Diagnostic qPCR Assay to Detect *Fusarium brasiliense*, a Causal Agent of Soybean Sudden Death Syndrome and Root Rot of Dry Bean

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Abstract

Species within clade 2 of the *Fusarium solani* species complex (FSSC) are significant pathogens of dry bean (*Phaseolus vulgaris*) and soybean (*Glycine max*), causing root rot and/or sudden death syndrome (SDS). These species are morphologically difficult to distinguish, and often require molecular tools for proper diagnosis to a species level. Here, a TaqMan probe-

based qPCR assay was developed to distinguish *F. brasiliense* from other closely related species within clade 2 of the FSSC. The assay displays high specificity against close relatives and high sensitivity, with a detection limit of 100 femtograms. This assay was able to detect *F. brasiliense* from purified mycelia, from infected dry bean roots, and from soil samples throughout Michigan. When multiplexed with an existing qPCR assay specific to *F. virguliforme*, accurate quantification of both *F. brasiliense* and *F. virguliforme* was obtained, which can facilitate accurate diagnoses and identify co-infections with a single reaction. The assay is compatible with multiple qPCR thermal cycling platforms, and will be helpful in providing accurate detection of *F. brasiliense*. Management of root rot and SDS pathogens in clade 2 of the FSSC is challenging and must be done proactively, as no mid-season management strategies currently exist. However, accurate detection can facilitate management decisions for subsequent growing seasons to successfully manage these pathogens.

Introduction

The *Fusarium solani* species complex (FSSC) is composed of three major clades, designated 1, 2, and 3 (Chitrampalam and Nelson 2016; O'Donnell 2000). Some *Fusarium* species within clade 2 are soil-borne plant pathogens that cause root rot of soybean (*Glycine max*) and dry bean (*Phaseolus vulgaris*) (Aoki et al. 2014; Chitrampalam and Nelson 2016). Macroscopic and microscopic morphological features of the species within clade 2 are very challenging to differentiate, as all produce similar pigments, and conidia lengths and widths overlap (Aoki et al. 2003). Of the species within this clade, six are known to cause sudden death syndrome (SDS) on soybean (Aoki et al. 2003, 2005; 2012a; 2012b; Tewoldemedhin et al. 2016). In South America, the SDS-causing pathogens include *F. virguliforme* O'Donnell & T.

Aoki*, F. brasiliense* T. Aoki & O'Donnell*, F. tucumaniae,* and *F crassistipitatum*, while in North America SDS is caused by *F. virguliforme* and the recently identified *F. brasiliense* (Wang et al. 2018b). *Fusarium brasiliense* and an undescribed *Fusarium* sp. cause soybean SDS in South Africa (Tewoldemedhin et al. 2016). The sixth SDS-causing species, *F. azukicola*, has only been isolated from adzuki bean (*Vigna angularis*), but when inoculated on soybeans it caused foliar SDS symptoms (Aoki et al. 2012b).

Soybean SDS is an annual threat in both the U.S. and Brazil (Aoki et al. 2003; O'Donnell et al. 2010). These countries dominate soybean production in North and South America, respectively, both producing an estimated 120 million metric tons of soybeans in 2017-2018 (World Agricultural Supply and Demand Estimates, 2018). Managing these SDS-causing FSSC clade 2 species is challenging, as few fungicides demonstrate efficacy (Kandel et al. 2018; Sang et al. 2018; Wang et al. 2017), genetic resistance is partial (Chang et al. 2018), and crop rotation practices take up to 3 or 4 years of non-host crops to lower SDS pressure (Leandro et al. 2018). However, some SDS-causing species like *F. tucumaniae* show increased sensitivity to certain chemistries (Sang et al. 2018), so it is important to know the species present in order to recommend proper management.

Monitoring the spread of fungal pathogens is also important to determine if management strategies are effective. Fungal pathogens can be spread through the transfer of infected materials, shared use of contaminated field equipment, and through wind-blown spores. The mechanism of *F. virguliforme* spread remains unknown, though the sexual stage has never been observed and only one mating type has been detected (Hughes et al. 2014), so spread through the discharge of sexual ascospores appears unlikely. A single mating type for *F. brasiliense* was identified in South Africa (Tewoldemedhin et al. 2016), but both mating types have been found

in South America (Hughes et al. 2014) and Michigan isolates (Oudman et al, *in prep*). In addition, *F. brasiliense* isolates with different mating types were identified in the same field (Oudman et al, *in prep*). This indicates that conventional fungal sexual reproduction is probable in *F. brasiliense*, providing an opportunity for the pathogen to produce perithecia and spread through the discharge of sexual ascospores into the air. Only one isolate of *F. brasiliense* had been identified in the U.S. prior to 2014 from an unknown host in California (Aoki et al. 2005), so it is unlikely that it has spread from California to Michigan without being detected in soybean-growing states in between. Population genetic studies of *F. virguliforme* suggest that it originated in South America and spread to North America (Wang 2016). It may be possible that *F. brasiliense* also originated in South America and spread to the U.S. through unknown mechanisms, but a native Michigan population of *F. brasiliense* cannot be ruled out.

As both soybeans and dry beans are hosts for *Fusarium* root rot pathogens, accurate identification of these species in these fields is critical to prevent significant inoculum increases, severe yield losses, and potential spread to neighboring fields. Accurate identification of *F. brasiliense* and monitoring its spread throughout the U.S. will allow growers to implement the few successful management strategies available. Additionally, with consumer trends increasing the demand of non-genetically modified foods, many dry bean growers are beginning to rotate non-genetically modified soybeans with dry beans (B. Glass, *personal communication*), potentially leading to even more significant inoculum increases. Our primary objective was to develop a highly specific and sensitive quantitative polymerase chain reaction (qPCR) assay to detect *F. brasiliense*. The second objective was to validate the performance of this qPCR assay in detecting *F. brasiliense* from purified mycelia, infected plant roots, and soil. Third, we examined the compatibility of this assay with a *F. virguliforme* qPCR assay for multiplexing

purposes, which provides fast and efficient diagnosis of closely related species or the presence of co-infections.

Materials and Methods

Assay Design

The intergenic spacer (IGS) region of the ribosomal RNA gene has been used to distinguish species within the FSSC (Wang et al. 2011), including SDS- and brown root rot-causing fusaria (O'Donnell et al. 2010), and was used as the target locus for this assay as in other qPCR assays (Chilvers et al. 2007; Okubara et al. 2013; Wang et al. 2015). IGS sequences from species within clade 2 of the FSSC were obtained from National Center for Biotechnology Information (NCBI); *F. azukicola* GenBank number JQ670149*, F. brasiliense* GenBank numbers KF706657 and FJ919512*, F. cuneirostrum* GenBank numbers FJ919511 and FJ919548*, F. crassistipitatum* GenBank numbers FJ919521 and FJ919554*, F. phaseoli* GenBank numbers FJ919498, FJ919510*,* and FJ919500*, F. tucumaniae* GenBank numbers FJ919507 and FJ919515*,* and *F. virguliforme* GenBank numbers FJ919499 and FJ919557. Sequences were aligned in MEGA7 (Kumar et al. 2016) using the MUSCLE algorithm (Edgar 2004).

