Soybean root rot caused by Fusarium oxysporum and Fusarium graminearum: interactions with biotic and abiotic factors

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**Fusarium oxysporum** *(Fo)* and **Fusarium graminearum** *(Fg)* are important components of the Fusarium root rot complex in soybean. *Fo* is one of the species most frequently associated with soybean root rot, and *Fg* isolates that colonize wheat and maize have been found to be highly pathogenic on soybean, in the United States. *Fo* and *Fg* cause seed decay, damping-off, crown and root rots and pod blight.

The goal of this research was to characterize the biology of *Fo* and *Fg* and determine their role as soybean seedling pathogens in the Fusarium root rot complex. The objectives were to: i) assess the phenotypic characteristics of *Fo* isolates from soybean, including the interaction between *Fo* isolates and soybean cultivars, growth characteristics in culture, and sensitivity to fungicides, ii) evaluate the effect of pH and temperature on the development of soybean root rot caused by *Fo*, and iii) determine the impact of soil texture, soil pH and soil water content on seedling disease caused by *Fg*.

For objective 1, pathogenicity of fourteen *Fo* isolates was evaluated on eleven soybean cultivars in rolled-towel and petri-dish assays. Our study revealed that cultivars differed in susceptibility to *Fo*, and there were significant isolate × cultivar interactions. These results suggests that the pattern of resistance or susceptibility for each soybean cultivar differs among isolates. In addition, soybean cultivars differed in susceptibility to *Fo*, illustrating the variability among *Fo* isolates from soybean and the potential for their management through cultivar selection.
Fo isolates also differed in radial growth on PDA. Pyraclostrobin and trifloxystrobin effectively reduced conidial germination, and ipconazole effectively reduced fungal growth, but fludioxonil was ineffective against Fo fungal growth. These results illustrate the variability among Fo isolates from soybean and the potential for their management through cultivar selection or seed treatment.

For objective 2, a growth chamber study was performed to assess the effects of pH and temperature on Fo fungal growth and seedling disease. Fo isolates were grown on artificial culture media at four pH levels (4, 5, 6, 7, 8), and incubated at four temperatures (15 20, 25, or 30°C). In a rolled-towel assay, seeds were inoculated with a suspension of a pathogenic or a non-pathogenic Fo isolate. We found that Fo isolates had the greatest radial growth at pH 6 and 25°C, and caused the most severe root rot at pH 6 and 25°C. In addition, a Gaussian model was performed to estimate optimal pH and temperature for fungal growth and disease severity. Optimal conditions estimated using a Gaussian model were pH 6.4 at 27.4 °C for maximal fungal growth, and pH 5.9 at 30°C for maximal root rot severity. These results indicate that optimal pH and temperature conditions for Fo growth are similar to optimal conditions for infection and disease in soybean seedlings, and suggest that Fo may be a more important seedling pathogen when soybeans are planted later, under warm conditions.

For objective 3, we tested the effect of four artificial soil textures (sand, loamy sand, sandy loam and loam), two levels of soil pH (6 and 8), and three levels of soil moisture (permanent wilting point, field capacity and saturation) on root rot of soybean caused by Fg. We found a significant interaction between soil moisture and soil texture for root rot. The greatest severity (~70%) was observed at pH 6 and permanent wilting point in sandy loam.
soils. In contrast, pot saturation resulted in the lowest levels of disease in sandy loam and loam soils (11.6 and 10.8%, respectively). Percentages of reduction on seedling growth parameters relative to the non-inoculated control, such as root length, foliar area, shoot and root dry weights and root tips were significantly higher in sandy loam soils. In contrast, there were no relative growth reductions in sandy soils. Our results suggest root disease caused by $Fg$ increases in water-stressed plants, resulting in detrimental effects on plant development.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation is organized in five chapters. The first chapter contains the general introduction, literature review, and research justification. Chapter two describes the phenotypic characterization of *Fusarium oxysporum* isolates collected in Iowa, including the interaction with soybean cultivars, fungal growth characteristics in culture, and sensitivity to fungicides. Chapter three describes the effects of pH and temperature on *Fusarium oxysporum* fungal growth and soybean seedling disease under growth chamber conditions. Chapter four is a greenhouse study to determine the effects of soil pH, soil texture and soil moisture on root rot of soybean caused by *Fusarium graminearum*. Finally, chapter five includes general conclusions and recommendations from this research project.

Literature Review

*Fusarium oxysporum*

*Fusarium oxysporum* (*Fo*) is a highly ubiquitous, anamorphic species that infects a wide range of hosts causing various diseases like vascular wilts, yellows, pre-emergence and post-emergence damping-off, and root rot. *Fo* is also considered a common constituent of fungal communities in the rhizosphere of plants (Fravel et al. 2003). All isolates of *Fo* are considered saprophytic since they can survive in the soil organic matter. Moreover, some
isolates are pathogenic to plant species and many other isolates do not invade the root tissues or cause disease (Alabouvette et al. 1993).

Fusarium wilt pathogens present a high level of host specificity, therefore intra-specific subdivision has been done mainly on the concept of *forma speciales* (f. sp.) based on the infected plant host (Laurence et al. 2014).

*Fo* is very diverse at the species level, with more than 120 f. sp. causing disease on a wide range of plant families (Michielse and Rep 2009). *Fo* causes vascular wilts in a wide variety of economically important crops including banana (*Fo* f. sp. *cubense*) (Groenewald et al. 2006), watermelon (*Fo* f. sp. *niveum*) (Wu et al. 2009), asparagus (*Fo* f. sp. *asparagi*) (Reid et al. 2002), beans (*Fo* f. sp. *fabae*) (Ivanovic et al. 1987), spinach (*Fo* f. sp. *spinaceae*) (Naiki and Morita 1983), guava (*Fo* f. sp. *psidii*) (Gupta et al. 2010), sugar beet (*Fo* f. sp. *betae*) (Webb et al. 2015), carnations (*Fo* f. sp. *dianthi*) (Harling et al. 1988), chickpea (*Fo* f. sp. *ciceris*) (Landa et al. 2004), lettuce (*Fo* f. sp. *latucae*) (Scott et al. 2010), tomato (*Fo* f. sp. *lycopersici*) (Borrego-Benjumea et al. 2014), cucumber (*Fo* f. sp. *cucumerinum*) (Chen et al. 2013), flax (*Fo* f. sp. *lini*) (Hoper et al. 1995), muskmelon (*Fo* f. sp. *melonis*) (Gordon and Okamoto 1990), cotton (*Fo* f. sp. *vasinfectu*) (Smith and Snyder 1975).

The paucity and variability of morphological characters of the asexual reproductive structures led to a short definition of *Fo* that did not reflect the inherent variability of this species. Molecular tools have provided a new insight in the taxonomic framework and species classification (Fravel et al. 2003). Even though, the use of molecular tools has allowed successful identification of pathogenic strains and races, determination f. sp. still widely relies on bioassays (Gordon and Martyn 1997).
Fo sexual stage has never been observed in nature or induced in the laboratory, therefore is considered to reproduce clonally. Fo has been hypothesized to undergo recombination besides sexual reproduction, such as parasexual recombination, although this has not been proven to occur (Gordon and Martyn 1997). Horizontal gene transfer may play an important role in generating new genetic diversity in Fo (Ma et al. 2010).

One interesting outcome of the genome sequence analysis of Fo f. sp. lycopersici is that pathogenicity-related genes seem to be non-randomly distributed. Chromosome 14 contains more avirulence genes in a region that is conserved between clonal linages of Fo f. sp. lycopersici than other genes on other chromosomes, indicating that chromosome 14 has been subjected to horizontal gene transfer (Michielse and Rep 2009; van der Does et al. 2008).

**Fusarium oxysporum species complex (FOSC)**

Due to the predominant asexual reproduction of Fo, it is regarded as a species complex; a collection of clonal lines or isolates within the genus Fusarium. Members of the FOSC collectively represent pathogenic, saprophytic and non-pathogenic depending on the interactions they have with host vegetation (Gordon and Martyn 1997). A number of these Fusarium are also clinically important, since they cause threatening infections in humans and other animals (O'Donnell et al. 2004b).

Distinguishing species boundaries within the FOSC is challenging due to the lack of taxonomic characters, its broad geographic distribution, the diverse biology of isolates, and finally the anthropogenic distribution of pathogens and its influence on fungal evolutionary aspects (Laurence et al. 2014; O'Donnell et al. 2009).
Four clades in the FOSC were identified based on the gene sequence information from two genes proven to be useful to distinguish among members of the FOSC, the translation elongation factor (tef1α) and the mitochondrial small subunit (mtSSU). The five clades were obtained including Fo isolates from a diverse number of plant species lettuce (Lactuca sativa L.), maize (Zea mays L.), banana (Musa spp.), cotton (Gossypium hirsutum L.), chickpea (Cicer arietinum L.), soybean (Glycine max (L.) Merr.) and other sources (Baayen et al. 2000; Ellis et al. 2014; O'Donnell et al. 1998; O'Donnell et al. 2004b).

**Fusarium oxysporum in soybeans**

There are several Fusarium species occurring on debris and soils samples in soybean fields, such as *F. oxysporum* *F. acuminatum*, *F. equiseti*, *F. moniliforme*, *F. graminearum*, *F. solani*, *F. semitectum*, *F. chamydosporium*, *F. compactum*, *F. merismoides*, and *F. proliferatum* (Leslie et al. 1990). Many of these species cause seed and seedling diseases, and vascular wilts in soybean in vegetative and reproductive developmental stages (Armstrong and Armstrong 1950; Broders et al. 2007; Díaz Arias et al. 2013a; Díaz Arias et al. 2013b; Killebrew et al. 1993a). However, Fo is the most common species associated to soybean roots in Iowa (Díaz Arias et al. 2013b).

One of the first reports of Fusarium root in Iowa was described by Dunleavy in 1953 (Niblack et al. 2002), identifying the causal agent as *F. orthoceras*. Later, Fo was reported as the predominant species isolated form symptomatic roots (French and Kennedy 1963). The causal agents of Fusarium root rot or wilt may act as primary pathogens, or secondary pathogens that colonize root tissues along with other soilborne pathogens, making it difficult to diagnose correctly. (Avanzato et al. 2008; Datnoff and Sinclair 1988; Farias and Griffin 1990; French and Kennedy 1963).
Soybean symptoms produced by *Foil* pathogenic isolates include pre and post emergence damping-off, vascular discoloration, necrosis of cotyledons, water-soaked lesions on the stems, wilting, and brown and black root rot (Backmand et al. 1993; Ellis et al. 2014; Nelson 1999).

Formae speciales have not been reported for *Foil* isolates causing seedling disease and root rot in soybean (Ellis et al. 2014). However, two other *Foil* forma speciales from cotton (*Foil* f. sp. *vasinfectum*, race 2) and cowpea (*Foil*. f. sp. *tracheiphilium*, race 1) have been reported to cause wilt in soybean in the cultivar ‘Yelredo’ (Armstrong and Armstrong 1958).

A genotypic and phenotypic characterization of *Foil* isolates collected in the Midwestern and southeastern United States from soybean roots, demonstrated a large genetic diversity in all five FOSC clades; three of these clades previously described by O’Donell et al. (2004b). Isolates from Iowa varied from highly aggressive to almost non-pathogenic to soybean seedling, but there was not a clear association between clades and level of pathogenicity; except for clade 2, in which many of the non-pathogenic isolates were classified (Cruz et al. 2013; Ellis et al. 2014).

*Fusarium graminearum*

*Fusarium graminearum* (synonym *Gibberella zeae* (Schwein.) Petch) is an important pathogen of plant cereal world-wide causing *Fusarium* head blight of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and oat (*Avena sativa* L.), and gibberella ear and stalk rot of corn (McMullen et al. 1997; Xue et al. 2007). In addition to head blight and stalk rot, *F. graminearum* is an important seedling pathogen of corn and wheat (Carter et al. 2002; Jones 1999). *F. graminearum* has also been reported to be a pathogen in non-cereal crops such as potato (*Solanum tuberosum* L.) (Ali et al. 2005), canola (*Brassica rapa* L.) (Chongo
et al. 2001), dry bean (*Phaseolus vulgaris* L.) (Bilgi et al. 2011), and sugar beet (*Beta vulgaris* L.) (Hanson 2006).

Since the year 2000 members of the *F. graminearum* species complex started being classified into different linages and are no longer considered as a single species (O'Donnell et al. 2008; O'Donnell et al. 2004a; Sarver et al. 2011). High phenotypic diversity has been reported within populations of *F. graminearum* in the USA and this has been associated with sexual outcrossing (Walker et al. 2001). *F. graminearum* commonly produce type B trichothecene mycotoxins, which are divided into three genotypes 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), or nivalenol (NIV) (Ellis and Munkvold 2014; Ward et al. 2008). Evidence of population subdivision within *F. graminearum* in the USA correlates with the trichothecene chemotype, most North American *F. graminearum* isolates predominantly produce 15-ADON, and a small number of isolates from Minnesota and North Dakota produce 3-ADON suggesting a division of the larger population from 15-ADON (Gale et al. 2003).

**Fusarium graminearum in soybeans**

*F. graminearum* is cosmopolitan and has been reported recently as a major necrotrophic pathogen of soybean (Broders et al. 2007; Xue et al. 2007), causing seed decay, pre- and post-emergence damping-off, crown and root rot, and pod blight in the United States, Canada and other temperate regions (Martinelli et al. 2004; Sella et al. 2014; Xue et al. 2007).

*F. graminearum* overwinters and colonizes corn residues, providing a source of primary inoculum for subsequent crops (Sutton 1982; Windels et al. 1988). *F. graminearum* has been isolated from various parts of the soybean plants including stems, seeds and roots
(Anderson et al. 1988). In the past *F. graminearum* was not considered to be pathogenic on soybean (Fernandez and Fernandes 1990; Garcia-Romera et al. 1998). However, subsequent studies have demonstrated that *F. graminearum* produces soybean seedling diseases in the USA (Broders et al. 2007; Xue et al. 2007), and soybean pod blight and root rot in south America (Martinelli et al. 2004).

Most of the soybean production areas in the USA and Canada are used in rotation with corn or wheat, in combination with reduced-tillage or no tillage systems (Miller et al. 1998). Reduced tillage practices substantially increase *F. graminearum* inoculum and keep the soil surface cool and wet, therefore increasing the amount of time required for the seed to germinate increasing the chances for soybean seedling diseases (Broders et al. 2007; Griffith et al. 1997; Vandoren and Triplett 1973). Xue et al. (2007) concluded that the emergence of *F. graminearum* as a soybean pathogen is may be due to selection pressure for highly aggressive isolates through crop rotation.

Even though, the emergence of *F. graminearum* as a soybean pathogen is linked to the scarce development and delivery of new cultivars by the industry and the limited diversity of soybean lines from some companies, disease resistance is promising for *F. graminiforum* management (Sneller 2003).

The type of disease resistance (innate or basal, qualitative and quantitative) often depends on the biology of the pathogen (Glazebrook 2005; Hammond-Kosack and Parker 2003; Poland et al. 2009). Due to the recent emergence of *F. graminearum* as an important pathogen of soybean there are a few studies characterizing the resistance in this pathosystem; however, in other pathosystems resistance to *F. graminearum* is quantitative (Bai et al. 1999; Gervais et al. 2003; Zhou et al. 2002).
Ellis et al. (2012) reported a wide range of reactions in soybean cultivars following inoculation with *F. graminearum*, finding cultivar that exhibit high levels of resistance suggesting that resistance to *F. graminearum* may be common in soybean and plant breeding effort sould be focusing on screeninig advance breeding lines.

**Fusarium life cycle**

The life cycle starts with a saprophytic phase of the chlamydospores surviving in soil (Gordon and Okamoto 1990). Pathogenic isolates haven been demonstrated to survive up to 25 years in the absence of a susceptible host (Smith and Snyder 1975). Nutrients released form the growing roots stimulate the dormant chlamydospores or conidia to germinate, producing hyphae from 6 to 8 hours if conditions are favorable. Fusarium then penetrates the epidermal cells and gets into the root system, colonizing the root cortex (Beckman and Roberts 1995). Penetration occurs directly through root epidermal cells or indirectly through wounds that naturally occur from secondary root development (Nelson et al. 1997; Nelson et al. 1981).

The fungus spreads intercellularly into the vascular bundle and proliferates into the xylem. In wilt causing strains, microconidia are transported into the xylem, germinate and blocks the vessels, producing a significant negative impact on the water economy of the host plant due to vessel clogging. The combined effect of fungal growth and the production of plant defenses accentuate the problem resulting in severe wilting and eventual death (Beckman and Roberts 1995).

In advanced stages of disease, the pathogen keeps growing in the vascular system in adjacent parenchyma cells, producing vast quantities of conidia. Some *Fusarium* species causing root rot specialized in infecting and destroying the cortical tissue of the host (Nelson
et al. 1997). In resistant hosts, penetration into root tissues by *Fusarium* occurs but further spread is blocked, limiting tissue colonization (Baayen et al. 1989; Harrison and Beckman 1982; Rodriguez-Molina et al. 2003).

**Abiotic factors contributing to pathogenesis in Fusarium wilts**

**Temperature**

Optimal growth temperature for *Fo* has been found between 25 to 28°C, with growth inhibition above 33°C and below 17°C (Cook and Baker 1983). Fusarium wilts are clearly influenced by soil temperature; for example, cabbage yellows, flax and tomato wilt are favored by high soil temperature, whereas and tobacco root rot are conditioned by low soil temperature (Jones 1924). Most of Fusarium wilts describe a parabolic trend in disease intensity, indicating that in low and high extremes of temperature symptoms do not develop. For example, in *Fusarium* wilt of carnation caused by *Fo f. sp. dianthi*, stems were highly colonized with severe symptoms in a range of temperatures between 23-26°C, but remained symptomless and with very little colonization in a range of temperatures between 14 to 20°C (Ben-Yephet and Shtienberg 1994).

Temperature and climate also determines the distribution and composition of *Fusarium* spp. in soil (Burgess and Summerell 1992). For example, in observational studies *F. compactum* occurs only in the warm areas of central and northern Australia, whereas *F. acuminatum* is restricted to the southern Australia (Backhouse and Burgess 1995). Similarly, in a 12-month field study, soils inoculated with five *Fusarium* species and subjected to different temperature regimes, displayed significant differences in community and structure in which *F. solani* and *F. compactum* presented a higher propagule density at high temperatures (25-30°C) (Saremi et al. 1999).
Soil moisture

Soil characteristics significantly influence most of the variation in disease intensity for soilborne pathogens (Dixon and Tilston 2010). Soil temperature and soil moisture have been recognized as important factors for disease development by *Fusarium* spp. (Brownell and Schneider 1985; Cook and Papendick 1972). Literature contains dissimilar results on the effects of soil moisture on Fusarium wilts, these discrepancies may depend on the *Fusarium* species, f. sp., physiological or geographical adaptations of isolates (Burgess et al. 1988; Jones 1924; Manshor et al. 2012; Walker and White 2005). However, there is strong evidence suggesting that *Fusarium* soil populations can be greatly reduced by maintaining high soil moisture conditions in the absence of a host (Stover 1953).

