogenicity assays were conducted using 42 *Fusarium* isolates., 13 of which were *F. brasiliense,* 24 *F. cuneirostrum* isolates, and 5 *F. phaseoli* isolates (Table S.1). *Fusarium* isolates were randomly distributed between 9 experiment sets. These experiment sets included *Fusarium* isolates from other species complexes such as *F. graminearum, F. oxysporum, and F. incarnatum-equiseti,* which are not included in this study. Each experiment set included 13 *Fusarium* spp. isolates, 1 non-inoculated control (NIC), 1 non-inoculated sterilized sorghum control, and 1 positive inoculated control (IC) that used a single-spored isolate of NRRL 22292 of *F. virguliforme*. The experiments were conducted on "Red Hawk" dark red kidney bean variety and "Zorro" black bean variety. Experiment sets were repeated twice on each respective dry bean cultivar and within each trial, there were 5 isolate replications.

Inoculum Production

Prior to inoculating sorghum grains with the respective isolates, sorghum was submerged in tap water overnight, 1.6-1.8 kg of drained sorghum were allocated into spawn bags (fungiperfecti.com), and the sorghum was autoclaved at 121℃ for 8 hours for sterilization then cooled to room temperature. Each *Fusarium* isolate was grown on 5 plates of PDA. An inoculum slurry was created with 500 mL of sterile water, 5 PDA plates containing no mycelial growth, and

the five, 14-day colonized PDA plates in a sterile stainless-steel blender. An approximate 100-mL aliquot of the inoculum slurry was added to each of the spawn bags containing sterilized sorghum grains, heat sealed, and incubated at room temperature for 4 weeks. Inoculum spawn bags were mixed 3X/week to ensure even colonization of the sorghum grains. Once the sorghum was completely colonized by the *Fusarium* isolate, the sorghum was dried by forced air at room temperature in drying ovens and stored at 4°C until use.

Experimental Conditions and Data Collection

Seedling pathogenicity assays used 354 mL paper cups (Solo Cup Company, Lake Forest, IL) filled with 250 mL of medium vermiculite and 12 g of inoculum that were thoroughly mixed in a quart zip-top closed bag and placed into the cup. Six bean seeds were planted on top of the vermiculite-inoculum mixture and covered with an additional 70 mL of medium vermiculite. The cups were placed into a growth chamber at 20°C at 85% relative humidity under 14 hours of light and 10 h of darkness photoperiod. Plants were watered regularly with half-strength Hoagland solution (Hoagland and Arnon 1938). After 14 days of growth the plants were removed and the roots were washed under running tap water. Roots were rated based on a 1-7 disease severity rating scale described in Schneider and Kelly (2000). In addition, the number of germinated and emerged beans in each cup was recorded. Emergence was determined by the development of true leaves. The roots and shoots were placed back into the cup and dried in ovens at 50°C. After 48 h, the roots and shoots were weighed and the mass of the dry weight was recorded.

Statistical Analysis.

Data was analyzed using RStudio statistical software, version 1.0.414 (RStudio Team 2016). Data was analyzed using least square means through packages “lme4”, “lmerTest”, and “emmeans” with multiple comparisons made using the Tukey adjustment. Least square means

were calculated using experimental set as the random factor. Root rot measurements were converted into a continuous disease severity index using the method from Li et al (2014). Germination and emergence data was converted into percentages of the number of plants that germinated or emerged divided by 6 seeds that were planted. Plant dry mass measurements were normalized by the number of plants in each replication that were emerged. Pairwise comparisons among species were conducted. For the analyses, species included all isolates of *F. brasiliense, F. cuneirostrum, F. phaseoli, the inoculated control, F. virguliforme,* and the non-inoculated control.

RESULTS

Species-level Identification, Distribution, and MAT characterization

From 2014-2016, *F. cuneirostrum* was the most abundantly isolated FSSC clade 2 dry bean root rot pathogen. Fifty *F. cuneirostrum* isolates were collected from seven counties while thirty-seven *F. brasiliense* isolates were collected from eight counties (Table 1.2). *F. phaseoli* was isolated 12 times from five of the eight counties. Based on Chi-square distributions tests, year was not a significant factor in the isolation of the three species, however county was a significant factor ($X^2 = 25.58$, df=14, $P = 0.0296$).

Table 1.2. Abundance and identification of 98 isolates collected throughout eight Michigan counties.

County	***F. brasiliense***	***F. cuneirostrum***	***F. phaseoli***
Bay	3	13	3
Gratiot	3	8	0
Huron	5	3	3
Ingham	1	1	0
Montcalm	5	15	0
Saginaw	18	9	4
Sanilac	1	1	1
Tuscola	1	0	1
Total	37	50	12

While previous studies only identified the presence of MAT1-1 in six *F. cuneirostrum* isolates from Japan, the U.S., and Canada (Hughes et al 2014), this study is the first detection of the MAT1-2 idiomorph in *F. cuneirostrum* isolates, suggesting the species is putatively heterothallic. The MAT1-2 idiomorph was present at a higher abundance in *F. brasiliense* isolates collected while MAT1-1 was present at a higher abundance for *F. phaseoli* isolates (Table 1.3). Using Chi-square distribution tests, mating type idiomorph was significantly related to county (X^2=17.27, df=7, P = 0.0157) and species identification (X^2=39.51, df=2, P < 0.0001), but not year.

Table 1.3. Mating type characterization for 93 FSSC clade 2 isolates.

	F. brasiliense		*F. cuneirostrum*		*F. phaseoli*	
	MAT1-1	MAT1-2	MAT1-1	MAT1-2	MAT1-1	MAT1-2
Bay	-	2	10	1	2	1
Gratiot	-	2	7	2	-	-
Huron	-	5	-	3	2	1
Ingham	1	-	1	-	-	-
Montcalm	2	3	11	3	-	-
Saginaw	1	17	5	3	3	-
Sanilac	-	1	-	1	-	1
Tuscola	1	-			1	-
Total	5	30	34	13	8	3

For *F. brasiliense* isolates, the MAT1-1 idiomorph was present in four counties: Ingham, Montcalm, Saginaw, and Tuscola. Isolates with MAT1-2 were also found in Montcalm and Saginaw counties, and were present at low frequencies in Bay, Gratiot, Huron, and Sanilac counties. Isolates with either MAT1-1 or MAT1-2 were collected from the same fields in Montcalm county in 2014 and 2015 and in Saginaw county in 2015 and 2016. *F. cuneirostrum* isolates of both idiomorphs were collected from Bay, Gratiot, Montcalm, and Saginaw counties with isolates from Huron and Sanilac counties only having MAT1-2 and one isolate from Ingham county only having MAT1-1. *F. cuneirostrum* isolates with either mating type idiomorph were

isolated from the same field in Montcalm county in 2014 and 2015. *F. phaseoli* isolates from Bay and Huron counties had both mating types and were isolated from the same field while isolates from Saginaw, Sanilac, and Tuscola counties only had MAT1-1 idiomorphs. Both idiomorphs, either MAT1-1 or MAT1-2 were recovered from all three species every year in this sample, except for 2014 when only one *F. phaseoli* isolate was collected in 2014 and identified as MAT1-1. The ability for *F. brasiliense, F. cuneirostrum*, or *F. phaseoli* isolates to produce perithecia or sexually reproduce was not examined in this study.

Phylogenetic Analysis

Sequences from the Intergenic Spacer region (IGS) were used to identify isolates collected from dry bean roots to the species level. Figure 1.1 shows *F. brasiliense* isolates (n=7), *F. cuneirostrum* isolates (n= 7), and *F. phaseoli* isolates (n=3) to be grouped among the respective NRRL strains.

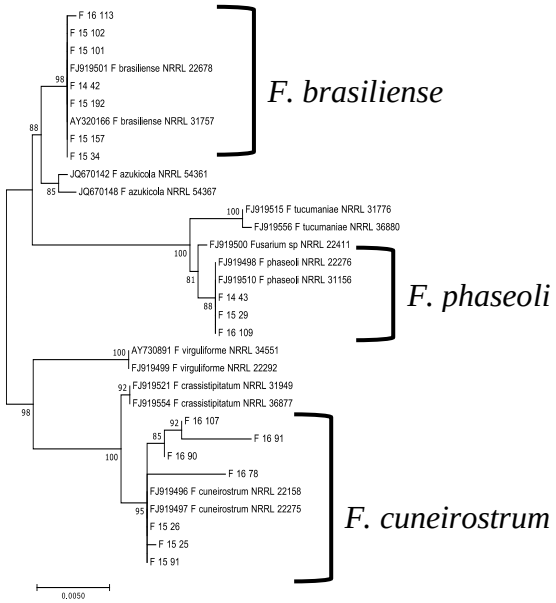


Figure 1.1. Maximum likelihood phylogenetic tree of IGS sequences showing the relationships between isolates within clade 2 of the FSSC. Model used was Hasegawa-Kishino-Yano. Bootstrap percentages out of 1000 bootstrap replicates are on nodes. Strains labeled NRRL are from ARS culture collection.

Seedling Pathogenicity Assays

Germination and Emergence

Percent germination of the non-inoculated control (NIC) on Red Hawk had a mean of 95.4% and none of the species tested significantly differed. On Zorro, species was a significant effect on percent germination ($P < 0.0001$) though only *F. phaseoli* caused significant reduction of germination, 83.5%, compared to the NIC, 90.8%. Percent emergence was significant by species on Red Hawk ($P = 0.0018$) with *F. cuneirostrum* significantly reducing percent emergence, 85.1%, compared to the NIC, 93.9% (Table 1.4). The species had a wider range of percent germination values on Zorro than on Red Hawk while the NIC had the same range on either variety. *F. brasiliense* had the widest range of impact as measured by percent emergence on Red Hawk, with some isolates causing a lack of emergence despite germination of the seed (Table 1.5). *F. virguliforme*, the inoculated control, consistently had the same or higher percent of germination and emergence than the NIC on both Red Hawk and Zorro.

Disease Severity Index

For the disease severity index, the interaction between species and variety was significant ($P <0.0001$). The effect of species was significant for both bean varieties (Red Hawk: $P < 0.0001$, Zorro: $P < 0.0001$). On both Red Hawk and Zorro, *F. brasiliense, F. cuneirostrum, F. phaseoli,* and the inoculated control, *F. virguliforme* all significantly increased the disease severity compared to the NIC (Table 1.4). *F. cuneirostrum* had the widest range of disease severity index values on both bean varieties, suggesting a variability of virulence between the individual isolates. *F. brasiliense* and *F. cuneirostrum* had the highest values of disease severity on both varieties.

Plant Dry Mass

The interaction of species and variety was significant on plant dry mass (P = 0.0002). Species varied significantly in their ability to reduce plant dry mass on Red Hawk (P < 0.0001) and Zorro (P < 0.0001). All four species significantly reduced the plant dry mass on Red Hawk compared to the NIC. *F. brasiliense, F. cuneirostrum* and *F. phaseoli* significantly reduced plant dry mass compared to the NIC on Zorro, but the inoculated control, *F. virguliforme* did not (Table 1.4). *F. phaseoli* had the highest maximum plant dry mass, 770 mg/emerged plants on Red Hawk, which was greater than the NIC. But on Zorro, *F. phaseoli* had the lowest maximum plant dry mass (Table 1.5). The plant dry mass for *F. brasiliense* and *F. phaseoli* significantly differed on Red Hawk. *F. brasiliense* had the lowest numerical plant dry mass averages on both varieties, suggesting *F. brasiliense* consistently reduced plant dry mass. *F. cuneirostrum* isolates were observed to cause foliar chlorosis (Figure 1.2).