A TaqMan probe using locked nucleic acids was designed to capitalize on the presence of two single nucleotide polymorphisms (SNPs) specific to *F. brasiliense* within eight base pairs of each other (Fig. 1). Primer and probe sequences are reported in Table 1. Each qPCR reaction was performed with two technical replicates, and contained 10 µL of 2X TaqMan Universal real-time PCR master mix (1X final concentration, Applied Biosystems), 250 nM Fb_Prb1, 500 nM Fb_F1 primer, 500 nM Fb_F2 primer, 400 ng BSA, 2 µL DNA template, and molecular grade water up to a total reaction volume of 20 µL. All assays were performed on the StepOnePlus real time

PCR system (v2.3, Applied Biosystems) and were set up in clear MicroAmp 96-well plates (Thermo-Fisher Scientific, Waltham, MA), except for multiplexed assays. Multiplexed reactions were performed with two technical replicates, each containing 10 µL of 2X TaqMan Universal real-time PCR master mix (Applied Biosystems), 250 nM Fb_Prb1, 500 nM Fb_F1 primer, 500 nM Fb_F2 primer, 100 nM FvPrb3, 500 nM F6-3, 500 nM R6, 400 ng BSA, 2 µL of each DNA template, and molecular grade water up to a total reaction volume of 20 µL.

qPCR Assay Specificity

Fusarium isolates used for testing the assay specificity were either collected from counties throughout Michigan or requested from the USDA ARS Culture Collection (NRRL) (Table 2). Isolates collected from Michigan were obtained from root tissues of infected soybean or dry bean plants. Briefly, plants displaying stunting or foliar SDS symptoms from fields throughout Michigan were uprooted and transported to the lab where soil was thoroughly washed off roots. Root sections displaying severe discoloration and rot were soaked in 5% bleach solution (0.4% NaOCl) for 4 minutes, rinsed thoroughly in sterile deionized water for 4 minutes, blotted dry with a sterile paper towel, and plated on WMS agar (2% agar, 15µg/mL metalaxyl, 300µg/mL streptomycin) to select for fungal growth. Plates were incubated for seven days at room temperature and observed under a dissecting microscope (Leica Microsystems, Buffalo Grove, IL, USA) for the formation of sporodochia. Using an insect pin, macroconidia from a sporodochium were transferred and spread on fresh WMS agar and allowed to grow for 24 hours. Finally, a single germinating macroconidium was aseptically transferred onto full strength potato dextrose agar (Neogen, Lansing, MI). Isolates were stored at room temperature on synthetic nutrient agar slants (1 g/L KH_2PO_4, 1 g/L KNO_3, 0.5 g/L $MgSO_4$, 0.5 g/L KCl, 0.2 g/L glucose,

0.2 g/L sucrose, 2% agar) in scintillation vials (Research Products International, Mount Prospect, IL, USA) for long-term storage.

Macroconidia from pure cultures were inoculated into potato dextrose broth and allowed to grow at room temperature for 48 hours with shaking at 100 rpm. DNA was extracted with two similar methods. For testing specificity at a standard 5 ng per reaction, mycelia were filtered from broth with two layers of Miracloth (Millipore Sigma, St. Louis, MO, USA) and rinsed with 200 mL of sterile deionized water. The fresh, rinsed mycelia were immediately transferred to a sterile, pre-chilled mortar and ground with a pestle in the presence of liquid nitrogen. After approximately 1 minute of tissue disruption, the ground mycelia were immediately transferred into microcentrifuge tubes containing a DNA extraction buffer (200 mM Tris-HCl, 1 M NaCl, 25 mM EDTA, 25 mM sodium dodecyl sulfate, 100 µg RNaseA) and incubated for 30 minutes at room temperature, with gentle inversions every 5 minutes. Proteinase K (200 µg) was added to each sample and incubated at room temperature for 20 minutes, with gentle inversions every 5 minutes. The samples were chilled on ice for 5 minutes, followed by an addition of 0.2 volumes of ice-cold 5 M potassium acetate, another 5-minute incubation, and then centrifuged at 5000 x g for 12 minutes at 4°C. The supernatant was transferred to fresh tubes, and an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol (Millipore Sigma) was added, mixed gently by inversion, and centrifuged. The phenol:chloroform step was repeated, followed by an additional step using chloroform only (Millipore Sigma). The DNA was precipitated by adding 0.1 volumes 5 M NaCl, 1 volume isopropanol, incubated on ice for 10 minutes, and pelleted at 10,000 x g in a refrigerated centrifuge at 4°C. The pelleted DNA was washed twice with freshly prepared 80% ethanol and resuspended in 10 mM Tris buffer pH 8.0 overnight. DNA quality was determined via 260/280 nm and 260/230 nm readings obtained from a Nanodrop 1000 (Thermo-Fisher

Scientific), and quantity was determined via the Qubit dsDNA BR Assay Kit (Thermo-Fisher Scientific). An aliquot of DNA for each strain was diluted to 2.5 ng/µL, with 2 µL used in qPCR assays for specificity testing (Fig. 2). For testing specificity of other isolates at various starting DNA concentrations (Table 2), mycelia were harvested with sterilized wooden stirrers (STR Supplies Inc., British Colombia, Canada) into sterile 1.5 mL tubes. The tube was plugged with a sterile cotton ball, and mycelia were lyophilized for 48 hours. Approximately 20 mg of lyophilized mycelia were ground with 3-5 2 mm glass beads, and subjected to the same extraction protocol as described above.

qPCR Assay Sensitivity

Extracted DNA from four *F. brasiliense* isolates were tested for sensitivity via serial dilutions. Concentrations tested were 10-fold dilutions beginning with 1,000,000 femtograms (1 nanogram) of *F. brasiliense* DNA to 10 femtograms (Fig. 3A). The Ct values for each concentration were averaged across isolates, since rDNA copy number can vary among strains of the same species (Balajee et al. 2009; Wang et al. 2015). The qPCR efficiency was calculated by finding the line of best fit on a plot with the logarithm of the DNA concentrations and their respective Ct value (Fig. 3B, Table 3). The limit of detection was determined by samples showing > 95% amplification at the lowest DNA concentration tested (Bustin et al. 2009).