Soil flooding produces physical, biological and chemical changes in the soil, anaerobic conditions and alterations in soil structure (Unger et al. 2010), increase the ammonia concentration, and decrease the nitrate nitrogen levels (Shelton et al. 2000). One or more of these physiochemical soil changes may play a role in the survival of soilborne pathogens. Stover (1953), observed significant reductions of *Foc*, *F. graminearum*, and *F. moniliforme* soil populations increasing soil moisture by 85% of saturation in a loam soil with the maximum bacterial soil population at 75% of saturation. Therefore, low soil moisture and light-textured soils with low bacterial populations favored the survival of *Fusarium* propagules in the soil. In similar studies, flooding for 15 days with the incorporation of maize or rice straw into the soil reduced propagules of *Foc* f. sp. *cubense* up to 90% (Wen et al. 2015).

In soybean, one of the first reports of the combined effects of temperature and soil moisture on *Foc* was made by French (1963), in which disease severity was dependent on
temperature when plants were subjected to saturated infested soils. Roots were healthy form 26 to 32°C, but symptoms developed and were more severe from 14 to 23°C. Soybeans are sensitive to flooding conditions (Komatsu et al. 2010; Russell et al. 1990). Common soilborne pathogens recovered from soybean diseased roots under flooding or anoxic conditions are Pythium spp., *Phytophthora sojae*, and *Rhizoctonia solani* (Brown and Kennedy 1966; Killebrew et al. 1993b; Rao et al. 1978). Conversely, pathogens such as *Fusarium* spp. and *Macrophomina phaseolina* were less frequently associated to root rot diseases under flood conditions (Kirkpatrick et al. 2006).

Fungal structures such as micro and macroconidia, chlamydospores have outer protective walls that maintain the cellular water potential above of the external dry surrounding environment, providing more tolerance to desiccation (Cook and Papendick 1972). Conversely, bacterial cells are less tolerant to low soil water potentials (Marshall 1975). As soil pores dry, water films become thinner and diffusion of substrate molecules become difficult, reducing the nutrient flux to the bacterial cell surface (Stark and Firestone 1995). In addition, solutes used by the cell to balance internal and external water potentials may interfere with specific essential biochemical processes (Csonka 1989).

**pH**

Soil pH influences plant disease development directly on the soilborne pathogen population and indirectly throughout the availability of nutrients to the plant host (Ghorbani et al. 2008). In *in vitro* conditions, fungi grow maximally over a narrow range of pH values, describing a parabolic trend in which fungal growth is negligible at high and low extremes; most plant pathogens grow best in media with pH from 5 to 6.5 (Cochrane 1958). In general,
acid soil conditions enhance spore germination, mycelial growth and production of conidiophores (Burpee 1990).

Physiological studies on plant pathogenic isolates in the *Fusarium* genus indicated that the most suitable pH for optimal vegetative growth is in the range of 6 and 6.5 (Cochrane 1958; Srobar 1978). However, some *Fusarium* spp. display optimal growth in acid pH media. For example, *F. graminearum*, *F. equiseti* and *F. solani* showed optimal growth in pH range from 3.5 to 4.5 (Agarwal and Sarbhoy 1978). *Fusarium* soil populations were observed to survive and grow in soils at a pH of 4.2; however, soil pH close to neutrality negatively affected this growth (Wilson 1946).

Raising soil pH appears to be a common cultural practice for the control of a number of Fusarium wilts, which is a disease commonly associated to acidic sandy dry soils (Woltz and Jones 1981). Comparisons between bacteria and fungi populations in a silty loam soil with a natural gradient of pH from 4 to 8.3 indicated a fivefold increase in bacterial growth and a fivefold decrease in fungal growth with high pH (Rousk et al. 2009). *In vitro* studies suggest that adverse effects of high pH on fungal populations may be due to microbial competition and bacterial antibiosis. In addition, bacteria and actinomycetes compete for nutrients at high pH (Marshall 1960).

Manipulation of the nutrient status of the soil by changing pH has demonstrated the role of microbial competition for nutrients and mechanisms of fungal soil suppression (Alabouvette 1999). Iron is an essential element for growth and development of living organisms, participating in a number of cellular processes including oxygen transport, ATP generation and detoxification; particularly for fungal pathogens iron is a major virulence factor (Symeonidis and Marangos 2012).
Iron bioavailability is reduced in alkaline soils (Expert 2009). The effect of soil fungal suppression at a given soil pH depends on the ability of the fungus to acquire iron compared to its antagonists under iron-limiting conditions (Hoper and Alabouvette 1996). For example, fluorescent pseudomonas produce high affinity iron siderophores that enhance the acquisition of iron (Scher and Baker 1980). High pH *Fusarium* suppressive soils contain siderophore-producing bacteria that complex iron, making it less available to microflora not capable of producing efficient iron-transport compounds, suggesting that management of iron availability in the soil through competition may induce suppressiveness of Fusarium wilt pathogens (Scher and Baker 1982).

**Soil texture**

Soil clay content appears to be an important abiotic factor that regulates microbial communities (Hoper et al. 1995). It has been demonstrated that addition of clay minerals such as kaolinite, illite or montmorillonite suppress the severity of Fusarium wilts (Hoper and Alabouvette 1996). Montmorillonite clays increase bacterial metabolic activity against soil fungi through a pH buffering effect related to a high cationic exchange capacity and rapid utilization of nutrients by the bacteria (Burpee 1990).

The spread of *Fusarium oxysporum* f. sp. *cubense* across Central America is a well-documented example of the effects of clay in *Fusarium* spp. Soils with high contents of montmorillonite clays maintain the fungus excluded by effective bacterial competition. Conversely, rapid disease spread was observed in soils lacking montmorillonite clays (Marshall 1975). Combined effects of soil pH and type of clay has been found to be effective in the suppression of Fusarium wilts, addition of illite in a sandy soil induced disease suppression of Fusarium wilt of flax in soils with a pH 7 or higher (Hoper et al. 1995).
The main physiochemical soil characteristics are completely interrelated. For example, there is a significant correlation between organic matter content and soil clay content (Chaussod et al. 1986). Modification of one factor such as clay content may result in the increase of the cationic exchange capacity, pore sizes, aggregation and level of organic matte that impacts microbial populations (Amir and Alabouvette 1993). For example, addition of organic amendments have positive effects in the suppression of *Fusarium* wilts (Fang et al. 2012).

**Thesis Justification**

*Fusarium* root rot complex of soybean is comprised by numerous *Fusarium* species capable of causing different soybean diseases, such as pre and post-emergence damping-off, root rot, seed rot and wilting of adult plants. Some of the most common *Fusarium* spp. in the root rot complex include *Fusarium oxysporum* and *F. graminearum*. However, the effects of abiotic factors such as soil texture, soil pH, soil moisture and temperature associated with disease development are unknown. The main goal of this research project was to make efforts to better understand which abiotic factors contribute to root rot development and are more deserving for further exploration. The results from these studies will contribute to increase understanding in the biology of *F. oxysporum* and *F. graminearum* and their role in the Fusarium root rot complex, which can help to effectively design management strategies in the future. The objectives of this research were to:

1. Phenotypic characterize *F. oxysporum* isolates associated with soybean root from Iowa.
2. Determine the effects of pH and temperature in the development of soybean root rot caused by *F. oxysporum*.

3. Measure the effects of important soil variables in the development of root rot of soybean caused by *F. graminearum*.

**Literature Cited**


CHAPTER 2. ISOLATE x CULTIVAR INTERACTIONS, \textit{IN-VITRO} GROWTH AND 
FUNGICIDE SENSITIVITY OF \textit{FUSARIUM OXYSPORUM} ISOLATES CAUSING 
SEEDLING DISEASE IN SOYBEAN

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Abstract

\textit{Fusarium oxysporum} Schlechtend:Fr (\textit{Fo}) is one of the species most frequently associated with soybean root rot in the United States. Information about pathogenicity and phenotypic characteristics of \textit{Fo} populations is limited. The goal of this study was to assess phenotypic characteristics of \textit{Fo} isolates from soybean, including the interaction between \textit{Fo} isolates and soybean cultivars, growth characteristics in culture, and sensitivity to fungicides. Pathogenicity of 14 isolates was evaluated in rolled-towel and petri-dish assays. In the rolled-towel assay, seeds were inoculated with a conidial suspension and rated for disease severity after 7 days. In the petri-dish assay, \textit{Fo} isolates were grown on 2\% water agar, seeds were placed on the \textit{Fo} colony, and seedling disease symptoms were rated. Soybean cultivars differed in susceptibility to \textit{Fo}, and there were significant isolate × cultivar interactions (P\leq0.05). \textit{Fo} isolates differed in their radial growth on PDA at 25°C. Pyraclostrobin and trifloxystrobin effectively reduced conidial germination with average EC$_{50}$ of 0.15 and 0.20
Ipconazole effectively reduced fungal growth with average EC\textsubscript{50} of 0.23 (µg a.i./ml), whereas fludioxonil was ineffective against \textit{Fo} fungal growth. These results illustrate the variability among \textit{Fo} isolates from soybean and the potential for their management through cultivar selection or seed treatment.

**Introduction**

Fusarium root rot is an important soybean disease in the United States and Canada. Common symptoms of Fusarium root rot vary from wilting, damping off, slow emergence, root rot, and vascular discoloration (Nelson 1999). Yield reduction caused by this disease has been particularly significant when cool and wet conditions are predominant during the planting season (Farias and Griffin 1990; Wrather and Koenning 2009; Wrather et al. 2001). In field conditions, \textit{Fusarium} spp. associated with soybean wilts and root rot show maximum disease at temperatures between 14 to 23\degree C (Carson et al. 1991), whereas several \textit{Fo} isolates have been reported to grow at a temperature range of 22 to 28\degree C \textit{in vitro} (Agarwal and Sarbhoy 1978; Balasu et al. 2015; Groenewald et al. 2006; Nelson et al. 1990b).

Diverse \textit{Fusarium} species have been associated with soybean root rot. Although fungal populations may vary due to geographic and climatic conditions, the most prevalent and frequent \textit{Fusarium} species found in roots, soil, or crop residues in a number of surveys performed across soybean fields in the United States were \textit{Fo}, \textit{F. solani} (Mart.) Sacc., and \textit{F. graminearum} Schwabe (Díaz Arias et al. 2013b; Leslie et al. 1990; Marburger et al. 2015; Nyvall 1976; Zhang et al. 2010). \textit{Fo} is composed of diverse cryptic species known as the \textit{Fusarium oxysporum} species complex (FOSC), members within this group are responsible to cause vascular wilts, damping-off and root rots in a wide range of economically important
crops (Michielse and Rep 2009; O'Donnell et al. 2009). In previous studies new *Fusarium* species have been identified within the FOSC, including *F. commune* Skovgaard, O’Donnell et Nierenberg (Skovgaard et al. 2003). In Iowa, *Fo* isolates associated to soybean seedling and root rot diseases correspond to five clades within the FOSC.

Soybean isolates within this species complex are genotypically and phenotypically diverse. Pathogenic capabilities of these isolates in soybeans range from highly pathogenic to non-pathogenic and saprophytic (Ellis et al. 2014; Laurence et al. 2014). Even though *Fo* is the species most commonly associated with soybean root rot, there have been inconsistent findings about its role and importance in the Fusarium root rot complex. For example, in greenhouse studies using artificially-infested soils, *Fo* caused less severe root rot compared to *F. graminearum*, *F. avenaceum* and *F. tricinctum* (REF). However, *Fo* produced the highest seed mortality (Zhang et al. 2010). In artificially-infested microplots, *Fo* did not significantly reduce yield but regression analysis performed on individual *Fo* isolates indicated a negative correlation between root rot severity and yield (Díaz Arias et al. 2013a). Similarly other reports estimated reductions up to 59% on yield under very similar field conditions (Leath and Carroll 1981).

Little information is available about the range of pathogenicity of *Fo* isolates toward different soybean cultivars. In one of the first reports about *Fo* and its interactions with soybeans cultivars, a differential response in susceptibility was evident across different soybean cultivars (Leath and Carroll 1981). However, this study was performed only with one *Fo* isolate, not addressing the vast diversity of the FOSC. For this reason, it is imperative to study the biology of FOSC associated with soybeans roots to elucidate its
unknown role as a part of the Fusarium root rot complex, and to reveal better management strategies.

Currently, one the most efficient disease management strategies to control seedling diseases in soybean is the use of fungicide seed treatments. These are typically used in circumstances where environmental conditions delay germination and prevent infection by soilborne pathogens that cause pre- and post-emergence damping-off, seedling blight and root rot. Although the use of seed treatments on soybeans is a relatively recent practice, it is becoming more common as many commercial seed treatments include a combination of nematicides and fungicides (Hartman et al. 1999; Mueller et al. 2013).

Among the most widely used active ingredients, are the quinone outside inhibitors (QoI), such as trifloxystrobin and pyraclostrobin, which are extensively used on soybeans and maize in combination with fludioxonil to improve efficacy against a wide range of soilborne pathogens including *Fusarium* spp. (Broders et al. 2007a; Munkvold 2009). However, QoIs have site-specific activity and represent a high risk for development of resistance (Mueller et al. 2013). Increased insensitivity to QoIs have been reported for *Fusarium* spp. in tomato (Chapin et al. 2006), *Alternaria alternata* in citrus and apple (Mondal et al. 2005; Reuveni and Sheglov 2002), and *Pythium* spp. in soybeans (Broders et al. 2007b).

The objectives of this research were to clarify the importance of *Fo* as a soybean seedling pathogen by: (i) studying the reactions of soybean cultivars to different *Fo* isolates collected from soybean production fields in Iowa, (ii) examining the impact of commonly
used fungicidal active ingredients on conidial survival and radial growth of *Fo* isolates, and (iii) comparing radial growth of *Fo* isolates under optimum temperature conditions.

**Materials and Methods**

**Inoculum and plant source**

Fourteen *Fo* isolates collected from symptomatic soybean roots across Iowa in 2007 (Díaz Arias et al. 2013a) were used in all experiments (Table 1). Isolates originated from single conidia and were maintained on silica gel beads at 4°C until use. The fourteen isolates had been tested previously in greenhouse studies and information regarding their aggressiveness in causing root rot and seedling mortality on the soybean cultivar Asgrow 2403 (SDS susceptible) (Monsanto Co., St. Louis, MO) was available (Díaz Arias et al. 2013a). In addition, the same isolates had also been previously characterized phenotypically in a rolled towel assay with the susceptible cultivar MN1805 (Ellis et al. 2014).

Each *Fo* isolate was grown on potato dextrose agar (PDA) for 7-14 days, in an incubator at 25°C with a 12 h photoperiod, in order to promote conidial formation. Sterile distilled water (SDW) was added to the culture which was gently scrubbed with a sterile stick to collect the macro and/or microconidia. The conidia suspension was passed through sterile cheesecloth to remove mycelial fragments. Inoculum concentration was calculated by counting conidia in a hemacytometer (Bright-line Hemacytometer, American Optical, Buffalo, NY) under the microscope, and conidial suspensions were adjusted to a concentration of $1 \times 10^4$ conidia/ml.
Eleven soybean cultivars were selected for these studies, including the susceptible cultivar MN1805. These cultivars had been tested previously for resistance to soybean death syndrome (SDS) and soybean cyst nematode (SCN) under greenhouse and field conditions, and represented a wide range of resistance and susceptibility to those pathogens across different soybean maturity groups (Table 2).

**Rolled towel pathogenicity assay**

Fifteen soybean seeds of the same cultivar were placed equidistantly in a row on a moist paper towel. Each seed was inoculated with 100 μl of the conidia $1 \times 10^4$ conidia/ml suspension. After inoculation, seeds were covered with another moist towel to keep them in place and to provide enough moisture for germination. There were three inoculated and one mock-inoculated rolls per cultivar. Inoculations were performed with one *Fo* isolate on eleven soybean cultivars at a time. The 44 rolls were placed in a 25 L bucket containing 300 ml of distilled water, and incubated for 7 days at room temperature (25°C). Mock-inoculated rolls were confined within the bucket with a plastic tray to avoid cross contamination. One roll was considered as an experimental unit with fifteen seeds or subsamples on each roll. Each of the fourteen *Fo* isolates were tested one at a time. The experimental design was a randomized complete block, in which each bucket was considered as a random factor for the statistical analysis. The experiment was conducted two times.

Disease intensity was evaluated by two methods as described by Ellis et al. (2011). Disease severity index (DSI) was calculated as the ratio between the length of discolored hypocotyl lesion and the total plant length, multiplied by 100. When seeds did not germinate and were completely colonized, they were assigned a DSI value of 100%. Seedlings were
also evaluated using a 1-5 severity scale, in which 1 = germination, no symptoms of disease or colonization on seedling; 2 = germination, little colonization of the seedling, 1 to 19% of the root with lesions; 3 = germination, some colonization of seedling, and 20 to 74% of the root with lesions; 4 = germination, complete colonization of the seedling, and 75% or more of the root with lesions; 5 = no germination, complete colonization of the seed (Fig. 1). Root length, root weight and plant length were also measured.

**Petri dish pathogenicity assay**

A petri dish assay was performed to test pathogenicity of fourteen *Fo* isolates and eleven soybean cultivars following a methodology similar to what has been previously published (Broders et al. 2007b; Muyolo et al. 1993; Zhang and Yang 2000). A 3-mm plug was transferred from a 7 day-old PDA culture of an isolate to the center of a petri dish containing 2% water agar, and incubated for 4 days under a 12 h photoperiod at 25˚C. Eight soybean seeds were surface disinfested in a 0.5% NaOCl solution for 2 min and rinsed twice with SDW for 2 minutes. Seeds were then placed and spaced evenly around the dish, approximately 1 cm from the edge of the fungal colony. Plates were incubated at 25˚C for 7 days with a 12 h photoperiod. Disease severity was rated using a 0 to 3 scale proposed by Broders et al. (2007): 0 = 100% germination with no symptoms of root infection; 1 = 70 to 99% germination with lesion formation on the roots; 2 = 30 to 69% germination with coalesced lesions; and 3 = 0 to 29% germination and all seed tissues were colonized (Fig. 2). The experimental design was arranged in incomplete blocks with 3 soybean cultivars each, and cultivars Jack and MN1805 as a control checks on each of the blocks. Therefore, there were 3 incomplete blocks that included eleven soybean cultivars. For each treatment
combination of cultivar and isolate there were three petri dishes and the experiment was conducted twice. Blocks were considered as random effect for the statistical analysis.

**Radial growth on PDA**

To compare the growth rate of the fourteen *Fo* isolates, 5 mm diameter mycelial plugs of each isolate were aseptically transferred from a 7 day-old PDA culture to the center of 90-mm diameter PDA petri dishes, and incubated for 4 days at 25°C, under a 12 h photoperiod (Balasu et al. 2015; Groenewald et al. 2006). Colony diameter was measured in two perpendicular directions on each plate, and an average was calculated, after subtracting 5-mm of the colonized plug from the final values. The experiment followed a completely randomized design with 4 replicate plates per isolate, and the experiment was conducted twice.

**Fungicide sensitivity assays**

The fourteen isolates were tested for sensitivity to growth inhibition by the fungicides ipconazole and fludioxonil. Active ingredients (a.i.) ipconazole (Bayer CropScience) and fludioxonil (Syngenta Crop Protection) were dissolved in acetone and added to PDA to obtain final concentrations of 0.01, 0.1, 1, 10, and 100 µg a.i./ml. The non-amended control contained acetone at 0.1% v/v only. A colonized 5 mm plug was transferred from the original PDA cultures to 90-mm diam petri dishes with the amended PDA.