Table 1.4. Means of percent germination, percent emergence, disease severity index (DIX), and plant dry mass (mg/emerged plants) on species used in seedling pathogenicity assays on both Red Hawk and Zorro.

	Red Hawk				**Zorro**			
Species	Germination (%)	Emergence (%)	DIX	Dry Mass (mg/emerged plants)	Germination (%)	Emergence (%)	DIX	Dry Mass (mg/emerged plants)
NIC	95.4	93.9 a	19.8 b	395.7 a	90.9 a	87.6	24.3 b	216.1 a
F. brasiliense	96.0	89.3 ab	71.7 a	300.2 c	91.1 a	88.1	69.6 a	167.7 b
F. cuneirostrum	93.1	85.1 b	72.1 a	315.5 bc	87.6 ab	83.4	73.0 a	176.6 b
F. phaseoli	93.5	86.8 ab	72.2 a	343.1 b	83.5 b	81.3	72.5 a	172.3 b
F. virguliforme	94.3	88.9 ab	74.7 a	318.0 bc	91.9 a	87.6	73.1 a	189.2 ab

Means within a column followed by the same letter do not differ significantly based on Tukey's HSD at P=0.05

Table 1.5. Minimum and maximum values for percent germination, percent emergence, disease severity index (DIX), and plant dry mass (mg/emerged plants) of species used in seedling pathogenicity assays on both Red Hawk and Zorro.

	Red Hawk				**Zorro**			
Species	Germination (%)	Emergence (%)	DIX	Dry Mass (mg/emerged plants)	Germination (%)	Emergence (%)	DIX	Dry Mass (mg/emerged plants)
NIC	50-100	50-100	14-57	120-705	50-100	33-100	9-71	102-346
F. brasiliense	66-100	0-100	42-100	158-520	33-100	33-100	38-100	10-448
F. cuneirostrum	50-100	16-100	28-100	91-614	16-100	16-100	17-100	45-530
F. phaseoli	50-100	16-100	45-97	160-770	16-100	16-100	54-92	40-305
F. virguliforme	66-100	50-100	50-92	86-561	50-100	50-100	47-97	28-398

Means within a column followed by the same letter do not differ significantly based on Tukey's HSD at *P*=0.05

Figure 1.2. Interveinal chlorosis symptoms (left) on Red Hawk induced by F. cuneirostrum isolate, F_16_107, non-inoculated control Red Hawk right.

DISCUSSION

F. brasiliense, *F. cuneirostrum*, and *F. phaseoli* were isolated from symptomatic dry beans in eight counties throughout Michigan at varying abundances. *F. brasiliense*, recently identified in Michigan, was isolated from all eight counties, while *F. cuneirostrum* was isolated at the greatest abundance from seven counties. All three species were all shown to be pathogenic on Red Hawk

and Zorro dry bean varieties by reducing emergence, increasing disease severity, and reducing plant mass, though the inoculated control, *F. virguliforme* also increased disease severity and decreased plant mass. Both mating types were found in isolates of all three species, at times in the same field, suggesting that there is potential for sexual recombination in the populations, though it has not been observed or examined.

Species-level Identification, Distribution, and MAT characterization

As dry beans were collected based on physical, above-ground symptoms, there may have been a sampling bias towards species that caused visually observed symptoms such as chlorosis or stunting. However, the sampling elucidated the distribution of common FSSC clade 2 root rot pathogens in Michigan. From this study, *F. brasiliense* has a confirmed presence in all eight counties surveyed in this study. It has also been isolated from soybeans in Michigan in several counties (Wang et al accepted). *F. virguliforme* has been shown to colonize corn residue and that the inoculum load can increase when using a corn-soybean crop rotation (Leandro et al 2018). As a related species, it is likely that *F. brasiliense* can also colonize corn. The ability to colonize corn residue may aid in the spread of the clade 2 FSSC species throughout Michigan however it is unknown whether *F. brasiliense* was recently introduced to Michigan or undetected until recently. More surveys need to be done throughout other states that produce dry beans in the U.S., such as North Dakota, Minnesota, Nebraska, and Washington to determine whether *F. brasiliense* is also present in those areas. *F. cuneirostrum* was isolated at the highest abundance from symptomatic dry beans in Michigan. *F. cuneirostrum* has a wide range, having been isolated from various locations in the U.S., Japan, Brazil, Uganda, and Canada, yet little is known about the species other than it having the widest host range of the FSSC clade 2 species as it is pathogenic on soybean, dry bean, and mung bean (Aoki et al 2005, O'Donnell et al 2010).

F. phaseoli, a commonly attributed dry bean root rot pathogen, was isolated in low abundance in this study.

This is the first identification of the MAT1-2 mating type in *F. cuneirostrum*. This study was further confirmation of putative heterothallism in *F. brasiliense* and *F. phaseoli*. Both mating types were found in the same county and even the same field, suggesting that there could be potential for sexual reproduction in each of the species. However, the ratio of MAT1-1 and MAT1-2 idiomorphs is skewed in favor of one mating type for each of the species, which could suggest sexual reproduction is rare or irregular (Ramirez-Prado et al 2008, Zhan et al 2002). In terms of the recent identification of *F. brasiliense,* a skewed mating type ratio could also suggest a recent introduction of the species as wild populations have not reached equilibrium

The mating types represented by *F. brasiliense* and *F. phaseoli* isolates from Michigan corresponds with Hughes et al (2014) that also identified both MAT idiomorphs in 12 *F. brasiliense* and 2 *F. phaseoli* NRRL strains characterized. However, Tewoldemedhin et al (2017) only identified the MAT1-1 idiomorph in nine *F. brasiliense* isolates collected in South Africa, possible evidence of a recent introduction of *F. brasiliense* of one mating type. While both mating types were detected in *F. brasiliense*, *F. cuneirostrum*, and *F. phaseoli*, no evidence of sexual reproduction has been uncovered and the pathogens may still be reproducing asexually. The X^2 test showed that county and species ID was significantly related to the proportions of mating types, suggesting that there is variation in the distribution of mating type idiomorphs. Kwon-Chung et al (1992) observed mating type and virulence to be related, but further studies would need to be conducted to determine if this relationship between mating type and virulence also exists in Michigan populations. If dry bean root rot causal agents are able to sexually

reproduce, there may be challenges to host resistance in dry beans or sensitivity to certain fungicide controls.

Seedling Pathogenicity

Pathogenicity varied between species and dry bean variety. Each species examined increased disease severity and decreased plant dry mass, though individual isolates varied in virulence. The species generally did not affect the germination or emergence of either cultivar. The inoculated control, *F. virguliforme*, strain 22292, was able to produce root rot symptoms on both cultivars, though it did not reduce the plant mass of Zorro. *F. brasiliense* and *F. phaseoli* isolates tested appeared to be highly pathogenic, while *F. cuneirostrum* isolates ranged from moderately to highly pathogenic. In greenhouse pathogenicity assays conducted on soybean by Aoki et al (2014), *F. brasiliense* and *F. cuneirostrum* appeared to have similar pathogenicity ranges on soybean, though several isolates of *F. brasiliense* were weakly pathogenic on soybean. Reports of *F. cuneirostrum* on dry beans in Canada and Uganda have found isolates to be moderately pathogenic in greenhouse assays (Henriquez et al 2014, Sang et al 2018). Due to the recent identification of *F. brasiliense* in Michigan, a field trial determining the pathogenicity if the pathogen on dry beans is in progress (Oudman et al unpublished), however studies examining *F. cuneirostrum* in the field should also be conducted to improve understanding of this global pathogen.

Conclusion

As literature shows, little is known about the dry bean root rot pathogens in FSSC clade 2. Since dry beans are the primary edible legume consumed worldwide, improving production through further understanding of the pathogens that infect dry beans is necessary. *F. brasiliense*, *F. cuneirostrum*, and *F. phaseoli* have high to moderate pathogenicity on dry bean and were

isolated in varying abundances from dry beans across Michigan. Determining if these species are present throughout other dry bean production regions in North America is a crucial next step in order to aid in dry bean breeding work and knowledge of these pathogens. The presence of both mating types in *F. brasiliense, F. cuneirostrum,* and *F. phaseoli* isolates from Michigan can help in determining the evolutionary history of the FSSC clade 2. This study can help elucidate the distribution, pathogenicity, and mating types of *F. brasiliense, F. cuneirostrum,* and *F. phaseoli,* three important soilborne dry bean pathogens.

CHAPTER 3. DRY BEAN ROOT ROT CAUSED BY *FUSARIUM BRASILIENSE* AND *F. VIRGULIFORME* IN FIELD TRIALS AND EFFICACY OF SEED TREATMENTS FOR MANAGEMENT

ABSTRACT

A dry bean (*Phaseolus vulgaris*) root rot survey conducted in Michigan isolated *Fusarium brasiliense* in high frequency. A related species, *F. virguliforme*, the primary causal agent of soybean sudden death syndrome (SDS) in North America, infects soybeans and has been reportedly spreading within the state. From previous studies, *F. virguliforme* has been shown to infect dry beans in greenhouse and field conditions and could prove to be an additional challenge to dry bean producers. To determine the effects of *F. brasiliense* and *F. virguliforme* on the development of dry beans, an inoculated field trial was conducted on two dry bean cultivars, a root rot susceptible kidney bean and a root rot tolerant black bean. Fusarium root rot in dry beans is generally managed through the use of partially resistant varieties and one of several commercially available seed treatments such as prothioconazole or azoxystrobin. Recently, the fungicide fluopyram has become a commercially available seed treatment (ILeVO) as a means to control *F. virguliforme* on soybeans. In order to determine the efficacy of seed treatments to control *F. brasiliense* or *F. virguliforme* in dry beans two seed treatments: a commercial base of prothioconazole, penflufen, and metalaxyl (CB) and the commercial base treatment plus fluopyram (CB +F) were compared to non-treated seed in artificially inoculated and naturally infested field trials. While both *F. brasiliense* and *F. virguliforme* significantly increased root rot on both cultivars in the inoculated trial, *F. virguliforme* was present in significantly decreased quantities in the roots. The seed treatment CB + F showed signs of early season phytotoxicity in the non-inoculated trial by reducing plant stand and plant mass, but it did also significantly decrease the amount of *F. brasiliense* present in the roots of both cultivars in both inoculated and

non-inoculated trials. Yield did not differ significantly between treatments, but the CB + F seed treatment appeared to numerically increase yield compared to non-treated, inoculated treatments. *F. brasiliense* appears to be a greater threat to dry beans in Michigan than *F. virguliforme*, but fungicide treatments including fluopyram may be efficacious against *F. brasiliense.*

Keywords: dry bean, fluopyram, fungicides, *Fusarium brasiliense, F. virguliforme,* root rot, seed treatments

INTRODUCTION

Dry beans (*Phaseolus vulgaris*) are an important legume crop having both high nutritional and commercial value. The United States is the sixth-largest producer of dry beans following Brazil, India, China, Burma, and Mexico. Within the United States, two states make up the majority of dry bean production, North Dakota and Michigan. Michigan is the second largest producer of dry beans in the United States, planting between 87,000 and 111,300 hectares annually between the years of 2014 to 2016 and contributing over $159 million dollars to the Michigan economy in 2015 (NASS 2017, USDA 2016). Diseases such as root rot and white mold are two primary yield-limiting factors facing dry bean producers. Root rot can be caused by a complex of organisms including *Fusarium* spp., *Pythium* spp., and *Rhizoctonia solani* (Schwartz and Pastor Corrales 1989). While *Pythium* spp. and *R. solani* typically infect dry beans early in the season and can cause seedling rot, *Fusarium* spp. can infect dry bean roots early in the season but continue to rot the dry bean taproot throughout the season. Fusarium root rot can cause chlorosis and stunting, however quantifying yield loss due to Fusarium root rot is challenging due to uneven disease pressure, unknown pathogen presence, microbial competition, and plant compensation (de Jensen 2002).

