Characterization of soybean seedborne *Fusarium* spp. in the state of Kansas, USA.

by

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B.S., Federal University of Lavras, 2006
M.S., Federal University of Lavras, 2009

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Plant Pathology
College of Agriculture

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Manhattan, Kansas

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Abstract

Fusarium spp. are among the most important pathogen groups on soybeans. However, information regarding this genus on soybean seeds in the state of Kansas remains underexplored. Therefore, the goal of this study was to characterize the identity, frequency, and pathogenicity of soybean seedborne *Fusarium* spp. in the state of Kansas. For the identification and frequency of seedborne *Fusarium* spp., culture-dependent (i.e. semi-selective medium) and -independent (i.e. DNA metabarcoding) approaches were used. Also, information regarding the pathogenicity of the most common seedborne *Fusarium* spp. from soybeans was assessed to better understand their role as soybean pathogens. Overall, eleven *Fusarium* spp. were identified in this study. Semi-selective media showed that approximately 33% of soybean seed samples were infected with *Fusarium* spp. Moreover, *Fusarium* spp. were isolated from seed sampled from 80% of the locations in Kansas. Furthermore, a low incidence of *Fusarium* spp. was observed within infected seed samples and averaged 2%. Nine *Fusarium* spp. were found in soybean seeds using the culture-dependent approach. *Fusarium semitectum* was the most frequent, followed by *F. proliferatum* and *F. verticillioides*. *Fusarium acuminatum*, *F. equiseti*, *F. fujikuroi*, *F. graminearum*, *F. oxysporum*, and *F. thapsinum* were found in lower frequencies among naturally infected seeds. DNA metabarcoding experiments showed that *Fusarium* spp. are more frequent in soybean seeds than previously known. All asymptomatic soybean seeds analyzed, using Illumina MiSeq platform, showed the presence of the genus *Fusarium* including two pathogenic species, *F. proliferatum* and *F. thapsinum*. *Fusarium acuminatum*, *F. merismoides*, *F. solani*, *F. semitectum*, and *Fusarium* sp. were also identified using the culture-independent approach. Preliminary results also showed that *F. proliferatum* and *F. thapsinum* were observed in all three major soybean seed tissues: seed coat, cotyledons, and the embryo axis. Depending on the

soybean genotype, inoculum potential and aggressiveness, *F. proliferatum*, *F. graminearum*, *F. fujikuroi*, *F. oxysporum*, *F. semitectum*, *F. thapsinum*, and *F. verticillioides* were pathogenic to soybean and negatively affect soybean seed quality, at different levels, in controlled conditions. Moreover, *F. equiseti* and *F. acuminatum* did not cause significant damage to soybean seeds and seedlings. Understanding seedborne *Fusarium* spp. and their influence on soybean seed and seedling diseases is critical for the development of effective disease control strategies, especially regarding early detection of pathogenic strains in seeds (i.e., seed health testing), ensuring the crop productivity, quality, and safety.

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Approved by:

Major Professor
Christopher R. Little

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“Ask, and you will receive; seek, and you will find; knock, and the door will be opened to you.”

Matthew 7:7

Dedication

This dissertation is dedicated to my beloved parents Sergio Donizete Pedrozo and Luzia Aparecida Bombo Pedrozo, my sister Bianca Pedrozo and my loving wife Gretchen Grace Pedrozo.

Chapter 1 - Introduction

Seeds are of immense importance for society. They represent the basic agricultural unit to ensure a sustained and improved food supply. Conversely, seed also provides an effective means of spreading plant diseases as numerous pathogens, especially fungi, are seed-transmitted. The movement of plant pathogens through infected seeds is a major concern for seed certification and quarantine programs, and represents an important challenge facing modern agriculture due to its potential to introduce exotic plant diseases into new hosts and areas. Hence, the seed and its movement are subjected to regulations and must undergo seed health testing for the presence or absence of seedborne pathogens to minimize the risk of spreading unwanted diseases among states, regions, countries, and continents.

For soybeans (*Glycine max* (L.) Merr.), the genus *Fusarium* represents one of the most important pathogen groups causing diseases including Fusarium wilt, caused by *F. oxysporum*; sudden death syndrome, caused by *F. virguliforme* in North America; and seed, seedling and root diseases caused by several *Fusarium* species. At the same time, some other species, such as *F. semitectum* (*F.* fc. *incarnatum*) and *F. equiseti*, are known to be saprophytes or endophytes and may not play an important role in any soybean disease process. Furthermore, most of the agriculturally important *Fusarium* spp. have frequently been reported in soybean seeds in North America and other parts of the world. Also, although it is not within the scope of this study, it is important to emphasize that *Fusarium* spp. can also produce mycotoxins, which represent a health risk to humans and livestock.

Overall, although the genus *Fusarium* represents a potential threat to soybean seed production as well as food, feed quality, and safety, many questions regarding the significance and influence of this important pathogenic group remains underexplored. For example, clear experiments are needed to answer the questions of impacts of *Fusarium* spp. on soybean seed and seedling diseases and how widespread seedborne pathogenic *Fusarium* spp. are. Thus, the correct identification and frequency of soybean seedborne *Fusarium* spp. and information regarding their pathogenicity to the crop is important for the development of effective disease control management strategies, improvements in seed certification and quarantine programs, and as a basis for making decisions to protect the future of agriculture.

Chapter 2 - Literature review

The role of seedborne pathogens in agriculture

Seeds are the basic material used for maintenance of ecosystems as well for agricultural practices. It is estimated that about 90% of the world's crops are sown by true seeds (Agarwal and Sinclair 1996; Neergaard 1979). Due to the significance of agriculture to modern society, using high-quality seeds from improved and adapted varieties is a fundamental element for agricultural productivity (Harman 1982; Neergaard 1986). Hence, improvements and development of new technologies that ensure the quality of seeds are essential for the future of agriculture and the survival of humankind.

Seedborne pathogens can affect seed quality and cause diseases that significantly impact yield or marketability of seed lots (Machado et al. 2002; Mathur and Kongsdal 2003; Van Gastel et al. 2002). By definition, seedborne pathogens are any infectious agent associated with seeds that have the potential to cause seed, seedling, and plant diseases (Agarwal and Sinclair 1996). Plant pathogenic bacteria, fungi, nematodes, and viruses occur with seed either as contaminants adhering to the seed surface, loosely mixed with seed or as an infection present inside the seed tissues (Neergaard 1979).

Most seed diseases and diseases related to seedborne pathogens of the major crops are caused by fungi. Numerous species of fungi are associated and have been reported in crop seeds (Agarwal and Sinclair 1996). Among those, many species are endophytes or saprophytes that do not adversely affect the performance of seeds or affect the health of the plant and do not affect

production and quality (Machado et al. 2002). On the other hand, several seedborne fungi have been reported to cause severe economic losses and represent a major threat to food production (Neergaard 1979). For example, losses due to Karnal bunt of wheat, incited by *Tilletia indica* (syn. *Neovossia indica*) in northwestern Mexico are estimated to average $7.5 million per year, in which the direct yield and quality losses account for 42.6% of the total (Brennan et al. 1992). Yield losses of 100% due to wheat loose smut, caused by *Ustilago tritici* (Pers.) Rostr., were reported in Georgia (Persons 1954). Rice blast, caused by *Magnaporthe oryzae*, was responsible for a famine in Japan during the 1930s and represents a US$ 55 million problem in South and Southeast Asia (Anderson et al. 2004).

Reduced germination by seed rot and increased post-emergent damping-off are common losses related to seedborne pathogens, which commonly affect the marketability of seed (McDonald 1998; McGee 1980). For example, soybean seedling emergence was reduced by 30% by *Cercospora kikuchii* (Singh and Agarwal 1986) and 59% by *Macrophomina phaseolina* (Gangopadhyay et al. 1971) depending on the level of infection observed in seeds. Barley and wheat seeds infected with *Bipolaris sorokiniana* did not germinate, or if they germinated, seedlings became infected (Al-Sadi and Deadman, 2010). However, reduced germination due to seedborne pathogens is complex and depends on many factors such as host genotype, environmental conditions, and the type, amount, and location of inoculum within the seeds (Agarwal and Sinclair 1996; Machado et al. 2013; Neergaard 1979). Due to this complex interaction, the relationships between most plant pathogenic fungi and seed production is still not well understood.

Roy et al. (2001) have reported that at least 63 genera and approximately 108 or more species occur in soybean seeds in North America. Although many of the soybean seedborne fungi are considered transients and are reported only sporadically in seed, accurate information regarding the widespread distribution of most soybean seedborne fungi is still lacking. Under normal conditions, species of *Alternaria*, *Cercospora*, *Fusarium*, and *Phomopsis* are the fungi most consistently and frequently isolated from seeds (Roy et al. 2001).

In the United States, seed and seedling diseases of soybean are a common and significant problem. Wrather and Koenning (2009) reported that seed and seedling diseases represent one of the most problematic diseases encountered in 28 soybean-growing states, including Kansas, during the 1998 to 2007 growing seasons. In 2007 for example, suppression of soybean yields caused by seed and seedling diseases reached 34,985,000 bushels followed by suppressions caused by soybean cyst nematode (SCN), which represented a decrease of yield in a magnitude of 93,981,000 bushels (Wrather and Koenning 2009). Several different pathogens cause soybean seed and seedling diseases. These include *Fusarium*, *Rhizoctonia*, *Macrophomina*, *Cercospora*, *Phomopsis*, *Phytophthora*, and *Pythium* and tend to be the most common fungal and oomycete genera in the Midwestern U.S. (Wrather and Koenning 2009). Most importantly, these pathogenic organisms may survive for an extended period in the soil and are often associated with plant debris or alternative hosts (Broders et al. 2007; French-Monar et al. 2006; Kmetz et al. 1979; Leslie et al. 2004; Payne and Waldron 1983; Ritchie et al. 2013; Singh et al. 1990). Those pathogens that persist in field soils or in alternative hosts are the most dangerous for introduction into new areas. Once established, it is difficult or virtually impossible to eliminate them

especially in the case of soil-infesting pathogens. The wider the host range of the pathogen, the more difficult eradication becomes as a cultural management strategy (Baker and Smith 1966).

The movement of plant pathogens through seeds

Due to the significant increase of international food trade, the risks of introducing exotic pathogens into new areas through infected or contaminated plant materials represent a major concern to agriculture (Stack et al. 2014; Strange et al. 2005). It is estimated that introduced pathogens are responsible for about 65% of U.S. crop losses, representing a cost of $137 billion annually (Fletcher et al. 2006). In Florida, citrus canker, caused by *Xanthomonas axonopodis* pv. *citri*, is suspected to be introduced via infected fruits. This pathogen has resulted in tremendous yield losses, which represent more than a US$ 200 million cost to citrus producers in the United States (Graham et al. 2004).

Another common means of spreading diseases are seeds. Seeds represent an efficient mechanism of spreading diseases among states, countries, and continents. Many plant pathogens can be asymptomatic in seed lots, which makes their detection extremely challenging (McGee 1995). For this reason, many countries have formulated legislation that helps to minimize or prevent the introduction of exotic pathogens or strains into new areas through infected or contaminated seeds and promote the improvement of seed certification as well as quarantine programs (Munkvold 2009).

The close association of plant pathogens with seeds facilitates their long-term survival and widespread dissemination (Mancini et al. 2016). For example, some fungi can survive

unfavorable conditions, normally encountered in dry seeds, by producing dormant mycelium or spores, such as chlamydospores (Neergaard 1979). Several studies have reported the incredible longevity that some plant pathogenic fungi can have in cereal seeds (Agarwal and Sinclair 1996). For instance, *Cochliobolus sativus* (formally known as *Helminthosporium sativum*) in barley seed was found to be alive after seven years (Machacek and Wallace 1952). *Fusarium verticillioides* (formally known as *F. moniliforme*) was isolated from maize seeds after 7 to 8 years of storage (Dungan and Koehler 1944).

The introduction of new pathogens into new areas may be disastrous to U.S. agriculture, and it may result in significant economic losses due to a ban placed on seed imports (Madden and Wheelis 2003). Depending on the pathogen and under favorable conditions, a few infected seeds may serve as a source of inoculum for the establishment of new epidemics (Neergaard 1979; Shaw and Osborne 2011). For example, as few as two cabbage seeds infected by *Xanthomonas campestris* pv. *campestris* per 10,000 are enough to cause an epidemic of black rot (Schaad et al. 1980). In beans, under favorable conditions, as little as 0.5% seed infection by *X c.* pv. *phaseoli* and 0.02% of *Pseudomonas syringae* pv. *phaseolicola* can produce epidemics caused by these pathogens (Walker et al. 1964; Wallen et al. 1965). Knowledge regarding seed infection and transmission rates is a crucial factor that affects the epidemiology and the effective control or management of diseases caused by seedborne pathogens (Agarwal and Sinclair 1996). Interestingly, for many soybean seedborne pathogenic fungi, especially *Fusarium* spp., only information on their seedborne nature is available, but infection and spread rates remain unknown.

Seed health testing

Infections resulting from diseased seeds are best avoided by protection (i.e. using treated seeds) or by exclusion (i.e. using pathogen-free seeds). At present, large quantities of seeds are routinely treated with chemical agents, especially fungicides (Tinivella et al. 2009). However, certain limitations (e.g. pathogen resistence to active ingredients) and environmental disadvantages (e.g. soil contamination), which have been associated with the use of chemicals as well as the uncertainties about the future availability of fungicides, call for the development of alternative methods for seedborne pathogen management (Gullino and Kuijpers 1994). Since the introduction of site-specific fungicides in the late 1960s, fungicide resistance in phytopathogenic fungi has become a major problem in crop protection (Ma and Michailides 2005). For example, no suppression of Fusarium head blight (FHB) and deoxynivalenol (DON) was observed in plants inoculated with the tebuconazole-resistant isolate following application of a commercial rate of tebuconazole when compared with sensitive isolates (Spolti et al. 2014).

Because of that, it has being suggested that the essential attribute to successfully control seed and seedling diseases is to plant seeds as free as possible of seedborne pathogens. Hence, accurate early diagnosis through seed health testing is the goal to manage seedborne pathogens and prevent their unwanted introduction and spread into new areas (McGee 1995). Therefore, the main objective of seed health testing is to accurately identify and quantify the presence of seedborne pathogens in seed (Machado et al. 2002). Ideally, seed health testing methods should be cheap, quick, reproducible, specific, and sensitive (Walcott 2003).

Depending on the pathogen, level of infection, and degree of damage, some fungal pathogens can be easily detected based on incubation methods followed by visual examinations (Mathur and Kongsdal 2003). For example, discoloration, shriveling, cracks, and the presence of fungal reproductive structures on soybean seeds are typical of *Phomospsis longicolla* and *Cercospora kikuchii*, and they can be commonly observed using these methods (Figure 1). Blotter test and agar plate method (i.e. semi-selective medium) are the two most common approaches used for identification of seedborne pathogens in commercial seed lots (Mathur and Kongsdal 2003). Overall, incubation methods and visual examinations are cheap, reproducible and easy to interpret. However, they are not specific and sensitive due to the difficulties in distinguishing relevant species based on morphological characters alone (i.e. *Fusarium* spp.) and considering that some fungi are not cultured in artificial media (Geiser et al. 2004; Mancini et al. 2016)

In general, detecting seedborne pathogens is a challenging task due to their complex interaction with the host (Mancini et al. 2016, Munkvold 2003; Walcott 2003). For example, latent or asymptomatic (cryptic) seedborne pathogens impose great challenges for the detection of contaminated seed lots at ports of entry and may explain the unintentional introduction of disease-causing microorganisms (Pimentel et al. 2000; Sinclair 1991). In addition to asymptomatic seeds, the pathogen amount of inoculum may be unevenly distributed in a seed lot, which could result in false negatives. For example, recently a wheat blast outbreak in Bangladesh has been reported, and it is suspected that the unwanted introduction of this pathogen was likely due to undetected infected wheat seeds from Brazil (Saharan et al. 2016). Moreover, seed health testing methods should guard against false positives as well. The presence of dead pathogens and

non-pathogenic strains are typical examples of false positives commonly encountered in seed lots (Agarwal and Sinclair 1996).

Among the tools available for plant pathogen detection, DNA-based techniques are widely recognized as one of the most useful and efficient. Diagnostic methods based on DNA significantly improved after the introduction of the polymerase chain reaction (PCR) in the mid-1980s (Narayanasamy 2011). DNA-based detection techniques that rely on PCR are quick, specific, and highly sensitive. However, proper implementation of these techniques poses further challenges that range from the high cost of the technology to the need to train personnel to interpret the results (Walcott 2003). Common PCR-based applications for seed health testing methods include conventional PCR, Bio-PCR, nested PCR, real-time PCR, magnetic capture hybridization PCR, and loop-mediated isothermal amplification (Mancini et al. 2016, Munkvold 2003; Walcott 2003).

Recently, next-generation sequencing (NGS) has gained more attention in the field of seed pathology, regarding its application and implications for seed health testing (Mancini et al. 2016). NGS is a relatively recent technology that allows for the generation of large amounts of sequence data from a given sample (Mardis 2008). The combination of NGS technology and metagenomics, i.e. DNA metabarcoding, offers new insights and many advantages for plant disease diagnosis including seed health testing (Coissac et al. 2012). For example, the screen for the presence of pathogen genomic fragments is captured by a genomic overview of everything in the sample; thus the identification of pathogen genomic material obtained from metabarcoding the microbiome would result in confirmation of a pathogen being present (Adams et al. 2009).

This methodology has been applied to several types of environmental samples including seawater, bilge water, marines, intestinal tracts of various animals, and contaminated water sources (Berg et al. 2014; Mardis 2008). Furthermore, DNA metabarcoding technique has been applied to plant disease diagnostics as a means to search for unknown pathogens such as plant viruses, bacteria, and fungi (Adams et al. 2009; Turner 2013). Although this method is not yet used for seed diagnostics, due to its enormous potential and increased availability, it will likely be applied for the detection of plant pathogens in seed lots in the near future (Mancini et al. 2016).

The soybean host

One of the most important cash crops in the world is soybean (*Glycine max* (L.) Merrill) due to its wide range of geographical adaptation, unique chemical composition, nutritional value, functional health benefits, and industrial applications (Masuda and Goldsmith 2009). The protein content in soybean seed is approximately 40%, and the oil content is around 20%. In fact, soybean represents the highest protein content and gross output of vegetable oil among the cultivated crops, providing around 60% of vegetable protein and 30% of the total vegetable oil production in the world (Medic et al. 2014). Additionally, soybean also improves soil fertility by adding nitrogen from the atmosphere. Biological nitrogen fixation (BNF) is one of the major features of soybean that makes it an attractive crop (Herridge et al. 2008). Because of the association of soybean with *Bradyrhizobium* in the root nodules, the soybean crop requires low nitrogen supplies (Herridge et al. 2001). Although the quantum of fixed nitrogen varies with the environment and soils conditions, agronomic practices, and genotype, for instance, it is estimated

that 50-60% of the nitrogen demand of soybean crop is met by BNF (Salvagiotti et al. 2008). This is a major benefit to exhausted soils considering that fertilizer availability is limited or are too expensive for many farmers around the globe, especially in undeveloped countries (Wilson 2008).

Because of its versatility, importance, and production potential, soybean is also known as the golden grain of the globe (Singh 2010). The use of soybean has increased in human nutrition and health, edible oil, livestock feed, biofuel, and other industrial and pharmaceutical applications (Chiu et al. 2004; Hammond and Vicini 1996). As a result, its production has increased more than ten times since the first decade of the 20th century, from 22 million tons to 313 million tons respectively (USDA website - http://www.globalsoybeanproduction.com/).

The first cultivation of soybean dated to China >5000 years ago (Singh 2010). Soybean was introduced into the United States in 1765 (Hymowitz and Harla 1983). However, its popularity and large-scale production began in the early 20th century due to the high demand for vegetable oil, especially during the two world wars (1914-1918 and 1939-1945). Afterward, the area planted to soybeans has expanded rapidly. Its success in the United States led to its introduction to South America, especially Argentina and Brazil. The United States is the country with the highest production (118.7 million tons), followed by Brazil (102 million tons), Argentina (57 million tons), China (12.5 million tons), India (9.7 million tons), Paraguay (9.1 million tons) and Canada (6 million tons). Last year production (2016/2017), reached 336.9 million tons, which represented an increase of 7.3% globally (USDA website - http://www.globalsoybeanproduction.com/).

The significant increase in production is the result of intensive and consistent breeding programs around the globe. Breeders continue to seek high-yielding genetic stocks to increase production, wider adaptation, and nutritional value as well as increased resistance to biotic and abiotic stresses (Kolhe and Hussian 2009; Nelson 2009). Therefore, germplasm, which includes primitive cultivars, landraces, and closely related wild species, genetic stocks, inbred and hybrids are the base of the crop improvement programs necessary to meet current and future demand (Singh 2010). It is estimated that around 147,000 (Kolhe and Hussian 2009) or 170,000 (Nelson 2009) soybean accessions, with some accessions duplicated, exist worldwide. In North America, extensive soybean collecting started in the 1920s, but systematic preservation did not occur until the United States Department of Agriculture (USDA) Soybean Collection was established in 1949 (Carter et al. 2004). Song et al. (2015) have recently reported that the USDA soybean germplasm collection contains approximately 19,700 soybean accessions.

Soybean crops have the potential to exhibit better productivity and quality in the coming years with provision of research back-up, technology transfer, and policy support from governments (Rao 2004). Among the major aspects to be improved on soybean crop to achieve higher productivity and quality, pest resistance, especially fungi and oomycetes, is one of the most imperatives (Rubiales et al. 2015; Yuan et al. 2002).

Soybean seedborne *Fusarium* spp.

Several pathogenic fungi are responsible for seed and seedling diseases in soybean. Among them, *Phomopsis*, *Fusarium*, *Rhizoctonia*, *Pythium*, and *Phytophthora* are the most common causal agent of seed and seedling diseases in growing states the United States. To date, only soybean varieties presenting complete or partial resistance to *Phomopsis*, *Rhizoctonia*, *Pythium*, and *Phytophthora* have been reported (Bates et al. 2008; Bradley et al. 2005; Dorrance et al. 2003; Jackson et al. 2005; Smith et al. 2008). Regarding *Fusarium* spp., although no resistant commercial variety is available, recent studies have shown that some varieties contain alleles conferring resistance to *F. graminearum* (Acharya et al. 2015; Ellis et al. 2012; Zhang et al. 2010).

Fusarium is a cosmopolitan genus of filamentous ascomycete fungi that represents a vast array of agronomically important plant pathogens (Geiser et al. 2013). Fusarium wilts or blights, seed, seedling, stem and root rots are typical diseases that cause economic losses in cash crops, horticultural, ornamental, and forest industries worldwide (Leslie and Summerell 2006). In fact, recently *Fusarium* was listed among the top 10 most important plant pathogenic fungi of crops (Dean et al. 2012). Native forest and grassland plants have also been reported as a natural reservoir of this important plant pathogenic group (Leslie et al. 2004; Leslie and Summerell 2006; McMullen and Stack 1988; Windels and Kommedahl 1974). Additionally, this genus has the potential to produce mycotoxins, such as deoxynivalenol (DON) and T-2 toxin (T-2)), zearalenone (ZEN) and fumonisin B1 (FB1) which can contaminate agricultural products resulting in depreciation of food and feed as well as harm to humans and livestock (Antonissen et al. 2014; Ma et al. 2013).

On soybeans, *Fusarium* can cause several diseases such as sudden death syndrome (SDS), caused by *F. virguliforme* in North America, and Fusarium blight or wilt, root and pod rot caused by several species (Table 2.1). Additionally, although the nature of *Fusarium* spp. as seedborne pathogens are not entirely understood and explored, several species have been reported to have the potential to cause seed and seedling diseases (Table 2.1). In North America, at least 14 *Fusarium* spp. have reported in soybean seeds (Miller and Roy 1982; Roy et al. 2001) including *F. acuminatum*, *F. avenaceum, F. culmorum*, *F. decemcellulare*, *F. equiseti*, *F. graminearum, F. oxysporum*, *F. proliferatum*, *F. semitectum* (*F.* fc. *incarnatum*), *F. solani, F. sporotrichioides*, *F. subglutinans*, *F. verticillioides*, and *F. tricinctum*. In general, the frequency of seedborne pathogens among and within seed samples may vary depending on geographical location, genotype, and agricultural practices (Harman 1982). For all of the seedborne *Fusarium* spp. known on soybean, accurate information regarding the frequency distribution of *Fusarium* spp. among and within soybean seed samples is lacking.

The most studied pathogenic soybean seedborne *Fusarium* spp. is *F. graminearum* followed by *F. oxysporum* (Barros et al. 2014; Broders et al. 2007; Ellis et al. 2011; Ellis et al. 2014; Schlub et al. 1981). As observed in several studies, *F. graminearum* and *Fusarium oxysporum* can impact soybean seed quality by decreasing seed germination and vigor as well as causing seedling damping-off and root rots (Barros et al., 2014; Broders et al. 2007; Ellis et al. 2011; Ellis et al. 2014; Hartman et al. 1999; Martinelli et al, 2004; McGee et al. 1980; Pioli et al., 2004).

F. graminearum Schwabe has been identified as a primary pathogen of soybean causing seed rot and seedling damping-off in North and South America (Barros et al, 2014; Broders et al.2007; Díaz Arias et al. 2013; Ellis et al., 2012; Martinelli et al. 2004; Pioli et al. 2004 Xue et al. 2007). In the United States, the first reported occurrence of infection of soybean seed by *F. graminearum* was recordered in the Midwestern in 1986 (Hartman et al. 1999). In comparison to other *Fusarium* species, *F.graminearum* was found to be highly aggressive in causing severe seed, seedling and root rots in soybean by several studies (Broders et al. 2007; Díaz Arias et al. 2013; Ellis et al. 2011; Jacobsen et al. 1995; Zhang et al. 2010). In the state of Ohio for example, *F. graminearum* was isolated from symptomatic soybeans seedlings collected in the field (Broders et al. 2007). In other surveys conducted in Iowa and Eastern Ontario, *F. graminearum* was also the most frequently recovered species of *Fusarium* in fields (Díaz Arias et al. 2013; Zhang et al. 2010). In artificially inoculated seeds and seedlings, symptoms of seed rot and seedling damping-off caused by this pathogen appear first as water-soaked lesions followed by light brown or pink discoloration around the inoculation point (Broders et al. 2007; Ellis et al. 2011; Xue et al. 2007). In South America, infections also observed to occur at pod filling, and symptoms include spreading of discoloration vertically on the stem, interveinal chlorosis of leaves leading to plant wilting, pod blight and death (Martinelli et al. 2004; Pioli et al. 2004).

Fusarium oxyporum Schlechtend emend. Snyder & Hansen has been traditionally associated with Fusarium wilt or blight in soybeans (Hartman et al. 1999). However, seed, seedling, root, and pod rots are also diseases caused by this species (Anwar et al. 1995; Shovan et al. 2008). Economic losses up to 59% resulting from wilt, 64% from root rot, and 50% from

reduced pod formation have been reported. Also, infected seeds can reduce germination up to 40% in the field (Hartman et al. 1999). In controlled conditions and depending upon the aggressiveness of the isolate, mortality of soybean seedlings by *F. oxysporum* reached 80% (Arias et al. 2013). Interestingly, Arias et al. (2013) also reported that some *F. oxysporum* were observed to be non-pathogenic to soybean. Interspecific variation in pathogenicity within *Fusarium* spp., especially regarding *F. oxysporum*, is well documented and isolates may range from highly aggressive to nonpathogenic (Leslie and Summerell 2006). Therefore, the presence of *Fusarium* spp. in a soybean seed lot does not always correspond to the development of seed and seedling diseases (Pawuk 1978; Graham and Linderman 1983; Axelrood et al., 1995).

Seeds heavily infected by *F. oxysporum* and *F. graminearum* appear shrunken, irregular in shape, often cracked and presenting salmon or pink to reddish discoloration in the seed coat (Agarwal and Sinclair 1996). Similar symptoms are also observed in soybean seeds infected by other *Fusarium* spp., such as *F. proliferatum*, *F. semitectum*, and *F. verticillioides* (Figure 2.3). However, depending on other factors, such as amount and position of inoculum, seed infected by not only these two pathogens, but other *Fusarium* spp. may be asymptomatic (Figure 2.4). In fact, the taxonomic array of plant species that host cryptic (asymptomatic) infection by *F. oxysporum* suggest that this mode of existence may be normal in plants (Stergiopoulos and Gordon 2014). Consistent with this view, *F. graminearum* have been recovered from asymptomatic soybean plants including seeds (Clear et al. 1989; Osorio and McGee 1992; Russo et al. 2016). Although not much is known regarding the relationship between asymptomatic soybean seeds and crop production, quality, and safety, several *Fusarium* spp., are commonly carried asymptomatically in seed lots.

Besides *F. graminearum* and *F. oxysporum*, limited information is known regarding the pathogenicity of other *Fusarium* spp. in soybean seeds. Understanding what seeds carry in and on them, especially regarding to pathogenic fungal strains, is a crucial first step towards significant improvement of seedborne pathogens control strategies. Therefore, preventive actions such as accurate early diagnosis, through specific and sensitive seed health testing, are the goal to effectively manage pathogenic seedborne *Fusarium* spp. and prevent their unwanted introduction and spread into new areas.

In the state of Kansas limited information is available regarding this important pathogenic seedborne genus. Until recently, besides the presence of the genus *Fusarium* in soybean seeds, no characterization of the isolates at the species level, their frequency in seeds or pathogenicity has been reported (Habermehl 1964; Jardine 1991).

Objectives

The goal of this research was to characterize the identity, frequency, and pathogenicity of soybean seedborne *Fusarium* spp. in the state of Kansas. To accomplish that, this study was divided into three major parts, with the following main objectives:

i) Use culture-dependent approach (i.e. semi-selective medium) for identification and frequency of seedborne *Fusarium* spp of soybean in the state of Kansas, USA

and evaluate their potential to decrease seed germination and vigor under laboratory and greenhouse conditions (Chapter 3; Appendix A; Appendix B);

ii) Use culture-independent approach in combination with next-generation sequencing (i.e. DNA metabarcoding) to identify and better understand the frequency distribution of pathogenic *Fusarium* spp. among and within soybean seed samples (Chapter 4; Appendix C);

iii) Understand the influence of inoculum potential of pathogenic seedborne *Fusarium* spp. on soybean seed quality (Chapter 5; Appendix D).

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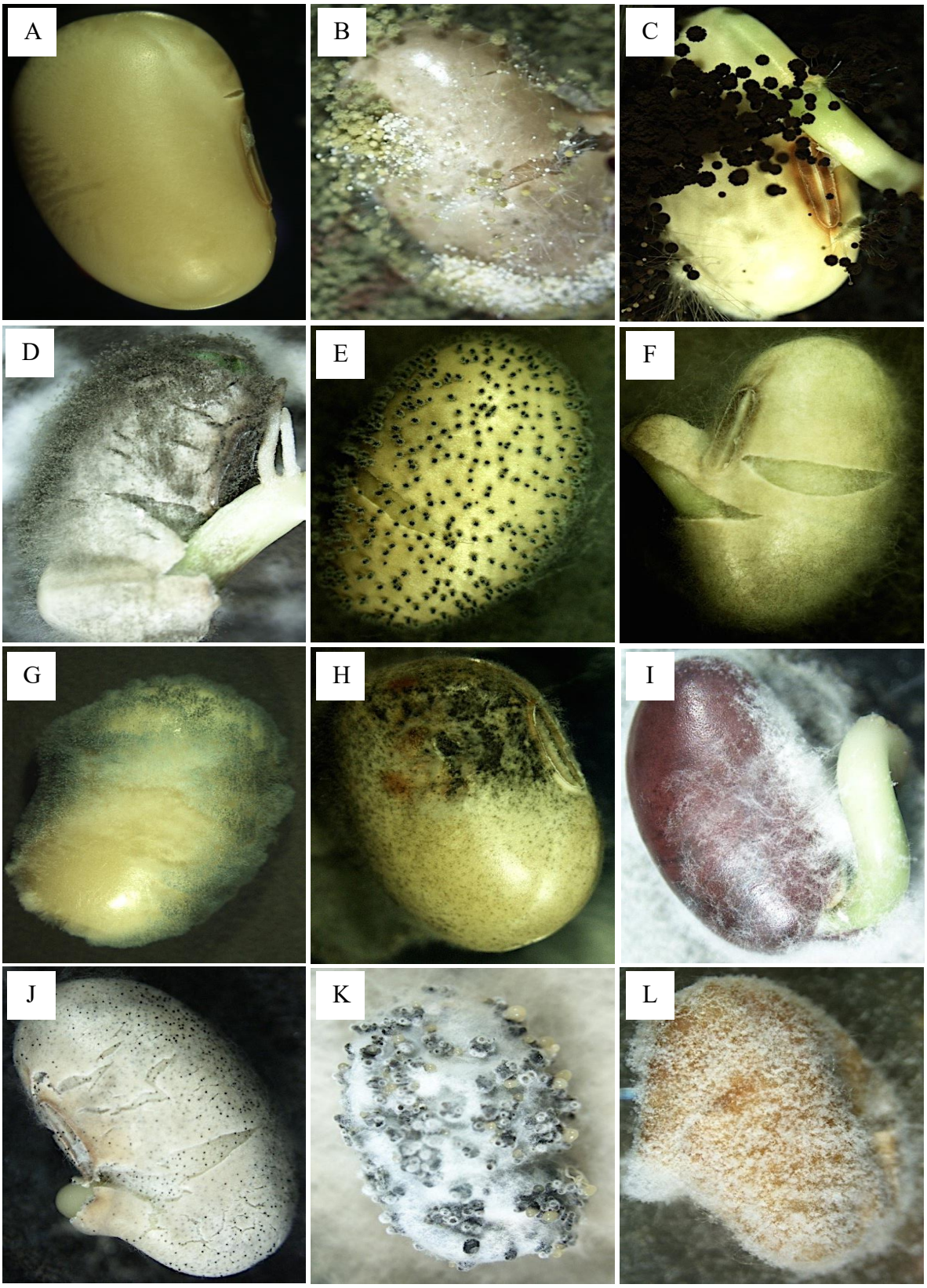
A
B
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Figure 2.1 Examples of soybean seedborne fungi after seed incubation in a moisture chamber after 7 days. Clean soybean seeds (A); *Aspergillus flavus* (C); *Aspergillus niger* (C); *Cladosporium* sp. (D); *Chaetomium* sp. (E); *Alernaria alternata* (F); *Penicillium* sp. (G); *Cercospora sojina* (H); *Cercospora kikuchii* (I); *Macrophomina phaseolina* (J); *Phomopsis longicolla* (K); *Fusarium* sp. (L).

Figure 2.2 Symptoms of *Fusarium proliferatum* (A), *F. semitectum* (B), and *F. verticillioides* (C) on artificially inoculated soybean seeds.

Table 2.1. Diseases caused by seedborne *Fusarium* spp. in soybean.

