THE EFFECT OF SEPTORIA BROWN SPOT ON SOYBEAN YIELD AND PHYLLOSPHERE MICROBIOME

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Crop Sciences
in the Graduate College of the
University of Illinois Urbana-Champaign, 2021

Urbana, Illinois

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ABSTRACT

Septoria brown spot (SBS), caused by *Septoria glycines* is the most prevalent soybean foliar disease in Illinois and often co-occurs with other late-season diseases, such as Cercospora leaf blight and frogeye leaf spot. Foliar fungicide applications during the reproductive stage is a common method to control these diseases. However, the application of fungicide does not always result in a yield increase. Furthermore, the effect of the fungicide applications on the phyllosphere fungal community needs further understanding. In this study, my research goals were to (i) characterize the development of SBS and its relationship with yield reduction, (ii) develop molecular markers for early identification and quantification of *S. glycines*, and (iii) characterize the effect of fungicide application on *S. glycines* and other phyllosphere organisms.

I conducted replicated multi-location inoculated field trials to characterize the disease development and evaluated the relationship between SBS and soybean yield. My results showed that the yield was negatively correlated with the percentage of the disease vertical progress and chlorotic area. From the stepwise regression analysis, the percentage of vertical progress was the best predictor variable for the model. As the vertical progress reached 30% at the R6 growth stage, there was a 10% predicted yield loss. Likewise, when the symptoms reached 80% of vertical progress, a 27% yield loss was predicted. There was no significant effect of the fungicide treatments on yield. The variance component analysis of the disease components data and yield data indicated that the location was the most critical factor that affected the experiment. Power analyses showed that at least eight locations are needed to reach 80% statistical power in small-plots studies with similar disease levels to my study to obtain statistically differences in fungicide treatments.

In chapter 3, I described the development and validation of a quantitative PCR (qPCR) method to accurately detect and quantify *S. glycines*. The assay designed with the actin gene (Ac) was specific to *S. glycines* for both conventional PCR and qPCR. The assay designed with the *β*-tubulin (Bt) gene was specific to *S. glycines* only on the qPCR. Both Ac and Bt assays had high qPCR reaction efficiency (95% and 98%) and sensitivity to detect 10 pg of *S. glycines* gDNA. The Bt assay was validated with field samples that had different necrotic areas. Symptoms of necrosis ranging from 0 to 30 % were significant and positively correlated ($r = 0.87$) to the *S. glycines* gDNA. The *S. glycines* gDNA was detected as early as 1-day post-inoculation in detached leaf assays.

In chapter 4, I used DNA metabarcoding to understand the dynamics between *Septoria* and non-target species using samples collected from the inoculated and fungicide-treated fields. Full-length ITS and partial LSU region were sequenced using oxford nanopore sequencer that yielded 3,342 unique OTUs. The cultivars had a significant difference of fungal communities at the V4 growth stage. Ten fungi were identified as core components of the leaves. Although possible interactions were identified between *Septoria* and other fungal organisms, the inoculation treatment did not significantly impact the entire communities according to the α and β diversity analyses. At R5 growth stage, the fungicide application significantly shaped the fungal communities. From the relative abundance analysis, the fungicide treatment significantly decreased the proportion of most fungi compared to the control samples, but the proportion of *Bipolaris*, and *Diaporthe* increased.

This study presents a comprehensive evaluation of SBS from multiple aspects. The results provide useful information for the estimation of the yield damage caused by SBS. I expect that the qPCR assays reported here could be used for disease diagnosis and to better characterize the

infection process of *S. glycines*. Finally, I demonstrated that metabarcoding could be a tool to quantify the effect of fungicide on target and non-target organisms. I believe that understanding the disease development, yield effect, and dynamics of the phyllosphere microbiome is necessary to untangle the late-season disease complex and develop better management practices.

ACKNOWLEDGMENTS

This work would not have been possible without the support of many people. First, I would like to thank my advisor Dr. Santiago Mideros for his continuous guidance and support throughout my Ph. D. program. Dr. Mideros has been a very helpful advisor in my research. He is always willing to talk to students and guide them to solve problems. I appreciate that he ensured that my research stayed on track. I could not have accomplished this degree without his support. I would like to thank my Ph.D. committee members, Dr. Glen Hartman, Dr. Maria Bonita Villamil, and Dr. Anthony Yannarell, for their critical advice and timely feedback for my research project. I would like to thank all the personnel from Dr. Mideros' lab and Dr. Jamann's lab for their support during the field seasons, greenhouse experiments, and lab meetings. I would like to thank Juan Pablo Granda, the former research specialist, for collecting samples, applying different treatments, and traveling with me during field seasons. I would like to thank the staff in the Department of Crop Sciences, the ACES plant care facility, and the HPCBio for making my life easier. I would like to thank all the friends I met here over the past five years. Life can be difficult for an international student; I appreciate that I can have friends to share good or bad things with them. Finally, I would like to thank my parents, husband, and daughter for giving me love and support over the years.

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CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Soybean (*Glycine max* (L.) Merr.) is a major oilseed crop produced and consumed worldwide and one of the most economically important crops in the United States. The seeds are valuable and have countless uses due to the high concentration of oil (20%) and protein (40%) (Singh, 2010). The mid-west area in the United States is the largest producer and exporter of soybean, with over 112 million metric tons produced in 2020 (USDA NASS, https://www.nass.usda.gov). Brazil, Argentina, China, and India are other leading producing countries.

One of the main yield-reducing factors for soybeans is biotic stresses. In 2016-2017, loss of soybean yield and quality caused by pests and pathogens worldwide were estimated at 21.4% (Savary *et al.*, 2019). In the United States, the total estimated economic loss due to diseases was 95.48 billion dollars from 1996 to 2016, with an average of 4.55 billion dollars per year (Bandara *et al.*, 2020). In descending order of importance, soybean cyst nematode, charcoal rot, seedling diseases, Phytophthora root rot, and stem root, root-knot and other nematodes, sudden death syndrome, Sclerotinia stem rot, Septoria brown spot, frogeye leaf spot, and Diaporthe-Phomopsis, were the 10 most destructive diseases in the United States (Bandara *et al.*, 2020). Among those diseases, this dissertation focuses in the most prevalent foliar disease in Illinois, soybean brown spot (Hobbs *et al.*, 2010), and associated foliar fungal microbiomes.

1.2 Brown spot of soybean – disease cycle, management, and yield impact

Brown spot, caused by *Septoria glycines* Hemmi, is a highly prevalent disease in most soybean growing areas. *Septoria glycines* belongs to Dothideomycetes class, Capnodiales order, and Mycosphaerellaceae Family. This pathogen was first described and named as *S. glycines* in

1915 in Japan (Hemmi, 1915). After that, it was reported in the United States (Wolf, 1923) and Canada (Koch & Hilderbrand 1944). This disease is also found in the soybean growing areas of Argentina, Brazil, China, Germany, Italy, Japan, Korea, Russia, Taiwan, and Yugoslavia (Hartman *et al.*, 2015b). The typical symptoms are irregular shaped, dark-brown necrotic spots on leaves often with a yellow halo, which can be observed as early as V1 toV4 stages in the field. The optimum temperature for disease development is 25 ºC, but the disease can develop in the range of 15-30 ºC (Schuh & Adamowicz, 1993).

Wolf and Lehman (1926) indicate that conidia are the only form that can overwinter in plant debris. However, it is reasonable to presume that the pathogen overwinters as pycnidia on infected plant debris in the soil and that conidia are the primary inoculum in the next season (Lim, 1980). A pycnidiospore-bearing droplet of *Septoria nodorum* (a wheat pathogen) can travel at least 70 cm far and 50 cm high with simulated rain and wind (Brennan *et al.*, 1985). *Septoria glycines*, as other Septoria species, presumably spreads by wind and splashing rain, but no dispersal studies have been conducted.

Septoria glycines develops from lower to upper canopies when soybeans reach maturity and might lead to premature defoliation (Hemmi, 1915; Pataky & Lim, 1981). Hot and dry conditions stop or slow the spread of the disease. The pathogen becomes active again when the environment is favorable for disease development (Hartman *et al.*, 2015b). Leaf wetness periods and temperature are the major factors that influence the number of lesions on leaves and disease severity (Schuh & Adamowicz, 1993; Ross, 1981). Increasing the post-inoculation mist period from 6 to 36 hours produces more lesions per cm^2 on the soybean cv. Essex (Ross, 1981). In another study, the disease severity was highest when treated with 24 hours of mist period on the soybean cv. Pella (Schuh & Adamowicz, 1993).

Histological experiments show that at 22 ºC and saturated moisture conditions, the conidia germinate within 15 hours after inoculation (MacNeill & Zalasky, 1957). No appressorium formation is observed during the invasion process. The pathogen penetrates through the stomata on the undersurface of the leaf. The invasive hyphae then develop upward to the palisade layer within 72 hours. The hyphae keep invading the surrounding cells until the pycnidia start to form in the necrotic area, which contains dead or dying cells. At this point, more distal cells become chlorotic (MacNeill & Zalasky, 1957). A host-specific toxin (20,000 Da polysaccharide) has been isolated from the culture filtrate of *S. glycines* that causes typical brown necrotic and chlorotic symptoms on leaves (Song *et al.*, 1993). This toxin may associate with the pathogenesis of the pathogen, but the structure of this toxin and mode of action have not been characterized.

The pathogen also penetrates pods through stomata and colonizes the intercellular region. Furthermore, the pathogen can invade placenta and funiculus, and then infect the seeds (MacNeill & Zalasky, 1957), but seedborne is not common in the field (Hartman *et al.,* 2015). The time from attachment to invasion has been clearly defined to be 72 hours; however, the incubation period (time between inoculation and symptom development) and the latent period (time between inoculation and reproduction of the pathogen) for this disease remain unclear. Young and Ross (1979) reported the differences in disease progression when they inoculated the soybeans at different developmental stages. The disease progression was more rapid and severe on the younger leaves. It took 60 days, which was twice the amount of time, for the R1 developmental stage soybeans to reach a similar disease level as the V2 developmental stage soybeans. This might indicate that the incubation period and latent period of this disease were

dependent on the stage of soybean maturity. High humidity on the leaf surface after infection can promote disease progression and decrease the length of the incubation period (Ross, 1981).

Brown spot can be considered part of the late-season disease complex (Carmona *et al.*, 2011). It usually co-occurs with *Cercospora kikuchii* (Cercospora leaf blight and purple stain), *Cerospora sojina* (frogeye leaf spot), *Colletotrichum truncatum* (anthracnose), and other pathogens between R1 and R6 developmental stages (Almeida *et al*., 2015; Carmona *et al*., 2011; Sinclair, 1991), which is a critical period for yield. The number of pods and seeds in soybeans are determined at the R1 developmental stage and extend through the R6 developmental stage (Kantolic & Slafer, 2007). The soybean green leaf area and its duration are also important for yield determination (Kantolic & Slafer, 2007). Therefore, controlling the late-season disease complex during the soybean reproductive growth stage is important to avoid yield loss. Brown spot and bacteria blight (*Pseudomonas savastanoi* pv. *glycinea*) may have an antagonistic relationship if infecting the soybean simultaneously (Williams & Nyvall, 1980; Basu & Butler, 1988). There are no reports about the effect of brown spot on other soybean pathogens.

Septoria glycines limits yield by reducing the green leaf duration area and decreasing seed size and seed weight (Young & Ross, 1979; Pataky & Lim, 1981). However, the effect of brown spot on yield is not consistent among studies. Lim (1979) reported yield losses ranging between 1% and 27%, which vary by variety, location, and inoculation treatment in a field survey with 1000 soybean plant introductions (PI's). Lim (1980) reported a yield reduction ranging from 12% and 34% in inoculated plots (cv. Wells and cv. Williams) and 8% in naturally infected plots. Kamicker and Lim (1985) reported no difference in yield and 300-seed weight on cv. Williams 79 between inoculated treatment and control, but the authors pointed out that this may be due to the late appearance of the symptoms and the relatively low disease severity. Cruz

et al. (2010) reported yield losses between 2.5% and 9.5% in Ohio. On average, the estimated economic losses caused by this disease across 28 states from 1996 to 2016 are 160 million US dollars (Bandara *et al.*, 2020).

Pathogenic variability among isolates has been reported from other *Septoria* spp., such as *Septoria tritici* (causing Septoria tritici blotch (STB) on wheat) (Kema, 1996), and *Septoria nodorum* (Septoria nodorum blotch (SNB) on wheat) (Krupinsky, 1997). Kamicker and Lim (1985) report significant differences in disease severity and vertical progress among thirty *S. glycines* isolates, but the results are not consistent among different trials in a two-year field study. The inconsistent results were associated with the weather condition during the growing seasons, and the cold and dry weather in the early growing season results in longer latent period of the disease (Kamicker and Lim, 1985).

Tillage, rotation with non-legume crops and fungicide application are the recommended methods to manage this disease (Hartman *et al*., 2015). No resistance genes in soybeans have been associated with this pathogen. In 1978, a total of 626 lines were evaluated for resistance at the seedling stage in the greenhouse and at the full-pod stage in the field. Only six lines produced non-chlorotic lesions at the seedling stage, and no resistance response was found (Young & Ross, 1978). In 1979, another screen was conducted in the field in Urbana, Illinois, with 1000 soybean PI's. Although two types of lesions were observed, and it was associated with the seed color, still, no resistance was found (Lim, 1979). A two-year experiment in Illinois shows that soybean cultivars (MG III) released after 1989 are relatively more resistant than older cultivars (Hartman *et al*., 2015), which indicated that there might be partial resistance in soybeans to brown spot.

Fungicide use in the United States has drastically increased since 2005 (Jiang *et al.*, 2019). Foliar fungicide application between R1 and R6 stages is recommended for the management of brown spot. However, the yield response after fungicide application varies among different studies (Cooper, 1989; Pataky & Lim, 1981; Cruz *et al*., 2010). Pataky and Lim (1981) applied benomyl (FRAC code: 1) on soybean cv. Williams at R1, R3 or R6 stage in Urbana during 1978-1979 and Brownstown in 1979. Benomyl is a systemic benzimidazole that binds to microtubules and interferes with mitosis and the transportation process in fungi. Disease severity significantly reduced in both naturally infected and artificially infected plots due to the fungicide application. Comparing the spraying time points and the number of applications, spraying at R3 and R3 plus R6 stages have the best effect on reducing the disease severity. However, significant differences between treatments in yield were only found in Urbana in 1979 (Pataky & Lim, 1981). Cooper (1989) tests the effect of benomyl with two determinate cultivars (Hobbit and Sprite) and two indeterminate cultivars (Pella and Williams 82). Benomyl is sprayed between R2 and R6 stages with 2-weeks interval. Yield increase significantly only in Hobbit and Pella (Cooper, 1989). In 2010, Cruz et al (2010) also evaluate strobilurin (FRAC code: 11) and triazole (FRAC code: 3) fungicides to control soybean brown spot with 0 to10 times of fungicide application. Several commercial lines (DeKalb DKB38-52, Pioneer 93M92, Midwest GR 3931, Asgrow AG3905, SC9384, Vistive RV2890, and Pioneer 93B82) were tested in this study. Application of strobilurins at R3 or combinations of pyraclostrobin at R3 with tebuconazole at R3 or R5 reduced disease severity significantly. However, the number of sprays does not have a consistent effect on yield (Cruz *et al.*, 2010). These studies suggest that applying fungicide only depending on the phenological growth stage may not be sufficient. The environmental conditions and disease development should also be taken into consideration. For example, Carmona et al (2011)

find that rainfall is a critical factor that affects the development of late-season disease complex at maturity. The yield is higher when applying fungicide (azoxystrobin+ triazole) between R3 to R5 stage only when there is a high level of accumulated rainfall during this same period (Carmona *et al.*, 2011). Also, it has been proposed that the fungicides should be applied during the early infection stage for controlling Cercospora leaf blight on soybean (Chanda *et al.*, 2014). This concept may also apply to other late-season diseases on soybean, including brown spot. For this to be possible, early detection, diagnostic, and quantification tools for *Fusarium virguliforme* (Wang *et al.*, 2014), *Sclerotinia sclerotiorum* (Botelho *et al.*, 2015), *Phialophora gregata* (Malvick & Impullitti 2007), *Phakopsora pachyrhizi* (Frederick *et al.*, 2002), and *Cercospora kikuchii* (Chanda *et al.*, 2014) through quantitative polymerase chain reaction (qPCR) have been developed. However, no specific qPCR primers have been reported for brown spot.

As indicated above, differences in yield between infected and not infected plants are highly variable. This variability could be due to multiple reasons, including a strong environmental effect. Also, the effect of the disease itself could be so small that some studies have been underpowered (Gent *et al.*, 2018). The possibility of a limited or even null effect on yield for an organism so commonly associated with cultivated soybeans highlights several gaps in the knowledge of this plant-microbe interaction.

1.3 Soybean microbiome

The collective communities of plant-associated microorganisms are referred to as the plant microbiome (Mendes *et al.*, 2013). It is considered an important factor related to plant development, plant health, and productivity (Turner *et al.*, 2013a). The plant microbiome resides in all parts of the plant, including the rhizosphere, the phyllosphere, and the endosphere.

The rhizosphere is the narrow zone surrounding the roots. Rhizosphere organisms like nitrogen-fixing bacteria, arbuscular mycorrhizal fungi (AM fungi), plant growth-promoting rhizobacteria (PGPR), and some biocontrol microorganisms are well-studied and known to be beneficial to plant growth and health (Mendes *et al.*, 2013). In the grass-clover field, the AM fungi increase 82 to 85% of productivity. Also, the number of the species of AM fungi is positively correlated with aboveground biomass (r^2 range from 0.11 to 0.48) (Wagg *et al.*, 2011). The diversity of the rhizosphere microbiome is majorly driven by the deposition of plant exudates and root residues (Mendes et al. 2013; Kent & Triplett 2002). For example, isoflavones (daidzein and genistein) released from the root can attract a symbiont: *Bradyrhizobium japonicum*, but also a pathogen: *Phytopthora sojae* (Morris *et al.*, 1998). Levels of these two isoflavones in the rhizosphere area affect the structure of the microbiome community (Guo *et al.*, 2011). Soybean maturity (growth stages: vegetative, flowering, and mature) also change the bacterial community in the soil (Sugiyama *et al.*, 2014). A recent study also shows that differences of soybean productivity are significantly associated with the soil microbiome (Chang *et al.*, 2017). The abundance of certain bacterial taxa (Bradyrhizobium and Gammaproteobacteria, which are involved in nodulation) are higher in high productivity areas in Illinois. Chang et al (2017) also use machine learning to predict the crop productivity based on the microbiome composition, reaching an accuracy of 0.787 at the order level.

The phyllosphere, which is the aerial part of the plants, contains relatively fewer nutrients and faces fluctuation of radiation, temperature, and moisture every day (Turner et al. 2013). Environmental variables, host genotype, crop growth stage, and geographical location may affect the composition of the phyllosphere microbiome. Copeland et al. (2015) report a dramatic change of relative abundance (RA) of microbiome after rain in Canola. On the other hand, the

RA of the microbiome in soybean and common bean are more associated with the sampling date and growth stage of the host (Copeland *et al.*, 2015). Although most of the bacteria identified from leaves are shared with the soil samples, there are some leaf specific microbiome that belongs to the phylum Actinobacteria, Firmicutes, Proteobacteria, and Thermi. The RA of the leaf-specific microbiome increase over time from 24% to 40% (Copeland *et al.*, 2015).

The effect of fungicide application on soybean's phyllosphere microbiome has not been reported. However, several studies investigated the effect of fungicides or organic farming practices on the phyllosphere microbiome on wheat (Karlsson *et al.*, 2014, 2017; Sapkota *et al.*, 2015). Karlsson et al. (2014) report that the composition of the fungal community at the order level is significantly different for fungicide-treated and untreated samples. Fungicide application also significantly affects the RA of some saprotrophs at the species level. The authors also point out that more research is needed to work on the fungicide-fungal interaction in the phyllosphere, which would be essential for the development of sustainable disease control strategies. Sapkota et al (2015) also study the fungicide effect, host genotype, and geographical region on the fungal community on the phyllosphere of different cereals, including wheat, oat, rye, and triticale. The results show that the host genotype explains the highest variance (43%), followed by fungicide treatment (13%), and location (4%). On the other hand, compared to conventional farming system, organic farming increases the richness of fungal taxa in the wheat phyllosphere (Karlsson *et al.*, 2017).

Microorganisms that reside outside the plant are called epiphytes, while those that reside inside the plant (endosphere) are endophytes. Endophytes can also promote plant growth. An endophyte, *Cladosporium sphaerospermum* isolated from roots of soybean (cv. Daemangkong), produces a high amount of gibberellins and could promote soybean (cv. Hwangkeumkong)

growth by increasing plant height and shoot length when applying the fungus filtrate at VC stage (Hamayun *et al.*, 2009), however, the effect of the fungal filtrate of this endophyte on final yield is unknown. Miller and Roy (1982), report no diversity of endophytes between leaves, pods, and seeds. However, this may be due to the cultural-dependent methodology. Impullitti and Malvick (2013) using both cultural-dependent and cultural-independent methods find no significant differences in the number of genera between cultivars. Sixty percent of isolates are obtained from dermal and vascular tissue, < 30% of isolates are obtained from the pith tissue. Most abundant genera are *Cladosporium, Alternaria, Diaporthe, and Epicoccum.* Some organisms, such as Alternaria, Diaporthe, and Phomopsis were known as opportunistic pathogens. The association of soybeans and the many other endophytic organisms detected in this study did not further verified (Impullitti & Malvick 2013).

The following chapters in this dissertation present a comprehensive evaluation of SBS from multiple aspects. Earlier studies reported variable results about the yield effect of SBS. Conducting follow-up studies and re-evaluating the yield impact of this disease in Illinois can provide useful information and improve current management practices. Therefore, the objectives of the second chapter was to characterize the impact of Septoria brown spot on soybean yield and understand the disease development in Illinois. A promising and stable molecular assay is useful for both fundamental research and applied purposes. The objective of the third chapter was to develope a molecular assay for the early diagnosis and detection of *S. glycines*. This molecular tool can be further applied for characterizing the incubation period and latent period of this disease and also screening for partial resistance for further studies. The objective of the last chapter was to use long reads sequencing platform to diagnose and detect the fungal organisms

in soybean phyllosphere, and to study the effect of fungicide application on *S. glycines* and other non-target fungal organisms.

CHAPTER 2. CHARACTERIZATION OF SEPTORIA BROWN SPOT DISEASE DEVELOPMENT AND YIELD EFFECTS ON SOYBEAN IN ILLINOIS[1]

2.1 Abstract

Brown spot, caused by *Septoria glycines* Hemmi, is a highly prevalent foliar disease of soybean (Glycine max (L.) Merr.). Despite its wide distribution, the development of Septoria brown spot and its relationship with yield reduction remain poorly characterized. In this study, I conducted replicated multi-location inoculated field trials to characterize the disease development and evaluated the relationship between Septoria brown spot and soybean yield. Multiple components of disease and yield were rated weekly to characterize disease development. Fungicide treatments had a significant effect on ratings of vertical progress of the disease and chlorotic area at the end of the season. There were also significant differences between the fungicide treatments for the Area Under the Disease Progress Curve (AUDPC) of all the disease components, including necrotic area and defoliation rate. Soybean yield was negatively correlated with the vertical progress of the disease ($r = -0.36$). The vertical progress was the best linear predictor of yield with an $R^2 = 0.08$ for the end of the season rating and an $R^2 = 0.2$ for the AUDPC. A variance component analysis of the data showed that location was the most critical factor, illustrating the large effect of local environmental conditions on the disease. There was no statistically significant effect of the fungicide treatments on yield. Power analyses indicated that at least eight locations are needed to detect an effect of 269 kg ha^{-1}. The results provide useful information in the characterization of the disease development and for estimations of yield damage

[1] This research has been published in Lin, H.-A., Villamil, M. B., and Mideros, S. X. 2021. Characterization of Septoria brown spot disease development and yield effects on soybean in Illinois. Can. J. Plant Pathol. 43:62–72.

2.2 Introduction

Soybean (*Glycine max* (L.) Merr.) is one of the most important crops in North America, with a cultivated area of 35.75 million ha and an average yield of 3.5 metric tons ha^{-1} in 2018 in the United States (USDA 2019). An estimated 12% of soybean yield is lost to diseases every year in the USA (Hartman *et al*., 2015). Brown spot caused by the fungal pathogen *Septoria glycines* Hemmi is one of the 10 most destructive diseases associated with yield losses (Allen *et al.*, 2017). This disease often occurs simultaneously with other late-season diseases such as frogeye leaf spot (*Cercospora sojina* Hara) and *Cercospora* leaf blight (*Cercospora kikuchii* (Mat and Tom) Gardner). Some authors even consider them to be a disease complex (Carmona *et al.*, 2011). These late-season diseases have not received much research attention, yet assessing their effect on soybean yield is critical to precision management practices and our understanding of disease development under field conditions.

In this study, I focus on Septoria brown spot of soybean, a highly prevalent disease in all soybean growing areas (Hartman *et al.*, 2015b). This disease was first reported in the US in 1922 (Wolf, 1923) and can be found in other soybean-producing countries such as Argentina, Brazil, and China (Hartman *et al.*, 2015b). In Illinois, Septoria brown spot is the most common soybean disease and is usually severe enough to affect yield (Eathington *et al.*, 1993). The typical symptoms are irregular, dark-brown necrotic spots with a chlorotic halo that in some cases can be observed as early as the V2 stage (Fehr *et al*,1971; Mueller *et al,* 2016). Under humid and warm environments, the pathogen produces pycnidia and new infections progress to the upper canopy, causing premature defoliation. The optimum temperature for disease development is

25℃, but the disease can develop between 15℃ to 30℃ (Hemmi, 1915; Pataky & Lim, 1981; Hartman *et al.*, 2015).

Several studies have evaluated the yield losses caused by Septoria brown spot. In a field survey with 1000 soybean plant introductions, Lim 1979 showed that yield losses due to Septoria brown spot ranged between 1% to 27%, depending on variety, location, and whether the plots were inoculated. Another study reported yield reductions ranging from 12% to 34% in inoculated plots, and from 8% to 8.7% in naturally infected plots (Lim, 1980). A more recent study in Ohio reported that yield losses ranged from 2.5% to 9.5% in naturally infected fields with a different number of chlorothalonil applications (Cruz *et al.*, 2010).

There is no resistance to Septoria brown spot. Exhaustive surveys of 1000 (Young & Ross 1978) and 626 soybean lines (Lim, 1979) identified no resistance to this disease, highlighting the importance of chemical management. There are no data on the resistance or susceptibility to brown spot in newly released soybean cultivars (Cruz *et al.*, 2010; Hartman *et al.*, 2015b), and no pathogenic variability among isolates has been found (Kamicker &Lim, 1985).

Application of chlorothalonil (FRAC CODE M05) or mixtures of demethylation inhibitor (FRAC CODE 3) and quinone outside inhibitor (FRAC CODE 11) compounds between R1 to R6 can effectively control Septoria brown spot (Cruz *et al.*, 2010). Fungicide treatments can significantly reduce disease severity at the end of the season; however, the reported impacts on yield varied among locations and years in several studies. Pataky and Lim (1981) reported that spraying benomyl (FRAC CODE 1) at R3 or R3 plus R6 stages had the best effect on reducing disease severity, but the yield increase due to the fungicide application was detected only in one year. Cooper (1989) detected yield increases only among two out of four cultivars when spraying benomyl in 2-weeks intervals, between R2 to R6 stages of soybean. Cruz et al. (2010) detected a

statistically significant effect of the application of strobilurins (pyraclostrobin or azoxystrobin (FRAC CODE 11)) or a combination of strobilurins and triazole (tebuconazole (FRAC CODE 3)) on reducing disease severity, but the yield increases were found only in three out of six locations/years.