While many *Fusarium* spp. can cause dry bean root rot, *F. phaseoli* and *F. cuneirostrum* are most commonly reported as causal agents throughout North America, eastern Africa, central Asia, and South America (Aoki et al 2003, 2014, Bogale et al 2009, Gossen et al 2016, Hajieghrari 2009, Henriquez et al 2014, Mwang'ombe et al 2008, Sang et al 2018). Recently, *F. brasiliense* was identified from root rot symptomatic dry beans in Michigan (Jacobs et al 2018). Being found throughout the dry bean growing region in Michigan, *F. brasiliense* may pose a threat to dry bean production. Another closely related and prevalent *Fusarium spp*. throughout the dry bean growing

region in Michigan is *F. virguliforme* (Chilvers et al 2016). *F. virguliforme* is the primary causal agent of soybean sudden death syndrome (SDS) in North America. Since the initial identification of *F. virguliforme* in Arkansas in 1971, the pathogen has spread into all of the major soybean states in the United States and into Ontario, Canada (Anderson and Tenuta 1998, Hartman et al 2015, Hirrel 1983, Leandro et al 2012). In 2009, *F. virguliforme* was identified causing SDS in Michigan (Chilvers and Brown-Rytelwski 2010). Aoki et al (2003, 2012) observed *F. virguliforme* to cause taproot rotting on dry beans grown in greenhouse conditions and since soybeans are often grown in rotation with dry beans, the widespread presence of *F. virguliforme* is thought to be a potential threat to dry bean production in Michigan.

Managing Fusarium root rot begins with planting resistant cultivars. Within the two gene pools of *P. vulgaris*, the small-seeded Middle American beans, such as black beans, have been observed to have greater inherent tolerance to root rot than the large-seeded Andean, or kidney beans (Beebe et al 1981, Kelly 2010, Roman-Aviles et al 2005, Schneider et al 2001). Fusarium root rot is encouraged by cool, moist soils and soil compaction so planting later in the season and using proper tillage is important to discourage disease development (Burke and Miller 1983). Managing Fusarium root rot through chemical means can be challenging. Often it is unclear which species are present, making fungicide selection difficult as fungicides have varying efficacies against the different root rot pathogens. Seed treatments containing fungicides such as prothioconazole, metalaxyl, azoxystrobin, fludioxonil, and mefenoxam are labeled for soybeans and dry beans for control of root rot (Bradley 2008). Azoxystrobin and fludioxonil specifically have activity against *Fusarium spp*. (Barlett et al 2002, Bradley 2008).

Another fungicide reported to control Fusarium root rot is fluopyram. Fluopyram (ILeVO; BASF, Research Triangle Park, NC) was labeled as a seed treatment for management of soybean

SDS in 2014. As a succinate dehydrogenase inhibitor, fluopyram inhibits cellular respiration and spore germination (Labourdette 2010). *F. virguliforme* has been shown to be sensitive to fluopyram in the lab as well as in the field (Kandel et al 2016, 2018, Wang et al 2017). *F. brasiliense* has also been shown to be sensitive to fluopyram in fungicide sensitivity assays in the lab, though its efficacy in the field is unknown (Sang et al 2018).

Due to recent identification of *F. brasiliense* on dry beans in Michigan and the presence of *F. virguliforme* in Michigan throughout the dry bean production region, it is important to understand the impact both pathogens may have on dry bean production. The objectives of this study were to i) determine the effects of *F. virguliforme* and *F. brasiliense* on dry bean production and ii) determine the effects of standard base seed treatment in reducing *Fusarium* infection and subsequent disease and iii) determine if fluopyram in addition to a base seed treatment is efficacious in managing *F. virguliforme* and *F. brasiliense* on dry beans in the field. This study examined the interaction between inoculum and seed treatment in an inoculated field trial and the efficacy of seed treatment in a field trial with a high natural *F. brasiliense* inoculum pressure by measuring variables such as stand count, dry plant mass, root rot, yield, and qPCR quantification of the pathogens present in the taproot tissue. Understanding how *F. brasiliense* and *F. virguliforme* influence dry bean development and the efficacy of seed treatments should improve management of Fusarium root rot.

METHODS AND MATERIALS

Impact of Pathogens and Seed Treatments in Field Trials

In 2017 and 2018, field experiments were completed in Ingham county and Montcalm county, Michigan. Dry bean cultivars used were “Red Hawk”, a root rot susceptible kidney bean,

and “Zorro”, a root rot tolerant black bean. Plots in both locations and years were planted with a four-row tractor-mounted planter with cone units and planted at a seedling rate of 15 seeds/m. Planting, harvest, and data collection dates are found in Table 2.1. The Ingham county location was artificially inoculated with *F. brasiliense* and *F. virguliforme* inoculum while the Montcalm county location was naturally infested with *F. brasiliense* and was not artificially inoculated with any pathogen (Wang et al accepted). Both seed treatments were tested at these locations to compare efficacy in artificially inoculated and naturally infested settings.

Table 2.1. Planting, data collection, and harvest dates for both trial locations in 2017 and 2018. Two harvest dates were needed in Montcalm in 2017 as the Red Hawk beans reached harvest maturity on 28 Sep and Zorro on 10 Oct.

County	Year	Planting Date	V2 Growth Stage	R2 Growth Stage	R6 Growth Stage	Harvest
Ingham	2017	7 Jun	26 Jun	17 Jul	10 Aug	11 Sep
Montcalm	2017	14 Jun	6 Jul	2 Aug	28 Aug	28 Sept/10 Oct
Ingham	2018	8 Jun	28 Jun	23 Jul	13 Aug	18 Oct
Montcalm	2018	15 Jun	9 Jul	25 Jul	22 Aug	27 Sep

The field experiment in Ingham county was a 3-way factorial, randomized complete block design with four replications in 2017 and six replications in 2018. Plots were four-row plots, 5.3m long with 76 cm spacing between rows. Pre-emergence herbicide was applied in both years of the trial and hand weeding was used for the remainder of the season. Fields were watered through overhead irrigation for the first four weeks to maintain moist soil conditions and later as needed to maintain dry bean development. Dry bean cultivar, inoculum, and seed treatment were the three factors in the study. The levels for inoculum were: non-inoculated control (NIC), *F. virguliforme* inoculum, or *F. brasiliense* inoculum. 100 mL of *Fusarium* colonized sorghum was applied to each row (5.3 m) in the planting furrow with the dry bean seed at planting. The levels of seed treatment were: non-treated control (NTC), a commercial base seed treatment (CB) and a commercial base plus fluopyram seed treatment (CB + F). The components and rates of application

of the seed treatments can be found in Table 2.2. For both years, the seed treatments were applied by BASF (Triangle Park, NC). The field used in Ingham county in 2017 was a Riddles-Hillsdale sandy loam and Conover loamy soil with a previous crop of soybeans. The field used in 2018 was a Colwood-Brookston loam and Conover loam with previous crop of wheat.

Table 2.2. Dry bean seed treatment components.

Seed Treatment (Code)	**Seed Treatment Trade Name**	**Active Ingredient**	**Application Rate mg ai/seed**
Non-treated control (NTC)	NA	-	-
Commercial Base (CB)	EverGol Energy	Prothioconazole	0.2 [R]/0.5 [Z]/ 0.019 [B]
		Penflufen	
		Metalaxyl	0.02 [B]
	Allegiance-FL	Metalaxyl	0.12 [B]
	Gaucho 600-FS	Imidacloprid	65 mL/100 kg [B]
	Precise F Finisher 1010		19.6 mL/100 kg [B]
	Red Colorant		
Commercial base + fluopyram (CB + F)	EverGol Energy	Prothioconazole	0.2 [R]/0.5[Z]/0.019[B]
		Penflufen	
		Metalaxyl	
	Allegiance-FL	Metalaxyl	0.02 [B]
	Gaucho 600-FS	Imidacloprid	0.12 [B]
	Precise F Finisher 1010		65 mL/100 kg [B]
	Red Colorant		19.6 mL/100 kg [B]
	ILeVO		0.15 [B]

[R]: Application rate on Red Hawk seeds in 2017;
[Z]: Application rate on Zorro seeds in 2017;
[B]: Application rate on both cultivars in 2017 and 2018

Inoculum for the Ingham county trial was created using single-spore isolates of *F. virguliforme* and *F. brasiliense*, Mont-1 (NRRL 22292) and F-14-42, respectively (Jacobs et al 2018). Isolates were grown on potato dextrose agar (PDA) for 14 d. Sorghum grains were soaked in tap water overnight, then drained, 1.6 – 1.8 kg of grain were distributed into spawn bags (Fungi Perfecti, Olympia, WA), and autoclaved for five hours at 121°C. An inoculum slurry was made by combining 5 PDA plates with 14 d growth of the respective isolates and 5 plates containing PDA

with no fungal growth and 500 mL of sterile water in an autoclaved stainless-steel blender. The homogenized mixture was evenly distributed into the spawn bags of room temperature sorghum and heat sealed. After 28 d of incubation at room temperature with ambient light and mixing by hand once a week, the sorghum grains were fully colonized by the respective pathogens and the inoculum was dried at room temperature with forced air and stored at 4°C until needed.

Inoculum colony forming units (CFU) were determined by making serial dilutions to the 10^{-5} dilution level and plating on nutrient agar medium in three replicates. In 2017, the *F. virguliforme* inoculum CFU was calculated as being 67,660 CFU/mL while the *F. brasiliense* inoculum was calculated as 2733 CFU/mL. In 2018, the CFU was 49,000 CFU/mL for *F. virguliforme* inoculum and 11,000 CFU/mL for *F. brasiliense* inoculum.

The field experiment in Montcalm county was a 2-way factorial in a randomized complete block design with four replications in 2017 and six replications in 2018. In 2017, early season flooding caused widespread plant death and left only three replications of each treatment. Only cultivar and seed treatments were factors. Seed treatments and application rates were the same as the Ingham county trial. The same field, with McBride sandy loam, was used in both years. Previous to the 2017 crop, soybeans were planted. Pre-emergence herbicide was applied in both years and glyphosate was sprayed between the rows with a hooded sprayer once mid-season in 2018, followed by hand-weeding where needed. Overhead irrigation was used throughout the season at a rate of 2.5 cm per week. The Montcalm county trial was naturally infested with Fusarium root rot pathogens with *F. brasiliense* being isolated in high abundance from previous trials in the same location (Jacobs et al 2018, Wang et al accepted).

Data collection techniques were the same for both trials. Plant stand was measured at the V2 growth stage by counting the total number of plants in the center two rows along the entire

length of the plot. Stand counts were normalized to plants/ha. At the V2, R2, and R6 growth stages, five plants from both outer rows in each plot were collected. Plants were washed and the roots were separated from the shoots. At the R2 and R6 growth stages, prior to being dried, five roots from each plot were randomly selected and measured on a 1-7 quantitative scale corresponding to the percent of rot and discoloration present on each root depicted in Schneider and Kelly (2000). Root rot rates of the five roots from each plot were averaged for a plot measurement. The roots and shoots were dried for three days in air dryers at 50°C and then weighed. Mass measurements for plant roots and shoots were combined in data analysis for total plant dry mass. Dried roots were kept in storage for later DNA extraction.