Colony diameter was measured after 4 days of incubation at 25°C with a 12 h photoperiod, as described above. Percent growth inhibition was obtained by dividing the diameter of the colonies in the fungicide treatments by the mean colony diameter of the non-amended control. The experiment followed a completely randomized factorial design with
two replicate petri dishes per fungicide x concentration x isolate combination. The experiment was repeated three times.

Isolates were also tested for sensitivity to conidial germination inhibition by the fungicides pyraclostrobin and trifloxystrobin. Active ingredients pyraclostrobin (BASF Corp.) and trifloxystrobin (Bayer CropScience) were dissolved in acetone to prepare stock solutions of $1 \times 10^4 \mu g$ a.i./ml. PDA was amended with each a.i, to obtain final concentrations of 0.01, 0.1, 1, 10, and 100 µg a.i./ml. Salicylhydroxamic acid (SHAM, Sigma Aldrich, St. Louis, MO) was dissolved in methanol at a rate of 100 µg a.i./ml to inhibit the alternative oxidase respiratory pathway. All dilutions, including the non-amended control, contained both acetone and methanol at 0.1% v/v and SHAM at 100 µg a.i./ml.

Conidia (macro and microconidia) were collected from the pure cultures by adding 10 ml of sterile distilled water per plate and dislodging conidia from the surface of the culture with a sterile glass rod. Culture suspensions were passed through three layers of cheesecloth in order to remove mycelial fragments, and conidia concentrations were adjusted to $2.0 \times 10^5$ conidia/ml using a hemacytometer (Bright-line Hemacytometer, American Optical, Buffalo, NY). One hundred µl of the adjusted conidia suspension were added to a single 60mm x 15mm petri dish with PDA. Plates were incubated at 25°C under continuous light for 18 h, then placed at 5°C and immediately scored for germination rate by direct observation under a light microscope at 100X magnification. One hundred arbitrarily selected conidia per plate were examined and considered germinated if the germ tube was twice the length of the conidium. The experiment followed a completely randomized factorial design with three replicate petri dishes per fungicide x concentration x isolate combination.
Data analysis

For the rolled towel assay, the percentage of DSI was arcsin transformed, and root length, plant length, and root weight were log transformed to meet the assumptions of the ANOVA. Levene’s test of homogeneity of variance was performed on the transformed data to compare the two experimental runs for each isolate separately using PROC GLM of SAS version 9.3 (SAS Institute Inc., Cary, NC). Since there were no significant differences in the response variables between the two experimental runs, all data were combined. Subsequently, ANOVA was performed on the transformed data using PROC GLIMMIX. In addition, averages of DSI and severity for the two experimental runs were used to calculate linear correlation coefficients using the PROC CORR statement. For the petri dish assay, the nonparametric disease data from the response variable severity were analyzed using the PROC GLIMMIX statement of SAS. For the radial growth on PDA, Levene’s tests of homogeneity and analysis of variance were performed using PROC GLM. Data for the two experimental runs were combined since there were no significant differences between the variances. For all experiments, mean separation analyses were performed using a Fisher’s protected least significant difference (LSD) test at $P = 0.05$.

EC$_{50}$s for the growth inhibitors ipconazole and fludioxonil were calculated in a probit regression analysis. For each $Fo$ isolate, the averaged percentage of growth inhibition on each dose was converted to a numerical equivalent of the probit function by using the normal inverse distribution function “NORMINV” in Excel (Microsoft). A linear equation was obtained from the regression between the logarithm of the doses and their respective probit values, and the EC$_{50}$ was calculated to the corresponding dose value where probit was equal to 0.5. For the conidial germination inhibitors pyraclostrobin and trifloxystrobin, EC$_{50}$
calculations were performed using the PROC PROBIT statement for each *Fo* isolate. Data for this analysis were based on the average of the percent inhibition as compared to the non-amended control.

**Results**

**Rolled-towel assay**

The analysis of variance indicated a significant effect for the main factors soybean cultivar and *Fo* isolate, as well as a significant cultivar × isolate interaction for DSI (P = 0.0140) and the visual severity scale (P = 0.0083). These significant effects indicate that the level of disease varied among all cultivars and also the level of aggressiveness differed among the *Fo* isolates (Table 3). Significant interactions between cultivar and isolate indicate that relative susceptibility of soybean cultivars was not uniform among *Fo* isolates.

Mean separation analysis for the main factors indicated a wide range of aggressiveness among isolates and susceptibility among cultivars. Not all the isolates were able to cause disease; in particular, FO36, FO42, and FO46 colonized the seed coat, but no lesions or fungal colonization were observed in the radicle or hypocotyls. In contrast, poor germination, fungal colonization of cotyledons and or large lesions on the hypocotyl and radicle were observed when soybean seeds were inoculated with isolates FO38, FO41, and FO40, which resulted in higher levels of DSI (Fig. 3A).

Soybean cultivars used in this experiment showed different degrees of susceptibility when inoculated with *Fo*. Cultivars Ripley and 299N had little or no symptoms of root rot
across all *Fo* isolates. Conversely, cultivars MN1805 and Jack displayed the highest mean levels of disease across *Fo* isolates (Fig. 3B). The DSI ranged from 2.6% on the soybean cultivar Ripley when inoculated with isolate FO46 to 60.8% on cultivar MN1805 when inoculated with isolate FO40.

The visual scale of severity presented results similar to those of the DSI. Visual severity ratings in the rolled-towel assay also indicated a significant interaction (*P* = 0.00083) between cultivars and isolates (Table 3). Furthermore, the two methods for root rot assessment (DSI and visual rating scale) were linearly related (*P* < 0.0001) (Fig 5). Pearson’s correlation coefficients for the two disease evaluation methods were highly significant for all cultivars (Table 4).

Pathogenic isolates negatively impacted plant development at different magnitudes. Plant length and root weight were significantly reduced for the plants inoculated with the highly aggressive isolates (FO38, FO41, FO40) when compared to the mock-inoculated controls. Conversely, non-pathogenic isolates (FO42, FO46, FO36, FO48), which showed the lowest levels of DSI, did not significantly affect the total plant length or root weight (Fig 4A, 4C).

Plant length was negatively affected for almost all cultivars when they were inoculated with pathogenic isolates of *Fo*, with the exceptions of CM396 and MN1805 (Fig 4B). On the other hand, only a few cultivars showed reductions in root weight or root length compared to non-inoculated controls; only highly susceptible cultivars H2494 and Jack exhibited significant reductions in root length (Fig 4D, 4F).
Pearson coefficients indicated a significant strong and negative linear correlation between DSI and plant length. In contrast, plant variables such as root length and root weight were not strongly correlated with DSI for most of the cultivars (Table 4).

**Petri-dish assay**

Based on the visual severity scale for the petri dish assay (Fig 2), cultivars varied in their degree of susceptibility to seed rot, and there was a significant interaction between the main factors cultivars and isolates ($P = 0.0455$) (Table 3). Overall, cultivars MN1805 and MAC02 were the most susceptible, showing poor germination and completely colonized seeds, with disease severity mean scores of 2.35 and 1.96, respectively. In contrast, cultivars 299N and MYC5171 presented the lowest disease severity mean scores of 1.35 and 1.40, respectively, with no symptoms or very small dark brown discoloration on seedling roots (Fig 3D).

Disease levels and type of symptoms varied widely among the isolates. Isolates such as FO36 and FO42 were almost non-pathogenic, producing small brown lesions on roots or no symptoms, such as in the cultivars MYC5171 and 299N. In contrast, isolates FO40, FO41 and FO43 were highly aggressive; they rapidly colonized the seed, causing pre-emergence damping-off. Cultivar MN1805 scored the highest severity average of 2.8 when inoculated with isolate FO40, and 2.7 with FO41. In addition, MN1805 was given the highest score for disease severity in 12 out of the 14 $Fo$ isolates.

**Radial growth on PDA**

Radial growth at 25°C differed significantly ($P < 0.001$) among the fourteen $Fo$ isolates. Radial growth after 4 days varied from 2.3 cm (FO46) to 4.3 cm (FO47). Isolates
FO47, FO42, and FO36 had significantly faster growth than the rest of the isolates. Average diameter among all isolates was 3.34 cm (Fig 6).

**Fungicide sensitivity assays**

Inhibition in radial growth *Fo* isolates due to fludioxonil was variable and did not exhibit a dose response; therefore, EC$_{50}$ values could not be calculated. Growth inhibition did not exceed 40% compared to the control (Fig 7). In contrast, ipconazole effectively reduced the growth of all isolates; at the highest two concentrations (10 and 100 µg a.i./ml), ipconazole inhibited growth by 97.3% and 99.3%, respectively (Fig 7). Ipconazole EC$_{50}$ values varied among isolates; however, isolates FO38 and FO41 showed the highest EC$_{50}$ values (0.747 and 1.667 µg a.i./ml, respectively) (Table 5).

QoIs effectively inhibited conidial germination for all *Fo* isolates. At the highest concentrations (10 and 100 µg a.i./ml), trifloxystrobin inhibited germination by 90% and 99%, respectively. Pyraclostrobin performed better than trifloxystrobin for the 10 µg/ml concentration (94.6% inhibition) (Fig 7). Even though QoIs displayed similar results, pyraclostrobin was more effective and had a mean EC$_{50}$ value lower than that of trifloxystrobin (Table 5). Interestingly, pyraclostrobin EC$_{50}$ values were high for individual isolates FO38 and FO41 (0.481, and 0.358 mg a.i./L, respectively), compared to the rest of the isolates. In addition, FO38 also showed a high EC$_{50}$ for trifloxystrobin (1.479 µg a.i./ml) (Table 5).
Discussion

This study provides information about the interaction of Fo with soybean cultivars and describes phenotypic diversity of Fo that may affect its role in the Fusarium root rot complex. The fourteen Fo isolates collected in Iowa and evaluated in this study displayed a high degree of variability in aggressiveness, ranging from almost non-pathogenic to very aggressive. Similarly, soybean cultivars displayed a wide range of susceptibility, with some cultivars showing only small lesions on seedling tissues and a few other cultivars experiencing extensive colonization leading to low germination.

Fo is an important component of the Fusarium root rot complex in soybean, and is usually found in significantly higher frequency than other Fusarium species in soybean roots (Diaz Arias et al. 2013b; Leslie et al. 1990; Nelson and Windels 1992). However, the role of Fo as a pathogen has been elusive due to the high degree of variability in disease severity caused by different strains. Although previous studies have reported significant differences on susceptibility among cultivars or aggressiveness among Fo isolates, they have included only one isolate across several cultivars at a time or vice versa. Therefore, conclusions from these studies were contradictory, proposing Fo as a primary pathogen of soybeans or as a secondary weak pathogen (Armstrong and Armstrong 1950, 1965; Ferrant and Carroll 1981; French and Kennedy 1963; Jester 1973; Killebrew et al. 1993; Zhang et al. 2010). Although we report a great variation in disease intensity as well, these data provide a more complete overview from the FOSC including interactions among several isolates and soybean cultivars.
In this study, we evaluated different aspects of *F. oxysporum* pathogenicity (root rot and damping-off), using two methodologies. The rolled towel and petri dish assays provide information on the ability of the isolates to cause disease at a controlled level of inoculum and when the seed is in contact with mycelium, respectively. Despite the difference in methodologies and the high degree of variation in disease levels, many isolates and cultivars showed consistent levels of susceptibility or aggressiveness in causing both root rot and damping-off in both assays. However, some isolates were aggressive causing either damping-off or root rot. This finding emphasizes the value of using assays that measure both root rot and damping-off, in order to fully understand the impact of specific isolate-cultivar interactions. More studies are necessary to determine how the interaction between different isolates may affect the expression of symptoms.

The significant cultivar x isolate interactions suggest that the pattern of resistance or susceptibility for each soybean cultivar differs among isolates. However, significant interactions were more attributable to differing magnitudes of disease severity ranges among cultivars, rather than changes in rank. Additionally, we report here two cultivars (Ripley and 299N) that consistently presented resistance to the isolates used in this study, and may serve as a future source for plant breeders.

The DSI and the visual scale of severity used in the rolled towel assay to measure disease intensity had a significant linear relationship suggesting they are similar indicators of disease across different soybean cultivars. The visual scale of severity could be a quicker technique to evaluate several cultivars in a short period of time as compared to the DSI. Although the visual scale is not as precise as the index based on lesion measurement, it was
accurate enough to present similar results in the ranking of the less susceptible cultivars and the most aggressive isolates compared to the DSI.

Correlation analysis between DSI and plant growth characteristics indicated the negative effects of *Fo* on seedling health. Plant length was negatively correlated with DSI and significant reductions in plant length occurred due to *Fo* inoculation. However, it is important to note that despite this high correlation, plant length was not indicative of susceptibility. Some soybean cultivars that exhibited resistance according to the DSI or visual severity still experienced a significant reduction in plant length. In addition, root weight and root length were highly variable within and among cultivars; therefore, it was difficult to observe significant differences between inoculated and control treatments.

Analysis of root weight reductions was, however, more useful when it was performed by isolate, since it pinpointed the 3 most aggressive isolates (FO38, FO41 and FO40) compared to the mock-inoculated controls. These results are consistent with Zhang et al., (2010) who reported no significant correlations between plant growth variables and root rot severity and reported that resistant soybean cultivars displayed reductions in fresh root weight and plant height.

*Fo* isolates differed in colony morphology and radial growth when growing on PDA media at 25°C. In similar previous studies, the optimum growth temperature has been reported at 25°C for a vast majority of members of the *Fusarium* genera and some *formaes speciales* of *Fo* from beans, spinach and banana (Groenewald et al. 2006; Ivanovic 1987; Naiki and Morita 1983; Nelson et al. 1990a). Our data did not suggest any significant correlation between levels of disease intensity and radial growth. For example, at 25°C
isolates FO41 and FO46 presented the highest and the lowest radial growth respectively and they are almost non-pathogenic on soybeans; in the same way, aggressive isolates FO41 and FO40 differed significantly in their relative radial growth. Similarly, temperature in our pathogenicity assays was close to 25°C and we obtained a wide range of variability in disease intensity; thus vegetative radial growth at 25°C was not an indicator of aggressiveness. However, it would be interesting to observe radial growth of these isolates at different temperatures and correlate it to pathogenicity data.

This study reports fungicide sensitivity analyses for Fo isolates obtained from soybean roots. Inhibition of spore germination by trifloxystrobin and pyraclostrobin indicated little variability in sensitivity across Fo isolates with EC$_{50}$ values ranging from 0.005 to 1.49 µg a.i./ml. These findings contrast with previous research showing that F. graminearum isolates collected from corn and soybean in Ohio have reduced sensitivity to trifloxystrobin; this fungicide inhibited growth of some isolates only up to 35% of the control at 100 µg a.i./ml (Broders et al. 2007a).

According to our sensitivity studies ipconazole significantly reduced the growth of all Fo isolates, these findings agree with previous reports in which ipconazole significantly reduced Fusarium wilt caused by Fo f. sp. niveum in watermelon field trials (Everts et al. 2014). It has also been shown that ipconazole used in combination with QoIs have positive effects on emergence in chickpea plants infected with Aschochyta rabiei (Wise et al. 2009).

Fo isolates tested in our studies were insensitive to fludioxonil. These results indicate that fludioxonil activity varies widely among Fusarium spp. For example, fludioxonil has been reported to be effective against F. graminearum from soybeans and maize providing
effective inhibition of mycelial growth when used as a seed treatment (Broders et al. 2007a; Ellis et al. 2011; Munkvold and O'Mara 2002). Fludioxonil significantly reduced plant death in asparagus plants inoculated with \textit{Fo f. sp. asparagi} and \textit{F. proliferatum} (Reid et al. 2002), and improved stands of wheat under field conditions (Mueller et al. 1997). However, fludioxonil-insensitive isolates in the genus \textit{Fusarium} have been reported in Canada and United States (Gachango et al. 2012; Peters et al. 2008; Vignutelli et al. 2002).

Although this is unknown, it is likely that the \textit{Fo} isolates tested in our studies had been previously in contact with the active ingredients used in the fungicide sensitivity analyses. These are widely used active ingredients, and have been used for a considerable period of time for the control of seedling disease in soybean and maize. Fludioxonil was introduced as a seed treatment for several crops including soybeans and maize in 1994, and trifloxystrobin and pyraclostrobin were approved for the same use in 1999 and 2008, respectively (Munkvold 2009). To the best of our knowledge, there are no previous reports of sensitivity profiles on \textit{Fo} isolates associated with soybean roots, and there is a lack of true baseline sensitivity data. These results serve as a reference for future studies and emphasize the need to include \textit{Fo} isolates from other locations and to test these active ingredients on \textit{in vivo} trials.

The sensitivity profiles for \textit{Fo} isolates provided here, in conjunction with previous research on fungicide sensitivity on other root rot pathogens associated with soybean root rot, such as \textit{Pythium} spp. and \textit{F. graminearum}, will provide insight into effective disease management strategies. For example, QoIs may provide excellent control for \textit{Fo} but not for \textit{Pythium} spp. or \textit{F. graminearum}. On the other hand, fludioxonil effectively reduced the growth of \textit{F. graminearum}, whereas \textit{Fo} is insensitive to it. All these differences in fungicide
sensitivity, and the diverse number of species that constitute the disease root rot complexes make it challenging to determine the best combinations of active ingredients to be included as seed treatments.

In this study we report the effect of fungicides on individual strains on amended plates, but more information should be added on the direct effect of these molecules on seed treatments under greenhouse and field conditions at different temperatures. Temperature may play an important role in the level of efficacy and relative control for this type of assay, as it has been demonstrated in the case of *Pythium* spp. (Matthiesen et al. 2016). This knowledge represents a strong motivation to further investigate the efficacy of these active ingredients at different temperatures.

FOSC is a genetically diverse group with polyphyletic origins (Ellis et al. 2014). Not surprisingly, a great deal of variability was observed regarding colony characteristics and pathogenicity in this study. It is important to remember that soybean seedling diseases and root rot always appear as disease complexes and, as a consequence, the interactions of the FOSC with other soil pathogens outside the *Fusarium* genus may influence the outcomes of seed treatments. In the future, it would be of great importance to generate information about the interaction between *Fo* isolates other genera and spp., not only for pathogenicity or disease intensity, but also for the implications it may have on fungicide seed treatments.

The most effective strategies to manage Fusarium root rot are crop rotation, high quality seed, delayed planting and fungicide seed treatments (Nelson 1999). However, cultivar resistance and pathogenicity information are a key point to explore possibilities to develop long-term strategies in plant breeding programs (Zhang et al. 2013). For example,
host transcriptome profiles have been done for soybean plants inoculated with Fo isolates FO40 and FO36, identified to be pathogenic and non-pathogenic respectively. Substantial differences were observed in the gene expression profiles in which FO40 activated stronger expression of defense-related genes compared to the non-pathogenic FO36, in response to which there was a very low number of differentially expressed genes (Lanubile et al. 2015). Pathogenicity information generated in this study along with transcriptome profiles could be used as a strategy for a more accurate selection of strains that may serve as biocontrol for Fo such as the soybean non-pathogenic isolate FO36. Although more studies on the effects of pathogenicity due to the interaction between pathogenic and non-pathogenic isolates must be done and the mode of actions of biocontrol must be elucidated (Fravel et al. 2003), there is a good chance that these studies will serve as a first step to develop biological seed treatments based on the accurate selection of Fo isolates in the near future.