The amount of photosynthate produced from flowering (R1) to seed formation (R5) is the critical factor that determines the seed number and seed weight in soybeans (Egli & Bruening 2001). Green leaf area duration during maturity is important for the plant to produce sufficient photosynthate. Therefore, disease control during this period is critical for the final yield. Carmona et al. (2011) reported a significant correlation between rain and yield response to fungicide application when rain occurred after pod formation (R3) and before seed formation (R5). These findings suggest that reducing foliar diseases, in general, could have a positive effect on yield under certain environmental circumstances.

While general rules for fungicide applications are useful, more detailed knowledge on microorganism-specific development and its effect on yield losses are necessary for the generation of precision disease management strategies. The ubiquity of Septoria brown spot symptoms suggests that it is a major component of the late-season disease complex (Carmona *et al.*, 2011). To accurately track the development of disease and to calculate the effect of plant pathogens on yield reductions, it is necessary to establish reliable methods to exert different levels of disease pressure on field trials. These experiments are often complicated by the large effect of environmental factors on disease development, thus requiring multiple diverse environments to reach valid conclusions. These experiments are expensive and time-consuming; thus, it is important to know the level of replication necessary to obtain useful results. The objectives were to i) develop protocols for field assays that generate different levels of Septoria

brown spot disease severity; ii) characterize the disease development in multiple environments; and iii) evaluate the relationship between Septoria brown spot disease severity and soybean yield. Finally, I conducted a power analysis that could be used for the development of future field trials to further evaluate the yield effects of Septoria brown spot on yield.

2.3 Materials and methods

2.3.1 Field management and experimental layout

Field trials were established at the Crop Sciences Research and Education Center near Urbana (N40.072170º, W88.220882º), the Orr Agricultural Research and Demonstration Center near Perry (N39.805006 º, W90.823208 º), and the Northwestern Illinois Agricultural Research and Demonstration Center near Monmouth, Illinois (N40.931887º, W90.725123º) in 2017. All fields were on corn-soybean rotation and fertilized according to local practices based on soil tests. At each location, the experiment was arranged in a randomized complete block design (RCBD) with four replications. The fungicide treatment had five levels: two controls without fungicide [control (0X): inoculated plots; and NIC: not-inoculated control plots]; and three fungicide treatments 3X, 6X, and 9X, that represent the number of weekly applications performed to obtain different disease severity in the field. Chlorothalonil (Echo™ 720 AG, Sipcam Agro USA, Inc.) was applied weekly at a rate of 1.05 kg ai ha^{-1}, starting one week after pathogen inoculation and continuing for 3, 6, or 9 weeks in each of the 3X, 6X, and 9X treatments, respectively. Fungicide was applied using a CO_2-pressurized backpack sprayer and a 0.48 m 601B-SST - four nozzle lightweight boom (R&D Sprayers Bellspray, Inc, USA) with four TJ60-11008 (50) nozzles (TeeJet Technology, USA). Each plot consisted of four 5.2 m long rows, and each row was 0.76 m wide. All trials were planted with soybean cultivar 'Williams' (Bernard & Lindahl 1972) between May 5 and May 18 at 348,480 seeds ha^{-1}. 'Williams' is a

public variety well adapted to central Illinois. I expected that using an older cultivar would exacerbate the yield effect of the treatments. The field in Urbana received supplemental irrigation starting at the maturity stage R1 using 14VH 1/2" inlet full circle brass wedge drive impact sprinklers (Rain Bird, USA) placed at 1.5 m height, and spaced every 9.14 m.

As indicated above, there are no reports on aggressiveness differences between isolates of this pathogen. Therefore, three randomly selected isolates (R3126, 16S006, and 16S012) that produced a large number of spores were chosen for inoculation. Isolate R3126 was collected in 2013, acquired from Dr. Carl Bradley (University of Kentucky), whilst 16S006 and 16S012 were collected in 2016 from a diseased soybean field in Urbana. To prepare the inoculum, three isolates of *S. glycines* were cultured on 3% malt extract agar (MEA) for two weeks at room temperature (~24 °C), with 12 h of fluorescent light. Sterile water was added to each plate, and 10 to 20 µL of the spore suspension was transferred and spread on 3% MEA plates. One week later, 5 to 10 mL water amended with 10 µg L^{-1} chloramphenicol was added to each plate, and three milliliters were transferred to a 1 L flask containing 135 g barley (*Hordeum vulgare* L.) and 15 g rice (*Oryza sativa* L.) grain. The grain was prepared by washing it with tap water three times and soaking in water overnight at 4°C. Excess water was drained before autoclaving. The water content of the substrate prepared in this way was approximately 60%. Following two weeks of incubation, the conidia were collected with water containing 0.05% (v/v) Tween 20 and filtered through sterilized, double-layered cheesecloth. The concentration of the spore suspension was adjusted to 10^6 spores mL^{-1} following quantification with a hemocytometer. The center two rows of each plot (except the NIC) were inoculated between the V3 and V4 developmental stages (Fehr et al. 1971) with a suprema bak-pak sprayer (Hudson Manufacturing Company, USA).

2.3.2 Disease and yield assessment

Visual ratings of the percent of four components of disease severity (vertical progress, defoliation rate, chlorotic, and necrotic area) were taken weekly from the center two rows of each plot. Vertical progress and defoliation rate were estimated by the percent height reached by brown spot symptoms, or removal of the leaves. The chlorotic and necrotic areas were the visual estimates of the percent of the whole plant foliage that was yellow or brown/black, respectively. To evaluate soybean yield components, 20 plants were collected randomly from each plot. After drying the plants at room temperature, the weight of 100-seeds, the number of pods per plant, and the number of seeds per plant were recorded. Field plots were harvested with a research plot combine between Oct 3 and Oct 18. Soybean yield data (kg ha^{-1}) were adjusted to 13% water content. Yield response was calculated by subtracting the yield from treatments (3X, 6X, or 9X) to the control plots (0X) in each block.

2.3.3 Data analysis

The area under the disease progress curve (AUDPC) values for the components of disease severity were calculated using the AUDPC function in the Agricolae package (De Mendiburu 2009) in R v. 3.5.2 (R Core Team 2018). Both simple and multi-location RCBD models were used to evaluate the experiment.

$$y_{ij} = \mu + B_i + \tau_j + \varepsilon_{ij}$$

$$y_{ijk} = \mu + L_i + B_{(i)j} + \tau_k + L\tau_{ik} + \varepsilon_{ijk}$$

In the simple RCBD model, y_{ij} is the observed data (yield components and AUDPC for each of the disease severity components) corresponding to the i^{th} block and j^{th} treatment. μ is the grand population mean, B_i is the random block effect, τ_j is the fixed treatment effect, and ε_{ij} is the random error term [NID$(0, \sigma_e^2)$]. In the multi-location RCBD model, location (L_i) is added

to the model as a random effect with block ($B_{(i)j}$) nested within it. $L\tau_{ik}$ is the random interaction effect between the i^{th} location and the k^{th} treatment.

Linear mixed models to evaluate the effects of fungicide treatments on yield and disease severity (raw data or AUDPC) were fitted using PROC MIXED in SAS v. 9.4 (SAS Institute Inc., Cary, NC). The covtest option was specified in the PROC MIXED statement to estimate the variance components. Least square means were separated using the PDIFF option of LSMEANS in SAS PROC MIXED and setting the probability of Type I error or α level to 0.05. Correlation among variables was evaluated with PROC CORR. A stepwise regression analysis (using the REG procedure) was conducted with the components of disease severity as dependent variables and yield as the response variables. Figures were generated in GraphPad Prism v. 6.0 for Windows (GraphPad Software, La Jolla, CA).

2.3.4 Statistical power analysis

I conducted a power analysis using the SIMR package (Green et al. 2016) in R (R Core Team 2018). The goal was to determine the appropriate number of locations and replicates needed to detect a significant yield response. SIMR produces Monte Carlo stochastic simulations with my empirical data in the linear mixed model indicated above and predetermined effect sizes. For this analysis, I used only the data from the control plots (0X) and the 3X treatment. The threshold of desired power was set as 0.8. The predetermined effect sizes used for the simulations with the yield data were 134.5, 269, and 403.5 kg ha^{-1} (two, four, and six bu ac^{-1}), which cover the range of 5-10% yield reduction for soybean cultivar 'Williams'. The range of 5-10% of yield reduction is also the reported yield response to fungicide without the disease (Kandel et al. 2016; Kandel et al. 2018), and with Septoria brown spot (Cruz et al. 2010). For the other yield components, the effects used for the simulations were 1.5, 2, and 4 pods per plant; 3,

6, and 9 seeds per plant and 0.2, 0.4, and 0.6 g of 100-seed weight. These values were based on a previous report with Septoria brown spot infected assays (Pataky & Lim 1981a).

2.4 Results

2.4.1 Environmental Conditions

Weather station data indicated that the Monmouth location received more precipitation at the vegetative stage (191.7mm) and more precipitation events through the season (Supplementary Figure 2.1, Supplementary Table 2.1). The precipitation in Monmouth also occurred at more regular intervals than in the other two locations. Perry received 123.3 mm precipitation which was lower than Monmouth during the vegetative stage of the crop, but several significant rain events occurred at the reproductive stage with a total precipitation of 139.2 mm. The lowest levels of precipitation occurred in Urbana, with most rain events occurring during the vegetative phase of the crop and providing < 40 mm of rain for the vegetative stage. The average temperature in Monmouth and Perry at V3 to V5 stage ranged from 24℃ to 26℃, which is the optimal temperature for disease development. The average temperature in Urbana at the same growth stage ranged from 27℃ to 30℃.

2.4.2 Disease development and response to fungicide treatments

Weekly evaluations produced nine observations from Perry and 11 from Urbana and Monmouth. The vertical progress of Septoria brown spot remained low for most of the season and did not reach halfway up the plant until 95 to 113 days after planting (approximately R5 to R6). It reached 100% for most of the treatments at the last rating at R7 (Figure 2.1). The percentage chlorotic area was < 20% for all treatments until 114 days after planting (dap; R6), but increased to > 50% for the last rating at 125 dap (R7; Figure 2.1). The necrotic area remained

< 20% throughout the study. The defoliation rate increased gradually, starting at R1, and reached close to 100% by the last rating at R7. Due to the differences in disease progression between the traits, the final rating for vertical progress was considered to be R6, while for the other disease components were R7. The AUDPC for vertical progress was calculated with the ratings obtained from V7 to R6; for the chlorotic and necrotic areas with the ratings from V7 to R7; and for defoliation rate with the ratings from R1 to R7.

At the end of the season, fungicide treatments significantly reduced the vertical progress at R6 ($p = 0.008$), the percent of chlorotic area at R7 ($p = 0.004$) (Figure 1.2), and showed a reduction trend for percent of the necrotic area at R7 ($p = 0.115$) (Table 1.1). Three, six, and nine weekly fungicide applications reduced the disease vertical progress by 17% to 33% compared with the plots that were not treated. Six and nine weekly fungicide applications reduced the chlorotic area by 18% and 21% compared with the non-treated controls. No differences were observed between the inoculated and not-inoculated control plots (Figure 2.2) and no other diseases were observed at any significant levels.

For the disease traits, the largest variance was due to the location effects (Table 2.2), which ranged between 15% to 78%. Only a maximum of 3% of the variance was due to the block within a location, and a maximum of 35% due to the location by treatment interaction. The one exception was for the percent of chlorotic area, which had no variance due to location and 52% variance due to location by treatment interaction (Table 2.2).

2.4.3 Correlation between brown spot disease and yield

All the disease components (vertical progress, necrotic area, chlorotic area, and defoliation rate) were significantly and positively inter-correlated ($r = 0.59$ to 0.88). Significant and negative correlations were observed between yield and vertical progress (both AUDPC r = -

0.45 and final rating at R6 r = -0.28), and a trend was observed between yield and the AUDPC of chlorotic area (r = -0.31; Table 2.3). Also, a negative correlation was observed between yield and 100-seed weight (r = -0.49). The AUDPC and the final rating of vertical progress (R6) were significantly correlated with the AUDPC of chlorotic area but not the final rating of the chlorotic area (Table 2.3). The 100-seed weight, in addition to being negatively correlated with yield, was also negatively correlated with the chlorotic area at R7 but positively correlated with the AUDPC of vertical progress. No significant correlations were found between yield response and yield, vertical progress, chlorotic area, or 100-seed weight. No correlations were significant when the analysis was done by location (not shown).

2.4.4 Linear regression analysis

Stepwise regression analysis identified the final rating and AUDPC of vertical progress to be significant predictor variables when included in the model independently. The estimated regression slope was -14.21 (p = 0.036) for vertical progress at R6, and -0.69 ($p < 0.001$) for AUDPC of vertical progress. The coefficient of determination (R^2) was 0.08 and 0.20, respectively (Figure 2.3). This indicated that an 8% to 20% variation of yield was explained by the vertical progress of Septoria brown spot.

2.4.5 Yield response to fungicide treatments

Fungicide treatment did not have a significant effect on yield, yield response, 100-seed weight, pods per plant, or seeds per plant. For yield and 100-seed weight, the largest percentage of variance was due to location effects (79% and 75% respectively). However, most of the variance was in the residual for pods per plant (51%) and seeds per plant (43%; Table 2.2). A

trend of higher yield on the plots treated nine times was observed in one of the locations (Perry). The yields in Perry and Urbana were similar but lower in Monmouth (Figure 2.4).

2.4.6 Power Analysis

Simulations based on my empirical data showed that the number of locations required to obtain > 80% statistical power to detect a yield reduction of 269 kg ha^{-1} (4 bu ac^{-1}) between the untreated control (NIC) and plots sprayed three times is 15 (Figure 2.5). Increasing the number of replicates within location did not result in any reasonable power increases (not shown). To obtain 80% power in a 3-location experiment, the difference between the NIC and the plots sprayed three times would have to be 1008.75 kg ha^{-1} (15 bsh acr^{-1}). The number of locations required for other yield components was 16 for pods per plant (effect of 4 pods/plant), 6 for seeds per plant (effect of 9 seeds/plant), and 8 for the 100-seed weight (effect of 0.6 g/100-seeds; not shown).

2.5 Discussion

Septoria brown spot is the most prevalent disease of soybean in Illinois (Eathington *et al.*, 1993). I conducted a detailed characterization of the development of the disease and its yield effect. I inoculated field plots in replicated experiments in three different locations and obtained significantly different levels of the disease following three, six, or nine applications of chlorothalonil. Weekly evaluations of four disease components (vertical progress, necrotic area, chlorotic area, and defoliation rate) allowed us to characterize the symptoms of this disease through the season, and the three locations provided an opportunity to observe the environmental effects on the disease development.

The disease was more severe in Monmouth than at the other two locations. Monmouth received more rain during the vegetative stage than the other two locations, probably explaining the increase of the vertical progress of the disease before the reproductive stage of the crop. The disease then continued its vertical progress during the reproductive stage even when rain was not as constant as during the vegetative stage. Supplemental irrigation provided in Urbana after R3 was probably applied too late to affect the epidemic. These observations suggest that rain conditions before flowering had a significant effect on the development of Septoria brown spot. It has been previously reported that the disease development (number of lesions on leaves and disease severity) is highly affected by the leaf wetness periods and temperature after infection (Ross 1981; Schuh & Adamowicz 1993). Precipitation is also known to be an important factor for the dispersal of the *Septoria* spp. Brennan et al. (1985) reported that a pycnidiospore-bearing droplet of *Septoria nodorum* (Berk.) Berk, a related wheat pathogen, can travel at least 70 cm far and 50 cm high with simulated rain and wind. Although there are no dispersal studies for *S. glycines*, it is presumably spread by splashing rain and wind to the upper canopy through the growing season. Field experiments also showed a correlation of yield responses to fungicide and rain between the R3-R5 maturity stages (Carmona *et al.*, 2011). My results indicate that precipitation before the reproductive stage also favours disease development.

Vertical progress, estimated visually as the percent height of the plant that presented Septoria brown spot symptoms, increased linearly and reached 100% of the plant for all the treatments at 'beginning maturity' (R7). Due to senescence, it is not surprising that all plants would present necrotic and chlorotic leaves at this stage, and I found no differences between treatments this late in the season. However, the previous developmental stage, 'full seed' (R6), presented a clear effect of the fungicide applications on vertical progress with the not sprayed

control (0X) showing > 60% vertical progress (Figure 2.2). Pataky & Lim (1981a) also evaluated vertical progress, and in their study, it reached 50% at R6 and > 90% at the last rating. With my data, we found significantly different levels of vertical progress of Septoria brown spot due to fungicide applications for both the rating of vertical progress at R6 and the AUDPCs calculated from V7 to R6. Most of the variance for vertical progress was due to the location, again pointing out the large environmental effect on the development of this disease. The analysis of the AUDPC data adjudicated a lower percent of the variance to residual and a larger F value than the stand-alone rating at R6, thus the AUDPC was a more robust measurement of disease. Strong and significant correlations of the AUDPC of vertical progress with AUDPCs of necrotic area, chlorotic area, and defoliation rate suggested that vertical progress could be sufficient for evaluation of Septoria brown spot.

Percent of diseased leaf area (DLA) is one of the standard methods used to evaluate brown spot severity (Young & Ross 1979; Lim 1979, 1980). To further our understanding of the symptom relationships in this disease, I chose to separate the percent of diseased leaf area into two components: percent of chlorotic and necrotic areas. These two symptoms had some similarities in their development in that both remained low (< 20% for the chlorotic area and < 10% for the necrotic area) through the season but spiked at the last rating ('beginning maturity' or R7). At this time (R7), the chlorotic area reached > 70% on the control plots and the necrotic area was > 15% in two of the three locations. Pataky & Lim (1981a) found that DLA reached 50-60% at the R6 stage, but Kamicker & Lim 1985 found DLA between 0 and 20% at R6. Cruz et al. 2010 used image analysis software and reported DLA reaching < 20% in both the lower and middle canopies at the reproductive stage. These reports highlight the variability of these traits for Septoria brown spot. In this study, at R7 there were significant differences between

treatments for the percent of chlorotic area, but these differences were detectable only for the AUDPC of the percent of necrotic area. Again, the AUDPC data produced a lower percentage of variances adjudicated to the residuals and higher F values than the single point ratings. For both the single point ratings and the AUDPCs, however, large percentages of variance were assigned to the residual or the location by treatment interaction (Table 2.2), showing how variably these symptoms are expressed by soybeans infected by the pathogen.

Premature defoliation is another symptom associated with this disease (Schuh & Adamowicz 1993). In my experiments, this was the least consistent trait among locations showing a linear increase in Monmouth that started at V9 and reached > 80% at R7 on the not treated control. At the other two locations, the defoliation remained low for most of the season and had a final spike at R7 when it also reached > 80%. Pataky & Lim (1981a) reported defoliation levels of 30% at R6, a value similar to my observations in Urbana. The final defoliation rating at R7 showed no significant effect for the fungicide treatments across locations, but the AUDPC from R1 to R7 did (Table 2.1). Similar to vertical progress, most of the variance for defoliation was explained by location. Percent defoliation was correlated strongly with the other disease traits: vertical progress, necrotic area, and chlorotic area. The significant differences observed for the AUDPC of defoliation across three field locations showed that fungicide treatments indeed prevented defoliation in the presence of Septoria brown spot.

To relate the yield crop loss to varying levels of disease, I applied fungicide treatments. Fungicide application was essential to obtain different levels of disease as there were no differences between the not inoculated controls with the not treated controls. Showing the high prevalence of this disease in Illinois, I found a moderate negative correlation between yield and

AUDPC of vertical progress (r = -0.45), weak but significant correlations between yield and vertical progress at R6 (r = -0.28), and yield with the AUDPC of chlorotic area (r = -0.30) (Table 3). The stepwise regression analysis identified two disease components that were significantly associated with yield: vertical progress at R6 and the AUDPC of vertical progress. For vertical progress at R6, the single point linear model explained 8% of the variation. For the AUDPC of vertical progress, the model explained 20% of the variation. Although the regression coefficients are low, the model suggests a 10%, 17%, and 27% yield loss when the vertical progress of Septoria brown spot at R6 reached the lower (30% vertical progress), middle (50% vertical progress), and upper canopies (80% vertical progress), respectively. In other words, for each increase in the vertical progress of Septoria brown spot at R6 by 10%, there was a decrease in the yield of 142.1 kg ha^{-1} (2 bu ac^{-1} or approximately 3.4%) (Figure 2.3). These yield loss predictions are in line with previous empirical measurements of yield loss due to the disease, and for the first time, I estimate different yield responses depending on the levels of vertical progress. Cruz et al. (2010) reported that the relationship between disease severity and yield had an R^2 ranging from 0.03 to 0.1 in Ohio in non-inoculated fields. Young & Ross (1979) found a negative correlation (r = -0.69) between yield and disease severity in one out of two years of field trials. Lim (1980) reported regression coefficients of linear models between disease severity and yield components to be between 0.10 and 0.42 while the AUDPC and yield components obtained regression coefficients between 0.03 and 0.27. All these results show a clear relationship of Septoria brown spot and yield reduction of soybeans. As I discussed above, however, this disease is influenced heavily by the environment.

In my data, the AUDPC of vertical progress was the best predictor for yield. Single visual ratings such as the vertical progress at R6 are more likely to be influenced by small errors than a

rating that is the product of several measurements throughout the season. Furthermore, the AUDPC is a representation of the epidemic development thus a better way to relate disease with yield. It is also possible that R6 is not the critical time for the effect of this disease on yield, and thus another reason that the AUDPC better reflects yield variations. From a practical point of view, however, evaluating the disease weekly is a significant investment of time and effort. Further investigations on the critical times for Septoria brown spot effects on soybean yield are warranted.

Although significantly different levels of disease severity were obtained and disease severity was correlated with yield, no significant difference in yield among the fungicide treatments was found in this study. Two previous studies reported consistent yield responses to fungicide application. Lim (1980) found a 12-34% yield increase between fungicide-treated and inoculated plots and an 8% yield increase between fungicide-treated and naturally infected plots in a two-year experiment. Cooper (1989) reported a 2.2% to 15.4% yield increase in the fungicide-treated plots in a 3-year experiment. However, other studies obtained a yield response only in one location/site or no yield response across any locations. Young & Ross 1979 reported a yield reduction of 13.4 to 17.8% between control and inoculated plots in one of two years of their study. Pataky & Lim (1981a) reported a 16% (30 mg) reduction of 100-seed weight in the inoculated plots only in one year out of two tested. Kamicker & Lim (1985) reported no yield reduction in inoculated plots in a two-year experiment. Cruz et al. (2010) reported that the yield increased between 183.7 to 490 kg ha^{-1} (~4-10% increase) with the application of fungicides in three of six locations/years. The inconsistent results among the studies may be due to low disease pressure (Paul et al. 2011), late appearance of the disease (Kamicker & Lim 1985; Henry et al. 2011), or insufficient statistical power for the experimental design (Kandel *et al.*, 2018).

Evidently, even three fungicide treatments are not a recommendable practice for field production, and in this study, the fungicide applications were used to obtain plots with different levels of disease.

The lack of significant effects for yield prompted us to conduct a more detailed analysis of the sources of variation and power analysis. Statistical power is the probability to detect a significant treatment effect in a study. Small-plots trials are commonly used since they are easier to manage (e.g., disease evaluation and sample collection). Kandel et al. (2018) compared the yield response between small-plots trials and on-farm replicated strip trials (without inoculation of any disease) and found that the trial type was not significantly different. However, Kandel et al. (2018) point out the importance of the statistical power in small-plots trials when the disease pressure is low. The results from my power analysis indicated that we needed between 8 to 13 environments to detect 269 to 403.6 kg ha^{-1} (7-10.5%) yield reduction effects from Septoria brown spot. My results agree with those of Kandel et al. (2018) in that a significant yield difference below 134-201 kg ha^{-1} (2-3 bu ac^{-1}) in small-plot trials was difficult to detect. Thus, it is apparent that my experiment was underpowered for the detection of differences between fungicide treatments.

In general, I found that vertical progress was the best trait to identify differences in the development of Septoria brown spot in soybeans. My results show that brown spot of soybean plays a role in limiting yield, but the yield response is highly related to location, which varies due to weather conditions. I estimated the levels of yield loss to be 10% when the disease is present in the lower canopy, 17% when the disease is present in the middle canopy and 27% when it reaches the upper canopy. While fungicide treatments were effective in producing different levels of disease, I did not detect significant differences in yield. Future studies should

be conducted to identify if a critical time for yield effects exists, and to define accurate thresholds for the timing of fungicide application in Illinois. For the latter, a minimum of eight different environments should be included to increase the chances of detecting yield differences in small plots.

CHAPTER 2 Tables and Figures

Table 2.1 Analysis of variance for the effect of fungicide treatments on multiple components of the severity of Septoria brown spot of soybean at the end of the season and their AUDPC values in Illinois.

Septoria brown spot severity component	F value	*p*-value
Vertical progress (R6)	7.67	0.0080*
AUDPC of vertical progress (V7 to R6)	17.81	0.0005*
Chlorotic area (R7)	4.47	0.0044*
AUDPC of chlorotic area (V7 to R7)	7.99	0.0086*
Necrotic area (R7)	1.99	0.1148
AUDPC of necrotic area (V7 to R7)	9.58	0.0047*
Defoliation rate (R7)	2.57	0.1196
AUDPC of defoliation rate (R1 to R7)	5.87	0.0166*

Table 2.2 Variance components for the response of Septoria brown spot of soybean to fungicide application, for disease and yield components evaluated at three locations in Illinois.

Disease or yield component	Location	Block (Location)	Location x treatment	Residual
Vertical progress (AUDPC)	78%	3%	0%	18%
Vertical progress (R6)	15%	2%	5%	78%
Chlorotic area (AUDPC)	0%	0%	52%	48%
Chlorotic area (R7)	23%	0%	5%	72%
Necrotic area (AUDPC)	33%	0%	35%	31%
Defoliation rate (AUDPC)	75%	0%	10%	15%
Yield	79%	0%	6%	16%
100-seed weight	75%	8%	8%	9%
Pods per plant	27%	17%	5%	51%
Seeds per plant	40%	17%	0%	43%

Table 2.3 Pearson correlation coefficients (top) and *p*-values (bottom) for selected components of Septoria brown spot of soybean, 100-seed weight, yield, and yield response for combined data from three locations in Illinois.