A 3.048 m section of the center two rows of each plot were harvested by hand at harvest maturity and run through a combine. In Ingham county in 2018, the entire length of the center two rows, 5.3 m, of Zorro plots were harvested by combine. Total mass of harvested beans and the mass of 100 seeds from each plot was recorded. A sub-sample of beans from each plot was used to calculate moisture using a GAC®2100 GI (DICKEY-john, Auburn, IL). Total mass and 100-seed weight for each plot were adjusted to 18% moisture. Using the adjusted total mass, the yield was calculated and adjusted to kg/ha.

Quantification of *F. virguliforme* and *F. brasiliense* in Taproot DNA

Lateral roots were hand removed and dried taproots from each plot were cut up and placed in a 15-mL polycarbonate grinding vial (OPS Diagnostics) with two 4 mm stainless steel balls. Roots were ground in a GenoGrinder for 2 minutes at 1500 rpm until they were ground to a fine powder. Using a Mag-Bind Plant DNA DS Kit M1130 from Omega, DNA was extracted from a 20-mg sample of the ground roots on a KingFisher Flex Purification System (ThermoFisher Scientific). DNA concentration of each root sample was calculated using the Quant-iT dsDNA

broad range assay kit (Invitrogen) on a 96-well SAFIRE microplate reader (TECAN). The DNA extracted from the roots was then used in real-time quantitative PCR (qPCR) to quantify the amount of pathogen in the root systems of each inoculated plot and expressed as a ratio of fg of pathogen DNA per total ng of DNA. A ratio was used to account for differences in DNA quantity from each sample. For the plots at the Ingham county trial inoculated with *F. virguliforme*, a qPCR primer and probe set designed by Wang et al (2015) for detection of *F. virguliforme* was used. A 20 µL total reaction volume and two technical replicates were performed using methods described by Wang et al (2015). Each 96-well PCR plate had twenty-four DNA samples with two technical replicates as well as non-template control and serial dilutions of the positive control (DNA of *F. virguliforme* isolate Mont-1) at final concentrations of 100 pg/µL, 10 pg/µL, 1 pg/µL, 100 fg/ µL, 10 fg/µL. Standard curves were created by plotting the cycle threshold number against the base10 log of the concentrations of the DNA standards. The standard curves were then used to estimate the quantity of *F. virguliforme* DNA in the test sample.

For the DNA from all plots at Montcalm county and those plots from Ingham county inoculated with *F. brasiliense,* quantification of *F. brasiliense* was preformed using TaqMan qPCR which consisted of 1 X of TaqMan Master Mix, 500 µM of each primer, 250 µM of the probe, 200 ng/ µL of BSA, 2 µL of templated DNA, and ddH_2O to a total reaction volume of 20 µL. A qPCR primer and probe set designed by for detection of *F. brasiliense* was used (Roth and Chilvers unpublished). Quantification of *F. brasiliense* was preformed using qPCR conditions as described above. Assays are described in Table 2.3.

Non-inoculated plots from Ingham county trials were tested using both *F. brasiliense* and *F. virguliforme* assays to determine if either pathogen was present or below the threshold of

detection. Quantification data did not meet assumptions of normality so data were adjusted and presented using the logarithmic scale.

Statistical Analysis

Data were analyzed in SAS using PROC GLIMMIX (SAS Institute Inc., Cary, NC, USA). Root rot ratings were adjusted to a continuous disease severity index (DIX) as described by Li et al (2014). Year, location, cultivar, seed treatment, and inoculum were treated as fixed values and replication was treated as a random factor. Lsmeans was used to look at the fixed-effects of variables and the multiple comparisons were compared using Tukey-Kramer adjustments. Figures were created with the "ggplot2" package in RStudio Version 1.0.136 (Wickham 2009, RStudio Team 2016).

Table 2.3. Primers and probes and annealing temperatures of *F. virguliforme* and *F. brasiliense*-specific quantitative PCR assays used in study.

Assay and Reference	Primer/ Probe Name	Sequence (5'-3')	Length (bp)	Tm (°C)
F. virguliforme (Wang et al 2015)	F6-3	GTAAGTGAGATTTAGTCTAGGGTAGGTGAC	30	58
	R6	GGGACCACCTACCCTACACCTACT	24	60
	FvPrb-3	6FAM-TTTGGTCTAGGGTAGGCCG-MGBNFQ	19	70
F. brasiliense (Roth and Chilvers unpublished)	Fb_F2	AGGTCAGATTTGGTATAGGGTAGGTGAGA	29	60
	Fb_R2	CGGACCATCCGTCTGGGAATTT	22	60
	Fb_Prb1	5HEX-TGGGATGCCCT+AATTTTT+ACGG-3IABkFQ	21	57

RESULTS

Impact of Pathogens and Seed Treatments in Inoculated Field Trial

Plant Stand

Plant stand was significantly lower for Zorro plants compared to Red Hawk plants in 2017 ($P < 0.0001$) as a result of low germination seed lot (Greg Varner *personal communication*). In

Ingham county in 2017, inoculum treatment had no significant effect on plant stands for Red Hawk or Zorro. In 2018 plots inoculated with *F. brasiliense* had significantly decreased stands compared to NIC or *F. virguliforme* inoculated treatments for both Red Hawk ($P = 0.0002$) and Zorro plants ($P = 0.0003$) (Table 2.3a).

In 2017 and 2018, the CB seed treatment significantly increased (2017: $P = 0.0094$, 2018: $P < 0.0001$) stands for Red Hawk compared to NTC. In 2018, both seed treatments significantly increased the stand of Zorro compared to the NTC ($P < 0.0001$) (Table 2.3b). The interaction between inoculum treatment and seed treatment approached significance in 2017 ($P = 0.0521$) and was significant in 2018 ($P = 0.0026$) for Red Hawk. The CB seed treatment increased stand for plants inoculated with *F. brasiliense* in 2017 and plants inoculated with *F. virguliforme* in 2018. The CB + F seed treatment also significantly increased stand for plants inoculated with *F. brasiliense* in 2018 (Table 2.4).

Table 2.4. Plant stand (plants/ha) response to inoculum treatment (A) and seed treatment (B) in Red Hawk (RH) and Zorro (Z) in Ingham county trial.

	Red Hawk		**Zorro**	
A Inoculum Treatment	**2017**	**2018**	**2017**	**2018**
NIC	194,930	179,900 a	132,600	181,600 a
F. virguliforme	186,530	181,700 a	139,100	188,700 a
F. brasiliense	195,610	153,900 b	136,980	168,500 b
B Seed Treatment	2017	2018	2017	2018
NTC	183,500 b	159,850 b	131,780	168,800 b
CB	197,540 a	187,730 a	137,150	188,400 a
CB + F	196,030 a	167,900 b	139,630	181,600 a

Means in columns with same letter do not significantly differ based on Tukey's HSD $P = 0.05$
NIC: non-inoculated control, NTC: non-treated control, CB: commercial base,
CB + F: commercial base + fluopyram

Table 2.5. Interaction of inoculum and seed treatments on Red Hawk and Zorro plant stand (plants/ha) in 2017 and 2018.

		Red Hawk		**Zorro**	
Inoculum Treatment	Seed Treatment	**2017**	**2018**	**2017**	**2018**
NIC	NTC	190,660 ab	180,480 ab	125,000	176,400 bc
	CB	200,150 ab	193,400 ab	134,540	187,900 ab
	CB + F	193,960 ab	165,900 c	138,350	180,480 b
F. virguliforme	NTC	181,990 b	170,850 bc	138,670	173,880 bc
	CB	182,400 b	200,000 a	134,950	203,000 a
	CB + F	195,200 ab	174,150 a-c	143,610	189,290 ab
F. brasiliense	NTC	177,870 b	128,200 d	131,640	156,270 c
	CB	210,060 a	169,750 bc	141,960	174,150 bc
	CB + F	198,920 ab	163,700 c	137,000	174,980 bc

Means in columns with same letter do not significantly differ based on Tukey's HSD $P = 0.05$
NIC: non-inoculated control, NTC: non-treated control,
CB: commercial base, CB + F: commercial base + fluopyram.

Plant Dry Mass

Only Red Hawk plant dry mass was significantly influenced by inoculum treatments in 2017. At the V2 growth stage, the plant dry mass of Red Hawk plants inoculated with *F. virguliforme* (16.8 g) was significantly decreased ($P = 0.0372$) as compared to the NIC plants (18.6 g). Later, at the R2 growth stage in 2017, the dry plant mass of Red Hawk plants inoculated with *F. virguliforme* and *F. brasiliense* were 110.8 and 98.6 g respectively, and both significantly decreased ($P = 0.0004$) compared to the NIC at 130.9 g. In 2018, at the V2 growth stage, Zorro plants inoculated with *F. brasiliense* had a significantly decreased ($P = 0.0003$) dry plant mass (6.0 g) compared to NIC (7.2 g) and *F. virguliforme*-inoculated (7.4 g) plants (Table 2.5). In both 2017 and 2018, there were no significant differences for dry plant mass at the R6 growth stage.

Table 2.6. Dry plant mass (g/10 plants) response of Red Hawk (RH) and Zorro (Z) to inoculum treatments at V2 and R2 growth stages in both years of Ingham county trial.

	Red Hawk				**Zorro**			
Year	**2017**		**2018**		**2017**		**2018**	
Growth Stage	**V2**	**R2**	**V2**	**R2**	**V2**	**R2**	**V2**	**R2**
NIC	18.6 a	130.9 a	11.1	71.4	7.4	63.4	72. a	143.0
F. virguliforme	16.0 b	110.8 b	10.5	66.2	6.9	52.1	7.4 a	128.4
F. brasiliense	1687 ab	98.6 b	10.1	92.3	6.4	54.5	6.0 b	129.5

Means within columns followed by same letter do not differ significantly based on Tukey's HSD $P = 0.05$

Disease Severity

Red Hawk had consistently higher disease severity compared to Zorro in 2017 ($P < 0.0001$) and 2018 ($P < 0.0001$). In 2017 Red Hawk plants inoculated with *F. brasiliense* and *F. virguliforme* had significantly higher ($P = 0.0361$) disease severity compared to NIC, while the disease severity of Zorro plants did not differ (Figure 2.1). In 2018, *F. brasiliense*-inoculated treatments had significantly higher disease severity compared to the NIC for both Zorro ($P = 0.0026$) and Red Hawk ($P = 0.0006$). Red Hawk plants inoculated with *F. virguliforme* also had significantly increased disease severity compared to NIC (Figure 2.1).