**Literature Cited**


Table 1. Description of *F. oxysporum* isolates collected from soybeans roots across Iowa in 2007 (Díaz Arias et al. 2013a), and genotypic characterization including mating type loci and clades within the *F. oxysporum* species complex (FOSC) (Ellis et al. 2014).

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<tr>
<th>Isolate ID</th>
<th>County</th>
<th>Growth stage&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mating type</th>
<th>FOSC clade</th>
<th>Type of conidia&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Microconidia</td>
<td>Macroconidia</td>
</tr>
<tr>
<td>FO36</td>
<td>Lyon</td>
<td>V3</td>
<td>MAT1-2</td>
<td>5</td>
<td>P A</td>
</tr>
<tr>
<td>FO37</td>
<td>Lyon</td>
<td>V3</td>
<td>MAT1-2</td>
<td>5</td>
<td>A P</td>
</tr>
<tr>
<td>FO38</td>
<td>Hamilton</td>
<td>R1</td>
<td>MAT1-2</td>
<td>5</td>
<td>P P</td>
</tr>
<tr>
<td>FO39</td>
<td>Lee</td>
<td>V3</td>
<td>MAT1-2</td>
<td>5</td>
<td>P A</td>
</tr>
<tr>
<td>FO40</td>
<td>Butler</td>
<td>V3</td>
<td>MAT1-1</td>
<td>3</td>
<td>P P</td>
</tr>
<tr>
<td>FO41</td>
<td>Jefferson</td>
<td>V3</td>
<td>MAT1-1</td>
<td>6</td>
<td>P A</td>
</tr>
<tr>
<td>FO42</td>
<td>Lee</td>
<td>V3</td>
<td>MAT1-2</td>
<td>2</td>
<td>P P</td>
</tr>
<tr>
<td>FO43</td>
<td>Crawford</td>
<td>V2</td>
<td>MAT1-2</td>
<td>5</td>
<td>P P</td>
</tr>
<tr>
<td>FO44</td>
<td>Winneshiek</td>
<td>V2</td>
<td>MAT1-2</td>
<td>5</td>
<td>P A</td>
</tr>
<tr>
<td>FO45</td>
<td>Lyon</td>
<td>V3</td>
<td>MAT1-2</td>
<td>5</td>
<td>P A</td>
</tr>
<tr>
<td>FO46</td>
<td>Crawford</td>
<td>V2</td>
<td>MAT1-2</td>
<td>5</td>
<td>A P</td>
</tr>
<tr>
<td>FO47</td>
<td>Dickinson</td>
<td>V3</td>
<td>MAT1-2</td>
<td>3</td>
<td>P A</td>
</tr>
<tr>
<td>FO48</td>
<td>Crawford</td>
<td>V2</td>
<td>MAT1-1</td>
<td>3</td>
<td>A P</td>
</tr>
<tr>
<td>FO49</td>
<td>Allamakee</td>
<td>V3</td>
<td>MAT1-2</td>
<td>6</td>
<td>P A</td>
</tr>
</tbody>
</table>

<sup>a</sup>Soybean developmental stage at the time of root sampling

<sup>b</sup>Type of conidia produced on PDA after 7 days of incubation in a 12h light period at 25°C

P present

A absent
Table 2. Description of eleven soybean cultivars used to study its interaction to *Fusarium oxysporum*

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Maturity Group</th>
<th>Pedigree</th>
<th>Year released</th>
<th>Developer reference</th>
<th>Disease reactions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forrest</td>
<td>V</td>
<td>Williams(7)</td>
<td>1981</td>
<td>Illinois AES and USDA</td>
<td>R R</td>
<td>(Hartwig and Epps 1973; Tatalović 2014)</td>
</tr>
<tr>
<td>MAC02</td>
<td>IV</td>
<td></td>
<td></td>
<td></td>
<td>S MR</td>
<td>(Luckew et al. 2012; Tatalović 2014)</td>
</tr>
<tr>
<td>MN1606</td>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td>R S</td>
<td>(Luckew et al. 2012; Tatalović 2014)</td>
</tr>
<tr>
<td>CM396</td>
<td>III</td>
<td></td>
<td></td>
<td></td>
<td>R S</td>
<td>(Schmidt et al. 2014; Tatalović 2014)</td>
</tr>
<tr>
<td>MYC5171</td>
<td>I</td>
<td>Williams(7) x Kingwa</td>
<td>1981</td>
<td>Illinois AES and USDA</td>
<td>MS S/MS</td>
<td>(Luckew et al. 2012; Tatalović 2014)</td>
</tr>
<tr>
<td>Williams</td>
<td>III</td>
<td>Fayette x Hardin</td>
<td>1989</td>
<td>Illinois AES</td>
<td>S S</td>
<td>(Bernard and Cremeens 1988; Schmidt et al. 2009; Tatalović 2014)</td>
</tr>
<tr>
<td>H2494</td>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td>S S</td>
<td>(Cianzio et al. 2016; Tatalović 2014)</td>
</tr>
<tr>
<td>MN1805</td>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td>MR S</td>
<td>(Luckew et al. 2012; Tatalović 2014)</td>
</tr>
</tbody>
</table>

*SDS Soybean death syndrome  †SCN Soybean cyst nematode  S susceptible  MS moderately susceptible  MR moderately resistant  PR partially resistant  R resistant*
Table 3. Analysis of variance indicating the effects of soybean cultivars and *F. oxysporum* isolates on disease severity index (DSI), visual severity rating, plant length (PL), root length (RL), and root weight (RW).

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>DSI*</th>
<th>Severity</th>
<th>Rolled towel</th>
<th>Severity</th>
<th>PL</th>
<th>Severity</th>
<th>RL</th>
<th>Severity</th>
<th>RW</th>
<th>Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>P &gt; F</td>
<td>F</td>
<td>P &gt; F</td>
<td>F</td>
<td>P &gt; F</td>
<td>F</td>
<td>P &gt; F</td>
<td>F</td>
<td>P &gt; F</td>
</tr>
<tr>
<td>Cultivar (C)</td>
<td>10</td>
<td>30.98</td>
<td>&lt;0.0001</td>
<td>23.05</td>
<td>&lt;0.0001</td>
<td>15.27</td>
<td>&lt;0.0001</td>
<td>46.95</td>
<td>&lt;0.0001</td>
<td>26.73</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Isolates (I)</td>
<td>13</td>
<td>65.27</td>
<td>&lt;0.0001</td>
<td>126.84</td>
<td>&lt;0.0001</td>
<td>35.67</td>
<td>&lt;0.0001</td>
<td>13.14</td>
<td>&lt;0.0001</td>
<td>9.72</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(C×I)</td>
<td>130</td>
<td>1.32</td>
<td>0.0140</td>
<td>1.36</td>
<td>0.0083</td>
<td>1.21</td>
<td>0.0717</td>
<td>1.64</td>
<td>&lt;0.0001</td>
<td>1.14</td>
<td>0.1587</td>
</tr>
</tbody>
</table>

*DSI was arcsin transformed. Visual scale of severity, plant length, root weight, and root length were log transformed. Experiments consisted of 3 replications (towels) for each treatment combination and two experiments for the rolled towel assay. Petri dish assay experiments consisted of 3 petri dishes per treatment combination in an incomplete block design, and using soybean cultivars Ripley and Jack as a control check for each block. Data are from 11 soybean cultivars (C) and fourteen *F. oxysporum* isolates (I).*
Table 4. Pearson correlation coefficients performed by soybean cultivar on disease severity index (DSI), Plant length (PL), root length (RL), and root weight (RW), and visual scale for severity.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>PL</th>
<th>RL</th>
<th>RW</th>
<th>SEVERITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ripley</td>
<td>-0.71</td>
<td>-0.44</td>
<td>-0.49</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>0.016</td>
<td>0.007</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>299N</td>
<td>-0.61</td>
<td>-0.39</td>
<td>-0.25</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>0.0005</td>
<td>0.038</td>
<td>0.189</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Forrest</td>
<td>-0.72</td>
<td>-0.42</td>
<td>-0.32</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>0.023</td>
<td>0.092</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MAC02</td>
<td>-0.69</td>
<td>-0.50</td>
<td>-0.36</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>0.006</td>
<td>0.056</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MN1606</td>
<td>-0.72</td>
<td>-0.63</td>
<td>-0.08</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>0.0003</td>
<td>0.654</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CM396</td>
<td>-0.73</td>
<td>-0.31</td>
<td>-0.21</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>0.098</td>
<td>0.272</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MYC5171</td>
<td>-0.41</td>
<td>-0.22</td>
<td>-0.13</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>0.027</td>
<td>0.258</td>
<td>0.484</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Williams82</td>
<td>-0.68</td>
<td>0.02</td>
<td>-0.08</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>0.881</td>
<td>0.659</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>H2494</td>
<td>-0.79</td>
<td>-0.59</td>
<td>-0.64</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001</td>
<td>0.0008</td>
<td>0.0002</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MN1805</td>
<td>-0.56</td>
<td>-0.48</td>
<td>-0.24</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>0.008</td>
<td>0.218</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Jack</td>
<td>-0.59</td>
<td>-0.26</td>
<td>-0.08</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>0.0008</td>
<td>0.178</td>
<td>0.669</td>
<td>&lt;0.0001</td>
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</table>
Table 5. Fungicide sensitivity analysis (EC$_{50}$) for fourteen *Fusarium oxysporum* isolates recovered from soybeans. EC$_{50}$ for the a.i. fludioxonil were not calculated since there was no apparent inhibition of mycelial radial growth with respect to the non-amended control.

<table>
<thead>
<tr>
<th></th>
<th>Pyraclostrobin</th>
<th>Trifloxystrobin</th>
<th>Ipconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC$_{50}$ Log10 (dose)</td>
<td>EC$_{50}$ µg a.i./ml</td>
<td>EC$_{50}$ Log10 (dose)</td>
</tr>
<tr>
<td>FO36</td>
<td>-0.777</td>
<td>0.166</td>
<td>-1.193</td>
</tr>
<tr>
<td>FO37</td>
<td>-0.991</td>
<td>0.102</td>
<td>-0.717</td>
</tr>
<tr>
<td>FO38</td>
<td>-0.317</td>
<td>0.481</td>
<td>0.170</td>
</tr>
<tr>
<td>FO39</td>
<td>-1.025</td>
<td>0.094</td>
<td>-1.040</td>
</tr>
<tr>
<td>FO40</td>
<td>-1.392</td>
<td>0.040</td>
<td>-1.331</td>
</tr>
<tr>
<td>FO41</td>
<td>-0.445</td>
<td>0.358</td>
<td>-1.560</td>
</tr>
<tr>
<td>FO42</td>
<td>-1.222</td>
<td>0.059</td>
<td>-1.661</td>
</tr>
<tr>
<td>FO43</td>
<td>-1.164</td>
<td>0.068</td>
<td>-1.238</td>
</tr>
<tr>
<td>FO44</td>
<td>-0.758</td>
<td>0.174</td>
<td>-0.519</td>
</tr>
<tr>
<td>FO45</td>
<td>-1.156</td>
<td>0.069</td>
<td>-2.236</td>
</tr>
<tr>
<td>FO46</td>
<td>-0.641</td>
<td>0.228</td>
<td>-1.126</td>
</tr>
<tr>
<td>FO47</td>
<td>-0.990</td>
<td>0.102</td>
<td>-0.521</td>
</tr>
<tr>
<td>FO48</td>
<td>-0.949</td>
<td>0.112</td>
<td>-1.469</td>
</tr>
<tr>
<td>FO49</td>
<td>-0.905</td>
<td>0.124</td>
<td>-0.761</td>
</tr>
<tr>
<td>Average</td>
<td>-0.909</td>
<td>0.155</td>
<td>-1.085</td>
</tr>
</tbody>
</table>
Fig 1. Fusarium root rot disease intensity evaluated by an ordinal severity scale 1 = germination, no symptoms of disease or colonization on seedling; 2 = germination, little colonization of the seedling, 1 to 19% of the root with lesions; 3 = germination, some colonization of seedling, and 20 to 74% of the root with lesions; 4 = germination, complete colonization of the seedling, and 75% or more of the seedling root with lesions; 5 = no germination, complete colonization of the seed.
**Fig 2.** Fusarium root rot disease intensity evaluated by a visual severity scale 0 = 100% germination with no symptoms of root infection; 1 = 70 to 99% germination with lesion formation on the roots; 2 = 30 to 69% germination with coalesced lesions; and 3 = 0 to 29% germination and all seed tissues were colonized.
**Fig 3.** Mean Disease severity index (DSI%) (A and B rolled towel assay) and visual severity rating (C and D petri dish assay) for the main effects of isolate and soybean cultivar for *Fusarium oxysporum* isolates from Iowa. Means with same letter are not significantly different using Fisher’s protected least significant difference (P = 0.05), on arcsin transformed data for the rolled towel assay. Data from the mock-inoculated seeds were not included in the analysis.
**Fig 4.** Differences in plant characteristics for the main effects of isolate and soybean cultivar.  

**A and B** Plant length (cm). **C and D** Root weight (g). **E and F** Root length (cm). * indicates significant differences from the non-inoculated control (P = 0.05) with a T-test for mean separation. Comparisons were made individually by isolate and soybean cultivar.
**Fig 5.** Relationship between the visual scale for severity and the disease severity index (DSI) for the rolled towel assay. Each point represents an average of 3 towels containing 15 soybean seeds each.
Fig 6. Radial growth of fourteen *Fusarium oxysporum* isolates on PDA after 4 d at 24°C.
Fig 7. Inhibitory effects of fungicides on radial growth (fludioxonil, ipconazole) and conidial germination (trifloxystrobin and pyraclostrobin) with respect to dose concentration on fourteen *Fusarium oxysporum* isolates.
CHAPTER 3. EFFECTS OF TEMPERATURE AND pH ON *FUSARIUM OXYSPORUM* AND SOYBEAN SEEDLING DISEASE

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

D. R. Cruz, L. F. S. Leandro and G. P. Munkvold

Department of Plant Pathology and Microbiology, Iowa State University, Ames. 50011.

Abstract

*Fusarium oxysporum* (*Fo*) is an important pathogen that reduces soybean yield by causing seedling disease and root rot. This study the effects of pH and temperature on *Fo* fungal growth and seedling disease were assessed. In an *in vitro* assay, 14 *Fo* isolates were grown on artificial culture media at five pH levels (4, 5, 6, 7, 8), and incubated at four temperatures (15, 20, 25, or 30°C). In a rolled-towel assay, seeds were inoculated with a suspension of a pathogenic or a non-pathogenic *Fo* isolate. The seeds were placed in rolled germination paper and the rolls were incubated in all combinations of buffer solutions at four pH levels (4, 5, 6, 7), and four temperatures (15, 20, 25, or 30°C). There was a significant interaction between temperature and pH (*P* < 0.05) for *in vitro* radial growth and root rot severity. Isolates showed the most *in vitro* radial growth after 5 days of incubation at pH 6 and 25°C. For the rolled-towel assay, the pathogenic isolate caused the most severe root rot at pH 6 and 25°C. Gaussian regression analyses were performed to estimate optimal pH and temperature for fungal growth and disease severity. Optimal estimated conditions were pH 6.4 at 27.4 °C for maximal fungal growth, and pH 5.9 at 30°C for maximal root rot severity.
These results indicate that optimal pH and temperature conditions are similar for *Fo* growth and disease in soybean seedlings, and suggest that *Fo* may be a more important seedling pathogen when soybeans are planted later in the planting season, when soil conditions tend to be warmer.

**Introduction**

*Fusarium oxysporum* Schltdl. (*Fo*) is one of the fungal species most commonly isolated from soybean roots in the soybean producing regions of North America (Díaz Arias et al. 2013a; Ellis et al. 2014; Zhang et al. 2010). *Fo* has been associated with soybean damping-off, seed and seedling rot, root rot, and vascular wilt (Armstrong and Armstrong 1965; Datnoff and Sinclair 1988; Díaz Arias et al. 2013a; Diaz Arias et al. 2013b; Leath and Carroll 1981). Significant differences in root rot severity have been observed among *Fo* isolates varying from weakly pathogenic to highly aggressive (Cruz et al. 2013; Díaz Arias et al. 2013a; Ellis et al. 2014). These variations in disease severity may be influenced by abiotic factors such as temperature and pH that alter host-pathogen dynamics, making it more difficult to understand the disease processes involved in soybean root rot development. However, the effects of abiotic factors on vegetative fungal growth and root rot severity of *Fo* on soybeans are unknown.

In the case of plant pathogenic isolates in the *Fusarium* genus, the most suitable pH for growth has been reported in the 6 to 6.5 range (Cochrane 1958; Srobar 1978). However, there are instances in which the greatest pH *in vitro* growth in *Fusarium* spp. has been found in more acidic ranges (Srivastava et al. 2011). For example, *F. graminearum* and *F. equiseti*
grew best at pH 3.5, and *F. solani* at pH 4.5 (Agarwal and Sarbhoy 1978). In addition, a number of *Fo forma speciales* (f. sp.) display a wide range of optimal pH for growth. For example, isolates from *Fo* f. sp. *cubense* grew optimally at pH 6.0 but no growth was observed at pH 8.0 (Groenewald et al., 2006). Gupta et al., (2010), on the other hand, observed a maximum growth of *Fo* f. sp. *psidii* at pH 5.5.

Maximal *in vitro* radial growth in *Fo* plant pathogenic isolates have been found at temperatures between 25 to 28°C (Brownell and Schneider 1985; Gupta et al. 2010; Marin et al. 1995; Nelson et al. 1990). However, there are isolates of many other *Fusarium* spp. and *Fo* f. sp. isolates, such as *F. beomiforme, F. proliferatum, F. verticillioides*, and *Fo* f. sp. *betae*, which display higher temperatures for maximal radial growth (≥30°C) (Miller and Burke 1985; Nelson et al. 1990; Webb et al. 2015). Although there is evidence of significant interactions between pH and temperature on fungal radial growth in *Fusarium* spp. (Chen et al. 2013; Marin et al. 1995; Schuerger and Mitchell 1992), interactions between these two factors are not often considered during Fusarium inoculum preparation for research experiments.

Temperature alters the expression of host defenses mechanisms favoring resistance or susceptibility reactions (Harling et al. 1988; Stuthman et al. 2007). For example, moderately resistant or highly resistant cultivars of banana, carnation, chickpea or lettuce, become susceptible under high temperatures (>25°C) after inoculation with their respective *Fo* f. sp. (Brake et al. 1995; Harling et al. 1988; Landa et al. 2006; Scott et al. 2010). The effects of temperature and pH in the soybean-*Fo* interaction are unknown, although there is some understanding of *Fo* genetic and phenotypic diversity, including the existence of pathogenic
and non-pathogenic isolates, and variable levels of resistance among soybean cultivars (Cruz et al. 2013; Ellis et al. 2014; Lanubile et al. 2015).