Seedborne *Fusarium* spp.	**Diseases on soybean**	**Reference**
F. acuminatum	Root rot	Arias et al. 2013
F. avenaceum	Seedling and root rot	Chang et al. 2015
F. culmorum	Seedling and root rot	Chang et al. 2015
F. equiseti	Root rot	Arias et al. 2013
F. graminearum	Seed, seedling and root rot	Arias et al. 2013
F. merismoides	Root rot	Anwar et al. 2013
F. oxysporum	Seed, seedling, root rot and wilting	Chang et al. 2015
F. proliferatum	Root rot	Chang et al. 2015
F. semitectum	Root rot	Arias et al. 2013
F. solani	Seed, seedling and root rot	Arias et al. 2013
F. sporotrichioides	Root rot	Arias et al. 2013
F. subglutinans	Root rot	Anwar et al. 2013
F. verticillioides	Seed and seedling rot	Broders et al. 2007
F. tricinctum	Root rot	Zhang et al. 2010

Chapter 3 - Identification, frequency, and pathogenicity of *Fusarium* spp. in soybean seeds in the state of Kansas.

Abstract

Although *Fusarium* spp. are one of the most important pathogen groups on soybeans, their identity and frequency in seeds as well as their importance as seedborne pathogens remain unclear. The objectives of this work were to characterize: *i)* the identity and frequency of *Fusarium* spp. present within 408 soybean seed samples in the state of Kansas during three growing seasons 2010, 2011, and 2012; and *ii*) to test the pathogenicity of the most commonly encountered seedborne *Fusarium* spp. on soybean seeds and seedlings under growth chamber and greenhouse conditions using artificial inoculation. A semi-selective medium (PCNB) was used for *Fusarium* isolation. Identification was based on morphological characters and PCR. The influence of *Fusarium* spp. on soybean seed germination and vigor was assessed by pathogenicity assays in laboratory and greenhouse. The three-year screening effort showed that 33% of the seed samples contained *Fusarium* spp. at some level. Nine *Fusarium* species were identified among the infected seed samples. *Fusarium semitectum* was the most frequently encountered species followed by *F. proliferatum, F. verticillioides, F. acuminatum, F. equiseti, F. thapsinum, F. fujikuroi, F. oxysporum,* and *F. graminearum*. Regarding pathogenicity, only soybean seeds artificially inoculated with *F. proliferatum, F. graminearum, F. fujikuroi, F. oxysporum,* and *F. thapsinum* significantly decreased seed germination ($p > 0.001$) and vigor ($p > 0.001$) as compared with mock-inoculated control. No significant reductions in seed quality were observed for seeds artificially inoculated with *F. semitectum, F. verticillioides, F. acuminatum,* or *F. equiseti*. Understanding the relationship between pathogenic *Fusarium* spp.

and soybean seeds will improve seed health testing methods, and ensure crop security, quality, and production.

Introduction

Fusarium spp. are among the most important pathogens on soybeans (*Glycine max* (L.) Merr.) causing diseases including Fusarium wilt, caused by *F. oxysporum*; sudden death syndrome, caused by *F. virguliforme* in North America; and root rots and seedling disease caused by several *Fusarium* spp. (Arias et al. 2013; Arias et al. 2011; Barros et al, 2014; Jordan et al. 1988; Osorio and McGeer 1992). Furthermore, *Fusarium* spp. have frequently been reported in soybean seeds (Hartman et al. 1999; McGee et al. 1980; Roy et al. 2001). However, although *Fusarium* is pathogenic to soybean, limited information is available regarding the significance and influence of this important genus on seed quality.

Seeds infected with pathogenic fungi can decrease seed germination and vigor, resulting in reduced seed quality (Agarwal et al. 1996; Munkvold 2009; McGee 1981; Pedrozo and Little 2014; Pedrozo et al. 2015). Moreover, infected and infested seeds may provide primary inoculum for the establishment of pathogens into new crops and hosts (Agarwal et al. 1996; Hartman et al. 1999; Neergaard 1977). Therefore, preventive actions such as accurate seed health testing methods are required and necessary to protect agricultural production, food quality, and safety (Machado et al. 2002; McGee 1995; Munkvold 2009).

Accurate diagnosis of seedborne pathogens is challenging (Machado et al. 2002; Munkvold 2009; Walcott 2003). For example, not all *Fusarium* spp. are known to be pathogenic

to soybean (Hartman et al. 1999; Leslie and Summerell 2006; McGee et al. 1980). Moreover, inoculum may be unevenly distributed within a seed or in a seed lot, which could result in false negatives due to sampling error or the use of non-sensitive diagnostic methods. Therefore, accurate identification and knowledge regarding the frequency distribution of *Fusarium* spp. among and within seed samples are essential for the accurate characterization of seedborne isolates and improvements on seed health methods (Leslie and Summerell 2006).

In North America, at least fourteen species have been observed in soybean seeds (Roy et al. 2001). Among these, the two most studied are *F. oxysporum* and *F. graminearum* (Barros et al., 2014; Broders et al. 2007; Ellis et al. 2011; Ellis et al. 2014). *Fusarium oxysporum* and *F. graminearum* have the potential to impact soybean seed quality by decreasing seed germination and vigor (Barros et al. 2014; Broders et al. 2007; Ellis et al. 2011; Ellis et al. 2014; Hartman et al. 1999; Martinelli et al. 2004; McGee et al. 1980; Pioli et al. 2004). For example, in controlled conditions and depending on the aggressiveness of the isolates, mortality of soybean seedlings by *F. oxysporum* and *F. graminearum* reached 80% (Arias et al. 2013) and 40% (Broders et al. 2007), respectively. However, besides these two species, little information is known regarding the pathogenicity of other soybean seedborne *Fusarium* species. Understanding what seeds carry in and on them, especially regarding pathogenic strains, is a crucial first step towards significant improvements of seed health testing methods.

In Kansas, limited information is known regarding this important pathogenic seedborne genus. No characterization of soybean seedborne *Fusarium* isolates at the species level, their frequency, and pathogenicity has been reported (Habermehl 1964; Jardine 1991). Thus, the

objectives of this study were to characterize: *i)* the identity and frequency of *Fusarium* spp. present within 408 soybean seed samples in the state of Kansas during three growing seasons 2010, 2011, and 2012; and *ii*) to test the pathogenicity of the most commonly encountered seedborne *Fusarium* spp. on soybean seeds and seedlings under growth chamber and greenhouse conditions using artificially inoculated seeds.

Materials and Methods

Soybean seed samples

Fusarium spp. were isolated from soybean seed samples obtained from eleven Kansas counties during the 2010, 2011, and 2012 growing seasons (Figure 1). A total of 408 soybean samples were analyzed over the three-year survey. Not all locations were sampled equally during the three years and the number of samples from each field and between years varied. In 2010, a total of 21 samples from nine counties were collected, 114 samples from six counties were collected in 2011, and 266 samples from 10 counties were collected in 2012 (Table 1). Seed samples were stored at 4°C in the Department of Plant Pathology at Kansas State University, Manhattan, Kansas, USA.

Isolation and morphological identification of seedborne *Fusarium* spp.

To avoid contaminants and to promote the isolation of internal *Fusarium* spp., seeds were surface-sterilized with a 1% sodium hypochlorite solution (v/v) for 10 min. Seeds were rinsed with sterile-distilled water and dried overnight at room temperature. One hundred arbitrarily selected seeds from each soybean sample were plated on Nash-Snyder medium, a semi-selective

medium for *Fusarium* spp. previously described by Leslie and Summerell (2006), and incubated at 23 ± 2°C for seven days (Leslie and Summerell 2006; Mathur et al. 2003). After incubation, plates were examined and colonies visually identified as *Fusarium* were single-spored by micromanipulation as described by Leslie and Sumerrell (2006) and then transferred to carnation leaf agar (CLA) and potato dextrose agar (PDA) for further morphological evaluation. CLA and PDA were used for species differentiation regarding colony pigmentation and morphology. CLA was used to characterize micromorphological features such as macroconidia, mesoconidia, and microconidia as well as conidial arrangement (shape, size, and formation), conidiogenous cell formation (mono- or polyphialides), and the formation and arrangement of chlamydospores (Leslie and Sumerrell, 2006). Isolates were grown on CLA for five days, and conidia were dislodged from the plate using 2 ml of a 10% glycerol solution to prepare single-spore cultures for long-term storage. The resulting conidial suspension and segments of medium containing mycelium ("blocks of medium") were removed from the plate, transferred to a 2 ml Eppendorf tube, and stored at -80°C. The entire collection was deposited and accessed in the *Fusarium* collection in the Department of Plant Pathology at Kansas State University (Table 3.2).

Molecular identification of seedborne *Fusarium* spp.

For molecular identification, the translation elongation factor 1-alpha region of the mitochondrial DNA of the isolates was sequenced using the TEF1 (forward: 5'-ATGGGTAAGGAGGACAAGAC-3') and TEF2 (reverse: 5'-GGAAGTACCAGTGATCAT GTT-3') primers (O'Donnell et al. 1998). To extract genomic DNA, mycelia was grown in nutrient broth (Difco™ Nutrient Broth, BD Diagnostics, Sparks, MD, USA) and incubated on a shaker at 124 rpm for four days at room temperature (~23°C). After vacuum filtration, mycelium

from each isolate was ground in a mortar to a fine powder with liquid nitrogen. Approximately 50 mg of the lyophilized mycelia was used for DNA extraction. A Master Pure^{TM} Yeast DNA purification kit (Biocentre, Madison, WI, U.S.A.) was used for DNA extraction following the manufacturer's instructions. After extraction, the DNA concentration was measured using a spectrophotometer (Nanodrop ND-1000, Thermo Scientific, Wilmington, DE, USA) and adjustments were made to achieve a final concentration of 20 ng DNA/µL per sample. PCR reactions consisted of 2 µL of the template DNA (40 ng DNA), 2 µL of each primer (TEF1 and TEF2; 5 pmol μL^{-1}), 2 µL of each deoxynucleotide triphosphate (dNTP, 2 mmol), 2 µL of 10x KCl with $MgCl_2$, 0.1 µL Taq DNA polymerase (Bioline USA Inc., Taunton, MA, USA), and 14.9 µL of sddH_2O resulting in a final volume of 25 µL. The PCR program consisted of an initial denaturation at 94°C for 1 min, then 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min. The final primer extension reaction consisted of incubation at 72°C for 7 min. As a result, a ~700 bp product was amplified. A negative PCR control in which templates were replaced with ddH_2O was used. PCR amplicons were mixed with one µL of loading dye (QIAGEN-GelPilot loading dye) and separated by electrophoresis in a 1.5% agarose gel in TB buffer at 70 V for 45 min. The PCR products were purified using the QIAquick after amplification. PCR purification kit (QIAGEN Inc., Valencia, CA, USA). TEF primers were used to sequence amplicons in both directions at the Kansas State University Sequencing and Genotyping Facility, Department of Plant Pathology, Manhattan, KS, USA. The sequences were edited using Bioedit software version 7.0.5.3 (Hall 1999) and blasted against the FUSARIUM-ID/FUNCBS (http://www.fusariumdb.org/) and The National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) databases.

Rolled-towel pathogenicity assay

The rolled-towel assay was used to evaluate the pathogenicity of all 69 soybean seedborne *Fusarium* isolates from 2010 and 2011 and their influence on soybean seed quality, herein measured by germination. The soybean variety used for this test was Asgrow 'AG3039' (SDS moderate susceptible) (Monsanto, Inc.; St. Louis, MO, USA). Prior to inoculation, seeds were surface disinfested with a 5% bleach solution (0.5% sodium hypochlorite v/v) for 1 min. Then seeds were dried overnight at room temperature. Physiological (% of normal germination) and phytosanitary (presence or absence of seedborne pathogens) parameters of the seeds were also evaluated before inoculation. As a result, ninety-four percent germination, and zero incidences of seedborne pathogens, especially *Fusarium*, were observed (*data not shown*). For inoculations, seeds were imbibed in a 25 ml conidial suspension for 1 min at 2.5×10^5 conidia ml^{-1}. Twenty-five artificially inoculated seeds were placed on two moistened sheets of germination paper (Anchor Paper Co., St. Paul, MN, USA). An additional sheet of moistened germination paper was placed over the inoculated seeds, the layers were rolled into a tube, secured by a rubber band, set upright in a plastic Rubbermaid® Cereal Keeper container (Newell Rubbermaid Co., Atlanta, GA, USA) and incubated in a growth chamber (Power Scientific Inc., St. Louis, MO, USA) at 25°C for seven days. During the growth chamber experiment, temperature and humidity within the plastic containers were measured using a data logger (MicroDAO Ltd, Contoocook, NH, USA) and averaged 25.4°C and 88.3%, respectively. For each *Fusarium* isolate, four rolled-towels were used, which corresponded to four replicates. After seven days, the pathogenicity and influence of *Fusarium* spp. on artificially inoculated soybean seeds was assessed by disease severity index (DSI), by percentage of normal germination (healthy seedlings), abnormal germination (symptomatic seedlings), dead seeds

(non-germinated seeds), and fresh seedling weight (g). DSI was calculated based on the rated seedlings, using a scale of 0 to 3 where: 0 = germinated seeds and healthy and normal seedlings with no symptoms (e.g., discoloration) on the primary and/or secondary roots or hypocotyl (A); 1 = seed germinates, and abnormal seedling shows minor discoloration and reduction on the primary and/or secondary roots as well as hypocotyl (B); 2 = seed germinates, and abnormal seedling shows heavy discoloration and reduction on the primary and/or secondary roots. Also, the hypocotyl is heavily discolored and girdled by the lesion (C); 3 = non-germinated seed (Figure 3.2). DSI was calculated based on the formula: DSI = ((A*0) + (B*1) + (C*2) + (D*3)) / Nt, where "A", "B", "C", and "D" are the number of seedlings presenting disease severity scores 0, 1, 2, and 3, respectively; and Nt = total number of seeds tested. Isolates having pathogenicity scores < 1, between 1 and 2, and > 2 were considered low, moderately, and highly aggressive, respectively.

Greenhouse pathogenicity assay

After screening the *Fusarium* isolates from 2010 and 2011 for pathogenicity and their influence on seed germination under laboratory conditions using the rolled-towel assay, one representative isolate from each species was used for greenhouse trials including *F. semitectum* (23565), *F. equiseti* (23567), *F. acuminatum* (23598), *F. verticillioides* (23625), *F. fujikuroi* (23560), *F. proliferatum* (23559), *F. thapsinum* (23623), *F. oxysporum* (23578), and *F. graminearum* (23577). The influence of *Fusarium* spp. on seed vigor, which reflects the ability of inoculated seeds to produce normal seedlings under less than optimum growing conditions like those that may occur in the field, were measured by the initial stand (IS), final stand (FS), and fresh aerial weight (FAW). The values for the IS and FS were measured at 10 and 25 days

after sowing, respectively, and the absolute value recorded at these two periods was converted in percentage. Values for FAW were obtained 25 days after sowing by weighing all fresh aerial mass of soybean plants from each pot in a semi-analytical balance. Results were expressed in grams. Seeds were artificially inoculated as previously described for the rolled-towel assay experiments. After inoculation, twenty-five seeds from each treatment were planted in 500 ml pots with autoclaved soil (reading silt clay loam) and vermiculite using 1:1 ratio. Mock-inoculated seeds (imbibed in sterile-distilled water) were used as the control.

Data analysis

The frequency of infected soybean samples (prevalence by samples; P_s) and locations presenting infected samples by *Fusarium* spp. (prevalence by location; P_l) were calculated based on the formulas: *i)* P_s = (Number of soybean samples having a *Fusarium* spp. / Total number of samples analyzed)*100; *ii*) P_l = (Number of soybean samples having a *Fusarium* spp. / Total number of locations presenting infected seeds)*100. Also, the percentage of infected seeds among infected samples (Incidence; In) was calculated based on (the number of isolates found in an infected sample) / (the total number of seed present in a sample) * 100. All variables were reported statewide for the overall data set. For the pathogenicity assays, analysis of variance was conducted using PROC MIXED of SAS (Version 9.3, SAS Institute). The influence among species or isolates of *Fusarium* spp. on soybean seed quality was measured using inoculated treatments (seeds artificially inoculated with seedborne *Fusarium* isolates) compared with the mock-inoculated control using Dunnett's test for both laboratory and greenhouse experiments. Treatments were significantly different at $p \leq 0.05$. Disease severity index (DSI) was used to measure the aggressiveness of pathogenic seedborne *Fusarium* species and isolates in laboratory

assays. Species/isolates having aggressiveness scores < 1, between 1 and 2, and > 2 were considered low, moderate, and highly aggressive, respectively.The experimental design used for the growth chamber and greenhouse pathogenicity tests was a completely randomized design. Each experiment was repeated three times. In addition, the seed quality variables measured in laboratory and greenhouse from inoculated seeds with seedborne *Fusarium* isolates were correlated using the package CORRPLOT of R (Version 0.98.987).

Results

Identification and frequency of soybean seedborne *Fusarium* spp.

During the three-year survey, 266 seedborne *Fusarium* isolates were collected and identified. One hundred thirty-nine isolates were identified based upon morphology alone and 127 isolates were identified based on morphology and PCR (Table 3.2). All species that were characterized based on morphological features showed considerable variation (Figure 3.1). *F. proliferatum, F. thapsinum,* and *F. semitectum* (*F.* fc. *incarnatum*) showed the most variable pigmentation range on PDA among seedborne isolates, ranging from light to dark violet, colorless to dark yellow, and light to dark brown, respectively (*data not shown*). A total of nine *Fusarium* spp. were identified within soybean seeds samples including *F. acuminatum*, *F. equiseti*, *F. fujikuroi*, *F. graminearum*, *F. oxysporum*, *F. proliferatum*, *F. semitectum*, *F. thapsinum,* and *F. verticillioides* (Table 3.3). The total number of *Fusarium* spp. from each year varied and increased as sample size increased (Table 3.3). Overall, *F. semitectum* (154 isolates) was the most frequently isolated species followed by *F. proliferatum* (44 isolates), *F. verticillioides* (38 isolates), *F. equiseti*, (7 isolates), *F. acuminatum* (7 isolates), *F. fujikuroi* (6

isolates), *F. thapsinum* (6 isolates), *F. oxysporum* (3 isolates), and *F. graminearum* (3 isolates) (Table 3.3).

Due to their agronomic importance and confusion regarding their correct identification, all *G. fujikuroi* species complex isolates, including *F. proliferatum*, *F. verticillioides*, *F. fujikuroi*, and *F. thapsinum* were identified based on DNA sequencing using *Fusarium*-specific translation elongation factor 1-α (TEF1, forward; TEF2, reverse) primers (Table 3.2). However, initial identification was based on morphological characteristics and was used to sort the species into smaller groups before molecular confirmation. Additionally, all *F. oxysporum* and *F. graminearum* isolates and some isolates of *F. acuminatum*, *F. equiseti*, and *F. semitectum* were identified by PCR regardless of their morphological identification. From the 127 isolates that required molecular confirmation, a ~700 bp band was amplified, and a BLAST search for similarity using the *Fusarium*-ID and the NCBI databases showed an identity of the isolates ranging from 98 to 100% (Geiser et al. 2004). Additionally, two members of the *F. incarnatum-equiseti* species complex (FIESC) showed lower identity (94%) with strains deposited in the *Fusarium*-ID. However, when blasted against the NCBI database, the identity was 100% (Table 3.2).

Thirty-three percent of the samples analyzed during the three-year survey contained *Fusarium* spp. at some level. Within infected samples, on average, 2% of the seeds contained *Fusarium* species. *F. semitectum* was the most frequently identified species among the infected samples in all three years (2010, 2011, and 2012) followed by *F. proliferatum*, *F. verticillioides*, and *F. equiseti* (Table 3.4). *F. acuminatum* and *F. thapsinum* were only identified from seed

samples acquired in 2011 and 2012 whereas *F. fujikuroi* was identified only from infected samples collected in 2010 and 2012 (Table 3.4). *F. oxysporum* and *F. graminearum* were identified only in 2010 samples (Table 3.4). Furthermore, approximately 80% of all locations sampled during the survey presented at least one soybean sample infected with *Fusarium* spp. during the three-year survey. The same trend was observed among species and *F. proliferatum*, *F. semitectum*, and *F. verticillioides* were present in most of the locations sampled statewide (Table 3.4). The rest of the species were present in <40% of the locations sampled during the three-year survey (Table 3.4).

Rolled-towel assay

Among the nine soybean seedborne *Fusarium* spp. found in this study, five species, including *F. fujikuroi*, *F. graminearum*, *F. oxysporum*, *F. proliferatum*, and *F. thapsinum* were identified as pathogenic to soybean (Table 3.5). The aggressiveness of pathogenic species and their influence on seed quality was measured by the disease severity index (DSI). *Fusarium graminearum* produced the highest DSI and was considered the most pathogenic species (Table 3.5). *Fusarium oxysporum*, *F. proliferatum*, *F. fujikuroi*, and *F. thapsinum* were identified as moderately aggressive (Table 3.5). *Fusarium acuminatum*, *F. equiseti*, *F. semitectum*, and *F. verticillioides* were classified as non-pathogenic (low aggressiveness) and were not able to significantly decrease normal soybean seed germination and fresh seedling weight when compared with mock-inoculated control (Table 3.5). Furthermore, no significant increase in the percentage of abnormal seed germination and dead seeds was observed with seed inoculated with *F. acuminatum*, *F. equiseti*, *F. semitectum*, and *F. verticillioides* (Table 3.5).

All pathogenic soybean seedborne *Fusarium* species significantly reduced normal soybean seed germination (healthy seedlings) of artificially inoculated seeds when compared with mock-inoculated control (Table 3.5). Reduced normal germination resulted from a significant increase in the percentage of abnormal germination (symptomatic seedlings) and an increase of dead seeds (non-germinated seeds) observed in artificially inoculated seeds when compared with mock-inoculated control (Table 3.5). Except for *F. thapsinum*, pathogenic soybean seedborne *Fusarium* species reduced the fresh weight of germinated seedlings when compared with mock-inoculated control (Table 3.5).

When DSI was analyzed within species, the *F. proliferatum* isolates presented the most variability regarding their pathogenicity. One isolate (23619) was identified as non-pathogenic (low aggressiveness), twelve isolates were identified as moderately aggressive (23559, 23592, 23603, 23605, 23606, 23608, 23612, 23613, 23615, 23618, 23620, 23621), and two isolates exhibited high aggressiveness (23602 and 23614) (Table 3.6). As expected, only moderately or highly aggressive *F. proliferatum* isolates reduced soybean seed quality by reducing seed germination and fresh seedling weight when compared to the mock-inoculated control (Table 3.6). *Fusarium oxysporum* showed similar behavior regarding pathogenicity and all three isolates were identified as pathogenic to soybean (Table 3.6). One *F. oxysporum* isolate (23578) was classified as highly aggressive whereas two isolates were identified as moderately aggressive (Table 3.6). None of the *F. acuminatum*, *F. equiseti*, *F. semitectum*, or *F. verticillioides* isolates reduced soybean seed germination and seedling fresh weight except for one isolate of *F. semitectum* (23574), which significantly reduced soybean fresh weight (Table 3.6).

Greenhouse assay

Seeds inoculated with moderately and highly aggressive isolates showed significant reduction of seed vigor, which resulted in a significant decrease of final stand of soybean plants when compared with mock-inoculated control (Table 3.7). Overall, there was a significant correlation between the aggressiveness of the isolates tested in laboratory conditions and the parameters tested in greenhouse assays (Figure 3.3). The vigor of the seeds artificially inoculated with *Fusarium* spp., measured by initial and final stand as well as fresh plant aerial weight, decreases as the DSI of the seedborne isolates increases.

Interestingly, only *F. graminearum* (23577), *F. proliferatum* (23559), *F. thapsinum* (23623), and *F. fujikuroi* (23560), but not *F. oxysporum* (23578), exhibited reduced seedling germination after ten days post-planting (initial stand) when compared with mock-inoculated control (Table 3.7). Post-emergent damping-off symptoms were observed among all the pathogenic *Fusarium* species (Figure 3.4).

Although the pathogenic *Fusarium* isolates tested reduced seed vigor in a significant manner, only isolates classified as highly pathogenic (aggressive) significantly reduced fresh aerial weight ($p < 0.001$) of inoculated soybean plants when compared to the mock-inoculated control (Table 3.7). Isolates with low pathogenicity (aggressiveness), such as *F. semitectum* (23565), *F. equiseti* (23567), *F. acuminatum* (23598), and *F. verticillioides* (23625) were not different from mock-inoculated seeds for any of the variables tested in the greenhouse (Table 3.7). This information confirms the results from laboratory assays regarding the limited potential of non-pathogenic (low aggressive) isolates to decrease seed germination.

Discussion

Infected and infested seeds may decrease seed quality as well as provide primary inoculum for the establishment of pathogens in new hosts and areas (Agarwal et al. 1996; Hartman et al. 1999; Neergaard 1977). The movement of plant pathogens through infected seeds is a major concern and represents an important challenge facing modern agriculture as seeds may travel around the globe (Munkvold 2009). Seedborne pathogens are difficult to control. Thus, preventive actions such as using sensitive and specific seed health testing methods are required and necessary to protect agricultural production, quality, and safety (Machado et al. 2002; McGee 1995; Munkvold 2009). Understanding what, and how much of a plant pathogen is carried in and on seed is crucial information and represents the first step toward significant improvements in seed health testing methods.

Overall, approximately 33% of soybean seed samples were infected with *Fusarium* spp. *Fusarium* spp. were isolated from seed sampled from 80% of the locations in Kansas. Furthermore, a low incidence of *Fusarium* spp. was observed within infected seeds and averaged 2%. Similar results were also observed in previous studies in Kansas where only 3 to 5% of soybean seeds were infected by *Fusarium* spp. (Habermehl 1964; Jardine 1991). Nine different species were present in soybean seeds in this study. Among these, *F. acuminatum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. proliferatum*, *F. semitectum*, and *F. verticillioides* have been reported previously by other authors in North America and in other parts of the world (Baird et al. 2001; Impullitti et al. 2013; Ivić et al. 2009; Jordan et al. 1988; Medić-Pap et al. 2007; Roy et

al. 2001). *Fusarium fujikuroi* and *F. thapsinum* were only recently reported in soybean seeds (Pedrozo & Little 2014; Pedrozo et al. 2015) (Appendix A & B). Among the species observed in this study, *F. semitectum* was the most frequent species found, followed by *F. proliferatum* and *F. verticillioides*. *Fusarium semitectum* is known to be a weak or a non-pathogenic species to the crop (Hartman et al. 1999; Leslie and Summerell 2006). The pathogenicity results from *F. semitectum*, from both laboratory and greenhouse studies, further confirm this information. *Fusarium semitectum* was not able to significantly decrease germination and vigor of soybean seeds.

The second most frequently identified seedborne species in this study was *F. proliferatum*. Although this species has recently gathered more attention due to its potential to cause soybean seed, seedling, and root rots on soybeans, limited information regarding its significance to soybean production is available (Arias et al. 2013; Arias et al. 2011, Pedrozo and Little 2016). The results obtained in this study further confirm that *F. proliferatum* has the potential to infect soybean seed and cause a significant reduction in the seed and seedling quality of artificially inoculated seeds. Although at a low incidence (~2%), most of the locations in this study presented seeds infected by this pathogenic species, which suggest that *F. proliferatum* is a pathogenic species to expect in soybean seed lots. Interestingly, *F. proliferatum* also showed the highest variability regarding its aggressiveness to soybean. Most of the *F. proliferatum* isolates reduced seed quality of artificially inoculated soybean seeds at some level, whereas yet one isolate was not able to cause any symptoms in seeds or seedlings. This variability in aggressiveness among *F. proliferatum* isolates is expected, and it was reported recently (Arias et al. 2013). Among four *F. proliferatum* isolates tested, Arias et al. (2013) reported that three

significantly increased seedling mortality (%), whereas one isolate did not reduce the emergence of soybean seedlings in greenhouse assays. These results suggest that current seed health testing methods should be improved to not only detect the presence or absence of seedborne pathogens but also to measure the potential variability in aggressiveness of pathogenic isolates.

Another frequently isolated species in this study was *F. verticillioides*. Although this species has never been recovered from soybean seeds in the state of Kansas, *F. verticillioides* was found in seeds from other geographical locations worldwide (Garcia et al. 2012; Ivić et al. 2009; Zelaya et al. 2013). Although *F. verticillioides* has the potential to decrease seed germination and vigor in maize (Munkvold et al. 1997), its influence on soybean seed quality is not well understood. In this study, the germination and emergence of soybean seedlings artificially inoculated by this pathogen were not significantly affected in laboratory and greenhouse assays. These results may be explained by the lack of sufficient inoculum present in the seeds.

Generally, besides other factors, such as the aggressiveness of the isolates and the susceptibility of the host, the amount of inoculum within a seed, i.e. “inoculum potential”, are most likely to significantly influence and cause damage to the seeds, seedlings, or adult plants (Agarwal et al. 1996; Neergard, 1979). In a recent study, Pedrozo & Little (2016) showed that the potential of *F. verticillioides* to decrease soybean seed quality is dependent upon the inoculum potential present in the seeds. Seeds inoculated with low inoculum potential of *F. verticillioides* did not influence seed germination, whereas seeds inoculated with high inoculum potential significantly reduced seed germination (Pedrozo & Little 2016). Although this

information is essential to the development of efficient disease management control strategies, the minimum amount of inoculum for a pathogenic species necessary to cause seed and seedling diseases in the field (inoculum threshold) is still unknown for *F. verticillioides* and other seedborne *Fusarium* spp. on soybeans. This is an area that deserves further attention. Because of increased international seed movement and the need for reasonable phytosanitary requirements, the need to establish minimum inoculum thresholds for seedborne pathogens is apparent (Agarwal and Sinclair 1996; Machado et al. 2002; McGee 1995; Munkvold 2009; Neergaard, 1979).

Fusarium oxysporum and *F. graminearum* are among the most studied seedborne species in soybean (Barros et al., 2014; Broders et al., 2007; Diaz et al., 2013; Ellis et al., 2011; Martinelli et al., 2004; Pioli et al., 2004; Xue et al., 2007). Both are well known seedborne species with the potential to impact soybean seed quality, especially on seeds that have experienced pre- or post-harvest damage (Agarwal et al. 1996; Barros et al. 2014; Neergaard 1979). *Fusarium graminearum* is a well-known pathogen of cereal crops worldwide (Barros et al. 2014; Broders et al., 2007; Martinelli et al., 2004; Pioli et al., 2004). In Kansas, *F. graminearum* causes head blight in wheat and stalk and ear rot of corn, which results in loss of seed and grain quality (Broders et al., 2007; Ellis et al., 2011). To date, *F. graminearum* is recognized as a primary pathogen of soybean in Argentina, Brazil, and the United States (Barros et al., 2014; Broders et al., 2007; Diaz et al., 2013; Ellis et al., 2011). In this study, *F. graminearum* was identified as highly aggressive to soybean seeds and seedling and reduced germination and vigor by 27% and 14%, respectively. Drastic reduction of soybean seed germination by this pathogen, ranging from 20 to 50%, was also observed by other authors

(Barros et al. 2014; Broders et al., 2007). In a similar way, a significant reduction of soybean seed quality was also observed in this study by *F. oxysporum*. The same behavior is also known for *F. oxysporum* where a significant decrease of seed germination and emergence of seedling was observed by other authors (Arias et al. 2013). Reduction of soybean seedling emergence was reported by Arias et al. (2013) and reached 80% in greenhouse assays. Also, in this study, both *F. oxysporum* and *F. graminearum* were observed to infect soybean seed at a low frequency, among and within samples.

In this study, two other pathogenic species found in low frequency, among and within samples, were *F. thapsinum* and *F. fujikuroi* (Pedrozo & Little 2014; Pedrozo et al. 2015). *Fusarium thapsinum* and *F. fujikuroi* were recently reported in soybean seeds in the United States (Pedrozo & Little 2014; Pedrozo et al. 2015). In sorghum and rice, they are well-known pathogens and can cause stalk rots as well as grain mold and Bakanae disease, respectively (Leslie et al. 2004; Leslie and Summerell 2006; Little et al. 2011; Pedrozo & Little 2014; Pedrozo et al. 2015; Tesso et al. 201, Suga et al. 2014). Soybean seedborne *F. fujikuroi* significantly reduced rice seed germination, promoted post-emergent damping off, and cause internode elongation, which is a typical Bakanae disease symptom (Pedrozo et al. 2015).

Fusarium acuminatum and *F. equiseti* were found less frequently in infected soybean seeds. They were identified as non-pathogenic under laboratory and greenhouse conditions. *Fusarium equiseti* is known to be endophytic and does not play a significant role in soybean seed and seedling disease development (Leslie and Summerell 2006; Park et al. 1999; Summerell et al. 2010). As previously mentioned, the inoculum potential present in the seeds plays an

important role and can influence seed and seedling disease development. However, even seeds inoculated with a high amount of *F. equiseti* inoculum did not significantly decrease soybean seed vigor (Pedrozo & Little 2014). *Fusarium acuminatum* was not pathogenic to soybean seeds or seedlings in this study. However, *F. acuminatum* has shown some potential to cause seedling damping-off and root rots in artificially inoculated plants in other studies suggesting that this species could be detrimental to soybean seed at higher inoculum levels (Arias et al. 2013). Thus, is it becomes evident that further investigation should be considered regarding this seedborne fungus to better understand its significance on soybean seed quality.

In summary, this study has shown that soybean seeds are commonly infected by both pathogenic and non-pathogenic *Fusarium* species. Nine *Fusarium* spp. were identified in naturally infected soybean seed in the state of Kansas. On average, low frequency of *Fusarium* spp. was observed among and within soybean seed samples. Moreover, the aggressiveness of pathogenic seedborne *Fusarium* spp. varied significantly among and within species. Hence, the collection of soybean seedborne *Fusarium* spp. yielded may contribute to advances on the development of more sensitive and specific seed health-testing methods, specifically designed to accurately detect pathogenic *Fusarium* isolates in commercial soybean seed lots, as well as helping breeders to develop resistant varieties against this important group of plant pathogens. To the best of our knowledge, this study provides the first complete documentation regarding the characterization of soybean seedborne *Fusarium* spp. in the state of Kansas, USA.