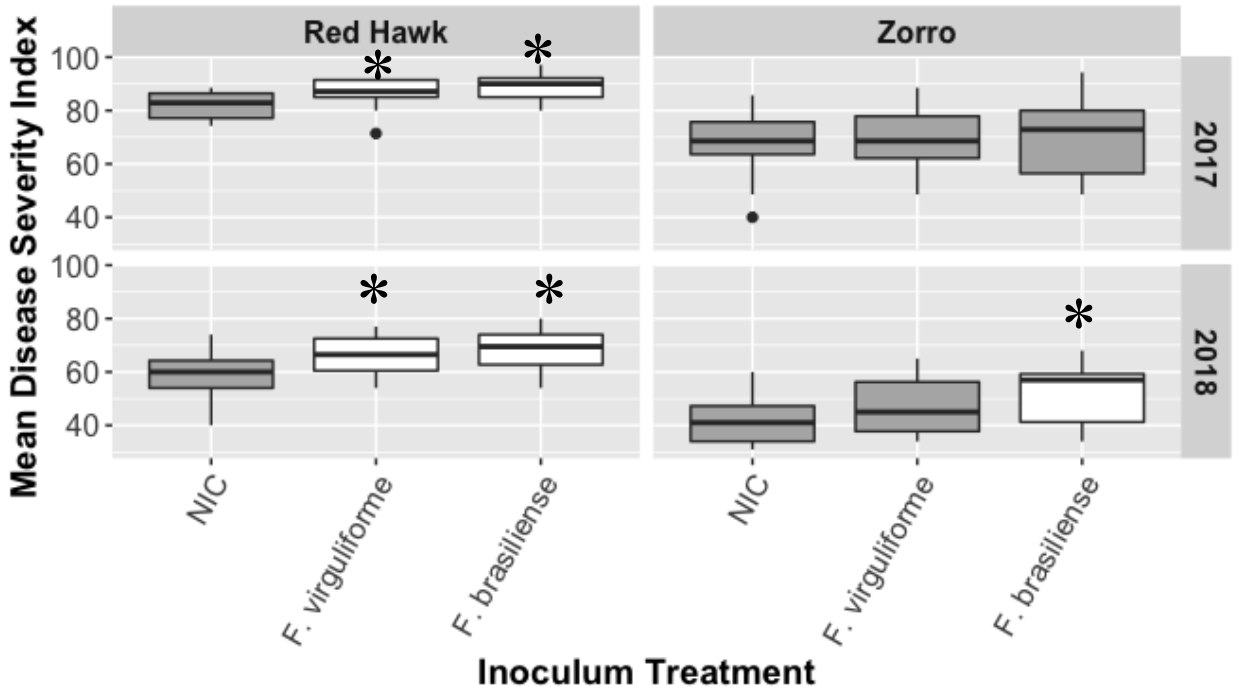


Figure 2.1. Disease severity index response to inoculum treatments on Red Hawk and Zorro in Ingham county trial. * Denotes significance from NIC by Tukey's HSD at P = 0.05.

Yield

Significant differences in yield was not observed between inoculum treatments or seed treatments for either cultivar or year in this study. However, the CB + F and CB seed treatments generally lead to a numerical increase in yield compared to the non-treated plots (Table S.2).

In both years, *F. brasiliense* significantly reduced plant stands, plant mass, and increased disease severity in Red Hawk and Zorro. *F. virguliforme* caused increased disease severity in Red Hawk in both years, however *F. virguliforme* induced plant dry mass reductions were only observed on Red Hawk in 2017. The CB seed treatment resulted in increased stand of Red Hawk in both years while both seed treatments increased Zorro stand in 2018.

Efficacy of Seed Treatment on Management of Naturally-infested *F. brasiliense* in Dry Beans

Stand

Significant differences were not observed for plant stand between any treatments within either cultivar for 2017 or 2018.

Plant Dry Mass

Plant dry mass did not differ significantly for treatments in 2017. A trend for plant dry mass in 2017 showed plants treated with CB + F had numerically lower plant dry mass than non-treated plants. At the V2 growth stage in 2018, the CB + F seed treatment led to significantly reduced plant dry mass for Red Hawk ($P = 0.0067$) and Zorro ($P = 0.0024$) (Table 2.6). At the R2 and R6 growth stages, there were no significant differences between seed treatments for plant dry mass.

Table 2.7. Dry plant mass (g/10 plants) of Red Hawk (RH) and Zorro (Z) from Montcalm county trial at V2 growth stage.

	2017		2018	
Seed Treatment	RH	Z	RH	Z
NTC	7.1	4.9	17.8 a	8.3 a
CB	7.2	4.0	19.3 a	10.8 a
CB + F	6.0	4.1	13.1 b	7.1 b

Means within columns followed by same letter do not differ significantly based on Tukey's HSD $P = 0.05$
NTC: non-treated control, CB: commercial base, CB + F: commercial base + fluopyram.

Disease Severity

Disease severity was not significantly different in 2017, Red Hawk and Zorro plants treated with the CB + F seed treatment had numerically decreased disease severity. Zorro plants treated with the CB + F seed treatment had significantly reduced disease severity ($P = 0.0017$) at R6 compared to CB treated and NIC plots. Red Hawk plants had consistently high disease

severity for all treatments at the R6 stage and experienced no significant differences (Figure 2.2). Red Hawk plants treated with CB + F had the lowest average disease severity of 87.7.

Yield

Yield was not significantly influenced by seed treatments in 2017 or 2018. Though yields were numerically greater for plots treated with CB + F seed treatment than the CB seed treatment for both years (Table S.3).

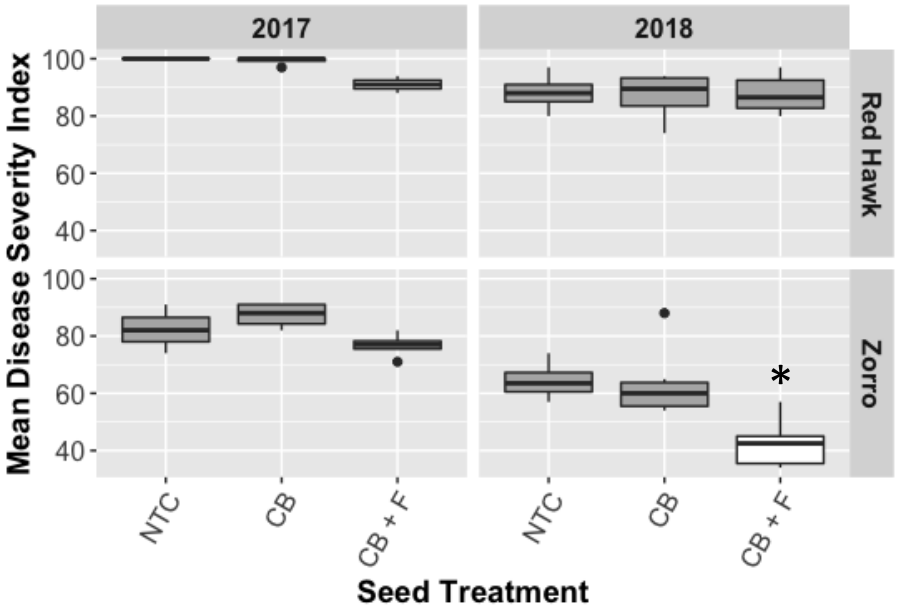


Figure 2.2. Disease severity response by seed treatments on Red Hawk and Zorro in Montcalm county trial. * Denotes significance from NIC by Tukey's HSD at P = 0.05. NTC: non-treated control, CB: commercial base, CB + F: commercial base + fluopyram.

Quantification of *F. virguliforme* and *F. brasiliense* in Dry Bean Root Systems

Quantification of Pathogens in Inoculated Trial

In both years of the Ingham county trial there were no significant difference in overall pathogen quantity between cultivars. There were significant differences in the pathogen quantity between growth stages for both cultivars. Pathogen quantity was significantly greater for both cultivars in 2017 as compared to 2018 (Red Hawk: $P < 0.0001$, Zorro: $P < 0.0001$). Growth stage

had a significant influence on the pathogen quantity for Red Hawk in 2017 ($P < 0.0001$) and Zorro in both years (2017: $P < 0.0001$, 2018: $P < 0.0001$).

The *F. brasiliense* and *F. virguliforme* assays had similar specificity and sensitivity so the pathogen quantities from both assays were compared to understand if *F. brasiliense* and *F. virguliforme* colonized root tissues differently. In both years, the amount of *F. brasiliense* present in the NTC plants was significantly higher than the amount of *F. virguliforme* in the NTC plants at the V2 growth stage for Red Hawk (2017: $P < 0.0001$, 2018: $P = 0.0008$) and Zorro (2017: $P = 0.0003$, 2018: $P < 0.0001$). The amount of *F. brasiliense* DNA in Zorro taproots was also significantly greater than the amount of *F. virguliforme* at the R2 stage in 2017 ($P = 0.0029$) and the R6 stage in 2018 ($P = 0.0169$). In 2018, the amount of *F. brasiliense* DNA in Red Hawk taproots was significantly increased ($P = 0.004$) compared to *F. virguliforme* at the R6 stage (Figure 2.3). Pathogen quantity in root tissue may have been related to root mass. Roots at the R2 growth stage for both cultivars had an average of five times more mass than the roots at the V2 stage, while between R2 and R6 stages, the roots for both cultivars only increased by a magnitude of two.

Interaction of Inoculum and Seed Treatment in Inoculated Trial

In the *F. brasiliense* inoculated treatment, the quantity of *F. brasiliense* in Zorro taproots was significantly influenced by seed treatment in V2 growth stage in 2018 ($P =0.0001$), in both years at the R2 growth stage (2017: $P = 0.0016$, 2018: $P = 0.0017$) and in both years at the R6 growth stage (2017: $P = 0.0293$, 2018: $P = 0.0001$). At these growth stages, the amount of *F. brasiliense* in Zorro taproots was significantly decreased when treated with CB + F seed treatment compared to the CB seed treatment and the NTC (Figure 2.4a). The quantity of *F. brasiliense* in Red Hawk taproots was significantly influenced by seed treatment at the V2

growth stage in both years (2017: *P* =0.008, 2018: *P* =0.0018), at the R2 growth stage in 2017 (*P* =0.038), and the R6 growth stage in 2018 (*P* =0.046). Again, the quantity of *F. brasiliense* was decreased significantly with the use of the CB + F seed treatment compared to the other two treatment levels (Figure 2.4a). The amount of *F. brasiliense* in either cultivar treated with the CB seed treatment did not significantly differ from the NTC at any growth stage in either year.

Though the CB + F seed treatment appeared to reduce the amount of *F. brasiliense* in taproots of both cultivars, it did not appear to do the same for *F. virguliforme* in taproots. The only significant interaction in *F. virguliforme* inoculated treatments due to seed treatments was observed at the R2 growth stage in 2018 (*P* = 0.0002) in Zorro plants. Beyond that one growth point, the quantity of *F. virguliforme* did not differ between seed treatments in the inoculated trial (Figure 2.4b).