Assay Validation

A field site in Ingham County, MI was planted with a randomized complete block design containing two dry bean cultivars (Zenith and Red Hawk), three treatments (non-inoculated, *F. brasiliense* inoculated, and *F. oxysporum* inoculated), with 4 plot replicates per cultivar x

treatment combination. Each plot was 6.1 m (20 ft) long with 4 rows of plants with 76 cm (30 inch) row spacing. The plots were planted on 8 June 2018 at 172,100 seeds ha^{-1} for Red Hawk and 193,700 seeds ha^{-1} for Zenith using a cone planter. Inoculum was produced on autoclaved sorghum grains, as described in Wang et al. 2018a. Inoculum was packaged into envelopes directly with seeds, and the entire contents were poured into the cone planter, sowing the seeds and inoculum in-furrow at an average rate of 7.1 mL of inoculum per foot. Three plants from each plot were sampled on 12 September 2018. The foliar tissues were removed from the roots in the field, and the roots were thoroughly washed clean of soil and allowed to dry at 55°C for 2 days. The dried roots from each sample were placed in a 50 mL plastic container (OPS Diagnostics, Lebanon, NJ, USA) with 2, 1 cm diameter steel balls and ground into a powder using a GenoGinder 2010 (SPEX Sample Prep, Metuchen, NJ, USA) at 1500 rpm for 5 minutes. DNA was extracted from these roots using the same phenol:chloroform procedure described above. Total DNA was quantified using a Nanodrop 1000 (Thermo-Fisher Scientific).

To validate the assay with soil samples, bulk soil was collected from ten soybean fields throughout Michigan during the spring and summer of 2018, using a 3 cm diameter, 15 cm deep soil core. Soil was placed into a 3.8 L (1-gallon) plastic bag, homogenized, subsampled into 2.3 kg (5 lb) paper bags, and dried at 55°C for 2 days. Approximately 500 mg of dried soil was used for DNA extraction using a FastDNA Spin Kit for Soil (MP Bio, Solon, OH). Two technical DNA extraction replicates were performed, and a third technical replicate was extracted after being spiked with approximately 5000 *F. brasiliense* conidia. An estimated number of conidia in non-spiked soil samples that showed positive qPCR results was obtained by performing the following calculation: 5000 spores * z = x spores * y, where 5000 is the number of spores spiked into each soil sample, z is the Ct of the spiked sample, and y is the Ct of the non-spiked sample.

Non-spiked samples that amplified at a Ct > 31 cannot be reliably quantified because they are below the limit of detection. Therefore, an estimated number of conidia were not calculated for these samples (Table 4).

To test the multiplexing capacity and transferability of this assay on other platforms, serial dilutions of *F. virguliforme* DNA and *F. brasiliense* DNA were amplified in a single reaction containing primers F6-3, R6, and probe FvPrb-3 (Wang et al. 2015) along with primers and probe developed in this study (Table 1). Reactions were set up in LightCycler 480 96-well plates (Roche Diagnostics, Mannheim, Germany) and performed on a CFX96 Real-Time PCR Detection System v3.1 (BioRad Laboratories, Hercules, CA). Multiplexed reactions were set up as described above.

Results

Assay Design

A 149 base pair segment in the IGS was identified containing *F. brasiliense* specific SNPs, and the primers and probe were designed in this region (Fig. 1). The forward primer encompasses one SNP shared by *F. brasiliense*, *F. cuneirostrum, F. crassistipitatum,* and *F. virguliforme*, but excludes *F. phaseoli* and *F. tucumaniae*. The reverse primer is conserved among all species used in the IGS alignment. The probe encompasses two SNPs unique and specific to *F. brasiliense*, containing two thymines instead of two cytosines. The probe associated with these residues was designed with locked nucleic acid bases to enhance specific binding at these sites (Fig. 1, asterisks). The primers and probe are not predicted to form any significant homo- or heterodimers ($\Delta G > -9$), and performs at an ideal anneal and extension temperature of 60°C.

231

232 *qPCR Assay Specificity*

233 Genomic DNA isolated from purified mycelia was used to show specificity across closely
234 related species within clade 2 of the FSSC (Table 2). The only isolates displaying amplification
235 were *F. brasiliense* and some members of FSSC clade 3 (Chitrampalam and Nelson 2016) (Fig.
236 2, Table 2). However, when tested with a standard 5 ng of starting DNA, *F. brasiliense* isolates
237 consistently had Ct values within 1 Ct of each other (13.94 - 14.92, Table 2). In contrast, FSSC
238 clade 3 isolates with positive reactions had higher Ct values. These results indicate that the assay
239 designed here can distinguish *F. brasiliense* from closely related clade 2 species and other
240 common soil-borne pathogenic fungi (Table 2). However, there may be some cross-reactivity
241 with clade 3 species. With pure cultures, *F. brasiliense* can readily be distinguished from clade 3
242 species based on growth rate and morphology (Fig. 4).

243

244 *qPCR Assay Sensitivity*

245 The *F. brasiliense* qPCR assay targeting the IGS region provided high sensitivity,
246 reproducibly amplifying target DNA as low as 10 femtograms of target DNA of multiple isolates
247 (Fig. 3). Although all four isolates tested here amplified at this low concentration, standard error
248 of detection was highest at this concentration. Therefore, the limit of detection was
249 conservatively determined to be 100 fg, or a Ct $\geq$ 31 (Table 3) (Bustin et al. 2009). Although
250 target DNA can be amplified at lower concentrations, quantities determined below this cycle
251 threshold may not be reliable.

252

253 *Assay Validation*

To determine the applicability of this assay to field trials, roots from artificially inoculated dry beans were harvested and subjected to DNA extraction and tested with the *F. brasiliense* qPCR assay developed here. *F. brasiliense* was detected consistently above the limit of detection in dry bean plots inoculated with *F. brasiliense* (average Ct = 29.6), but not in non-inoculated plots or plots inoculated with *F. oxysporum* (average Ct = 36.3) (Fig. 5).

Soil samples obtained from ten counties throughout Michigan were screened for the presence of *F. brasiliense*. All soil samples spiked with *F. brasiliense* conidia were successfully detected with Ct values ranging from 17.29 to 21.97 indicating relatively pure DNA samples lacking PCR inhibitors (Table 4). Additionally, *F. brasiliense* was detected in non-spiked soil samples from six counties, although only two were confidently above the limit of detection of 100 fg (Table 4). Using a known quantity of spores in the spiked samples, an estimated 2804 and 3051 spores were present in Saginaw County and Sanilac County, respectively, per 500 mg of soil.

The primers and probes for the *F. brasiliense* assay and a *F. virguliforme* assay (Wang et al. 2015) were used simultaneously in a multiplexed reaction (Table 1). Amplification for each assay was only observed when target DNA was present, indicating no cross-reactivity between the assays (Table 5). Combinations of serial dilutions of *F. brasiliense* and *F. virguliforme* DNA were successfully amplified in pooled reactions, and an accurate quantification of each target was obtained (Table 5).