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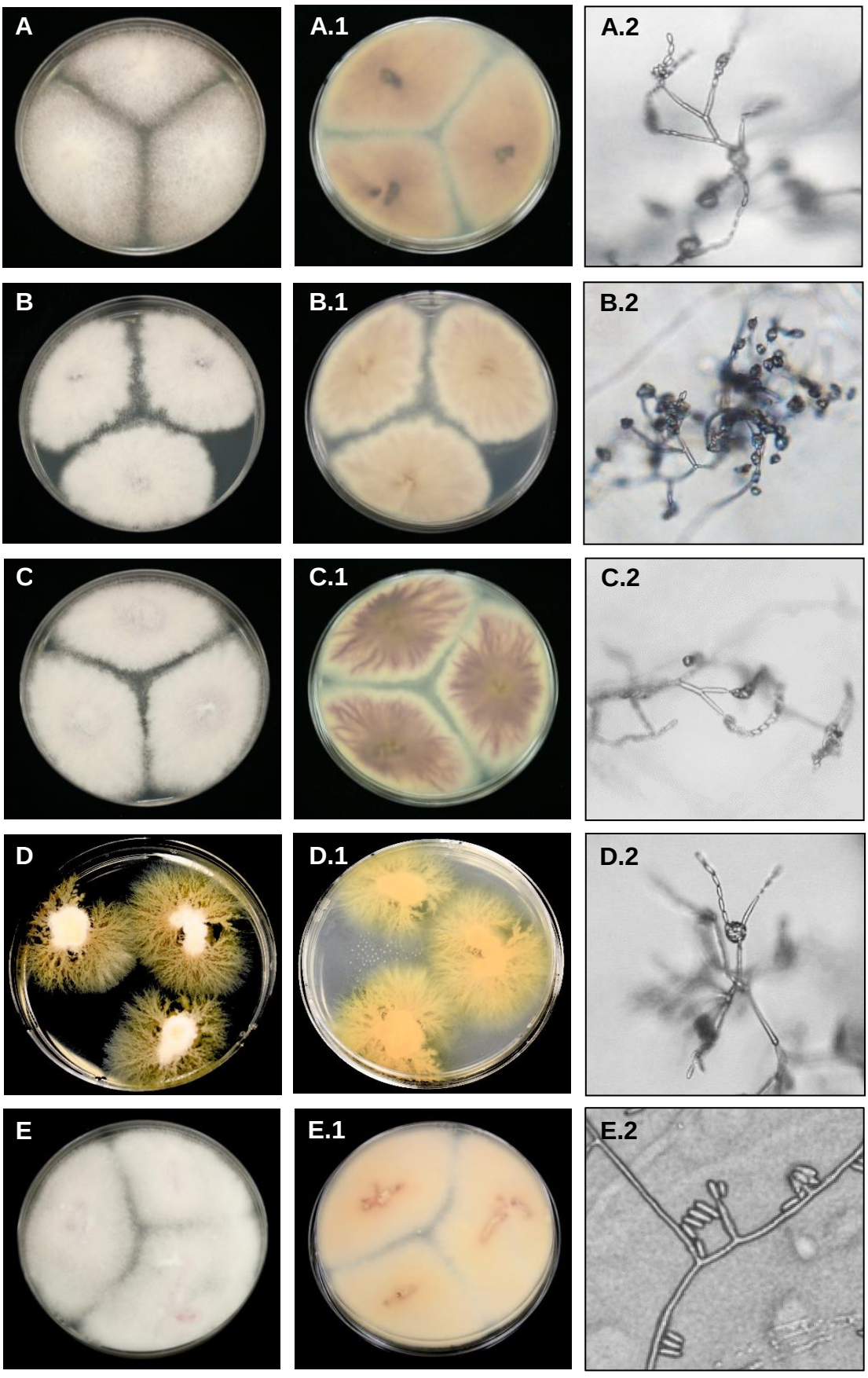
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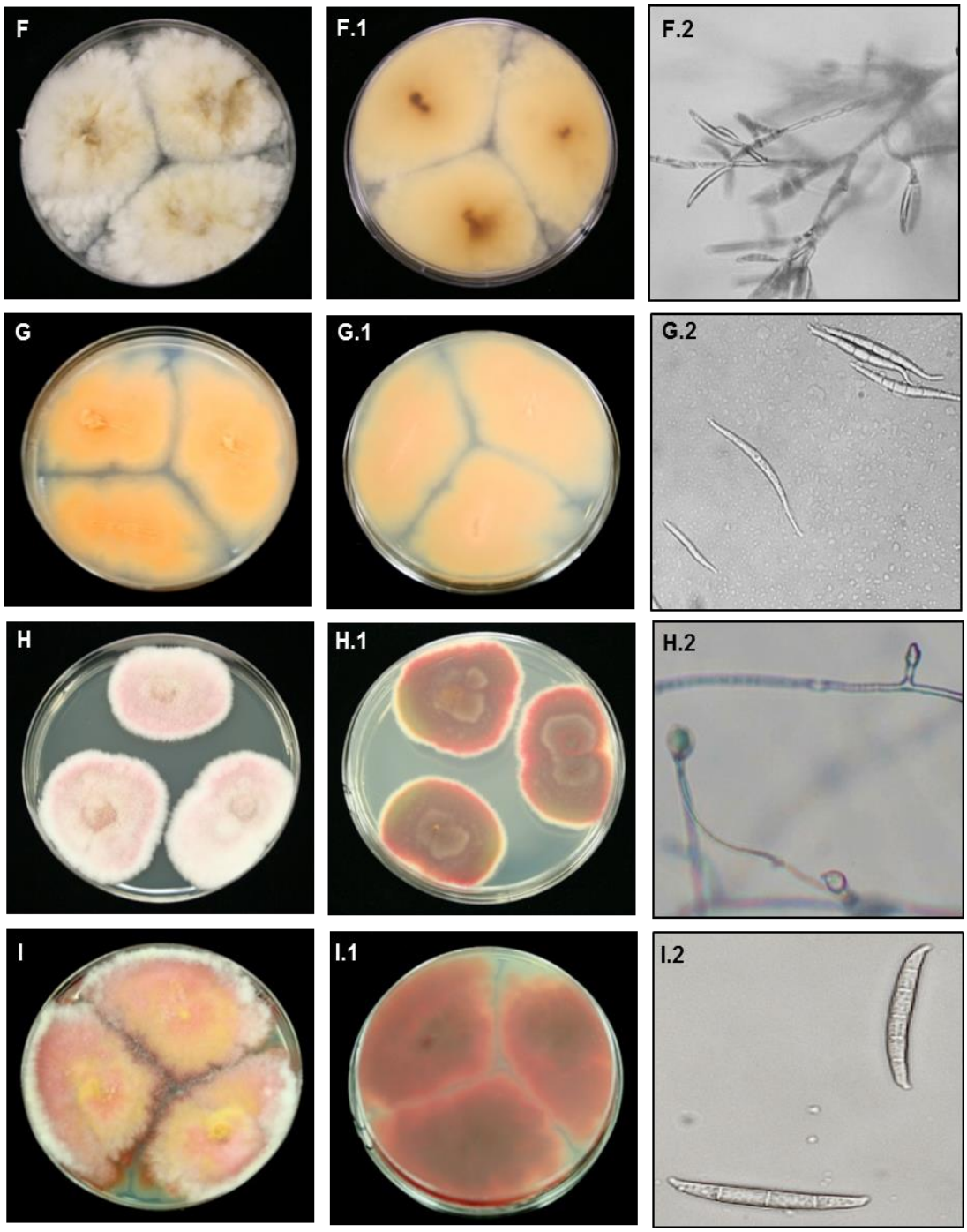

Figure 3.1 Morphological characteristics of the nine seedborne *Fusarium* spp. recovered from soybean seed in the state of Kansas during the 2010, 2011, and 2012 surveys. Colony shape and pigmentation on PDA medium (left and middle columns) and asexual reproductive structures (right column). *Fusarium verticillioides* (23564; A, A.1, A.2); *F. proliferatum* (23602; B, B.1, B.2); *F. fujikuroi* (23560; C, C.1, C.2); *F. thapsinum* (23623; D, D.1, D.2); *F. oxysporum* (23563; E, E.1, E.2); *F. semitectum* (23569; F, F.1, F.2); *F. equiseti* (23585; G, G.1, G.2); *F. acuminatum* (23598; H, H.1, H.2); *F. graminearum* (23577; I, I.1, I.2).

Figure 3.2 Disease severity index (DSI) was calculated based on seedling ratings using a scale of 0 to 3 where: 0 = germinated seeds and healthy and normal seedlings with no symptoms on the primary and/or secondary roots or hypocotyl (A); 1 = seed germinates and abnormal seedling presents minor discoloration and reduction on the primary and/or secondary roots as well as hypocotyl (red arrow) (B); 2 = seed germinates and abnormal seedling presents heavy discoloration and reduction on the primary and/or secondary roots. Also, the hypocotyl is heavily discolored and/or girdled by the lesion (red arrow) (C); 3 = non-germinated seed/seeds initially germinated and dead (D).

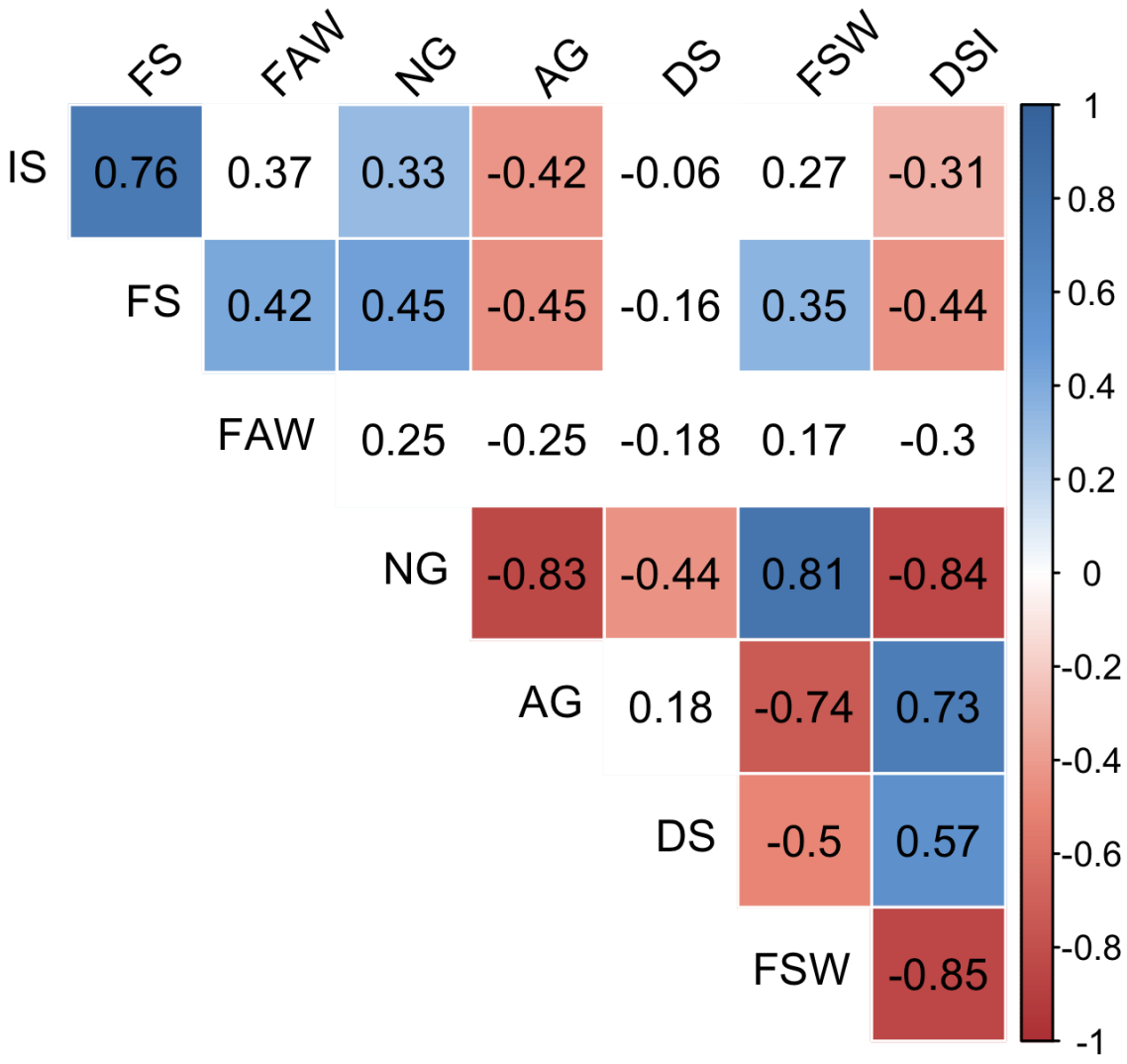

Figure 3.3 Linear correlations among soybean seed germination and vigor characteristics. The strength of the correlations is color labeled. Blue indicates strong and significant positive correlation of parameters; and red strong and significant negative correlation. White represents no significant correlation at $p < 0.05$. Parameters observed in the laboratory: Disease severity index (DSI); Normal germination (NG); Abnormal germination (AG); Dead seed (DS); Fresh seedling weight (FSW). Parameters observed in the greenhouse: Initial stand (IS); Final stand (FS); Fresh aerial weight (FAW).

Figure 3.4 Example of reduced of soybean seed quality by decreasing seed germination and vigor under laboratory and greenhouse conditions, respectively. Non-pathogenic *F. semitectum* isolate (23565) compared with a pathogenic isolate of *F. proliferatum* (23606) (A and B). Mock-inoculated seeds (control) presented healthy and abundant aerial plant mass when compared with seeds artificially inoculated with a pathogenic isolate of *F. proliferatum* (23606) (C and D). Post-emergent damping-off symptoms caused by *F. proliferatum* (23606) (E).

Table 3.1 Soybean seed samples used for isolation and characterization of seedborne *Fusarium* species during three growing seasons (2010, 2011, and 2012) in the state of Kansas.

County	Number of samples			Total [a]
	2010	2011	2012	
Republic	4	32	--	36
Pottawatomie	1	8	26	35
Shawnee	2	--	21	23
Franklin	2	--	34	36
Saline	1	--	26	27
Reno	1	--	38	39
Cherokee	10	8	18	36
Neosho	4	13	23	40
Crawford	3	18	25	46
Labette	--	35	31	66
Finney	--	--	24	24
Total [b]	28	114	266	408

[a]Total number of soybean seed samples collected during the three-year survey from each county studied; [b]Total number of soybean seed samples collected during the three-year survey from each year studied.

Table 3.2 Identification of *Fusarium* isolates collected during the survey.

Year	Isolate [a]	Locations [b]	Morph. ID [c]	Molecular ID (*Fusarium* - ID / NCBI) [d]			Final ID [e]	References [f]
				Accession numbers	Identity (%)	ID		
2010	23558	CK	FSE	FD_01635 / JX268971	100 / 100	FIESC / FSE	FSE	(1)
2010	23559	CK	FPR	FD_01389 / KM462975	100 / 100	FPR / FPR	FPR	(1)
2010	23560	CK	FSP	FD_01369 / JN695742	98 / 100	FFU / FFU	FFU	(2)
2010	23561	CK	FSE	*	*	*	FSE	(3)
2010	23562	NO	FSE	FD_01635 / JX268971	100 / 99	FIESC / FSE	FSE	(4)
2010	23563	NO	FOX	FD_01141 / JF740817	100 / 100	FOX / FOX	FOX	(5)
2010	23564	CK	FVE	FD_01388 / FN179343	100 / 99	FVE / FVE	FVE	(6)
2010	23565	CK	FSE	*	*	*	FSE	(3)
2010	23566	CK	FSE	*	*	*	FSE	(3)
2010	23567	CK	FEQ	*	*	*	FEQ	(3)
2010	23568	CK	FSE	*	*	*	FSE	(3)
2010	23569	CK	FSE	FD_01635 / JF270198	100 / 99	FIESC / FSE	FSE	(7)
2010	23570	CK	FSE	FD_01643 / KF962948	100 / 100	FIESC / FSE	FSE	(8)
2010	23571	CK	FEQ	*	*	*	FEQ	(3)
2010	23572	CK	FSE	*	*	*	FSE	(3)
2010	23573	CK	FSE	*	*	*	FSE	(3)
2010	23574	FR	FSE	FD_01635 / JF270296	100 / 100	FIESC / FSE	FSE	(7)
2010	23575	CK	FOX	FD_01141 / JF740817	100 / 100	FOX / FOX	FOX	(5)
2010	23576	PT	FSE	*	*	*	FSE	(3)
2010	23577	RP	FGR	FD_0005 / CM002652	100 / 100	FGR / FGR	FGR	(9)
2010	23578	CK	FOX	FD_01141 / JF740817	100 / 100	FOX / FOX	FOX	(5)
2011	23579	LB	FSE	*	*	*	FSE	(3)
2011	23580	LB	FSE	FD_01659 / JF270275	100 / 100	FIESC / FSE	FSE	(7)

2011	23581	LB	FSE	FD_01659 / JF270269	100 / 100	FIESC / FSE	FSE	(7)
2011	23582	LB	FSE	FD_01635 / JF270296	100 / 100	FIESC / FSE	FSE	(7)
2011	23583	LB	FSE	*	*	*	FSE	(3)
2011	23584	LB	FSE	*	*	*	FSE	(3)
2011	23585	LB	FEQ	FD_01694 / JN127347	100 / 99	FIESC / FEQ	FEQ	(10)
2011	23586	LB	FSE	*	*	*	FSE	(3)
2011	23587	LB	FSE	FD_01659 / JF270275	100 / 100	FIESC / FSE	FSE	(7)
2011	23588	LB	FSE	*	*	*	FSE	(3)
2011	23589	LB	FSE	*	*	*	FSE	(3)
2011	23590	LB	FSE	*	*	*	FSE	(3)
2011	23591	LB	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2011	23592	LB	FPR	FD_01389 / KM462975	100 / 100	FPR / FPR	FPR	(1)
2011	23593	LB	FSE	*	*	*	FSE	(3)
2011	23594	LB	FSE	*	*	*	FSE	(3)
2011	23595	LB	FSE	*	*	*	FSE	(3)
2011	23596	LB	FSE	*	*	*	FSE	(3)
2011	23597	LB	FSP	FD_01659 / JF270275	100 / 100	FIESC / FSE	FFU	(7)
2011	23598	LB	FAC	FD_01726 / JX397865	99 / 100	FAC / FAC	FAC	(12)
2011	23599	LB	FSE	*	*	*	FSE	(3)
2011	23600	LB	FSE	*	*	*	FSE	(3)
2011	23601	LB	FSE	*	*	*	FSE	(3)
2011	23602	RP	FPR	FD_01389 / KM462975	100 / 99	FPR / FPR	FPR	(1)
2011	23603	PT	FPR	FD_01378 / KM462975	100 / 99	FPR / FPR	FPR	(1)
2011	23604	PT	FVE	FD_01388 / KJ481244	100 / 99	FVE / FVE	FVE	(11)
2011	23605	CR	FPR	FD_01378 / KM462975	99 / 99	FPR / FPR	FPR	(1)
2011	23606	RP	FPR	FD_01378 / KM462975	100 / 100	FPR / FPR	FPR	(1)
2011	23607	CK	FSE	*	*	*	FSE	(3)
2011	23608	PT	FPR	FD_01378 / JX268968	100 / 100	FPR / FPR	FPR	(4)
2011	23609	CR	FSE	*	*	*	FSE	(3)

2011	23610	CR	FSE	*	*	*	FSE	(3)
2011	23611	CR	FSE	FD_01635 / JF270296	100 / 100	FIESC / FSE	FSE	(7)
2011	23612	CR	FPR	FD_01378 / JX268968	100 / 100	FPR / FPR	FPR	(4)
2011	23613	RP	FPR	FD_01378 / JX268968	99 / 100	FPR / FPR	FPR	(4)
2011	23614	RP	FPR	FD_01378 / JX268968	99 / 99	FPR / FPR	FPR	(4)
2011	23615	RP	FPR	FD_01378 / KM462975	100 / 99	FPR /FPR	FPR	(1)
2011	23616	RP	FSE	*	*	*	FSE	(3)
2011	23617	CK	FEQ	*	*	*	FEQ	(3)
2011	23618	NO	FPR	FD_01378 / KM462975	100 / 100	FPR / FPR	FPR	(1)
2011	23619	NO	FPR	FD_01389 / JX268968	100 / 100	FPR / FPR	FPR	(4)
2011	23620	LB	FPR	FD_01389 / KM462975	100 / 100	FPR / FPR	FPR	(1)
2011	23621	LB	FPR	FD_01389 / KM462975	100 / 100	FPR / FPR	FPR	(1)
2011	23622	LB	FSE	*	*	*	FSE	(3)
2011	23623	LB	FTH	FD_01177 / KM463006	99 / 100	FTH / FTH	FTH	(1)
2011	23624	LB	FSE	*	*	*	FSE	(3)
2011	23625	LB	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23626	LB	FSE	*	*	*	FSE	(3)
2012	23627	LB	FSE	*	*	*	FSE	(3)
2012	23628	LB	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23629	LB	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23630	LB	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23631	LB	FSE	*	*	*	FSE	(3)
2012	23632	LB	FSE	*	*	*	FSE	(3)
2012	23633	LB	FSE	*	*	*	FSE	(3)
2012	23634	LB	FSE	*	*	*	FSE	(3)
2012	23635	LB	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23636	LB	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23637	LB	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23638	LB	FSE	*	*	*	FSE	(3)

2012	23639	LB	FSE	*	*	*	FSE	(3)
2012	23640	LB	FSE	*	*	*	FSE	(3)
2012	23641	LB	FSE	*	*	*	FSE	(3)
2012	23642	LB	FSE	*	*	*	FSE	(3)
2012	23643	LB	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23644	RN	FSE	*	*	*	FSE	(3)
2012	23645	RN	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23646	LB	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23647	LB	FPR	FD_01378 / JX268968	99 / 99	FPR / FPR	FPR	(4)
2012	23648	NO	FSE	*	*	*	FSE	(3)
2012	23649	LB	FPR	FD_01389 / JX268968	99 / 99	FPR / FPR	FPR	(4)
2012	23650	LB	FSE	*	*	*	FSE	(3)
2012	23651	SA	FTH	FD_01177 / KM463006	100 / 100	FTH / FTH	FTH	(1)
2012	23652	LB	FSE	*	*	*	FSE	(3)
2012	23653	NO	FSE	FD_01694 / JF270184	94 / 100	FIESC / FSE	FSE	(7)
2012	23654	NO	FSE	*	*	*	FSE	(3)
2012	23655	LB	FVE	FD_01388 / KJ481244	99 / 99	FVE / FVE	FVE	(11)
2012	23656	NO	FSE	*	*	*	FSE	(3)
2012	23657	NO	FSE	*	*	*	FSE	(3)
2012	23658	LB	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23659	NO	FSE	*	*	*	FSE	(3)
2012	23660	NO	FSE	*	*	*	FSE	(3)
2012	23661	NO	FSE	*	*	*	FSE	(3)
2012	23662	NO	FPR	FD_01389 / KM462975	100 / 100	FPR / FPR	FPR	(1)
2012	23663	NO	FSE	*	*	*	FSE	(3)
2012	23664	NO	FSE	*	*	*	FSE	(3)
2012	23665	NO	FSE	*	*	*	FSE	(3)
2012	23666	NO	FSE	*	*	*	FSE	(3)
2012	23667	NO	FSE	*	*	*	FSE	(3)

2012	23668	RN	FPR	FD_01378 / JX268968	99 / 99	FPR / FPR	FPR	(4)
2012	23669	RN	FPR	FD_01389 / KM462975	100 / 99	FPR / FPR	FPR	(1)
2012	23670	RN	FPR	FD_01378 / JX268968	99 / 99	FPR / FPR	FPR	(4)
2012	23671	RN	FPR	FD_01378 / KM462975	100 / 100	FPR / FPR	FPR	(1)
2012	23672	RN	FPR	FD_01389 / KM462975	100 / 99	FPR / FPR	FPR	(1)
2012	23673	RN	FPR	FD_01378 / JX268968	99 / 99	FPR / FPR	FPR	(4)
2012	23674	RN	FPR	FD_01389 / KM462975	100 / 99	FPR / FPR	FPR	(1)
2012	23675	RN	FPR	FD_01389 / KM462975	100 / 99	FPR / FPR	FPR	(1)
2012	23676	LB	FSE	*	*	*	FSE	(3)
2012	23677	CR	FSE	*	*	*	FSE	(3)
2012	23678	CR	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23679	CR	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23680	CR	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23681	NO	FSE	*	*	*	FSE	(3)
2012	23682	NO	FSE	*	*	*	FSE	(3)
2012	23683	NO	FSE	*	*	*	FSE	(3)
2012	23684	SA	FTH	FD_01177 / KM463006	99 / 100	FTH / FTH	FTH	(1)
2012	23685	LB	FVE	FD_01388 / KJ481244	100 / 99	FVE / FVE	FVE	(11)
2012	23686	PT	FSE	*	*	*	FSE	(3)
2012	23687	PT	FSE	*	*	*	FSE	(3)
2012	23688	PT	FSE	*	*	*	FSE	(3)
2012	23689	PT	FPR	FD_01378 / JX268968	100 / 100	FPR / FPR	FPR	(4)
2012	23690	PT	FSE	*	*	*	FSE	(3)
2012	23691	PT	FSE	*	*	*	FSE	(3)
2012	23692	PT	FSE	*	*	*	FSE	(3)
2012	23693	PT	FSE	*	*	*	FSE	(3)
2012	23694	PT	FSE	*	*	*	FSE	(3)
2012	23695	PT	FSE	*	*	*	FSE	(3)
2012	23696	PT	FSE	*	*	*	FSE	(3)

2012	23697	PT	FSE	*	*	*	FSE	(3)
2012	23698	PT	FSE	*	*	*	FSE	(3)
2012	23699	PT	FSE	*	*	*	FSE	(3)
2012	23700	PT	FSE	*	*	*	FSE	(3)
2012	23701	PT	FSE	*	*	*	FSE	(3)
2012	23702	PT	FSE	*	*	*	FSE	(3)
2012	23703	PT	FPR	FD_01378 / JX268968	100 / 99	FPR / FPR	FPR	(4)
2012	23704	PT	FAC	*	*	*	FAC	(3)
2012	23705	PT	FAC	*	*	*	FAC	(3)
2012	23706	PT	FSE	*	*	*	FSE	(3)
2012	23707	PT	FSE	*	*	*	FSE	(3)
2012	23708	PT	FSE	*	*	*	FSE	(3)
2012	23709	PT	FSE	*	*	*	FSE	(3)
2012	23710	PT	FSE	*	*	*	FSE	(3)
2012	23711	FR	FSE	*	*	*	FSE	(3)
2012	23712	FR	FSE	*	*	*	FSE	(3)
2012	23713	PT	FSE	*	*	*	FSE	(3)
2012	23714	PT	FSE	*	*	*	FSE	(3)
2012	23715	PT	FSE	*	*	*	FSE	(3)
2012	23716	PT	FSE	*	*	*	FSE	(3)
2012	23717	PT	FSE	*	*	*	FSE	(3)
2012	23718	PT	FSE	*	*	*	FSE	(3)
2012	23719	PT	FSE	*	*	*	FSE	(3)
2012	23720	PT	FSE	*	*	*	FSE	(3)
2012	23721	PT	FSE	*	*	*	FSE	(3)
2012	23722	PT	FSE	*	*	*	FSE	(3)
2012	23723	PT	FSE	*	*	*	FSE	(3)
2012	23724	PT	FSP	FD_01369 / JN695742	98 / 100	FFU / FFU	FFU	(2)
2012	23725	PT	FSE	*	*	*	FSE	(3)

2012	23726	PT	FSE	*	*	*	FSE	(3)
2012	23727	PT	FSE	*	*	*	FSE	(3)
2012	23728	PT	FSE	*	*	*	FSE	(3)
2012	23729	RN	FPR	FD_01389 / KM462975	100 / 99	FPR / FPR	FPR	(1)
2012	23730	FR	FSE	*	*	*	FSE	(3)
2012	23731	FR	FPR	FD_01378 / JX268968	100 / 100	FPR / FPR	FPR	(4)
2012	23732	RN	FSE	*	*	*	FSE	(3)
2012	23733	NO	FSE	*	*	*	FSE	(3)
2012	23734	PT	FPR	FD_01389 / KM462975	100 / 100	FPR / FPR	FPR	(1)
2012	23735	SA	FPR	FD_01389 / KM462975	100 / 100	FPR / FPR	FPR	(1)
2012	23736	PT	FAC	*	*	*	FAC	(3)
2012	23737	PT	FSE	*	*	*	FSE	(3)
2012	23738	PT	FSE	*	*	*	FSE	(3)
2012	23739	PT	FSE	*	*	*	FSE	(3)
2012	23740	PT	FPR	FD_01389 / KM462975	100 / 100	FPR / FPR	FPR	(1)
2012	23741	LB	FVE	FD_01388 / KJ481244	100 / 99	FVE / FVE	FVE	(11)
2012	23742	LB	FVE	FD_01388 / KJ481244	100 / 99	FVE / FVE	FVE	(11)
2012	23743	LB	FVE	FD_01388 / KJ481244	100 / 99	FVE / FVE	FVE	(11)
2012	23744	LB	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23745	LB	FVE	FD_01388 / KJ481244	100 / 99	FVE / FVE	FVE	(11)
2012	23746	SA	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23747	SH	FPR	FD_01388 / KJ481244	100 / 100	FVE / FVE	FPR	(11)
2012	23748	FR	FAC	*	*	*	FAC	(3)
2012	23749	SA	FPR	FD_01389 / KM462975	100 / 100	FPR / FPR	FPR	(1)
2012	23750	FR	FSE	*	*	*	FSE	(3)
2012	23751	NO	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23752	NO	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23753	NO	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23754	PT	FSE	*	*	*	FSE	(3)

2012	23755	PT	FEQ	*	*	*	FEQ	(3)
2012	23756	NO	FSE	*	*	*	FSE	(3)
2012	23757	NO	FSE	*	*	*	FSE	(3)
2012	23758	NO	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23759	NO	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23760	NO	FSE	*	*	*	FSE	(3)
2012	23761	NO	FSE	*	*	*	FSE	(3)
2012	23762	NO	FSE	*	*	*	FSE	(3)
2012	23763	NO	FEQ	*	*	*	FEQ	(3)
2012	23764	NO	FSE	*	*	*	FSE	(3)
2012	23765	NO	FSE	*	*	*	FSE	(3)
2012	23766	NO	FSE	*	*	*	FSE	(3)
2012	23767	NO	FSE	*	*	*	FSE	(3)
2012	23768	CR	FSE	*	*	*	FSE	(3)
2012	23769	CR	FPR	FD_01380 / JX268968	100 / 99	FPR / FPR	FPR	(4)
2012	23770	NO	FSE	*	*	*	FSE	(3)
2012	23771	SA	FSE	*	*	*	FSE	(3)
2012	23772	SA	FTH	FD_01177 / KM463006	100 / 100	FTH / FTH	FTH	(1)
2012	23773	SA	FPR	FD_01389 / KM462975	99 / 99	FPR / FPR	FPR	(1)
2012	23774	PT	FSE	*	*	*	FSE	(3)
2012	23775	PT	FPR	FD_01389 / KM462975	99 / 100	FPR / FPR	FPR	(1)
2012	23776	PT	FSE	*	*	*	FSE	(3)
2012	23777	PT	FSE	*	*	*	FSE	(3)
2012	23778	PT	FSE	*	*	*	FSE	(3)
2012	23779	PT	FSE	*	*	*	FSE	(3)
2012	23780	PT	FSE	*	*	*	FSE	(3)
2012	23781	SA	FSE	*	*	*	FSE	(3)
2012	23782	RN	FVE	FD_01388 / KJ481244	100 / 99	FVE / FVE	FVE	(11)
2012	23783	PT	FSE	*	*	*	FSE	(3)

2012	23784	PT	FSE	*	*	*	FSE	(3)
2012	23785	PT	FPR	FD_01378 / JX268968	100 / 99	FPR / FPR	FPR	(4)
2012	23786	PT	FSP	FD_01369 / HF679028	98 / 99	FFU / FFU	FFU	(13)
2012	23787	PT	FSE	*	*	*	FSE	(3)
2012	23788	PT	FSE	*	*	*	FSE	(3)
2012	23789	PT	FPR	FD_01378 / JX268968	100 / 99	FPR / FPR	FPR	(4)
2012	23790	PT	FSP	FD_01369 / HF679028	98 / 99	FFU / FFU	FFU	(13)
2012	23791	FR	FVE	FD_01388 / KJ481244	100 / 99	FVE / FVE	FVE	(11)
2012	23792	PT	FAC	*	*	*	FAC	(3)
2012	23793	PT	FSP	FD_01369 / HF679028	98 / 99	FFU / FFU	FFU	(13)
2012	23794	PT	FAC	*	*	*	FAC	(3)
2012	23795	PT	FSE	*	*	*	FSE	(3)
2012	23796	PT	FSE	*	*	*	FSE	(3)
2012	23797	PT	FSE	*	*	*	FSE	(3)
2012	23798	PT	FSE	*	*	*	FSE	(3)
2012	23799	PT	FSE	*	*	*	FSE	(3)
2012	23800	PT	FSE	*	*	*	FSE	(3)
2012	23801	PT	FSE	*	*	*	FSE	(3)
2012	23802	PT	FSE	*	*	*	FSE	(3)
2012	23803	PT	FSE	*	*	*	FSE	(3)
2012	23804	PT	FSP	FD_01369 / HF679028	98 / 99	FFU / FFU	FFU	(13)
2012	23805	PT	FEQ	FD_01694 / JN127347	100 / 99	FIESC / FEQ	FEQ	(10)
2012	23806	PT	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23807	PT	FSE	*	*	*	FSE	(3)
2012	23808	PT	FSE	*	*	*	FSE	(3)
2012	23809	PT	FVE	FD_01388 / KJ481244	100 / 99	FVE / FVE	FVE	(11)
2012	23810	PT	FVE	FD_01388 / KJ481244	100 / 99	FVE / FVE	FVE	(11)
2012	23811	RN	FAC	*	*	*	FAC	(3)
2012	23812	FR	FPR	FD_01389 / KM462975	99 / 99	FPR / FPR	FPR	(1)

2012	23813	RN	FSE	*	*	*	FSE	(3)
2012	23814	SA	FTH	FD_01177 / KM463006	100 / 100	FTH / FTH	FTH	(1)
2012	23815	CR	FSE	*	*	*	FSE	(3)
2012	23816	CR	FSE	*	*	*	FSE	(3)
2012	23817	RN	FPR	FD_01389 / KM462975	100 / 99	FPR / FPR	FPR	(1)
2012	23818	RN	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23819	SA	FTH	FD_01177 / KM463006	100 / 100	FTH / FTH	FTH	(1)
2012	23820	LB	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)
2012	23821	SA	FPR	FD_01389 / KM462975	100 / 99	FPR / FPR	FPR	(1)
2012	23822	NO	FPR	FD_01389 / KM462975	100 / 99	FPR / FPR	FPR	(1)
2012	23823	LB	FVE	FD_01388 / KJ481244	100 / 100	FVE / FVE	FVE	(11)

[a]Soybean seedborne *Fusarium* isolates were deposited and accessed in the fungal collection from the Department of Plant Pathology at Kansas State University; [b]Locations of soybean seed samples infected by *Fusarium* spp. in Kansas (County codes: Cherokee (CK), Crawford (CR), Franklin (FR), Labette (LB), Neosho (NO), Pottawatamie (PT), Reno (RN), Republic (RP), and Saline (SA)); [c]*Fusarium* isolates were analyzed based upon specific morphological features as described in Leslie and Summerell (2006) (Species codes: *F. acuminatum* (FAC), *F. equiseti* (FEQ), *F. fujikuroi* (FFU), *F. graminearum* (FGR), *F. incarnatum-equiseti* species complex (FIESC), *F. oxysporum* (FOX), *F. proliferatum* (FPR), *F. semitectum* (FSE), *F. thapsinum* (FTH), *F. verticillioides* (FVE), and an unidentified *Fusarium* sp. (FUS)); [d]BLAST searches to known sequences in the *Fusarium*-ID and NCBI databases were used for molecular identification of *Fusarium* isolates; [e]*Fusarium* spp. assigned based upon morphological and molecular identification;. [e]Morphological and molecular literature references: (1) Funnell-Harris et al. 2011;(2) Suga et al. 2014; (3) Leslie and Summerell 2006; (4) Funnell-Harris et al. 2013; (5) O'Donnell et al. 2012; (6) Wulff et al. 2009; (7) Funnell-Harris and Pedersen 2011; (8) Castella and Cabanes 2014; (9) Gardiner et al. 2014; (10) Garibaldi et al. 2011; (11) Babič et al. 2015; (12) Niessen et al. 2012; (13) Wiemann et al. 2013*;* **Fusarium* isolates assigned to species based upon morphology only.