	Vertical Progress		Chlorotic area			
	AUDPC	R6	AUDPC	R7	100-SW[a]	Yield response
Yield	-0.45	-0.28	-0.31	0.08	-0.49	0.19
	<0.001	0.034	0.0232	0.546	<0.001	0.220
Vertical Progress | AUDPC	-	0.85	0.74	0.11	0.34	-0.03
	-	<0.001	<0.0001	0.43	0.009	0.871
Vertical progress | R6	-	-	0.62	0.21	0.12	-0.05
	-	-	<0.001	0.128	0.371	0.768
Chlorotic area | AUDPC	-	-	-	0.66	0.03	-0.1
	-	-	-	<0.001	0.804	0.561
Chlorotic area | R7	-	-	-	-	-0.38	0.03
	-	-	-	-	0.005	0.838
100-SW[a]	-	-	-	-	-	0.03
	-	-	-	-	-	0.859

[a] Weight of 100 seeds

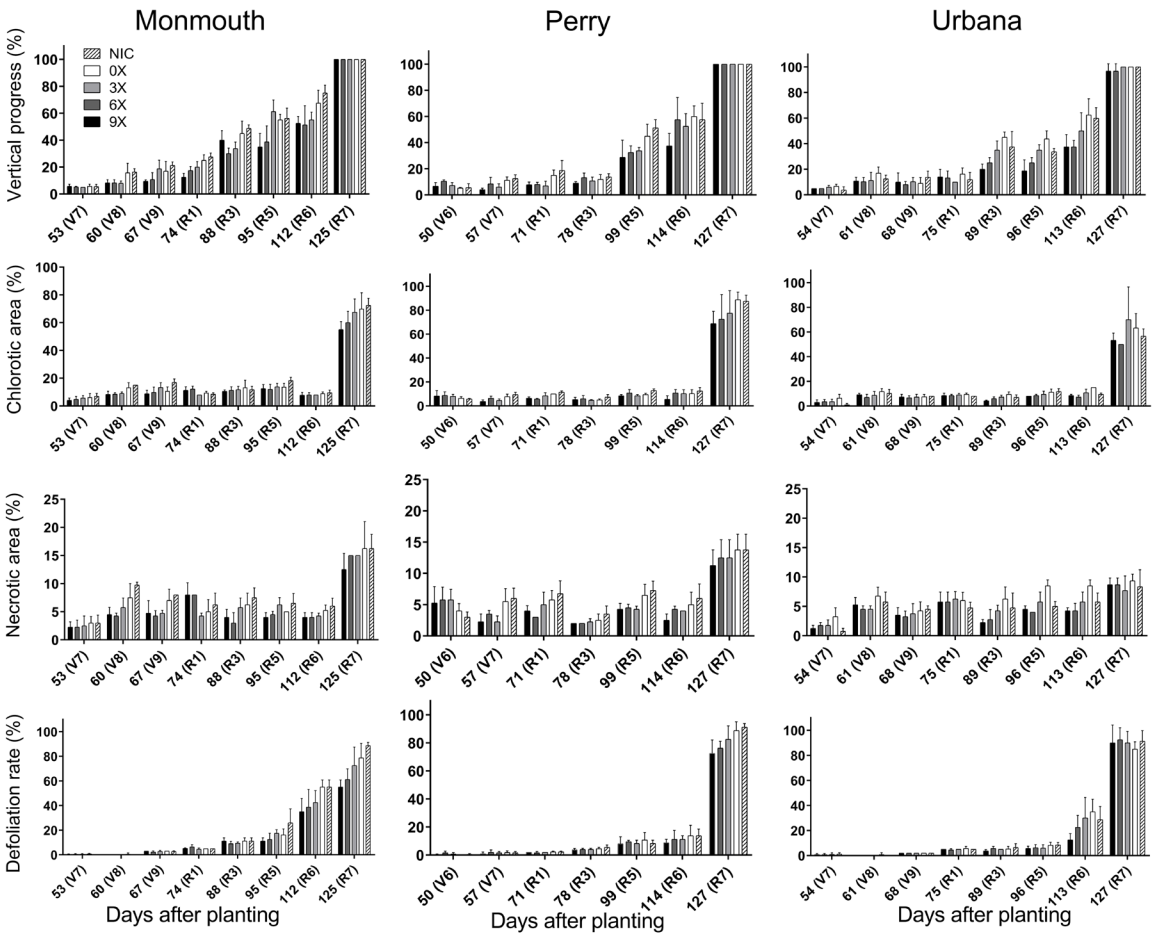


Figure 2.1 Development of Septoria brown spot of soybean in three locations in Illinois. Five treatments were applied: NIC means not-inoculated control; 0X is no fungicide application; 3X means three applications of chlorothalonil; 6X means six applications of chlorothalonil; 9X means nine applications of chlorothalonil. The per cent of four disease components (y-axes) were evaluated through the season. Bars are the standard error.

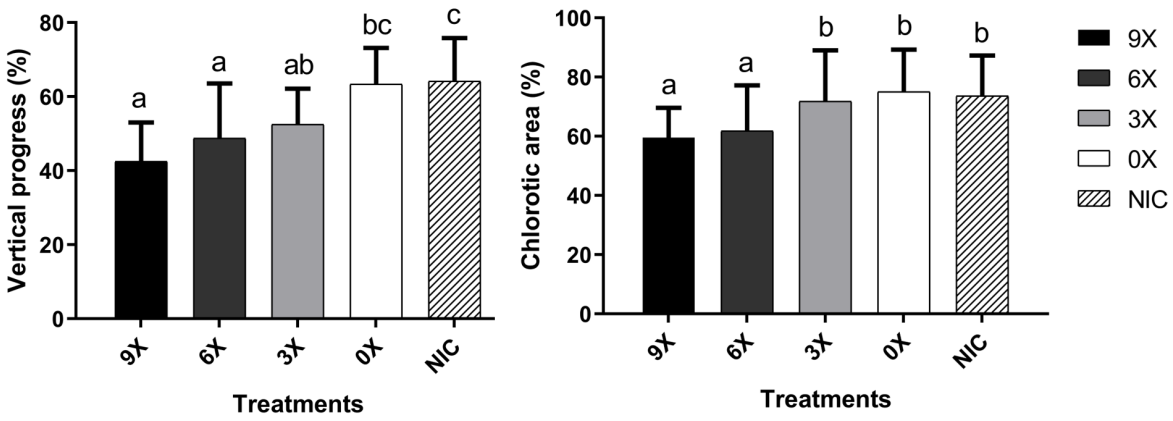


Figure 2.2 Mean values of vertical progress at R6 and chlorotic area at R7 caused by Septoria brown spot of soybean with five treatments and three locations in Illinois. Error bars are standard errors. Bars with the same letters indicate no significant difference according to Fisher's Least Significant Difference (LSD) pairwise comparisons ($p < 0.05$).

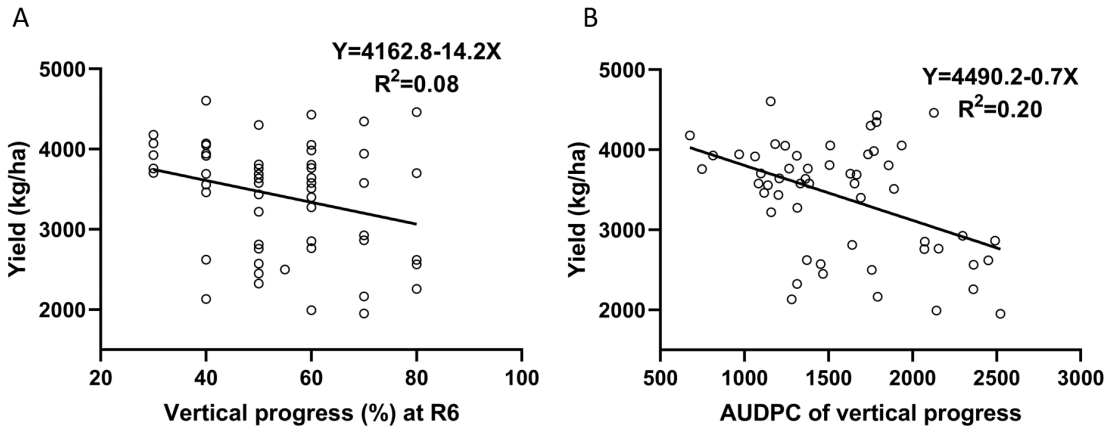


Figure 2.3 Linear relationships between two components of Septoria brown spot of soybean severity and yield. (a) Percent of vertical progress at R6. (b) AUDPC (V7 to R6) of vertical progress. Trials were held at three locations in Illinois.

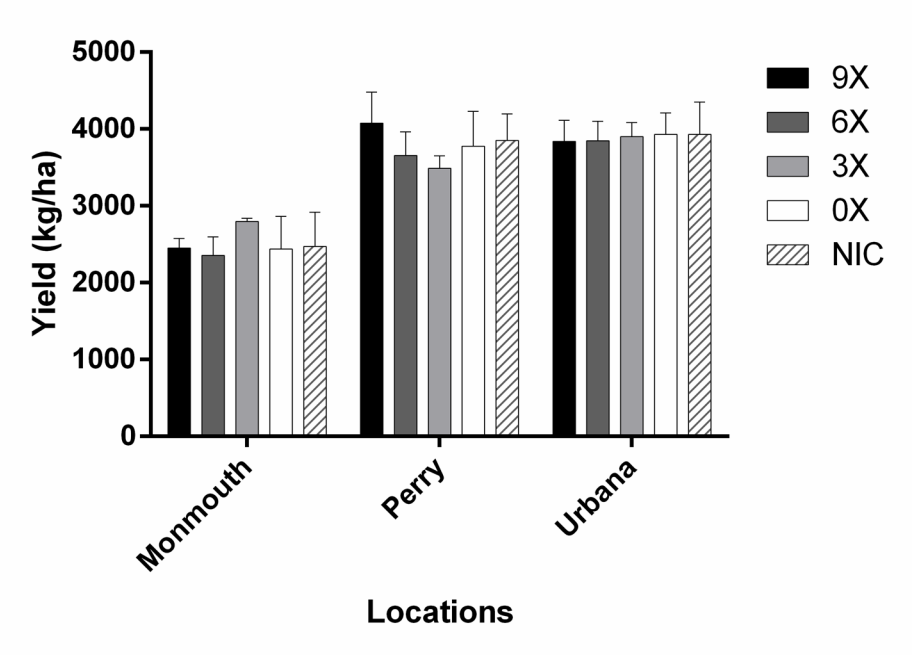


Figure 2.4 Mean yield (kg ha^{-1}) of soybean field trials inoculated with *Septoria glycines* and treated with fungicide sprays 9 times (9X), 6 times (6X) and 3 times (3X), or not-sprayed (0X) or not- inoculated control (NIC), at three locations in Illinois. Bars show the standard error. There were no significant differences between treatments ($\alpha = 0.05$).

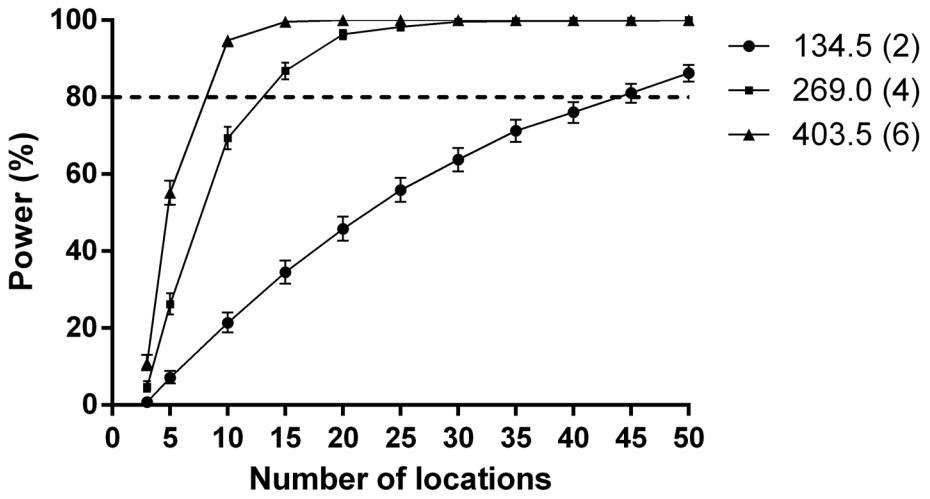
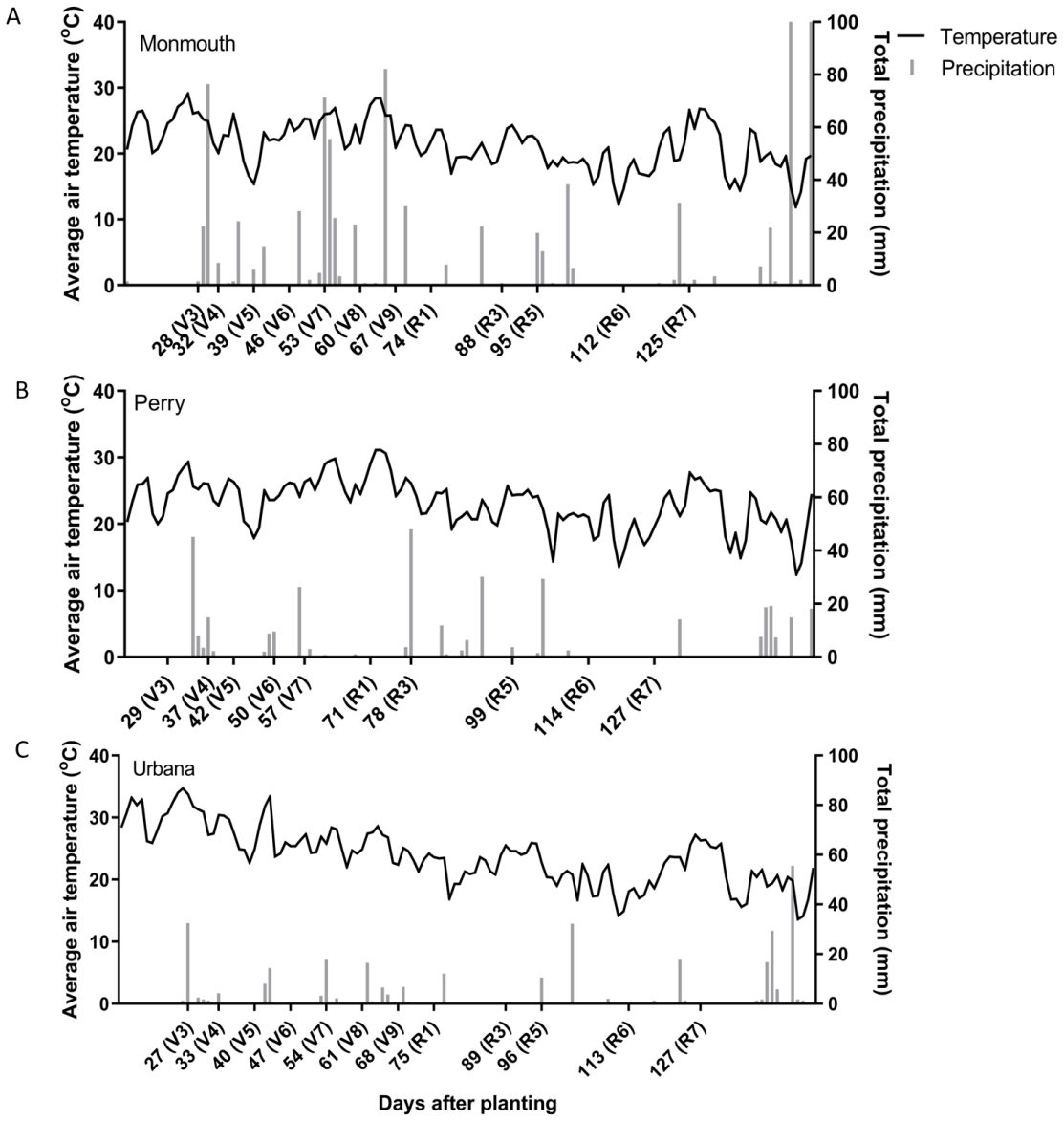


Figure 2.5 Power curves simulated based on my data for the required number of locations to detect statistically significant yield response (80% power) for treatment differences (effect) of 134.5 (2), 269.0 (4) and 403.5 (6) kg ha^{-1} (bu ac^{-1}), in experiments with soybean inoculated with *Septoria glycines* in Illinois.

Supplementary Table 2.1 Average temperature and total precipitation for different soybean growth stages at three locations in 2017 in Illinois.

Monmouth			Perry			Urbana		
Stage	Average Temp (℃)	Total precipitation (mm)	Stage	Average Temp (℃).	Total precipitation (mm)	Stage	Average Temp (℃).	Total precipitation (mm)
V3-V4	24.5	39.9	V3-V4	26.5	56.1	V3-V4	30.4	37.1
V4-V5	21.4	13.7	V4-V5	24.8	16.8	V4-V5	27.2	4.6
V5-V6	20.8	8.1	V5-V6	22.2	10.7	V5-V6	27.6	22.1
V6-V7	24.4	13.7	V6-V7	25.0	35.6	V6-V7	25.7	3.1
V7-V8	24.2	71.1	V7-R1	26.6	4.1	V7-R1	25.4	19.6
V8-V9	26.0	33.3				V8-V9	26.5	27.2
V9-R1	21.9	11.9				V9-R1	23.4	7.1
Vegetative stage	23.3	191.7	Vegetative stage	25.02	123.3	Vegetative stage	26.6	120.8
R1-R3	20.3	11.9	R1-R3	28.3	3.8	R1-R3	21.6	12.4
R3-R5	22.7	0.3	R3-R5	22.5	98.8	R3-R5	25.0	1.0
R5-R6	18.2	31.0	R5-R6	22.0	36.6	R5-R5	19.4	44.2
R6-R7	19.0	13.7	R6-R7	18.8	0.0	R6-R7	21.3	19.6
Reproductive stage	20.05	56.9	Reproductive stage	22.9	139.2	Reproductive stage	21.825	77.2
All growing season	22.1	248.6	All growing season	24.1	262.5	All growing season	24.9	198.0



Supplementary Figure 2.1 Average temperature and precipitation for different soybean growth stages at three locations in 2017 in Illinois.

CHAPTER 3: ACCURATE QUANTIFICATION AND DETECTION OF SEPTORIA GLYCINES IN SOYBEAN USING QUANTITATIVE PCR[1]

3.1 Abstract

Septoria brown spot, caused by *Septoria glycines*, is a highly prevalent foliar disease of soybean in the United States. Accurately identifying and quantifying the pathogen in soybean can provide valuable information for disease management. In this study, I describe the development and validation of a quantitative PCR (qPCR) method to detect and quantify *S. glycines* accurately. Three sets of primers and assays were designed based on the polymorphic regions on the β-tubulin, the calmodulin, and the actin genes of *S. glycines*. Assays designed with the actin gene (Ac) were specific to *S. glycines* for both conventional PCR and qPCR. The assay designed with the β-tubulin (Bt) gene was specific to *S. glycines* only on the qPCR. The qPCR reaction efficiency of the Ac and Bt assays was 95 % and 98 %, respectively. The sensitivity of both Ac and Bt assays was 10 pg of *S. glycines* gDNA. The Bt assay was validated with field samples that had different necrotic areas. Symptoms of necrosis ranging from 0 to 30 % were significant and positively correlated (r = 0.87) to the *S. glycines* gDNA. The *S. glycines* gDNA was detected as early as 1-day post-inoculation in detached leaf assays. I expect that the assays reported here could be used for disease diagnosis and to better characterize the infection process of *S. glycines*.

[1] Part of this chapter has been published in Lin, H.-A., and Mideros, S. X. 2021. Accurate quantification and detection of Septoria glycines in soybean using quantitative PCR. Curr. Plant Biol. 25:100-192.

3.2 Introduction

Septoria brown spot, caused by *Septoria glycines* Hemmi, is a highly prevalent foliar disease in the United States (Allen *et al.*, 2017) and other soybean (*Glycine max*) production areas, such as Argentina, Brazil, and China (Hartman *et al.*, 2015b). The estimated yield losses in the northern United States between 2010 to 2014 ranged from 173,625 to 688,786 metric tons each year (Allen *et al.*, 2017). In Illinois, Septoria brown spot is the most predominant soybean disease (Hobbs *et al.*, 2010). I have previously shown that when the symptoms of the disease reach 30% vertical progress of the plant at the R6 physiological state there is a 10% yield loss, but if the symptoms reach 80% vertical progress then the there is a 27% yield loss (Lin *et al.*, 2020). This disease limits the yield by causing premature defoliation and reducing seed weight when severe infection occurs (Pataky & Lim 1981; Young & Ross 1979).

The typical symptoms of Septoria brown spot are dark and irregular spots surrounded by chlorosis on the leaves. The pathogen infects the plant through stomata without forming an appressorium (MacNeill & Zalasky 1957). It can also infect pods and seeds, but the pathogen is rarely seed-borne (MacNeill & Zalasky 1957; Hartman et al. 2015). In the field, symptoms on leaves can be observed as early as V2 to V3 stage (Fehr et al. 1971; Mueller et al. 2016), and the disease gradually develops to the upper canopy throughout the growing season (Lin *et al.*, 2020). The incubation period (the time between infection to showing visible symptoms) of *S. glycines* has been reported to vary depending on host maturity (Young & Ross 1979).

This disease often co-occurs with other foliar diseases. In the early season, the symptoms might be confounded with bacterial blight (*Pseudomonas syringae* pv. *glycinea*) (Basu & Butler 1988; Williams & Nyvall 1980). In the late-season, Septoria brown spot, frogeye leaf spot (*Cercospora sojina* Hara), and Cercospora leaf blight (*Cercospora kikuchii* (Mat and Tom)

Gardner) have been considered to be a "disease complex" (Carmona *et al.*, 2011) because they often occur together. To properly design a management strategy for Septoria brown spot, it is imperative to detect and quantify the pathogen accurately.

Management of Septoria brown spot relies on foliar fungicides applied during the reproductive stage (Cruz *et al.*, 2010). However, yield responses from fungicide applications vary among years and locations (Cruz *et al.,* 2010; Lin *et al.,* 2020; Pataky & Lim, 1981; Cooper, 1989). The inconsistent yield improvement after fungicide application is due to the late appearance of the disease (Kamicker & Lim, 1985; Henry *et al.,* 2011), low disease pressure (Paul *et al.*, 2011), or lack of statistical power in small field trials (Kandel *et al.*, 2018; Lin *et al.*, 2020). No genetic source of resistance to this pathogen is available in soybean (Young & Ross, 1978; Lim, 1979). Thus, management relies only on fungicide application, or the disease is assumed to be a minor concern.

Despite the prevalence of the disease and the proven although non-devastating yield effects, little is known about the infection and colonization of the pathogen on soybeans, which is indispensable to model and predict disease development. To accurately diagnose and quantify the levels of colonization of *S. glycines*, a sensitive and specific molecular-based tool would be helpful. Molecular technologies have been intensively used for detection, quantification, and diagnosis of plant pathogens. Quantitative polymerase chain reaction (qPCR) is a well-developed technique to detect and quantify the target sequences. This technology provides a fast and high-throughput approach, with a wide dynamic range of quantification (Kralik & Ricchi, 2017). Many qPCR assays have been developed for early detection, diagnosis, and quantification for soybean pathogens such as *Fusarium virguliforme* O'Donnell & T. Aoki (Sudden death syndrome) (Wang *et al.*, 2014), *Sclerotinia sclerotiorum* (Lib.) de Bary (Sclerotinia stem rot)

(Botelho *et al.*, 2015), *Phialophora gregata* f. sp. *sojae* (Allington & Chamberlain) Gams (Brown stem rot) (Malvick &Impullitti, 2007), *Phakopsora pachyrhizi* Syd. (soybean rust) (Frederick *et al.*, 2002), and *Cercospora kikuchii* (Tak. Matsumoto & Tomoy.) M. W. Gardner (Cercospora leaf blight and purple seed stain) (Chanda *et al.*, 2014). To my best knowledge, no qPCR protocol has been reported for *S. glycines*.

I hypothesize that this disease has a different length of infection periods that are associated with the host developmental stages. Soybean may have adult plant resistance or partial resistance against this pathogen. Thus, the objectives of this study were to (i) design a qPCR protocol for *S. glycines*; (ii) validate the qPCR protocol by comparing it to the standard disease evaluation methods from field and greenhouse samples; (iii) correlate *S. glycines* biomass estimated by qPCR to visual disease symptoms; and (iv) characterize the incubation period and latent period of this disease.

3.3 Materials and Methods

3.3.1 Fungal isolates

Diseased soybean leaves were collected from the field in 2016. The leaves were immersed in 75% EtOH for 30 s, 5% bleach for 5 min, and rinsed three times with sterilized dH_2O. The leaves were then placed in a humidity chamber to induce sporulation. The pycnidia were transferred to 3% malt extract agar (MEA) with 50 ppm penicillin and streptomycin (Verkley *et al.*, 2013) and incubated at 24-25℃, 12/12 hour light/dark period. Hyphal tips of germinated colonies were then transferred to 3% MEA without fungicide. All the isolates were preserved in 20% glycerol at -80℃. Internal transcribed spacer (ITS) sequence of these isolates was amplified with ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') primers (White *et al.*, 1990) and sequenced for

identification. In total, seven out of 26 collected *S. glycines* isolates were used in this study. Two *Colletotrichum gloeosporioides* isolates (C001 and C002) were isolated in the field in 2016 following the same procedure mentioned above. Four *Cercospora sojina* (CS1303-3, CS1355-1, CS1334-3, and CS1321-2) and three *S. glycines* (R3101, R3216, and ATCC 38699) were acquired from Dr. Carl Bradley (University of Kentucky) and used in this study as controls.

3.3.2 Sample preparation and DNA extraction

The genomic DNA of fungal isolates and soybean leaves was extracted by following a modified CTAB method (Chung *et al.*, 2010). The *S. glycines* isolates were cultured in 3% malt extract (ME) liquid medium for 3 to 5 days. Fungal hyphae were collected through sterilized filter papers on a Büchner funnel connected to a vacuum suction system. The hyphae were then lyophilized for three days and stored at -20℃. Isolates of other fungal species were cultured on PDA following the same procedures. Leaf samples collected from the field were rinsed under tap water to remove the soil on the leaf surface, then lyophilized for three days and stored at -20℃. A total of 10 mg of lyophilized hyphae or leaf tissue were placed in a 2 mL FastPrep® tube containing a 6.35 mm ceramic bead, and were then homogenized in a FastPrep®-24 Classic Instrument (MP BIOMEDICALS, USA) for 60 s, speed 5.5. Then 500 µL CTAB buffer (with 0.2% (v/v) of 2-mercaptoethanol) was added to each microcentrifuge tube and incubated at 60℃ for 20 min. After cooling the samples on ice for 10 min, they were centrifuged at 13.8 G for 15 min. The supernatant was transferred to a new tube. A total of 400 µL CIA [phenol: chloroform: isoamyl alcohol= 25: 24: 1] was added to the supernatant, inverted for 3 to 5 min and centrifuged at 9.6 G for 12 min. The supernatant was then transferred to a new tube. This step was repeated twice to obtain high-quality DNA. A total of 300 µL isopropanol was added to the supernatant and stored at -20℃ for at least two hours. For DNA precipitation, samples were centrifuged at

13.8 G for 10 min. After decanting the supernatant, the pellet was sequentially washed with 300 µL 70% EtOH and 100% EtOH, followed by centrifugation for 5 min and 3 min, respectively. To remove excess polysaccharide, 250 µL of Tris-EDTA [10 mM Tris-HCl, 1 mM EDTA, 1.5 M NaCl, PH 8.0] was used to dissolve the pellet (Fang *et al.*, 1992) and then centrifuged at 2.6 G for 5 min. The supernatant was transferred to a new tube. Then, 500 µL of 100% EtOH was added to the tube, and the tube was stored at -20°C overnight. The DNA precipitation steps were the same as mentioned above. Finally, a total of 30 µL of nuclease-free water (with 0.5% 10mg/mL RNase) was added to dissolve the air-dried pellet. The extracted DNA was visualized by electrophoresis on a 2% agarose gel (containing 0.01% of GelRed (Phenix, USA)) in 1X TAE buffer for 1 hour at 90 V, following analysis on a NanoDrop spectrophotometer (Thermo Scientific™, USA). All DNA samples were stored at -20°C.

3.3.3 Probe and primer design

Specific primers (Table 2.1) were designed based on the β-tubulin (DQ026384.1), calmodulin (KF254081.1), and actin (KF253733.1) gene sequences of *S. glycines* obtained from Genbank. Polymorphic regions were identified by aligning the target sequences with those of other pathogens of field crops, including *Zymoseptoria tritici* and *Parastagonospora nodorum,* as well as common foliar pathogens of soybean, including *Colletotrichum truncatum, Cercospora kikuchii, Cercospora sojina, Phakopsora pachyrhizi, Microsphaera diffusa,* and *Alternaria solani*. The primers and probes were designed in Primer3 [30]. Annealing temperatures (Tm) for the designed primers ranged from 59°C to 61°C. For the probes, the Tm ranged from 68°C to 70°C. The product size was set between 90 to 100 base pairs. Other parameters were set as the default. The primers specificity was tested on DNA from pure cultures first by gel electrophoresis and then by sequencing the PCR amplicons. The sequences were

aligned to the NR database of National Center for Biotechnology Information (NCBI) (O'Leary *et al.*, 2015) using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990).