Discussion

Many of the clade 2 species in the FSSC are economically important pathogens capable of causing root rot and/or soybean SDS, and significant yield losses. They are very similar

277 morphologically with a slow growth rate and are taxonomically distinguished by conidia
278 measurements and number of septa (Aoki et al. 2014). However, since a pure culture is required
279 to obtain conidia, and conidia measurements of these species overlap (Aoki et al. 2003), it can be
280 very difficult to obtain accurate identifications in a timely manner, if at all, using morphology
281 alone. DNA extractions and qPCR assays can be performed in a single day, expediting accurate
282 diagnoses. High quality qPCR assays have high sensitivity, capable of detecting very low
283 quantities of target DNA. Designing an assay around a multi-copy target like the IGS region can
284 increase sensitivity. Previous assays designed around the IGS region have provided sensitivity to
285 as low as 100 femtograms of target DNA (Rojas et al. 2017; Wang et al. 2015). The assay
286 developed here has validated SNPs specific to *F. brasiliense* compared to other species within
287 the FSSC clade 2 (Fig. 1, Table 2). The assay performs at a high efficiency of 99.6%, and
288 consistently detected 10 fg of target DNA when obtained from pure culture, and has a confident
289 limit of detection of 100 fg (Fig. 3, Table 3).
290 Nomenclature within clade 3 of the FSSC is complicated, but recent progress has been
291 made by incorporating multilocus genotyping and formal typification efforts (O'Donnell et al.
292 2010; Schroers et al. 2016). For instance, FSSC clade 3 species in this study represent FSSC 5,
293 the true *F. solani*, and FSSC 11, an unnamed species. Interestingly, amplification of *F. solani*
294 (FSSC 5) isolates and FSSC 11 isolates showed variation among isolates, suggesting some IGS
295 sequence diversity within these species. Sanger sequencing of the 149 bp IGS region where this
296 qPCR assay was designed showed that FSSC 11 isolate MI-Mtc-C3FS (Wht) and *F. solani*
297 isolates F-15-118 and MI-Mtc-C17FS share SNPs with other non-*F. brasiliense* species,
298 confirming their negative reactions. Sanger sequencing of this region for FSSC 11 isolate MI-
299 Mtc-A2FS and *F. solani* isolate MI-Mtc-B10 IGS showed SNPs shared with *F. brasiliense*, thus

300 explaining the positive reactions. Phylogenetic analyses of *F. solani* clade 3 isolates commonly
301 use other molecular markers such as *TEF1*, ITS rDNA, and *RPB2* (Chitrampalam and Nelson
302 2016, Wang et al. 2018b), perhaps due to the difficulty of sequencing and assembling the
303 repetitive IGS region. However, this assay provides specificity to *F. brasiliense* within *F. solani*
304 clade 2, and qPCR positive FSSC clade 3 strains can be distinguished from *F. brasiliense* though
305 morphology (Fig. 4). However, the identity of all isolates should be confirmed through PCR and
306 Sanger sequencing of multiple loci (Wang et al. 2018b).
307 When pure cultures and collections are not being obtained or maintained, this assay can
308 still provide an initial screen for *F. brasiliense* in soil and root samples. The assay is not likely to
309 produce false-positive results by cross-amplification of FSSC clade 3 species because some do
310 not cross react even at high concentrations, and others that do cross-react do so with much lower
311 efficiency, producing significantly higher Ct values (Table 2, Fig. 2). For instance, with five
312 nanograms of purified fungal DNA, cross-reactive FSSC clade 3 species showed higher Ct
313 values by at least 6 cycles (Table 2). Therefore, obtaining a sufficient concentration of FSSC
314 clade 3 DNA from root or soil samples for cross-reactivity is unlikely. In contrast, all *F.*
315 *brasiliense* isolates from both the U.S. and Brazil were consistently detected with high accuracy
316 and efficiency. Therefore, obtaining a sufficient concentration of *F. brasiliense* from root or soil
317 samples for successful amplification and detection is possible, as shown in Table 4 and Figure 5.
318 Initial screens with this qPCR assay can help guide when and where to begin isolation attempts
319 for pure cultures, and also help generate an understanding of the global distribution of *F.*
320 *brasiliense*. For example, the *F. brasiliense* assay gave Ct values < 31 from soil samples from
321 Saginaw and Sanilac, counties, where *F. brasiliense* has already been successfully isolated from
322 plant roots (Oudman et al, *in prep*). The assay also gave Ct values > 31, but < 36 with DNA from

soil samples from Montcalm and Ingham counties, where *F. brasiliense* has also been successfully isolated (Oudman et al, *in prep*). Finally, the assay gave Ct values > 31, but < 36, from Lenawee and St. Joseph soils, where *F. brasiliense* has not yet been isolated. Therefore, the assay provides evidence that warrants further investigation and isolation attempts for *F. brasiliense* from these counties.

Soybean and dry bean are valuable crops in Michigan worth a combined $1.08 billion in 2017 (USDA-NASS, 2017). Fusarium root rot and SDS consistently rank in the top 10 most destructive soybean diseases in northern states, including Michigan (Allen et al. 2017). To date, genetic resistance to Fusarium root rot is partial, and only few fungicides are effective against it (Sang et al. 2018; Wang et al. 2017). Therefore, accurate detection and integrating successful management practices like using seed treatments (Kandel et al. 2018), crop rotations (Leandro et al. 2018), tillage practices, and planting partially resistant cultivars are key to preventing yield loss. Further understanding of a possible host preference is also needed in order to inform management strategies, as *F. brasiliense* is more commonly found in areas of dry bean production while *F. virguliforme* is more commonly found in areas of soybean production in Michigan (Oudman et al, *in prep*). Preliminary experiments examining host preference have shown that both *F. brasiliense* and *F. virguliforme* cause disease on both soybean and dry bean, and that disease severity or root colonization is not significantly different from one another in a growth chamber setting (Roth and Chilvers, *unpublished*). Symptomatic roots infected by *F. brasiliense* and *F. virguliforme* are indistinguishable from one another, and co-infections may also be possible. Therefore, the ability to multiplex the *F. brasiliense* and *F. virguliforme* assays can also save time and effort in accurate diagnoses.

345 Partial genetic resistance will continue to be an important part of integrated management
346 for Fusarium root rot pathogens. Though more research has been done with resistance to
347 *Fusarium* spp. in soybean, resistance is complicated and partial (Chang et al. 2018). Dry beans
348 (*Phaseolus vulgaris*) originated from Mesoamerica (Bitocchi et al. 2012), but were traded
349 through Central America and experienced a bottle neck while reaching South America.
350 Therefore, gene pools in current bean breeding lines originate from Mesoamerica or the northern
351 Andean region of South America (Bellucci et al. 2014; Miklas et al. 2006; Singh 2001; Singh
352 and Schwartz 2010). Dry bean varieties from Mesoamerican gene pools, like cultivar Zenith, are
353 smaller in size and typically have higher resistance to root rot diseases (Román-Avilés and Kelly
354 2005; Schneider et al. 2001). Another Mesoamerican variety, Zorro, was shown to have more
355 resistance to *F. virguliforme* than the Andean variety Red Hawk, a large-seeded red kidney bean
356 cultivar (Dry Bean Research Report, 2017). Interestingly, more *F. brasiliense* was detected in
357 inoculated Zenith roots than Red Hawk roots in this study. However, root colonization and
358 disease severity are not always strongly correlated, particularly among the clade 2 FSSC species
359 (Wang et al. 2018a). A formal study should be conducted to determine the inherent resistance
360 levels of multiple varieties from the two different gene pools to *F. brasiliense*.
361

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Table 1. Primers and probes used in this study. Tm = melting temperature, bp = base pairs
[a] Probes contain 5' fluorophores "5HEX" or "6FAM", and 3' quenchers "3IABkFQ" or "MGBNFQ"
[b] Calculated with the OligoAnalyzer tool from IDT, using the default "qPCR" parameter settings.