Table 3.3 Number of soybean seedborne *Fusarium* isolates recovered during three growing seasons (2010, 2011, and 2012) in the state of Kansas.

Species	2010 (N †= 28)	2011 (N = 114)	2012 (N = 266)	Total* (N = 408)
F. semitectum (FSE)	12	26	116	154
F. proliferatum (FPR)	1	15	28	44
F. verticillioides (FVE)	1	3	34	38
F. equiseti (FEQ)	2	2	3	7
F. acuminatum (FAC)	0	1	6	7
F. fujikuroi (FFU)	1	0	5	6
F. thapsinum (FTH)	0	1	5	6
F. oxysporum (FOX)	3	0	0	3
F. graminearum (FGR)	1	0	0	1
Fusarium spp. ** (*n*)	21	48	197	266

*Total number of *Fusarium* spp. isolates recovered during the three-year survey; **Total number of *Fusarium* spp. isolates recovered by year; † Number of soybean seed samples.

Table 3.4 Prevalence of soybean seedborne *Fusarium* spp. by samples (P_s) and location (P_l) and incidence of infected seeds among infected samples (In) observed during the survey three from 2010, 2011, and 2012 seasons in Kansas.

Species	2010 (N † = 28; n†† = 9)			2011 (N = 114; n = 6)			2012 (N = 266; n = 10)		
	P_s (%) [a]	P_l (%) [b]	In (%) [c]	P_s (%)	P_l (%)	In (%)	P_s (%)	P_l (%)	In (%)
F. semitectum	21.4	44.4	2.0	11.4	66.7	1.0	18.1	70.0	2.3
F. proliferatum	3.6	11.1	1.5	11.4	83.3	1.0	7.1	80.0	1.5
F. verticillioides	3.6	11.1	1.0	2.6	33.3	1.0	7.1	70.0	1.8
F. equiseti	3.6	22.2	1.5	1.8	33.3	1.0	1.1	20.0	1.0
F. acuminatum	0.0	0.0	0.0	0.9	16.7	1.0	1.5	30.0	1.3
F. fujikuroi	3.6	11.1	1.0	0.0	0.0	0.0	1.5	10.0	1.3
F. thapsinum	0.0	0.0	0.0	0.9	16.7	1.0	1.5	10.0	1.3
F. oxysporum	7.1	11.1	1.5	0.0	0.0	0.0	0.0	0.0	0.0
F. graminearum	3.6	11.1	1.0	0.0	0.0	0.0	0.0	0.0	0.0

[a]Prevalence by seed sample (P_s, %) = (Number of soybean samples containing a *Fusarium* species / Total number of samples analyzed)*100; [b]Prevalence by location (P_l, %) = (Number of locations containing a seed samples infected by a *Fusarium* species / Total number of locations analyzed)*100; [c]Incidence (In, %) = (the number of isolates found in a seed sample) / (the number of seed samples) *100. † Number of soybean seed samples studied by a given year. †† Number of locations sampled by a given year.

Table 3.5 Pathogenicity and aggressiveness of seedborne *Fusarium* spp. and their effect on soybean seed normal germination (NG), abnormal germination (AG), dead seeds (DS), and fresh seedling weight (FSW) under laboratory conditions[†].

Species (no. of isolates)	Aggressiveness [a]			NG	AG	DS	FSW
	Low	Moderate	High				
F. acuminatum (1)	+			91.0	3.3	5.7	22.5
F. equiseti (4)	+			88.5	4.6	6.9	23.3
F. fujikuroi (1)		+		71.1 ***	16.7 ***	12.2 ***	19.5 ***
F. graminearum (1)			+	65.5 ***	24.0 ***	10.5 **	17.9 ***
F. oxysporum (3)		+		71.8 ***	16.9 ***	11.2 **	17.6 ***
F. proliferatum (15)		+		71.9 ***	15.2 ***	13.0 ***	18.7 ***
F. semitectum (38)	+			89.5	4.2	6.3	23.3
F. thapsinum (1)		+		77.7 ***	12.7 **	9.7 **	22.8
F. verticillioides (4)	+			89.5	3.9	6.6	22.8
Mock-inoculated control	+			92.5	4.8	2.7	23.4

[a] Species having aggressiveness scores < 1, between 1 and 2, and > 2 were considered low, moderate, and highly aggressive, respectively; **, *** Significantly different from mock-inoculated control at the $P \leq 0.05$, $P \leq 0.001$, and $P \leq 0.0001$ levels using Dunnett's test. [†]Results are the means of three experiments.

Table 3.6 Pathogenicity of seedborne *Fusarium* spp. among individual isolates and their effect on soybean normal seed germination (NG), abnormal germination (AG), dead seeds (DS) and fresh seedling weight (FSW) on soybean seed artificially inoculated under laboratory conditions[†].

Species (no. of isolates)	Isolate Code	Aggressiveness [a]			NG	AG	DS	FSW
		Low	Moderate	High				
F. acuminatum (1)	23598	+			91.0	3.3	5.7	22.5
F. equiseti (4)	23567	+			88.0	5.7	6.7	24.5
	23571	+			88.7	4.0	7.3	24.0
	23617	+			88.0	4.0	8.0	22.4
	23585	+			89.3	4.7	6.0	22.5
F. fujikuroi (1)	23560		+		71.1 ***	16.7 ***	12.2 **	19.5 ***
F. graminearum (1)	23577			+	65.5 ***	24.8 ***	9.7	17.9 ***
F. oxysporum (3)	23575		+		77.8 ***	17.0 ***	11.0 **	17.5 ***
	23578			+	63.7 ***	25.3 ***	11.0 **	17.1 ***
	23563		+		74.2 ***	20.3 ***	5.5	18.2 ***
F. proliferatum (15)	23559		+		70.0 ***	21.0 ***	9.0	18.9 ***
	23614			+	68.0 ***	20.3 ***	11.7 **	18.1 ***
	23602			+	65.0 ***	13.0 ***	22.0 ***	17.5 ***
	23606		+		72.7 ***	17.3 ***	10.0 **	18.9***
	23613		+		72.7 ***	17.0 ***	10.3 **	19.6 ***
	23615		+		73.7 ***	19.7 ***	6.7	18.3 ***
	23612		+		71.0 ***	14.7 ***	14.3 **	18.0 ***
	23605		+		82.7 **	9.7 **	7.7	19.6 ***
	23603		+		61.0 ***	20.7 ***	18.3 ***	17.4 ***
	23608		+		76.7 ***	13.0***	10.3 **	19.2***
	23618		+		65.7 ***	20.3 ***	14.0 **	17.5 ***
	23619	+			87.0	5.0	8.0	22.3
	23592		+		67.0 ***	15.7 ***	17.3 ***	17.9 ***
	23620		+		67.7 ***	18.3 ***	14.0 **	18.6 ***

	23621		+	71.7 ***	17.0 ***	11.3 **	20.4 ***
F. semitectum (38)	23576	+		90.3	4.0	7.3	25.0
	23574	+		90.2	3.5	6.3	20.8 ***
	23565	+		87.2	7.2	5.7	22.3
	23566	+		89.3	6.5	4.7	25.3
	23568	+		90.3	3.7	5.0	24.3
	23569	+		92.5	3.8	3.7	23.3
	23570	+		91.0	4.7	4.3	24.6
	23572	+		91.7	3.0	5.3	24.1
	23573	+		89.2	6.5	4.3	24.7
	23562	+		87.5	5.0	8.3	22.3
	23561	+		88.0	5.0	7.0	23.2
	23616	+		88.0	4.3	7.7	23.7
	23607	+		87.3	6.0	6.7	23.0
	23609	+		92.3	1.4	6.3	23.6
	23610	+		89.7	3.7	6.7	23.4
	23611	+		87.7	5.3	7.0	23.9
	23584	+		87.7	4.3	8.0	22.6
	23586	+		86.3	6.4	7.3	22.3
	23587	+		90.3	2.7	7.0	23.2
	23624	+		91.3	2.0	6.7	22.7
	23600	+		91.7	3.7	4.7	24.4
	23601	+		91.3	1.4	7.3	23.3
	23595	+		93.3	3.4	3.3	23.6
	23596	+		92.0	1.7	6.3	23.9
	23597	+		89.0	5.7	5.3	23.7
	23599	+		87.7	5.3	7.0	22.9
	23622	+		86.3	5.7	8.0	22.8
	23593	+		85.3	4.0	10.7 *	22.2
	23594	+		90.7	3.0	6.3	22.7
	23579	+		88.3	4.7	7.0	22.7

	23580	+		89.3	6.7	4.0	23.4
	23581	+		90.7	3.0	6.3	22.8
	23583	+		92.0	2.7	5.3	23.1
	23582	+		88.7	5.7	5.7	22.5
	23590	+		91.3	4.4	4.3	24.3
	23588	+		89.3	3.4	7.3	22.0
	23589	+		89.0	5.7	5.3	22.9
	23558	+		89.9	4.4	5.7	24.6
F. thapsinum (1)	23623		+	77.7 ***	12.7 ***	9.7	22.8
F. verticillioides (4)	23564	+		93.0	4.3	2.7	24.0
	23604	+		87.7	6.0	6.3	22.0
	23625	+		90.0	4.3	5.7	23.5
	23591	+		87.3	3.7	9.0	21.8
Mock-inoculated control	NA	+		92.5	4.8	2.7	23.4

[a] Isolates having scores < 1, between 1 and 2, and > 2 were considered low, moderate, and highly aggressive, respectively; *, **, ***Significantly different from mock-inoculated control at the $P \leq 0.05$, $P \leq 0.001$, and $P \leq 0.0001$ levels using Dunnett's test. [†]Results are the means of three experiments.

Table 3.7 Effect of seedborne *Fusarium* isolates on soybean seed vigor characteristics including initial stand (%), final stand (%), and fresh aerial plant weight (FAW, g) of soybean seeds artificially inoculated under greenhouse conditions[†].

Isolate Code	Species	Aggressiveness	Initial stand (%) [a]	Final stand (%) [b]	FAW (g) [c]
23577	*F. graminearum*	High	86.0***	82.3***	19.0***
23614	*F. proliferatum*	High	87.7***	85.3***	17.0***
23578	*F. oxysporum*	High	91.0	86.3***	20.7**
23623	*F. thapsinum*	Moderate	86.3***	86.3***	26.3
23560	*F. fujikuroi*	Moderate	89.7*	85.3***	23.7
23625	*F. verticillioides*	Low	92.0	91.7	22.6
23567	*F. equiseti*	Low	92.7	90.7	25.5
23565	*F. semitectum*	Low	94.7	94.7	27.1
23598	*F. acuminatum*	Low	94.0	94.7	27.3
Mock-inoculated control	MCO	--	96.3	96.3	24.5

[a]Percentage of seedlings emerged 10 days post-inoculation (d.p.i.); [b]Percentage of seedlings emerged at 25 d.p.i.; [c]Fresh aerial weight (FAW) of soybean plants at 25 d.p.i. [*; **; ***]Significantly different from mock-inoculated control at the $P \leq 0.05$, $P \leq 0.001$, $P \leq 0.0001$ levels using Dunnett's test. [†]Results are the means of three experiments.

Chapter 4 - Metabarcoding pathogenic *Fusarium* spp. in soybean seeds

Abstract

The goal of this study was to identify *Fusarium* spp. and better understand their frequency distribution among and within naturally infected and asymptomatic soybean seed samples using DNA metabarcoding. A total of nine soybean seed samples were used in this study. The soybean seedborne fungal DNA (i.e., soybean seed mycobiome) was extracted from five individual asymptomatic seeds from each sample. Forward fITS7 and reverse ITS4-barcoded primers were used for the amplification of the fungal ITS2 region. After library construction, amplicons were sequenced using the Illumina platform. Approximately 291,000 high-quality reads were produced from all soybean seed samples analyzed. Overall, 66 operational taxonomic units (OTUs) representing 29 fungi genera, were identified in this study. The BLAST search showed that the genus *Fusarium,* including known pathogenic groups such as *F. proliferatum* and *F. thapsinum*, was observed in all seed analyzed, including in the high-quality seed control. Overall, *F. proliferatum* (OTU02; 44,429 reads) was the most abundantly amplified species followed by *F. thapsinum* (OTU03; 11,820 reads), *F. acuminatum* (OTU08; 4,609 reads), *F. merismoides* (OTU13; 4,302 reads), *F. solani* (OTU35; 254 reads), *Fusarium* sp. (OTU55; 19 reads), and *F. semitectum* (OTU57; 17 reads). Accurate information regarding the identity and frequency of what seed lots carry among and within them is crucial for significant improvements towards seed and seedling disease management strategies, especially regarding the detection of pathogenic seedborne fungi.

Introduction

Although seed and seedling diseases caused by *Fusarium* spp. is documented in the United States, accurate information regarding the identity and frequency distribution of most pathogenic species among and within naturally infected and asymptomatic soybean seed lots remain underexplored. Infected soybean seeds may present poor germination and emergence of seedlings (Hartman et al. 1999; McGee et al. 1980; Pedrozo and Little 2014; Pedrozo et al. 2015; Pedrozo and Little 2016). Besides, infected seeds may serve as a source of inoculum to new hosts and areas, which represent a concern for global food production, quality, and safety due to constant international seed trade.

Seedborne pathogens are difficult to control and thus, preventive actions such as accurate diagnosis, which can be accomplished by using appropriate seed health testing methods, is one of the most effective ways to manage them (Machado et al. 2002; McGee 1995; Munkvold 2009, Walcott 2003). Accurate information regarding the identification and frequency of what is carried among and within seed lots is crucial for significant improvements towards seed and seedling disease management strategies, especially regarding the detection of pathogenic seedborne groups (Agarwal and Sinclair 1996; Neergaard, 1979; Mathur and Kongsda 2003).

In previous studies, nine *Fusarium* spp. were identified including two new species reported in soybean seed for the first time in the United States, *F. thapsinum* and *F. fujikuroi* (see Chapter 3; Pedrozo and Little 2014; Pedrozo et al. 2015). Six species, *F. oxysporum*, *F. graminearum*, *F. proliferatum*, *F. thapsinum*, *F. fujikuroi*, and *F. verticillioides* significantly reduced the percentage of germination and vigor of artificially inoculated seeds in laboratory and

greenhouse assays (see Chapter 3; Pedrozo and Little 2014. Pedrozo et al. 2015; Pedrozo and Little 2016). Moreover, the overall amount of inoculum present among and within naturally infected soybean seed samples (prevalence and incidence) of pathogenic seedborne *Fusarium* spp. was low, averaging 33 and 2%, respectively (see Chapter 3). Besides other factors that might affect the accurate quantification of seedborne pathogens in seed lots, the methodology (i.e. culture-dependent or culture-independent approaches) as well as technology (i.e. next-generation sequencing) used for detection play a crucial role (Agarwal and Sinclair 1996; Machado et al. 2002).

Novel approaches such as DNA metabarcoding, which couples culture-independent methodology plus next-generation sequencing technology, have been used for improved detection and characterization of fungal communities associated with diverse plant species (Begerow et al. 2010; Coissac et al. 2012; Cuadros-Orellana et al. 2013; Glenn et al. 2011; Lundberg et al. 2015; Nam et al. 2012). Although this approach has a tremendous potential for the detection of seedborne pathogens in crops, no information has been reported for soybean seeds. Thus, the main objective of this study was to use DNA metabarcoding to identify and better understand the frequency distribution of *Fusarium* spp. in naturally infected soybean seed samples.

Materials and Methods

Soybean seed samples

To access the soybean seed core mycobiome, eight soybean samples, representing two genotypes, Midland 4263 (Sylvester Ranch INC, Ottawa, KS, USA) and Pioneer 94Y01 (Du Pont Pioneer, Johnston, IA, USA), from four different locations in the state of Kansas (Franklin, Finney, Neosho, and Reno counties) were used in this study (Table 4.1). Also, the variety Asgrow 3039 (Monsanto, Inc.; St. Louis, MO, USA) was used as a high-quality seed control. Poor quality seeds carry a higher percentage (prevalence and incidence) of seedborne pathogens than good quality seeds (Agarwal and Sinclair 1996).

Seed sample quality testing

The soybean samples used in this study were further analyzed regarding the quality of the seeds. The physical condition of the seeds, measured by the percentage of damaged seeds, were observed and reported as well as physiological and sanitary parameters (Table 4.2). The percentage of damaged seeds was calculated based on the formula: DS (%) = (A / Nt) * 100, where "A" was the number of seeds presenting some type of physical damage (Figure 4.2); and Nt = total number of seeds tested. Physiological conditions of the seed samples were measured based on the tetrazolium and warm germination tests according to standard protocols for soybean seeds from the Association of Official Seed Analysis (AOSA). For seed health testing, which accounts for the presence or absence of seedborne pathogens in a seed lot, a semi-selective medium was used. One hundred arbitrarily selected seeds from each soybean seed sample were plated on semi-selective Nash-Snyder media (Leslie and Sumerrell, 2006) and incubated at 23 ± 2°C for seven days (Mathur and Kongsdal 2003). To promote the isolation of internal seed seedborne fungi, especially *Fusarium* spp., seeds were surface-sterilized with a 10% sodium

hypochlorite solution (v/v) for 10 minutes. Seeds were rinsed with sterile-distilled water and dried overnight at room temperature. Identification of soybean seedborne species such as *Alternaria alternata, Cercospora kikuchii, Phomopsis longicolla* and *Fusarium* spp. was made based on morphological features observed on the seeds under a stereomicroscope (Mathur and Kongsdal 2003). The frequency of infected soybean samples (sanitary aspect of the samples) was calculated based on the formula: Sanitary = (Number of soybean samples infected by fungi/ Total number of samples analyzed)*100. After the first screening, *Fusarium*-like colonies were isolated, purified (single-spored) and further identified based on morphological characters (Leslie and Summerell, 2006) and polymerase chain reaction (PCR) (Geiser et al. 2014). The translation elongation factor 1-alpha region of the mitochondrial DNA of the isolates was amplified using TEF1 (forward: 5'-ATGGGTAAGGAGGACAAGAC-3') and TEF2 (reverse: 5'-GGAAGTACCAGTGATCAT GTT-3') primers set (Geiser et al. 2014; O'Donnell et al. 1998). After identification, the soybean seedborne *Fusarium* isolates used in this study were deposited and accessed in the *Fusarium* collection in the Department of Plant Pathology at Kansas State University, USA (Table 4.3).

Soybean seedborne fungal DNA extraction

Only healthy individual soybean seeds were used to access the seed fungal community from each sample tested, and any physically damaged seeds (Figure 4.2) were discarded. DNA extraction of the seeds was performed using DNeasy Mini Plant Kit (Qiagen, USA) accordantly to the manufactured protocol. Before the DNA extraction, seeds were surface sterilized using a 5% bleach solution (0.5% NaOCl, v/v) for 5 minutes to minimize external contamination, and dried overnight at room temperature. After DNA extraction, DNA pools (plant + fungal DNA)

obtained from each experimental unit (45 total) were quantitated with an ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). After quantification, the DNA of the samples was standardized to 5.0 ng μl^{-1} for subsequent PCR amplification. DNA was stored at -20°C until PCR amplification.

ITS2 amplification and sequencing

The internal transcribed spacer 2 (ITS2) region of the ribosomal RNA gene, proposed as the universal barcode for fungi, was chosen for amplification of the soybean seedborne mycobiome (Schoch et al., 2012) The amplification was performed in a two-step nested PCR process following the protocol recommended by Berry et al. (2011) using the forward primer fITS7 (50-GTGARTCATCGAATCTTTG-30 and reverse primer ITS4 (50-TCCTCCGCTTAT TGATATGC-30) (Ihrmark et al., 2012; White et al., 1990) for the first run. PCR reactions were performed in 25 µl reaction volumes with three technical replicates of each of the 45 experimental units, and a negative control was also used to avoid and check potential cross-contamination (molecular biology grade water). The primary PCRs contained the following amounts/concentrations: 25 ng DNA template (5 µl), 200 lM dNTPs, 1 lM of both forward (fITS7) and reverse (ITS4) primers, 5 µl Phusion Green HF Buffer containing 1.5 mM $MgCl_2$, 7.3 µl molecular biology grade water, and 0.5 unit (0.25 µl) of the proof-reading Phusion Green Hot Start II High-Fidelity DNA polymerase (Thermo Scientific, Pittsburgh, USA). PCR cycling parameters included an initial denaturing at 98^{o}C for 30 s, followed by 35 cycles of denaturing at 94^{o}C for 30 s, annealing at 58^{o}C for 1 min and extension at 72^{o}C for 2 min, and a final extension at 72^{o}C for 8 min. Unique sample-specific 12-base-pair sequence barcodes were used in a secondary PCR as ITS4-barcoded primers (Table 4.4). Identical conditions were used in the

secondary PCR except that they included five µl of the primary PCR product as template, tagged reverse primers (ITS4), and the number of PCR cycles was reduced to seven. The secondary PCR amplicons were cleaned using Agencourt AmPure XP magnetic 96-well SPRIplate system (Beckman Coulter, Indianapolis, Indiana) following the manufacturer's protocol with 1:1 AmPure XP solution to amplicon ratio. The three technical replicates per experimental unit (45 in total) were combined and the experimental units equimolarly pooled into one amplicon library. Size selection (180 to 400 bp) was also performed using Pippin Prep (Sage Science). The libraries were AmPure cleaned again to remove any residual short DNA contaminants and submitted to the Integrated Genomics Facility at Kansas State University (Manhattan, KS, USA). Illumina specific adapters and indices were ligated using QIAGEN GeneRead Library Prep (QIAGEN, USA) and then sequenced using MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA) with 600 cycles.

Sequencing analyses

After sequencing, the data (reads) were analyzed using the program Mothur (v. 1.37.5; Schloss et al., 2009). After initial contig construction, paired-end read library with less than 100 bp overlap, ambiguous bases, any disagreements with primer or DNAtag sequences, sequences shorter than 250 bp, or homopolymers longer than 8 bp were screened and discarded. Identical sequences were preclustered to reduce potential sequencing bias (Huse et al., 2008) and screened for chimeras (uchime; Edgar et al., 2011). After quality control and removal of chimeras, sequences were normalized, and 6,600 high-quality sequences (reads) were subsampled from each experimental unit. The pairwise distance matrix was calculated and sequences clustered into operational taxonomic units (OTUs) at 99% sequence similarity. As suggested by previous

studies, OTUs presenting low frequency of reads (<10 sequences) were removed from the dataset (Brown et al., 2015; Oliver et al. 2015; Tedersoo et al., 2010).

The soybean seed mycobiome

A representative sequence for each OTU was picked and then taxonomically assigned at the UNITE (https://unite.ut.ee/; Kõljalg et al. 2005) and GeneBank taxonomy references ((http://www.ncbi.nlm.nih.gov/) using the PlutoF Biodiversity platform (https://plutof.ut.ee/). A minimum of 97% identity threshold for positive confirmation of the OTUs as species was used in this study.The seedborne frequency distribution of the soybean seed mycobiome, including the genus *Fusarium*, among and within naturally and asymptomatic soybean seed samples were calculated based on prevalence and incidence. The frequency of infected soybean samples (Prevalence; Pr) was calculated based on the formula: Pr = (Number of soybean samples having a fungi group / Total number of samples analyzed)*100. Besides, the percentage of infected seeds among infected samples (Incidence; In) was calculated based on: In = (the number of infected seeds found in an infected sample) / (the total number of seeds present in a sample) *100.

Results

Seed sample quality tests

The physical, physiological, and sanitary quality aspects of the nine soybean seed samples was analyzed and physical, physiological, and sanitary aspects were measured (Table 4.2). Overall, the results showed that all the samples were significantly different from the high-

quality seed check regarding their physical and physiological parameters except sample no. 3 (S3) (Table 4.2). Regarding the sanitary aspect of the samples, which considers the amount of plant pathogens present in a seed lot, on average 77% of the samples were infected by seedborne fungi (Table 4.2). On average 44% of the samples were naturally infected by *Fusarium* spp. (Table 4.2). Among the *Fusarium* infected-samples, an average of 3% of the seeds were infected. Overall, eleven seedborne *Fusarium* isolates were obtained, where ten isolates were identified as *F. proliferatum* and one as *F. semitectum* (*Fusarium* fc. *incarnatum*) based on morphological and molecular identification (Table 4.3). Interestingly, the only sample that showed similarities regarding physical and physiological parameters with the high-quality seed control, S3, was the only sample to present a high incidence of pathogenic *F. proliferatum* (Table 4.2). Besides *Fusarium* spp., three other fungal genera were observed and identified based on morphological features of the soybean seed samples, *Alternaria alternata*, *Cercospora kikuchii*, and *Diaporthe* (*Phomopsis*) *longicolla* (Table 4.2). Using culturable methods, the high-quality check did not present seedborne fungi (Table 4.2).

The soybean seed mycobiome

Independent of the quality of the samples used in this study, only healthy/asymptomatic soybean seeds were used to access the seed fungal community from each sample. Any physically damaged seeds were discarded (Figure 4.2). Overall, a broad range of seedborne fungi was observed among the seed samples tested (Table 4.5). Among the 45 experimental units (single soybean seeds), a total of 291,194 high-quality sequences (reads) were obtained (Table 4.5). Most of the sequences obtained in this study were identified as ascomycetes (237,397 reads), followed by basidiomycetes (33,325 reads), zygomycetes (44 reads), and unclassified fungi

(11,552 reads) (Table 4.5). Furthermore, reads presenting < 97% identity were classified as unidentified sequences ("others") (8,876 reads; Table 4.5; Table 4.6). As a result, most the OTUs identified in this study are ascomycetes (45.5%), followed by basidiomycetes (27.0%), unclassified fungi (17%), unidentified sequences ("others") (9.0%), and zygomycetes (1.5%) (Table 4.5).

Considering all the seed samples, a total of 66 OTUs and 29 major seedborne fungal genera plus unclassified fungi and unidentified sequences (classified as "other") were identified in this study (Tables 4.6 and 4.7). In general, the genus *Fusarium* was the second most frequent genus found among the samples (Table 4.7). Additionally, the genus *Fusarium* was found not only among all the samples (prevalence) but also in every seed analyzed (incidence) from all the samples studied (Table 4.7). Besides the genus *Fusarium*, fifteen other genera were also commonly identified within the soybean seed mycobiome and were also present in 100% of the seeds analyzed (Table 4.7). Interestingly, the groups representing the unclassified fungi as well as unidentified sequences ("others") also showed representative OTUs (i.e. OTU05 and OTU25) to be commonly present in the seed mycobiome (Tables 4.6 and 4.7). Although not present in all the samples or seeds analyzed, ten other genera including yeasts (*Bulleromyces*, *Tilletiopsis*), filamentous ascomycetes (*Aspergillus*, *Biappendiculispora*, *Cladosporium*, *Exserohilum*), and filamentous basidiomycetes (*Baeospora*, *Marasmius*, *Phlebiella*, *Resinicium*) were commonly identified within the soybean seed samples, and their presence and incidence ranged from 77 to 100% and 31 to 53%, respectively (Table 4.7).

Interestingly, other important soybean seedborne and plant pathogenic groups such as *Diaporthe, Erysiphe*, and *Exserohilum* were also observed to be common inhabitants of the soybean seed mycobiome (Table 4.7). Even though DNA was extracted from healthy seed, one of the most important soybean seedborne species, *Diaporthe* (*Phomopsis*) *longicolla*, the causal agent of seed decay, was observed among and within all the samples analyzed (Table 4.7). Other well-known seedborne genera such as *Cercospora* and *Macrophomina* were not as commonly identified in soybean seeds, and they were present in only 33% and 11% of the samples, respectively (Table 4.7).

Seven seedborne *Fusarium* species (OTUs) were identified in this study (Figure 4.3). Overall, *F. proliferatum* (OTU02; 44,429 sequences) was the most abundantly amplified species followed by *F. thapsinum* (OTU03; 11,820 sequences), *F. acuminatum* (OTU08; 4,609 sequences), *F. merismoides* (OTU13; 4,302), *F. solani* (OTU35; 254 sequences), *Fusarium* sp. (OTU55; 19 sequences), and *F. semitectum* (OTU57; 17 sequences) (Table 4.8). The BLAST search for similarity showed an identity of the isolates ranging from 97 to 100% (Table 4.8). Among the species identified, *F. proliferatum* (OTU02), *F. thapsinum* (OTU03), *F. acuminatum* (OTU08), and *F. merismoides* (OTU13) were present in 100% of the samples analyzed (Table 4.8). *Fusarium solani* (OTU3) was identified in most of the soybean samples and seeds whereas *Fusarium* sp. (OTU55), and *F. semitectum* (OTU57) were identified in a few soybean seed samples (Table 4.8).

Discussion

The results of this chapter demonstrate that seven *Fusarium* spp., *F. proliferatum, F. thapsinum, F. acuminatum*, *F. merismoides, F. solani, Fusarium* sp. (unclassified), and *F. semitectum* were identified among the nine soybean seed samples analyzed using DNA metabarcoding. *Fusarium proliferatum, F. thapsinum, F. acuminatum*, and *F. merismoides* were the most commonly identified species among and within naturally infected seed samples. In fact, *F. proliferatum, F. thapsinum*, and *F. acuminatum* were observed in all the asymptomatic soybean seeds that were analyzed. Interestingly, *F. proliferatum* and *F. thapsinum* were previously reported to have the potential to decrease soybean seed germination and vigor as well as cause post-emergent damping-off (Pedrozo and Little 2014; Pedrozo et al. 2015; Pedrozo and Little 2016). These results suggest that the presence of pathogenic *Fusarium* spp. associated with soybean seed samples is higher than previously recognized (see Chapter 3), where only a small percentage of infection among and within seed samples was observed.

Recent studies using DNA metabarcoding have revealed the presence of *Fusarium* spp. in other seed crops such as wheat, canola, sorghum, and peanuts (Nicolaisen et al. 2014; Links et al. 2015; Stokholm et al. 2016; Xing et al. 2016). In wheat and canola, *F. equiseti* and *F. graminearum* were commonly found (Links et al. 2015). An important pathogenic *Fusarium* species of sorghum, *F. thapsinum*, was identified as one of the most abundant species on sorghum seed and seedlings (Stokholm et al. 2016). In another study, although no further information was given at the species level, Xing et al. (2016) reported that the genus *Fusarium* was commonly found within peanut seeds. This information suggests that important plant pathogenic groups, such as *Fusarium,* are commonly present in the seed mycobiome of a broad array of agriculturally important crops.

The findings of this study have demonstrated that DNA metabarcoding can contribute to increased detection sensitivity of seedborne pathogens in soybean seed. For example, DNA metabarcoding offers mass parallelization of sequencing reactions, which significantly increases the amount of DNA that can be sequenced in one run (Heather and Chain 2016). As a result, in addition to the two pathogenic *Fusarium* spp. (*F. proliferatum* and *F. thapsinum*), three other soybean seedborne pathogenic species *Diaporthe* (*Phomopsis*) *longicolla*, *Diaporthe caulivora*, and *Erysiphe polygoni* were also present among and within all the soybean seed samples analyzed. These results agree with previous studies in which these important seedborne pathogenic species have been found in asymptomatic soybean seeds (Walcott 1998). Other authors have also reported that *F. graminearum*, another important species pathogenic to soybean, have been recovered from asymptomatic soybean plants and seeds (Russo et al. 2016). Although more studies are required to better understand the implications and significance of having important pathogenic seedborne fungi in healthy soybean seeds, these current findings reinforce the challenges facing accurate detection of plant pathogens in seed lots. These findings highlight the need for the development of new and more advanced molecular seed health testing methods that relies on quantification of seedborne pathogens rather than just presence or absence.

Several studies have reported the successful use of molecular techniques to identify pathogenic or toxigenic *Fusarium* spp. in soybean plants (Arias et al. 2011; Arias et al. 2013; Chandra et al. 2011; Watanabe et al. 2011). However, molecular techniques developed to detect *Fusarium* spp. in soybean seeds, specifically, are still underexplored. Seedborne pathogens are

difficult to control and thus, preventive actions such as accurate early diagnosis, which can be accomplished by using appropriate seed health testing methods, is one of the most effective management tools (Machado et al. 2002; McGee 1995; Munkvold 2009). Detecting seedborne pathogens is a challenging task due to the presence of asymptomatic seeds, which makes visual detection impossible (Walcott 2003). Besides, the pathogen inoculum in seeds may be low or unevenly distributed in the seed lot, which could result in false negatives (Mathur and Kongsda 2003). Thus, sensitive seed health testing methods, which account for those adversities, is required and necessary.

Besides pathogenic species, a diversity of yeasts and other fungi were also found within the soybean seed mycobiome. Sapkota et al. (2015) studied the composition of cereal grain leaves and found that among the most abundant taxa, some pathogens were identified together with a range of non-pathogenic yeasts and filamentous fungi. The current study shows that 29 fungal genera were present in healthy/asymptomatic soybean seeds. Interestingly, it is known from metagenomics studies that the number of sequences (reads) obtained from a species is dependent upon several factors including the relative abundance of the species, DNA extraction efficiency, genome copy number, primer set, analysis of the data, and specificity and accuracy of the datasets (Caporaso et al. 2012; Joshi et al. 2014; Leach and Board 2015; Valentini et al. 2009). Thus, it is possible and reasonable to think that more fungal genera than currently observed including pathogens, mycotoxin producers, endophytes, and yeasts are present among naturally infected and asymtomatic soybean seeds. Additional studies that use new technologyies for DNA extraction, amplification and sequencing are necessary for a deeper understanding of the fungal composition of soybean seeds and their influence on productivity, quality, and safety.

Using new molecular approaches, such as DNA metabarcoding, for identification and to gain accurate information regarding the frequency distribution of seedborne fungi among naturally infected and asymptomatic seeds is important and necessary. Understanding what crop seeds carry within them (i.e., the seed microbiome) is a crucial first step towards the development of accurate seed health testing methods. Crop seeds microbial prolifing can help us to better address and estimate inoculum thresholds of important pathogenic groups, such as *Fusarium* spp., which can improve commercial seed certification as well as quarantine programs. DNA metabarcoding may lead to the design of innovative methods for detection and identification of all classes of seedborne pathogens in a single assay.