3.3.4 Conventional PCR assay

Conventional PCR was performed on an Eppendorf Thermo Cycler Eco (Eppendorf, USA) with a total reaction volume of 25 µL. Each reaction included 10 µL of Gotaq Green master mix (Promega, USA), 1 µL of forward and reverse primer (10 µM), 3 µL of DNA template (25 ng/µL) and 7.5 µL of dH_2O. The PCR program was: 94℃ for 2 min; followed by 35 cycles of 94℃ for 15 s, 60℃ for 60 s and 72℃ for 30 s; the final extension step for 72℃, 60 s. The PCR products were visualized by electrophoresis on a 2% agarose gel (containing 0.01% of GelRed) in 1X TAE buffer for 1 hour at 90 V.

3.3.5 Quantitative PCR assay

The qPCR assay was performed on an ABI Prism7000 sequence detection system (Applied Biosystems, Foster City, CA) with a total reaction volume of 20 µL. Each reaction included 10 µL of PerfeCTa FastMix II (QuantaBio, Beverly City, MA); 0.1 µL of forward primer, reverse primer and TaqMan probe (10 µM); 5 µL of DNA template (25 ng/µL for pure fungal isolates, 100 ng/µL for leaf samples) and 4.7 µL of dH_2O. The amplification program was: 50℃ for 120 s, 95℃ for 10 min and followed by 40 cycles of 95℃ for 15 s; and then 60℃ for 60 s. Pure *S. glycines* DNA (extracted from strain ATCC 38699) was 10-fold serially diluted from 100000 pg to 10 pg to build the standard curve. Primer efficiency (E%) was calculated with the formula:

$E\% = (-1+10^{-\frac{1}{slope}}) \times 100\%$. Three technical replicates were conducted for each sample.

3.3.6 Plot level field samples for qPCR validation

A field experiment was conducted in Urbana in 2018. The experimental design was a split-plot design with three replicates. Two treatments (inoculated and control) were set as the main plot and three soybean lines were set as subplots. On the inoculated plots, the plants from center of two rows were treated with a spore suspension at a concentration of 10^6 spores/mL and (0.05% Tween 20) between V3 to V4 stages. The inoculation was carried out with a Suprema bak-pak sprayer (Hudson Manufacturing Company, USA). Spores of three isolates (16S012, 16S006, and R3216) were produced as previously described (Lin *et al.*, 2020). The control plots were not inoculated and were instead sprayed with fluxapyroxad and pyraclostrobin (Priaxor, BASF, Research Triangle Park, NC) at the R2-R3 stage following label directions. The application of fungicide was carried out with a CO_2-pressurized backpack sprayer equipped with a 0.48 m 601B-SST - four nozzle light weight boom (R&D Sprayers Bellspray, Inc, USA) and four TJ60-11008 (50) nozzles (TeeJet Technology, USA).

On the subplots, the soybean lines Williams, LD12-8677, and LD13-14071R2 were planted at 348,480 seeds/hectare. Each subplot contained four 5.2 m rows with 76.2 cm alleys.

The foliage of ten plants was collected from each subplot at V4-R1 stage (one week after inoculation), R2-R3 (one week after fungicide application), and R5 stage. At the same time as sample collections, the necrotic area, chlorotic area, vertical progress, and defoliation rate were evaluated as a percentage for each plant. All the leaves from ten plants were pooled, lyophilized, ground, and stored at -20°C for DNA extraction and qPCR quantification as indicated above. Three technical replicates of the qPCR were performed for each sample. The quantity of *S. glycines* gDNA for each technical replicate was calculated using a standard curve created from control DNA included in each plate.

3.3.7 Trifoliate level field samples for qPCR validation

Leaf samples with different levels of visually estimated necrotic area (0%, 5-10%, 10-20%, and 20-30%) were collected from the inoculated plot of Williams at the R2-R3 stage from the experiment indicated above. Four sets of samples were collected, and each set was collected on the same day.

A digital image of each one of the leaves was captured using an Epson Expression 10000XL scanner (Epson, USA) and stored as a TIF file. Necrotic area of each trifoliate was then calculated using APS ASSESS 2.0 image analysis software (The American Phytopathological Society, St. Paul, MN). In Assess 2.0, RGB files were transformed into hue-saturation-intensity files (HSI). The HSI image was then used for disease quantification. The disease quantification in Assess 2.0 is conducted by differentiating the hue between lesions (yellow, red, and brown) and healthy tissue (green) (Lamari, 2008). After image analysis, each trifoliate was lyophilized and stored at -20℃ for DNA extraction and qPCR quantification.

3.3.8 Detached leaf experiments for qPCR validation

A detached leaf experiment was performed in 2019. Soybean line Williams was grown in the greenhouse to produce leaves. Briefly, the surface of the seeds were disinfected with 0.05% NaClO for 30 seconds and washed with distilled water a total of three times. Five to six seeds were planted in 24 cm diameter pots with a steam sterilized mix of soil:sand:perlite=1:1:1 v/v. Seven grams of 13-13-13 Osmocote were added to each pot when planting the seed. The greenhouse temperature settings were set to 23 – 25℃ with 10/14 hours of dark/light period. Plantings were repeated at three week intervals to generate samples of different leaf age. The soybean leaves were collected from the lower canopy at V3 and R1 stage. The detached leaves were disinfected with 75% EtOH and 0.05% NaClO for 30 s, and then washed with distilled

water three times. Then, the leaves were placed into a plastic tray and inoculated with a MAS KIT-VC16-B22 portable mini airbrush air compressor kit (Master Airbrush, USA) with 10^5 spores/mL of spore suspension (0.05% Tween 20) collected from the 16S012 *S. glycines* isolate. The control group was sprayed with sterilized water. Sterilized wet paper towels were placed on the bottom of the trays to maintain humidity. After inoculation, the plastic trays were covered with black plastic bags and incubated for 24 hours, then covered with transparent plastic bags to maintain high humidity. The symptoms were evaluated daily using the following rating scale: (0) no symptoms observed; (1) to (3) <5%, 5 to 25%, or >50% of small brown dot (needle-like) lesions observed, respectively; (4) typical brown spot symptoms (irregular necrotic lesion surrounded by chlorotic tissue) observed; and (5) ozing pycnidiospores observed. Leaves were collected at 1, 3, and 5 days postinoculation (dpi) for microscopy observation, and were collected at 0, 1, 3, 5, and 7 dpi for DNA extraction and qPCR quantification. The rating data were used for survival analysis.

3.3.9 Microscopy observations

Microscopy observations were performed with a modified trypan blue stain assay [34]. The leaf samples were cut into 1x1 cm^2 pieces and placed in 24-well plates. The samples were cleared in acetic acid: ethanol = 1:3 v/v solution overnight, and then acetic acid: ethanol: glycerol = 1:5:1 v/v/v, for at least three hours. The samples were then submerged in staining solution (0.01% trypan blue in lactophenol) overnight. To remove excess trypan blue, the samples were washed with lactophenol and stored in 60% glycerol until examination. The samples were observed with Leica DMLS Binocular Microscope (Leica Microsystems Inc, USA). The photographs were captured by ZEISS Axiocam ERc 5s microscope camera and edited with the ZEN3.0 software (Zeiss, Oberkochen, Germany).

3.3.10 Data analysis

The area under the disease progress curve (AUDPC) values for the vertical progress (VP), necrotic area (N), chlorotic area (C), and defoliation rate (D) were calculated using the AUDPC function in the Agricolae package (Mendiburu, 2010) in R 3.6.1 (R Core Team 2018). To test if there were differences between the treatments, linear mixed models were fit using PROC MIXED in SAS v9.4 (SAS Institute Inc., Cary, NC). In the split-plot model for the analysis of plot level disease in the field experiment:

$$y_{ijk} = \mu + B_i + C_j + \varepsilon_{ij} + T_k + CT_{jk} + \varepsilon_{ijk}$$

y_{ij} was the disease data (VP, N, C, and D) corresponding to the i^{th} block, j^{th} cultivar, and k^{th} treatment. μ was the grand population mean, B_i was the random block effect, C_j was the fixed term for cultivars, T_k was the fixed treatment effect, and CT_{jk} was the fixed interaction term for j^{th} cultivar and k^{th} treatment. ε_{ij} and ε_{ijk} were random error terms [NID$(0, \sigma_e^2)$]. Correlation analysis and linear regression analysis were performed in SAS v9.4 with PROC CORR and PROC REG. Figures were generated in GraphPad Prism Version 6.0 for Windows (GraphPad Software, La Jolla, CA).

3.3.11 Survival analysis

To determine the length of the incubation period (IP) and latent period (LP) of the Septoria brown spot, the rating data of the detached leaf experiment were used for this analysis. The detached leaf inoculations were repeated four times for V3 stage samples and three times for R1 stage samples. Leaves with rating scores less than 4 on the last day of evaluation were excluded from the survival analysis. A total of 35 V3 stage leaves (18 leaves from LD13-14071R2 and 17 leaves from Williams) and 22 of R1 leaves (12 leaves from LD13-14071R2 and 10 leaves from

Williams) were used for survival analysis. The survival analysis was performed with "survival" package (Therneau, 2015) in R 3.6.1 (R Core Team 2011). The event of interest was defined as disease scale from 1 to 5, respectively. Scale 4 indicate the incubation period and scale 5 indicate the latent period. The median of incubation period and latent period were produced by utilizing the Kaplan-Meier method with "survfit" function. The log rank test was performed with "survdiff" function to compare the survival curves.

3.4 Results

3.4.1 Specificity of conventional PCR assay

Primers designed from β-tubulin gene (Bt) amplified an 89 bp product. However, it also amplified a faint band in the absence of the *S. glycines* template (Figure 3.1.A). Primers designed from actin gene (Ac) amplified a 97 bp product only in the presence of the genomic DNA of *S. gly*cines (Figure 3.1.B). Primers designed from the calmodulin gene (cal) were not specific to *S. glycines* gDNA.

3.4.2 Sensitivity and specificity of qPCR assay

The designed primers and probe combinations (Ac, Bt, and Cal) were first tested with the gDNA from pure fungal isolates, healthy soybean leaves, and no-template control (NTC). The Bt and Ac primers and probe combinations amplified the product under the presence of the *S. glyci*nes gDNA. The cycle threshold (Ct) value of other non-target organisms, soybean gDNA and NTC, was at least over 35 or undetectable (Table 3.2). DNA of *S. glycines* was serially diluted in 10 folds from 10^5 to 10 pg to evaluate the sensitivity and efficiency of the primers. There was a linear relationship between Ct values and serially diluted DNA (log-transformed)

for Ac and Bt primers and probe sets (Ac: R^2=0.997; Bt: R^2=0.998) with PCR efficiency of 95 and 98%, respectively (Figure 3.2).

3.4.3 Validation of qPCR assay with field samples at the plot level

The disease was not observed in the field until R2-R3 stage at both inoculated and control treatment plots. Treatment by variety interactions were not significant except for necrotic area (p=0.043). There were significant differences between inoculated and control plots (treatments) for the AUDPC of vertical progress (p<0.0001), necrotic area (p<0.0001), chlorotic area (p<0.0001), but not for defoliation rate (p=0.0505) (Supplementary table 3.1). There were no differences on the components of disease between varieties. For all the varieties at R2-R3 and R5 stage, the vertical progress of the disease on inoculated plots was about two-fold higher than in the control plots. The necrotic area and chlorotic area remained below 5%. The defoliation rates were about 20% at the R5 stage for both treatments (Table 3.3).

A total of 48 whole-plant-samples were tested with the Bt primers and probe set. There was no *S. glycines* detected at the early vegetative stage in any of the samples. Highly variable levels of *S. glycines* gDNA were detected at the R2-R3 stage on the inoculated plots of the three cultivars (Table 3.3). One sample in the control group for line Williams detected pathogen (Ct=29.64) at R2-R3 stage. At R5 stage, the pathogen was detectable (Ct=28.29) only on the inoculated LD13-14071R2 plots. After fungicide application (R5 on control plots), the pathogen was detected in Williams control plot (Ct=37.77) but with a lower amount compared to R2-R3 stage (Ct=29.64) (Table 3.3). Overall, qPCR on the foliage of whole plants from field samples produced highly variable results with the pathogen being undetectable on at least one replicate of each treatment (Table 3.3).

3.4.4 Correlation between symptoms and pathogen gDNA at the trifoliate level

Four sets of leaf samples with different levels of necrotic area were collected in the field. The leaf samples were then scanned and analyzed with Assess 2.0 to precisely measure the symptomatic area (Figure 3.3). The quantity of *S. glycines* gDNA increased from 0.2 pg to 1000 pg as the necrotic area increased from below 5% to above 20% (Table 3.4). One outlier value in replicate three was removed for the correlation and linear regression analysis. There was a significant (p <0.0001) and strong positive correlation (r=0.87) between the necrotic area and the quantity of *S. glycines* gDNA. There was also a linear relationship between the necrotic area and quantity of *S. glycines* gDNA with adjusted R^2=0.74 (p < 0.0001) (Figure 3.4).

3.4.5 qPCR validation on detached leaves

After inoculation, the needle-like necrotic dots could be observed at 1 dpi. The typical symptoms were observed at 5 dpi, and sporulation was observed at 7 dpi (Table 3.5). Low amounts of pathogen gDNA were detected in both V3 and R1 stage leaves (cv. Williams) at 1 dpi with the Bt primers and probe set. The detected gDNA of *S. glycines* increased in the V3 leaves and reached its highest amount at 7 dpi, while the gDNA of *S. glycines* remained low in the R1 leaves (Table 3.5, Figure 3.5).

3.4.6 Microscope observations

Microscope observations were conducted on V3 leaves at 1, 3, and 5 dpi. Although the needle-like dot symptoms (<5%) were observed at 1 dpi, I did not obtain good images at this time point. At 3 dpi, spores (Figure 3.6.A), germinated spores (Figure 3.6.B), and penetration events (Figure 3.6.C) were observed. The hyphae penetrated the host through stomata without

producing an appressorium (Figure 3.6.C). At 5 dpi, branching hyphae were observed on the surface of the leaf and growing from stomate to stomate (Figure 3.6.D).

3.4.7 Survival analysis results

The log rank test indicated there were significant differences between growth stage for IP (p=0.002) and LP (p=0.0007). The median lengths of IP for V3 and R1 leaves were ranged between 6-7 and 7-10 days, while the median length of LP for V3 and R1 leaves were ranged between 7-8 days and 7.5-11 days, respectively. The overall trend showed that the lengths of IP and LP were longer in the R1 leaves than the V3 leaves. The difference between IP/LP may also depend on the soybean lines because when the data were analyzed by soybean lines, the significant difference of IP and LP between growth stage were only observed in LD13-14071R2 (p=0.001 for IP and 0.0003 for LP). Additionally, the IP (p=0.05) and LP (p=0.003) only showed significant differences between cultivars at R1 stage, not at the V3 stage (Table 3.6).

3.5 Discussion

Septoria brown spot is the most prevalent soybean foliar disease disregard in Illinois (Eathington *et al.*, 1993), and one of the most prevalent in the whole of the United States (Hartman *et al.*, 2015b). Despite the disease having been discovered and reported in the United States in the early 1900s (Wolf, 1923), little is known about the epidemiology of this disease. The pathogen often infects the plants at V2 to V3 stage and remains at the low canopy until reproductive stage (Hartman *et al.*, 2015b). The vertical progress of the disease was highly correlated with the yield losses (Lin *et al.*, 2020), and the application of fungicide during the reproductive stage helped to reduce the disease progression (Cruz *et al.*, 2010). However, fungicide application does not always result in a yield increase (Lin *et al.*, 2020; Pataky & Lim,

1981; Cruz *et al.,* 2010; Cooper, 1989). The development of specific and sensitive molecular tools for detection, quantification, and diagnosis of Septoria brown spot is necessary to manage the application of fungicide, better understand the epidemics caused by this disease as well as to characterize the interactions of this pathogen with other late-season diseases of soybean.

In this study, I developed three sets of primers and probe combinations for qPCR assay based on the polymorphic regions of actin, β-tubulin, and calmodulin genes of *S. glycines*. The primers and probe designed based on the calmodulin gene were not specific to *S. glycines*. The Ac primer set could distinguish *S. glycines* from non-target fungal pathogens and soybean gDNA with conventional PCR, which provided a low-cost tool for pathogen diagnosis and identification. As for qPCR assay, both Ac and Bt primers and probe sets were specific and sensitive to the *S. glycines* gDNA. I was able to repeatedly detect as little as 10 pg/µl of pathogen DNA while the qPCR reactions maintained over 95% efficiency.

The Bt primers and probe set were validated with a series of experiments to demonstrate the sensitivity and reproducibility for detecting and quantifying *S. glycines* in soybeans. The qPCR assay developed in this study could detect the pathogen gDNA with a visible necrotic area as low as 1-5% on the R2-R3 stage trifoliate field samples (Table 3.4, Fig.3.3.B) and as early as 1 dpi on the detached leave samples (Table 3.5). The early symptoms of Septoria brown spot are small necrotic lesions with a diameter of less than 4 mm (Hartman *et al.*, 2015b), which can be confounded with the symptoms of bacterial blight and the early infection of other foliar fungal diseases. My Bt primers and probe set is sensitive and specific for early detection and diagnosis of the disease before the typical symptoms show up.

There was a significant and positive linear relationship between brown spot necrotic area and gDNA of *S. glycines* (adjusted R^2=0.74) on the field samples at the trifoliate level (Fig. 3.4).

Interestingly, there was an outlier in this experiment and was removed from the regression analysis. That sample only had a necrotic area of 15.5%, but the necrotic area coalesced and localized in one of the leaflets. It is possible that the coalesced lesions contained more pathogen biomass and had started to produce pycnidia, thus resulting in an unusually high level of pathogen gDNA (Table 3.4). Taking the one outlier out of the analysis, the experiment demonstrated that the necrosis symptoms (from 1.4% to 28.6%) and the biomass of the pathogen are linearly related. However, the linear relationship appears to break above 30% necrosis. One possibility is that necrotic and chlorotic areas may contain a high level of polyphenol compounds and decrease the quality of extracted DNA, which influenced pathogen quantification [39]. From another point of view, under favorable microclimate conditions, if the pathogen produced pycnidia, the amounts of extractable pathogen gDNA increase dramatically. A nonlinear relationship between pathogen's gDNA and visual symptoms (disease incidence and number of pycnidia per leaf) at the late infection stage has been reported in the wheat-*Zymoseptoria tritici* pathosystem (Guo *et al.*, 2006). It has been reported that when *Z. tritici* moved from the latent phase to the necrotrophic phase, the pathogen accelerated the growth rates and formed mature pycnidia and pycnidiospores (Steinberg, 2015; Rahman *et al.*, 2020).

The amount of pathogen gDNA was difficult to quantify from the whole plant. The Bt primers and probe set were not able to consistently detect and quantify the pathogen from the plot-level field samples. A low amount (Ct >29) of the pathogen was only detected at R2-R3 stage from the inoculated plots (Table 3.3). Although the corresponding disease rating data showed that the vertical progress of the disease remained between 30% and 50% from R2 to R5 stage, the necrotic area was only around 5%. Since the soybean lines used in this study were indeterminate, the constant vegetative growth after flowering may have diluted the amount of

pathogen and made it harder to detect. I might have also lost some pathogen gDNA due to the 10 to 20% defoliation rate observed in the field. In practical terms, to reduce false negative detection results, the sampling should be focus on symptomatic soybean leaves instead of whole plants.

Rahman et al. (2020) developed a qPCR assay and reported the correlation between the accumulation of *Zymoseptoria tritici* biomass, production of pycnidia, host genotype, and duration of the latent period in wheat (Rahman *et al.*, 2020). The growth rate of *Z. tritici* in the host was reported as a good predictor for modeling the length of the latent period and the coverage of pycnidia (Rahman *et al.*, 2020). Several foliar fungal pathogens in soybean, including *Cercospora kikuchii*, *Cercospora sojina*, and *Colletotrichum* spp. have been reported to have a latent period in soybean plants with a possible range from V1 to R4/R5 or even R7 stage (Sinclair, 1991). Although the information about the latent period of *S. glycines* is unclear, it has been previously reported that the incubation period of *S. glycines* varied depending on the host maturity. Observation of potted plants suggested that the incubation period on cv. Essex for V2, R1, and R3 plants were 21, 40, and 21 days, respectively (Young & Ross, 1979). More research is needed to explore the interaction between soybean and *S. glycines*, and my qPCR assay can be a strong tool to characterize the incubation period and the latent period of *S. glycines*.

Young et al. (1979) suggested that the longer incubation period at R1 stage than V3 and R3 stage plant may indicate there were undiscovered resistance mechanisms in a particular stage of soybeans against brown spot (Young & Ross, 1979). The resistance that is only effective at the older tissue or late-stage has been named as adult plant resistance (APR) or age-related resistance (Whalen, 2005). Most of the adult plant resistance are partial resistance with broad-spectrum to

multiple pathogens. This kind of resistance usually gradually increases with the plant age, but some only happen at a particular stage. In my detached leaf experiment results, the amount of *S. glycines* gDNA showed a huge difference between V3 and R1 stage leaves at 7 dpi (Fig. 3.5). Both samples at 7 dpi had typical symptoms (score=5), but the percentage of diseased area observed was smaller on R1 stage leaves than V3 stage leaves. The R1 stage leaves may be more resistant than the V3 leaves and slow down the colonization of the hyphae in leaves. Similar phenomenon was reported on the powdery mildew of soybeans. Gonçalves et al. (2002) mentioned that the V1 leaves were more susceptible than V3 leaves, and the leaves from the lower canopies were more susceptible than the leaves from the higher canopies to powdery mildew (Gonçalves *et al.*, 2002). My qPCR tools can be used to assist the characterization of the possible adult plant resistant in soybeans in further research.

The presence of *S. glycines* in the soybean trifoliate was confirmed by the microscopy observations. The microscopy observation on the cv. Williams' V3 stage leaves indicated that the spores germinated and penetrated the host within 3 dpi without forming an appressorium. The form of penetration of this pathogen matched the description from previous histological research (MacNeill & Zalasky, 1957).

The survival analysis characterized the range of median IP and LP for this disease from the detached leaf samples and found that the IP/LP had significant different between leaf stages. The length of IP and LP of this disease may also be different in the field conditions. I observed longer (13 to 20 dpi) IP and LP at V3 to V4 stage in the field in 2018, which may due to low humidity and high temperature that were unfavorable for disease development. Young and Ross (1979) also reported diverse rate of disease development when inoculated the plants at V2 (21 days), R1 (40 days) and R3 (21 days) stage.

Identification and diagnosis at the early infection stage on the host is important for the proper application of management strategies. Usually, it is too late to apply a fungicide when people observe the visual symptoms. The qPCR assay developed in this study allows for diagnosing and detecting *S. glycines* rapidly in soybean leaves at the early infection stages. It can also assist the characterization of the latent period and to screen for quantitative resistance in soybean germplasm.

CHAPTER 3 Tables and Figures

Table 3.1 Lists of the primers and probes used in this study.

Primers	Sequence	Target gene	T_m	Length	Reference
Ac1(f)	ACAATCCAGGGACCACAATC	Actin	59.64	20	This study
Ac2(r)	ATGGCTGATCGCATACCC	Actin	59.44	18	This study
Ac(probe)	6FAM-AGAGCTGACCAGGACCCAGCATCCA-TAM	Actin	73.09	25	This study
Bt1(f)	GGTCGAGAACTCCGATGAGA	Beta-tubulin	60.35	20	This study
Bt2(r)	TACGAGGGGTTGTTGAGCTT	Beta-tubulin	59.73	20	This study
Bt(probe)	6FAM-CGACATATGCATGCGCACCCT-TAM	Beta-tubulin	68.47	21	This study
Cal1(f)	TCTTCGTACGTGTCCCTTGA	Cal gene	59.29	20	This study
Cal2(r)	TCCTTGTCCTGTTGTGGTCA	Cal gene	60.13	20	This study
Cal(probe1)	6FAM-CCGAGACACGGCAGCAGGCA-TAM	Cal gene	72.56	20	This study
Cal(probe2)	6FAM-TCACGCCGAGACACGGCAGC-TAM	Cal gene	72.61	20	This study
ITS 5	GGAAGTAAAAGTCGTAACAAGG	ITS	52		White et al. (1990)
ITS 4	TCCTCCGCTTATTGATATGC	ITS	52		White et al. (1990)

Table 3.2 Validation of qPCR assay with 25 ng/µl of DNA from pure fungal isolates and soybean.

Isolate	Species	Bt		Ac	
		Mean Ct[1]	Standard error	Mean Ct	Standard error
R3101	*S. glycines*	22.85	0.45	22.9	0.25
R3216	*S. glycines*	22.02	0.65	23.01	0.22
16S007	*S. glycines*	23.67	0.37	23.86	0.11
16S008	*S. glycines*	23.04	0.41	23.15	0.29
16S009	*S. glycines*	22.03	0.55	22.21	0.17
16S011	*S. glycines*	23.1	0.16	23.26	0.16
16S006	*S. glycines*	23.43	0.53	22.92	0.31
16S010	*S. glycines*	23.14	0.38	22.65	0.09
16S012	*S. glycines*	23.4	0.34	23.15	0.04
ATCC 38699	*S. glycines*	23.23	0.22	22.82	0.24
10^5 pg/µl	*S. glycines*[1]	20.14	0.35	21.4	0.07
10^4 pg/µl	*S. glycines*[1]	23.91	0.43	25.14	0.13
10^3 pg/µl	*S. glycines*[1]	27.05	0.45	28.32	0.21
10^2 pg/µl	*S. glycines*[1]	30.74	0.29	31.43	0.97
10 pg/µl	*S. glycines*[1]	33.8	0.11	34.85	0.81
C001	*C. gloeosporioides*	36.38	0.89	UN[2]	UN
C002	*C. gloeosporioides*	36.18	1.73	UN	UN
CS1303-3	*C. sojina*	UN	UN	37.46	0.26
CS1355-1	*C. sojina*	UN	UN	38.26	0.7
CS1334-3	*C. sojina*	UN	UN	37.86	0.42
CS1321-2	*C. sojina*	UN	UN	38.4	0.18
water		36.67	0.22	UN	UN
Soybean	*Glycine max*	35.98	0.54	37.75	1.07

[1]Ct mean values were calculated from three separate qPCR assays. There were three technical replicates within each assay.

[2]UN was referred to undetermined Ct value.

Table 3.3 Validation of qPCR assay with leaf samples collected from the field plots at different growth stages. Samples were collected from each plot at V4-R1 stage (one week after inoculation), R2-R3 (one week before fungicide application), and R5 stage (after fungicide application). The vertical progress (VP), necrotic area (N), chlorotic area (C), and defoliation rate (D) were evaluated as a percentage.