Table 2. Panel of isolates used to determine specificity of *F. brasiliense* qPCR assay. + indicates positive amplification, while – indicates no amplification. n.d. = not determined.
[a]Isolate IDs with only numbers were obtained from the USDA ARS Culture Collection (NRRL). All other isolates were obtained by J. Jacobs and Dr. Martin Chilvers.
[b]FSSC = *Fusarium solani* species complex. FSSC 5 is named *F. solani*, while FSSC 11 is an unnamed species within clade 3.

Table 3. Serial dilutions for four *F. brasiliense* isolates used to determine assay sensitivity.
[a]Isolate 34938 was obtained from the USDA ARS Culture Collection (NRRL). All other isolates were obtained by J. Jacobs and Dr. Martin Chilvers.
[b]Concentration in femtograms of DNA per reaction
[c]Ct = cycle threshold, or cycle at which fluorescent signal crossed the detection threshold.

Table 4. Amplification of *F. brasiliense* from soils across Michigan. Values with an asterisk (*) are estimates, calculated as indicated in the materials and methods. Dashes indicate no detection, and n.d. indicates not determined since detection was below the limit of detection.
[a]Ct = cycle threshold, or cycle at which fluorescent signal crossed the detection threshold.
[b]Quantity in femtograms
[c]Estimated by taking solving for x, using the equation: 5000 spores * z = x spores * y, where 5000 is the number of spores spiked into each soil sample, z is the Ct of the spiked sample, and y is the Ct of the non-spiked sample.

Table 5. Amplification of serial dilutions of *F. brasiliense* and/or *F. virguliforme* DNA. Assays display specificity towards target species and can be distinguished and quantified in a multiplexed system. Dashes represent no detection.
[a]Quantity in femtograms
[b]Ct = cycle threshold, or cycle at which fluorescent signal crossed the detection threshold for *F. virgulforme*.
[c]Ct = cycle threshold, or cycle at which fluorescent signal crossed the detection threshold for *F. brasiliense*.

Figure 1. Sequence alignment of 149 base pairs of the intergenic spacer (IGS) of species within clade 2 of the *Fusarium solani* species complex. Primers (blue) and probe (green) sequences are displayed as arrows at their annealing location within the IGS. Asterisks represent locked nucleic acids designed in the qPCR probe.

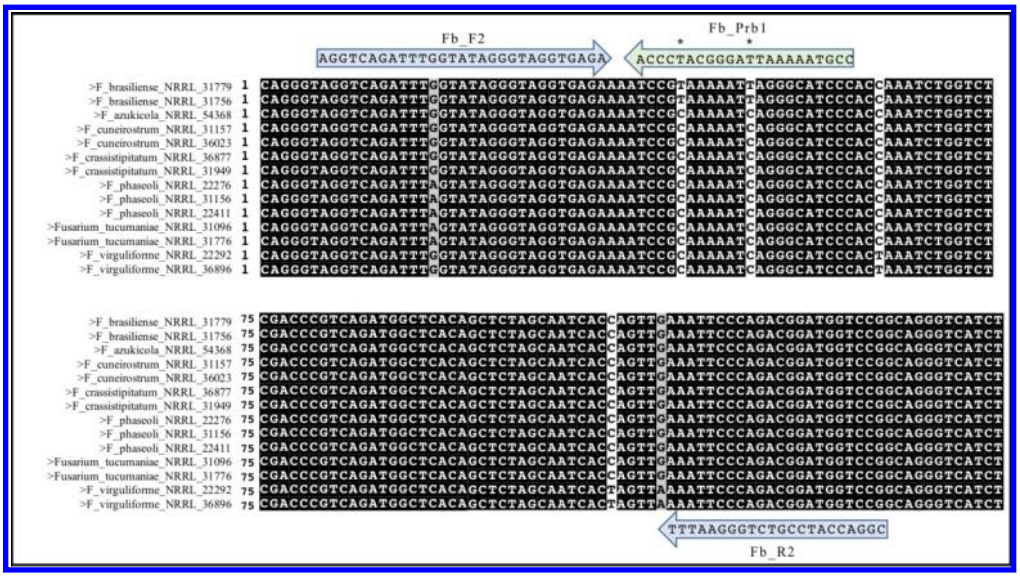
Figure 2. Amplification results of 5 ng DNA of select isolates across different species within the *Fusarium solani* species complex. Isolates represented are *F. azukicola* isolates NRRL 54361 and 54362, *F. brasiliense* isolates F-14-42, F-15-158, F-16-137, NRRL 34938, MI-Mtc-C1, *F. solani* (FSSC 5) isolates MI-Mtc-B10 and F-15-118, *F. crassistipitatum* isolate NRRL 31949, no-template (water) control, *F. cuneirostrum* F-14-52, *F. phaseoli* isolates MI-Mtc-A12 and

NRRL 22411, *F. tucumaniae* isolates NRRL 43334 and 31777, and *F. virguliforme* isolates DB P28 R13 and Vb-2a.

Figure 3. Detection of serial dilutions of 4 *F. brasiliense* isolates to determine assay sensitivity (**A**). These isolates consistently amplified at all concentrations tested, with an average of 99.6% efficiency (**B**). Fb = *Fusarium brasiliense*

Figure 4. Representative isolates of (**A**) *F. brasiliense* and (**B**) *Fusarium solani* species complex (FSSC) clade 3 isolates plated on potato dextrose agar, 7 days post inoculation. *Fusarium brasiliense* isolates represented are (i) MI-Mtc-C1, (ii) MI-Mtc-A13, (iii) F-16-137, and (iv) F-15-158. *Fusarium solani* (FSSC 5) isolates represented are (i) MI-Mtc-C17FS, (ii) MI-Mtc-B16FS, and (iii) MI-Mtc-B10, while FSSC 11 isolate MI-Mtc-A2FS is also represented (iv).

Figure 5. Detection of *F. brasiliense* within root tissues of dry bean cultivar (**A**) Zenith or (**B**) Red Hawk. Values listed over bars indicate the average femtograms of *F. brasiliense* DNA detected within roots of 12 plants per treatment. Non-inoculated and *F. oxysporum* inoculated samples were not detected above the limit of detection of 100 fg.