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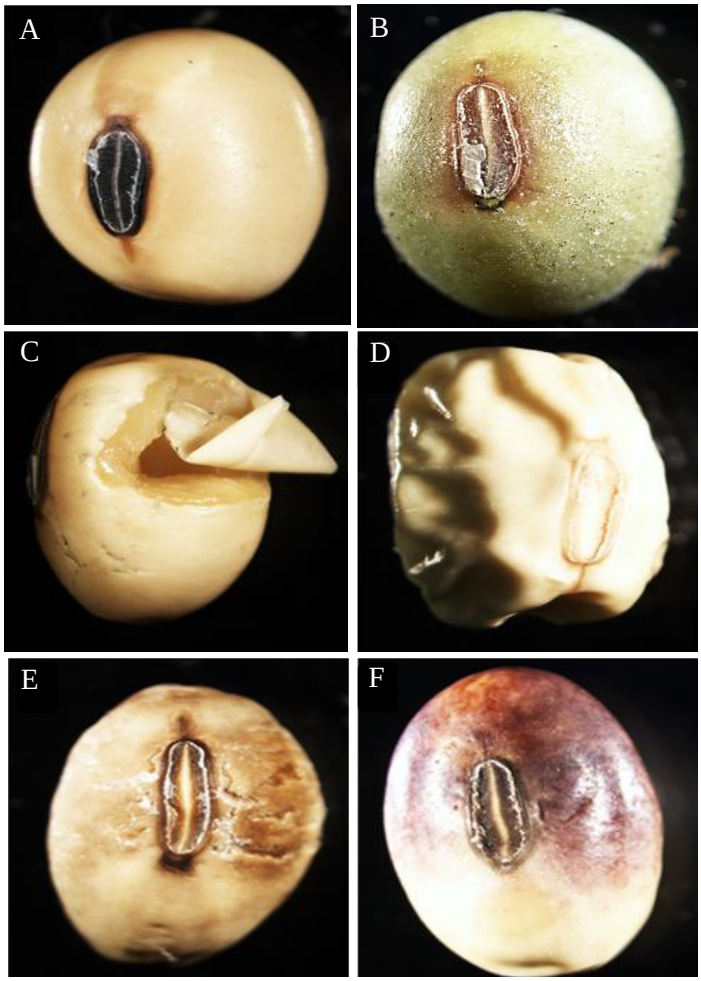

Figure 4.1 Healthy (asymptomatic) seeds (A); shriveled seeds (C); mechanically damaged seeds (D) and stained seeds (E and F). Only healthy seeds (A) were used for identification of pathogenic seedborne *Fusarium* spp. All the damaged seeds (B, C, D, E, and F) were discarded.

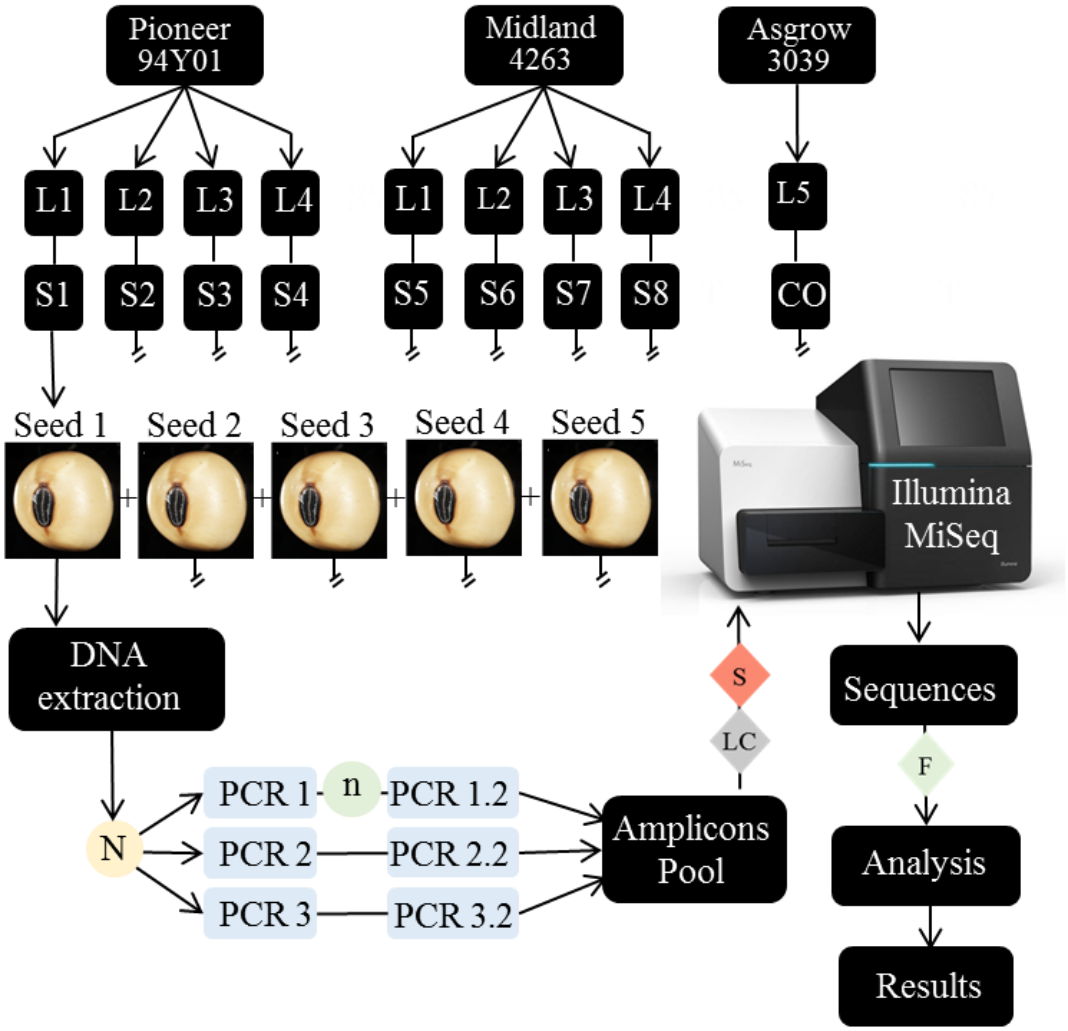

Figure 4.2 The soybean seed samples (S1-S8) used for identification of pathogenic seedborne *Fusarium* spp. within the soybean seed core mycobiome represent three soybean varieties (Pioneer 94Y01, Midland 4263, and Asgrow 3039) as well as four locations (Franklin-Kansas (L1), Finney-Kansas (L2), Neosho-Kansas (L3), and Reno-Kansas (L4), Idiana (L5)). Asgrow 3039 was used as a high-quality seeds (CO) check. Five biological replicates (individual soybean seeds) were used for each sample. After extraction and quantification, the DNA pool (plant + fungi DNA) was normalized (N) to 5.0 ng μl^{-1}. Three technical replicates were used for amplification of the ITS2 region (PCR 1, 2, 3). Nested PCR (n) was conducted after the first round of PCR using barcoded reverse ITS4 primers (Table 3.4). After library construction (LC), the library as sequenced (S), using the Illumina MiSeq platform.

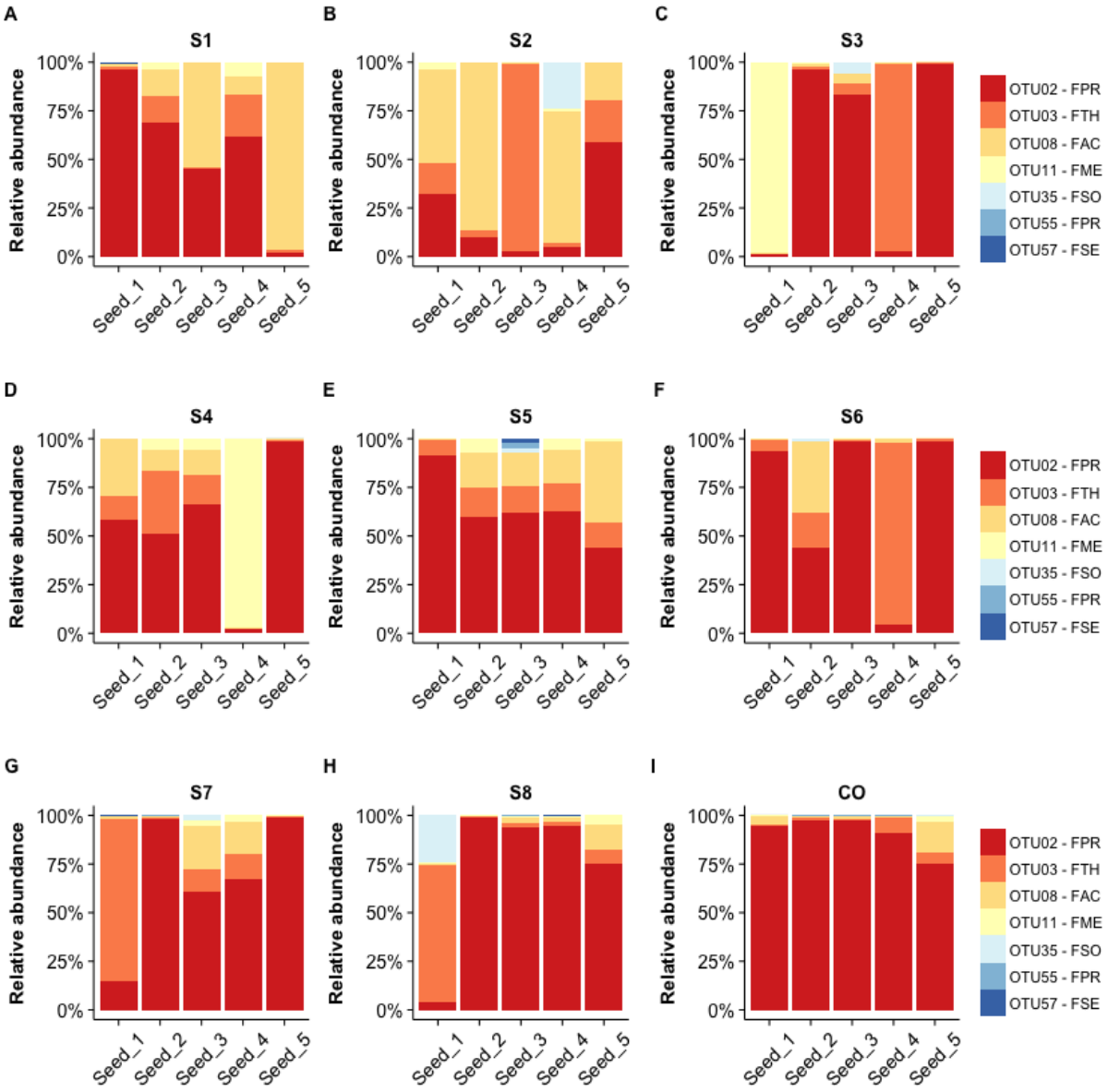

Figure 4.3 Major OTUs classified as *Fusarium* spp. observed among soybean seed samples analyzed in this study. The OTU002, OTU003, OTU008, OTU011, OTU035, OTU055, and OTU057 were identified as *F. proliferatum* (FPR), *F. thapsinum* (FTH), *F. acuminatum* (FAC), *F. merismoides* (FME), *F. solani* (FSO), *Fusarium* sp. (FSP), and *F. semitectum* (FSE), respectively. S1 (Franklin, KS; Midland 4263); S2 (Neosho, KS; Midland 4263); S3 (Reno, KS; Midland 4263); S4 (Finney, KS; Midland 4263); S5 (Franklin, KS; Pioneer 94Y01); S6 (Neosho, KS; Pioneer 94Y01); S7 (Reno, KS; Pioneer 94Y01); S8 (Finney, KS; Pioneer 94Y01); CO (Indiana; Asgrow 3039).

Table 4.1 Soybean seed samples used for isolation and molecular identification of seedborne fungi.

		Location		
Sample	Genotype	Field	County	State
S1	Midland 4263	Ottawa	Franklin	KS
S2		Erie	Neosho	KS
S3		Hutchinson	Reno	KS
S4		Garden city	Finney	KS
S5	Pionner 94Y01	Ottawa	Franklin	KS
S6		Erie	Neosho	KS
S7		Hutchinson	Reno	KS
S8		Garden city	Finney	KS
CO	Asgrow - 3039	--	--	IN

Table 4.2 Physical, physiological and sanitary aspects of the soybean seed samples used in this study.

	Physical (%)	Physiological (%)		Sanitary (%) – Culture-dependent †			
Sample	DS [a]	Tz [b]	Germ. [c]	AAL [d]	CKI [e]	PHO [f]	FSP [g]
S1	9.0 **	95.0	89.2	4.0	2.0	--	1.0
S2	7.0 *	95.0	82.5 **	6.0	10.0	5.0	1.0
S3	1.5	97.5	95.8	1.0	--	--	8.0
S4	5.0	90.0	85.0 **	4.0	9.0	1.0	--
S5	11.5 ***	85.0	72.5 ***	11.0	3.0	--	1.0
S6	3.0	87.5	80.8 ***	2.0	7.0	2.0	--
S7	3.0	87.5	85.0 **	1.0	--	--	--
S8	20.5 ***	47.5 ***	20.8 ***	--	--	--	--
CO	1.5	95.0	95.8	--	--	--	--

[a]The physical quality of the samples was calculated based on the percentage of damaged seeds (DS) accordingly to the formula: DS = (A / Nt) * 100, where "A" was the number of seeds presenting mechanical damage; and Nt = total number of seeds tested. [b]Tetrazolium test (Tz); [c]Percentage of normal geminated seedlings (Germ.); [d]*Alternaria alternata* (AAL); [e]*Cercospora kikuchii* (CKI); [f]*Phomopsis longicolla* (PHO); [g]*Fusarium* sp. (FSP). [*, **, ***]Significantly different from mock-inoculated control at the $P \leq 0.01$, $P \leq 0.001$, and $P \leq 0.0001$ levels using Dunnett's test (α=0.05). [†]Culture-dependent approach (semi-selective medium).

Table 4.3 Identification and cataloging of *Fusarium* isolates collected among the soybean seed samples used in this study.

		(*Fusarium* - ID / GeneBank)		Final	
Isolate [a]	Sample [b]	Accession strains [c]	Identity (%)	ID [d]	Ref. [e]
23812	S1	FD_01389 / KM462975	99 / 99	FPR	(3)
23648	S2	FD_01659 / JF270275	100 / 100	FSE	(1)
23668	S3	FD_01378 / JX268968	99 / 99	FPR	(3)
23669	S3	FD_01389 / KM462975	100 / 99	FPR	(3)
23670	S3	FD_01378 / JX268968	99 / 99	FPR	(2)
23671	S3	FD_01378 / KM462975	100 / 100	FPR	(3)
23672	S3	FD_01389 / KM462975	100 / 99	FPR	(3)
23673	S3	FD_01378 / JX268968	99 / 99	FPR	(2)
23674	S3	FD_01389 / KM462975	100 / 99	FPR	(3)
23675	S3	FD_01389 / KM462975	100 / 99	FPR	(3)
23731	S5	FD_01378 / JX268968	100 / 100	FPR	(2)

[a]Soybean seedborne *Fusarium* isolates were deposited and accessed in the fungal collection from the Department of Plant Pathology at Kansas State University; [b]Soybean seed samples used in this study (Further information can be found in the Table 3.1 and Table 3.2); [c]BLAST searches for comparison to known sequences in the *Fusarium*-ID and NCBI databases were used for molecular identification of *Fusarium* isolates; [d]Species codes: *F. proliferatum* (FPR), and *F. semitectum* (FSE); [e]Literature references: (1) Funnell-Harris and Pedersen 2011; (2) Funnell-Harris and Prom 2013; (3) Funnell-Harris et al. 2015.

Table 4.4 Primers and Multiplexing IDentifiers (MIDs) sequences used for sample identification in Illumina MiSeq ITS2 amplicon library. The MIDs were combined with the ITS4 primer and the sample specific MIDs were incorporated into the amplicons in secondary PCRs. Sample ID includes (1) individual soybean seed replicate (SS1-SS5); (2) soybean seed sample (S1, S2, S3, S4, S5, S6, S7, S8, CO*). *High quality soybean seed sample (CO).

Forward primer (ITS1F) - CTTGGTCATTTAGAGGAAGTA		
Forward primer (f7ITS1) - GTGARTCATCGAATCTTTG		
Reverse primer (ITS4) - TCCTCCGCTTATTGATATGC		
Sample_ID	MIDs †	Primers (5' - MIDs + ITS4 - 3')
SS1_S1	ACTCCTTGTGTT	ACTCCTTGTGTTTCCTCCGCTTATTGATATGC
SS2_S1	CCAATACGCCTG	CCAATACGCCTGTCCTCCGCTTATTGATATGC
SS3_S1	ACTTGGTGTAAG	ACTTGGTGTAAGTCCTCCGCTTATTGATATGC
SS4_S1	TCACCTCCTTGT	TCACCTCCTTGTTCCTCCGCTTATTGATATGC
SS5_S1	CAAACAACAGCT	CAAACAACAGCTTCCTCCGCTTATTGATATGC
SS1_S2	GCAACACCATCC	GCAACACCATCCTCCTCCGCTTATTGATATGC
SS2_S2	GCACACCTGATA	GCACACCTGATATCCTCCGCTTATTGATATGC
SS3_S2	CGAGCAATCCTA	CGAGCAATCCTATCCTCCGCTTATTGATATGC
SS4_S2	AGTCGTGCACAT	AGTCGTGCACATTCCTCCGCTTATTGATATGC
SS5_S2	GCGACAATTACA	GCGACAATTACATCCTCCGCTTATTGATATGC
SS1_S3	CGAGGGAAAGTC	CGAGGGAAAGTCTCCTCCGCTTATTGATATGC
SS2_S3	TCATGCTCCATT	TCATGCTCCATTTCCTCCGCTTATTGATATGC
SS3_S3	AGATTGACCAAC	AGATTGACCAACTCCTCCGCTTATTGATATGC
SS4_S3	AGTTACGAGCTA	AGTTACGAGCTATCCTCCGCTTATTGATATGC
SS5_S3	GCATATGCACTG	GCATATGCACTGTCCTCCGCTTATTGATATGC
SS1_S4	CAACTCCCGTGA	CAACTCCCGTGATCCTCCGCTTATTGATATGC
SS2_S4	GAGAGCAACAGA	GAGAGCAACAGATCCTCCGCTTATTGATATGC
SS3_S4	TACGAGCCCTAA	TACGAGCCCTAATCCTCCGCTTATTGATATGC
SS4_S4	CACTACGCTAGA	CACTACGCTAGATCCTCCGCTTATTGATATGC
SS5_S4	TGCAGTCCTCGA	TGCAGTCCTCGATCCTCCGCTTATTGATATGC
SS1_S5	ACCATAGCTCCG	ACCATAGCTCCGTCCTCCGCTTATTGATATGC
SS2_S5	TCGACATCTCTT	TCGACATCTCTTTCCTCCGCTTATTGATATGC
SS3_S5	GAACACTTTGGA	GAACACTTTGGATCCTCCGCTTATTGATATGC
SS4_S5	GAGCCATCTGTA	GAGCCATCTGTATCCTCCGCTTATTGATATGC
SS5_S5	TTGGGTACACGT	TTGGGTACACGTTCCTCCGCTTATTGATATGC
SS1_S6	CGTGCTTAGGCT	CGTGCTTAGGCTTCCTCCGCTTATTGATATGC
SS2_S6	CACTCATCATTC	CACTCATCATTCTCCTCCGCTTATTGATATGC
SS3_S6	TATCTATCCTGC	TATCTATCCTGCTCCTCCGCTTATTGATATGC
SS4_S6	TTGCCAAGAGTC	TTGCCAAGAGTCTCCTCCGCTTATTGATATGC
SS5_S6	CATACCGTGAGT	CATACCGTGAGTTCCTCCGCTTATTGATATGC
SS1_S7	TACTACGTGGCC	TACTACGTGGCCTCCTCCGCTTATTGATATGC
SS2_S7	GGCCAGTTCCTA	GGCCAGTTCCTATCCTCCGCTTATTGATATGC

SS3_S7	GATGTTCGCTAG	GATGTTCGCTAGTCCTCCGCTTATTGATATGC
SS4_S7	CTATCTCCTGTC	CTATCTCCTGTCTCCTCCGCTTATTGATATGC
SS5_S7	ACTCACAGGAAT	ACTCACAGGAATTCCTCCGCTTATTGATATGC
SS1_S8	ATGATGAGCCTC	ATGATGAGCCTCTCCTCCGCTTATTGATATGC
SS2_S8	GTCGACAGAGGA	GTCGACAGAGGATCCTCCGCTTATTGATATGC
SS3_S8	TGTCGCAAATAG	TGTCGCAAATAGTCCTCCGCTTATTGATATGC
SS4_S8	CATCCCTCTACT	CATCCCTCTACTTCCTCCGCTTATTGATATGC
SS5_S8	ATGTGTGTAGAC	ATGTGTGTAGACTCCTCCGCTTATTGATATGC
SS1_CO	TTCTCTCGACAT	TTCTCTCGACATTCCTCCGCTTATTGATATGC
SS2_CO	ACAATAGACACC	ACAATAGACACCTCCTCCGCTTATTGATATGC
SS3_CO	CGGTCAATTGAC	CGGTCAATTGACTCCTCCGCTTATTGATATGC
SS4_CO	GCTCTCCGTAGA	GCTCTCCGTAGATCCTCCGCTTATTGATATGC
SS5_CO	GCTCGAAGATTC	GCTCGAAGATTCTCCTCCGCTTATTGATATGC

† Multiplexing IDentifiers (Barcodes)

Table 4.5 Number of sequences (reads) and OTUs observed among the overall seedborne fungi groups identified within infected soybean seed samples.

Groups	Reads	RA (%) [a]	OTUs	RA (%)
Ascomycetes	237,397	81.53	30	45.45
Basidiomycetes	33,325	11.44	18	27.27
Zygomycetes	44	0.02	1	1.52
Fungi (Unclassified)	11,552	3.97	11	16.67
Others [†]	8,876	3.05	6	9.09
Total	291,194	100	66	100

[a]Relative abundance (RA); [†] Reads presenting less than 97% identity with accessed strains from UNITE and GeneBank were identified as "others", which represents unidentified sequences.

Table 4.6 Taxonomic assignment of representative ITS2 OTUs according to UNITE and GenBank reference sequences.

						UNITE	GenBank	
OTUs	Reads	RA (%) [a]	In (%) [b]	Genus	Species	Accession strain	Accession strain	Identity (%) [c]
OTU01	149,384	51.301	100	*Alternaria*	*A. alternata*	SH215493.07FU	LT560139	100
OTU02	44,429	15.258	100	*Fusarium*	*F. proliferatum*	SH219673.07FU	X94171 (1)	99
OTU03	11,820	4.059	100	*Fusarium*	*F. thapsinum*	SH219673.07FU	KX171659 (2)	100
OTU04	8,236	2.828	100	*Alternaria*	*A. infectoria*	SH216783.07FU	Y17067	100
OTU05	8,113	2.786	98	Fungi (unclassified)	Fungi (unclassified)	SH199073.07FU	KC753422	99
OTU06	6,072	2.085	100	Others	Others	SH211110.07FU	DQ421255	95
OTU07	5,770	1.981	91	*Malassezia*	*M. sympodialis*	SH188402.07FU	KM454159	100
OTU08	4,609	1.583	100	*Fusarium*	*F. acuminatum*	SH219674.07FU	KU382624 (3)	100
OTU09	4,585	1.575	49	*Baeospora*	*B. myosura*	SH187911.07FU	LN714524	100
OTU10	4,397	1.510	100	*Diaporthe*	*D. longicolla*	SH185492.07FU	U97658	100
OTU11	4,302	1.477	84	*Fusarium*	*F. merismoides*	SH175278.07FU	AB586998 (4)	100
OTU12	3,988	1.370	89	*Phlebia*	*P. chrysocreas*	SH192450.07FU	KP135358	99
OTU13	3,795	1.303	80	*Wallemia*	*Wallemia* sp.	SH216454.07FU	FR682244	97
OTU14	3,616	1.242	64	*Schizophyllum*	*S. commune*	SH190191.07FU	LC068797	100
OTU15	3,401	1.168	76	*Clitocybe*	*C. vibecina*	SH218334.07FU	JF907821	100
OTU16	2,968	1.019	71	*Aureobasidium*	*A. namibiae*	SH195774.07FU	KT693730	100
OTU17	2,650	0.910	96	*Cryptococcus*	*Cryptococcus* sp.	SH197623.07FU	LC018794	100
OTU18	2,011	0.691	67	*Erysiphe*	*E. polygoni*	SH187440.07FU	AF011308	100
OTU19	1,965	0.675	60	*Nigroporus*	*N. vinosus*	SH190478.07FU	JX109857	100
OTU20	1,841	0.632	36	Others	Others	SH179952.07FU	KT581876	85
OTU21	1,658	0.569	29	Fungi (unclassified)	Fungi (unclassified)	SH215453.07FU	KF800580	100
OTU22	1,465	0.503	78	*Penicillium*	*P. brevicompactum*	SH199400.07FU	LN833549	100
OTU23	1,318	0.453	53	*Bulleromyces*	*B. albus*	SH215453.07FU	HE650882	100
OTU24	1,003	0.344	36	*Biappendiculispora*	*B. japonica*	SH532144.07FU	LC001730	99
OTU25	924	0.317	64	Fungi (unclassified)	Fungi (unclassified)	SH210215.07FU	KT202892	100

OTU26	809	0.278	47	*Resinicium*	*R. friabile*	SH204932.07FU	DQ826545	98
OTU27	713	0.245	24	*Phlebia*	*Phlebia* sp.	SH525658.07FU	KJ654591	100
OTU28	686	0.236	13	Others	Others	SH179952.07FU	KT581876	87
OTU29	563	0.193	100	*Diaporthe*	*D. caulivora*	SH185506.07FU	KT895390	99
OTU30	500	0.172	27	*Phlebiella*	*P. borealis*	SH532759.07FU	KP814210	99
OTU31	445	0.153	40	*Epicoccum*	*E. nigrum*	SH207241.07FU	KU204774	100
OTU32	401	0.138	38	*Penicillium*	*Penicillium* sp.	SH207150.07FU	KP016820	100
OTU33	384	0.132	9	*Aureobasidium*	*Aureobasidium* sp.	SH195774.07FU	LC018807	100
OTU34	349	0.120	16	Fungi (unclassified)	Fungi (unclassified)	SH010431.07FU	AB828221	97
OTU35	254	0.087	22	*Fusarium*	*F. solani*	SH205225.07FU	KJ541492 [(5)]	99
OTU36	247	0.085	100	Others	Others	SH208383.07FU	KP814441	78
OTU37	173	0.059	73	Fungi (unclassified)	Fungi (unclassified)	--	KM247468	97
OTU38	163	0.056	13	*Acremonium*	*A. fusidioides*	SH203375.07FU	HF680234	100
OTU39	145	0.050	31	*Aspergillus*	*Aspergillus* sp.	SH182491.07FU	GU910689	100
OTU40	123	0.042	93	Fungi (unclassified)	Fungi (unclassified)	SH200466.07FU	KF800626	100
OTU41	121	0.042	71	*Alternaria*	*Alternaria* sp.	SH215493.07FU	GU721735	100
OTU42	101	0.035	87	Fungi (unclassified)	Fungi (unclassified)	SH190991.07FU	JX675046	100
OTU43	79	0.027	40	*Malassezia*	*M. restricta*	SH176394.07FU	NR:103585	100
OTU44	75	0.026	2	*Alternaria*	*Alternaria* sp.	SH215493.07FU	EF504668	99
OTU45	58	0.020	9	*Cercospora*	*C. apiicola*	SH206769.07FU	KU870468	100
OTU46	53	0.018	71	Fungi (unclassified)	Fungi (unclassified)	SH203201.07FU	KC785574	100
OTU47	46	0.016	60	*Wallemia*	*Wallemia* sp.	SH216453.07FU	KF800096	100
OTU48	44	0.015	4	*Rhizopus*	*R. arrhizus*	SH193530.07FU	LC149790	100
OTU49	34	0.012	42	*Epicoccum*	*E. nigrum*	SH207241.07FU	KU254609	100
OTU50	34	0.012	49	Fungi (unclassified)	Fungi (unclassified)	SH197623.07FU	KU515728	100
OTU51	29	0.010	51	*Marasmius*	*M. tubulatus*	SH010949.07FU	FJ936151	97
OTU52	26	0.009	33	*Wallemia*	*Wallemia* sp.	SH216453.07FU	JF497133	100
OTU53	24	0.008	16	*Alternaria*	*Alternaria* sp.	SH215493.07FU	GU721735	98
OTU54	20	0.007	24	*Cladosporium*	*C. subuliforme*	SH212842.07FU	LN834396	100
OTU55	19	0.007	16	*Fusarium*	*Fusarium* sp.	SH219673.07FU	KJ466111	97
OTU56	19	0.007	33	*Tilletiopsis*	*T. washingtonensis*	SH186666.07FU	HQ115649	99

OTU57	17	0.006	13	*Fusarium*	*F. semitectum*	SH204421.07FU	KU881904	100
OTU58	16	0.005	31	*Wallemia*	*Wallemia* sp.	SH216453.07FU	GU370753	100
OTU59	16	0.005	7	Others	Others	SH219673.07FU	X94174	96
OTU60	16	0.005	2	*Macrophomina*	*M. phaseolina*	SH182425.07FU	KU863545	100
OTU61	14	0.005	4	Others	Others	SH193582.07FU	GQ922553	96
OTU62	13	0.004	4	Fungi (unclassified)	Fungi (unclassified)	SH198034.07FU	JX984706	100
OTU63	12	0.004	27	*Exserohilum*	*E. rostratum*	SH211295.07FU	KU945863	100
OTU64	11	0.004	9	*Pseudopithomyces*	*P. chartarum*	SH186930.07FU	LK936369	100
OTU65	11	0.004	4	Fungi (unclassified)	Fungi (unclassified)	SH176396.07FU	GU327512	100
OTU66	11	0.004	20	*Aspergillus*	*A. ruber*	SH179237.07FU	U18357	100

[a]Relative abundance (RA). [b]Percentage of infected seeds (Incidence; In). [c]BLAST searches to known sequences in the NCBI databases were used for molecular identification of soybean seedborne fungi. [(1)]Waalwijk et al. 1996; [(2)]Stokholm et al. 2016; [(3)]Scruggs and Quesada-Ocampo 2016; [(4)]Watanabe et al. 2011; [(5)]Schuck et al. 2016.

Table 4.7 Taxonomic assignment of soybean seedborne fungi groups (phylum; unclassified fungi; and others) and distribution shown in % of total reads (relative abundance; RA), number of OTUs taxonomically assigned to a group (OTUs), % of infected samples (prevalence; Pr), and % of infected seeds among infected samples (incidence; In).

		Soybean seeds (*N* = 291,194) [a]				
Groups	Genus	Reads	RA (%)	OTUs	Pr (%)	In (%)
Ascomycetes	*Acremonium*	163	0.06	1	55	23
(*N* = 237,397)	*Alternaria*	157,840	54.2	5	100	100
	Aspergillus	156	0.05	2	100	38
	Aureobasidium	3,352	1.15	2	100	73
	Biappendiculispora	1,003	0.34	1	88	43
	Cercospora	58	0.02	1	33	27
	Cladosporium	20	0.01	1	77	31
	Diaporthe	4,960	1.70	2	100	100
	Epicoccum	479	0.16	2	100	64
	Erysiphe	2,011	0.69	1	100	67
	Exserohilum	12	0.00	1	77	34
	Fusarium	65,450	22.48	7	100	100
	Macrophomina	16	0.01	1	11	20
	Penicillium	1,866	0.64	2	100	87
	Pseudopithomyces	11	0.00	1	33	20
Basidiomycetes	*Baeospora*	4,585	1.57	1	100	49
(*N* = 33,325)	*Bulleromyces*	1,318	0.45	1	100	53
	Clitocybe	3,401	1.17	1	100	76
	Cryptococcus	2,650	0.91	1	100	96
	Malassezia	5,849	2.01	2	100	100
	Marasmius	29	0.01	1	100	49
	Nigroporus	1,965	0.67	1	100	60
	Phlebia	4,701	1.61	2	100	91
	Phlebiella	500	0.17	1	88	33
	Resinicium	809	0.28	1	88	53
	Schizophyllum	3,616	1.24	1	100	64
	Tilletiopsis	19	0.01	1	100	33
	Wallemia	3,883	1.33	4	100	96
Zygomycetes	*Rhizopus*	44	0.02	1	11	40
Fungi	Unclassified	11,552	3.97	11	100	100
Others [b]	--	8,876	3.05	6	100	100

[a]Total number of reads (sequences) observed among all nine soybean seed samples analyzed; [b]Reads presenting less than 97% identity with accessed strains from UNITE and GeneBank were identified as “others,” which represents unidentified sequences.

Table 4.8 Taxonomic assignment, relative abundance (RA), % of infected samples (Prevalence; Pr), and % of infected seeds among infected samples (Incidence; In) of soybean seedborne *Fusarium* OTUs.

Species	OTUs	Reads	RA (%)	Pr (%)	In (%)
F. proliferatum	OTU02	44,429	67.88	100	100
F. thapsinum	OTU03	11,820	18.06	100	100
F. acuminatum	OTU08	4,609	7.04	100	100
F. merismoides	OTU11	4,302	6.57	100	84
F. solani	OTU35	254	0.39	89	22
Fusarium sp.	OTU55	19	0.03	55	15
F. semitectum	OTU57	17	0.03	67	13

Chapter 5 - Effects of *Fusarium proliferatum* on soybean seed quality

Abstract

Although *Fusarium proliferatum* has shown the potential to cause soybean seed, seedling and root diseases, the conditions necessary to negatively affect seed quality are still underexplored. The objectives of this study were to evaluate the aggressiveness of *F. proliferatum* and the influence of its inoculum potential on soybean seed quality. Aggressiveness screens were conducted using laboratory and greenhouse assays. Eight *F. proliferatum* isolates were used and the results, from all of parameters tested, were compared with mock-inoculated controls. Overall, all *F. proliferatum* isolates significantly affected ($p < 0.001$) seed quality varibles in laboratory assays. In greenhouse assays, most *F. proliferatum* isolates tested reduced seed vigor ($p < 0.001$) when compared with mock-inoculated control. Using the rolled-towel assay, two *F. proliferatum* isolates were used to study the influence of inoculum potential and its interaction with aggressiveness on soybean seed quality. There was a significant interaction between isolate aggressiveness and inoculum potential ($p < 0.001$). The effects of seedborne *F. proliferatum* isolates on soybean seed quality parameters increases as the inoculum potential in contact with the seeds increases and it was more severe with the higly aggressive isolate. Moreover, no significant effects on the seed quality was observed when soybean seeds were treated with low inoculum potential (2.5×10^1 conidia ml^{-1}) with either moderate or highly pathogenic isolates. The findings of this study may serve as a baseline for future experiments

addressing the establishment of inoculum thresholds for pathogenic *Fusarium* spp. in soybean seed lots. These experiments may contribute to advances on the development of accurate diagnostic tools (i.e, seed health testing methods) especifically designed to detect pathogenic *F. proliferatum* strains in naturally infected and asymptomatic commercial soybean seed lots.