Line	Treatment[a]	Growth stage	Dpi[b]	VP	N	C	D	pg of *S.g* DNA[c]	pg of *S.g* DNA per ng of soybean DNA[3]
LD12-8677	Inoculated	V4-R1	6-9	0	0	0	0	0/0/0	0/0/0
		R2-R3	35-37	40.0	4.5	3.9	19.6	16/0/0	0.159/0/0
		R5	48-50	36.0	4.3	2.7	23.0	0/0	0/0
	Control	V4-R1	6-9	0	0	0	0	0/0/0	0/0/0
		R2-R3	35-37	21.3	0.8	2.7	13.3	0/0/0	0/0/0
		R5	48-50	19.4	0.4	1.2	16.3	0/0	0/0
LD13-14071R2	Inoculated	V4-R1	6-9	0	0	0	0	0/0/0	0/0/0
		R2-R3	35-37	37.4	5.6	5.1	15.4	11/1085/0	0.11/10.85/0
		R5	48-50	38.8	5.3	3.2	23.0	958/0	0.96/0
	Control	V4-R1	6-9	0	0	0	0	0/0/0	0/0/0
		R2-R3	35-37	13.7	0.3	2.6	7.9	0/0/0	0/0/0
		R5	48-50	26.7	0.9	1.3	17.7	12/0	0.12/0
Williams	Inoculated	V4-R1	6-9	0	0	0	0	0/0/0	0/0/0
		R2-R3	35-37	39.3	4.9	4.4	19.9	12/368/0	0.12/3.68/0
		R5	48-50	34.8	4.1	2.4	23.5	0/0	0/0
	Control	V4-R1	6-9	0	0	0	0	0/0/0	0/0/0
		R2-R3	35-37	4.9	0.2	2.8	13.6	387/0/0	3.87/0/0
		R5	48-50	18.8	0.6	1.3	19.5	3/0	0.03/0

[a] Inoculated = The plants were inoculated with S. glycines at V3 to V4 stage; Control = The plants were not inoculated but sprayed with fluxapyroxad and pyraclostrobin (Priaxor, BASF, Research Triangle Park, NC) at R1 stage.

[b] Dpi = days post inoculation, VP = vertical progress (%), N = necrotic area (%), C = chlorotic area (%), and D = defoliation rate (%). The disease evaluation value for four disease components were the mean of three replicates.

[c] Each number represented the qPCR result (amount of detected S.g. DNA) for one sample. Each sample was a composite of ten plants in one field plot. There were three samples per treatment except at R5 when there were only two samples.

Table 3.4 Validation of qPCR assay with trifoliate samples (cv. Williams) collected from the field with different necrotic area.

Sample	Necrotic area[1] (%)	pg of *S.g.* DNA	pg of *S.g.* DNA per ng of soybean DNA
Rep1	0	0	0
	7.28	66	0.66
	14.64	121	1.21
	28.61	690	6.9
Rep2	0	0	0
	1.39	44	0.44
	13.69	114	1.14
	17.84	481	4.81
Rep3	0	0	0
	4.06	211	2.15
	15.47	1753	17.53
	25.92	829	8.29
Rep4	0	0	0
	2.82	71	0.71
	10.51	476	4.76
	22.3	1022	10.22

[1] Necrotic area was determined using image analysis software (Assess 2.0)

Table 3.5 Relationship between *S. glycines* infection stage, visual symtpoms and qPCR results at V3 and R1 stage of cv. Williams.

Stage	Dpi[a]	Infection stage [b]	Disease scale	pg of *S.g.* DNA	StdDev Qty	pg of *S.g.* per ng of soybean DNA
V3	0	Inoculation	0	20	7.6	0.2
	1	Attach/germination	1	27	11.6	0.27
	3	Germination/Penetration	1	534	668.0	0.53
	5	Colonization	3	2362	309.8	23.62
	7	Typical symptoms/sporulate	5	5506	1281.9	55.06
R1	0	Inoculation	0	37	4.4	0.04
	1	-	1	44	11.5	0.44
	3	-	1	1057	729.2	10.57
	5	-	4	474	85.6	4.74
	7	Typical symptoms/sporulate	5	461	50.4	4.61

[a] Days post inoculation.

[b] Samples from 3 and 5 dpi were comfirm with microscopy observation on cv. LD13-14071R2 and cv. Williams at V3 stage. No difference in infection stage were found between cultivars.

[c] The Ct value for control were >35, except for samples collected at R1 (5 dpi), Ct=27.72/ 28.35/ 28.62, and mean Qty = 462.93.

Table 3.6 Descriptive statistics of the inbubation period and latent period of soybean leaves collected at two physiological stages (V3 and R1) from two soybean lines inoculated with *S. glycines.*

Stage	Soybean line	No. of leaves	Incubation period			Latent period		
			Median (days)	χ^2	*p*	Median (days)	χ^2	*p*
V3	-	35	7	9.2	0.002*	8	11.4	0.0007*
R1	-	22	7			11		
V3	LD13-14071R2	18	7	10.8	0.001*	7	13.2	0.0003*
R1		12	10			11		
V3	Williams	17	6	20.6	0.4	8	0.1	0.8
R1		10	7			7.5		

[1] The chi-square statistic and p-value given above are for the log rank test with the null hypothesis of no difference between the two survival curves.

[2] Samples with disease severity < 4 was excluded from the analysis

Supplementary Table 3.1 Analysis of variance for the effect of treatments on disease severity of *Septoria* brown spot on their AUDPC values.

	Source	DF	F value	*p*-value
Vertical progress	Treatment	1	75.04	<0.0001
	Variety	2	2.16	0.1657
	Variety*Treatment	2	1.82	0.2119
	Source	DF	F value	*p*-value
Necrotic area	Treatment	1	430.17	<0.0001
	Variety	2	2.22	0.1589
	Variety*Treatment	2	4.38	0.043
	Source	DF	F value	*p*-value
Chlorotic area	Treatment	1	41.91	<0.0001
	Variety	2	0.99	0.4049
	Variety*Treatment	2	1.67	0.2375
	Source	DF	F value	*p*-value
Defoliation area	Treatment	1	4.96	0.0505
	Variety	2	0.77	0.4867
	Variety*Treatment	2	0.11	0.9855

[1] The data for necrotic area and chlorotic area were square root transfomed to meet the assumption of ANOVA test.

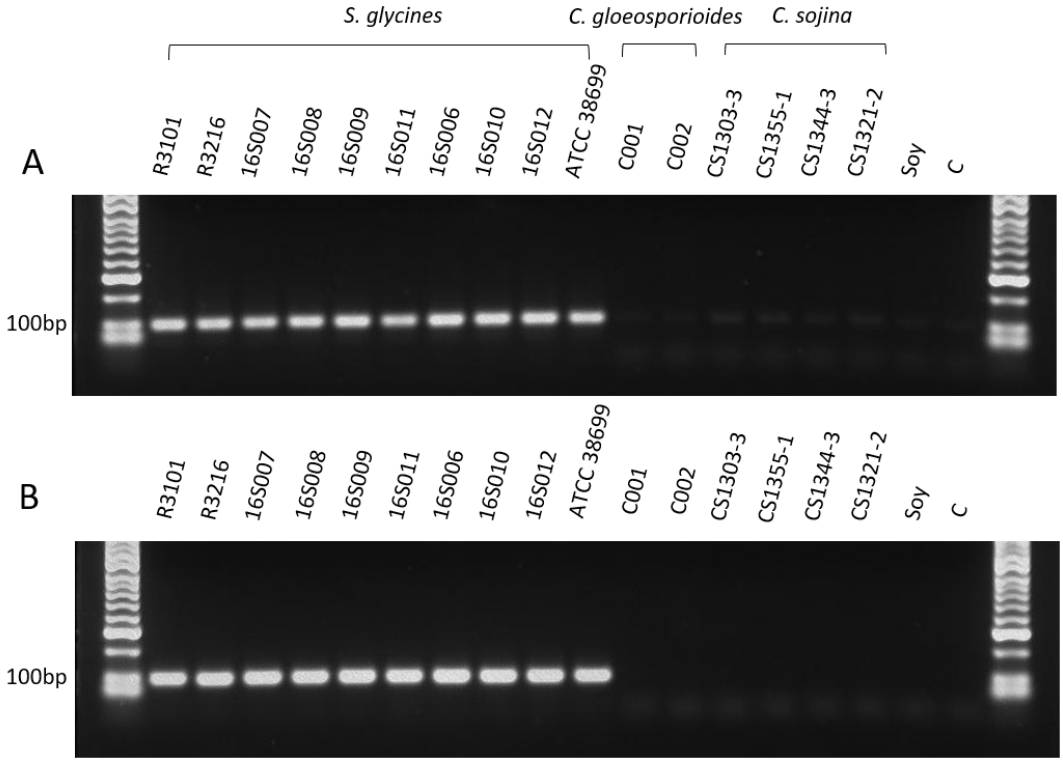


Figure 3.1 Conventional PCR results of A) β-tubulin (Bt) and B) actin (Ac) primers. The Bt primers produced a faint band without S. glycines template, and the Ac primers were highly specific to S. glycines. soy: soybean DNA; c: water control.

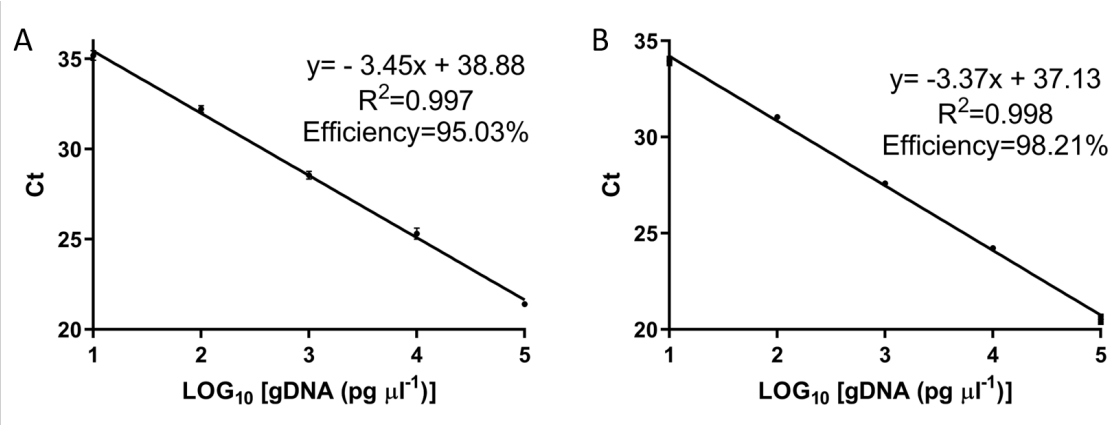


Figure 3.2 Standard curve for absolute quantification of S. glycines genomic DNA. A) Actin primers B) β-tubulin primers. The cycle threshold (Ct) is the average of three replicates of each dilution, and the error bars indicate standard deviation. The gDNA of *S. glycines* were 10- fold diluted from 105 pg to 10 pg and were log- transformed. All samples were tested three times in one plate (technical replicates) and the assays were replicated three times in different plates.

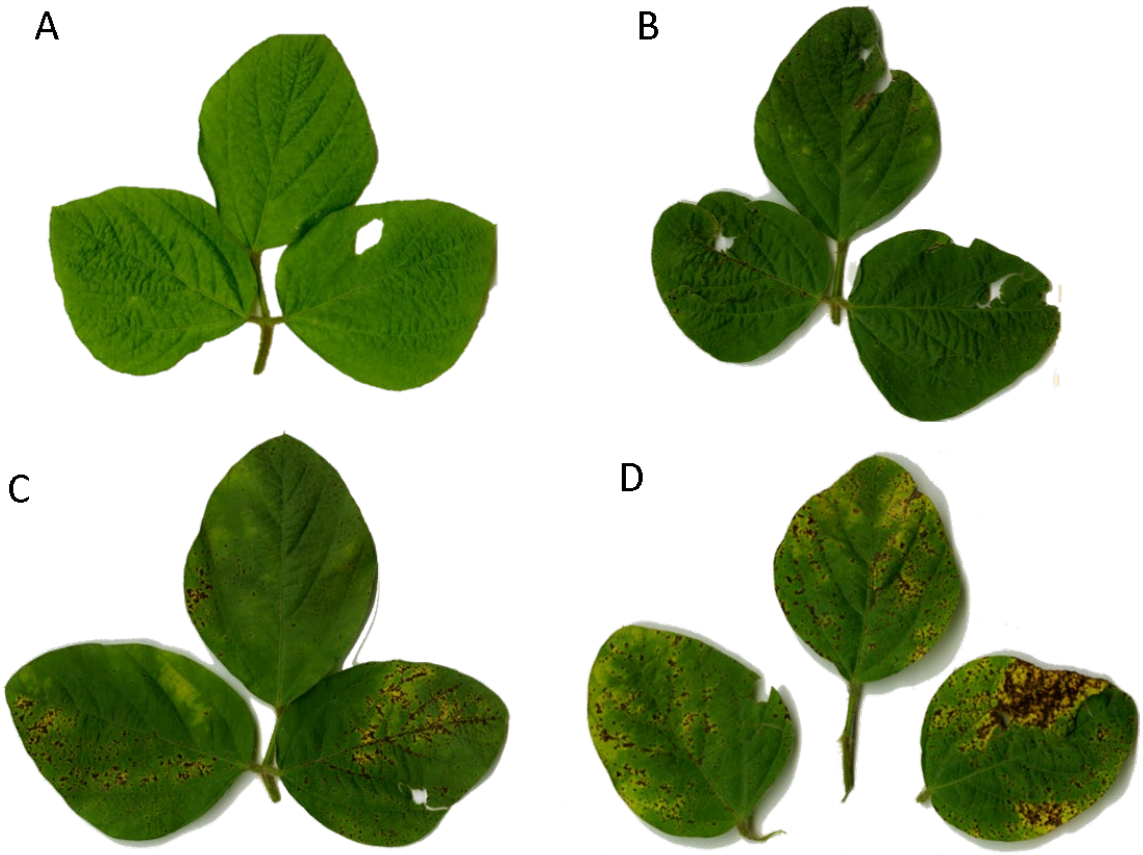


Figure 3.3 Example field trifoliate samples with a different range of necrotic area of Septoria brown spot determined with Assess 2.0. A) = 0%, B) = 7.3 %, C) = 14.6 %, and D) = 28.6 %.

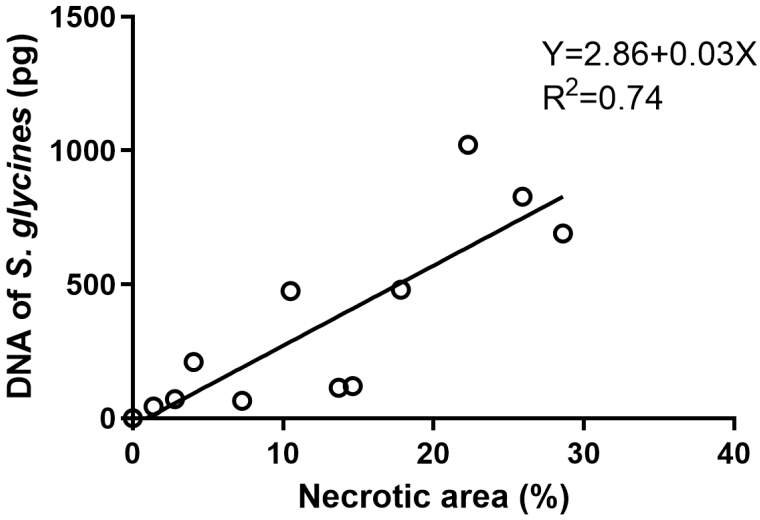


Figure 3.4 Linear relationship between the necrotic area and *S. glycines* gDNA.

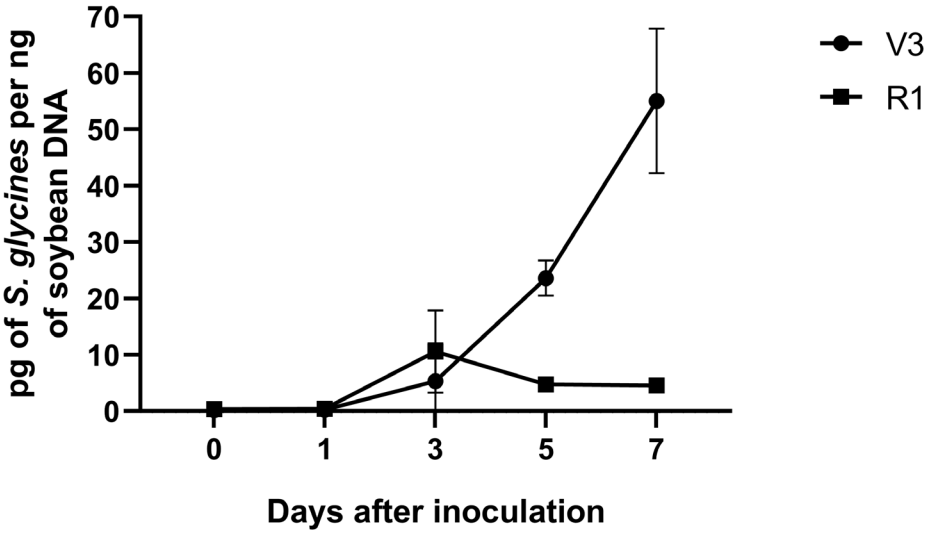


Figure 3.5 Quantification of *S. glycines* after 0, 1, 3, and 7 days post-inoculation on detached leaves collected from plants at two different physiological stages (V3 and R1).

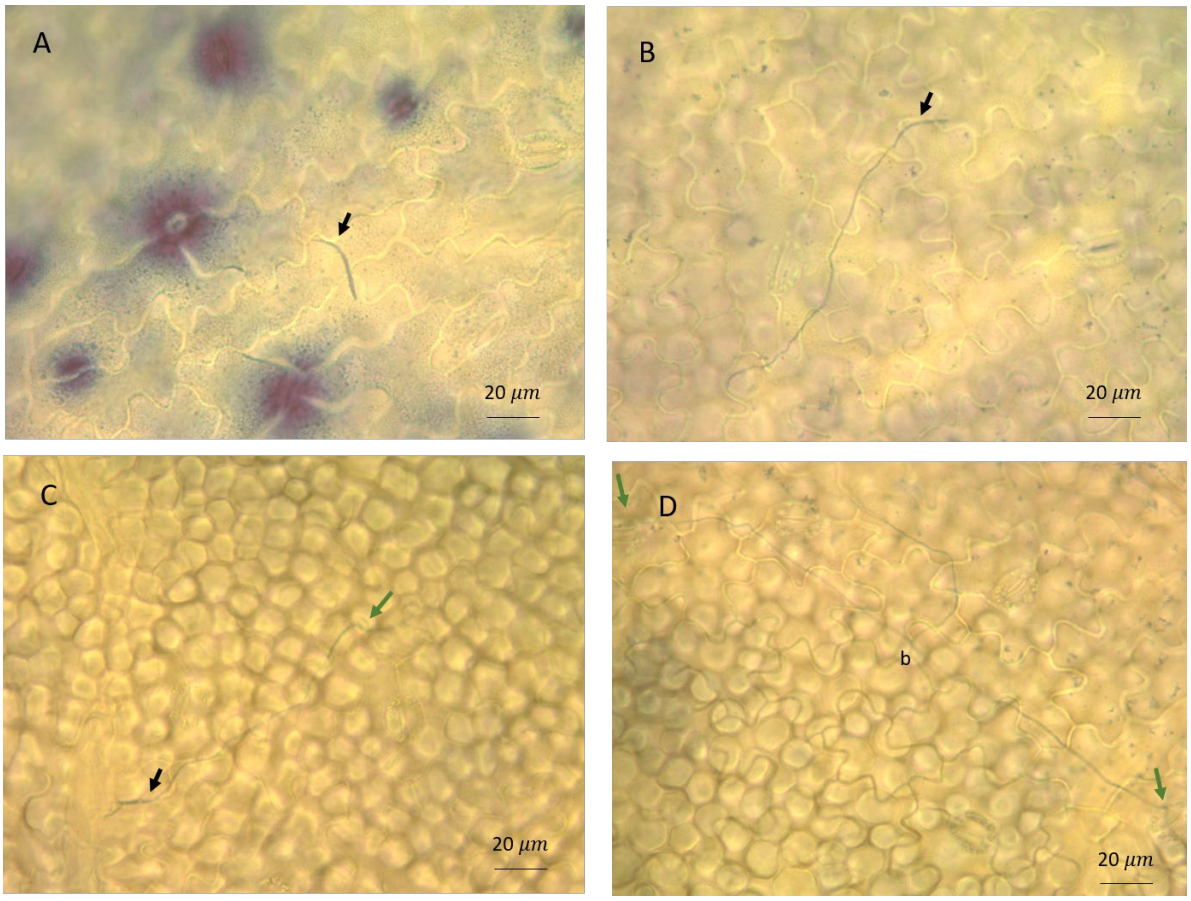


Figure 3.6 Microscopy observations of the infection of *S. glycines*. A) Conidia (black arrowhead) of *S. glycines* at 3 dpi. B) Conidia germinated on the leaf surface at 3 dpi. C) Conidia germinated and penetrated through stomata (green arrowhead) at 3 dpi. D) Branching hyphae (b) were growing on the leaf surface from stomate to stomate at 5 dpi.

CHAPTER 4 THE EFFECT OF *SEPTORIA GLYCINES* AND FUNGICIDE APPLICATION ON SOYBEAN PHYLLOSPHERE MICROBIOME

4.1 Abstract

Septoria brown spot is the most prevalent foliar disease in Illinois. It is common to use a foliar fungicide during the reproductive stage to control Septoria brown spot and other foliar diseases. However, the effects of fungicide on non-target organisms in the phyllosphere are not well characterized. In this study, I use DNA metabarcoding to understand the fungal community composition in soybean leaves and the dynamic of Septoria and non-target fungi under different treatments and growth stages. Full-length ITS and partial LSU region were sequenced using oxford nanopore technologies. After alignment to Unite and RDP databases, there were 3,342 operational taxonomic units (OTUs). The cultivars had a significant effect on the microbiome communities at the V4 stage. *Gibberella* (33.7%), *Alternaria* (24.24%), *Didymella* (11.77%), *Cladosporium* (9.02%), *Plectosphaerella* (7.67%), *Colletotrichum* (2.59%) and *Bipolaris* (2.20%) were identified as core microbiome in soybean leaves. Few possible interactions were identified between Septoria and other fungal organisms. The inoculation with Septoria did not significantly affect the entire fungal communities from the alpha and beta diversity analysis. At R5 stage, the fungicide application was an important factor that shaped the fungal communities. Surprisingly, from the Deseq2 analysis, the abundance of Septoria did not significantly decrease in the fungicide treated samples. The fungicide treatment significantly decreased the proportion of *Epicoccum*, *Didymella*, *Cladosporiaceae_sp,* and other fungi, but on the other hand, it increased the proportion of *Septoria, Bipolaris*, and *Diaporthe.* In this study, I set *Septoria* as target organism and demonstrated that metabarcoding could be a tool to quantify the effect of fungicide on target and non-target organisms. I believe that understanding the dynamics of the

phyllosphere microbiome is necessary to start untangling the late-season disease complex and provide information for better control of the disease.

4.2 Introduction

Septoria brown spot, caused by *Septoria glycines* Hemmi, is the most prevalent fungal foliar diseases of soybean (*Glycine max* L. Merrill) in Illinois, USA (Hobbs *et al.*, 2010). The estimated yield loss caused by this disease ranged between 1,736,077 to 6,901,851 metric tons from 2010 to 2014 in the northern USA and Ontario, Canada (Allen *et al.*, 2017). Although the susceptibility of the disease varies among cultivars, there are no resistance sources or genes have been associated with this disease (Hartman *et al.*, 2015b). The fungal pathogen, *S. glycines*, overwinters in the soybean residue by forming pycnidia. The pycnidiospores produced in the next growing season spread by splashing rain to the seedlings and start the initial epidemics from the low canopy. The typical symptoms of this disease are irregular brown necrotic lesions surrounded by chlorotic areas. The vertical progress of the disease development at the late stage is negatively associated with the final yield. Lin et al. (2020) reported that when the disease symptoms present up to 30% of the plant height at the R6 stage, it may lead to a 10% of estimated yield loss, and if it is present at 80% of the plant height, it may lead to a 27% of estimated yield loss. Septoria brown spot can co-occur with other foliar diseases, such as Cercospora leaf blight and anthracnose, and they are considered as late-season disease complex in some references (Carmona *et al.*, 2011). However, the interaction between *S. glycines* and other organisms in the soybeans is unclear.

The collective communities of plant-associated microorganisms are referred to as the plant microbiome (Mendes *et al.*, 2013). The members of the plant microbiome can be beneficial, neutral, or pathogenic microorganisms, including bacteria and fungi. All parts of the

plants can provide a habitat for microorganisms. Based on where those organisms reside, it can be characterized as phyllosphere (the aerial parts of plants), rhizosphere (the belowground of the plant), and endosphere (inside of the plant) microbiome. The plant microbiome is considered an important factor that can be directly or indirectly related to plant nutrient uptake, productivity, disease resistance, and stress tolerance (Turner *et al*., 2013; Trivedi *et al.*, 2020; Berendsen *et al*., 2012; Wagg *et al*., 2011). The microbiome composition differs between plant species, genotype (Sharaf *et al.*, 2019), and developmental stage (Copeland *et al.*, 2015). The communities can also be affected by different agricultural management, such as tillage, fertilization, and chemical treatment (Karlsson *et al.*, 2014; Wattenburger *et al.*, 2019; Longley *et al.*, 2020). Understanding the effect of agricultural practices on the microbiome is important for the development of sustainable management strategies.

Several studies have investigated the effect of different factors on the microbiome communities in the aboveground of soybeans. The main factors that drive the phyllosphere microbiome communities in soybeans include sampling location, plant growth stage (Copeland *et al.*, 2015; Longley *et al.*, 2020), and agricultural systems (conventional, no-till, or organic farming system) (Longley *et al.*, 2020). Some microbes were abundant in leaf and stem across all management systems, such as *Alternaria*, but some microbes were associated with specific management systems. For example, *Phoma* and *Davidiella* were more abundant in the aboveground samples from the organic farming system (Longley *et al.*, 2020). The application of foliar fungicides is another critical factor that shapes the phyllosphere fungal microbiome. While the desired effect of chemicals on the target organisms was important, more studies start to investigate the impact of those chemicals on the non-target organisms since they might act as competitors or antagonisms in the community. In wheat, fungicide application had a moderate

but significant effect on the fungal community composition in the phyllosphere (Karlsson *et al.*, 2014). In Karlsson et al (2014), the fungicide application reduced the evenness of the communities. However, the relative abundance of common pathogens in wheat, such as *Mycosphaerella graminicola* and *Parastagonospora nodorum* were not significantly different between control and fungicide treated samples. The relative abundance of those pathogens tend to have higher variation in the fungicide treated samples. In soybeans, Batzer and Mueller (2020) reported the effect of foliar fungicide on the soybean endophyte using a cultural-dependent method. They reported that spraying the fungicide decreased the amount of *Alternaria* but increased the proportion of *Diaporthe*, which is the causal agent of soybean pot and stem blight, stem canker, and seed decay. Although using a cultural-dependent method ensured that the isolates were truly endophyte, they also admitted that only low diversity of organisms was detected using this method. Using sequencing technologies can overcome the limitation mentioned above and better understand the impact on overall communities in the phyllosphere.

Amplicon sequencing has become a widely used approach for studying the plant microbiome (Lucaciu *et al.*, 2019). The fungal organisms are typically characterized by the internal transcribed spacer (ITS) region, which comprises ITS1 region, 5.8S rRNA gene, and ITS2 region (Raja *et al.*, 2017). The analysis of the fungal microbiome has mainly been based on sequencing ITS1 or ITS2 regions using the second-generation sequencing platform (e.g., Illumina MiSeq). However, the short amplicons (250 to 450 bp) limited the resolution of phylogenetic information. The entire length of the ITS region can vary from 300 to 1200 bp, with an average length of 600 bp (Heeger et al. 2018; Op De Beeck et al. 2014). Several studies using the third-generation sequencing technologies that generate longer amplicons, showing that using

full-length ITS regions or even longer regions (SSU and LSU) for fungal metabarcoding can significantly improve the taxonomic resolution (Heeger *et al*., 2018; Tedersoo *et al.,* 2018).