One approach to understand the interactions of abiotic factors on pathogens is to model. Gaussian and quasi-Gaussian models are used to describe spatial dispersion of spores over long distances (Skelsey et al. 2008; Soubeyrand et al. 2008). Gaussian models have been used in plant pathology to simulate spore wind dispersal of *Phytophthora infestans* in potatoes (Spijkerboer et al. 2002), and describe spatial movement of wind-dispersed spores of *Gibberella zeae* foci on wheat plots (Paulitz et al. 1999; Prussin et al. 2015). With FO, a few studies have modeled the effects of the interaction between pH and temperature on Fusarium wilts caused by Fusarium spp. Polynomial models have been used to describe temperature and pH effects on Fusarium wilts under greenhouse and hydroponic conditions in cucumber and mung bean (Chen et al. 2013; Schuerger and Mitchell 1992). Development of a Gaussian model may be useful to describe how these factors impact disease development.

The objectives of this research were to evaluate the importance of pH and temperature in the development of soybean root rot caused by *Fo* by studying (i) the effects of the pH and temperature interaction on *in vitro* radial growth, (ii) the effects of the pH and temperature interaction on soybean root rot under growth chamber conditions, and (iii) the differences between a pathogenic and non-pathogenic *Fo* isolate in causing disease under different pH and temperature conditions.
Materials and Methods

Effects of pH and temperature on *in vitro* radial growth

Fourteen single-spore *Fo* isolates collected from symptomatic soybean roots in Iowa in 2007 (Díaz Arias et al. 2013a) were maintained on dry silica beads at 4˚C until use. To prepare inoculum for the experiments, the *Fo* isolates were grown on potato dextrose agar (PDA) at 39g/L (Difco, Becton, Dickinson and Co, Spark, MD, USA) at 25˚C for seven days, with 12 h photoperiod. A 5-mm plug was punched from the PDA culture and transferred to the center of a 90 mm diameter petri dish containing differential media with one of five pH levels (4, 5, 6, 7, 8), and additionally full strength PDA media (pH ~ 6.5).

The media differing in pH levels were prepared as described by Groenewald et al. (2006). Citrate – phosphate buffer was prepared by titrating 0.1 M citric acid (Armesco, Ohio, USA) with 0.2 M Na₂HPO₄ 12H₂O (Acros, New Jersey, USA) to reach a pH of 4 or 5. Phosphate buffer was prepared by titrating 0.2 M Na₂HPO₄ 12H₂O (Acros, New Jersey, USA) with 0.2 M NaH₂PO₄ 2H₂O (Acros, New Jersey, USA) to reach a pH of 6 or 7. Boric acid buffer was prepared by titrating 0.2 M of boric acid (Fisher, Illinois, USA) with 0.05M of sodium tetraborate Na₂B₄O₇ 10H₂O (Fisher, Illinois, USA) to reach a pH of 8. Once the buffers for pH 4, 5, 6, 7, and 8 were prepared, a basal medium was added to each liter of buffer. The basal medium consisted of 45g sucrose (C&H, Crocket, CA, USA), 3g of NaNO₃ (Sigma-Aldrich), 1.5g K₂HPO₄ (Fisher, Illinois, USA), 0.75g MgSO₄ 7H₂O (Sigma-Aldrich), 0.75 KCl (Fisher) and 0.015g FeSO₄ 7H₂O (Fisher, Illinois, USA). Agar (Difco, Becton, Dickinson and Co, Spark, MD, USA) was added at a rate of 18 g/L to the pH 4 buffer and 15 g/L of agar for pH buffers 5, 6, 7, 8. In addition to the pH differential media a full strength PDA media at 39g/L (pH ~6.5) was included in the experiments.
Fo cultures on the pH differential media and full strength PDA media (pH ~6.5), were incubated at four different temperatures (15, 20, 25, 30°C) for five days, in an incubator (Hoffman manufacturing Inc. Oregon, USA) at 65% relative humidity and in the dark. Diameter of each colony was then measured in two perpendicular directions, and an average of the two measurements was calculated after subtracting the 5-mm diameter of the colonized plug. The experiment followed a split-plot design, with temperature as the main plot and media and Fo isolates as sub-plot factors. Each petri plate was considered as an experimental unit, and there were four replicate petri dishes per isolate, pH level, and temperature combination (14 isolates x 6 media x 4 temps x 4 reps = 1,344 experimental units). The experiment was conducted twice.

Effects of pH and temperature on seedling disease

Fo isolates FO38 and FO42, previously identified as pathogenic and non-pathogenic to soybeans, respectively, (Cruz et al. 2013; Díaz Arias et al. 2013a; Ellis et al. 2014), were grown on PDA for 7 days at 25°C, with a 12 h photoperiod, to promote conidial formation. Macro and microconidia were collected by rinsing the plates with sterile distilled water and scraping the surface of the cultures with a sterile stick. The suspension was filtered through sterile cheesecloth to remove mycelial fragments. Inoculum concentration was calculated by counting conidia in a hemocytometer (Bright-line Hemocytometer, American Optical, Buffalo, NY) under the microscope, and conidial suspensions were adjusted to a concentration of $1\times10^6$ conidia/ml.

Fifteen seeds of Fo-susceptible cultivar Jack were placed on moist towels and individually inoculated with 200 µl of the spore solution of FO38 or FO42. Paper towels were moistened with a buffer solution adjusted to pH levels 4, 5, 6, or 7 using citric acid
(Amresco, Ohio, USA) and sodium hydroxide (NaOH) (Fisher, Illinois, USA). Towels were rolled up and placed in 1500 ml glass jars containing 250 ml of the respective buffer and incubated during 8 days in growth chambers (Hoffman manufacturing Inc. Oregon, USA) at four different temperatures (15, 20, 25, and 30°C) at 65% relative humidity, and with a 12 h photoperiod.

Seedlings were rated for root rot using two methods: a disease severity index (DSI) described by Ellis et al. (2011) and a visual disease severity scale (1-5). Disease severity index (DSI) was calculated as the ratio between the lesion length (discolored or colonized hypocotyl and radicle) and the total plant length, multiplied by 100. When seeds did not germinate and were completely colonized, they were assigned a DSI value of 100%. The visual 1-5 disease severity scale was assessed according to the following ratings: 1 = germination, no symptoms of disease or colonization on seedling; 2 = germination, little colonization of the seedling, 1 to 19% of the root with lesions; 3 = germination, some colonization of seedling, and 20 to 74% of the root with lesions; 4 = germination, complete colonization of the seedling, and 75% or more of the root with lesions; 5 = no germination, complete colonization of the seed. Seedling growth variables (root weight, root length and shoot length) were also evaluated.

Each towel roll was considered as an experimental unit with fifteen seeds or subsamples on each roll. There were three inoculated rolls for each of the *F. oxysporum* isolates and three mock-inoculated rolls for each pH and temperature treatment combination (4 temps x 4 pH x 3 inoculation trts x 3 reps = 144 experimental units). The experiment followed a split plot design with temperature as the main-plot factor, and pH buffers and *F. oxysporum* isolates as sub-
plot factors. Treatment combinations of pH levels and isolates were assigned randomly on each growth chamber. The experiment was conducted twice.

**Data analysis**

Variances of the raw data for the two experiments of radial growth were compared by using Lavene’s test for homogeneity under the general linear model procedure (PROC GLM) of SAS version 9.3 (SAS Institute Inc., Cary, NC). Variances of the two experiments were not statistically different according to the test ($P = 0.95$), therefore the raw data for the two experiments were combined for subsequent analysis. In addition, normal distribution of the residuals was tested under the PROC UNIVARIATE procedure. Analysis of variance was performed under PROC GLIMMIX procedure, in which the two experimental replications and its interaction with temperature were considered as random effects. pH differential media and *Fo* isolates and full strength PDA media were considered as fixed factors for the statistical analysis. Mean separation analyses were performed using a Fisher’s protected least significant difference (LSD) test at $P = 0.05$.

The disease severity index (DSI) for the pathogenicity assay was arcsin transformed and the disease severity scale was square-root transformed. In addition, root weight, root length and plant length were log transformed. Analysis of variance was performed under the PROC GLIMMIX procedure in which the two experimental replications and its interaction with temperature were considered as random effects. pH buffers and *Fo* isolates were considered as fixed factors for the statistical analysis. Mean separation analyses on the transformed data were performed using a Fisher’s protected least significant difference (LSD) test at $P = 0.05$.
Linear correlation coefficients were calculated using the PROC CORR statement on the average raw data for DSI and the visual scale for severity.

Exponential, polynomial, logistic, Gompertz and Gaussian models were tested to describe the radial growth and root rot of Fo under the pH and temperature levels selected for the experiments. Model selection was based on the following criteria described by Byamukama et al. (2011), low standard error of the estimate (SEE), significant F statistics ($P \leq 0.05$) and high coefficients of determination ($R^2$). The model that best described and fit to the data was selected to obtain parameter estimates.

Gaussian regression analyses for fungal radial growth and disease severity index, were performed for each individual Fo isolate using SigmaPlot version 13 (Systat Software, Inc., San Jose, CA) under the following function: 

$$f = a \exp \left( -5 \left( \frac{x - k_a}{b} \right)^2 + \left( \frac{y - Y_a}{c} \right)^2 \right)$$

Estimated values of temperature and pH for maximal fungal radial growth and disease severity were obtained by using a non-linear optimization procedure (solver add-in) from Excel (Microsoft) under the following constraints: pH range between 4 and 7 and a temperature range between 15°C and 30°C.

**Results**

**Effects of pH and temperature on in vitro radial growth**

Analysis of variance indicated that main effects of temperature, pH and Fo isolate and their interactions were highly significant ($P < 0.0001$) for fungal radial growth. The greatest mean growth averaged among isolates (5.18 cm) was observed in the combination of 25°C
and pH 6, and the lowest radial growth (0.96 cm) was observed in the combination of 15°C at pH 5. No growth was observed at pH 8 for any of the *Fo* isolates evaluated in this study.

Maximal radial growth was observed at pH 6 at 25°C and 30°C, for isolates FO41 and FO49 (6.39 cm and 6.30 cm, respectively). Minimal radial growth was observed at 15°C at pH 4 for isolate FO38 (0.3 cm) (Fig. 1). When mean separation analyses were performed for radial growth across temperatures for each pH level, growth was significantly higher at 25°C, followed by 30°C, 20°C and 15°C. This trend was observed across all pH buffer media and on full strength PDA, which had pH of around 6.5.

There was a strong effect of temperature and isolate on radial growth on full strength PDA and a significant interaction between these two factors (*P* < 0.0001). Although individual isolates had significant differences in their final radial growth, maximum growth across all isolates occurred at 25°C, except for FO42, which had greater growth at 30°C.

The Gaussian model best described the changes in radial growth across levels of pH and temperature for all *Fo* isolates, with significant *F* statistics ranging from 6.8012 to 37.3336, indicating there was a strong relationship between temperature, pH and radial growth (Table 1). Coefficients of determination (*R*²) from the Gaussian regression analyses varied among isolates, explaining up to 93.1% of the variation in fungal radial growth. In addition, the Gaussian model had lower standard error of the estimates with values ranging from 0.3907 to 0.9157 (Table 1), compared to exponential, logistic and Gompertz models (data not shown).

Estimated values of optimal pH and temperature for maximal radial growth obtained from the Gaussian regression analysis indicated variation among isolates. pH estimated
values varied between 5.6 and 6.8, and temperatures varied between 25.4°C and 30°C. A separate regression was performed on the average data of fungal radial growth across Fo isolates, the average estimate of pH and temperature for maximal radial growth over all Fo isolates was 6.3 and 27.1°C, respectively (Table 1) (Fig 2).

**Effects of pH and temperature on seedling disease**

According to the analysis of variance, pH, temperature and their interaction had significant effects ($P = 0.0005$) on DSI. In addition, there was a significant effect of isolate ($P < 0.0001$), indicating that disease severity differed between the pathogenic isolate FO38 and the nonpathogenic isolate FO42 (Table 2).

Disease severity index varied across levels of pH and temperature. For the pathogenic isolate, the highest level of root rot was observed at pH 6 and 30°C (67%), and the lowest levels of disease severity were observed at 15°C at pH 4 and 5 (4.2%) (Fig. 3A). Interestingly, when the mean separation analyses were performed across temperatures separately for each pH buffer media, 30°C and 25°C had significantly higher levels of disease in all pH levels. Root rot severity at 15 and 20°C displayed the lowest levels of disease for all pH levels (Fig. 3A).

Disease levels for the non-pathogenic FO42 isolate were significantly lower than the pathogenic isolate, but showed similar trends. The highest disease severity (17.2%) was observed at the same level of pH and temperature as the pathogenic isolate; pH 6 at 30°C (Fig. 3B). Mean separation analysis indicated no differences in disease severity between 15, 20, and 25°C for all pH levels (Fig. 3B).
Root rot symptoms differed between the pathogenic and non-pathogenic *Fo* isolates. Soybean seed inoculations performed with the pathogenic isolate FO38 caused extended colonization of radicles, hypocotyls, cotyledons and tissue maceration. Subsequently, germination was lower, and soybean seedlings had poor root and shoot development (Fig. 4A-D). Conversely, inoculations performed with the non-pathogenic isolate FO42 caused a few small spots of tissue discoloration on the hypocotyls and radicles but tissue structure and consistency were intact with no maceration or softening (Fig. 4E-H).

Analysis of variance also revealed highly significant effects of isolate for the plant variables root weight, root length, and plant length (Table 2) (Fig. 5). Mean separation analysis indicated that the pathogenic isolate FO38 produced significant detrimental effects on plant length and root weight compared to the mock-inoculated control. Conversely, overall means for root length and plant length were significantly higher when seeds were inoculated with the non-pathogenic FO42 isolate when compared to the pathogenic isolate and to the non-inoculated control.

The highest root weight reductions were observed at 30°C ranging from 35 to 47%, and the lowest reductions at 15°C, ranging from 8.2 to 22% when seeds were inoculated with the pathogenic isolate FO38 (Fig. 5A). Conversely, seeds inoculated with the non-pathogenic FO42 isolate presented significantly lower reductions in root weight (<20%) (Fig. 5B).

The highest root length reductions were observed when seeds were inoculated with the pathogenic isolate FO38 and incubated at 25°C and pH 5, 6, and 7; ranging from 16 to 21%. Similarly, seeds incubated at 25°C and pH 4 displayed a root length reduction of 20%. The lowest root length reductions were observed at 15°C, fluctuating from 6 to 8% (Fig. 5B).
In contrasts, seeds inoculated with the non-pathogenic FO42 isolate showed significantly lower reductions in root length (<13%) (Fig. 5D).

Plant length reductions were the highest when seeds were inoculated with the pathogenic isolate FO38 and incubated at 30°C and pH 4 and 5 (25%). The lowest plant length reductions were observed at 15°C ranging from 4.1 to 9.3% (Fig. 5C). Reductions in plant length were <9% when seeds were inoculated with the non-pathogenic FO42 isolate, with a few observations presenting no reductions (Fig. 5F).

The visual scale of severity presented similar results to DSI. The main effects of pH, isolate, and the interaction between temperature and isolates were highly significant ($P < 0.0001$). In addition, pH and its interaction with temperature were significant ($P = 0.0021$) (Table 2). Disease severity index and the visual scale for severity were linearly related. Pearson’s correlation coefficients performed separately by $Fo$ isolates were highly significant ($P < 0.0001$) indicating a strong positive linear relationship (Fig. 6).

Although there were highly significant differences in the disease intensity caused by the $Fo$ isolates, the Gaussian regression analysis described with high precision the variation in disease across pH levels and temperatures. Gaussian regressions performed separately on each $Fo$ isolate had low standard errors of the estimates, indicating higher precision for predicted values of disease, compared to other standard models (data not shown). In addition, regression analysis showed significant $F$ statistics ($P < 0.0001$), and high coefficients of determination ($R^2$) explaining 98% and 96% of the variation in disease for the pathogenic and non-pathogenic isolate, respectively (Table 1).
Estimated optimal values of pH and temperature for maximal root rot obtained from the Gaussian regression analysis were equivalent for the pathogenic and non-pathogenic *Fo* isolates. Interestingly, estimated values of pH at 5.9 and 30°C produced the highest level of root rot for both isolates. Under these optimal pH and temperature conditions, the pathogenic *Fo* isolate FO38 had a maximal estimated root rot of 64% and the non-pathogenic FO42 had a maximal estimated root rot of 17%. (Table 1) (Figure 7).

**Discussion**

This study provides evidence that pH and temperature have significantly effects on *Fo* fungal radial growth and severity of Fusarium root rot of soybean seedlings, increasing our understanding of the epidemiology of the disease. The combination of pH 6 and 25°C resulted in the fastest *in vitro* radial growth and most severe root rot development. Using Gaussian models, the optimal estimated conditions were pH 6.4 at 27.4 °C for maximal fungal growth, and pH 5.9 at 30°C for maximal root rot severity, showing that *Fo* growth and seedling disease are favored by similar conditions.

The results from this study are similar to the findings of earlier reports for *in vitro* radial growth of other *Fusarium* species and *formae speciales*. For example, *Fo* f. sp. *cubense* displayed a maximum radial growth at pH 6 at 25°C, with a minimum growth at pH 4 (Groenewald et al. 2006). Similar results for optimal fungal growth at 25°C were reported for *F. moniliforme* (Shahadat et al. 2015), *Fo* f. sp. *lactucae* (Scott et al. 2010), *Fo* f. sp. *fabae* (Ivanovic et al. 1987), and *Fo* f. sp. *spinaciae* (Naiki and Morita 1983).
Our findings were similar to those on *Fo f. sp. cubense* (Groenewald et al. 2006), using the same citrate – phosphate buffer media in which the most radial growth was observed at pH 6 at 25°C. However, in our studies, radial growth was significantly lower on PDA at pH 6.5 at 25°C compared to the buffered media at pH 6 and 7 at the same temperature. These results suggest that studies to determine optimal temperature for fungal growth must be viewed with some caution when PDA is used. Growth of fungi affects the pH of the media due to the production of secondary metabolites, pigments, absorption of anions and production of ammonia (Cochrane 1958). These metabolic activities tend to acidify the medium as has been demonstrated in *Fo* (Srivastava et al. 2011), making it complex to draw conclusions when poorly buffered growth media such as PDA is used. If the fungus is able to change the pH media in order to favor its growth, then conclusions about the optimal pH for growth could be misleading and a buffered media must be used.

pH is not a unitary factor influencing the mechanisms of growth; other physical parameters such as temperature, water potential and relative humidity may interact to a certain degree (Cochrane 1958; Pehrson 1948). Optimal pH for fungal growth can change along with increasing or decreasing temperatures. For example, optimal pH for growth of *Phacidium infestans* on liquid buffered medium decreased with temperature (Pehrson 1948). This contrasts with our findings on *Fo* in which the optimal pH remained at 6.0 for growth across all temperatures.

Fungal growth also depends on genetic background and physiological adaptations to predominant temperatures of geographical areas (Burgess et al. 1988; Manshor et al. 2012; Walker and White 2005). Isolates from tropical regions may have higher optimal growth temperature than isolates from temperate and subtropical regions (Cochrane 1958; Nelson et
al. 1990). For example, greatest fungal radial growth tends to occur at 25°C for Fo f. sp. *betae* isolates collected from temperate regions in the United States (Webb et al. 2015), as well for Fo f. sp. *psiidi* and *F. solani* isolates collected from subtropical regions in India (Gupta et al. 2010). In contrast, isolates of different *Fusarium* species occurring in arid and tropical regions of Australia grow the best at 30°C (Nelson et al. 1990; Sangalang et al. 1995). It would be interesting to include Fo isolates from tropical regions and compare them to those from Iowa in future pH and temperature studies.