Introduction

Fusarium proliferatum (Matsushima) Nirenberg ex Gerlach & Nirenberg is a fungal plant pathogen isolated from a vast array of hosts and geographic locations (Leslie and Summerell 2006). It can colonize and cause diseases in important crops such as asparagus (Seefelder et al. 2002), banana (Jimenez et al. 1993), garlic and onion (Stankovic et al. 2007), orchids (Kim et al. 2002), maize (Munkvold 2003), rice (Amatulli et al 2012), sorghum (Leslie and Summerell 2006), and wheat (Desjardins et al. 2007). Furthermore, some isolates of this fungus can produce potent mycotoxins, including beauvericin, fumonisins, fusaproliferin, fusaric acid, fusarins, and moniliformin. Some of these secondary metabolites are associated with serious animal and human diseases (Bacon et al. 1994; Bacon et al. 1996; Chelkowski et a. 1990; Leslie et al. 2004; Miller et al. 1995).

On soybeans, the first report of *Fusarium proliferatum* as a disease agent was described in the United States by Arias et al. (2011). Under artificial conditions, *F. proliferatum* can cause seedling and root rots (Arias et al. 2011; Arias et al. 2013; Chang et al. 2015). Symptoms include pre- and post-emergent damping-off, water-soaked lesions on the stems, stunting, chlorosis and necrosis of cotyledons, wilting, and brown to black root rot in both the lower taproot and lateral

roots, with cortical decay or vascular discoloration (Nelson 1999). In general, yield losses by seedling and root rot diseases were estimated as 177,000 tons per year in the USA from 2003 to 2005 (Wrather and Koenning 2006). However, losses caused by *F. proliferatum,* specifically, have not been specified.

The seedborne nature of *F. proliferatum* has also been observed and reported in soybeans (Medić-Pap et al. 2007; Roy et al. 2001). Another important consideration is that infected seeds with pathogenic species and strains of *Fusarium* can serve as a source of inoculum dispersal, providing primary inoculum for infestation and establishment of the pathogens into new hosts and fields. This represents a significant threat to the future of food production, quality, and safety (Agarwal and Sinclair 1996; Machado et al. 2002; Neergaard 1979; Stack et al. 2014). However, besides its seedborne nature, little is known regarding the potential and conditions necessary for isolates of *F. proliferatum* to negatively affect soybean seed quality.

In previous study (see Chapter 4), *F. proliferatum* was identified in every single asymptomatic soybean seed analysed. Moreover, this pathogenic species was found in all three major soybean seed tissues, seed coat, cotyledons and embryo axis, from high quality seeds (Appendix C). The effect of a pathogen on seed germination and vigor can be influenced by different variables, of which, inoculum potential and aggressiveness of the pathogen plays an important role in addition to the incidence of the organism in the seed lot (Agarwal and Sinclair 1996; Neergaard 1979). Hence, the inoculum potential and aggressiveness of *F. proliferatum* may explain the presence of this pathogenic species of *Fusarium* in naturally and asymptomatic infected soybean seeds.

Because the aggressiveness of soybean seedborne *F. proliferatum* as well as the effects of the inoculum potential of this pathogen on soybean seed quality are still poorly understood and underexplored, the objectives of this study were: *i*) screening eight soybean seedborne *F. proliferatum* isolates, previously isolated from asymptomatic seeds, for pathogenicity under laboratory and greenhouse conditions, and *ii*) to evaluate the effects of *F. proliferatum* inoculum potential on soybean seed germination.

Materials and methods

Soybean seedborne *F. proliferatum* isolates

A total of eight soybean seedborne *F. proliferatum* isolates were used in this study. The isolates were previously isolated from asymptomatic soybean seeds (viability >97% and germination >95%) using culture-dependent approach (Nash-Snyder medium) and identified based on morphological features and PCR as previously reported (see Chapter 4).

Screening for pathogenicity:

Rolled-towel assay

The rolled-towel assay to evaluate pathogenicity and aggressiveness of seedborne *Fusarium* spp. on soybean seeds and seedlings (see Chapter 3) was used to compare the aggressiveness of eight seedborne *F. proliferatum* isolates to mock-inoculated control. The soybean variety used this study was Asgrow 'AG3039' (SDS moderate susceptible) (Monsanto, Inc.; St. Louis, MO, USA). Prior to inoculation, seeds were surface disinfested with a 5% bleach

solution (0.5% sodium hypochlorite v/v) for 1 min and dried overnight at room temperature. For inoculations, seeds were imbibed within a 25 ml conidial suspension at 2.5×10^5 conidia ml^{-1} for 1 min. Twenty-five artificially inoculated seeds were placed on two moistened sheets of germination paper (Anchor Paper Co., St. Paul, MN, USA). An additional sheet of moistened germination paper was placed over the inoculated seeds, the layers were rolled into a tube, secured by a rubber band, set upright in a modified plastic Rubbermaid® Cereal Keeper container (Newell Rubbermaid Co., Atlanta, GA, USA) and incubated in a growth chamber (Power Scientific Inc., St. Louis, MO, USA) at 25°C for seven days. For each *F. proliferatum* isolate, four rolled-towels were used, which corresponded to four replicates. After 7 days, the quality of the artificially inoculated soybean seeds was accessed by germination of normal seedlings (%), abnormal seedlings (%), dead seeds (%), and fresh seedling weight (g). The aggressiveness of the *F. proliferatum* isolates was based on the disease severity index (DSI) (Broders et al. 2007). DSI was calculated based on the formula: DSI = ((A*0)+(B*1)+(C*2)+(D*3)) / Nt, where A, B, C, and D are the number of seedlings presenting disease severity scores 0, 1, 2, and 3, respectively; and Nt = total number of seeds tested. The scale used for DSI ranged from 0 to 3 where: 0 = germinated seeds and healthy and normal seedlings with no symptoms on the primary and/or secondary roots or hypocotyl (A); 1 = seed germinates and the abnormal seedling shows minor discoloration and reduced primary and/or secondary roots as well as hypocotyl (B); 2 = seed germinates and abnormal seedling shows heavy discoloration and reduced primary and/or secondary roots. Also, the hypocotyl is heavily discolored and girdled by the lesion (C); 3 = non-germinated seed. Isolates having pathogenicity scores < 1, between 1 and 2, and > 2 were considered low, moderately, and highly aggressive, respectively.

Greenhouse assay

After screening the seedborne *F. proliferatum* isolates for their aggressiveness under laboratory conditions using the rolled-towel assay, all *F. proliferatum* isolates were screened in greenhouse assays to evaluate their influence on soybean seed vigor. The vigor of artificially inoculated soybean seeds was measured by the percentage of germinated seedlings at 10 days post-inoculation (d.p.i.) (i.e., initial stand), and at 25 d.p.i (i.e, final stand). In addition, dry plant aerial mass and root weight of artifially inoculated soybean plants were measured at 25 d.p.i. To assess the dry plant aerial mass, the seedlings were cut at 2 cm above substrate line and then subjected to the drying process in a forced air circulation oven, at 50ºC temperature, until reaching constant weight. After 96 h, the dried material was weighed in a semi-analytical balance. Results were expressed in grams. After cutting the aerial part of the plants, the remaining roots in the pots were washed with water and then dried and measured using the same approach describied previously. Results were also expressed in grams.The methodology used for seed inoculation was the same as previously described for the rolled-towel assay experiments. After inoculation, twenty-five seeds from each treatment were planted in 500 ml pots with autoclaved soil and vermiculite (1:1) in the greenhouse.

Effects of *F. proliferatum* inoculum potential and aggressiveness on soybean seed quality

One moderate (23675) and one highly aggressive (23670) seedborne *F. proliferatum* isolate was used to study the effects of inoculum potential of this pathogen on soybean seed quality. Six inoculum potential treatments (0 to 5) were used in this study. Mock-inoculated seeds with ddH_20 (0) and 2.5×10^1 conidia ml^{-1} (1) to 2.5×10^5 conidia ml^{-1} (5). As before,

AG3039 was the genotype used for this pathogenicity screen. After 7 days, the quality of artificially inoculated soybean seeds treated with different inoculum potentials was accessed by germination of healthy seedlings (percentage of normal germination), abnormal seedlings (percentage of symptomatic seedlings, dead seeds (percentage of non-germinated seeds) and fresh seedling weight (g).

Data analysis

Screening for pathogenicity: Analysis of variance was conducted using PROC MIXED of SAS (Version 9.3, SAS Institute). Among isolates, means of inoculated treatments (seeds inoculated with seedborne *F. proliferatum* isolates) were compared with the mock-inoculated control using Dunnett's test. Treatments were significantly different at $P \leq 0.05$. Furthermore, variables measured in laboratory and greenhouse assays were correlated using the SAS PROC CORR procedure.

Effects of *F. proliferatum* inoculum potential and aggressiveness on soybean seed quality: For the interaction between aggressiveness and inoculum potential assays, analysis of variance was conducted using PROC MIXED of SAS (Version 9.3, SAS Institute) and the treatments were arranged in a factorial sheme 2 x 6 (2 *F. proliferatum* isolates representing two aggressiveness levels (moderate and highly aggressive) and 6 inoculum potentials (mock-inoculated seeds with ddH_20 (IP0) and 2.5×10^1 conidia ml^{-1} (IP1) to 2.5×10^5 conidia ml^{-1} (IP5)). Moreover, data from the inoculum potential experiments were submitted to orthogonal polynomial contrast analysis of SAS (Version 9.3, SAS Institute) to determine the relationship between classes of inoculum potential and percentage of normal and abnormal germination, dead

seed and fresh seedling wieght of artificially inoculated seeds with moderate and higly aggressive isolates considered in laboratory trials. In addition, the influence of inoculum potential treatments (IP1-IP5) within isolates on soybean seed quality variables was also compared with the mock-inoculated control (IP0) using Dunnett's test for both laboratory and greenhouse experiments. Treatments were significantly different at $p \leq 0.05$.

The experimental design used for the pathogenicity assays was a completely randomized design, and all experiments were repeated three times.

Results

Rolled-towel assay

All eight *F. proliferatum* isolates tested were identified as pathogenic to soybean seed and seedlings under laboratory conditions (Table 5.1). There was a significant decrease ($p < 0.001$) in the percentage of normal seed germination for artificially inoculated seeds when compared to mock-inoculated control for all isolates (Table 5.1). The percentage of abnormal seedlings ($p < 0.001$; symptomatic seedlings) as well as dead seeds ($p < 0.001$; non-germinated seeds) was also affected when inoculated seeds were compared with mock-inoculated control (Table 5.1). All of the isolates tested, but the isolates 23668 and 23675, were able to significantly increase the percentage of abnormal seedlings (Table 5.1). However, all isolates tested significantly increased ($p < 0.001$) the percentage of dead seeds when compared with mock-inoculated seeds (Table 5.1). Except for isolate 23675, seed artificially inoculated with *F. proliferatum* isolates presented a significant decrease in fresh seedling weight when compared to

the mock-inoculated control (Table 5.1). Six isolates were identified as moderately aggressive (23668, 23660, 23671, 23672, 23674, and 23675) and two as highly aggressive (23670 and 23673) to soybean seeds and seedlings (Table 5.1).

Greenhouse assay

In addition to the laboratory assay, the *F. proliferatum* isolates were also tested in the greenhouse to evaluate their influence on seedling vigor. Most of the isolates tested significantly reduced initial ($p < 0.001$) and final stand ($p < 0.001$) when compared to mock-inoculated control. Isolates 23668, 23674, and 23675 did not reduce soybean seedling vigor after artificial inoculation of seeds (Table 5.2). Only isolates 23669, 23670 and 23673 significantly reduced dry aerial weight of artificially inoculated soybean plants when compared with the mock-inoculated control ($p < 0.001$; Table 5.2). Interestingly, all eight seedborne *F. proliferatum* isolates significantly reduced ($p < 0.001$) dry root weight when compared with mock-inoculated control (Table 5.2; Figure 5.1).

Overall, there was a significant correlation between the aggressiveness of the isolates tested under laboratory conditions and the parameters tested in greenhouse assays (Table 5.3). Overall, the vigor of the seeds artificially inoculated with *F. proliferatum*, measured by initial and final stand as well as dry plant aerial and root weight decreases as the DSI of the seedborne isolates increases (Table 5.3). Under laboratory conditions, all parameters tested were also affected by the aggressiveness of the isolates (i.e., DSI; Table 5.3). As DSI of the isolates increases, germination and fresh seedling weight of artificially inoculated seeds decrease.

Effects of *F. proliferatum* inoculum potential on soybean seed quality

Overall, the inoculum potential treatments of the *F. proliferatum* isolates significantly reduced seed quality and were influenced by the aggressiveness of the isolates of artificially inoculated seeds in laboratory assays (Table 5.4). The effect of both moderate and higly aggressive seedborne *F. proliferatum* isolates on soybean seed germination decreases as the inoculum potential in contact with the seeds decreases (Figure 5.2 and Figure 5.3). No reduction of seed germination was observed when soybean seeds were treated with the low inoculum potential treatment (2.5×10^1 conidia ml^{-1}) and seeds inoculated with both moderately and highly pathogenic isolates were not significaly affected (Table 5.5). The fresh weight of soybean seedlings, the percentage of abnormal soybean seedlings and dead seeds were also not significantly affected hen soybean seeds were treated with the low inoculum potential treatment (2.5×10^1 conidia ml^{-1}) (Table 5.5). In the same manner, both moderate and highly aggressive isolates decrease the fresh weight of soybean seedlings as the inoculum potential increases (Figure 5.3). Moreover, the percentage of abnormal soybean seedlings and dead seeds increases as the inoculum potential increases (Figure 5.3.

Discussion

Although *Fusarium proliferatum* has shown the potential to cause soybean seed rot, seedling damping-off and root rots, the conditions necessary for this species to negatively affect seed quality remain underexplored. The findings of this study confirm and complement previous results (see Chapter 3 and Appendix D), in which *F. proliferatum* significantly reduced soybean seed quality. Overall, *F. proliferatum* significantly decreased soybean seed germination and vigor under laboratory and greenhouse conditions. In addition, *F. proliferatum* caused seedling

damping-off and negatively affected the roots, by significantly reducing its mass, of artificially inoculated soybean plants, suggesting their potential importance as a seedborne pathogen in soybean growing regions. These results are consistent with previous studies where *F. proliferatum* isolates decreased seedling emergence and caused root disease (Arias et al. 2011; Chang et al. 2015). For example, seedling mortality from 40 to 78% was observed on soybean plants infected by *F. proliferatum* isolates in greenhouse experiments (Arias et al. 2013, Chang et al. 2015).

F. proliferatum is a soil inhabitant (Leslie and Summerell 2006). There is evidence that this fungus can colonize organic matter and persist on the soil surface or remain buried in the field for an extended period (Cotton and Munkvold 1998; Leslie et al. 1990; Gaige 2016). Therefore, in addition to the direct effect of *F. proliferatum* on soybean seeds, seedling and plant quality, infected soybean seeds represent a risk for the introduction and establishment of aggressive isolates into new areas (Gamliel 2008). Furthermore, *F. proliferatum* has been reported to cause diseases in other important cash crops such as sorghum, rice, wheat, and maize (Leslie et al. 1990; Bashyal et al. 2016; Molnár 2016; Munkvold 2003). Once established into new fields, *F. proliferatum* present in the soil can build up over time and serve as a primary source of inoculum to new crops. Because of that, the use of traditional plant diseases management strategies such as rotation of crops between host and non-host plant species may become restricted. Hence, early detection of *F. proliferatum* in commercial soybean seed lots is necessary to minimize the spread of pathogenic strains among crop growing areas.

The current study also showed that the effects of *F. proliferatum* on soybean seed quality increased as inoculum potential increased. Moreover, the effects on soybean seed quality of seed inoculated with a high inoculum potential of *F. proliferatum* were more severe with highly aggressive isolate than moderately aggressive isolate. Most importantly, soybean seeds inoculated with low *F. proliferatum* inoculum potential showed no significant decrease of seed quality parameters for moderately and highly aggressive isolates. Thus, considering that the amount of inoculum present within seeds plays a crucial role in seed and seedling diseases development (Agarwal and Sinclair 1996; Neergaard, 1979), it is tempting to speculate that soybean seedborne pathogenic groups, such as *Fusarium* spp. for example, are perhaps present in low inoculum levels in the seeds in order to not incite seed and seedling diseases. This may explain the presence of pathogenic *Fusarium* species, such as *F. proliferatum*, within naturally infected and asymptomatic soybean seeds observed in previous studies (see Chapter 4 and Appendix C).

Similar results have also been observed regarding the effects of *F. verticillioides* inoculum potential on soybean seed quality (Pedrozo and Little 2016). Pedrozo and Little (2016) suggested that *F. verticillioides* has the potential to reduce soybean seed quality, depending on the amount of inoculum present in seeds (i.e. inoculum potential). At low inoculum potential, *F. verticillioides* was not able to significantly reduce soybean seed quality (Pedrozo and Little 2016). This inoculum potential phenomenon of seedborne *Fusarium* spp. has been observed to negatively affect the germination and vigor of other crop seeds, such as cotton and maize seeds (Araujo et al. 2016; Machado et al. 2013). Araujo et al. (2016) observed that the influence of *Fusarium oxysporum* f. sp. *vasinfectum* on the germination of cotton seeds was higher when the

amount of inoculum in seeds was increased. Machado et al. (2013) reported that the most severe effects of *F. verticillioides* on the development of maize seed and seedling diseases were observed at the highest level of inoculum present in seeds. Overall, the most severe effect of these two *Fusarium* spp. on cotton (Araujo et al. 2016) and maize (Machado et al. 2013) seedlings were observed when high inoculum potential was present in seeds. In contrast, no decrease of seed quality parameters such as germination and vigor was observed when low inoculum potential was present within seeds (Araujo et al. 2016; Machado et al. 2013). For both studies, seeds were inoculated with different inoculum potentials using the osmoconditioning method described previously by Machado et al. (2012).

From these results, it becomes clear that the presence of *Fusarium* spp., per se, among and within seeds does not fully translate the potential of this pathogenic genus to significantly affect the quality of soybean seed. The findings of this study showed that the inoculum potential as well as the aggressiveness of *F. proliferatum* isolates present in the seeds may play a significant role for soybean seed and seedlings diseases. Therefore, these are important factors that influence seed quality. Further studies are necessary to better understand and estimate the significance of infected seeds by *F. proliferatum* to soybean seed production and quality. Considering that the presence of asymptomatic plant pathogens in seed lots makes their accurate identification extremely challenging (Sousa et al. 2015; Stergiopoulos and Gordon 2014), this study may serve as a baseline for future experiments addressing the mechanisms used by pathogenic *Fusarium* spp. to colonize plants and seeds asymptomatically as well as the establishment of an inoculum threshold for pathogenic *Fusarium* spp. in soybean seed lots.

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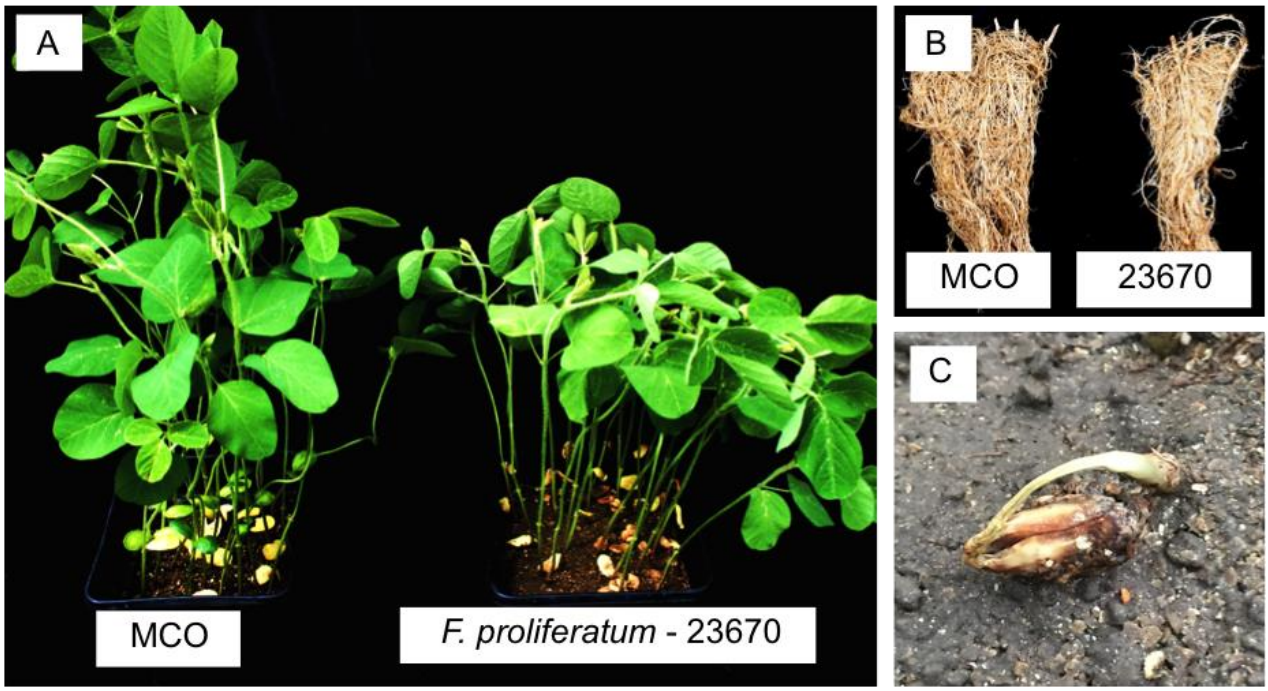

Figure 5.1 Influence of the highly aggressive seedborne *F. proliferatum* isolate (23670) on seed-inoculated soybean plants compared to mock-inoculated control (MCO) (A). Healthy and abundant root masses develop from mock-inoculated plants (MCO), compared to those where the seed was imbibed with *F. proliferatum* (B). Characteristic post-emergent damping-off of seedlings inoculated with a highly aggressive seedborne isolate (23670) of *F. proliferatum* (C).

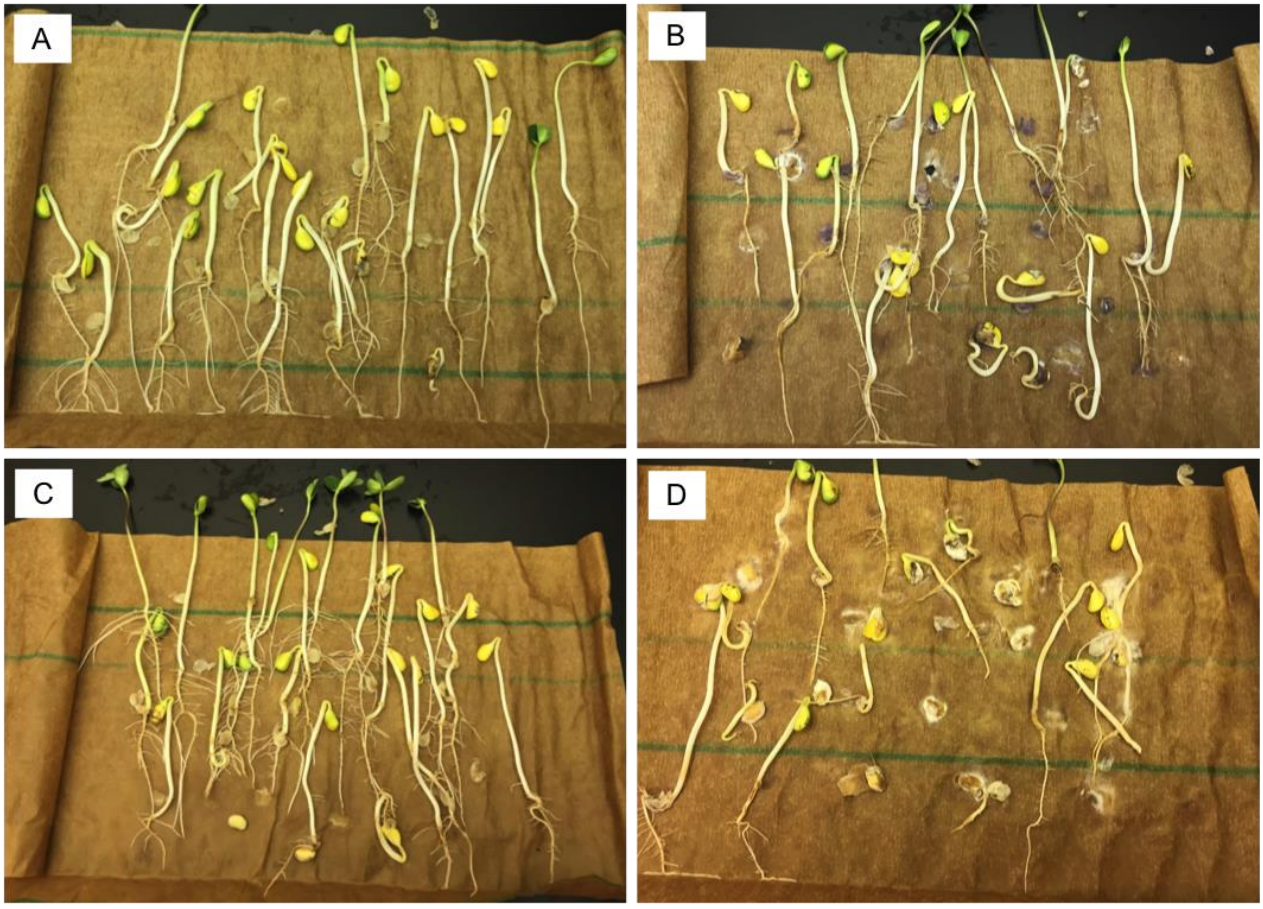

Figure 5.2 Influence of inoculum potential treatment by moderately and highly aggressive seedborne *F. proliferatum* isolates (23675 and 23670) on soybean seed quality. Comparisons of soybean seeds inoculated with *F. proliferatum* isolate (23675) using the lowest (A) and highest inoculum potential (B). Soybean seeds inoculated with *F. proliferatum* isolate (23670) using the lowest (C) and highest inoculum potential (D).

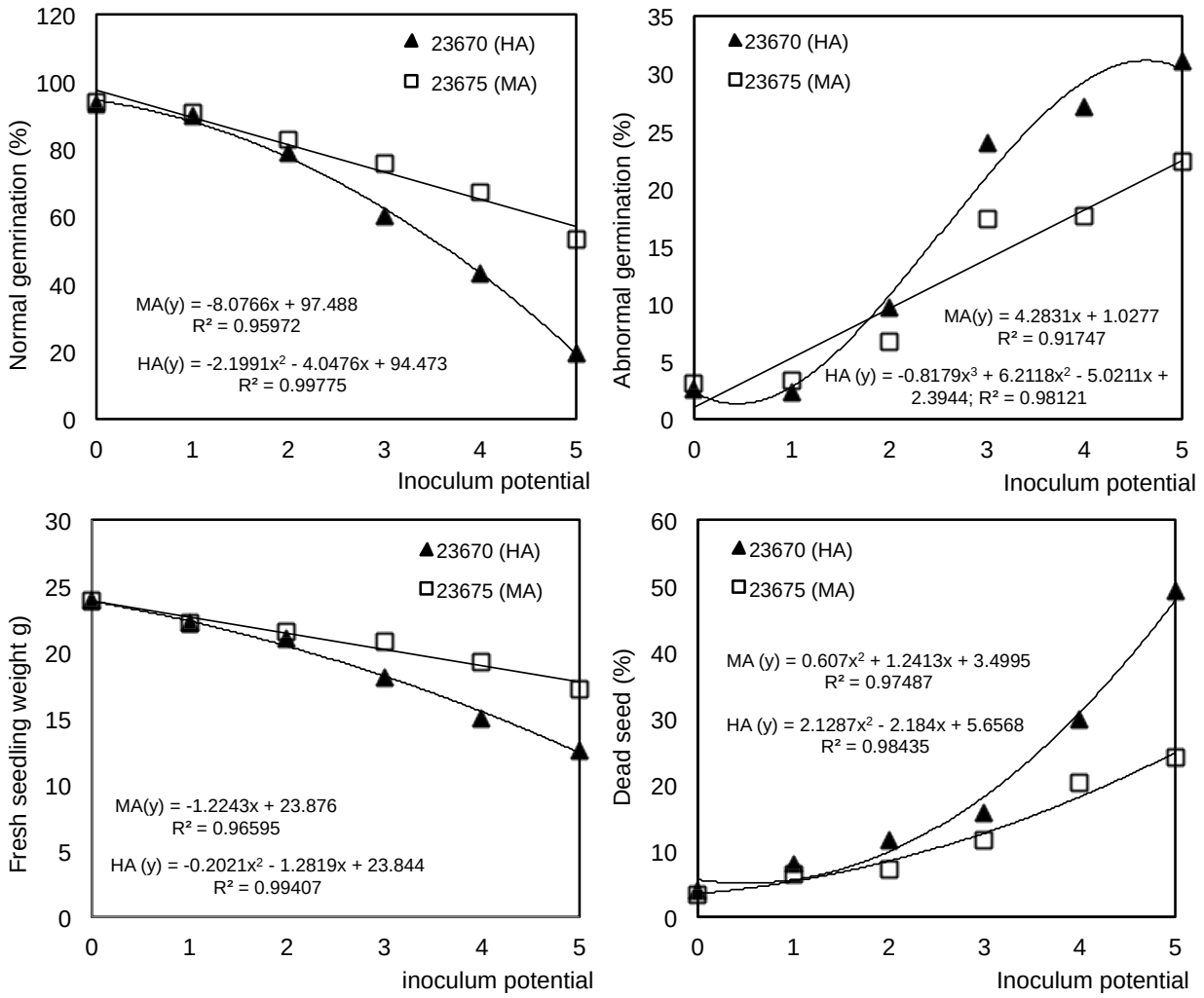

Figure 5.3 Relationship between germination (%) and inoculum potential treatments (0 to 5) in contact with the soybean seeds for each of the seedborne *F. proliferatum* isolates tested (highly aggressive [HA], 23670; moderately aggressive [MA], 23675). Mock-inoculated seeds, treatment 0; Inoculum potential, treatment 1 (2.5×10^1 conidia ml^{-1}); Inoculum potential, treatment 2 (2.5×10^2 conidia ml^{-1}); Inoculum potential, treatment 3 (2.5×10^3 conidia ml^{-1}); Inoculum potential, treatment 4 (2.5×10^4 conidia ml^{-1}); Inoculum potential, treatment 5 (2.5×10^5 conidia ml^{-1}).

Table 5.1 Effect of seedborne *Fusarium proliferatum* isolates on soybean seed germination, and fresh seedling weight under laboratory conditions.

Treatments	Isolates	Aggressiveness [a]			Normal Germ. (%)	Abnormal Germ. (%)	Dead Seed (%)	F.S.W. (g)
		LA	MA	HA				
Mock-inoculated control	NA	0.2	--	--	93.7	1.7	4.6	20.3
F. proliferatum	23668	--	1.7	--	61.7 ***	7.3	31.0 ***	15.8 **
	23669	--	1.7	--	61.0 ***	8.0	31.0 ***	16.6 *
	23670	--	--	2.1	36.0 ***	21.3 ****	42.7 ***	14.8 ***
	23671	--	1.8	--	55.3 ***	14.3 **	30.4 ***	17.2 *
	23672	--	--	2	49.0 ***	15.3 **	35.7 ***	15.7 **
	23673	--	--	2.1	41.3 ***	18.7 ***	40.0 ***	13.5 ***
	23674	--	--	2	50.7 ***	9.3	40.0 ***	14.7 **
	23675	--	1.2	--	78.0 **	5.0	17.0 *	21.0

[a] Isolates having pathogenicity scores < 1, between 1 and 2, and > 2 were identified with low (LA), moderate (MA), and high aggressiveness (HA), respectively; $^{*, **, ***}$ Significantly different from control (mock-inoculated seeds) at the $P \leq 0.05$, $P \leq 0.001$, and $P \leq 0.0001$ levels using Dunnetts test. [†]Results are the means of three experiments. Normal Germ. = Normal germination; Abnormal Germ. = Abnormal germination; Dead Seed = Dead seed; F.S.W.=Fresh Seedling weight.

Table 5.2 Effect of seedborne *Fusarium proliferatum* isolates on soybean seedling vigor measured by the percentage of initial and final stand as well as dry aerial and root weight of plants.[†]

Isolates	Code	I.S. (%)	F.S. (%)	D.A.W. (g)	D.R.W. (g)
Mock-inoculated control	MCO	95.7	95.0	2.9	1.5
F. proliferatum	23668	75.3	75.0	2.4	1.1*
	23669	50.7 **	51.7 **	1.6 **	0.8 **
	23670	54.3 **	55.4 **	1.7 **	0.6 ***
	23671	59.7 *	58.3 **	1.9	0.9 **
	23672	61.3 *	60.7 *	2.0	0.9 **
	23673	52.0 **	53.3 **	1.7 **	0.8 *
	23674	80.7	81.0	2.6	0.9 **
	23675	76.0	78.7	2.5	0.9 *

*, **, ***Significantly different from mock-inoculated control at the $P \leq 0.05$, $P \leq 0.001$, and $P \leq 0.0001$ levels using Dunnett's test. [†]Results are the means of three experiments. I.S. = Initial stand; F.S. = Final stand; D.A.W. = Dry aerial weight; D.R.W. = Dry root weight.

Table 5.3 Correlation coefficients (*r*) and probabilities (*P*) for linear correlations among soybean seed germination and vigor characteristics in plants with inoculated seeds with *F. proliferatum* isolates in laboratory and greenhouse assays. †

Variable	Final stand GH - (%)	Root weight GH - (g)	Aerial weight GH - (g)	Normal Germ. LAB - (%)	Abnormal Germ. LAB - (%)	Dead Seed LAB - (%)	F.S.W. LAB - (g)	DSI LAB
Initial final GH (%) (*r*)	0.9888	0.7727	0.9749	0.4610	-0.4248	-0.4021	0.2510	-0.4815
P	< 0.0001	< 0.0001	< 0.0001	0.01550	0.0272	0.0376	0.2066	0.011
Final stand GH (%)	…	0.77732	0.96395	0.43087	-0.4105	-0.36806	0.21853	-0.44509
	…	< 0.0001	< 0.0001	0.02490	0.0334	0.0589	0.2735	0.02
Root weight GH (g)	…	…	0.71531	0.47087	-0.46355	-0.39376	0.2368	-0.50687
	…	…	< 0.0001	0.01320	0.0149	0.0421	0.2343	0.007
Aerial weight GH (g)	…	…	…	0.41867	-0.38772	-0.36398	0.26069	-0.41526
	…	…	…	0.02970	0.0457	0.062	0.1737	0.0312
Normal Germ. LAB (%)	…	…	…	…	-0.80071	-0.94048	0.81077	-0.96107
	…	…	…	…	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Abnormal Germ. LAB (%)	…	…	…	…	…	0.54947	-0.52844	0.70497
	…	…	…	…	…	< 0.0001	< 0.0001	< 0.0001
Dead Seed LAB (%)	…	…	…	…	…	…	-0.083101	0.9405
	…	…	…	…	…	…	< 0.0001	< 0.0001
Fresh seedling weight LAB (g)	…	…	…	…	…	…	…	-0.76669
	…	…	…	…	…	…	…	< 0.0001

†Data combined for three laboratory (LAB) and greenhouse (GH) experiments.