Unlike the rhizosphere microbiome that are most studied and elaborate, we have little information about the phyllosphere microbiome in most plants (Berg *et al.*, 2017). In this study, my goals were to: 1) use long-read sequencing platform (Oxford Nanopore) to characterize the fungal microbiome in soybean phyllosphere, 2) determine the effect of fungicide application and *S. glycines* on the soybean phyllosphere microbiome, and 3) determine the differences between fungal communities at different developmental stage and cultivars. I hypothesize that the composition of the fungal communities will change not only by growth stages but also change after fungicide application. Septoria brown spot of soybeans is only one of many fungal species that affect soybean at maturity, but it is the most commonly observed foliar pathogens in soybean in Illinois. Understanding the interaction of *S. glycines* with other foliar fungal organisms and the effect of foliar fungicide on them is necessary to start untangling the late-season disease complex and provide information for better control of the disease.

4.3 Materials and Methods

4.3.1 Field trial and sample collection

A field trial was conducted in 2018 at the Crop Sciences Research and Education Center near Urbana (N40.070431º, W88.218929º) in Illinois, USA. The field was on corn-soybean rotation and fertilized according to local practices. The experimental design followed a split-plot design with four treatments as the main plots and three soybean lines (Williams, LD12-8677, and LD13-14071R2) as the sub-plots. Soybeans were planted on 30 April at 348,480 seeds hec^{-1}, and each sub-plot consisted of four 5.2 m long rows with 76.2 cm alleys.

The treatments were 1) Inoc: inoculated with *S. glycines* at V3 and R2 stage (Fehr *et al.*, 1971); 2) Inoc/Fun: inoculated with *S. glycines* as for treatment 1 and fungicide application at R3 stage; 3) NIC/Fun: fungicide application at R3 stage, and (4) NIC/No_Fun: received no inoculation nor fungicide application as control. Each treatment had three replicates. Three *S. glycines* isolates (16S006, 16S012, and R3216) were used for inoculation (Lin *et al.*, 2020). The culture condition of the fungal isolates, preparation of the inoculum, and inoculation procedure followed the description in Lin et al. 2020. The center two rows of the assigned plots were inoculated with a spore suspension 10^6 mL^{-1} spores at V3 stage on 5 June. A second inoculation was applied in those plots at R2 stage on 3 July to increase the disease severity. Fluxapyroxad and pyraclostrobin (Priaxor, BASF, USA) were sprayed at the R3 stage (17 July) at the assigned plots with a CO2-pressurized backpack sprayer equipped with a 0.48 m 601B-SST-four nozzle light weight boom (R&D Sprayers Bellspray, Inc, USA) and four TJ60-11008 (50) nozzles (TeeJet Technology, USA). Ten plants were randomly collected from each subplot at three timepoints, (1) One week after inoculation treatment (V4 stage, collected on 11 June, 13 June, and 14 June), (2) One week before fungicide application (R3 stage, collected on 10 July, 11 July, and 12 July), and (3) One week after fungicide application (R5 stage, collected on 23 July, 24 July, and 25 July) stage. The samples were collected in the morning by each replicate and processed within the same day. Percentage of necrotic area, chlorotic area, vertical progress, and defoliation rate were evaluated for each plant (Lin et al. 2020). After disease evaluation, all leaves were collected from each plant. In total, 108 pooled leaves samples were lyophilized and stored at -20°C for DNA extraction.

4.3.2 DNA extraction

The lyophilized leaf samples were submerged in the liquid nitrogen and then

mixed in the laboratory blender (Waring Commercial, USA). A total of 100 mg of mixed leaf tissue from each sample were placed in a 2 mL FastPrep® tube containing a 6.35 mm ceramic bead (MP BIOMEDICALS, USA) for homogenized and DNA extraction. The DNA was extracted following a modified CTAB method as described in Lin and Mideros (2021). The extracted DNA was analyzed on a NanoDrop spectrophotometer (Thermo Scientific™, USA).

4.3.3 Library preparation and sequencing

Ninety-six out of 108 samples with good quality of DNA were sent to Roy J. Carver biotechnology center for library preparation and sequencing (Supplementary Table 4.1). The sequenced samples consisted of three replicates for each treatment collected at the V4 and R3 stage, and two replicates for each treatment at the R5 stage. The amplicons were produced with the Fluidigm Access Array system (Fluidigm, USA) using primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and LR6 (5'- CGCCAGTTCTGCTTACC-3') (Walder *et al.*, 2017). The ITS1F/LR6 primer set targeted the fungal internal transcribed spacer (ITS) and the partial region of the large subunit (LSU). The pooled amplicons were converted into an Oxford Nanopore library with the SQK-LSK109 library kit (Oxford Nanopore, UK). The library was sequenced on a SpotON R9.4.1 FLO-MIN106 flow cell for 72 hr, using a GridIONx5 sequencer. Base-calling of the fastq files was performed with Guppy 3.0.3 (https://community.nanoporetech.com). The filtering of reads with average Q scores < 7 was done with NanoFilt (DeCoster *et al.*, 2018). Demultiplexing and library adaptor trimming were done with Porechops 0.2.3 (Wick *et al.*, 2018).

4.3.4 Bioinformatics analysis

The reads that contained adaptors in the middle of the sequence were removed using Porechop 0.2.3 (Wick *et al.*, 2018). The reads were then filtered by length using Nanofilt (DeCoster *et al.*, 2018), and the read length between 250 to 2000 bp has remained for further analysis. Transformation of the files from fastq to fasta format was performed using FASTX-Toolkit 0.0.14 (Gordon & Hannon, 2010). The reads were then pre-clustered to operational taxonomic units (OTUs) within each sample using VSEARCH 2.4.3 (Rognes *et al.*, 2016) at 95% similarity and clustered again in the merged files at 92% similarity. The 92% similarity criteria was determined as the optimal clustering criteria after testing with 95%, 92%, 90%, 88%, and 86% similarity (Supplementary Table 4.2). Multiple sequence alignment was performed within each OTUs, and a consensus sequence was extracted from each OTU to eliminate the potential sequencing random error (Mafune *et al.*, 2020). After filtering out the singletons and doubletons, the ITS and LSU regions in each consensus sequence were detected using ITSx 1.1.1 (Bengtsson-Palme *et al.*, 2013). To assign the taxonomy to each OTU, the ITS and LSU data set was aligned using BLAST to the UNITE v8.2 general fasta release (Nilsson *et al.*, 2018) and the RDP v2.9.0 database (Cole *et al.*, 2014), respectively. The ITS and LSU sequences were aligned to the database using BLAST+ 2.9.0 (Camacho *et al.*, 2009) with an E-value set as 10^{-5}. The classification of each OTU was determined with the lowest E-vale, highest bit score, and highest identity (Supplementary Table 4.3).

4.3.5 Statistical analysis for visual assessment of disease development

The area under the disease progress curve (AUDPC) values for the percentage of vertical progress, necrotic area, chlorotic area, and defoliation rate were calculated in R using package Agricolae (De Mendiburu 2010). A split-plot model was used to evaluate the experiment.

$$y_{ijk} = \mu + B_i + T_j + \varepsilon_{1(ij)} + C_k + CT_{jk} + \varepsilon_{2(ijk)}$$

In the model, y_{ijk} is the AUDPC for each disease severity components corresponding to the i^{th} block, j^{th} treatment, and k^{th} cultivars. μ it the grand population mean, B_i is the random block effect, T_j is the fixed treatment effect, C_k is the fixed cultivar effect, CT_{jk} is the fixed interaction effect between the j^{th} treatment and k^{th}cultivar. The $\varepsilon_{1(ij)}$ and $\varepsilon_{2(ijk)}$ are the random error terms [NID (0,σ_e^2)]. The model was fitted using PROC MIXED in SAS v.9.4 (SAS Institute., Cary, NC).

4.3.6 Statistical analysis for sequencing data

The OTU frequency table that gives the number of reads per sample per OTU and the taxonomy table were transformed into proper format and imported into R 4.0.2 (R Core Team, 2017). The alpha diversity and beta diversity analyses were performed using R package phyloseq (McMurdie and Holmes 2013) at both OTU and genus levels. The data was separated into three growth stages (V4, R3, and R5) to study the effect of treatments on the fungal communities. The V4 and R3 stage data were used to assess the effect of inoculation treatment. The R5 stage data were used to assess the effect of both inoculation and fungicide application treatments. The OTU frequency data were used to calculate richness, Shannon index, and Simpson index. Kruskal-Wallis test and Wilcoxon rank-sum test were used to identify the differences between treatments and cultivars and calculate *p* values. For beta-diversity analysis, the data were further separated into ITS and LSU dataset. The reads were normalized using the rarefaction method (Willis, 2019). To generate the weighted Unifrac distance matrix (Lozupone *et al.*, 2011), phylogenetic trees were constructed using R package phangorn (Schliep, 2011). The principal coordinate analysis (PCoA) plots were generated from the weighted Unifrac distance matrix. A permutational multivariate analysis of variance (PERMANOVA) analysis was performed using the adonis function in R package vegan (Dixon, 2003). The OTU differential abundance analysis

was performed using DeSeq2 (Love *et al.*, 2014) to identify the specific OTUs at the genus level that were affected by the treatments. The core microbiome was defined with the prevalence of 0.5 and abundance larger than 1% using R package microbiome (Lahti *et al.,* 2017), and the analysis was performed with control samples (NIC/No_Fun) and all samples.

4.3.7 Network analysis

The co-occurrence network analysis was performed using SParse InversE Covariance Estimation for Ecological ASsociation Inference (SPIEC-EASI) method (Kurtz *et al.*, 2015) in R. The unnormalized data were merged to the genus levels and subsetted based on the sampling stage (V4, R3 and R5) and treatments. The V4 data set include 18 inoculated and 18 not-inoculated samples; R3 data set include 17 inoculated and 17 not-inoculated samples; and R5 data set included 12 samples for each treatment combinations (Inoc/Fun, NIC/Fun, Inoc/No_Fun, and NIC/No_Fun). In each dataset, OTUs with a frequency of less than 6 were considered infrequent OTUs and excluded from the analysis (Kerdraon *et al.*, 2019). The SPIEC-EASI pipeline first transformed the OTU frequency data to compositional data. Then, the Meinshausen-Bühlmann's neighborhood selection method (MB method) was used as the graphical inference model with the minimum lambda ratio set at 10^{-2} and 50 repetitions. The adjacency matrix and centrality metrics (alpha centrality and betweenness centrality) were extracted from the speiec.easi object (Birt & Dennis, 2021). The edge stability of each network was extracted with the getOptMerge function in R package SPIEC-EASI. The networks were visualized and drawn with Cytoscape V. 3.6.1 (Shannon *et al.*, 2003).

4.4 Results

4.4.1 Disease development

Symptoms of Septoria brown spot were first observed at R3 stage. At R3 stage, a higher percentage of vertical progress (40%), necrotic area (4-6%), chlorotic area (4-6%), and defoliation rate (20-30%) were observed on the inoculated plots. Disease evaluation on the non-inoculated plots was 20% for vertical progress, 4-6% for necrotic and chlorotic area, and 20% for defoliation rate. At R5 stage, the disease development did not significantly decrease due to fungicide application, but overall, the disease severity on the non-inoculated plots was lower than the inoculated plots (Supplementary Table 4.4). The ANOVA analysis indicated that the treatment combinations significantly (p<0.05) affected the AUDPC of the percentage of vertical progress, chlorotic area, and necrotic area. The soybean cultivars also significantly affected the AUDPC of the percentage of vertical progress and necrotic area (Supplementary Table 4.5).

4.4.2 Sequencing and OTU classification summary

The Oxford Nanopore GridIONx5 sequencer resulted in a total of 4,354,575 reads, with a mean read length of 1,819 bp and in the range between 300 bp and 9,941 bp. Two out of 96 samples were discarded from the analysis due to low reads number (642) or failure to amplify the amplicon. The quality reads number was 4,184,494, with an average of 44,047±36,773 (SD) reads for each sample (Supplementary Table 4.1). There were a total of 1,490,827 unique OTUs identified, and 3,342 OTUs (2,668,341 reads) remained after filtered out the singletons (35%) and doubletons (0.09%) (Supplementary Table 4.2). I successfully extracted 2,842 ITS sequences and 2,976 LSU sequences out of 3,342 OTUs' consensus sequences. After BLAST alignment, there were 2840 hits and 2972 hits for ITS and LSU sequences, respectively (Supplementary table 4.3). For the OTU classification using the ITS sequences, 94.37% were classified as Ascomycota, followed by Basidiomycota (3.98%), Chytridiomycota (0.07%), Glomeromycota (0.18%), Mortierellomycota (0.04%), and unclassified (1.37%). For the OTU

classification using the LSU sequences, 95.49% were classified as Ascomycota, followed by Basidiomycota (3.77%), Chytridiomycota (0.20%), Blastocladiomycota (0.07%), and unclassified (0.47%). Overall, about 99% of ITS and LSU sequences were classified to a taxonomy level with the identity ranging between 80 to 100% and a mean of 97% (Supplementary table 4.3).

4.4.3 Relative abundance (RA) of Septoria

At V4 stage, the relative abundance (RA) of *Septoria* ranged between 0-7% and 0-3% in the inoculated samples and non-inoculated samples, respectively. The RA of *Septoria* was significantly different between inoculated and non-inoculated samples at V4 stage within cultivars except for cv. LD13-14071R2. The relative abundance of *Septoria* increased at R3 stage in both inoculated (0-23%) and non-inoculated samples (0-3%). The significant difference between treatments indicated that the inoculation was successful, and the pathogen colonized the soybean leaf tissue after inoculation. A low amount of *Septoria* was also detected in the non-inoculated samples (0-3%), showing that the disease naturally occurred in the field or cross contamination between plots. At R5 stage, the cultivars were merged to calculate the RA since there was no significant difference for cultivars from the diversity analysis. Surprisingly, more *Septoria* was identified in the fungicide treated samples. The RA of Septoria was still higher in the inoculated samples compared to the non-inoculated samples. Although the fungicide application did not decrease the RA of *Septoria*, there was higher variability of *Septoria* on the samples that treated with fungicide (5$\pm$6% SD) (Figure 4.1).

4.4.4 Community analysis – Alpha diversity

The alpha diversity was estimated with richness, Shannon index, and Simpson index using the OTU- and genus-level data. The results showed a similar trend between the two data sets, but more significant comparisons were identified from the genus-level data. Significant differences between cultivars was only detected at V4 stage for all diversity indices. The diversity was also showing a significant difference between the three growth stages. The richness slightly increased at R3 and R5 stage. The distribution of the diversity index had the widest range at R3 stage in both inoculated and non-inoculated samples. At R5 stage, although the diversity was higher in the fungicide treated samples regardless of inoculation or not, the fungicide treatment reduced the variation between the replicates within the fungicide treated samples. The significant difference between inoculated and non-inoculated treatments was only identified in richness in cv. Williams at V4 stage. This result indicated that the inoculation of the *Septoria* did not affect the overall fungal alpha diversity (Figure 4.2, Table 4.1 & 4.2).

4.4.5 Community analysis - Beta diversity

The PERMANOVA test showed that the cultivars were a significant factor that structured the fungal community at V4 stage at both OTU- and genus levels (ITS and LSU, p=0.001). A minor effect for inoculation treatment (ITS, p=0.03) was only detected at V4 stage at the OTU level. While the inoculation treatment explained 1% to 6% of the variation, the cultivars explained 13% to 37% of variation at V4 stage. No significant factors were identified from the analysis at the R3 stage. At R5 stage, there was no significant difference between cultivars. The treatments at R5 stage were only significant at the OTU level and explained 40% of the variation (ITS, p=0.091) (Table 4.3). The principal coordinates analysis (PCoA) also suggested that the community were clustered by cultivars at V4 stage and the inoculation treatment only had a minor effect on the community (Figure 4.3 A-B). No apparent patterns were observed from the

PCoA results for the R3 stage data. At R5 stage, the application of fungicide had a larger effect than cultivars and inoculation treatment, suggesting that fungicide application can shape the fungal community in the phyllosphere (Figure 4.3 C-D).

4.4.6 Core microbiome

The soybean phyllosphere was dominated by Ascomycota and accounted for 97% of classified reads. A total of 10 OTUs were identified as the core microbiome in the soybean phyllosphere. Eight out of 10 OTUs were enriched and continuously presented across samples when using the entire data set or the subset data set (NIC/No_Fun) for analysis. Of note, the OTU17 (*Septoria,* 1.6%) was identified as a core microbiome when using all samples for analysis. The OTU45 (unclassified_Pleosporales, 0.14%) was identified as a core microbiome when using the LSU data set for analysis. Overall, the most abundant genus was *Gibberella* with 33.7% of classified reads, followed by *Alternaria* (24.24%), *Didymella* (11.77%), *Cladosporium* (9.02%), *Plectosphaerella* (7.67%), *Colletotrichum* (2.59%), *Bipolaris* (2.20%) (Table 4.4). Although those core microbiomes comprised 92% to 98% RA of the fungal community in each sample, the dynamics of those abundant organisms were observed across growth stages (Figure 4.4, Supplementary Figure 4.1.).

4.4.7 OTU differential analysis using Deseq2

At V4 and R3 stage, when comparing the inoculated and non-inoculated samples, only *Septoria* (OTU17) and *Didymella* (OTU7) were significantly different in the inoculated samples,. Six comparisons between the treatments at the R5 stage resulted in 28 and 19 significantly differential abundant OTUs using ITS and LSU data sets. One of the comparisons (Inoc/Fun vs Not_inoc/Fun) resulted in 0 significantly differential abundant OTUs. Fifteen out of 19 OTUs

identified from the LSU data set had consistent and similar differential patterns with the ITS data set. The taxa assigned for the LSU data set were at the higher taxonomy level but mostly agreed with the assigned results from the ITS data. At R5 stage, the inoculation of *S. glycines* increased the abundance of *Septoria* (OTU17) and *Tausonia* (OTU23) when compared the inoculated samples with non-inoculated samples (Inoc/No_Fun vs NIC/No_Fun). The fungicide application affected most of the core microbiome, except for *Plectosphaerella* (OTU4) and *Colletotrichum* (OTU10). After fungicide application, most of the significant OTUs such as *Epicoccum* (OUT278), *Didymella* (OUT7), and Cladosporiaceae_sp (OTU76025) decreased, but surprisingly, the amount of *Septoria* (OTU17), *Bipolaris* (OTU14), and *Diaporthe* (OTU11) increased (Figure 4.5, Supplementary Table 4.6).

4.4.8 Network analysis

The data was subset by growth stages and treatments, resulting in 11 data sets for network analysis. After filtering out the low abundance OTUs, the remaining OTUs for analysis ranged between 95 to 187 (Table 4.5). There were more positive edges in all networks than negative edges (Fig 4.6, Supplementary Figure 4.2-4.5). The networks at the later growth stage had higher numbers of nodes and edges (Table 4.5, Supplementary Figure 4.2). The average degree and betweenness centrality increased throughout the seasons, indicating more interactions between organisms, and the complexity of the network was higher at the late stage (Table 4.5). Microorganisms with high betweenness centrality in the network, such as *Alternaria, Bipolaris, Cladosporium, Didymella,* and *Gibberella* were also identified as the core microbiome from the previous analysis (Figure 4.6). The consistent results between the two analysis suggesting that those organisms play an important role in the community. Only a few direct interactions were identified between *Septoria* and other organisms. At V4 and R3 stage, *Septoria* was predicted to

have positive interaction with an unknown OTUs. At R5 stage, after filtering for edge stability (> 0.5), *Septoria* was predicted to have positive interaction with *Diaporthe*, *Bipolaris*, and an unidentified OTUs, and negatively interacted with *Hypocreales* (Figure 4.6). From the Deseq2 analysis, *Septoria, Diaporthe,* and *Bipolaris* had similar differential patterns (they were all promoted after fungicide application). The results were consistent between the network analysis and Deseq2 analysis.

4.5 Discussion

In this study, I assessed the effect of Septoria brown spot and fungicide application on the dynamics of phyllosphere fungal communities at V4 (after Inoculation), R3 (before fungicide application), and R5 (after fungicide application) stage on soybean. By sequencing 96 samples using oxford nanopore technologies, a total of 3,342 OTUs (649 genus) were obtained. I identified the organisms that significantly increased or decreased after the inoculation or fungicide application treatments. The potential interactions between those organisms were estimated using network analysis. I also characterized the core microbiome associated with soybean phyllosphere. My results reveal the dynamic of target and un-target organisms that were related to the disease and agricultural practices, which may provide information to improve foliar disease management.

In this study, the phyllosphere fungal communities were significantly associated with the soybean cultivars only at the early growth stage. The difference between cultivars were not detected in the R3 and R5 samples, which may due to the treatments and other environmental factors. Sapkota et al. 2015 also observed the fungal community clustered by the cultivars in the non-fungicide treated samples in cereals. The association between plant genotype and phyllosphere microbiome has been reported in grape species (Singh *et al.*, 2019). Singh et al

(2019) reported that the bacterial and fungal communities in the phyllosphere were significantly associated with grape species and growing years. However, they only collected the samples one time each year. It is unclear if this association can persist though out the growth stage. More evidence suggested that the microbiome communities in the rhizosphere were associated with plant genotypes in many crops, such as maize, wheat, pea, and oat (Turner *et al.*, 2013a). In soybean, the genotypes had a significant but mild effect on the rhizosphere microbiome when comparing five soybean genotypes, including cv. Williams, a non-nodulating mutant of Williams, cv. Williams 82, a drought-resistant cultivar, and a cyst nematode-resistance line (Liu *et al.*, 2019). In these soybean lines, cv. Williams, cv. Williams 82, and the mutant of Williams shared similar genetic backgrounds, and they cluster together on the beta diversity analysis (Liu *et al.*, 2019). Soybean may also have indigenous phyllosphere microbiota associated with cultivars as I observed at the V4 stage samples, some beneficial communities may become host trait and integrate into the breeding program.

The core microbiome can be defined as a set of stable and persistent microorganisms associated with specific status or environmental conditions (Berg *et al.*, 2020). In this study, a total of 8 OTU were identified as core microbiome. Many of those organisms have been identified as endophytes in soybeans using the cultural-dependent method, such as *Alternaria*, *Cladosporium*, *Colletotrichum*, *Fusarium*, and *Didymella* (Impullitti and Malvick 2013; Batzer and Mueller 2020). Some abundant OTUs, such as *Diaporthe, Fusarium,* and *Hannaella* were consistent with the results reported by Zeng et al (2019) using the amplicon sequencing method. Although those microbes were consistently identified and enrich across all samples, the dynamic composition of those core microbes between growth stage and treatments were observed. Lemanceau et al. 2017 have reviewed examples of functional core microbiota (mostly bacteria)

that can enhance host fitness regardless of plant nutrient intake, drought tolerance, and defense response. The function of these core microbes on the soybeans is unclear and need further characterization.

Priaxor (14.3% fluxapyroxad and 28.6% pyraclostrobin) is a broad-spectrum foliar fungicide which recommended to applied between R2 to R4 stage for targeting multiple foliar diseases, including Alternaria leaf spot, Anthracnose, Septoria brown spot, Cercespora leaf blight, Frogye leaf spot, Pod and stem blight, and Rhizoctonia. It is reasonable to expect that fungicide application will reduce the richness and other diversity indices, but I did not see these results in the R5 stage samples. However, a trend of reduced variation within fungicide treated samples than the untreated samples were observed in other studies. Both Knorr et al (2019) and Karlsson et al (2014) reported higher variation within untreated samples than fungicide treated samples in fungal phyllosphere in wheat. According to the PERMANOVA analysis and PCoA plots, the fungicide treatment had an effect on the fungal communities on soybean leaves. The Deseq2 analysis suggested that the fungicide application significantly reduced the amount of *Alternaria*, *Epicoccum*, *Didmylla,* and other fungi. However, it also significantly increased the amount of *Bipolaris* and *Diaporthe*. Some of the patterns I observed were consistent with the results in Batzer and Mueller (2020). Both results highlight the possibility of increased the risk of another disease when controlling the target organisms, which will be an important factor to consider in disease management.

Surprisingly, the fungicide did not have an effect on the relative abundance of *Septoria* at the R5 stage samples. However, this non-significant fungicide effect on the target pathogens were also observed in other fungal phyllosphere microbiome studies in cereal (Sapkota *et al.*, 2015) and wheat leaves (Karlsson *et al.*, 2014). Karlsson et al 2014 reported that the fungicide

did not significantly affect the common wheat pathogens identified from their study, such as *Mycosphaerella graminicola*, *Blumeria graminis, Puccinia striiformis, Phaeosphaeria nodorum* (*Parastagonospora nodorum*), *Monographella spp,* and *Pyrenophora tritici-repenti.* Sapkota et al. 2015 also reported that the fungicide did not have a significant on the fungicide target organisms, *Zymoseptoria tritici* and *Blumeria graminis.* One of the possible reasons mentioned in Sapkota et al (2015) was the fast resilience of the fungal phyllosphere communities after fungicide application. The time from fungicide application to sample collection might enable the pathogens to recover. In my case, when considering the characteristics of the *S. glycines*, the pathogen mostly remains on the lower canopy and gradually develop to the upper canopy. While I apply fungicide from the top canopy, it is possible that the pathogen escape from the application, and because the fungicide reduced the organism that occupied the space in leaves, which may allow Septoria to replicate in leaves.

This study used oxford nanopore sequencing technology to generate long amplicons covering the full-length ITS and partial LSU regions to study the phyllosphere microbiome in soybeans. I highlight the effect of cultivars and fungicide treatment on the fungal communities on soybean leaves. I also investigated the potential interaction between *Septoria* and other fungal organisms. Although I am still unable to identify OTUs to the species level due to the limitation of the database, this may be resolved in the near future when third-generation sequencing technologies applied to more studies, and more full-length ITS and LSU sequences become available in the database. I also believed that understanding the dynamic of the microbiome in the phyllosphere can facilitate the strategies for disease management.

CHAPTER 4 Tables and Figures

Table 4.1 Analysis of variance (ANOVA) to assess the effect of treatments (inoculation and no inoculation and cultivars on the alpha diversity index. Only statistically significant results were listed.

Taxonomy level	Growth stage	Alpha diversity index	Factor	F value	*p* value[2]
OTUs	V4	Richness[1]	Inoculation	0.23	NS
			Cultivars	4.29	0.0237**
			Inoculation*Cultivars	1.5	NS
		Shannon	Inoculation	0	NS
			Cultivars	4.37	0.0987*
			Inoculation*Cultivars	1.19	NS
		Simpson	Inoculation	0.13	NS
			Cultivars	8.74	0.0347**
			Inoculation*Cultivars	0.12	NS
Genus	V4	Richness	Inoculation	0.43	NS
			Cultivars	5.31	0.0749*
			Inoculation*Cultivars	1.36	NS
		Shannon	Inoculation	0.01	NS
			Cultivars	6	0.0625*
			Inoculation*Cultivars	0.71	NS
		Simpson	Treatment	0.15	NS
			Cultivars	9.35	0.031**
			Inoculation*Cultivars	0.12	NS

[1]The value was log-transformed to meet the assumption of normality and homogeneity of variance for the ANOVA test.

[2] NS: not significant, *p<0.1, **p<0.05, ***p<0.005

Table 4.2 Kruskal Wallis test to assess the effects of treatments[1] and cultivars on the alpha diversity index. Only statistically significant results were listed.