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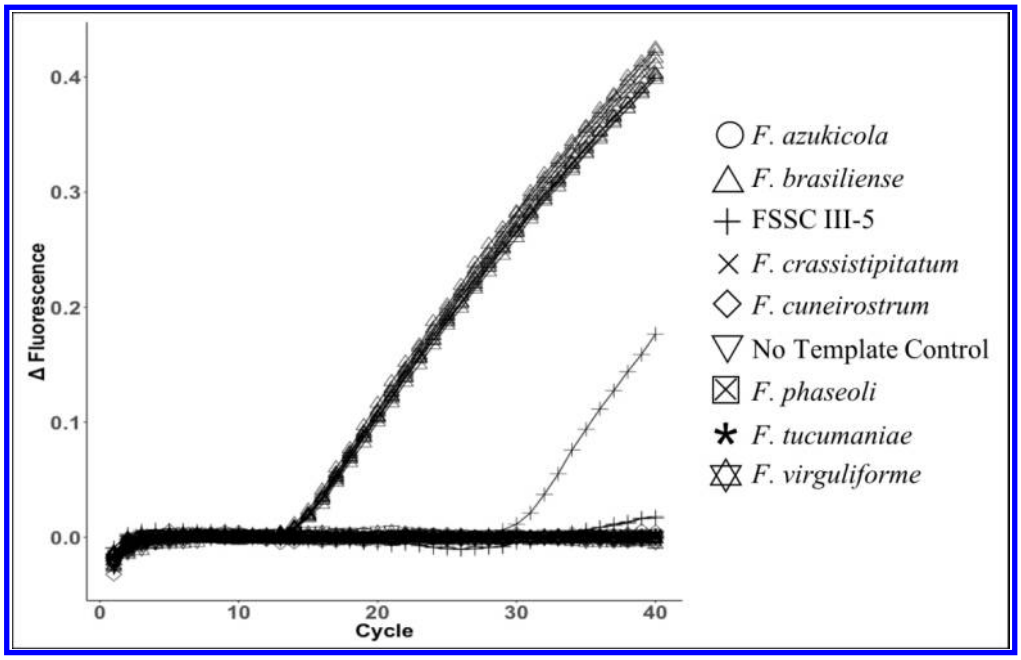

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151x96mm (300 x 300 DPI)

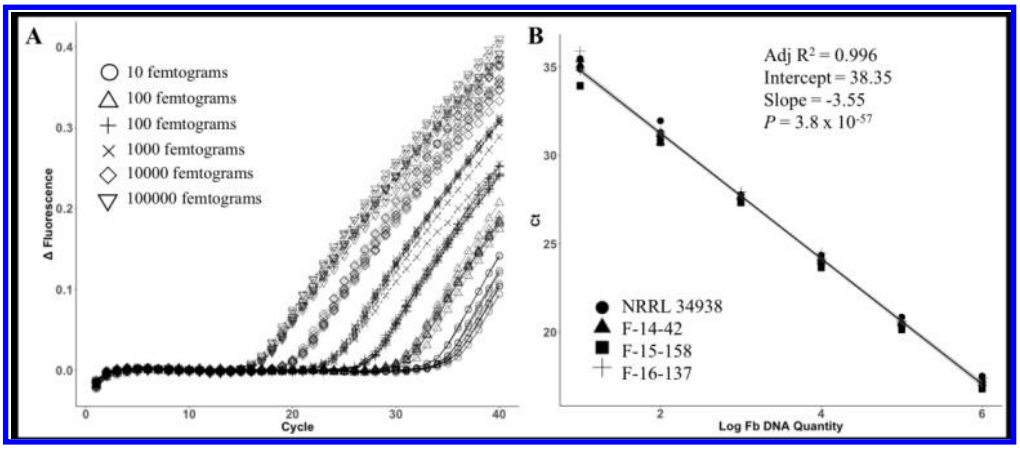
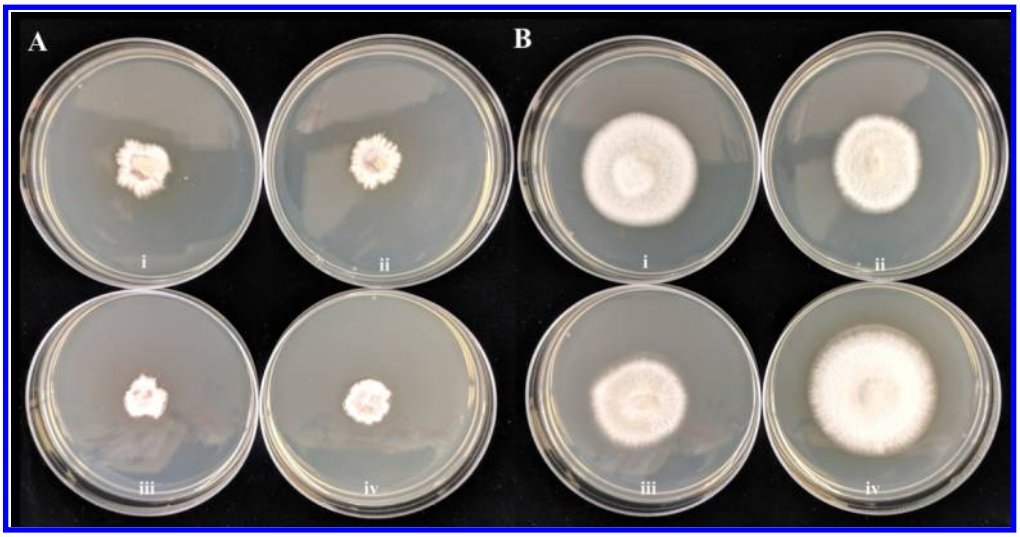


Figure 3. Detection of serial dilutions of 4 *F. brasiliense* isolates to determine assay sensitivity (**A**). These isolates consistently amplified at all concentrations tested, with an average of 99.6% efficiency (**B**). Fb = *Fusarium brasiliense*

170x72mm (300 x 300 DPI)



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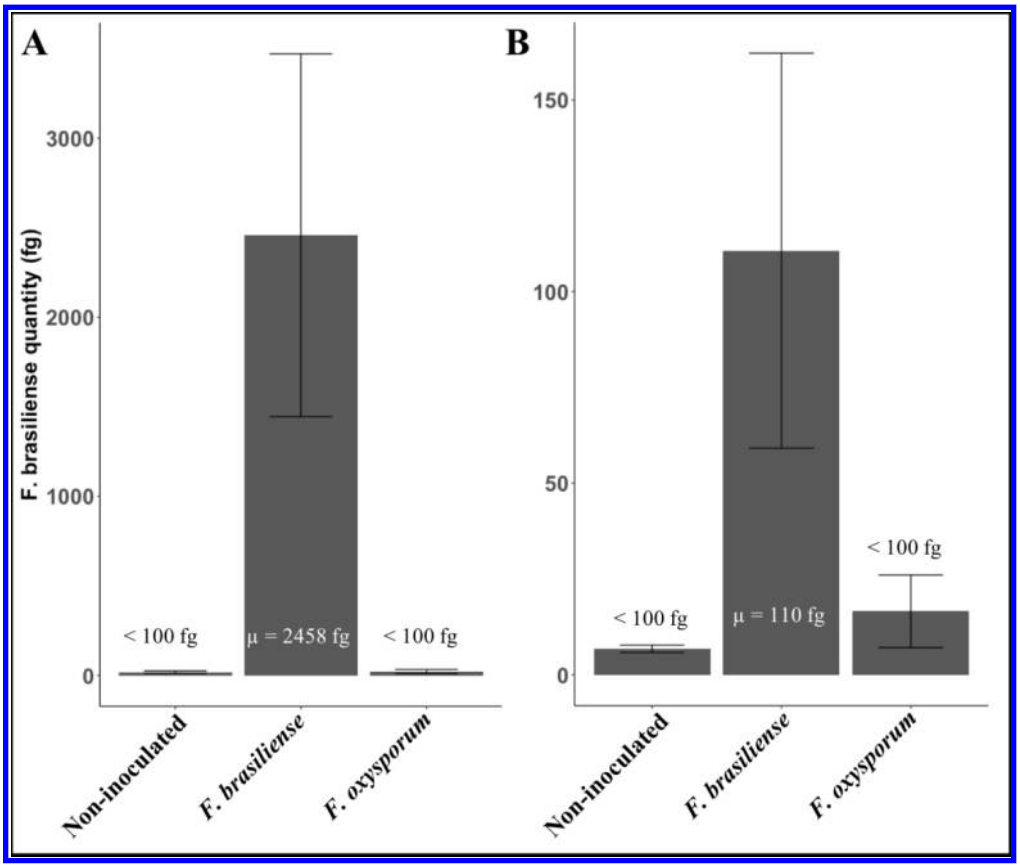
170x87mm (300 x 300 DPI)