Table 5.4 *P* values of *F* tests from analysis of variance for soybean seed quality variables as measured with six inoculum potentials and two soybean seedborne *Fusarium proliferatum* isolates ($\alpha = 0.05$).

	Df	Normal Germ. (%)	Abnormal Germ. (%)	Dead Seed (%)	F.S.W. (g)
Agressiveness	1	< 0.001	0.0061	< 0.001	< 0.001
Inoculum potential (IP)	5	< 0.001	< 0.001	< 0.001	< 0.001
Agressiveness * IP	5	0.0002	0.0190	0.0048	0.0026

Normal Germ. = Normal germination; Abnormal Germ. = Abnormal germination; Dead Seed = Dead seed; F.S.W.=Fresh Seedling weight.

Table 5.5 Influence of inoculum potential treatment (IP) of two-soybean seedborne *Fusarium proliferatum* isolates on soybean seed quality variables, in laboratory condition ($\alpha = 0.05$).

	Normal Germ. (%)		Abnormal Germ. (%)		Dead seed (%)		F.S.W. (g)	
IP[a]	23670[b]	23675[c]	23670	23675	23670	23675	23670	23675
IP0	93.5	93.7	2.7	3.1	3.9	3.3	23.8	23.9
IP1	89.7	91.1	2.3	3.3	8.0	6.3	22.2	22.2
IP2	78.7**	83.0	9.7	6.7	11.7	7.3	21.0	21.5
IP3	60.3***	76.0**	24.0**	17.3**	15.7*	11.7**	18.1**	20.8*
IP4	43.3***	67.0***	27.0***	17.7**	29.7**	20.3***	15.0***	19.3**
IP5	19.7***	53.0***	31.0***	22.3**	49.3***	24.0***	12.6***	17.2**

[a]Inoculum potential treatments (IP); Mock-inoculated seeds, treatment IP0; Inoculum potential, treatment IP1 (2.5×10^1 conidia ml^{-1}); Inoculum potential, treatment IP2 (2.5×10^2 conidia ml^{-1}); Inoculum potential, treatment IP3 (2.5×10^3 conidia ml^{-1}); Inoculum potential, treatment IP4 (2.5×10^4 conidia ml^{-1}); Inoculum potential, treatment IP5 (2.5×10^5 conidia ml^{-1}). [b]Highly aggressive (HA) *F. proliferatum* isolate (23670). [c]Moderately aggressive (MA) *F. proliferatum* isolate (23675). $^{*, **, ***}$ Significantly different from mock-inoculated seeds (IP0) at the $P \leq 0.05$, $P \leq 0.001$, and $P \leq 0.0001$ levels using Dunnetts test. Normal Germ. = Normal germination; Abnormal Germ. = Abnormal germination; Dead Seed = Dead seed; F.S.W. = Fresh Seedling weight.

Chapter 6 - Conclusions and future work

The main objective of this dissertation was to characterize the identity, frequency, and pathogenicity of soybean seedborne *Fusarium* spp. in the state of Kansas.

The first objective of this study (Chapter 3; Appendix A; Appendix B) was to survey soybean seed samples throughout the state of Kansas using a culture-dependent approach (semi-selective medium) for characterization of *Fusarium* species. Overall, nine *Fusarium* spp. were found in naturally infected soybean seeds. Two new soybean seedborne *Fusarium* spp., *F. thapsinum* and *F. fujikuroi*, were detected and reported for the first time in the United States. *Fusarium semitectum, F. proliferatum,* and *F. verticillioides* were the three most frequently identified *Fusarium* spp. observed during the three-year survey. Besides identification and frequency, pathogenicity tests were conducted in laboratory and greenhouse environments to better understand the potential of *Fusarium* spp. to decrease soybean seed quality. Using artificially infested seed, *F. proliferatum, F. thapsinum, F. fujikuroi, F. oxysporum*, and *F. graminearum* decreased soybean seed germination and vigor. Also, those *Fusarium* spp. were able to significantly incite seedling damping-off in greenhouse assays. *Fusarium acuminatum, F. equiseti, F. semitectum,* and *F. verticillioides* were identified as non-pathogenic to soybean in both environments (Chapter 3). Also, seedborne isolates of *F. proliferatum* exhibited significant variation regarding aggressiveness ranging from low to high. Along with soybeans, *F. fujikuroi* was also tested for pathogenicity against rice plants and results suggest that soybean seedborne *F. fujikuroi* causes reduced seed germination and seedling damping-off in rice and elongation of plant internodes. Additional screening for pathogenicity should include more soybean genotypes

and seedborne *Fusarium* isolates of pathogenic as well as non-pathogenic species. It would be important to investigate the influence of soybean seedborne *Fusarium* spp. in other commonly planted crops in Midwest such as sorghum, wheat, and sunflower. In addition to laboratory and greenhouse screening, future studies should also include field experiments to investigate the relationship between the presence of pathogenic *Fusarium* spp. in seeds and yield losses.

In Chapter 4, the frequency of *Fusarium* spp. among and within asymptomatic soybean seed samples was investigated using DNA metabarcoding. Using this sensitive technology, the genus *Fusarium* was identified in each of the seed samples tested. Seven *Fusarium* spp. were identified using Illumina MiSeq platform including *F. acuminatum, F.proliferatum, F.thapsinum, F. merismoides, F. solani*, *Fusarium* sp., and *F. semitetum*. Interestingly, two known pathogenic seedborne groups such as *F. proliferatum* and *F. thapsinum* were identified in every single asymptomatic soybean seed analyzed in this study. In addition, preliminary experiments (Appendix C) also showed the presence of these two pathogenic species within the three major soybean seed tissues, seed coat cotyledons, and embryo axis of high quality seeds analyzed. However, the significance and implications of healthy or asymptomatic soybean seeds being inhabited by plant pathogens remain to be elucidated in future studies. More soybean seed samples representing different genotypes, physiological, physical, biochemical, and environmental conditions should be considered. Moreover, it would be interesting to test and use multiple methodologies for DNA extraction of the fungal community from soybean seeds. Also, future experiments should consider using different primer sets and databases, specifically designed for plant pathogenic *Fusarium* spp. These experiments would help us to gain more knowledge regarding the pathogenic fungal community inhabiting commercial seed lots.

DNA metabarcoding has tremendous potential in seed pathology studies and may expand and refine our understanding regarding the influence and significance of seedborne pathogens on seed quality, mainly due to its potential to unveil the set of fungi that commonly live within the seed, independently of host genotype, physical, physiological, and environmental conditions.i.e. the seed *core mycobiome*. The findings of this study for example, suggest that at least two pathogenic seedborne *Fusarium* spp., *F. proliferatum* and *F. thapsinum,* are common inhabitants of the soybean seed core mycobiome. The reasons why and how important pathogenic groups are, or become, common inhabitants of the seed core mycobiome is an interesting question that should be further investigated. Each newly identified pathogen inhabiting the seed core mycobiome, comprises a step towards improvement and development of new seed and seedling disease management strategies. Understanding the soybean seed core mycobiome can help us to better address and estimate inoculum thresholds for pathogenic species, which can contribute to the development of new seed health testing methods.

The objectives of Chapter 5 were to understand the influence of inoculum potential (i.e., amount of inoculum) and the aggressiveness of pathogenic seedborne *Fusarium* spp., in particular, *F. proliferatum*, on soybean seed quality. The presence of pathogenic *F. proliferatum* within asymptomatic soybean seeds (Chapter 4 and Appendix C) and its aggressiveness variability (Chapter 3) led us to hypothesize that the influence of this pathogen on soybean seed quality is dependent on the amount and aggressiveness of inoculum present in the seed. Results from laboratory pathogenicity assays showed that the amount of inoculum (i.e. inoculum potential) significantly influenced the decrease of soybean seed quality. Moreover, the decline in soybean seed quality was also influenced by the aggressiveness of the *F. proliferatum* isolate.

Most importantly, at low inoculum potential, none of the *F. proliferatum* isolates were able to significantly reduce seed quality. Similar responses were also observed with *F. verticillioides* and *F. semitectum,* and their ability to decrease soybean seed quality were also influenced by the amount of inoculum present in the seed (Appendix D). These results may explain the presence of pathogenic *Fusarium* species, such as *F. proliferatum*, within naturally infected and asymptomatic soybean seeds observed in previous studies (see Chapter 4 and Appendix C). In addition, preliminary results found in Appendix D also indicate that soybean variety plays a major role in soybean seed, and likely seedling, disease development. Ideally, more detailed experiments are necessary to unveil the mechanisms involved in the pathogenisis of soybean seedborne *Fusarium* spp. For example, RNAseq experiments should be considered to dissect the molecular mechanisms governing the lifestyle switch (non-pathogenic to pathogenic) present among soybean seedborne *Fusarium* spp. Gene expression profiling could serve as a baseline for the search of biomarkers to be used in next-generation seed health testing.

The movement of plant pathogens throughout infected seeds is a concern and represents a significant challenge facing modern agriculture due to its potential to introduce exotic plant diseases into new hosts and areas. The correct identification and frequency of soybean seedborne *Fusarium* spp. as well as information regarding their potential pathogenicity to the crop is essential for the development of effective disease control management strategies, improvements in seed certification and quarantine programs, and as a basis for making decisions to protect the future of agriculture.

Appendix A - Pedrozo and Little (2014)

This study has been published as the following: Pedrozo, R., and Little, C. (2014). First report of seedborne *Fusarium thapsinum* and its pathogenicity on soybean (*Glycine max*) in the United States. Plant Disease, 98:1745.

Abstract

A three-year survey from 2010 to 2012 was conducted in Kansas to investigate the identity and diversity of seedborne *Fusarium* spp. in soybean. A total of 408 soybean seed samples from 10 counties were tested. One hundred arbitrarily selected seeds from each sample were surface-sterilized for 10 min in a 1% sodium hypochlorite solution to avoid contaminants and promote the isolation of internal fusaria. Seeds were rinsed with sterile distilled water and dried overnight at room temperature (RT). Surface-sterilized seeds were plated on modified Nash-Snyder medium and incubated at 23 ± 2°C for 7 days. *Fusarium* isolates were single-spored and identified by morphological characteristics on carnation leaf agar (CLA) and potato dextrose agar (PDA) (Leslie and Summerell 2006). From 276 seedborne *Fusarium* isolates, six were identified as *F. thapsinum* (Klittich et al. 1997). On CLA, *F. thapsinum* isolates produced abundant mycelium and numerous chains of non-septate microconidia produced from monophialides. Microconidia were club-shaped and some were napiform. No chlamydospores were found. On PDA, three of the isolates presented characteristic dark yellow pigmentation and three were light violet. Confirmation of the isolates to species was based on sequencing of an elongation factor gene (EF1-α) segment using primers EF1 and EF2 and the beta-tubulin gene using primers Beta1 and Beta2 (Geiser et al. 2004). Sequence results (~680 bp, EF primers; ~600 bp, beta-tubulin primers) were confirmed by using the FUSARIUM-ID database (Geiser et al. 2004). All isolates matched *F. thapsinum* for both genes sequenced (Accession No. FD01177) at 99% identity. Koch's postulates were completed for two isolates of *F. thapsinum* under greenhouse conditions. Soybean seeds (Asgrow AG3039) were imbibed with 2.5×10^5 conidia ml^{-1} for 48 h. After inoculation, seeds were dried for 48 h at RT. One isolate each of *F. equiseti* and *F. oxysporum* were used as the non-pathogenic and pathogenic inoculation controls,

respectively. In addition, non-inoculated seeds and seeds imbibed in sterile distilled water (mock) were also used. Twenty-five seeds from each treatment were planted in pots (500 ml) with autoclaved soil and vermiculite (1:1). The experiment was a completely randomized design with three replicates (pots) per isolate. The entire experiment was repeated three times. After 21 days, aggressiveness of both *F. thapsinum* isolates was assessed using initial stand (%), final stand (%), and seed mortality (% of non-germinated seeds). Both seedborne *F. thapsinum* isolates caused reduced emergence and final stand, and increased seedling mortality when compared to the non-inoculated and *F. equiseti* controls ($P< 0.0001$). No significant difference was observed between *F. thapsinum* isolates and *F. oxysporum. F. thapsinum* isolates were re-isolated from wilted seedlings and non-germinated seeds, but not from the control treatments. Typically, *F. thapsinum* is considered a pathogen of sorghum, but it has also been recovered from bananas, peanuts, maize, and native grasses (Leslie and Summerell 2006). However, its presence on soybean plant tissues and its pathogenicity has never been reported. To our knowledge, this is the first report of seedborne *F. thapsinum* and its pathogenicity on soybean in the United States.

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Appendix B - Pedrozo *et al.* 2015

This study has been published as following: Pedrozo, R., Fenoglio, J., and Little, C.R. (2015). First report of seedborne *Fusarium fujikuroi* and its potential to cause pre- and post-emergent damping-off on soybean (*Glycine max*) in the United States. Plant Disease, 99:1865.

Abstract

To investigate the identity and diversity of soybean seedborne *Fusarium* spp. in the state of Kansas, a 3-year survey was conducted. A total of 408 soybean samples obtained from nine counties in 2010, six counties in 2011, and 10 counties in 2012 were analyzed for *Fusarium* isolates during the survey. Seeds were surface-sterilized for 10 min with a 1% sodium hypochlorite solution to reduce contaminants and to enhance the isolation of *Fusarium* spp. from internal seed tissues. After incubation, plates were examined and colonies visually identified as *Fusarium* were single-spored by micromanipulation for confirmatory evaluation as described by Leslie and Summerrell (2006). All seedborne *Fusarium* isolates that were identified morphologically and grouped into the *Gibberella fujikuroi* species complex (GFSC) were confirmed based on identity with the translocation elongation factor 1-alpha (TEF-1α) gene. From 94 seedborne GFSC *Fusarium* isolates, six isolates were identified as *F. fujikuroi* using PCR. In order to further confirm the identity of the *F. fujikuroi*-like isolates, five additional markers were used including β-tubulin (tub-2), RNA polymerase second largest subunit (RPB2), histone 3 (H3), calmodulin (cmd), and mitochondrial small subunit (mtSSU). A BLAST search of GenBank (NCBI) showed that the sequences of all markers matched those of *F. fujikuroi* (Accession Nos. JN695742, HF679028, AF158332, and JX910420) with 99 to 100% identity. Koch's postulates were fulfilled using all six isolates under greenhouse conditions. Soybean seeds (AG3039, Asgrow) were imbibed in sterile distilled water with 2.5×10^5 conidia/ml for 48 h. After inoculation, seeds were dried for 48 h at room temperature. Additionally, non-inoculated seeds and seeds mock-inoculated with sterile distilled water were used as controls. Artificially inoculated seeds were planted in pots (500 ml) with autoclaved soil (Reading silt clay loam) and vermiculite (1:1) in the greenhouse. The pathogenicity of the *F.*

fujikuroi isolates was assessed based upon initial stand (% of seedlings germinated after 10 days), final stand (% of seedlings germinated after 25 days), and seed mortality (% of pre- and post-emergence damping-off after 25 days). The experiment was a completely randomized design with three replicates (25 seeds/pot) per isolate and the entire experiment was repeated three times. Since *F. fujikuroi* is known to cause bakanae disease on rice (Leslie and Summerell 2006), the isolates were also tested against rice seedlings (cv. Koshihikari, Kitazawa Seed Co.) using the methodology described above. *Fusarium fujikuroi* isolates were able to reduce emergence ($P < 0.0001$), final stand ($P < 0.0001$), and significantly increased seed mortality (pre- and post-emergence damping-off) ($P < 0.0001$) when compared with the controls for both soybean and rice. Furthermore, only on rice, typical bakanae symptoms such as elongation of seedlings were observed. *F. fujikuroi* isolates were reisolated from symptomatic seedlings and non-germinated seeds in both crops, but not from the control treatments. *F. fujikuroi* has been recovered from crops other than rice, including maize, wheat, strawberries, and water grass (*Echinochloa* spp.) (Carter et al. 2008; Suga et al. 2014; Wiemann et al. 2013). However, its presence and pathogenicity on soybean have not been previously reported. To our knowledge, this is the first report of soybean seedborne *F. fujikuroi* and its potential to cause pre- and post-emergent damping-off on soybean in the United States.

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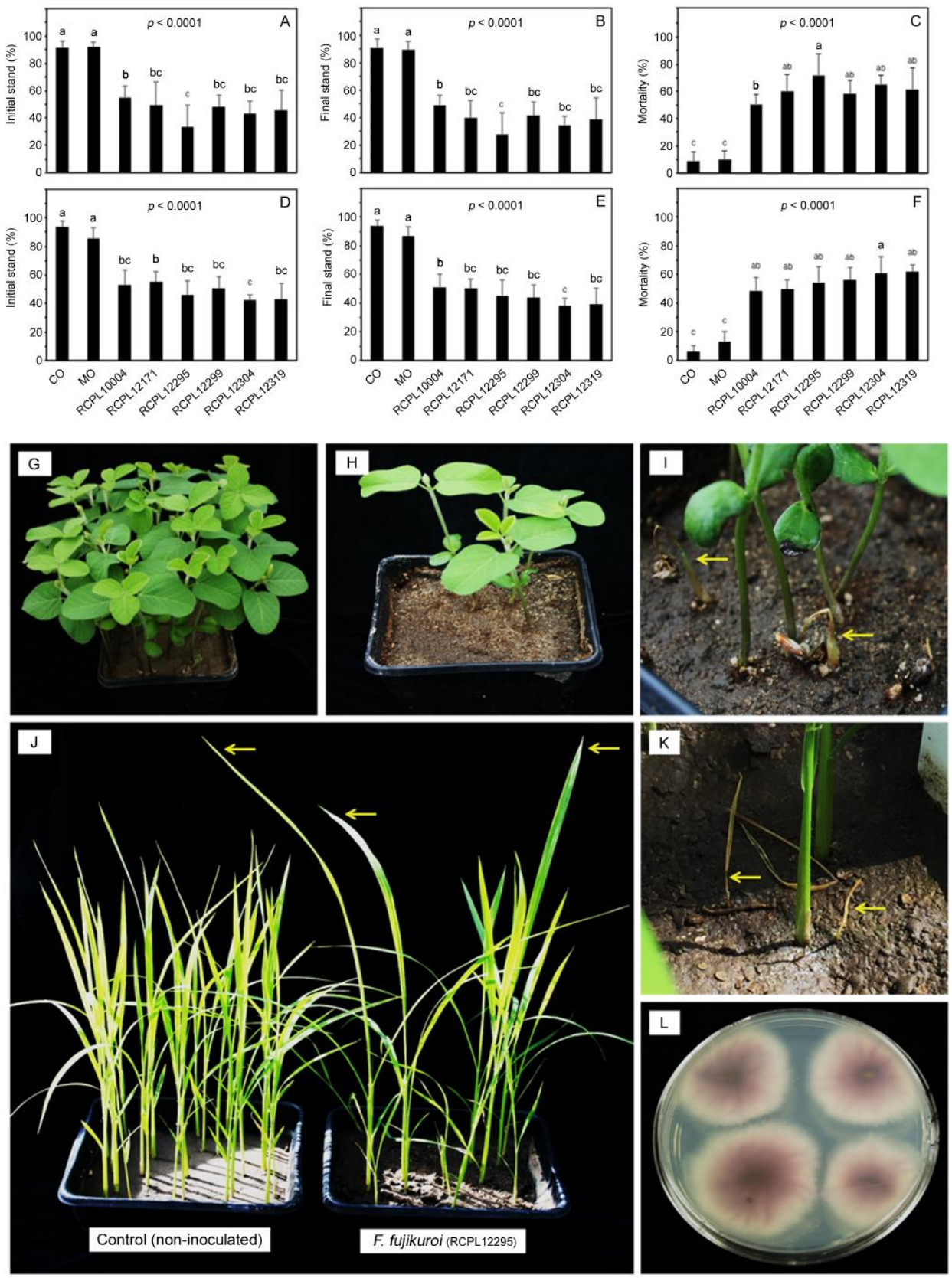
A
B
C
D
E
F
G
H
I
J
K
L
p < 0.0001
Initial stand (%)
Final stand (%)
Mortality (%)
CO
MO
RCPL10004
RCPL12171
RCPL12295
RCPL12299
RCPL12304
RCPL12319
Control (non-inoculated)
F. fujikuroi (RCPL12295)

Figure 1. Effects of seedborne soybean *Fusarium fujikuroi* isolates on soybean and rice seeds artificially inoculated under greenhouse conditions. A, B, and C, response of soybean seedlings inoculated with *F. fujikuroi* isolates. D, E, and F, response of rice seedlings inoculated with *F. fujikuroi* isolates. CO = control, MO = mock-inoculated seeds; RCPL10004, RCPL12171, RCPL12295, RCPL12299, RCPL12304, RCPL12304, and RCPL12319 = *F. fujikuroi* isolates. Means with the same letter are not significantly different according to Tukey-Kramer test (P = 0.05). G and H, non-inoculated seeds (control) presented normal final stand (% of germinated seeds) when compared with *Fusarium*-infected seeds (RCPL12295) 25 days post-inoculation. I, characteristic blighted seedling symptoms (yellow arrows) inoculated with pathogenic seedborne *F. fujikuroi* isolates. J, typical bakanae symptom of internode elongation on rice seedlings (yellow arrows). K, post-emergence damping-off on rice seedlings (yellow arrows). Successful re-isolation of *F. fujikuroi* from symptomatic tissues from soybean and rice seedlings was obtained. L, example of pure culture of *F. fujikuroi* (RCPL12295) isolated from symptomatic soybean seedling tissues.

Table 1. Molecular identification of six *Fusarium fujikuroi* isolates collected during a 3-year survey (2010 to 2012) of soybean seedborne fungi in the state of Kansas, USA.

	TEF 1-α[a]		β-tubulin[b]		RPB2[c]		Histone[d]		Calmodulin[e]		mtSSU[f]	
FFU Isolates	ID (%)[g]	Accession number[h]	ID (%)	Accession number	ID (%)	Accession number	ID (%)	Accession number	ID (%)	Accession number	ID (%)	Accession number
RCPL10004	100	JN695742	100	HF679028	100	HF679028	100	HF679028	99	HF679028	100	JX910420
RCPL12171	100	JN695742	100	HF679028	100	HF679028	100	HF679028	99	HF679028	100	JX910420
RCPL12295	99	HF679028	100	HF679028	99	HF679028	99	HF679028	100	AF158332	100	JX910420
RCPL12299	99	HF679028	100	HF679028	99	HF679028	100	HF679028	99	AF158332	100	JX910420
RCPL12304	99	HF679028	100	HF679028	100	HF679028	100	HF679028	100	AF158332	99	JX910420
RCPL12319	99	HF679028	100	HF679028	99	HF679028	100	HF679028	99	HF679028	100	JX910420

[a]Translocation elongation factor 1-alpha primer set (TEF1: 5'-ATGGGTAAGGA(A/G)GACAAGAC and TEF2: 5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT); [b]beta-tubulin (β-tub T01: 5'-AACATGCGTGAGATTGTAAGT and T02: 5'-TCTGGATGTTGTTGGGAATCC; [c]RNA polymerase second largest subunit (5F2: 5'-GGGGWGAYCAGAAGAAGGC and 7cR: 5'-CCCATRGCTTGYTTRCCCAT); [d]Histone 3 primer set (H3-1a: 5'-ACTAAGCAGACCGCCCGCAGG and H3-1b: 5'–GCGGGCGAGCTGGATGTCCTT); [e]Calmodulin (CL1: 5'-GA(GA)T(AT)CAAGGAGGCCTTCTC and Cl2: 5'-TTTTTGCATCATGAGTTGGAC; [f]Mitochondrial small subunit (MS1: 5'-CAGCAGTCAAGAATATTAGTCAATG and MS2: 5'-GCGGATTATCGAATTAAATAAC; [g,h]Percent identity (ID) to the reference GenBank (NCBI) strain and accession number.

Appendix C - Position of *Fusarium* spp. within the three major soybean seed tissues: seed coat, cotyledons, and embryo axis.

This supplementary material shows preliminary results regarding the exploration of the fungal community, with focus on *Fusarium* spp., within the three major soybean seed tissues, seed coat, cotyledons and embryo axis of asymptomatic seeds. Two soybean genotypes were used to explore the position of *F. proliferatum* within soybean seed tissues using DNA metabarcoding, Asgrow 'AG3039' (Monsanto, Inc.; St. Louis, MO, USA) and Midland 4263 (Sylvester Ranch INC, Ottawa, KS, USA). The position of *Fusarium* spp. in naturally infected and asymptomatic soybean seed samples were based on the amplification of the ITS2 region of seedborne fungi following methodology described in Chapter 4.

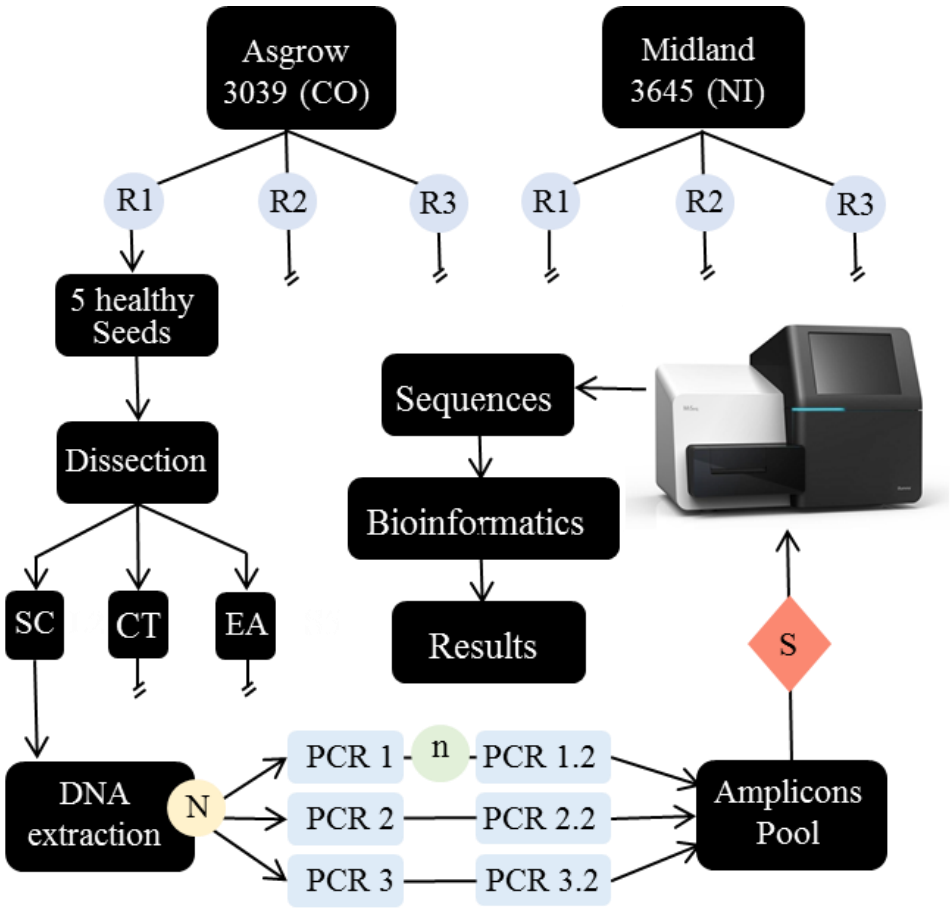

Figure C.1 Midland 4263 (Naturally infected, NI) and AG3039 (High quality seeds, CO), were used to explore the location of *Fusarium* spp. among three major soybean seed tissues, seed coat (SC), cotyledons (CT) and embryo axis (EA). Three biological replicates were used from each genotype (R1, R2, and R3). Each replicate was composed of five healthy seeds (asymptomatic seeds). Before DNA extraction, seed tisues were surface sterilized using a 5% bleach solution (0.5 sodium hypochlorite v/v) for 5 minutes and dried overnight at room temperature. After extraction and quantification, the DNA was normalized (N) to 5.0 ng μl^{-1}. Three technical replicates were used for the amplication of the ITS2 region (PCR 1, 2, 3). Nested PCR (n) was conducted after the first round of PCR using barcoded reverse ITS4 primers. Sequencing (S), using Illumina V3. Technology, was used after library construction.

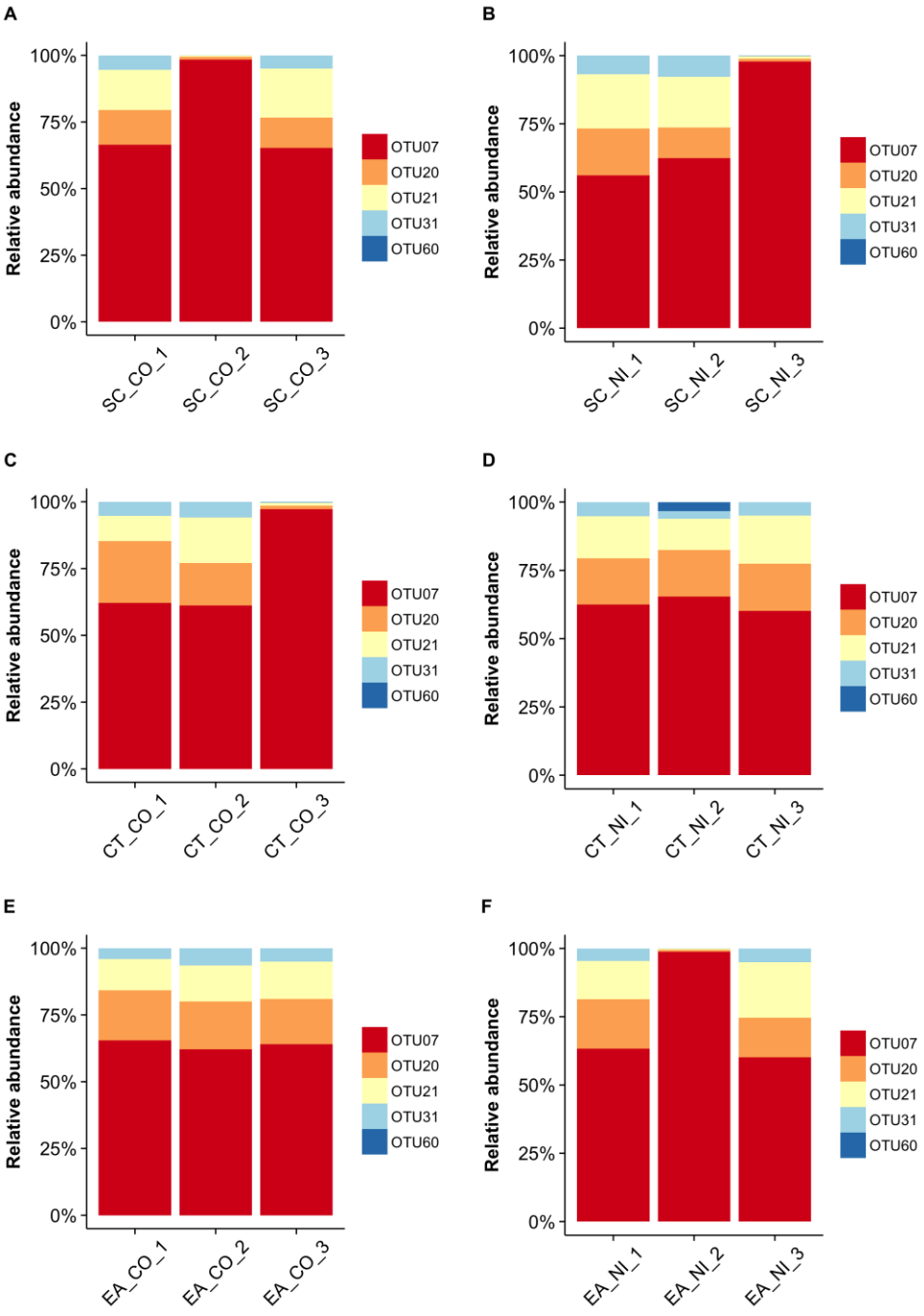

Figure C.2 Presence of *Fusarium* species (OTU07, OTU20, OTU21, OTU31, OTU60) among three major soybean seed tissues, seed coat (CT), cotyledons (CT) and embryo axis (EA). Among the OTUs observed, two were identified as *F. proliferatum* (OTU07), one as one as *F. thapsinum* (OUT20), one as *F. acuminatum* (OTU21), one as *F. merismoides* (OTU31), and once as *Fusarium* sp. (OTU60). Two genotypes, Midland 4263 (Naturally infected, NI) and AG3039 (High quality seeds, CO), were used in this study. Three replicates (1, 2, and 3) were observed from CO (A, C and E) and from NI seed tissues (B, D, and F).

Table C.1 Physical, physiological and sanitary quality of the soybean seed samples Midland 4264 and Asgrow (3039) used in this study.

Code	Genotype	Physical (%)	Physiological (%)		Sanitary (%)	
			Tz [a]	Germ. [b]	AAL [c]	FPR [d]
NI [e]	Midland - 4263	1.5	96.7	95.8	1.0	8.0
CO [f]	Asgrow - 3039	1.5	97.4	94.0	0.0	0.0

[a] Tetrazolium test (Tz); [b] Germination test (Germ.); [c] *Alternaria alternaria* (AAL); [d] *Fusarium proliferatum* (FPR); [e] Naturally infected soybean seeds (NI); [f] High quality commercial seeds (CO).

Table C.2 Primers and Multiplexing IDentifiers (MIDs) sequences used for sample identification in Illumina MiSeq ITS2 amplicon library. Sample ID includes (1) individual soybean seed tissue replicate (seed coat, SC1-SC3; cotyledons, CT1-CT3; embryo axis, EA1-EA3); (2) soybean seed sample (S3 and CO*). *High quality soybean seed sample (CO).