Optimal pH and temperature conditions for soilborne plant diseases may or may not mimic optimal conditions for *in vitro* radial growth (Cochrane 1958). Our results showed the highest root rot severity occurred at pH 6 at 30°C, followed by pH 5 at 25°C. These values correspond to the highest *in vitro* radial growth, suggesting that pH and temperature have similar effects on Fo growth and soybean root rot severity caused by Fo.

Our results on root rot severity are similar to those reported for Fusarium root rot in other *Fusarium* species, such as Fo f. sp. *dianthi, cucumerinum, cubense, ciceris, spinaceae, phaseoli,* and *lactucae,* and *F. graminearum,* (Ben-Yephet and Shtienberg 1994; Chen et al. 2013; Ellis et al. 2011; Groenewald et al. 2006; Landa et al. 2006; Naiki and Morita 1983; Schuerger and Mitchell 1992; Scott et al. 2010), in which the highest disease severity was observed at temperatures between 25 to 30°C. Interestingly, the majority of these species were predominantly from humid subtropical climatic zones.

Our results indicate that pathogen fungal growth and root rot severity temperature curves have similar trends and follow each other closely. Similarities in these curves might indicate that disease development and severity are mainly influenced by the effect of temperature on the pathogen (Dickson 1923; Jones 1924).
In addition to the slower growth of *Fo* at lower temperatures, soybean seedling resistance to *Fo* may be increased. Low temperatures have been reported to favor plant defenses mechanisms making soybean seedlings more resistant to *Fo*. There is evidence of overexpression of resistance related proteins (esterase and PR-2) after soybean cultivars were inoculated with pathogenic *Fo* isolates at low temperatures. *Fo*-inoculated seedlings incubated at 8°C had higher 1,3-β-glucanases activity compared to the seedlings incubated at 24°C (Koretsky 2001). Therefore, low temperatures may favor resistance to *Fo* on soybeans, agreeing with our observations of low root rot severity and low percentage of reduction in seedling growth variables, such as root weight, root length and plant length, at 15 and 20°C under growth chamber conditions. Root rot at temperatures between 25 to 30°C may be influenced by a lack of soybean resistance, which, combined with faster growth and colonization of *Fo* at its optimal growth temperature, resulted in more severe root rot. However, these temperature dependent defense mechanisms need further study.

Our findings regarding optimal temperature for disease development conflicts with some greenhouse and field observations in which Fusarium root rot of soybean occurs at low temperatures (<18°C) and disease severity was reduced when temperature increases from 26 to 32°C in *Fo* infested saturated soils (Carson et al. 1991; Farias and Griffin 1990; French 1963). A possible reason for these conflicting results is the variability in soil temperature and soil moisture under field conditions. For example, in Iowa, soybean is planted in late April or early May and emergence usually takes between 7 to 14 days when average soil temperatures are 10°C (De Bruin and Pedersen 2008); seedling disease risk increases before emergence if temperature drops and soils are wet. In our experiments, seeds were maintained at a constant temperature and incubated in soilless pH media for only 8 days making it difficult to
compare with the variable temperatures that occur in the field. Another factor that may explain these conflicting results is the fact that lower soil temperatures and high soil moisture are associated with no-till soybean production, which may result in greater inoculum survival that favors seedling root rot (Bockus and Shroyer 1998). Furthermore, seedling disease under cool, moist conditions is most often due to other pathogens, such as *Pythium* spp. (Rizvi and Yang 1996).

The ideal soybean germination and emergence temperatures are between 25 to 30°C (Hatfield and Egli 1974). However, these temperatures only occur during late-May or early-June (4 or 5 weeks after planting) when soybean plants are already in active vegetative growth (V3-V5) (Licht et al. 2013). In the field in Iowa, soybean planting would rarely take place when soils are at 25 or 30°C. However, in other regions, especially where soybean is double-cropped with winter cereals, temperatures in this range may be common at planting (Dillon 2014). It is important to observe the effect of various temperatures on disease development at early soybean stages. Likewise, these observations could lead to a new research to describe root rot disease development in relation to soybean growth stages.

*Fo* is an important component of the *Fusarium* rot root complex in soybeans, which includes other *Fusarium* spp. such as *F. graminearum*, *F. pseudograminearum*, *F. commune*, *F. proliferatum*, *F. redolens*, and *F. solani* (Broders et al. 2007; Díaz Arias et al. 2013a; Ellis et al. 2013; Ellis et al. 2014; Killebrew et al. 1993; Skovgaard et al. 2003). In addition, *Pythium* and *Rhizoctonia* spp. are primary soybean pathogens and may interact with the members of the *Fusarium* root rot complex to cause disease (Datnoff and Sinclair 1988; Pieczarka and Abawi 1978). The effect of interaction among these species and soil microbial populations on *Fusarium* root rot is still not well understood.
Our findings of the effect of pH on fungal growth and disease severity agreed with a number of studies on *Fusarium* spp. in which slightly acidic growth media at around pH 6 promotes fungal growth and sporulation (Agarwal and Sarbhoy 1978; Cochrane 1958; Groenewald et al. 2006; Gupta et al. 2010; Wu et al. 2009). According to our results, it appears that there were inhibitory effects on disease severity at pH 4 and 7. Given the results of this and other pH studies performed under similar conditions (Schuerger and Mitchell 1992), we propose that under growth chamber conditions, root rot severity is mainly influenced by effects of pH and temperature on fungal growth, combined with the effect of temperature in host resistance.

Interestingly, alkalizing soil amendments to rise soil pH may result in a transitory or partial suppression of *Fusarium* wilts by reducing fungal biomass and availability of iron to the pathogen (Gatch and du Toit 2017; Hoper et al. 1995; Peng et al. 1999). On the other hand; in the case of soybeans, high soil pH levels could lead to iron-deficiency chlorosis, high soybean cyst nematode infections, and more severe root rot symptoms, resulting in yield losses (Charlson et al. 2004; Chen et al. 2007; Hansen et al. 2004; Naeve 2006; Rogovska et al. 2007). Soil pH as a management strategy for root rot of soybeans is still difficult to elucidate, since optimal soil pH from 6.2 to 7 is important for root nodulation (Hartman et al. 1999). However, the evidence that none of the *Fo* isolates grew at pH 8 could provide a motivation to explore the effects of alkaline soils on root rot of soybeans under greenhouse and field conditions.

Disease severity and root rot symptoms varied significantly between the pathogenic and non-pathogenic *Fo* isolates. Lanubile et al. (2015) demonstrated significant differences in soybean defense gene expression profiles in response to pathogenic and non-pathogenic
Pathogenic *Fo* isolates induced a strong defense reaction compared to non-pathogenic, suggesting that the non-pathogenic isolates did not induce a response in the host to infection. These descriptions agreed with our observations in which the non-pathogenic *FO42* isolate produced small patches of root discoloration but not evident damage to root tissues or evident signs of root colonization.

The non-pathogenic isolate *FO42* showed a notable ability to promote plant growth; seedlings inoculated with this isolate showed significantly higher root weight and total plant length compared to the mock-inoculated control. Plant growth stimulation by non-pathogenic *Fo* isolates has also been observed in other hosts (Fracchia et al. 2000; Thongkamngam and Jaenaksorn 2017). This effect has been associated with the interaction of certain fungal metabolites and volatiles with the host hormone transport and signaling of auxins (Bitas et al. 2015), and the overexpression of PR genes along with the activation of jasmonic and salicylic acid pathways upon inoculation (Saldajeno and Hyakumachi 2011; Shcherbakova et al. 2016; Veloso et al. 2016). Additional research on the interaction between pathogenic and non-pathogenic *Fo* isolates must be done in order to identify the potential for biocontrol and how biocontrol efficacy might be influenced by abiotic factors such as pH and temperature in soybeans.

Although, disease severity between *FO38* and *FO42* isolates had similar trends in response to environmental factors, severity for the non-pathogenic *FO42* isolate remained low in all combinations of pH and temperature. In addition, estimates of percent reduction in seedling growth variables for the non-pathogenic *FO42* isolate were significantly lower in all the pH and temperature combinations in which the pathogenic isolate *FO38* displayed the highest levels of reductions. These results suggest it is unlikely that the non-pathogenic *Fo*
isolates become pathogenic to soybean at least under the environmental conditions set in our studies. Our results suggest that abiotic factors influence the level of disease caused by *Fo* pathogenic isolates; however, pathogenicity may be conferred by other mechanisms, such as horizontal gene transfer of genomic regions rich in transposons or pathogenicity-related genes that contributes to the origin of pathogenic genotypes (Ma et al. 2010).

This is the first report quantifying and modeling the effects of abiotic factors on *Fo* in soybeans. We found that the Gaussian model best explained the effects of temperature and pH on fungal radial growth and disease severity. Previous studies on Fusarium wilts in other pathosystems have used polynomial models to describe the interaction between temperature and pH for fungal growth in soil (Chen et al. 2013), and spore attachment under hydroponic conditions (Schuerger and Mitchell 1992). In addition, Navas-Cortes et al. (2007) developed exponential models to describe effects of soil temperature and inoculum density on Fusarium wilt of chickpea. Interestingly, these polynomial and exponential models describe trends and optimal temperatures similar to our studies of *Fo* on soybeans. The models developed for *Fo* growth and root rot will give insights on high and low risk scenarios to growers and farmers. This simple quantitative model captures crucial features of host-pathogen interactions on key abiotic variables such as pH and temperature. Additional variables could be added to create more complex and representative models.

The information generated in this research will increase knowledge about the epidemiology of *Fo* and may help to predict the risk of Fusarium root rot. This knowledge may assist breeders to better identify soybean resistant cultivars, taking into account that temperature significantly influences disease development and host susceptibility. Quantifying the impact on environmental factors on root rot disease development by the use of
quantitative models may help predict scenarios in which root rot may be more severe. However, we acknowledge that the predictability of our model will decrease if the effects of soil moisture, soil temperature or soil pH and their interaction are considered. For these reasons these soil variables must be considered in future studies.

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Table 1. Estimates of pH and temperature obtained from the non-linear optimization procedure from the Gaussian model for *in vitro* fungal radial growth and root rot on *Fusarium oxysporum* isolates.

### Gaussian model parameters and statistics for *in vitro* radial growth

<table>
<thead>
<tr>
<th>Isolates</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Radial growth (cm)</th>
<th>SEE$^y$</th>
<th>$F$ statistics</th>
<th>$P &gt; F$</th>
<th>Gaussian regression $R^2_z$</th>
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<td>6.2</td>
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<td>5.3</td>
<td>0.3907</td>
<td>37.3336</td>
<td>&lt;0.0001</td>
<td>0.9314</td>
</tr>
<tr>
<td>FO41</td>
<td>6.0</td>
<td>25.4</td>
<td>5.8</td>
<td>0.8204</td>
<td>11.8469</td>
<td>0.0006</td>
<td>0.8116</td>
</tr>
<tr>
<td>FO42</td>
<td>6.4</td>
<td>27.1</td>
<td>4.9</td>
<td>0.5791</td>
<td>17.1491</td>
<td>0.0001</td>
<td>0.8618</td>
</tr>
<tr>
<td>FO43</td>
<td>6.8</td>
<td>27.0</td>
<td>5.0</td>
<td>0.9150</td>
<td>6.8012</td>
<td>0.0052</td>
<td>0.7121</td>
</tr>
<tr>
<td>FO44</td>
<td>6.4</td>
<td>26.6</td>
<td>5.1</td>
<td>0.7169</td>
<td>13.9697</td>
<td>0.0003</td>
<td>0.8355</td>
</tr>
<tr>
<td>FO45</td>
<td>6.4</td>
<td>28.9</td>
<td>5.2</td>
<td>0.6895</td>
<td>16.4253</td>
<td>0.0001</td>
<td>0.8566</td>
</tr>
<tr>
<td>FO46</td>
<td>6.7</td>
<td>30.0</td>
<td>4.5</td>
<td>0.8193</td>
<td>7.2480</td>
<td>0.0041</td>
<td>0.7249</td>
</tr>
<tr>
<td>FO47</td>
<td>6.5</td>
<td>26.7</td>
<td>5.4</td>
<td>0.5334</td>
<td>23.8329</td>
<td>&lt;0.0001</td>
<td>0.8966</td>
</tr>
<tr>
<td>FO48</td>
<td>6.5</td>
<td>27.4</td>
<td>4.9</td>
<td>0.6307</td>
<td>15.3397</td>
<td>0.0002</td>
<td>0.8480</td>
</tr>
<tr>
<td>FO49</td>
<td>6.4</td>
<td>26.0</td>
<td>6.0</td>
<td>0.8846</td>
<td>11.1288</td>
<td>0.0007</td>
<td>0.8019</td>
</tr>
<tr>
<td>Average</td>
<td>6.3</td>
<td>27.1</td>
<td>4.9</td>
<td>0.5792</td>
<td>17.1491</td>
<td>0.0001</td>
<td>0.8618</td>
</tr>
</tbody>
</table>

### Gaussian model parameters and statistics for root rot seedling disease

<table>
<thead>
<tr>
<th>Isolates</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>DSI (%)$^x$</th>
<th>SEE$^y$</th>
<th>$F$ statistics</th>
<th>$P &gt; F$</th>
<th>Gaussian regression $R^2_z$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FO38</td>
<td>5.9</td>
<td>30</td>
<td>64.0</td>
<td>2.9389</td>
<td>168.7979</td>
<td>&lt;0.0001</td>
<td>0.9840</td>
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<tr>
<td>FO42</td>
<td>5.9</td>
<td>30</td>
<td>17.1</td>
<td>1.0859</td>
<td>70.2363</td>
<td>&lt;0.0001</td>
<td>0.9623</td>
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</tbody>
</table>

$^x$Maximal radial growth and maximal disease severity index estimates were obtained by using a non-linear optimization procedure including pH range from 4 to 7 and temperature range from 15 to 30°C for each isolate.

$^y$SEE Standard error of estimate

$^z$R$^2$ Coefficients of determination
Table 2. Analysis of variance indicating the effects of pH and temperature on seedling disease after inoculation of soybean seeds with pathogenic (FO38), and non-pathogenic (FO42) *Fusarium oxysporum* isolates in a rolled-towel assay.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>DSI^\text{c} F value</th>
<th>P &gt; F</th>
<th>Severity F value</th>
<th>P &gt; F</th>
<th>Root Weight F value</th>
<th>P &gt; F</th>
<th>Root length F value</th>
<th>P &gt; F</th>
<th>Plant length F value</th>
<th>P &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>3</td>
<td>24.14</td>
<td>&lt;0.0001</td>
<td>24.85</td>
<td>&lt;0.0001</td>
<td>9.09</td>
<td>&lt;0.0001</td>
<td>148.19</td>
<td>&lt;0.0001</td>
<td>74.71</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Temperature (T)</td>
<td>3</td>
<td>7.19</td>
<td>0.0434</td>
<td>11.45</td>
<td>0.0197</td>
<td>2.24</td>
<td>0.2255</td>
<td>4.72</td>
<td>0.0084</td>
<td>8.80</td>
<td>0.0310</td>
</tr>
<tr>
<td>pH×T</td>
<td>9</td>
<td>3.52</td>
<td>0.0005</td>
<td>3.07</td>
<td>0.0021</td>
<td>2.39</td>
<td>0.0148</td>
<td>6.18</td>
<td>&lt;0.0001</td>
<td>2.42</td>
<td>0.0135</td>
</tr>
<tr>
<td>Isolate (I)</td>
<td>1</td>
<td>600.83</td>
<td>&lt;0.0001</td>
<td>532.04</td>
<td>&lt;0.0001</td>
<td>12.22</td>
<td>0.0006</td>
<td>14.04</td>
<td>0.0003</td>
<td>24.76</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>pH×I</td>
<td>3</td>
<td>0.20</td>
<td>0.8947</td>
<td>1.24</td>
<td>0.2961</td>
<td>1.39</td>
<td>0.2480</td>
<td>1.40</td>
<td>0.2443</td>
<td>0.32</td>
<td>0.8113</td>
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<tr>
<td>T×I</td>
<td>3</td>
<td>39.88</td>
<td>&lt;0.0001</td>
<td>66.82</td>
<td>&lt;0.0001</td>
<td>6.17</td>
<td>0.0005</td>
<td>0.76</td>
<td>0.5982</td>
<td>2.97</td>
<td>0.0337</td>
</tr>
<tr>
<td>pH×T×I</td>
<td>9</td>
<td>0.51</td>
<td>0.8646</td>
<td>1.60</td>
<td>0.1206</td>
<td>1.24</td>
<td>0.2753</td>
<td>0.98</td>
<td>0.4610</td>
<td>0.72</td>
<td>0.6920</td>
</tr>
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</table>

^\text{c}DSI (disease severity index) was arcsin transformed. Visual scale of severity was square root transformed. Seedling growth variables root weight, root length and plant length were log transformed. ANOVA do not include the mock-inoculated controls data. Experiments consisted of 3 replications (towels) for each treatment combination and two experiments for the rolled towel assay. Analyses of variance for all response variables included the mock-inoculated controls.
Figure 1. Radial growth of 14 *Fusarium oxysporum* isolates with respect to temperature and pH of the growth medium. **A**, **B**, **C**, **D** isolates growing in Citrate – phosphate buffer set at pH 4, 5, 6, and 7, respectively. Data represent the mean values of two experiments with four replicate petri dishes for each treatment combination.
Figure 2. Estimates of fungal radial growth (cm) from the Gaussian regression analysis across the main factors pH at 4, 5, 6, and 7 and temperature at 15, 20, 25, 30°C for the average growth of 14 *Fusarium oxysporum* isolates. Data represent the mean values of two experiments with four replicate petri dishes for each treatment combination.
Figure 3. Bar graph for disease severity index (DSI) with respect to pH buffer and incubation temperature for A, FO38 pathogenic *Fo* isolate and B, FO42 non-pathogenic *Fo* isolate. Comparisons were made separately by pH buffer. Means with the same letter are not significantly different according to Fisher’s protected least significant difference (*P* < 0.05).
Figure 4. Soybean seedlings incubated at 30°C for 8 days after seed inoculation with *Fusarium oxysporum* isolates with a spore suspension of $1 \times 10^6$ conidia/ml. A-D, seedlings inoculated with pathogenic isolate FO38. A, B, C, D seedlings growing in citric acid and NaOH buffer set at pH 4, 5, 6, and 7, respectively. E-H, seedlings inoculated with the non-pathogenic isolate FO42. E, F, G, H seedlings growing in citric acid and NaOH buffer set at pH 4, 5, 6, and 7, respectively.
Figure 5. Percentage of reduction on seedling growth variables relative to the mock-inoculated control with respect to pH buffer and incubation temperature for A, C, E plants inoculated with FO38 pathogenic *Fo* isolate and B, D, F plants inoculated with FO42 non-pathogenic *Fo* isolate. Means with the same letter are not significantly different according to Fisher’s protected least significant difference ($P < 0.05$).
**Figure 6.** Linear relationship between visual scale for severity disease severity index (DSI).