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128x108mm (300 x 300 DPI)

Primer/Probe Name[a]	Sequence (5'-3')	Length (bp)	Tm (°C)[b]	Reference
Fb_F2	AGGTCAGATTTGGTATAGGGTAGGTGAGA	29	67.4	This study
Fb_R2	CGGACCATCCGTCTGGGAATTT	22	66.3	This study
Fb_Prb1	5HEX-TGGGATGCCCT+AATTTTT+ACGG-3IABkFQ	22	64.7	This study
F6-3	GTAAGTGAGATTTAGTCTAGGGTAGGTGAC	30	57.8	Wang et al. 2014
R6	GGGACCACCTACCCTACACCTACT	24	59.6	Wang et al. 2014
FvPrb-3	6FAM-TTTGGTCTAGGGTAGGCCG-MGBNFQ	19	70.0	Wang et al. 2014

Table 1. Primers and probes used in this study. Tm = melting temperature, bp = base pairs
[a] Probes contain 5' fluorophores "5HEX" or "6FAM", and 3' quenchers "3IABkFQ" or "MGBNFQ"
[b] Calculated with the OligoAnalyzer tool from IDT, using the default "qPCR" parameter settings.

Isolate[a]	Species[b]	Host	Location isolated	Assay Result	Ct with 5 ng starting DNA
54361	*F. azukicola*	*Vigna angularis*	Obihiro, Hokkaido, Japan	–	n.d.
54362	*F. azukicola*	*V. angularis*	Obihiro, Hokkaido, Japan	–	n.d.
34938	*F. brasiliense*	Unknown	Vila Maria, Rio Grande do Sul, Brazil	+	14.92
22678	*F. brasiliense*	*Phaseolus vulgaris*	California, USA	+	
MI-Mtc-C1	*F. brasiliense*	*Glycine max*	Montcalm County, MI, USA	+	14.21
MI-Mtc-A13	*F. brasiliense*	*G. max*	Montcalm County, MI, USA	+	14.43
F-14-12	*F. brasiliense*	*P. vulgaris*	Ingham County, MI, USA	+	
F-14-42	*F. brasiliense*	*P. vulgaris*	Montcalm County, MI, USA	+	14.71
F-14-44	*F. brasiliense*	*P. vulgaris*	Montcalm County, MI, USA	+	
F-15-33	*F. brasiliense*	*P. vulgaris*	Montcalm County, MI, USA	+	
F-15-101	*F. brasiliense*	*P. vulgaris*	Huron County, MI, USA	+	
F-15-102	*F. brasiliense*	*P. vulgaris*	Huron County, MI, USA	+	
F-15-144	*F. brasiliense*	*P. vulgaris*	Montcalm County, MI, USA	+	
F-15-158	*F. brasiliense*	*P. vulgaris*	Saginaw County, MI, USA	+	14.47
F-15-162	*F. brasiliense*	*P. vulgaris*	Saginaw County, MI, USA	+	
F-15-174	*F. brasiliense*	*P. vulgaris*	Gratiot County, MI, USA	+	
F-15-192	*F. brasiliense*	*P. vulgaris*	Michigan, USA	+	
F-16-21	*F. brasiliense*	*G. max*	Montcalm County, MI, USA	+	
F-16-59	*F. brasiliense*	*G. max*	Montcalm County, MI, USA	+	13.94
F-16-118	*F. brasiliense*	*P. vulgaris*	Saginaw County, MI	+	
F-16-119	*F. brasiliense*	*P. vulgaris*	Saginaw County, MI	+	
F-16-124	*F. brasiliense*	*P. vulgaris*	Saginaw County, MI	+	
F-16-125	*F. brasiliense*	*P. vulgaris*	Saginaw County, MI	+	
F-16-127	*F. brasiliense*	*P. vulgaris*	Saginaw County, MI	+	
F-16-128	*F. brasiliense*	*P. vulgaris*	Saginaw County, MI	+	
F-16-131	*F. brasiliense*	*P. vulgaris*	Saginaw County, MI	+	
F-16-136	*F. brasiliense*	*P. vulgaris*	Saginaw County, MI	+	
F-16-137	*F. brasiliense*	*P. vulgaris*	Saginaw County, MI, USA	+	14.52
31949	*F. crassistipitatum*	*G. max*	Cristalina, Goias, Brazil	–	n.d.
46170	*F. crassistipitatum*	*G. max*	Las Lajitas, Salta, Argentina	–	
46175	*F. crassistipitatum*	*G. max*	Las Lajitas, Salta, Argentina	–	