Forward primer (ITS 1F) - CTTGGTCATTTAGAGGAAGTA

Forward primer (f ITS1 7) - GTGARTCATCGAATCTTTG

Reverse primer (ITS 4) - TCCTCCGCTTATTGATATGC

Sample_ID	MIDs	Primers (5' - MIDs + ITS4 - 3')
SC1_S3	AGGCTTACGTGT	AGGCTTACGTGTTCCTCCGCTTATTGATATGC
SC2_S3	TCTCTACCACTC	TCTCTACCACTCTCCTCCGCTTATTGATATGC
SC3_S3	ACTTCCAACTTC	ACTTCCAACTTCTCCTCCGCTTATTGATATGC
CT1_S3	CTCACCTAGGAA	CTCACCTAGGAATCCTCCGCTTATTGATATGC
CT2_S3	GTGTTGTCGTGC	GTGTTGTCGTGCTCCTCCGCTTATTGATATGC
CT3_S3	CCACAGATCGAT	CCACAGATCGATTCCTCCGCTTATTGATATGC
EA1_S3	TATCGACACAAG	TATCGACACAAGTCCTCCGCTTATTGATATGC
EA2_S3	GATTCCGGCTCA	GATTCCGGCTCATCCTCCGCTTATTGATATGC
EA3_S3	CGTAATTGCCGC	CGTAATTGCCGCTCCTCCGCTTATTGATATGC
SC1_CO	GGTGACTAGTTC	GGTGACTAGTTCTCCTCCGCTTATTGATATGC
SC2_CO	ATGGGTTCCGTC	ATGGGTTCCGTCTCCTCCGCTTATTGATATGC
SC3_CO	TAGGCATGCTTG	TAGGCATGCTTGTCCTCCGCTTATTGATATGC
CT1_CO	AACTAGTTCAGG	AACTAGTTCAGGTCCTCCGCTTATTGATATGC
CT2_CO	ATTCTGCCGAAG	ATTCTGCCGAAGTCCTCCGCTTATTGATATGC
CT3_CO	AGCATGTCCCGT	AGCATGTCCCGTTCCTCCGCTTATTGATATGC
EA1_CO	GTACGATATGAC	GTACGATATGACTCCTCCGCTTATTGATATGC
EA2_CO	GTGGTGGTTTCC	GTGGTGGTTTCCTCCTCCGCTTATTGATATGC
EA3_CO	ATGCCATGCCGT	ATGCCATGCCGTTCCTCCGCTTATTGATATGC

Table C.3 Taxonomic assignment of representative ITS2 OTUs according to UNITE and GenBank reference sequences.

			ID		UNITE	GenBank	
OTUs	Reads	In (%)[a]	Genus	Species	Accession No.	Accession No.	Ident. (%)[b]
OTU01	123,746	100.0	*Alternaria*	*A. alternata*	SH215493.07FU	LT560139	100
OTU02	29,408	100.0	Others*	Others	SH208383.07FU	KP814441	78
OTU03	19,771	100.0	*Diaporthe*	*D. longicolla*	SH185492.07FU	U97658	100
OTU04	18,790	100.0	Unclassified fungi	Unclassified fungi	SH190991.07FU	JX675046	100
OTU05	18,450	100.0	*Wallemia*	*Wallemia* sp.	SH216453.07FU	KF800096	100
OTU06	18,311	100.0	*Cryptococcus*	*Cryptococcus* sp.	SH197623.07FU	LC018794	100
OTU07	18,033	100.0	*Fusarium*	*F. proliferatum*	SH219673.07FU	X94171	99
OTU08	17,810	100.0	Unclassified fungi	Unclassified fungi	SH203201.07FU	KC785574	100
OTU09	8,478	77.8	Unclassified fungi	Unclassified fungi	SH216453.07FU	KT799189	100
OTU10	8,344	77.8	Others	Others	|SH010949.07FU	FJ936151	92
OTU11	7,760	100.0	*Wallemia*	*Wallemia* sp.	SH216454.07FU	FR682244	97
OTU12	7,144	38.9	*Rhodotorula*	*R. yarrowii*	SH198363.07FU	NR:073328	100
OTU13	7,104	66.7	Unclassified fungi	Unclassified fungi	SH197623.07FU	KU515728	100
OTU14	3,777	72.2	*Exserohilum*	*E. rostratum*	SH211295.07FU|	KU945863	100
OTU15	3,726	50.0	*Tilletiopsis*	*T. washingtonensis*	SH186666.07FU	HQ115649	99
OTU16	2,894	88.9	*Epicoccum*	*E. nigrum*	SH207241.07FU	KU254609	100
OTU17	1,156	55.6	*Tilletiopsis*	*T. washingtonensis*	SH186666.07FU	KC460875	100
OTU18	729	27.8	*Penicillium*	*P. roqueforti*	SH207151.07FU	NR:103621	100
OTU19	579	61.1	*Aspergillus*	*A. ruber*	SH179237.07FU	U18357	100
OTU20	564	100.0	*Fusarium*	*F. thapsinum*	SH219673.07FU	KM589051	100
OTU21	497	100.0	*Fusarium*	*F. acuminatum*	SH219674.07FU	KU382624	100
OTU22	312	100.0	*Alternaria*	*A. infectoria*	SH216783.07FU	Y17067	100
OTU23	310	100.0	*Diaporthe*	*D. caulivora*	SH185506.07FU	KT895390	99
OTU24	261	100.0	Unclassified fungi	Unclassified fungi	SH199073.07FU	KC753422	99
OTU25	249	100.0	Others	Others	SH211110.07FU	DQ421255	95
OTU26	226	100.0	*Malassezia*	*M. restricta*	SH176394.07FU	NR:103585	100

OTU27	219	100.0	*Malassezia*	*M. sympodialis*	SH188402.07FU	KM454159	100
OTU28	192	27.8	Others	Others	SH216453.07FU	JF497133	95
OTU29	172	100.0	Unclassified fungi	Unclassified fungi	SH200466.07FU	KF800626	100
OTU30	159	100.0	*Phlebia*	*P. chrysocreas*	SH192450.07FU	KP135358	99
OTU31	154	100.0	*Fusarium*	*F. merismoides*	SH175278.07FU	AB586998	100
OTU32	115	100.0	*Clitocybe*	*C. vibecina*	SH218334.07FU	JF907821	100
OTU33	108	83.3	*Penicillium*	*P. brevicompactum*	SH199400.07FU	LN833549	100
OTU34	107	100.0	*Schizophyllum*	*S. commune*	SH190191.07FU	LC068797	100
OTU35	100	100.0	*Erysiphe*	*E. polygoni*	SH187440.07FU	AF011308	100
OTU36	90	94.4	*Aureobasidium*	*A. namibiae*	SH195774.07FU	KT693730	100
OTU37	78	22.2	*Wallemia*	*Wallemia* sp.	SH216453.07FU	FJ524297	100
OTU38	64	55.6	*Aspergillus*	*Aspergillus* sp.	SH182491.07FU	GU910689	100
OTU39	63	83.3	*Bulleromyces*	*B. albus*	SH215453.07FU	HE650882	100
OTU40	61	61.1	Others	Others	--	KM247468	95
OTU41	52	94.4	*Baeospora*	*B. myosura*	SH187911.07FU	LN714524	100
OTU42	47	83.3	*Nigroporus*	*N. vinosus*	SH190478.07FU	JX109857	100
OTU43	38	88.9	*Biappendiculispora*	*B. japonica*	SH532144.07FU	LC001730	99
OTU44	33	11.1	Unclassified fungi	Unclassified fungi	--	KM493544	98
OTU45	32	77.8	Unclassified fungi	Unclassified fungi	|SH215453.07FU	KF800580	99
OTU46	31	94.4	Unclassified fungi	Unclassified fungi	SH210215.07FU	KT202892	100
OTU47	28	11.1	Unclassified fungi	Unclassified fungi	SH190991.07FU	JX675046	98
OTU48	27	50.0	*Cladosporium*	*C. subuliforme*	SH212842.07FU	LN834396	100
OTU49	26	72.2	*Resinicium*	*R. friabile*	SH204932.07FU	DQ826545	98
OTU50	25	5.6	Unclassified fungi	Unclassified fungi	--	KP892326	100
OTU51	25	55.6	*Penicillium*	*Penicillium* sp.	SH207150.07FU	KR905616	100
OTU52	22	5.6	Unclassified fungi	Unclassified fungi	SH216453.07FU	KT799189	97
OTU53	22	16.7	*Alternaria*	*Alternaria* sp.	SH215493.07FU	GU721735	99
OTU54	22	77.8	Others	Others	SH179952.07FU	KT581876	85
OTU55	21	88.9	Unclassified fungi	Unclassified fungi	SH190991.07FU	JX67504	99
OTU56	20	27.8	*Cladosporium*	*C. sphaerospermum*	SH216250.07FU	LN834390	100
OTU57	19	66.7	*Phlebiella*	*P. borealis*	SH532759.07FU	KP814210	99

OTU58	19	66.7	*Epicoccum*	*E. nigrum*	SH207241.07FU	KU204774	100
OTU59	18	16.7	*Wallemia*	*Wallemia* sp.	SH216453.07FU	GU370753	98
OTU60	16	22.2	*Fusarium*	*Fusarium* sp.	SH219673.07FU	X94171	97
OTU61	12	16.7	*Diaporthe*	*D. longicolla*	SH185492.07FU	U97658	98
OTU62	12	11.1	Unclassified fungi	Unclassified fungi	SH190991.07FU	JX675046	98
OTU63	12	22.2	*Wallemia*	*Wallemia* sp.	SH216453.07FU	JF497133X	97
OTU64	12	33.3	*Alternaria*	*Alternaria* sp.	SH215493.07FU	GU721735	97
OTU65	11	44.4	Unclassified fungi	Unclassified fungi	SH179952.07FU	KT581876	87
OTU66	11	11.1	*Wallemia*	*Wallemia* sp.	SH216453.07FU	FJ770080	98

[a]Percentage of infected seed tissues (Incidence; In). [b]BLAST searches to known sequences in the NCBI databases were used for molecular identification of soybean seedborne fungi.*Reads presenting less than 97% identity with accessed strains from UNITE and GeneBank were identified as “others,” which represents unidentified sequences.

Table C.4 Taxonomic assignment of soybean seedborne fungi groups (phylum; unclassified fungi; and others), number of OTUs taxonomically assigned to a group (OTUs), % of infected seed tissues (incidence; In).

				Reads (n = 320,724)[a]		
Goups	Genera	In (%)	OTUs[b]	SC	CT	EA
Ascomycetes	*Alternaria*	100	4	36,602	50,333	37,157
(N = 171,919)	*Aspergillus*	58	2	130	505	8
	Aureobasidium	100	1	28	31	31
	Biappendiculispora	83	1	16	11	11
	Cladosporium	50	2	20	12	15
	Diaporthe	100	3	15,554	4,242	297
	Epicoccum	100	2	39	2,859	15
	Erysiphe	100	1	32	35	33
	Exserohilum	66	1	3,146	7	624
	Fusarium	100	5	8,839	5,281	5,144
	Penicillium	83	3	78	433	351
Basidiomycetes	*Baeospora*	100	1	14	17	21
(N = 57,699)	*Bulleromyces*	83	1	24	24	15
	Clitocybe	100	1	33	39	43
	Cryptococcus	100	1	81	9,976	8,254
	Malassezia	100	2	98	226	121
	Nigroporus	83	1	18	10	19
	Phlebia	100	1	49	60	50
	Phlebiella	50	1	4	9	6
	Resinicium	66	1	9	8	9
	Rhodotorula	33	1	3	3,764	3,377
	Schizophyllum	100	1	30	40	37
	Tilletiopsis	50	2	3,817	865	200
	Wallemia	100	6	7,030	4,962	14,337
Others[c]	--	100	6	17,036	279	20,961
Unclassified Fungi	--	100	15	14,157	22,894	15,779

[a]Total number of reads (sequences) among three major soybean seed tissues, seed coat (SC), cotyledons (CT) and embryo axis (EA); [b]Total number of OTUs identified among SC, CT, and EA in both genotype studied; [c]Reads presenting less than 97% identity with accessed strains from UNITE and GeneBank were identified as "others," which represents unidentified sequences.

Table C.5 Identification of *Fusarium* spp. identified among three major soybean seed tissues.

Species	OTUs	Number of reads		
		Seed Coat	Cotyledons	Embryo Axis
F. proliferatum	OTU07	8,449	4,782	4,802
F. thapsinum	OTU20	166	232	166
F. acuminatum	OTU21	170	195	132
F. merismoides	OTU31	53	58	43
Fusarium sp.	OTU60	1	14	1

Table C.6 Incidence of *Fusarium* spp. observed among three major soybean seed tissues, seed coat (SC), cotyledons (CT), and embryo axis (EA).

		Incidence (%)					
		SC		CT		EA	
Fusarium spp.	OTUs	CO [a]	NI [b]	CO	NI	CO	NI
F. proliferatum	OTU07	100	100	100	100	100	100
F. thapsinum	OTU20	100	100	100	100	100	100
F. acuminatum	OTU21	100	100	100	100	100	100
F. merismoides	OTU31	100	100	100	100	100	100
Fusarium sp.	OTU60	0	33	33	33	0	33

[a] High-quality seeds (CO; Asgrow 3039); [b] Naturally infected seeds (NI; Midland 4263)

Appendix D - Pedrozo and Little (2016)

This study has been published as following journal article: Pedrozo, R., & Little, C. R. (2016). *Fusarium verticillioides* inoculum potential influences soybean seed quality. European Journal of Plant Pathology, doi:10.1007/s10658-016-1127-z. Supplementary information can be accessed online.

Abstract

F. verticillioides (FVE) is an important *Fusarium* species that has been recovered from soybean seeds. In other crops, such as maize, it has the potential to decrease seed germination and vigor. The objective of this study was to evaluate the influence of seed inoculum potential (amount of inoculum present in seeds) of FVE on soybean seed quality, as measured by germination of artificially inoculated seeds. Seeds were inoculated with 2.5×10^5 FVE conidia/ml for one minute as the low inoculum potential seed treatment. For the high inoculum potential seed treatment, inoculated seeds were inoculated with the same conidia suspension, but osmoconditioned on potato dextrose agar +8% mannitol for 48 h. Two soybean genotypes, 'AG3039' and 'KSU3406' were tested. Analysis of seeds inoculated with the low inoculum potential treatment showed that none of the FVE isolates tested and only the positive controls were able to significantly reduce soybean seed germination ($P < 0.001$) when compared with the mock-inoculated control for both genotypes tested. Under the high inoculum potential treatment, all three FVE isolates were able to decrease seed germination when compared with the mock-inoculated control treatment for both genotypes. This study suggests that *F. verticillioides* has the potential to reduce soybean seed quality, depending on the amount of inoculum present in seeds (inoculum potential), which affects pathogenicity and negatively influences soybean seed germination as well as the establishment of a uniform and healthy stand in the field.

Fusarium spp. are among the most important plant pathogens (Leslie and Summerell 2006). For soybeans, *Fusarium* spp. can range from non-pathogenic to pathogenic with the potential to cause diseases that result in significant economic losses, reduce yield, and impact seed quality (Agarwal and Sinclair 1996; Neergaard 1979). Furthermore, infected seeds can serve as a source of inoculum dispersal, which provides primary inoculum for pathogen infestation and establishment into new hosts and fields (Agarwal and Sinclair 1996; Neergaard 1979). Although the importance of *Fusarium* spp. to soybean crop production has been documented, the role of seedborne *Fusarium* spp. on soybean disease development, especially as seedling pathogens, remains poorly understood and underexplored (Agarwal and Sinclair 1996; Barros et al. 2014).

F. verticillioides (Sacc.) Nirenberg has been recovered from soybean seeds worldwide (Garcia et al. 2012; Ivić et al. 2009; Zelaya et al. 2013; Pedrozo and Little 2015). Although *F. verticillioides* (FVE) has the potential to decrease seed germination and vigor in maize, its influence on seed germination and vigor in soybean remain unexplored. Some studies report that maize seed lots with a high incidence of this fungus experience little or no reduction in germination or seedling growth, while others may be seriously affected by the fungus (Machado et al. 2013; Munkvold et al. 1997; Oren et al. 2003). Besides the incidence of a pathogen in a seed lot, other variables should be considered to evaluate the effect of microorganisms on seed germination and vigor, of which the amount of inoculum (inoculum potential) present in the seed plays an important role (Agarwal and Sinclair 1996; Neergard 1979). Machado et al. (2013) reported that the most severe effects of *F. verticillioides* on the development of maize seed and seedlings and adult plants were observed at the highest levels of inoculum potential. On soybean

seeds, no information regarding the pathogenicity of *F. verticillioides* and its impact upon seed quality is available. Thus, the objective of this study was to evaluate the ability of *F. verticillioid*es to decrease soybean seed quality, measured here by germination of artificially inoculated soybean seeds using low and high inoculum potential treatments.

The *Fusarium* isolates used in this study were previously identified, deposited, and accessed in the *Fusarium* collection in the Department of Plant Pathology at Kansas State University, USA. Three seedborne *F. verticillioides* isolates (K-State accession nos. 23591, 23604, and 23625) were tested for their pathogenicity in soybean seeds under growth chamber conditions. The rolled-towel method, used to evaluate the pathogenicity of *F. graminearum* on soybean and corn by Ellis et al. (2011), was used to compare the pathogenicity of each *F. verticillioides* isolate and its influence on seed germination. For negative controls, three non-pathogenic seedborne isolates of *F. semitectum* were used (K-State accession nos. 23586, 23590, and 23616). *F. semitectum* is normally classified as a weak or non-pathogen of soybean seed and seedlings (Leslie and Summerell 2006; Pedrozo and Little 2015). As positive controls, three pathogenic seedborne *F. proliferatum* isolates were used (K-State accession nos. 23592, 23602, and 23621). *F. proliferatum* has the potential to decrease soybean seed germination and vigor (Pedrozo and Little 2015) and has been reported as a potential source of inoculum for soybean seedling and root diseases (Arias et al. 2011). Interestingly, *F. semitectum* and *F. proliferatum* are both commonly reported on soybean seeds (Leslie and Summerell 2006; Pedrozo and Little 2015; Roy et al. 2001).

The soybean varieties used for this study were Asgrow ‘AG3039’ (Monsanto, Inc.; St. Louis, MO, USA) and ‘KSU3406’ (Kansas State University, Manhattan, KS, USA). Prior to

inoculation, seeds were surface disinfested with a 5% bleach solution (0.5 sodium hypochlorite v/v) for 1 min and dried overnight at room temperature. The germination and sanitary aspects of the seeds were also evaluated prior to inoculation, and 94% and 91% germination and zero incidence of *Fusarium* in the soybean seeds were observed for both genotypes used (data not shown). Two inoculum potential treatments were used, “low” and “high”. In this study, the inoculum potential is empirically referring to the physical amount of inoculum present in the seed. The low inoculum potential treatment was achieved by imbibing the seeds with a 25 ml conidial suspension for 1 min at 2.5 × 105 conidia ml−1 (Supplementary Fig. 1). The high inoculum potential treatment was achieved by using osmoconditioning (i.e., the “water restriction” method) (Machado et al. 2004). After imbibement in the conidia suspension, seeds were incubated in direct contact with the isolates inoculum for 48 h at 30 °C in potato dextrose agar (PDA) medium amended with 81.3 g of mannitol (osmotic restrictor) per liter, to provide an osmotic potential of −1.2 MPa preventing the germination of the seeds. After inoculation, seeds were dried at room temperature for 24 h and then used for the germination test (Supplementary Fig. 1).

Twenty-five artificially inoculated seeds, with low and high inoculum potential treatment, were placed on two moistened sheets of germination paper (Anchor Paper Co., St. Paul, MN, USA). An additional sheet of moistened germination paper was placed over the inoculated seeds, the layers were rolled into a tube, secured by a rubber band, set upright in a modified plastic Rubbermaid® Cereal Keeper container (Newell Rubbermaid Co., Atlanta, GA, USA) and incubated in a growth chamber (Power Scientific Inc., St. Louis, MO, USA) at 25 °C for seven days. During the growth chamber experiment, temperature and humidity within the plastic

containers were checked using a data logger (MicroDAO Ltd., Contoocook, NH, USA) and averaged 25 °C and 88%, respectively. After 7 days of incubation, seed germination (%) and disease severity index (DSI) were measured (Broders et al. 2007; Ellis et al. 2011). DSI was calculated using a scale of 0 to 3 where: "0" = germinated seeds and healthy and normal seedlings with no symptoms on the primary and/or secondary roots or hypocotyl; "1" = seed germinates and abnormal seedling shows minor discoloration and reduction on the primary and/or secondary roots as well as hypocotyl; "2" = seed germinates and abnormal seedling shows heavy discoloration and reduction on the primary and/or secondary roots. Also, the hypocotyl is heavily discolored and girdled by the lesion; and "3" = non-germinated seed (Fig. 1). DSI was calculated based on the formula: DSI = ((A*0) + (B*1) + (C*2) + (D*3)) / Nt, where "A", "B", "C", and "D" are the number of seedlings presenting disease severity scores 0, 1, 2, and 3, respectively; and Nt = total number of seeds tested. Isolates having pathogenicity scores <1, between 1 and 2, and >2 were considered non-, moderately, and highly pathogenic, respectively (Broders et al. 2007; Ellis et al. 2011).

For seeds inoculated with the low inoculum potential treatment only, *F. proliferatum* isolates (i.e., the positive controls) were able to significantly reduce seed germination ($P < 0.001$) when compared to the mock-inoculated controls for both varieties tested (Fig. 2). Furthermore, all three *F. proliferatum* isolates were classified as moderately pathogenic to soybean variety 'AG3039' and highly pathogenic to 'KS3406' (Fig. 3). The *F. verticillioides* test isolates, however, and the *F. semitectum* isolates (negative disease controls) were classified as non-pathogenic and did not differ significantly from each other in either variety (Fig. 3). Interestingly, under the high inoculum potential treatment, all three *F. verticillioides* isolates were also able to significantly ($P < 0.001$) decrease soybean seed germination when compared

with mock-inoculated controls for both varieties tested (Fig. 2). This result suggests that the decrease in seed germination was due to the inoculum potential of *F. verticillioides* present in the seeds, which influences pathogenicity of the seedborne pathogen. When soybean seeds were inoculated with the high inoculum potential treatment of *F. verticillioides*, the pathogenicity of the isolates was significantly different from mock-inoculated controls and was classified as moderately and highly pathogenic on 'AG3039' and 'KS3406', respectively (Fig. 3). As expected, even after the high inoculum potential treatment, *F. semitectum* isolates were not able to significantly reduce soybean seed germination when compared with mock-inoculated control and DSI remained <1 for 'AG3039' (Fig. 2). Similar results were observed by Pedrozo and Little (2014) where no influence on seed germination was observed on soybean seeds artificially inoculated using a high inoculum potential treatment of a non-pathogenic *F. equiseti* isolate. Interestingly, FSE isolates were able to significantly decrease seed germination of 'KS3406' seeds inoculated with the high inoculum potential treatment compared to mock-inoculated control ($P < 0.001$). *F. semitectum* was classified as moderately pathogenic on 'KS3406' (Fig. 3). Therefore, isolate pathogenicity, inoculum potential, and host genotype are important for seed and seedling diseases (Agarwal and Sinclair 1996; Machado et al. 2013; Neergaard 1979).

In summary, this study shows that *F. verticillioides* has the potential to decrease soybean seed quality depending on the amount of inoculum (inoculum potential) present in the seeds. Thus, soybean seed lots infected by pathogenic *Fusarium* spp., such as *F. verticillioides*, may play a significant role as a source of inoculum to new hosts and areas. In addition to inoculum potential, more studies evaluating environmental conditions (field experiments) and more host genotypes (varieties) are needed to better estimate and understand the significance of *F.*

verticillioides on soybean seed quality. This information can contribute to the development of more precise and accurate seed health testing methods designed to detect pathogenic *Fusarium* species and strains in seed lots, as well as helping breeders to select for resistance against *Fusarium* spp.

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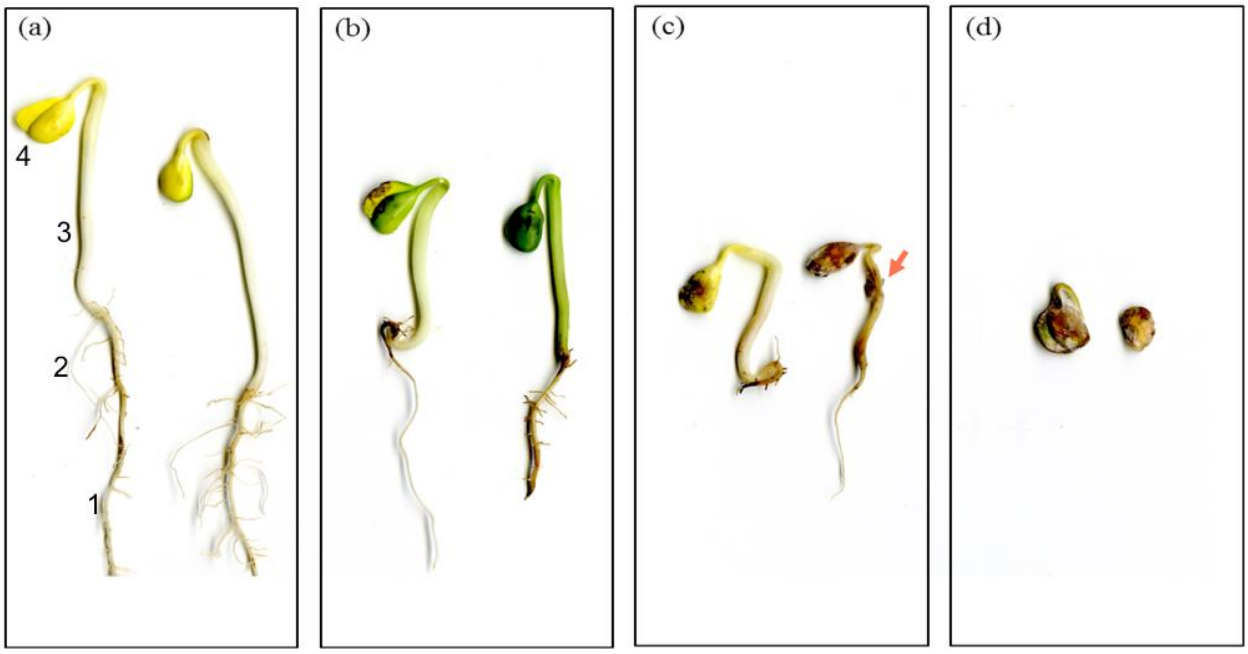

Fig. 1.The disease severity index (DSI) was calculated using a scale of 0 to 3 where: 0 = germinated and normal seedlings with no symptoms on the primary or secondary roots, hypocotyl and cotyledons (**a**); 1 = seed germinates and abnormal seedling shows minor discoloration and reduction of the primary and/or secondary roots, hypocotyl, and cotyledons (**b**); 2 = seed germinates and abnormal seedling shows heavy discoloration, reduced primary and/or secondary roots, the hypocotyl and cotyledons are discolored and girdled (*red arrow*) by the lesion (**c**); and 3 = non-germinated seeds (**d**). In the first panel (**a**), the primary and secondary roots, hypocotyl and cotyledons are labeled 1, 2, 3 and 4 respectively

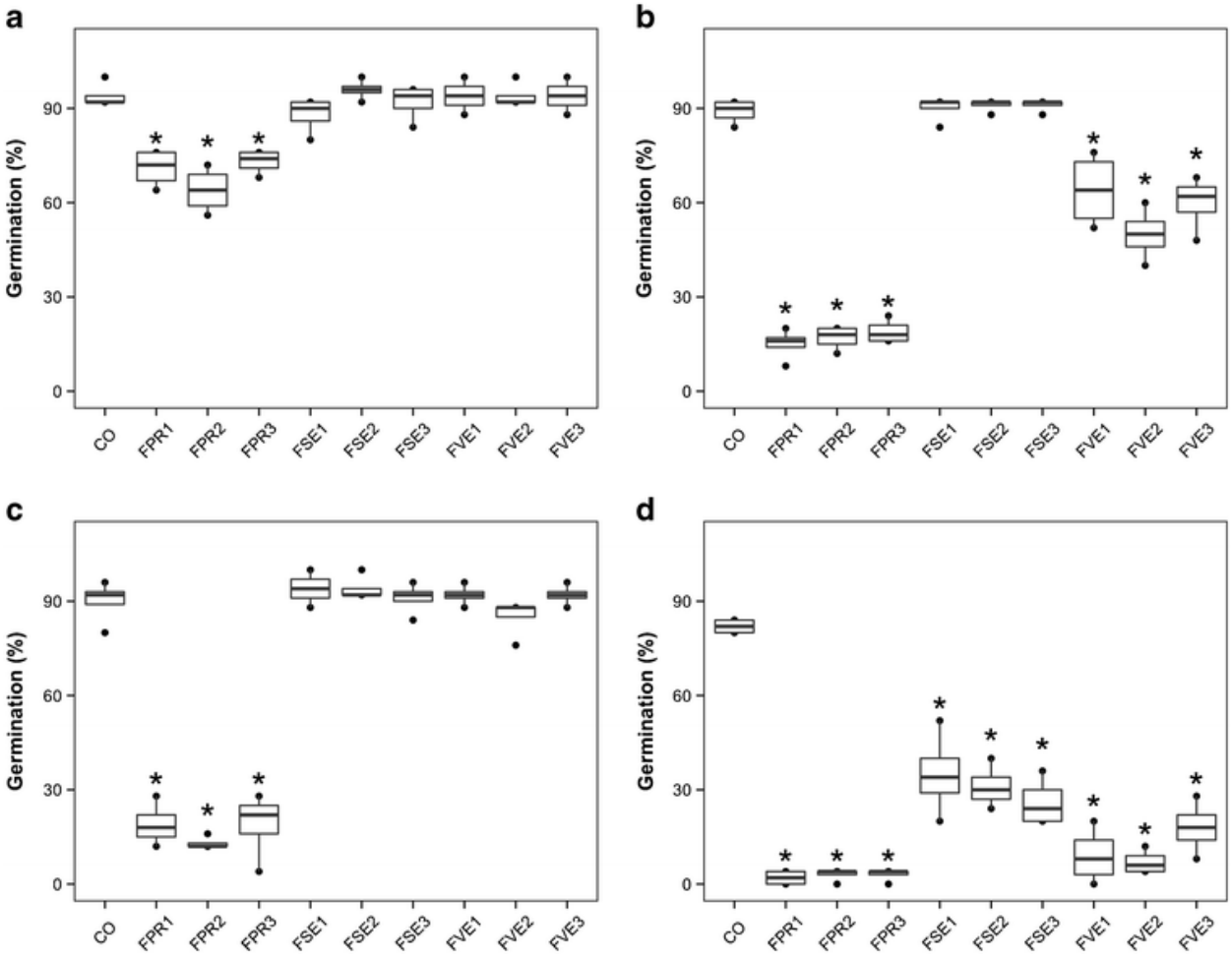

Fig. 2. The influence of seedborne *F. verticillioides* isolates (FVE1, FVE2, and FVE3) on 'AG3039' (**a** and **b**) and 'KS3406' (**c** and **d**) soybean seed germination after low and high inoculum potential inoculations. Low and high inoculum potentials are represented in panels (**a** and **c**) and (**b** and **d**) respectively. Positive (*F. proliferatum*; FPR1, FPR2, and FPR3) and negative (*F. semitectum*; FSE1, FSE2, and FSE3) pathogenicity controls. "*" Significantly different from mock-inoculated seeds (CO) using Dunnett's *t*-test ($P < 0.001$).

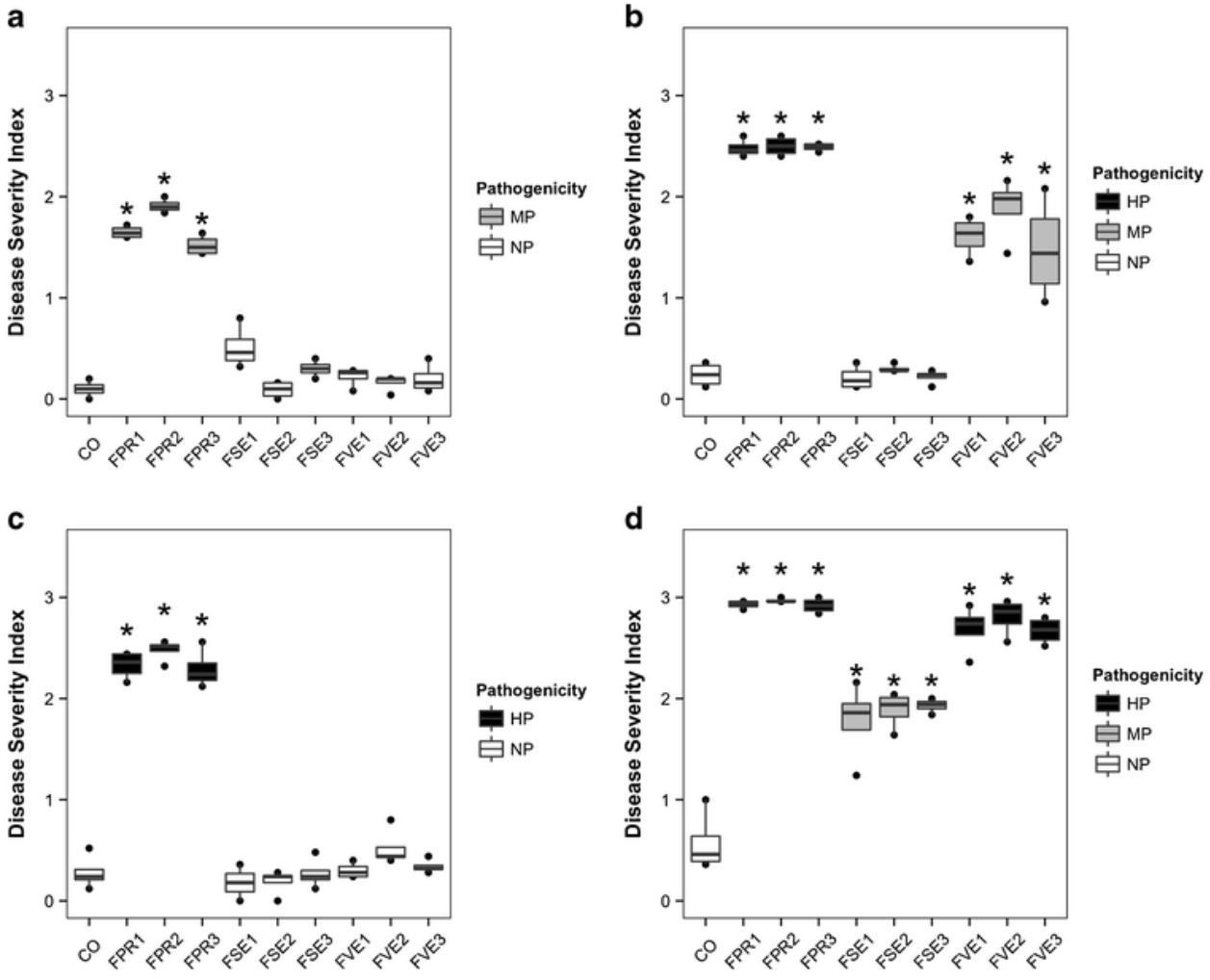

Fig. 3. Influence of *Fusarium* spp. inoculum potential on its pathogenicity behaviour on soybean seeds as measured by disease severity index (DSI). Soybean seeds were artificially inoculated with low (**a** and **c**) and high inoculum potential (**b** and **d**) treatments. Two varieties were used in this study, 'AG3039' (**a** and **b**) and 'KS3406' (**c** and **d**). Three *F. verticillioides* isolates (FVE1, FVE2, and FVE3), positive controls (*F. proliferatum*; FPR1, FPR2, and FPR3), and negative controls (*F. semitectum*; FSE1, FSE2, and FSE3) were used. Isolates having pathogenicity scores <1, between 1 and 2, and >2 were classified as non- (NP), moderately (MP), and highly pathogenic (HP), respectively. "*" Significantly different from mock-inoculated seeds (CO) using Dunnett's *t*-test ($P < 0.001$).