Each data point represents the average disease of one towel containing 15 seeds inoculated with **A**, FO38 pathogenic *Fo* isolate and **B**, FO42 non-pathogenic *Fo* isolate. *r* = Pearson’s linear correlation coefficients.
Figure 7. Estimates of disease severity index (DSI) from the Gaussian regression analysis across the main factors pH at 4, 5, 6, and 7 and temperature at 15, 20, 25, 30°C for A, FO38 pathogenic *Fo* isolate and B, FO42 non-pathogenic *Fo* isolate. Data represents the disease mean values of two experimental replicates for each *Fo* isolate.
CHAPTER 4. EFFECTS OF SOIL CONDITIONS ON ROOT ROT OF SOYBEAN CAUSED BY *FUSARIUM GRAMINEARUM*

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

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Department of Plant Pathology and Microbiology, Iowa State University, Ames. 50011.

**Abstract**

*Fusarium graminearum (Fg)* is an important soybean pathogen that causes seedling disease, root rot, pre- and post-emergence damping-off. However, effects of soil conditions on the disease are not well understood. The objective of this greenhouse study was to observe the impacts of soil texture, pH, and soil moisture on seedling root rot symptoms and detrimental effects on seedling development caused by *Fg*. *Fg*-infested millet was added (10% v/v) to soil with four different textures (sand, loamy sand, sandy loam, and loam). Soil moisture was maintained at saturation, field capacity or permanent wilting point at soil pH levels of 6 or 8. Seedlings were evaluated 4 weeks after planting for root rot, root length, root and shoot dry weights, and leaf area. There was a significant interaction between soil moisture and soil texture for root rot assessed visually (*P*<0.0001). Highest severity (~70%) was observed at pH 6 and permanent wilting point in sandy loam soils. In contrast, pot saturation resulted in the lowest levels of disease in sandy loam and loam soils (11.6 and 10.8%, respectively). Percent reductions in seedling growth parameters, including root length, foliar area, shoot and root dry weights and root tips, relative to the non-infested
control, were significantly greater in sandy loam soils. In contrast, there were no relative growth reductions in sandy soils. This study showed that levels of root rot increased under moisture-limiting conditions, producing detrimental effects on plant development.

**Introduction**

*Fusarium graminearum* (*Fg*) (synonym *Gibberella zeae* (Schwein.) Petch) is a major necrotrophic pathogen of soybean, causing seed decay, damping-off, crown and root rots, and pod blight in the United States, Canada and other temperate regions (Martinelli et al. 2004; Sella et al. 2014; Xue et al. 2007). Soybean is often included in rotation with wheat, maize and other cereal crops that are hosts of *Fg*. *Fg* colonizes and overwinters on host crop residues, thus the residues provide a source of inoculum for subsequent seasons (Barros et al. 2014; Leslie et al. 1990; Windels et al. 1988). *Fg* isolates pathogenic on wheat and maize have been also found to be highly pathogenic on soybean (Broders et al. 2007). Xue et al. (2007) suggested that soybean in rotation with cereal crops provide strong selection pressure for the development of highly aggressive *Fg* isolates on all crops.

*Fg* is highly prevalent in Iowa and has been associated with soybean seedlings with severe root rot (Díaz Arias et al. 2013a; Díaz Arias et al. 2013b). Seedling diseases are caused by diverse *Fusarium* and *Pythium* spp. and are often more prevalent when soils are cool and wet at planting. Delayed germination and emergence in such conditions increase the chances for seed and seedling infection (Broders et al. 2007; Licht et al. 2013). Disease severity for some pathogens may also be linked to soil physiochemical conditions, and biodiversity in soil microbial populations (Ghorbani et al. 2008). There is a lack of detailed
knowledge about soil environment and soil conditions such as pH, soil texture and soil moisture that influence disease development, incidence and severity of Fusarium root rot of soybean caused by \( F_g \).

Soil pH influences root rot disease severity by directly affecting soil microbial communities and the balance between pathogens and their specific antagonists (Hoper and Alabouvette 1996). Evidence suggests that lowering soil pH from 8 to 6 in Fusarium suppressive soils drastically reduces the viability of \( Pseudomonas \) spp. and eliminates the suppressive effects on Fusarium wilt diseases (Scher and Baker 1980). Similarly, changes in soil pH indirectly affect nutrient availability to the pathogen and the plant host (Ghorbani et al. 2008).

Soybeans are sensitive to changes in soil pH; greatest soybean yields in Iowa and Minnesota have been reported in soil pH levels between 6.2 and 7.0 (McLean and Brown 1984; Rogovska et al. 2007). Altered nutrition induced by soil acidity can increase or decrease the susceptibility of the plant host, affecting disease severity and incidence (Fang et al. 2012; Ghorbani et al. 2008). Under high soil pH, iron availability is reduced and pathogen establishment will depend on the ability to acquire and compete for iron. \( F_g \) has two types of iron acquisition systems, secretion and uptake of iron-chelating molecules and production of cell wall iron reductases to reduce ferric iron to ferrous iron (Greenshields et al. 2007; Kosman 2003; Philpott 2006). Iron availability also affects soybean plants and can lead to iron deficiency chlorosis (IDC) in high pH soils (Rogovska et al. 2007). Soybean plants with IDC symptoms often display root rot symptoms, but the basis of this association is not clear.

Soil texture can also affect pathogen distribution in soil and plant resistance to disease. For example, Fusarium wilts have been commonly associated with sandy soils
Field observations revealed Fusarium wilts caused by *F. oxysporum* are more severe in coarse sandy soils or loamy-textures soils, while clayey soils seem to be root rot suppressive (Hoper and Alabouvette 1996; Scher and Baker 1980; Yuen et al. 1983). An association between Fusarium root rot of soybean caused by *Fg* and soil texture components have not been documented. Soil texture and type of clay have been correlated to disease incidence and severity in a diverse number of Fusarium wilts on muskmelon, banana, flax, barley, and tobacco (Alabouvette et al. 1979; Dominguez et al. 1996; Hoper et al. 1995; Strunnikova et al. 2015; Stutz et al. 1989; Toussoun 1975). Soil clay minerals actively interact with bacteria due to the high cationic exchange capacity (CEC), surface area and variable negative charge (Hoper and Alabouvette 1996); these interactions consist mainly of buffering soil pH, providing available nutrients that can be rapidly used. Similar to alkaline soil pH, the addition of clay minerals into the soil reduces the availability of iron to fungal pathogens, promoting bacterial growth (Hoper et al. 1995). It has been demonstrated that high clay CEC stimulates the metabolism of bacteria (Stotzky and Rem 1966). In contrast silt-sand minerals are considered more inactive (Robert and Chenu 1992).

Depending on the pathosystem, low and high soil water potential may affect on root rot differently. For example, in *Fusarium oxysporum* infested soils near saturation, soybean root rot severity increased, but only at low soil temperatures (French 1963). A three or seven day duration of flooding at soybean emergence increased the incidence and severity of *Pythium* spp. while *Fusarium* spp. were less frequently associated with seedling infection under flooded conditions (Kirkpatrick et al. 2006). On the other hand, low water potentials may predispose the host to infection and favor diseases such as dry rot of beans caused by *F.*
solani, and seedling blight of cereals caused by *F. roseum* (Ghorbani et al. 2008; Hoper and Alabouvette 1996). Fusarium root rot of sweet potatoes, peas and beans are also more severe in dry soil conditions (Cook and Papendick 1972). Several Fusarium wilt diseases are also favored by dry soil conditions, including Fusarium wilt of chickpea caused by *F. oxysporum* f. sp. *ciceris* (Landa et al. 2006; Navas-Cortes et al. 2007), seedling blight of clover caused by *F. roseum* (Graham et al. 1957), root and stem rot of peas and chickpeas caused by *F. solani* f. sp. *pisi* (Bhatti and Kraft 1992).

The objectives of this research were to measure the effects of important soil variables on the development of root rot of soybean caused by *Fg* by studying (i) the effects of soil pH and soil water content interaction on root rot, (ii) the effects of artificial soil textures and its interaction with soil pH and soil water content on root rot, and (iii) possible detrimental effects on soybean seedling growth parameters under various edaphic conditions on *Fg* infested soils.

**Materials and Methods**

**Inoculum production**

*Fg* isolate FG5 was collected from symptomatic soybean roots in Iowa in 2007 (Díaz Arias et al. 2013b). FG5 was single spore and maintained on dry silica gel beds at 4°C until use. Aggressiveness and pathogenicity of the FG5 isolate was tested on soybean cultivar Asgrow 2403 (SDS susceptible) (Monsanto Co., St. Louis, MO) under greenhouse conditions (Díaz Arias et al. 2013a).
Millet seeds were soaked for 24 h in water, drained and autoclaved at 121°C for 1 h in autoclave bags with a micro-porous filter patch on two consecutive days. Isolate FG5 was grown on potato dextrose agar (PDA) 39g/L (Difco, Becton, Dickinson and Co, Spark, MD, USA) for 14 days in an incubator at 25°C with a 12 h photoperiod, in order to promote conidial formation. Plugs (~1 cm²) from cultures of FG5 were added to the sterile millet in a biosafety cabinet, and then sealed with a rubber band. Bags were then placed in an incubator (Hoffman manufacturing Inc. Oregon, USA) for 8 days, at 65% relative humidity with a 12 h photoperiod, and mixed by hand every day. FG5-infested millet was mixed with soil at 10% concentration by volume. Non-infested control treatments contained autoclaved sterile millet mixed with soil.

**Artificial soil textures**

Four artificial soil textures (sand, loamy sand, sandy loam and loam) were created by mixing sand and silt loam in different proportions by volume (Table 1). Resulting textural classes on the artificial soils were analyzed for particle size distribution following the protocol by the USDA (1996). Soils were pasteurized in a soil steamer for 1.5 h, dried on a greenhouse bench for 4 days and kept in plastic containers until use.

**Soil pH**

Initial soil pH for each soil textural class was measured by the electrometric standard method, using a 1:1 soil/water ratio (Watson and Brown 1997). Ten ml of distilled water were added to 10 g of soil in a 50 ml falcon tube, which was shaken for 10 min and settled for 5 min, placing the pH meter electrode in the slurry. After the initial pH measurements, soil pH was adjusted to 6 or 8 by gradually adding Al₂(SO₄)₃ (Bonide Products, Inc. New
York, USA) and CaCO$_3$ (Fisher, Illinois, USA) to the soil, respectively. Soils were then wet, dried and mixed twice a day during two weeks to allow the soil react with the chemical compounds. Soil pH measurements were performed periodically until the soil remained at constant pH equilibrium (Islam et al. 2004).

**Soil moisture content**

Three levels of soil moisture including pot saturation (PS), field capacity (FC) and permanent wilting point (PWP) were set for each soil texture, derived from the volumetric water content ($\theta_v$) points for different textural classes described by Rowell (1994), and Bradly and Weil (2004) (Table 2). For each soil textural class, 230 ml of dried soil were weighed in a Styrofoam cup and a calculation the of volume of water needed for each type of soil texture was performed by using the following formula

$$V_w = V_s \times \theta_v$$

Where $V_w$ is the volume of water contained in a soil, $V_s$ is the total volume of soil, and $\theta_v$ is the soil volumetric water content point. The weight of 230 ml of soil for each soil textural class and the weight of the water needed to reach the desired water content point were added to have a final weight per cup that was subsequently maintained by adding water once a day.

**Soil infestation and experimental design**

After two weeks of incubation, inoculum was mixed with soil at 10% concentration by volume. Non-infested control treatments contained 10% autoclaved sterile millet. Seeds of soybean cultivar Jack were surface disinfested in 0.5% NaOCl for 2 min, rinsed twice for 2 min in sterile distilled water, and dried for 20 min in a laminar flow hood before planting.
Three seeds per cup were planted into the infested or non-infested soil mix and watered daily for 2 weeks. Soil moisture levels PS, FC, and PWP were imposed 2 weeks after planting and maintained for 2 weeks. Four weeks after planting, seedlings were visually rated for root rot severity (%), roots were scanned using a flatbed scanner (EPSON Expression, 10000XL, Epson America, Inc.), and root images were analyzed using WinRhizo software 2008 (Regent Instruments Inc., Quebec, QC, Canada) to obtain estimates of root length, surface area, root volume, root tips, and forks on each individual plant. In addition, foliar area was estimated on individual plants using Assess 2.0 (The American Phytopathological Society (APS), Saint Paul, Minnesota). Shoot and root dry weights were measured on each individual plant after oven drying at 80°C for 24 h. The experiment was organized in a split-plot design with soil moisture (PS, FC, PWP) as the main-plot factor, and soil texture (sand, loamy sand, sandy loam and loam), soil pH (6 or 8) and Fg infested or non-infested soils as sub-plot factors. One cup was considered as an experimental unit with three seeds or subsamples in each cup. The experiment had 8 replications and was conducted twice.

**Data analysis**

Visual root rot severity data (%) was arccsin transformed. Root length, surface area, root volume, root tips, forks, foliar area, shoot and dry weight were square-root transformed to meet the assumptions of the ANOVA. In addition, normal distribution of the residuals was tested for all the transformed data under the PROC UNIVARIATE procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC). Separate analysis of the two experimental replications showed similar trends, so data were combined for subsequent analyses. Analysis of variance was performed on the transformed data using PROC GLIMMIX in which the main-plot
effect; soil moisture, was tested against the whole-plot variation error, and sub-plot effects were tested against the residual variability. Mean separation analyses on the transformed data were performed using a Fisher’s protected least significant difference (LSD) test at $P = 0.05$.

**Results**

**Effects on root rot severity**

The analysis of variance indicated a significant effect for the main factors soil moisture content ($\theta_v$), soil pH, soil texture, as well as the interaction between $\theta_v$ and soil texture ($P < 0.0001$) for the visual evaluation of root rot (Table 3). These significant interactions indicate that the level of disease varied across the soil textures and the effect of soil moisture content also varied among soil textures, primarily because soil moisture content did not have an effect on root rot in sandy soil.

Soil pH significantly influence disease severity; soil at pH 6 showed more disease in all treatment combinations compared to pH 8 (Fig. 1). Overall, across soil textures, the highest level of root rot was observed at pH 6 and PWP (55.3%); and the lowest level of disease was observed at pH 8 and PS (15.4%) (Fig. 1A).

The effects of soil moisture and soil texture on root rot severity displayed similar trends for pH 6 and 8. Root rot severity increased from sandy to clayey soils at PWP; conversely, disease severity decreased from sandy to clayey soils at PS; therefore, PWP and PS revealed inverse trends on root rot severity. Disease severity at FC remained between the highest and lowest disease levels in each soil texture. In addition, the sandy soil showed no significant differences in the levels of disease across the soil moisture treatments (Fig. 2).
The highest levels of disease corresponded to loamy sand and sandy loam soils at PWP at both pH levels (Fig. 2). At soil pH 6, the highest levels of disease were 52.6% and 67%, in loamy sand and sandy loam soils, respectively, while at pH 8 they were 51.7% and 49.8%. In contrast, the lowest levels of root rot were found in sandy loam and loam soils (10.2% and 9.7%, respectively) at PS and soil pH 8 (Fig. 2B), followed by sandy loam and loam soils (21.5% and 19.9%, respectively) at PS and soil pH 6 (Fig. 2A).

**Effects on seedling growth variables**

Comparisons between *Fg* infested and non-infested treatments revealed detrimental effects on shoot and root morphological characteristics, especially in loamy sand and sandy loam soils. Plants growing in *Fg* infested soils had significantly shorter roots compared to the non-infested control in loamy sand and sandy loam soil textures at all soil moisture levels. In contrast, root length was not affected by *Fg* when plants were grown in sandy soil (Fig. 3A).

There were significant detrimental effects on foliar area for *Fg* infested sandy loam soil at all soil moisture content levels, and loam soil at PS. Conversely, foliar area was significantly higher in the infested treatments compared to non-infested treatments in sandy soils at FC and PWP (Fig 3B).

Root and shoot dry weight were negatively impacted by *Fg* in sandy loam soil at PS and FC. However, there were no detrimental effects on root and shoot dry weight for the plants growing in infested sandy soil (Fig 3C-D).

Root volume was significantly affected by *Fg*; there were significant differences in root volume between infested and non-infested plants for all soil textures and soil moisture treatments except for sand (Fig. 3E). Number of root tips was significantly affected by *Fg*
infection in sandy loam and loam soils but no adverse effects were observed in sand or loamy sand soils (Fig. 3F).

Sandy loam soils generate the greatest percentages of reduction in the root and shoot morphological characteristics (Fig. 4). The highest percentages of reduction in root length were observed in sandy loam soils in all levels of soil moisture ranging from 26 to 21% followed by loam soil at FC (18.6%) (Fig. 4A). Foliar area was significantly reduced in sandy loam soil at PWP (53.2%) followed PS (23.7%). Loamy sand and sandy soils had no reductions in foliar area (Fig. 4B).

Greatest percentages of reduction in root dry weight were observed in sandy loam and loam soils at PS and FC ranging from 27 to 2%. In addition, there was no reduction in root dry weight at PWP across all soil textures (Fig. 4C). Similarly, there was a significant reduction in shoot dry weight in sandy loam and loam soils ranging from 19 to 3%. Seedlings growing in loamy sand and sand soils did not displayed reductions in shoot dry weight (Fig 4D).

Greatest reductions in root volume were observed in plants growing in sandy loam soils in all soil moisture treatments, followed by loamy and sandy loam soils at FC and PS (Fig. 4E). Root tips reductions were significantly higher in plants growing in sandy loam soils ranging from 16.2 to 6.5% (Fig. 4F). Sandy soils did not show reductions in the shoot and root morphological characteristics measured in this study.
Discussion

In this study, we provided the first analysis concerning the interactions among several abiotic soil factors (soil moisture, soil pH, and soil texture) in the development of root rot of soybean seedlings caused by *Fg*. The results suggest that edaphic characteristics have a considerable influence on disease severity and seedling development. Acidic soils (pH 6) were more conductive for root rot than alkaline soils (pH 8). Root rot severity was significantly different across the soil moisture treatments, in which PWP displayed the highest levels of root rot and PS the lowest.

Our findings that disease was most severe in acidic soils (pH 6) and least severe in alkaline soils (pH 8) agree with several similar studies. For example, sorghum rot infection by *Fg* was significantly higher at soil pH 5.1 than 6.1 on seedlings 36 days after planting under field conditions (Davis et al. 1994). Strawberries infected by *Fo f. sp. fragariae* displayed more severe root rot and reduced plant size at pH 5.2 than at pH 7.5 (Fang et al. 2012). Increasing soil pH has also been suggested as an alternative for disease management in other Fusarium wilt diseases such as banana, spinach, tomato, cotton and melons (Domínguez et al. 1996; Gatch and du Toit 2017; Groenewald et al. 2006; Jones et al. 1989; Woltz and Jones 1973). Agarwal and Sarbhoy (1978) reported *Fg* grew best at pH 3.5 in *in vitro* studies. Therefore, it seems plausible that increasing soil pH with CaCO₃ might reduce growth of *Fg*, decreasing the chances to effectively infect soybean roots.

Previous reports suggest that increasing soil pH by liming reduces soil fungal populations and at the same time improves bacterial growth (Alabouvette 1999; Muhlbachova and Tlustos 2006; Scher and Baker 1980). According to our observations, liming with CaCO₃ had a significant reduction effect on root rot of soybean caused by *Fg*.
during a four-week period. In addition, the adverse effects of liming on fungi are more significant under limited iron in soil (Expert 2009; Ghini et al. 2000). These observations may explain the low root rot severity at soil pH 8 in our studies, and suggest that disease suppression is proportional to the reduction of available iron in the soil, as it has been reported in other Fusarium wilts (Hoper et al. 1995).