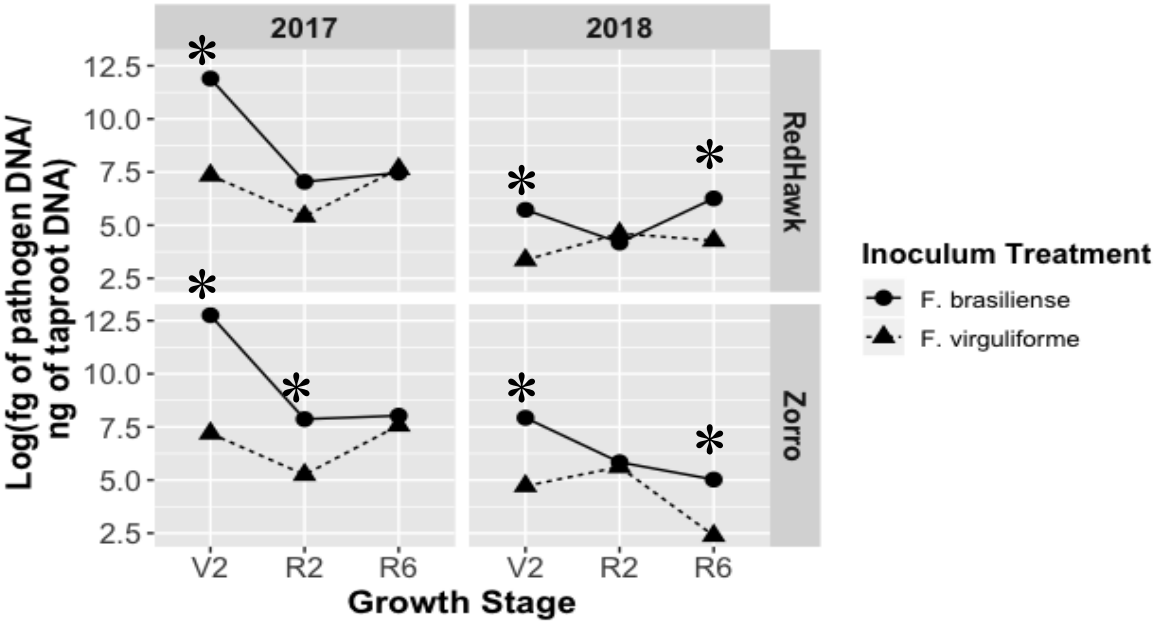
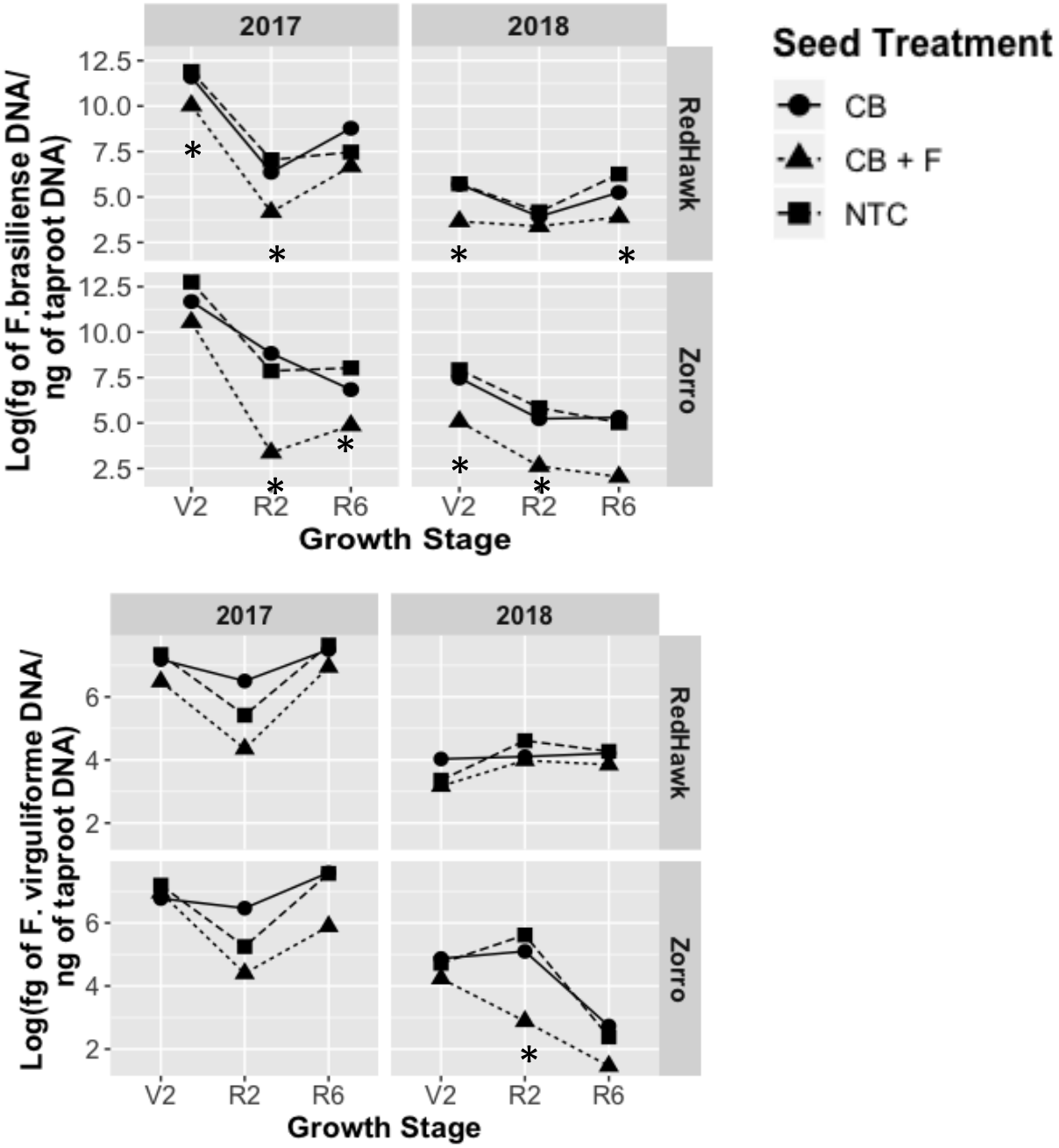


Figure 2.3. Temporal comparison of quantity of *F. brasiliense* and *F. virguliforme* in taproot DNA in non-treated Red Hawk and Zorro in Ingham county trial. * denotes significance between inoculum treatments within cultivar and year by Tukey's HSD at P = 0.05.



Figures 2.4a and b. Temporal response to seed treatment of quantity of (A) F. brasiliense and (B) F. virguliforme in Red Hawk and Zorro taproot DNA in Ingham county trial. * denotes significance from NTC within cultivar and year by Tukey's HSD at P = 0.05.

Influence of Seed Treatment in Naturally Infested Trial

In 2017, there were no significant differences of *F. brasiliense* quantity in the taproots between seed treatments or cultivars in the non-inoculated trial. In 2018, there was a significant interaction of *F. brasiliense* quantity in Red Hawk and Zorro taproots by growth stage (Red Hawk: P =0.0016, Zorro: $P < 0.0001$). In 2018, at the V2 growth stage, Red Hawk and Zorro plants treated with the CB + F seed treatment had significantly reduced *F. brasiliense* compared to the other two treatments (Red Hawk: $P = 0.0002$, Zorro: $P = 0.0001$). At the R2 and R6 growth stages, Zorro plants treated with the CB + F seed treatment had significantly increased quantities of *F. brasiliense* compared to the NTC (R2: $P = 0.006$, R6: $P = 0.002$) (Figure 2.5). There were no significant differences between seed treatments observed at the R2 or R6 growth stages for Red Hawk. For both cultivars, the quantity of *F. brasiliense* increased at the R2 growth stage, even when treated with CB + F. Despite the increase in quantity of *F. brasiliense* in the roots, the disease severity was still significantly decreased compared to the NTC. The CB seed treatment did not significantly influence the quantity of *F. brasiliense* in either cultivar compared to the NTC.

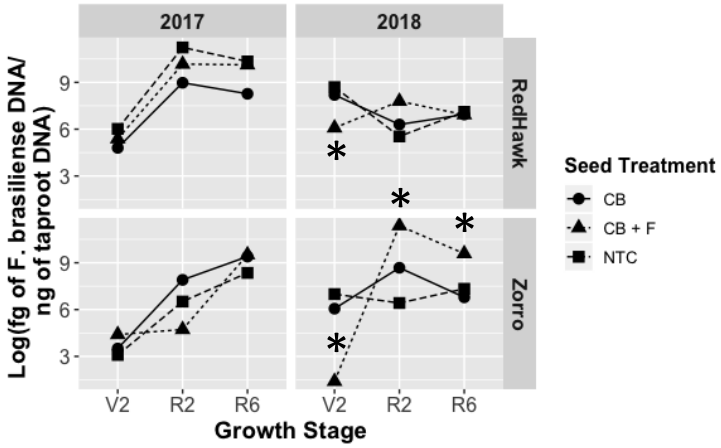


Figure 2.5. Temporal comparison of *F. brasiliense* quantity in taproot DNA by seed treatment on Red Hawk and Zorro in Montcalm county trial. * denotes significance from NTC by Tukey's HSD at P = 0.05.

DISCUSSION

The *F. brasiliense* and *F. virguliforme* inoculated field trial demonstrated that *F. brasiliense* can affect dry bean development and production. While *F. virguliforme* did cause root rot on both cultivars and affected dry plant mass of Red Hawk plants in 2017, its effects appear to be limited in scope and virulence. The CB seed treatment did offer some early season protection by significantly increasing the stand of both cultivars in the field. However, CB + F seed treatment decreased the quantity of pathogens, specifically *F. brasiliense*, in the root tissue, though it did show signs of early season phytotoxicity in the non-inoculated field trial. While yield was not significantly influenced by either inoculum or seed treatment, the seed treatments numerically increased yield compared to non-treated plots.

F. brasiliense and *F. virguliforme* Root Infection in the Field.

This is the first field study documenting the virulence of *F. brasiliense* on dry beans. While previously only known to be found in Brazil and Argentina, *F. brasiliense* was identified in 2017 in South Africa on SDS symptomatic soybeans and identified recently on dry beans and soybeans in Michigan (Aoki et al 2005, Jacobs et al 2018, O'Donnell et al 2010, Tewoldemedhin et al 2017, Wang et al accepted). The recent identification of *F. brasiliense* demands further exploration of this pathogen. Prior greenhouse pathogenicity trials by Aoki et al (2014) show *F. brasiliense* is capable of reducing plant mass and increasing root rot on soybeans. In a seedling pathogenicity study conducted by Oudman et al (unpublished) on several clade 2 FSSC isolates, *F. brasiliense* caused significantly increased disease severity and reduced plant mass on two dry bean cultivars. These observations were confirmed on dry beans in the inoculated field trial as both Red Hawk and Zorro experienced reduced plant mass, increased root rot, and reduced plant stand. There was no significant effect of *F. brasiliense* on yield. The CFU for the *F. brasiliense*

inoculum used in the inoculated trial was much less than the CFU for the *F. virguliforme* inoculum in both years. Despite this *F. brasiliense* colonized both dry bean cultivar taproots to a greater extent compared to *F. virguliforme* and caused consistent root rot symptoms. *F. brasiliense* has been isolated from dry beans throughout the dry bean production region, however *F. virguliforme* has yet to be isolated from dry beans though it has been isolated from soybeans in the same region (Jacobs et al 2018, Wang et al accepted).

This is the first study examining *F. virguliforme* on dry beans in the field. While *F. virguliforme* did cause some stunting in Red Hawk plants in 2017 and root rot in Red Hawk in both years of the experiment, the pathogen had little other effect on the dry beans, specifically on Zorro dry beans. According to the qPCR assays, *F. virguliforme* was found in significantly lower amounts than *F. brasiliense*. Kolander et al (2012) showed *F. virguliforme* to be a symptomatic host of two dry bean varieties, pinto and navy, by consistently causing root rot symptoms on the dry beans in a growth chamber. While pinto and navy beans derive from the generally more root rot tolerant germplasm of the Middle American landrace, disease may be enhanced in growth chambers due to inoculum rates compared to field settings that are subject to much wider environmental conditions (Singh et al 2007). This study offers further evidence that dry beans are symptomatic hosts of *F. virguliforme*.

The number of hectares in soybean production in Michigan have risen in the past two decades with the production in close proximity to that of dry beans(NASS 2011, 2017, USDA 2016). Because the same land is in production for both dry beans and soybeans, there is cause for concern for both crops. *F. brasiliense* can cause stunting and severe root rot to dry beans and soybean SDS. *F. virguliforme* can cause rotting of dry bean roots and build up soil inoculum which may present greater challenges to subsequent soybean crops. *F. virguliforme* can colonize

dry bean roots, therefore crop rotation between soybeans and dry beans may be an ineffective means of management of soybean SDS or dry bean Fusarium root rot. To better understand the impact of *F. brasiliense* on dry bean production in the U.S., more surveys must be done to determine the range of this pathogen and conclude if it is a causal agent of Fusarium root rot on dry beans beyond Michigan. Finding effective management of *F. brasiliense*, either through tolerant cultivars or fungicidal seed treatments, is crucial for the dry bean industry in Michigan.