Taxonomy level	Growth stage / Cultivars	Factor	Alpha diversity index	Chi-squared	*p* value[2]
OTUs	V4	Cultivars	Richness	7.19	0.027**
			Shannon	7.96	0.019**
			Simpson	11.28	0.004***
	V4/Williams	Inoculation	Richness	5.04	0.024**
			Shannon	2.08	NS
			Simpson	0.03	NS
Genus	V4	Cultivars	Richness	9.92	0.007**
			Shannon	9.54	0.008**
			Simpson	11.07	0.004***
	V4/Williams	Inoculation	Richness	4.35	0.037**
			Shannon	2.08	NS
			Simpson	0.03	NS
	R5	Treatments	Richness	1.29	NS
			Shannon	7.79	0.051*
			Simpson	8.29	0.04**

[1] Treatments at V4 stage: inoculation and no inoculation; Treatments at R5 stage: inoculation/fungicide, inoculation/no fungicide, no inoculation/fungicide, and no inoculation/no fungicide.

[2] NS: not significant, *p<0.1, **p<0.05, ***p<0.005

Table 4.3 Permutation multivariate analysis of variance (PERMANOVA) to access the effect of treatments and cultivars at each growth stage at OTU- and genus level. The weighted Unifrac distance matrix was generated from the rarefied data set. Only statistically significant results were listed in the table.

Taxonomy level	Target region	Growth stage	Factors	F value	R^2	*p* value
OTU	ITS	V4	Inoculation	2.25	0.06	0.03**
			Cultivars	2.54	0.13	0.001***
			Inoculation*Cultivars	0.27	0.01	NS
		R5	Treatment	2.80	0.40	0.091*
			Cultivars	0.05	0.00	NS
			Treatment*Cultivars	0.07	0.02	NS
	LSU	V4	Inoculation	0.55	0.01	NS
			Cultivars	7.77	0.33	0.001***
			Inoculation*Cultivars	0.33	0.01	NS
Genus	ITS	V4	Inoculation	0.52	0.01	NS
			Cultivars	7.25	0.32	0.001**
			Inoculation*Cultivars	0.40	0.02	NS
	LSU	V4	Inoculation	0.48	0.01	0.623
			Cultivars	9.36	0.37	0.001***
			Inoculation*Cultivars	0.40	0.02	0.81

[1] NS: not significant, *p<0.1, **p<0.05, ***p<0.001

Table 4.4 List of core microbiome (Genus level) in soybeans. OTUs except OTU17 and OTU45 were identified as core microbiome with the entire data set or the subset data set (control samples).

OTU	Phylum	Class	Order	Family	Genus	Reads number	% of classified reads
OTU1	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Gibberella	899301	33.70
OTU2	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria	646797	24.24
OTU7	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Didymella	314157	11.77
OTU8	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	240713	9.02
OTU4	Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	Plectosphaerella	204665	7.67
OTU10	Ascomycota	Sordariomycetes	Glomerellales	Glomerellaceae	Colletotrichum	69035	2.59
OTU14	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Bipolaris	58795	2.20
OTU17[1]	Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	Septoria	44915	1.68
OTU28	Ascomycota	Dothideomycetes	Pleosporales	unidentified	Pleosporales_sp	42599	1.60
OTU45[2]	Ascomycota	Dothideomycetes	Pleosporales	unclassified_Pleosporales	unclassified_Pleosporales	3745	0.14

[1] Present when using all samples as input data
[2] Present only in the LSU data set

Table 4.5 Number of OTUs differentially abundant for each data set and properties of correspondent network.

Data set			Deseq2		
Stage	Sample size	All taxa	Number of taxa promoted in inoculated condition	Number of taxa promoted after fungicide application	Number of taxa supressed after fungicide application
V4	36	649	1	-	-
R3	34	649	1	-	-
R5	24	649	2	7	21
V4_Inoc	18	649	-	-	-
V4_NIC	18	649	-	-	-
R3_Inoc	17	649	-	-	-
R3_NIC	17	649	-	-	-
R5_Inoc (Inoc/Fun + Inoc/noFun)	12	649	-	3	10
R5_NIC (NIC/fun + NIC/noFun)	12	649	-	3	16
R5_Fun (Fun/Inoc + Fun/NIC)	12	649	-	-	-
R5_noFun (noFun/Inoc + noFun/ NIC)	12	649	2	-	-

Table 4.5 (conc)

Data set			Network analysis								
						All edges					
Stage	Sample size	All taxa	Filtered taxa (reads >6)	Positive edge	Negative edge	Sg interaction	Alpha centrality	sd	Betweeness centrality	sd	Average Degree (total edge/total node)
V4	36	649	120	129	24	1	1.58	0.78	76.55	143.72	1.19
R3	34	649	187	285	67	3	1.04	4.69	376.30	386.62	1.90
R5	24	649	187	308	61	5	3.26	3.66	337.20	335.18	1.99
V4_Inoc	18	649	135	188	62	0	1.24	0.32	248.20	352.40	1.05
V4_NIC	18	649	95	100	44	0	1.14	0.30	47.64	103.04	0.91
R3_Inoc	17	649	117	67	18	1	1.11	0.18	27.38	62.02	0.99
R3_NIC	17	649	165	171	45	1	1.54	0.72	367.39	499.93	1.35
R5_Inoc (Inoc/Fun + Inoc/noFun)	12	649	134	105	18	1	1.19	0.20	210.30	416.78	1.06
R5_NIC (NIC/fun + NIC/noFun)	12	649	158	176	60	2	1.67	0.69	358.80	400.12	1.43
R5_Fun (Fun/Inoc + Fun/NIC)	12	649	131	82	33	1	1.13	0.23	129.60	230.18	1.02
R5_noFun (noFun/Inoc + noFun/ NIC)	12	649	140	107	30	1	1.20	0.22	232.50	385.56	1.08

Table 4.5 (conc)

Data set			Network analysis					
			Edge stability >0.5			Edge Stability >0.8		
Stage	Sample size	All taxa	Positive edge	Negative edge	Sg interaction	Positive edge	Negative edge	Sg interaction
V4	36	649	110	15	1	61	6	0
R3	34	649	228	46	2	119	17	2
R5	24	649	246	27	4	128	11	2
V4_Inoc	18	649	-	-	-	-	-	-
V4_NIC	18	649	-	-	-	-	-	-
R3_Inoc	17	649	-	-	-	-	-	-
R3_NIC	17	649	-	-	-	-	-	-
R5_Inoc (Inoc/Fun + Inoc/noFun)	12	649	-	-	-	-	-	-
R5_NIC (NIC/fun + NIC/noFun)	12	649	-	-	-	-	-	-
R5_Fun (Fun/Inoc + Fun/NIC)	12	649	-	-	-	-	-	-
R5_noFun (noFun/Inoc + noFun/ NIC)	12	649	-	-	-	-	-	-

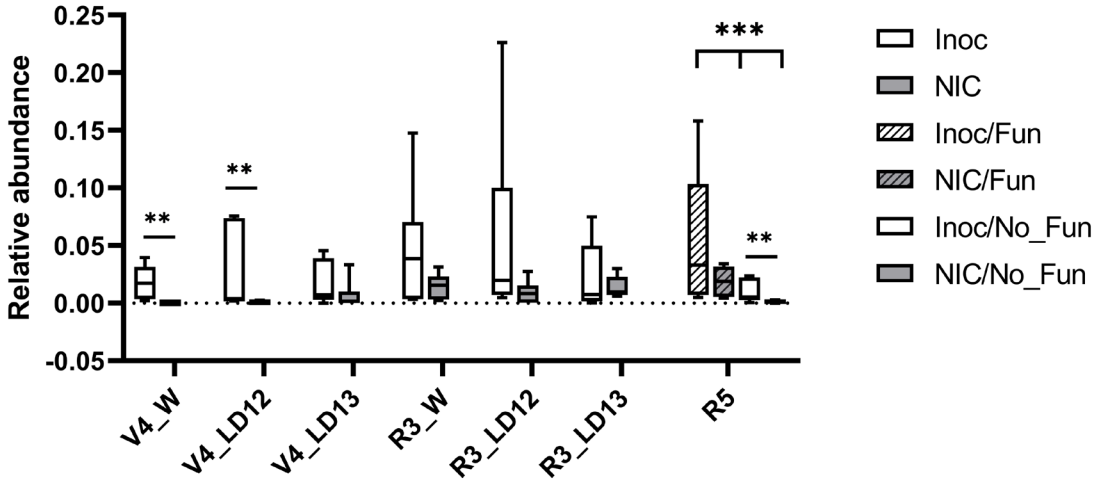


Figure 4.1 The relative abundance of *Septoria* at V4, R3, and R5 stage. At V4 and R3 stage, each box consisted of three replicates for W(illiams), LD12(-8677), and LD13(-14071R2). At R5 stage, the cultivars were merged for analysis since there was no significant difference between cultivars, and each box consisted of 6 replicates. The Wilcoxon rank-sum tests were performed for treatments (Inoc vs NIC, Fun vs No_Fun).(*p<0.1, **p<0.05, ***p<0.005

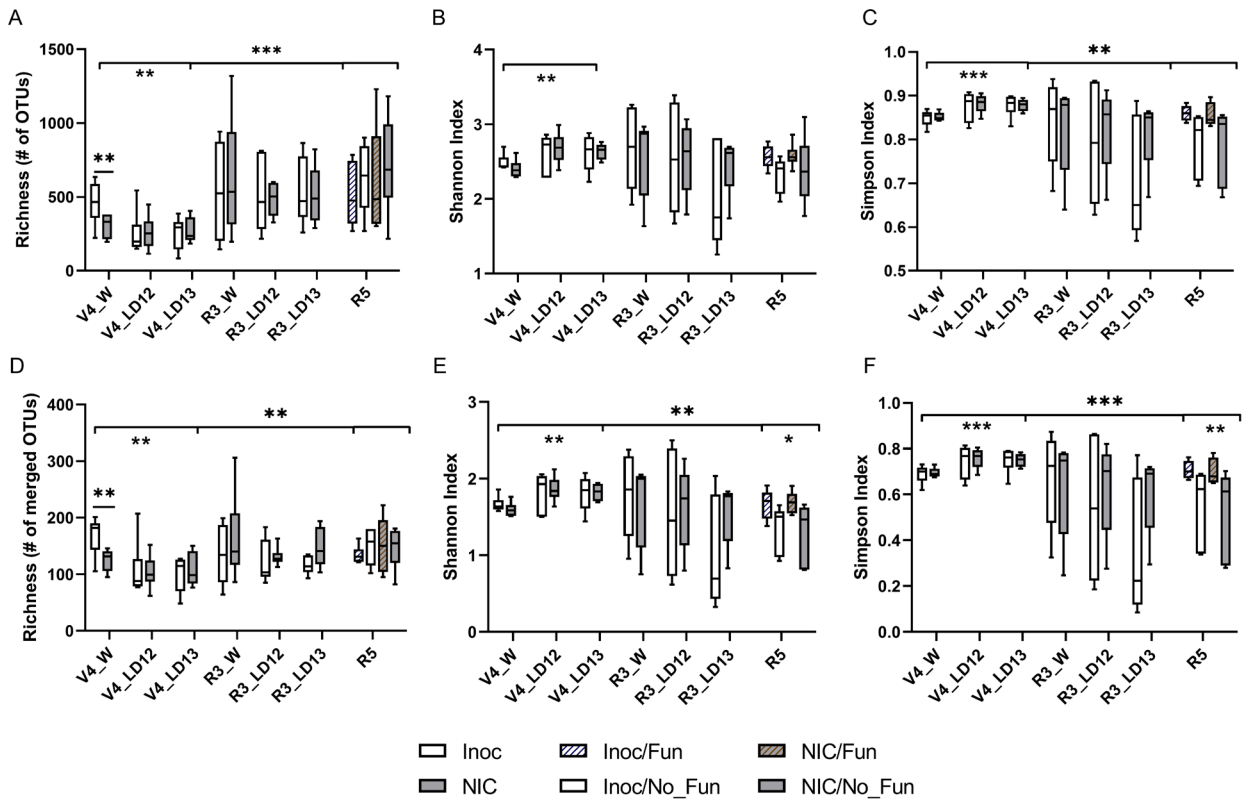


Figure 4.2 Boxplots showing the differences of three alpha diversity indices (Richness, Shannon index, and Simpson index) between treatments, cultivars, and growth stage using data at (A)-(C) OTU level and (D)-(F) Genus level. At V4 and R3 stage, each box consisted of three replicates for W(illmas), LD12(-8677), and LD13(-14071R2). At R5 stage, the cultivars were merged for analysis since there was no significant difference between cultivars, and each box consisted of 6 replicates. The Kruskal-Wallis test s were performed for treatments, cultivars, and growth stage. (*p<0.1, **p<0.05, ***p<0.005)

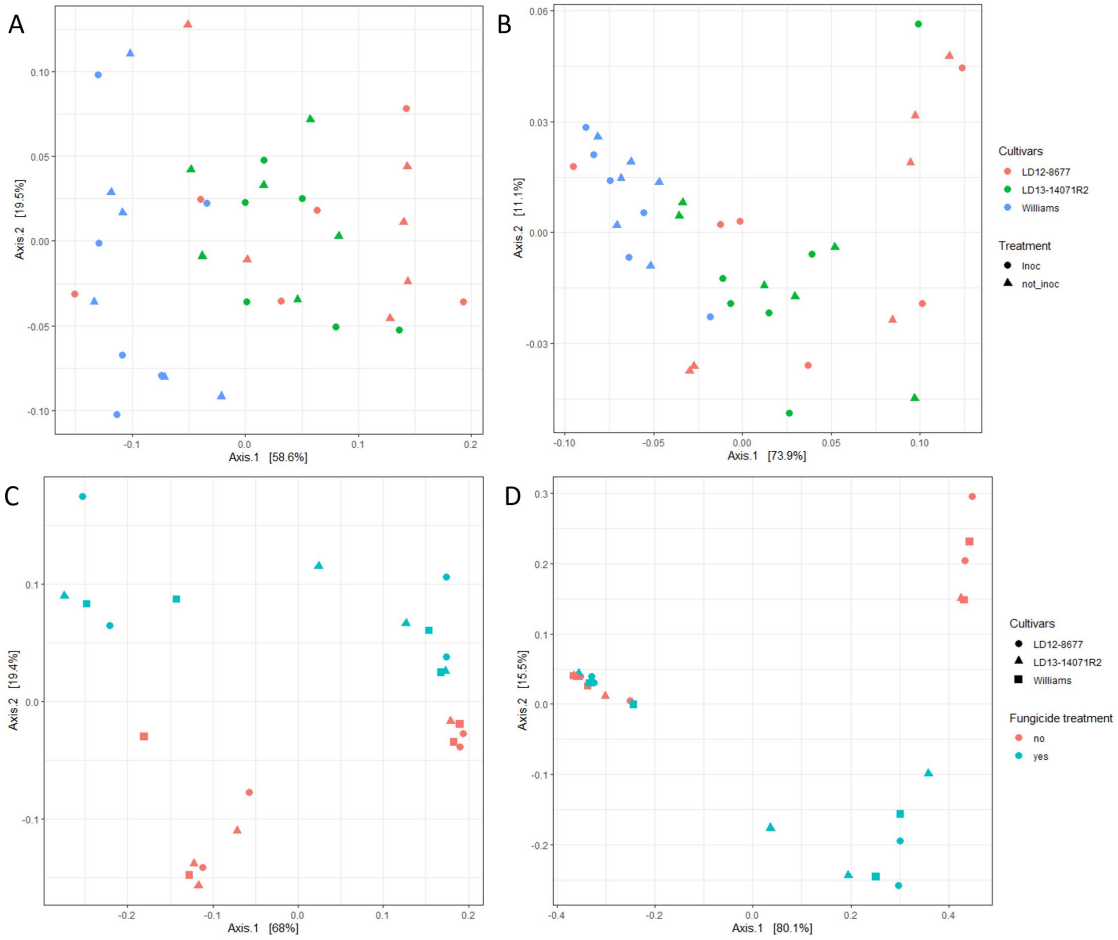


Figure 4.3 Principal coordinates analysis (PCoA) of weighted unifrac distance matrix. The distance matrices were calculated with the rarefied data (ITS or LSU data set) at the genus level by the growth stage. (A) V4, ITS data (B) V4, LSU data (C) R5, ITS data, and (D) R5, LSU data

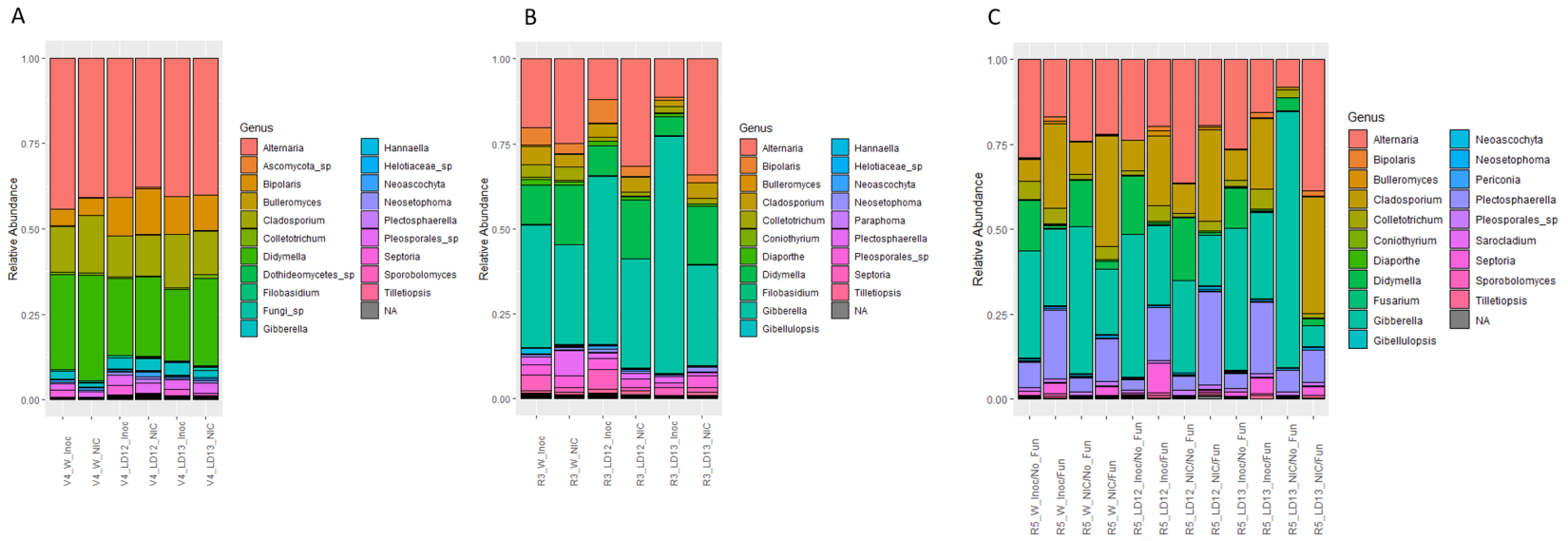


Figure 4.4 Stacked bar plots showing the relative abundance of fungal community across growth stage, cultivars, and treatments. (A) V4 (B) R3 and (C) R5. The top 20 most abundant taxa were listed on the legends. At V4 and R3 stage, each bar consisted of three replicates for W(illiams), LD12(-8677), and LD13(-14071R2). At R5 stage, each bar consisted of two replicates.

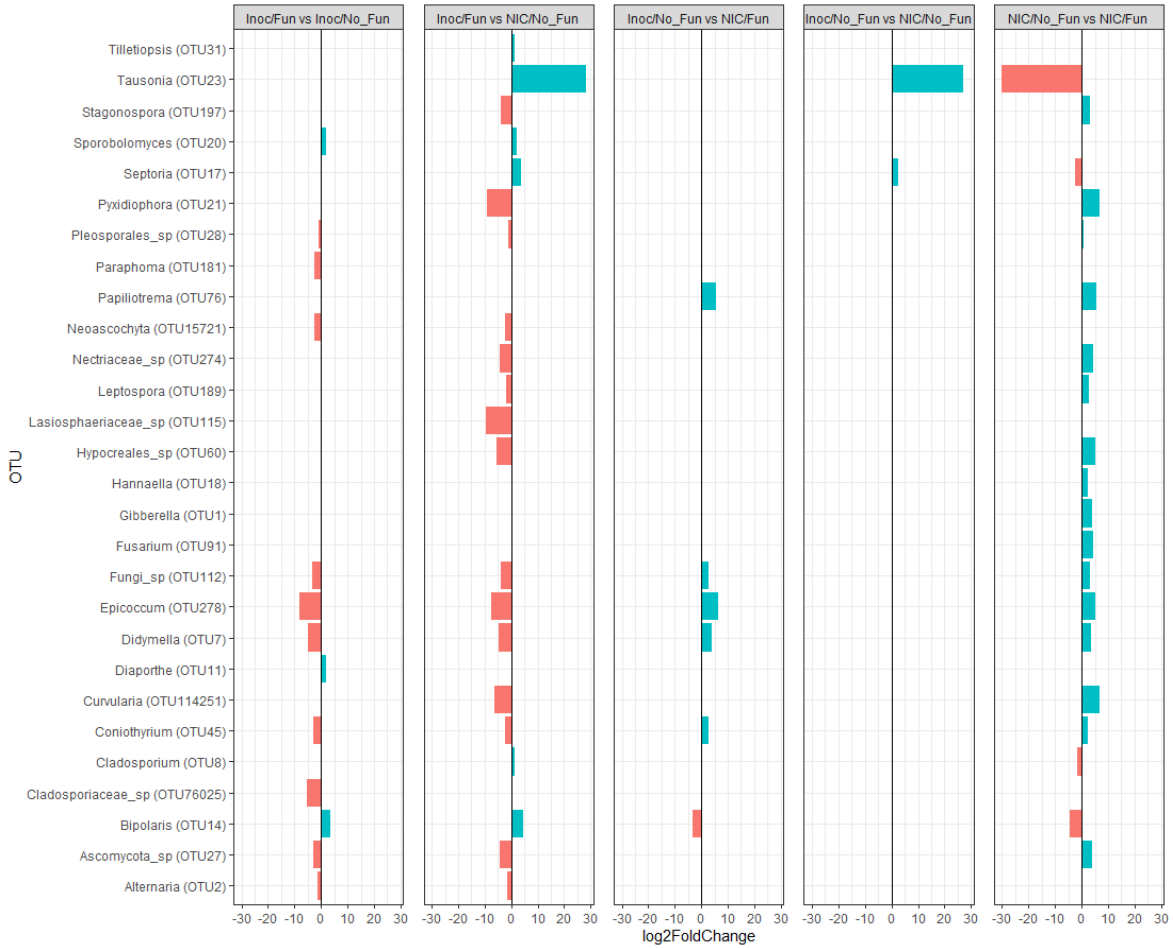


Figure 4.5 OTU differential abundance between treatments at R5 stage. The analysis was performed at the genus level, and only the OTUs with adjusted p-value <0.05 were shown. Each column represents a comparison between treatments combinations with abbreviations: Inoc(ulated), NIC(non-inoculated), Fun(gicide), and No_Fun(gicide). A blue bar indicates a higher abundance of OTUs, and a red bar indicates a lower abundance of OTUs in the former data set in each comparison.

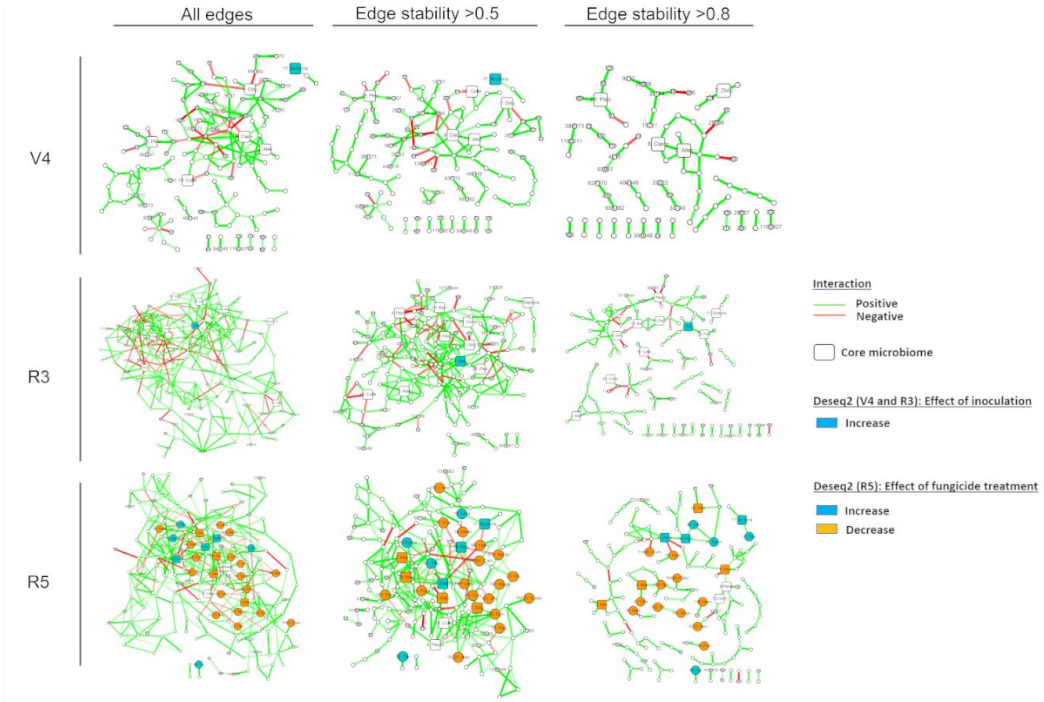


Figure 4.6 Network analysis for V4, R3, and R5 data. The networks were presented with all edges and filtered by the edge stability >0.5 and >0.8. The squared node represents that the OTUs were identified as core microbiome from the core microbiome analysis. The node color corresponds to the results of OTU differential analysis using Deseq2. Edges color corresponds to potential positive (green) and negative (red) interactions between OTUs. The nodes plotted with OTU numbers were classified to a known organism at the Genus level. The nodes without labels were unclassified OTUs. The OTUs that were differentially abundant from the Deseq2 results were labeled with OTU numbers followed by the abbreviations of the genus name. Alter(naria), Bipo(laris), Clado(sporiaceae), Clado(sporium), Colle(totrichum), Coni(othyrium), Curv(ularia), Diap(orthe), Didy(mella), Epic(occum), Fusa(rium), Gibb(erella), Hann(aella), Hypo(creales), Lasio(sphaeriaceae), Lepto(spora), Nectri(aceae), Neo(ascochyta), Papi(liotrema), Para(phoma), Pleo(sporales), Pyxi(diophora), Septoria, Sporo(bolomyces), Stago(nospora), Tau(sonia), and Tille(tiopsis).

Supplementary Table 4.1 Sample information and sequencing results.