Iron availability is affected in calcareous soils with high pH; the solubility of iron $\text{Fe}^{3+}$ decreases 1,000-fold with each unit increase in pH (Expert 2009). In addition, $\text{CaCO}_3$ reduces the bioavailability of iron (Loeppert 1988). Iron is a limiting nutrient for fungi metabolism and it is involved in mechanisms of disease virulence (Scher and Baker 1982; Symeonidis and Marangos 2012; Weinberg 2009). From our results, low root rot severity at soil pH 8 suggests that pathogenicity can be significantly influenced by the relative ability of $Fg$ to obtain essential iron under the limiting conditions imposed by high soil pH. High soil pH can lead to IDC in soybean, which is associated with root rot symptoms often attributed to $Fusarium$ spp. However, our results do not support a role for $Fg$ in enhanced root rot symptoms under high pH conditions. Root rots associated with IDC are likely caused by other fungal species.

Although several studies have demonstrated that clays favor bacterial activity and are less favorable to fungal growth (Marshall 1975; Stotzky 1966; Stotzky and Rem 1967), in our studies the effect of soil texture was highly dependent on soil moisture content. At PS, root rot decreased with increasing clay content; at FC, sand had the most severe root rot but it was similar among loamy sand, sandy loam, and loam; and at PWP, root rot was most severe in sandy loam or loamy sand. Together, these results suggest that root rot was enhanced under moisture-stress conditions; in sand, plant moisture stress may have been similar in all
the moisture content treatments, due to rapid drainage of water added to the cups. The interaction of soil texture with soil pH was not significant, but a trend toward lower root rot severity with higher clay content was more evident at pH 8 than at pH 6 (Fig. 1B). This observation is consistent with previous reports made by Hoper et al. (1995) and Amir and Alabouvette (1993) on Fusarium wilts, which demonstrated that addition of clay (montmorillonite or illite) provides the most disease suppression at high soil pH.

Soil texture affects plant disease due to the correlation with water holding capacity, nutrient availability, porosity and root growth (Ghorbani et al. 2008). In our studies, we observed that the combined effect of soil texture and soil moisture displayed a high variation in disease severity. For example, sandy soils did not present significant differences in root rot across soil moisture contents. However, effects of soil moisture treatment on root rot severity became more accentuated with incremental clay content across soil textures, suggesting that the water holding capacity effect of clay plays an important role on seedling root rot development.

The lowest root rot severity and the greatest seedling growth were observed at PS in the sandy loam and loam soils. The results suggest that high soil moisture given by the higher water holding capacity of high clay content soils may have been detrimental for the *Fg* inoculum viability. Previous studies on inoculum survival in loam soils indicated that soil moisture content higher than 85% of saturation reduced soil populations of *Fg*, *F. moniliforme* and *F. oxysporum*, and stimulated soil bacterial populations (Stover 1953).

Our observations of reduced root rot on high moisture soils agrees with reports in other pathosystems. For example, Fusarium root rot of wheat caused by *F. roseum* f. sp.
cerealis was less severe in high moisture fine-textured soils under field conditions (Papendick and Cook 1974).

Root rot was significantly higher at PWP soil moisture treatments in all soil textures except sand. Similar results regarding drought stress predisposing wheat seedlings to higher \( F_g \) infection have been described by Beddis and Burgess (1992). In cereals, \( F_g \) tends to be the most predominant Fusarium species in the warmer regions of the USA, and warm and dry weather are considered to be the optimal conditions for foot rot development (Doohan et al. 2003; Vigier et al. 1997). It is likely that there is an association between dry climatic conditions optimal for \( F_g \) to produce disease and our observation on root rot of soybean.

Soybean seedlings growing in soils at PWP displayed reduced root and shoot growth. The effects of low water stress in soybean include inhibition of cell division, reduction in foliar area, suppression of shoot, root growth and root volume, and decrease in photosynthetic rate (Hossain et al. 2014; Krizek et al. 1985). Although studying the adverse effects of water stress on soybean seedling growth parameters was not our main objective, it is important to note that the highest levels of root rot were observed on water-stressed seedlings with reduced growth in root and shoot. For these reasons, it was more informative to draw conclusions on the effects of plant growth variables by making comparisons between the infested and non-infested seedlings in each treatment combination.

Soybean seedlings growing in \( F_g \) infested soils displayed significant reduction in plant growth parameters. These findings are in general agreement with (Zhang et al. 2010) and (Xue et al. 2007), in which seed or seedling \( F_g \) inoculation reduced root dry weight and plant height up to 24 and 28\%, respectively. Reductions in root length, root volume, number of tips and forks by \( F_g \) soil inoculation have also been reported (Díaz Arias et al. 2013a).
Differences in all plant growth parameters between infested and non-infested plants in sandy soils were negligible. This effect may be due in part to the reduction of inoculum potential associated with low amount of nutrients provided to $F_g$ inoculum from the soil and the low CEC of sand minerals (Robert and Chenu 1992). Moreover, the low impedance for root elongation offered by the high sand content allows the root to scavenge, reducing detrimental effects of $F_g$, also evident in the shoot weight and foliar area (Baligar et al. 1980).

Highly significant differences were observed between the infested and non-infested treatments for all the root morphological variables in the PS and FC sandy loam soils, even though these treatment combinations had the lowest root rot severity. A possible explanation for the correlation between low root rot severity and high percentage of growth reduction is that the visual evaluation of disease does not take into account root internal colonization and the possible damage in the vascular system that might affect root growth and development.

Root morphological characteristics analyzed on WinRhizo are useful indicators of root health. Estimates of root length, and root volume, root forks and root tips helped to interpret the detrimental effects of $F_g$ and its interactions with soil moisture, soil texture and soil pH. Root volume however, seems to be the most sensitive variable in estimating detrimental effects of root rot. For example, there were significant differences in root volume comparing infested and non-infested treatments in all soil textures and soil moisture treatment combinations except for sand.

The results from this study have provided information on abiotic factors of importance for root rot of soybean caused by $F_g$. We demonstrated that changes in edaphic factors and their interactions influence disease severity. The soil parameters studied the most in relation to soilborne pathogens are soil pH, clay content and nitrogen content (Janvier et
al. 2007). There are not many reports on Fusarium wilts that included several soil factors in the same study and a combined approach is needed due to the possible number of biotic and abiotic factors related to soilborne diseases.

Additional variables such as temperature fluctuation and interactions between and within fungal species should be included in future studies. For example, Tu (1994) performed a study of the effects of soil moisture and temperature on the Fusarium root rot complex of pea, including *F. solani* f. sp. *pisi* and *F. oxysporum* f. sp. *pisi* and found that both lack of moisture and saturation increased disease severity. It is important to study these types of interactions in the future since the soybean root rot complex is not only associated with *Fg* but include a diverse number of *Fusarium* species (Díaz Arias et al. 2013a; Leslie et al. 1990).

**Literature cited**


Table 1. Artificial soil textural classes generated by sand and silt loam mix.

<table>
<thead>
<tr>
<th>Soil mix by volume</th>
<th>Textural class</th>
<th>Particle size distribution analysis$^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand (%)</td>
<td>Silt loam (%)</td>
<td>Sand (%)</td>
</tr>
<tr>
<td>90</td>
<td>10</td>
<td>95.90</td>
</tr>
<tr>
<td>75</td>
<td>25</td>
<td>87.14</td>
</tr>
<tr>
<td>25</td>
<td>75</td>
<td>61.94</td>
</tr>
<tr>
<td>10</td>
<td>90</td>
<td>42.10</td>
</tr>
</tbody>
</table>

$^\dagger$Particle size distribution analyses were performed by the pipette method by the USDA 1996

Table 2. Values of volumetric water content of soils of varying soil textures

<table>
<thead>
<tr>
<th>Soil textures</th>
<th>Soil volumetric water content ($\theta_v$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PS</td>
</tr>
<tr>
<td>Sand</td>
<td>0.13</td>
</tr>
<tr>
<td>Loamy sand</td>
<td>0.16</td>
</tr>
<tr>
<td>Sandy loam</td>
<td>0.25</td>
</tr>
<tr>
<td>Loam</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Soil moisture levels PS = pot saturation, FC = field capacity, PWP = permanent wilting point

Table 3. Analysis of variance indicating the effects of soil moisture content, soil pH and soil texture on root rot visual evaluation (%), following soil infestation with *Fusarium graminearum*.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>$F$ value</th>
<th>$P &gt; F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil moisture content ($\theta_v$)</td>
<td>2</td>
<td>73.09</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Soil pH (pH)</td>
<td>1</td>
<td>50.42</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$\theta_v\times$ pH</td>
<td>2</td>
<td>0.57</td>
<td>0.5675</td>
</tr>
<tr>
<td>Soil texture (ST)</td>
<td>3</td>
<td>14.51</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$\theta_v\times$ ST</td>
<td>6</td>
<td>16.66</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>pH$\times$ST</td>
<td>3</td>
<td>1.40</td>
<td>0.2406</td>
</tr>
<tr>
<td>$\theta_v\times$ pH$\times$ST</td>
<td>6</td>
<td>0.77</td>
<td>0.5956</td>
</tr>
</tbody>
</table>

Analysis of variance was performed only on plants growing on soils infested with *Fusarium graminearum*. Visual root rot disease data (%) were arcsine transformed. The experiment consisted of eight reps in a split-plot design, with soil moisture content as the main-plot factor, and soil pH and soil texture as the sub-plot factors.
Table 4. *F* values for comparisons of seedling growth parameters between *F. graminearum* infested and non-infested treatments on the main effects of soil texture and soil moisture (PS= pot saturation; FC= field capacity; PWP= permanent wilting point) at different soil pH.

* indicates significant differences from the non-infested control (*P* ≤ 0.05) with a T-test for mean separation.

<table>
<thead>
<tr>
<th>ST</th>
<th>ωv</th>
<th>RL</th>
<th>FA</th>
<th>RDW</th>
<th>SDW</th>
<th>RV</th>
<th>RT</th>
<th>SA</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>PS</td>
<td>1.71</td>
<td>0.04</td>
<td>0.83</td>
<td>0.85</td>
<td>0.05</td>
<td>1.18</td>
<td>0.62</td>
<td>2.65</td>
</tr>
<tr>
<td></td>
<td>FC</td>
<td>2.24</td>
<td>2.65</td>
<td>2.48</td>
<td>3.06</td>
<td>0.37</td>
<td>0.95</td>
<td>1.08</td>
<td>3.05</td>
</tr>
<tr>
<td></td>
<td>PWP</td>
<td>2.06</td>
<td>0.50</td>
<td>0.85</td>
<td>0.32</td>
<td>1.94</td>
<td>1.77</td>
<td>2.05</td>
<td>5.06*</td>
</tr>
<tr>
<td>LS</td>
<td>PS</td>
<td>3.27</td>
<td>0.01</td>
<td>3.30</td>
<td>0.41</td>
<td>2.86</td>
<td>0.14</td>
<td>3.56</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>FC</td>
<td>1.76</td>
<td>0.49</td>
<td>0.83</td>
<td>0.17</td>
<td>3.88</td>
<td>0.07</td>
<td>2.95</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>PWP</td>
<td>7.06*</td>
<td>0.66</td>
<td>2.61</td>
<td>0.06</td>
<td>15.75*</td>
<td>0.01</td>
<td>11.37*</td>
<td>0.31</td>
</tr>
<tr>
<td>SL</td>
<td>PS</td>
<td>33.75*</td>
<td>22.67*</td>
<td>26.38*</td>
<td>23.25*</td>
<td>50.74*</td>
<td>9.40*</td>
<td>45.05*</td>
<td>18.17*</td>
</tr>
<tr>
<td></td>
<td>FC</td>
<td>14.98*</td>
<td>1.66</td>
<td>2.06</td>
<td>5.90*</td>
<td>12.76*</td>
<td>7.38*</td>
<td>14.37*</td>
<td>13.33*</td>
</tr>
<tr>
<td></td>
<td>PWP</td>
<td>9.27*</td>
<td>15.20*</td>
<td>3.53</td>
<td>3.92</td>
<td>22.67*</td>
<td>0.66</td>
<td>16.01*</td>
<td>2.85</td>
</tr>
<tr>
<td>L</td>
<td>PS</td>
<td>10.45*</td>
<td>8.95*</td>
<td>13.51*</td>
<td>5.93*</td>
<td>15.97*</td>
<td>3.25*</td>
<td>13.98*</td>
<td>1.63</td>
</tr>
<tr>
<td></td>
<td>FC</td>
<td>5.38*</td>
<td>0.36</td>
<td>5.08*</td>
<td>0.65</td>
<td>12.98*</td>
<td>1.24</td>
<td>9.13*</td>
<td>2.19</td>
</tr>
<tr>
<td></td>
<td>PWP</td>
<td>2.32</td>
<td>6.52*</td>
<td>0.71</td>
<td>1.78</td>
<td>4.77</td>
<td>0.38</td>
<td>3.84</td>
<td>0.01</td>
</tr>
</tbody>
</table>

ST = soil texture, S = sand, LS = loamy sand, SL = sandy loam, L = loam, RL = root length (cm), FA = Foliar area (cm²), RDW = root dry weight (g), SDW = shoot dry weight (g), RV = root volume (cm³), RT = root tips, SA = surface area. Data represent values for two experiments with a total of 16 observations per treatment combination. Analyses were performed on square-root transformed data.
Figure 1. Effects of soil pH on root rot of soybean caused by *F. graminearum*. Average values of visual disease evaluations over A, soil moisture content. B, soil texture. Means with the same letter are not significantly different according to Fisher’s protected least significant difference (*P* < 0.05).

PS = pot saturation, FC = field capacity, PWP = permanent wilting point

Comparisons were made separately by pH level on the arcsin transformed data. Data represents values of two experimental replications. Analysis did not included data from non-infested plants.
Figure 2. Combined effects of soil texture and soil moisture content on root rot of soybean caused by *F. graminearum*. Average values of visual disease evaluations on **A**, soil at pH 6. **B**, soil at pH 8. Means with the same letter are not significantly different according to Fisher’s protected least significant difference ($P < 0.05$).

PS= pot saturation, FC= field capacity, PWP= permanent wilting point

Comparisons were made separately by pH level on the arcsin transformed data. Data represents values for two experimental replications and do not include data from non-infested plants.
Figure 3. Comparisons between infested and non-infested treatments on plant characteristics on the main effects of soil texture, and soil moisture. A, root length (cm). B, foliar area (cm²). C, root dry weight (g). D, shoot dry weight (g). E, root volume (cm³). F, root tips. * indicates significant differences from the non-infested control (P = 0.05) with a T-test for mean separation.
PS= pot saturation, FC= field capacity, PWP= permanent wilting point

Non-infested control contained sterile non-infested millet (10% v/v)

Mean comparisons were performed individually by soil moisture on each soil texture. Analyses were performed on the square-root transformed data. Data represents values for two experimental replications.
Figure 4. Percentage of growth reduction relative to the non-infested control on plant variables. Differences in plant characteristics for the interaction between the main effects of soil texture and soil moisture. **A**, root length. **B**, foliar area. **C**, root dry weight. **D**, shoot dry weight. **E**, root volume. **F**, root tips. Means with the same letter are not significantly different according to Fisher’s protected least significant difference ($P < 0.05$).
PS = pot saturation, FC = field capacity, PWP = permanent wilting point

Non-infested control = soil containing sterile non-infested millet (10% v/v)

Analyses were performed on the square-root transformed data. Data represents values for two experimental replications.
CHAPTER 5. CONCLUSIONS AND RECOMMENDATIONS

The objectives of this dissertation were to characterize the biology of *Fusarium oxysporum* and determine its role as a soybean seedling pathogen in the Fusarium root rot complex. Additionally, these studies had the objective to determine the impact of environmental factors, including pH, temperature, soil texture, and soil water content, on the development of soybean root rot caused by *F. oxysporum* and *F. graminearum* under laboratory and greenhouse conditions.

Pathogenicity data collected from a rolled towel and a petri dish assay, including 11 soybean cultivars and 14 *F. oxysporum* isolates provided valuable information on the diversity of *F. oxysporum* collected from soybean roots in Iowa, demonstrating that isolates varied phenotypically. Significant variation in cultivar susceptibility and isolate aggressiveness was observed. Our research is novel in that it tested the interaction of different cultivars and isolates. Previous reports of root rot of soybean caused by *F. oxysporum* included only one cultivar or one isolate on different cultivars, which in most cases bring incomplete, conflicting or wrong conclusions.

Although results of the petri dish and the rolled towel assays were similar, the symptoms produced by each isolate sometimes differed between assays. For example, some isolates caused only root rot symptoms in the rolled towel assay but caused both root rot and damping-off symptoms in the petri dish assay. This observation raises the questions about how the presence and interaction of various isolates in each field affects the predominance of damping-off or root rot symptoms. In addition, there is no information about other Fusarium spp. and other pathogens such as *Pythium* or *Rhizoctonia* interacting with *F. oxysporum*
causing soybean seedling disease. Future research may need to include within and among species interactions, and determine how abiotic factors influence disease expression.

In growth chamber experiments investigating the effect of temperature and pH on *F. oxysporum* fungal growth and soybean root rot, a significant interaction of temperature and pH was found, with the combination of pH 6 and 25°C resulting in the greatest fungal growth and the most severe root. Interestingly, our results for temperature agreed with other studies on Fusarium wilts performed under growth chamber conditions, but conflicted with previous studies conducted in field conditions. These findings suggest that temperature fluctuations and natural edaphic factors, such as soil texture, soil moisture, organic matter, nutrient availability, and soil microbial populations, may also play an important role in the development and severity of Fusarium wilts.

A Gaussian model helped to further describe the effects of pH and temperature on fungal radial growth and root rot severity under controlled conditions. According to the Gaussian regression analysis, the estimated pH and temperature optimal values for maximal root rot were equivalent for the pathogenic and non-pathogenic *F. oxysporum* isolates. However, inferences can only be made on the *F. oxysporum* isolates included in our studies. Therefore, more isolates should be included in this type of pathogenicity studies to validate these results. In addition, the level of predictability of the model could increase as more significant variables are added, helping to explain or predict phenomena that occur under more complex conditions.

Finally, greenhouse experiments testing the effects of soil pH, soil texture and soil moisture on root rot caused by *F. graminearum* suggested that disease may be inhibited in alkaline soils with pH levels around 8. In addition, soil moisture had a significant effect on
disease development. Under moisture-limiting conditions, levels of root rot increased significantly. Conversely, seedlings subjected to soil saturation displayed significantly low levels of disease. No correlation between disease severity and reduction in plant growth parameters were observed in our studies. This may be due to the inability of visual disease evaluations to take into account non-symptomatic root colonization or damage to internal root vascular tissues. In future studies, it may be useful to measure or quantify root colonization to estimate endophytic fungal biomass that can be correlated to other plant growth variables and help to understand detrimental effects of Fusarium wilts on plant hosts.

Overall, the evidence collected in the different experiments contained in this thesis demonstrated clear effects of temperature, soil pH, soil texture, and soil moisture on Fusarium root rot. However, more biotic and abiotic variables and their interactions must be studied in the future to generate useful information for designing management strategies that can realistically fit in soybean production systems.