Management of Fusarium Root Rot

Fusarium root rot in dry beans is typically managed through cultural techniques, tolerant cultivars, or fungicide seed treatments. The most reliable management tool is planting tolerant cultivars (Abawi et al 1985). Dry beans with origins in the Middle American landrace, such as Zorro black beans, are more tolerant to root rot than are dry beans of Andean origin, such as Red Hawk variety (Cichy et al 2015, Kelly 2010, Singh et al 1991). Although increased disease severity was observed in Red Hawk in both years compared to that of Zorro, the amount of *F. brasiliense* colonizing the roots showed that the amount of *F. brasiliense* in the two cultivars was not significantly different. A similar finding in Wang et al (2018) saw that the colonization amount of *F. virguliforme* in soybean cultivars of varying resistance was not significantly different, though foliar symptoms differed. Rather than conclude susceptibility of cultivar purely on root rot ratings, it may be more beneficial to utilize additional phenotypes to identify QTL associated with resistance to Fusarium root rot.

The efficacies of the commercial base and commercial base + fluopyram seed treatments were also assessed in this study. While the efficacy of the CB and fluopyram have been studied in soybeans, the efficacy of fluopyram on dry beans has not been examined. The fluopyram formulation used in this study, ILeVO, is not registered for dry beans. However, Propulse

(BASF, Research Triangle Park, NC), a fungicide with fluopyram as an active ingredient is registered for in-furrow use on dry beans. A one-year study of the efficacy of Propulse on Fusarium root rot was completed in the same field as the Montcalm field trial revealing similar results to this study such as early phytotoxicity on Zorro beans specifically and a reduction in the colonization of *F. brasiliense* in both Red Hawk and Zorro (Oudman et al unpublished). Even if ILeVO is not registered for dry beans, this study can further the understanding of the efficacy of fluopyram against Fusarium root rot. Since the CB seed treatment is a standard treatment applied to dry beans, this study can determine its efficacy as a management tool against Fusarium root rot.

Data from the naturally infested Montcalm county trial in 2017 did not reveal any significant differences. This may have been due too few of replications caused by early-season flooding. However, the significant differences observed in 2018 for plant dry mass and disease severity supported numerical trends in 2017. Other studies have reported early season phytotoxicity of soybeans, this study also noted early season stunting at the V2 growth stage for both cultivars in 2018 in the Montcalm county trial (Kandel et al 2016, Wise et al 2015). This study also reported decreased stand in non-inoculated Red Hawk beans treated with CB + F in 2018 in the Ingham county trial. Despite early season phytotoxicity, this study showed that stunting was not observed past the V2 growth stage. Kandel et al (2018) also observed that soybeans were able to compensate after initial phytotoxicity and that the observed phytotoxicity had not effect on yield. There was evidence that fluopyram was still efficacious on dry beans by decreasing the colonization of *F. brasiliense* in the root system. In both inoculated and natural-inoculum field settings, CB + F significantly decreased the amount of *F. brasiliense* as compared to the NTC for both cultivars. *F. virguliforme* is known to be sensitive to fluopyram, however *F.*

brasiliense has been shown to have a similar sensitivity *in vitro* to fluopyram (Wang et al 2017, Sang et al 2018). As *F. brasiliense* was present in a higher baseline amount in the root systems than *F. virguliforme*, the significant decrease when plants were treated with CB + F is notable. Many studies have shown a positive increase in yield with the application of fluopyram seed treatment on soybeans, while not a significant trend, this study did observe a numerical increase in yield with the use of the CB + F seed treatment compared to the NTC in the inoculated trial and the CB seed treatment in the non-inoculated trial (Kandel et al 2016, 2018, Gaspar et al 2016).

Studies with EverGol Energy, the primary fungicide component in the CB seed treatment, have been conducted on soybeans for various disease control with mixed results (Batzer et al 2016, Gaspar et al 2014). With the use of EverGol Energy, an increase in plant stand has been observed, but no significant effects on yield, similar to this trial (Batzer et al 2016). Furthermore, no other significant improvements on the dry beans were seen in terms of reducing root rot or colonization of the pathogens suggesting the CB is effective at maintaining plant stand and may be efficacious against other rhizosphere pathogens, but doesn't offer full season control against *F. brasiliense* or *F. virguliforme.* Fluopyram may be effective in early season management of Fusarium root rot, however combining seed treatments with tolerant cultivars can provide greater protection.

Conclusion

This study provides further evidence from inoculated and natural infested settings that *F. brasiliense* presents a threat to dry bean growers in Michigan. The risk of soybean SDS may not be reduced through crop rotation if the rotation includes dry beans as *F. virguliforme* can colonize dry beans. While the commercial base seed treatment may confer early plant stand

protection, it was not observed to be efficacious as a means of management for dry bean Fusarium root rot. The addition of fluopyram to the commercial base seed treatment however appears to decrease root rot and root colonization of *F. brasiliense*, despite early season phytotoxicity. To better understand the impact of *F. brasiliense* on dry beans, further survey work should be done outside of Michigan to determine if *F. brasiliense* infects dry beans elsewhere in the United States. Further work to determine effective fungicide management should also be completed to protect dry beans against *F. brasiliense*.

APPENDICES

APPENDIX A

Supplementary Tables

Table S.1. List of isolates, county of origin, year of isolation, and mating type. * denotes use in seedling pathogenicity assays, # denotes use in phylogenetic analysis.

Isolate	Location	Year	Mating Type	GenBank Number
F. brasiliense				
F_14_12*	Ingham	2014	MAT1-1	
F_14_42*#	Montcalm	2014	MAT1-2	MK227762
F_14_44*	Saginaw	2014	MAT1-2	
F_14_51*	Montcalm	2014	MAT1-1	
F_15_30	Huron	2015	MAT1-2	
F_15_33*	Montcalm	2015	MAT1-1	
F_15_34#	Sanilac	2015	MAT1-2	MK227767
F_15_46*	Huron	2015	MAT1-2	
F_15_50	Huron	2015	MAT1-2	
F_15_85	Saginaw	2015	MAT1-2	
F_15_101#	Huron	2015	MAT1-2	MK227769
F_15_102#	Huron	2015	MAT1-2	MK227770
F_15_144	Montcalm	2015	MAT1-2	
F_15_147	Montcalm	2015	MAT1-2	
F_15_157*#	Saginaw	2015	MAT1-2	MK227771
F_15_158	Saginaw	2015	MAT1-2	
F_15_162	Saginaw	2015	MAT1-2	
F_15_166	Saginaw	2015	MAT1-2	
F_15_174*	Gratiot	2015	MAT1-2	
F_15_192*#	Gratiot	2015	MAT1-2	MK227772
F_15_201	Saginaw	2015	MAT1-2	
F_15_202*	Saginaw	2015	MAT1-2	
F_15_203	Saginaw	2015	MAT1-2	
F_16_80*	Bay	2016		
F_16_93	Gratiot	2016		
F_16_100	Bay	2016	MAT1-2	
F_16_113*#	Bay	2016	MAT1-2	MK227778
F_16_118	Saginaw	2016	MAT1-1	
F_16_119	Saginaw	2016	MAT1-2	
F_16_122	Saginaw	2016	MAT1-2	
F_16_124	Saginaw	2016	MAT1-2	
F_16_125	Saginaw	2016	MAT1-2	
F_16_126	Saginaw	2016	MAT1-2	

Table S.1 (cont'd)

F_16_127	**Saginaw**	**2016**	**MAT1-1**	
F_16_128	Saginaw	2016	MAT1-2	
F_16_133	Saginaw	2016	MAT1-2	
F_16_136	Saginaw	2016	MAT1-2	
Isolate	**Location**	**Year**	**Mating Type**	**GenBank Number**
F. cuneirostum				
F_14_3*	Ingham	2014	MAT1-1	
F_14_40*	Montcalm	2014	MAT1-1	
F_14_41*	Montcalm	2014	MAT1-2	
F_14_52*	Montcalm	2014		
F_15_25#	Montcalm	2015	MAT1-2	MK227764
F_15_26*#	Montcalm	2015	MAT1-1	MK227765
F_15_31	Huron	2015	MAT1-2	
F_15_32	Huron	2015	MAT1-2	
F_15_35*	Sanilac	2015	MAT1-2	
F_15_47	Huron	2015	MAT1-2	
F_15_91*#	Saginaw	2015	MAT1-1	MK227768
F_15_92*	Saginaw	2015	MAT1-2	
F_15_113*	Montcalm	2015	MAT1-1	
F_15_142	Montcalm	2015	MAT1-1	
F_15_145	Montcalm	2015	MAT1-1	
F_15_146*	Montcalm	2015	MAT1-1	
F_15_148	Montcalm	2015	MAT1-2	
F_15_150	Montcalm	2015	MAT1-1	
F_15_152	Montcalm	2015	MAT1-1	
F_15_154	Montcalm	2015	MAT1-1	
F_15_155	Montcalm	2015	MAT1-1	
F_15_159*	Saginaw	2015	MAT1-1	
F_15_165	Saginaw	2015	MAT1-2	
F_15_199	Montcalm	2015	MAT1-1	
F_15_204	Saginaw	2015	MAT1-1	
F_15_205	Saginaw	2015	MAT1-1	
F_15_206	Saginaw	2015	MAT1-1	
F_15_207	Saginaw	2015	MAT1-1	
F_16_73	Bay	2016	MAT1-2	
F_16_74*	Bay	2016		

Table S.1 (cont'd)

F_16_77*	**Bay**	**2016**	**MAT1-1**	
F_16_78#	Bay	2016	MAT1-1	MK227773
F_16_84*	Bay	2016	MAT1-1	
F_16_86*	Gratiot	2016		
F_16_87	Gratiot	2016	MAT1-1	
F_16_88	Gratiot	2016	MAT1-1	
F_16_89*	Gratiot	2016	MAT1-1	
F_16_90#	Gratiot	2016	MAT1-1	MK227774
F_16_91#	Gratiot	2016	MAT1-1	MK227775
F_16_92*	Gratiot	2016	MAT1-1	
F_16_94*	Gratiot	2016	MAT1-1	
F_16_96	Bay	2016	MAT1-1	
F_16_97*	Bay	2016		
F_16_98	Bay	2016	MAT1-1	
F_16_102	Bay	2016	MAT1-1	
F_16_107*#	Bay	2016	MAT1-1	MK227776
F_16_108	Bay	2016	MAT1-1	
F_16_111	Bay	2016	MAT1-1	
F_16_114	Bay	2016	MAT1-1	
F_16_121	Saginaw	2016		
F_16_135	Saginaw	2016	MAT1-2	
Isolate	**Location**	**Year**	**Mating Type**	**GenBank Number**
F. phaseoli				
F_14_43*#	Saginaw	2014	MAT1-1	MK227763
F_15_24*	Huron	2015	MAT1-2	
F_15_29*#	Huron	2015	MAT1-1	MK227766
F_15_38	Sanilac	2015	MAT1-1	
F_15_103	Huron	2015	MAT1-1	
F_15_161*	Saginaw	2015	MAT1-1	
F_16_75	Bay	2016	MAT1-1	
F_16_105*	Bay	2016	MAT1-2	
F_16_109*#	Bay	2016	MAT1-1	MK227777
F_16_129	Saginaw	2016	MAT1-1	
F_16_130	Saginaw	2016		
F_16_132	Saginaw	2016	MAT1-1	

Table S.2. Yield (kg/ha) by inoculum and seed treatments in Ingham county trial for Red Hawk (RH) and Zorro (Z).