F-14-52	*F. cuneirostrum*	*P. vulgaris*	Montcalm County, MI, USA	–	n.d.
Fsp3	*F. cuneirostrum*	*P. vulgaris*	Uganda, Africa	–	
F5	*F. oxysporum*	*Solanum tuberosum*	Michigan, USA	–	
F-16-16	*F. phaseoli*	*G. max*	Montcalm County, MI, USA	–	
MI-Mtc-A12	*F. phaseoli*	*G. max*	Montcalm County, MI, USA	–	n.d.
22158	*F. phaseoli*	*P. vulgaris*	Rockville, MD, USA	–	
22276	*F. phaseoli*	*P. vulgaris*	Rockville, MD, USA	–	
MI-Mtc-A4	*F. phaseoli*	*G. max*	Montcalm County, MI, USA	–	
22411	*F. phaseoli*	*P. vulgaris*	California, USA	–	n.d.
36549	*F. proliferatum*	*Gladiolus sp.*	The Netherlands	–	
MI-Mtc-C3FS (Wht)	FSSC 11	*G. max*	Montcalm County, MI, USA	–	
MI-Mtc-A2FS	FSSC 11	*G. max*	Montcalm County, MI, USA	+	21.13
F-15-118	*F. solani* (FSSC 5)	*P. vulgaris*	Montcalm County, MI, USA	–	n.d.
MI-Mtc-C17FS	*F. solani* (FSSC 5)	*G. max*	Montcalm County, MI, USA	–	
MI-Mtc-B16FS	*F. solani* (FSSC 5)	*G. max*	Montcalm County, MI, USA	–	
MI-Mtc-B10	*F. solani* (FSSC 5)	*G. max*	Montcalm County, MI, USA	+	30.43
31777	*F. tucumaniae*	*G. max*	Vila Maria, Rio Grande do Sul, Brazil	–	n.d.
43334	*F. tucumaniae*	*G. max*	Armstrong, Sante Fe, Argentina	–	n.d.
31096	*F. tucumaniae*	Unknown	Argentina	–	
DB P28 R13	*F. virguliforme*	*P. vulgaris*	Van Buren County, MI, USA	–	n.d.
Vb-2a	*F. virguliforme*	*G. max*	Van Buren County, MI, USA	–	n.d.
F-14-77	*F. virguliforme*	*G. max*	Ingham County, MI, USA	–	
DB P27 R13	*F. virguliforme*	*P. vulgaris*	Van Buren County, MI, USA	–	
DB P30 R5	*F. virguliforme*	*P. vulgaris*	Van Buren County, MI, USA	–	
22292 (Mont-1)	*F. virguliforme*	*G. max*	Illinois, USA	–	
36897	*F. virguliforme*	*G. max*	Argentina	–	
54291	*F. virguliforme*	*G. max*	Argentina	–	
VB-1	*F. virguliforme*	*G. max*	Van Buren County, MI, USA	–	
	Rhizoctonia solani (AG2-2)	*G. max*		–	
	Rhizoctonia solani (AG4)	*G. max*		–	
	Phialophora gregata (genotype A)	*G. max*		–	
	Phialophora gregata	*G. max*		–	

(genotype B)		
Macrophomina phaseolina	*G. max*	–
Sclerotinia sclerotiorum	*G. max*	–

Table 2. Panel of isolates used to determine specificity of *F. brasiliense* qPCR assay. + indicates positive amplification, while – indicates no amplification. n.d. = not determined.
[a]Isolate IDs with only numbers were obtained from the USDA ARS Culture Collection (NRRL). All other isolates were obtained by J. Jacobs and Dr. Martin Chilvers.
[b]FSSC = *Fusarium solani* species complex. FSSC 5 is named *F. solani*, while FSSC 11 is an unnamed species within clade 3.

Isolate[a]	DNA Concentration[b]	Ct[c]	Mean Ct	Standard Error
F-14-42	1000000	17.20	17.22	0.099
F-15-158	1000000	16.82		
F-16-137	1000000	17.37		
NRRL 34938	1000000	17.50		
F-14-42	100000	20.50	20.49	0.075
F-15-158	100000	20.22		
F-16-137	100000	20.57		
NRRL 34938	100000	20.69		
F-14-42	10000	24.08	24.08	0.097
F-15-158	10000	23.72		
F-16-137	10000	24.33		
NRRL 34938	10000	24.17		
F-14-42	1000	27.65	27.66	0.079
F-15-158	1000	27.35		
F-16-137	1000	27.92		
NRRL 34938	1000	27.72		
F-14-42	100	30.96	31.18	0.139
F-15-158	100	30.86		
F-16-137	100	31.28		
NRRL 34938	100	31.62		
F-14-42	10	35.26	34.93	0.249
F-15-158	10	33.93		
F-16-137	10	35.36		
NRRL 34938	10	35.16		

Table 3. Serial dilutions for four *F. brasiliense* isolates used to determine assay sensitivity.
[a]Isolate 34938 was obtained from the USDA ARS Culture Collection (NRRL). All other isolates were obtained by J. Jacobs and Dr. Martin Chilvers.
[b]Concentration in femtograms of DNA per reaction
[c]Ct = cycle threshold, or cycle at which fluorescent signal crossed the detection threshold.

Location in MI	Type	Ct[a]	Quantity DNA detected[b]	Spores / 500 mg soil[c]
Clinton County	Unknown	–	–	–
	Spiked	21.26	57083	5000
Hillsdale County	Unknown	–	–	–
	Spiked	18.10	346132	5000
Lenawee County	Unknown	36.92	7	n.d.
	Spiked	20.14	108360	5000
Montcalm County	Unknown	32.03	137	n.d.
	Spiked	18.90	226150	5000
Ingham County	Unknown	34.13	39	n.d.
	Spiked	17.29	547582	5000
Saginaw County	Unknown	30.83	246	2804*
	Spiked	18.01	363252	5000
Sanilac County	Unknown	29.51	529	3052*
	Spiked	17.41	512226	5000
St. Joseph County	Unknown	32.95	74	n.d.
	Spiked	19.72	137118	5000
Van Buren County, Field 1	Unknown	–	–	–
	Spiked	19.90	123579	5000
Van Buren County, Field 2	Unknown	–	–	–
	Spiked	21.97	70167	5000

Table 4. Amplification of *F. brasiliense* from soils across Michigan. Values with an asterisk (*) are estimates, calculated as indicated in the materials and methods. Dashes indicate no detection, and n.d. indicates not determined since detection was below the limit of detection.
[a]Ct = cycle threshold, or cycle at which fluorescent signal crossed the detection threshold.
[b]Quantity in femtograms
[c]Estimated by taking solving for x, using the equation: 5000 spores * z = x spores * y, where 5000 is the number of spores spiked into each soil sample, z is the Ct of the spiked sample, and y is the Ct of the non-spiked sample.

F. virguliforme DNA added[a]	*F. brasiliense* DNA added[a]	*F. virguliforme* Ct[b]	*F. brasiliense* Ct[c]
1,000,000	0	17.35	–
100,000	0	20.95	–
10,000	0	24.46	–
1,000	0	28.10	–
100	0	31.53	–
0	1,000,000	–	17.57
0	100,000	–	21.02
0	10,000	–	24.17
0	1,000	–	26.60
0	100	–	31.80
1,000,000	1,000,000	17.49	18.86
10,000	1,000,000	24.74	17.26
10,000	100,000	24.52	20.73
100	100,000	33.34	20.87
1,000	10,000	28.02	23.61
100	1,000	31.82	27.00
0	0	–	–

Table 5. Amplification of serial dilutions of *F. brasiliense* and/or *F. virguliforme* DNA. Assays display specificity towards target species and can be distinguished and quantified in a multiplexed system. Dashes represent no detection.
[a]Quantity in femtograms
[b]Ct = cycle threshold, or cycle at which fluorescent signal crossed the detection threshold for *F. virgulforme*.
[c]Ct = cycle threshold, or cycle at which fluorescent signal crossed the detection threshold for *F. brasiliense*.