		2017		2018	
Inoculum Treatment	Seed Treatment	RH	Z	RH	Z
NIC	NTC	1535.8	1679.9	1603.2	2252.9
	CB	1578.8	2204.18	1315.1	2256.9
	CB + F	1633.2	2120.7	1463.8	2720.7
F. virguliforme	NTC	1540.8	2431.6	1187.2	2373.9
	CB	1637.3	1990.7	1400.4	2751.5
	CB + F	1722.7	2046.3	1547.1	2469.5
F. brasiliense	NTC	1567.4	2415.8	1284.7	2570.5
	CB	1648.9	1766.7	1362.8	2356.0
	CB + F	1583.4	1986.1	1665.5	3235.13

Table S.3. Yield (kg/ha) by seed treatments in Montcalm county trial for Red Hawk (RH) and Zorro (Z).

	2017		2018	
	RH	Z	RH	Z
NTC	3413.7	2178.6	1724.4	1724.4
CB	2457.3	2071.3	1651.2	1651.2
CB + F	2737.6	2272.6	1575.8	1575.8

APPENDIX B

Plant Disease Management Report

Evaluation of in-furrow Propulse application for Fusarium root rot control in dry beans in Michigan, 2018

Submitted November 2018

DRY BEAN (*Phaseolus vulgaris* "Red Hawk" and "Zorro")
Fusarium Root Rot; *Fusarium brasiliense*

K.A. Oudman, A.M. Byrne, J.F. Boyse, M.I. Chilvers

Department of Plant, Soil and Microbial Sciences

Michigan State University
East Lansing, MI 48824

Evaluation of in-furrow Propulse® application for Fusarium root rot control in dry beans in Michigan, 2018

The field experiment was conducted at the Montcalm Research Center in Montcalm county, Michigan where *F. brasiliense* has been isolated in high abundance from previous dry bean and soybean crops. The previous crop was dry bean and soil type a Montcalm sandy loam. Dry beans were planted on 15 Jun 18 in 30-in. rows at a rate of 80,000 seeds/A. Plots were 17.5 ft. long. Seeds were treated by BASF with EverGol Energy, Allegiance-FL, Gaucho 600-FS, Precise S Finisher 1010, and red colorant. An in-furrow application was made at 9.59 G/A volume of Propulse at a 6 fl oz/A rate with furrow jet nozzles attached to the four-row seed cone planter. The experimental design was a complete randomized block design with two treatments and six replications. Stand counts were taken on 9 Jul on the center two harvest rows at the V2 growth stage. At the V2 (9 Jul), R2 (25 Jul), and R6 (22 Aug) growth stages, 5 plants from both outer rows were collected from each plot, for a total of 10 plants. Root rot ratings were taken on 5 random roots per plot using a 1-7 scale described in Kelly and Schneider (2000) where 1 describes a healthy, non-rotted root and 7 describes a dead root. Root rot ratings were converted into a continuous disease severity index (DIX) using a formula described in Li et al (2014). Plants were washed under tap water and dried in a drying oven for 3 d at 145°F for dry plant measurements. Dried taproots were ground in 15 mL vials with two 4 mm stainless steel balls in a GenoGrinder for 2 min at 1500 rpm. DNA was extracted from a 30-mg aliquot of ground, homogenized roots using a Mag-Bind Plant DNA DS Kit M1130 on the KingFisher™ Flex Purification System (ThermoFisher Scientific). DNA concentration was calculated using the Quant-IT dsDNA broad range kit (Invitrogen) in a 96-well SAFIRE microplate reader (Tecan). *F. brasiliense* DNA in taproots was quantified using a specific qPCR assay (Roth and Chilvers, unpublished). Yield was calculated by hand-harvesting 10 bed ft. of the center two rows, combining, weighing the seed, and adjusting to 18% moisture. Data were analyzed using PROC GLIMMIX in SAS. Cultivar and treatment were treated as classification variables while replication was used a random factor with multiple comparisons using the Tukey-Kramer adjustment (SAS Institute, Cary, NC).

The stand of Zorro dry beans was significantly lower with an in-furrow application of Propulse compared with the non-treated, while the stand counts of Red Hawk were not significantly different. Plant mass at the V2 growth stage was significantly lower for Zorro plants treated with Propulse, but not Red Hawk, perhaps suggesting early season phytotoxicity. At the R2 growth stage, the DIX was significantly decreased for Red Hawk plants treated with Propulse. The DIX was significantly diminished for Zorro plants treated with Propulse compared to the non-treated at the R6 stage. The quantity of *F. brasiliense* DNA in the taproot was significantly lower for both Red Hawk and Zorro plants treated with Propulse at the V2 growth stage. However, at the R2 growth stage, the Red Hawk and Zorro plants treated with Propulse had significantly higher quantities of *F. brasiliense* in the taproot. Yield was not significantly different for Red Hawk nor Zorro. Although Propulse is not currently registered for use as an in-furrow treatment for dry bean root rot, this study demonstrates that Propulse may aid in the reduction of root rot and root infection by *F. brasiliense*.

Table S.4. Fusarium root rot development variables in Propulse in-furrow trial conducted on Red Hawk and Zorro in 2018.

Cultivar	Treatment	V2 Stand Count (plants/A)	V2 Plant Mass (g/10 plants)	R2 DIX	R6 DIX	V2 *F. brasiliense* Quantity (Log(fg of *F. brasiliense* DNA/ng of taproot DNA)	R2 *F. brasiliense* Quantity (Log(fg of *F. brasiliense* DNA/ng of taproot DNA)	Yield (cwt/A)
Red Hawk	Non-treated	58,743	20.05	82.4 a	86.6	8.3 a	7.2 b	12.07
	Propulse	52,272	18.9	72.3 b	80.4	6.2 b	8.5 a	9.34
	p-value	*0.1817*	*0.4845*	*0.0305*	*0.2213*	*0.0444*	*0.0143*	*0.2962*
Zorro	Non-treated	59,325 a	12.9 a	59.5	55.7 a	5.0 a	8.3 b	17.97
	Propulse	49,782 b	10.4 b	48.6	45.7 b	1.08 b	11.7 a	16.85
	p-value	*0.006*	*0.0209*	*0.0788*	*0.0167*	*0.0184*	*0.0069*	0.6561

Column numbers followed by different letters are significantly different at p=0.05 within cultivar, as determined by least square means comparison

APPENDIX C

Plant Disease Management Report

Evaluation of Quadris in-furrow application and fungicide seed treatments for dry beans, 2017 and 2018 in Michigan

Submitted November 2018

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K.A. Oudman, A.M. Byrne, J.F. Boyse, M.I. Chilvers

Department of Plant, Soil and Microbial Sciences

Michigan State University
East Lansing, MI 48824

Evaluation of Quadris® In-furrow application and fungicide seed treatments for dry beans, 2017 and 2018 in Michigan

In 2017 and 2018, field experiments were completed in the same field at the Montcalm Research Center in Montcalm County, Michigan. The soil was a Montcalm sandy loam and the field had a high natural inoculum pressure of *F. brasiliense*, a newly-identified soybean sudden death syndrome (SDS) and dry bean root rot pathogen in Michigan, though previously known soybean SDS pathogen in South America. Prior to the 2017 crop, soybeans were grown. The trial was planted on 14 Jun 2017 and 15 Jun 2018, planted at a seeding rate of 80,000 seeds/A. Plots were four rows with 30-inch spacing between each row and were 17.5 ft. long. The plots were set up in a complete randomized block design with three treatments (non-treated, base seed treatment plus Quadris, and base + ILeVO seed treatment plus Quadris) and four replications in 2017 and six replications in 2018. In 2017, Quadris in-furrow was applied at a rate of 10 fl oz/A in 15 G/A spray volume of water using a hand-held spray boom pressurized with CO2 at 40 psi. In 2018 Quadris was applied in the planting furrow at a rate of 8.7 fl oz/A at 9.5 G/A spray volume of water using furrow jet nozzles attached a four-row cone seed planter. The base seed treatment contained EverGol Energy, Allegiance-FL, Gaucho 600-FS, Precise S Finisher 1010, and red colorant at standard rates. The beans with base seed treatment plus ILeVO were treated with 0.15 mg ai/seed of ILeVO at 600 g ai/L in addition to the base seed treatment for both cultivars. At the V2 growth stage (6 Jul 17 and 9 Jul 18), plant stand was measured by counting plants in the center two rows. Ten plants, five plants from either outer row in a plot, were collected at the V2, R2 (2 Aug 17, 25 Jul 18), and R6 (28 Aug 17, 22 Aug 18) growth stages. Five random roots per plot were used to take root rot ratings using a 1-7 scale from Kelly and Schneider (2000) where roots are progressively more rotted with each subsequent number. Using a method described by Li et al (2014), root rot ratings were converted to a continuous disease severity index (DIX). After being washed under tap water, plants were dried at 145°F for three d in a drying oven prior to collecting dry plant mass measurements. 10 bed feet of the center two rows were hand-harvested, combined, weighed, and adjusted for 18% moisture to calculate yield. Data analysis was conducted using PROC MIXED in SAS (SAS Institute, Cary, NC).

Plots were flooded shortly after 2017 planting, leaving only three replications of each treatment in 2017. Poor germination of Zorro plants was observed in 2017 due to seed originating from a poor-quality seed lot. Due to the small sample size and reduced stand of Zorro plants, there is no significance in either cultivar for any variable tested in 2017. The 10 oz/A of Quadris used led to observed phytotoxicity foliar symptoms in both Red Hawk and Zorro plants. The reduced rate of 8.7 oz/A of Quadris in 2018 did not lead to any observed phytotoxicity symptoms. In 2018, The Red Hawk and Zorro plants treated with the base seed treatment and Quadris had significantly increased stands compared to plants treated with Quadris and the base + ILeVO seed treatment. Red Hawk plants treated with base + ILeVO and Quadris had significantly decreased dry plant mass at the V2 stage compared to the non-treated and base seed treatment. However, at the R2 growth stage, Red Hawk plants treated with base + ILeVO and Quadris had a significantly lower DIX than the non-treated or base seed treatment with Quadris beans. In the Zorro beans, the base seed treatment with Quadris also in addition to the base + ILeVO with Quadris had significantly decreased DIX compared to the non-treated Zorro beans. In both years, yield was not significantly different between treatments for either cultivar. This study demonstrates that at a rate of 8.7 fl oz/A, Quadris in-furrow applications and a base + ILeVO seed treatment may reduce root rot infection by *F. brasiliense*.

Table S.5. Fusarium root rot development variables in Quadris in-furrow trial conducted on Red Hawk and Zorro in 2017 and 2018.

Cultivar	Treatment	2018 Stand Count (plants/A)	2018 V2 Dry Plant Mass (g/10 plants)	2018 R2 DIX	2018 R6 DIX	2017 Yield (Cwt)	2018 Yield (Cwt)
Red Hawk	Non-treated	39,328 b	17.8 ab	85.2 a	88.5	7.64	10.8
	Base + Quadris	56,254 a	20.7 a	81.4 a	86.6	10.2	8.78
	Base + ILeVO + Quadris	41,319 b	14.6 b	70.4 b	81.9	8.4	9.61
	p-value	*0.0275*	*0.0142*	*0.0013*	*0.5331*	*0.5122*	*0.3791*
Zorro	Non-treated	54,761 a	8.4	70.9 a	64.7 a	6.11	11.07
	Base + Quadris	56,752 a	9.9	60.9 b	55.2 ab	7.78	11.05
	Base + ILeVO + Quadris	48,787 b	8.6	53.3 c	44.7 b	8.17	10.9
	p-value	*<0.0001*	*0.3268*	*<0.0001*	*0.0124*	*0.8172*	*0.9947*

Column numbers followed by different letters are significantly different within cultivar at p=0.05, determined by least squares mean comparison

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