Sample Name	Barcode	Plot ID	Seed	Rep	Treatment	Growth stage	Collection date	Bases	Reads	Filtered reads
QM001	BC01	18QM1011	LD13-14071R2	1	Inoc/No_Fun	V4	6/11/2018	10,873,922	6,597	6,585
QM002	BC13	18QM1012	Williams	1	Inoc/No_Fun	V4	6/11/2018	35,732,459	21,923	21,907
QM003	BC25	18QM1013	LD12-8677	1	Inoc/No_Fun	V4	6/11/2018	16,066,850	9,788	9,780
QM004	BC37	18QM1021	LD12-8677	1	Inoc/Fun	V4	6/11/2018	177,783,271	108,059	106,746
QM005	BC49	18QM1022	LD13-14071R2	1	Inoc/Fun	V4	6/11/2018	14,893,285	9,107	9,094
QM006	BC61	18QM1023	Williams	1	Inoc/Fun	V4	6/11/2018	120,507,402	73,701	73,540
QM007	BC73	18QM1031	LD13-14071R2	1	NIC/Fun	V4	6/11/2018	45,777,644	27,941	27,816
QM008	BC85	18QM1032	LD12-8677	1	NIC/Fun	V4	6/11/2018	22,669,418	13,759	13,740
QM009	BC02	18QM1033	Williams	1	NIC/Fun	V4	6/11/2018	52,640,125	32,290	32,254
QM010	BC14	18QM1041	LD13-14071R2	1	NIC/No_Fun	V4	6/11/2018	20,626,871	12,434	12,218
QM011	BC26	18QM1042	Williams	1	NIC/No_Fun	V4	6/11/2018	60,077,337	36,665	36,350
QM012	BC38	18QM1043	LD12-8677	1	NIC/No_Fun	V4	6/11/2018	32,402,149	19,619	19,596
QM013	BC50	18QM2011	LD13-14071R2	2	Inoc/Fun	V4	6/13/2018	32,185,711	19,672	19,629
QM014	BC62	18QM2012	LD12-8677	2	Inoc/Fun	V4	6/13/2018	16,340,842	9,790	9,744
QM015	BC74	18QM2013	Williams	2	Inoc/Fun	V4	6/13/2018	96,643,696	59,507	59,456
QM016	BC86	18QM2021	LD13-14071R2	2	Inoc/No_Fun	V4	6/13/2018	27,508,509	16,817	16,786
QM017	BC03	18QM2022	LD12-8677	2	Inoc/No_Fun	V4	6/13/2018	25,290,160	15,488	15,475
QM018	BC15	18QM2023	Williams	2	Inoc/No_Fun	V4	6/13/2018	115,547,826	71,167	71,061
QM019	BC27	18QM2031	LD13-14071R2	2	NIC/No_Fun	V4	6/13/2018	21,387,283	13,007	12,972

Supplementary Table 4.1 (con.)

Sample Name	Barcode	Plot ID	Seed	Rep	Treatment	Growth stage	Collection date	Bases	Reads	Filtered reads
QM020	BC39	18QM2032	LD12-8677	2	NIC/No_Fun	V4	6/13/2018	7,190,195	4,318	4,256
QM021	BC51	18QM2033	Williams	2	NIC/No_Fun	V4	6/13/2018	28,293,676	17,367	17,306
QM022	BC63	18QM2041	LD13-14071R2	2	NIC/Fun	V4	6/13/2018	17,862,079	10,629	10,197
QM023	BC75	18QM2042	Williams	2	NIC/Fun	V4	6/13/2018	56,276,012	34,403	34,247
QM024	BC87	18QM2043	LD12-8677	2	NIC/Fun	V4	6/13/2018	30,713,951	18,305	17,906
QM025	BC04	18QM3011	LD12-8677	3	Inoc/Fun	V4	6/14/2018	39,500,452	23,728	23,242
QM026	BC16	18QM3012	LD13-14071R2	3	Inoc/Fun	V4	6/14/2018	29,454,395	17,892	17,724
QM027	BC28	18QM3013	Williams	3	Inoc/Fun	V4	6/14/2018	60,191,278	36,846	36,750
QM028	BC40	18QM3021	Williams	3	NIC/Fun	V4	6/14/2018	35,769,183	21,943	21,780
QM029	BC52	18QM3022	LD13-14071R2	3	NIC/Fun	V4	6/14/2018	48,796,126	29,711	29,531
QM030	BC64	18QM3023	LD12-8677	3	NIC/Fun	V4	6/14/2018	42,210,047	25,882	25,799
QM031	BC76	18QM3031	Williams	3	Inoc/No_Fun	V4	6/14/2018	84,569,967	52,104	52,041
QM032	BC88	18QM3032	LD13-14071R2	3	Inoc/No_Fun	V4	6/14/2018	57,429,042	34,282	33,543
QM033	BC05	18QM3033	LD12-8677	3	Inoc/No_Fun	V4	6/14/2018	17,379,181	10,462	10,407
QM034	BC17	18QM3041	Williams	3	NIC/No_Fun	V4	6/14/2018	44,622,533	27,395	27,264
QM035	BC29	18QM3042	LD13-14071R2	3	NIC/No_Fun	V4	6/14/2018	16,912,015	10,282	10,178
QM036	BC41	18QM3043	LD12-8677	3	NIC/No_Fun	V4	6/14/2018	16,010,742	9,790	9,736
QM037	BC53	18QM1011	LD13-14071R2	1	Inoc/No_Fun	R3	7/10/2018	1,127,355	642	574
QM038	BC65	18QM1012	Williams	1	Inoc/No_Fun	R3	7/10/2018	13,184,914	7,814	7,464
QM039	BC77	18QM1013	LD12-8677	1	Inoc/No_Fun	R3	7/10/2018	17,216,849	9,648	8,371

Supplementary Table 4.1 (con.)

Sample Name	Barcode	Plot ID	Seed	Rep	Treatment	Growth stage	Collection date	Bases	Reads	Filtered reads
QM040	BC89	18QM1021	LD12-8677	1	Inoc/Fun	R3	7/10/2018	21,326,813	12,857	12,532
QM041	BC06	18QM1022	LD13-14071R2	1	Inoc/Fun	R3	7/10/2018	46,699,986	28,330	28,089
QM042	BC18	18QM1023	Williams	1	Inoc/Fun	R3	7/10/2018	22,142,482	13,402	13,146
QM043	BC30	18QM1031	LD13-14071R2	1	NIC/Fun	R3	7/10/2018	114,413,707	70,281	70,092
QM044	BC42	18QM1032	LD12-8677	1	NIC/Fun	R3	7/10/2018	37,806,777	23,066	22,992
QM045	BC54	18QM1033	Williams	1	NIC/Fun	R3	7/10/2018	13,663,604	8,368	8,341
QM046	BC66	18QM1041	LD13-14071R2	1	NIC/No_Fun	R3	7/10/2018	38,982,342	23,858	23,832
QM047	BC78	18QM1042	Williams	1	NIC/No_Fun	R3	7/10/2018	36,610,596	22,071	21,433
QM048	BC90	18QM1043	LD12-8677	1	NIC/No_Fun	R3	7/10/2018	36,389,146	21,843	21,383
QM049	BC07	18QM2011	LD13-14071R2	2	Inoc/Fun	R3	7/11/2018	135,858,816	84,492	84,373
QM050	BC19	18QM2012	LD12-8677	2	Inoc/Fun	R3	7/11/2018	47,934,230	29,921	29,862
QM051	BC31	18QM2013	Williams	2	Inoc/Fun	R3	7/11/2018	199,473,703	123,042	122,895
QM052	BC43	18QM2021	LD13-14071R2	2	Inoc/No_Fun	R3	7/11/2018	91,795,942	56,429	56,254
QM053	BC55	18QM2022	LD12-8677	2	Inoc/No_Fun	R3	7/11/2018	68,656,119	42,323	42,270
QM054	BC67	18QM2023	Williams	2	Inoc/No_Fun	R3	7/11/2018	90,383,255	55,590	55,512
QM055	BC79	18QM2031	LD13-14071R2	2	NIC/No_Fun	R3	7/11/2018	29,914,951	18,188	18,096
QM056	BC91	18QM2032	LD12-8677	2	NIC/No_Fun	R3	7/11/2018	71,797,941	44,186	44,152
QM057	BC08	18QM2033	Williams	2	NIC/No_Fun	R3	7/11/2018	72,853,795	44,610	44,430
QM058	BC20	18QM2041	LD13-14071R2	2	NIC/Fun	R3	7/11/2018	-	-	-

Supplementary Table 4.1 (con.)

Sample Name	Barcode	Plot ID	Seed	Rep	Treatment	Growth stage	Collection date	Bases	Reads	Filtered reads
QM059	BC32	18QM2042	Williams	2	NIC/Fun	R3	7/11/2018	364,643,893	224,933	224,695
QM060	BC44	18QM2043	LD12-8677	2	NIC/Fun	R3	7/11/2018	38,039,978	23,385	23,341
QM061	BC56	18QM3011	LD12-8677	3	Inoc/Fun	R3	7/12/2018	130,668,084	81,321	81,278
QM062	BC68	18QM3012	LD13-14071R2	3	Inoc/Fun	R3	7/12/2018	103,233,059	64,547	64,515
QM063	BC80	18QM3013	Williams	3	Inoc/Fun	R3	7/12/2018	187,501,708	116,913	116,856
QM064	BC92	18QM3021	Williams	3	NIC/Fun	R3	7/12/2018	102,345,259	63,602	63,566
QM065	BC09	18QM3022	LD13-14071R2	3	NIC/Fun	R3	7/12/2018	160,959,791	99,797	99,607
QM066	BC21	18QM3023	LD12-8677	3	NIC/Fun	R3	7/12/2018	70,643,416	43,951	43,935
QM067	BC33	18QM3031	Williams	3	Inoc/No_Fun	R3	7/12/2018	31,110,210	19,244	19,236
QM068	BC45	18QM3032	LD13-14071R2	3	Inoc/No_Fun	R3	7/12/2018	96,482,331	60,312	60,265
QM069	BC57	18QM3033	LD12-8677	3	Inoc/No_Fun	R3	7/12/2018	48,685,890	30,347	30,335
QM070	BC69	18QM3041	Williams	3	NIC/No_Fun	R3	7/12/2018	121,003,723	75,534	75,489
QM071	BC81	18QM3042	LD13-14071R2	3	NIC/No_Fun	R3	7/12/2018	87,441,266	54,424	54,387
QM072	BC93	18QM3043	LD12-8677	3	NIC/No_Fun	R3	7/12/2018	77,696,964	48,495	48,477
QM074	BC10	18QM1012	Williams	1	Inoc/No_Fun	R5	7/23/2018	218,578,144	136,094	136,007
QM075	BC22	18QM1013	LD12-8677	1	Inoc/No_Fun	R5	7/23/2018	57,985,430	36,157	36,145
QM076	BC34	18QM1021	LD12-8677	1	Inoc/Fun	R5	7/23/2018	99,075,232	61,543	61,507
QM077	BC46	18QM1022	LD13-14071R2	1	Inoc/Fun	R5	7/23/2018	100,868,249	62,425	62,404

Supplementary Table 4.1 (con.)

Sample Name	Barcode	Plot ID	Seed	Rep	Treatment	Growth stage	Collection date	Bases	Reads	Filtered reads
QM079	BC58	18QM1031	LD13-14071R2	1	NIC/Fun	R5	7/23/2018	32,194,765	19,875	19,851
QM080	BC70	18QM1032	LD12-8677	1	NIC/Fun	R5	7/23/2018	258,067,103	160,379	160,233
QM081	BC82	18QM1033	Williams	1	NIC/Fun	R5	7/23/2018	137,529,672	85,450	85,402
QM082	BC94	18QM1041	LD13-14071R2	1	NIC/No_Fun	R5	7/23/2018	102,151,173	63,739	63,697
QM083	BC11	18QM1042	Williams	1	NIC/No_Fun	R5	7/23/2018	126,955,800	79,275	79,237
QM084	BC23	18QM1043	LD12-8677	1	NIC/No_Fun	R5	7/23/2018	110,315,607	68,041	68,007
QM087	BC35	18QM2013	Williams	2	Inoc/Fun	R5	7/24/2018	122,878,329	75,701	75,664
QM088	BC47	18QM2021	LD13-14071R2	2	Inoc/No_Fun	R5	7/24/2018	122,294,232	76,175	76,144
QM091	BC59	18QM2031	LD13-14071R2	2	NIC/No_Fun	R5	7/24/2018	113,716,522	70,531	70,489
QM097	BC71	18QM3011	LD12-8677	3	Inoc/Fun	R5	7/25/2018	83,863,196	51,257	51,192
QM098	BC83	18QM3012	LD13-14071R2	3	Inoc/Fun	R5	7/25/2018	44,491,707	27,191	27,120
QM099	BC95	18QM3013	Williams	3	Inoc/Fun	R5	7/25/2018	64,000,552	39,154	39,081
QM100	BC12	18QM3021	Williams	3	NIC/Fun	R5	7/25/2018	112,579,602	69,409	69,294
QM101	BC24	18QM3022	LD13-14071R2	3	NIC/Fun	R5	7/25/2018	29,723,064	18,275	18,252
QM102	BC36	18QM3023	LD12-8677	3	NIC/Fun	R5	7/25/2018	83,058,106	51,028	50,939
QM103	BC48	18QM3031	Williams	3	Inoc/No_Fun	R5	7/25/2018	24,448,583	15,055	15,048
QM104	BC60	18QM3032	LD13-14071R2	3	Inoc/No_Fun	R5	7/25/2018	116,827,508	71,705	71,660
QM105	BC72	18QM3033	LD12-8677	3	Inoc/No_Fun	R5	7/25/2018	115,650,733	71,241	71,199
QM106	BC84	18QM3041	Williams	3	NIC/No_Fun	R5	7/25/2018	105,975,405	65,288	65,200
QM108	BC96	18QM3043	LD12-8677	3	NIC/No_Fun	R5	7/25/2018	19,556,082	11,998	11,991

Supplementary Table 4.2 Number of OTUs from different cluster criteria after pre-cluster with 95% identity.

Cluster criteria (identity %)	Number of OTUs	Number of singletons[1]	Number of doubletons[1]	Number of OTUs after removing singletons	Number of OTUs after removing singletons and doubletons
95	3,097,478	3,096,255 (74%)[2]	836 (0.02%)	1,223	387
92	1,490,827	1,483,709 (35%)	3,776 (0.09%)	7,118	3,342
90	866,645	858,726 (21%)	3,861 (0.09%)	7,919	4,058
88	487,483	478,301 (11%)	3,998 (0.1%)	9,182	5,184
86	263,004	253,800 (6%)	3,766 (0.09%)	9,204	5,438

[1] Singleton: sequences that occurred one time across all samples; Doubletons: sequences that occurred two times across all samples.

[2] Percentage=number of singletons or doubletons/filtered reads (reads number=4,184,494) x 100%.

Supplementary Table 4.3 The number of hits, identity, and coverage after BLAST to the database.

Target region	Query sequence	Database	hits	Identity (%)				Coverage			
				Min.	Med.	Mean	Max.	Min.	Med.	Mean	Max.
ITS	2,842	Unite v8.2	2,840	82.34	97.75	97.47	100	29	100	98.78	100
LSU	2,976	RDP v11.5	2,972	80.27	97.53	97.36	100	23	99	97.93	100

Supplementary Table 4.4 Disease development for each soybean cultivars and four treatments at V4, R3, and R5 stage.

Cultivars	Treatment	Growth stage	Vertical progress (%)	Necrotic area (%)	Chlorotic area (%)	Defoliation rate (%)	qPCR (pg of *S.g* DNA)
LD12-8677	Inoc/ No_Fun	V4	0	0	0	0	0/0/0
		R3	40	5	4	20	16/0/0
		R5	36	4	3	23	0/0
	Inoc/ Fun	V4	0	0	0	0	-
		R3	41	5	4	23	-
		R5	38	4	3	27	-
	NIC/ No_Fun	V4	0	0	0	0	-
		R3	20	1	3	12	-
		R5	22	0	1	18	-
	NIC/ Fun	V4	0	0	0	0	0/0/0
		R3	21	1	3	13	0/0/0
		R5	19	0	1	16	0/0
LD13-14071R2	Inoc/ No_Fun	V4	0	0	0	0	0/0/0
		R3	37	6	5	15	11/1085/0
		R5	39	5	3	23	958/0
	Inoc/ Fun	V4	0	0	0	0	-
		R3	41	6	5	17	-
		R5	41	5	3	26	-
	NIC/ No_Fun	V4	0	0	0	0	-
		R3	14	0	3	7	-
		R5	21	1	1	16	-
	NIC/ Fun	V4	0	0	0	0	0/0/0
		R3	14	0	3	8	0/0/0
		R5	27	1	1	18	12/0

Supplementary Table 4.4 (con.)

Cultivars	Treatment	Growth stage	Vertical progress (%)	Necrotic area (%)	Chlorotic area (%)	Defoliation rate (%)	qPCR (pg of *S.g* DNA)
Williams	Inoc/ No_Fun	V4	0	0	0	0	0/0/0
		R3	39	5	4	20	12/368/0
		R5	35	4	2	23	0/0
	Inoc/ Fun	V4	0	0	0	0	-
		R3	40	5	4	26	-
		R5	37	5	4	25	-
	NIC/ No_Fun	V4	0	0	0	0	-
		R3	9	0	3	11	-
		R5	26	1	1	20	-
	NIC/ Fun	V4	0	0	0	0	0/0/0
		R3	5	0	3	14	387/0/0
		R5	19	1	1	19	3/0

Supplementary Table 4.5 Analysis of variance for the effect of treatments on disease severity of Septoria brown spot on their AUDPC values.

	Source	DF	F value	*p*-value
Vertical progress	Treatment	3	17.87	0.0021***
	Cultivar	2	4.06	0.0374**
	Cultivar*Treatment	6	1.17	NS
Chlorotic area (log-transformed)	Treatment	3	19.7	0.0017***
	Cultivar	2	0.55	NS
	Cultivar*Treatment	6	0.98	NS
Defoliation rate	Treatment	3	3.19	0.0849*
	Cultivar	2	4.41	0.0855*
	Cultivar*Treatment	6	0.21	NS
Necrotic area (square root transformed)	Treatment	3	163.4	<.0.0001***
	Cultivar	2	3.86	0.0429**
	Cultivar*Treatment	6	2.87	0.043**

NS: not significant, *p<0.1, **p<0.05, ***p<0.01

Supplementary Table 4.6 OTU differential test using DeSeq2 with the data collected at R5 stage (after fungicide application). The data were merged at Genus level for this analysis. All listed OTUs were significantly different between contrast with adjusted *p*-value < 0.05.

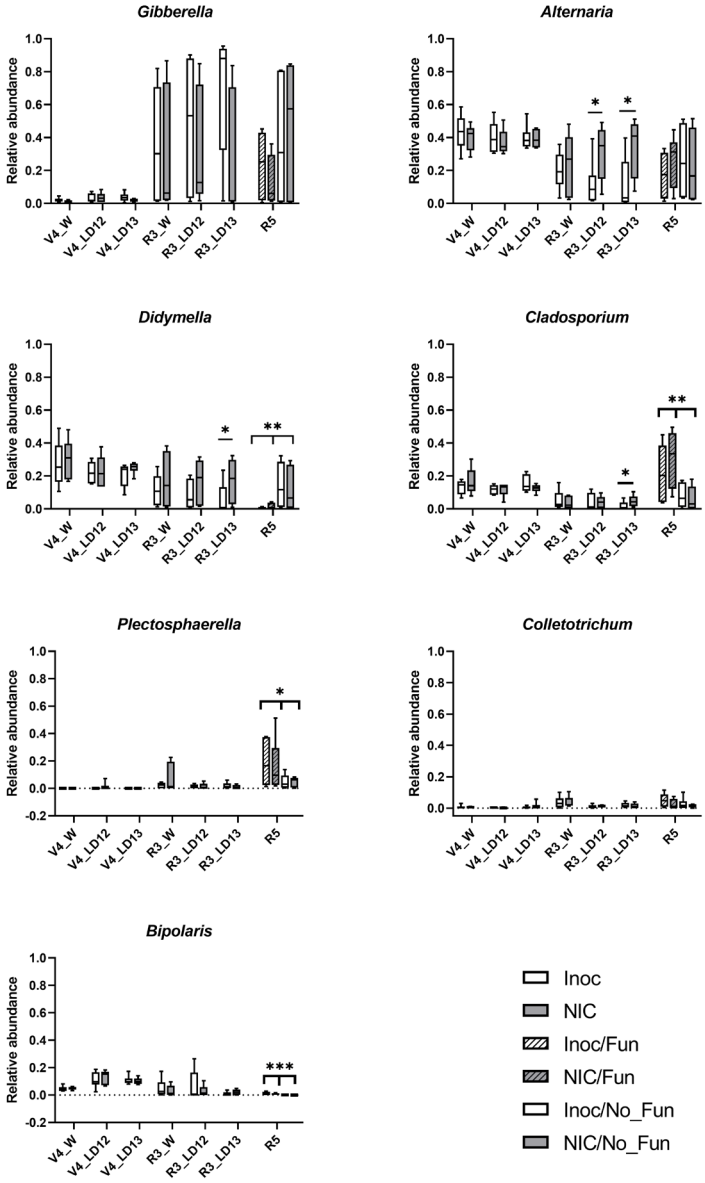
Target	ITS			LSU		
Contrast	OTU	log2FoldChange	Genus	OTU	log2FoldChange	Genus
Inoc/Fun vs Inoc/No_Fun	OTU2	-1.35	Alternaria	-	-	-
	OTU7	-5.02	Didymella	OTU7	-4.77	unclassified_Didymellaceae
	OTU11	1.93	Diaporthe	OTU11	2.27	Phomopsis
	OTU14	3.68	Bipolaris	OTU14	4.21	Tremella
	OTU20	1.82	Sporobolomyces	-	-	-
	OTU27	-2.84	Ascomycota_sp			
	OTU28	-0.89	Pleosporales_sp	OTU141	-4.28	unclassified_Dothideomycetes
	OTU45	-2.93	Coniothyrium	OTU45	-1.89	unclassified_Pleosporales
	OTU112	-3.42	Fungi_sp	-	-	-
	OTU181	-2.55	Paraphoma	-	-	-
	OTU278	-8.28	Epicoccum	OTU278	-5.94	Davidiella
	OTU15721	-2.53	Neoascochyta	-	-	-
	OTU76025	-5.34	Cladosporiaceae_sp			
Inoc/No_Fun vs NIC /No_Fun	OTU17	2.42	Septoria	OTU17	2.40	
	OTU23	27.25	Tausonia	OTU23	17.37	
Inoc/No_Fun vs NIC /Fun	OTU7	3.84	Didymella	OTU7	3.47	
	-	-	-	OTU11	-2.04	
	OTU14	-3.41	Bipolaris	OTU14	-4.13	
	OTU45	2.89	Coniothyrium	-	-	
	OTU76	5.61	Papiliotrema	-	-	
	-	-	-	OTU104	-1.86	
	OTU112	2.58	Fungi_sp	-	-	
	-	-	-	OTU141	3.67	
	OTU278	6.18	Epicoccum	-	-	

Supplementary Table 4.6 (con.)

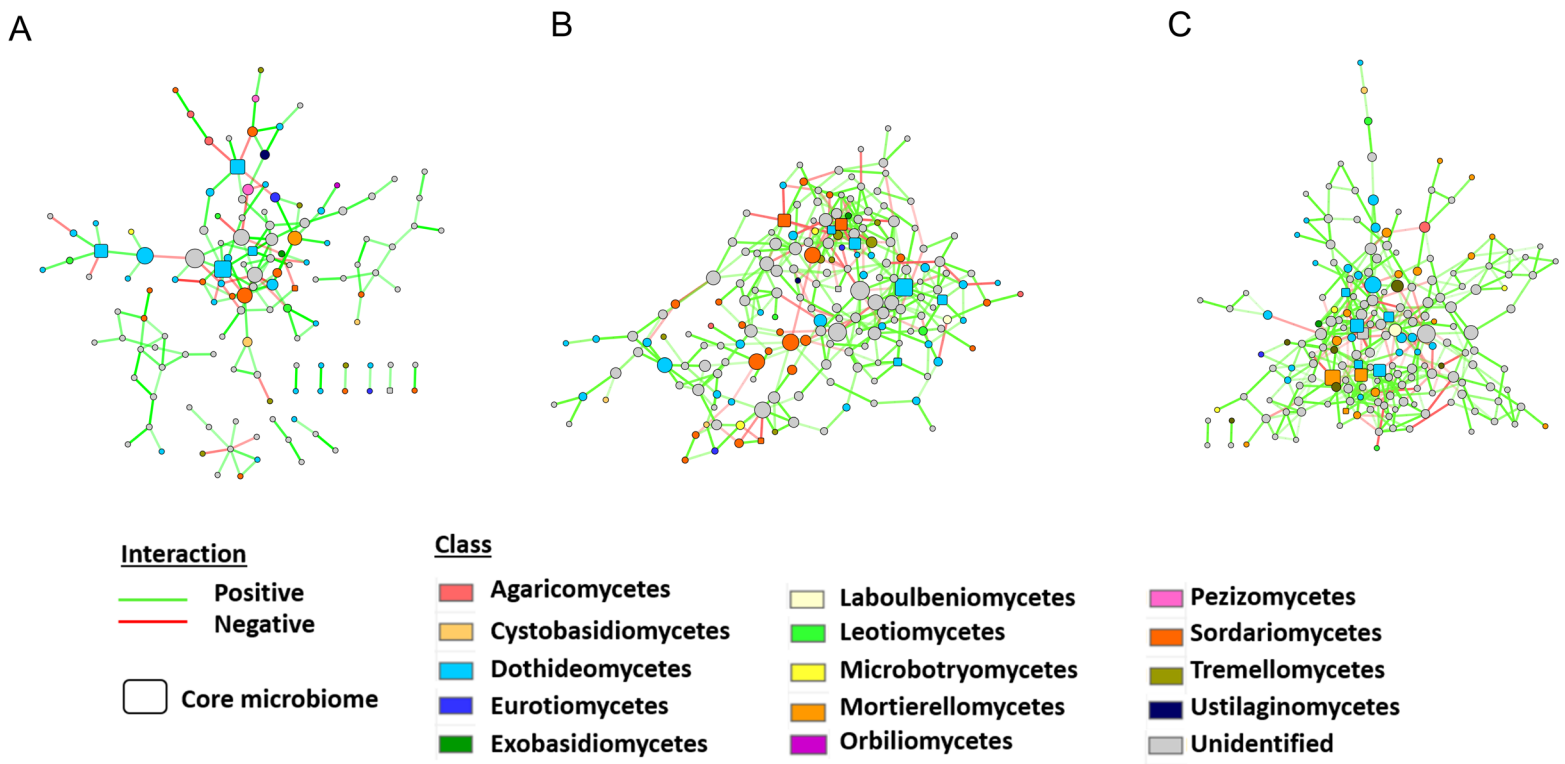
Target		ITS			LSU	
Contrast	OTU	log2FoldChange	Genus	OTU	log2FoldChange	Genus
Inoc/Fun vs NIC/No_Fun	OTU2	-1.42	Alternaria			
	OTU7	-4.77	Didymella	OTU7	-4.40	unclassified_Didymellaceae
	OTU8	1.39	Cladosporium	OTU8	1.80	unclassified_Davidiellaceae
	-	-	-	OTU11	1.53	Phomopsis
	OTU14	4.70	Bipolaris	OTU14	5.47	Tremella
	OTU17	3.75	Septoria	OTU17	4.23	Septoria
	OTU20	2.22	Sporobolomyces	OTU20	2.71	Leucosporidium
	OTU21	-9.00	Pyxidiophora	OTU21	-8.36	Pyxidiophora
	OTU23	28.09	Tausonia	OTU23	18.37	Guehomyces
	OTU27	-4.48	Ascomycota_sp	-	-	-
	OTU28	-1.14	Pleosporales_sp	-	-	-
	OTU31	1.24	Tilletiopsis	OTU31	1.68	unclassified_Exobasidiomycetes
	OTU45	-2.47	Coniothyrium	OTU45	-1.62	unclassified_Pleosporales
	OTU60	-5.48	Hypocreales_sp	-	-	-
	-	-	-	OTU69	-2.39	Phaeodothis
	OTU112	-3.98	Fungi_sp	-	-	-
	OTU115	-9.37	Lasiosphaeriaceae_sp	OTU115	-8.61	Cercophora
	-	-	-	OTU141	-3.74	unclassified_Dothideomycetes
	OTU189	-2.06	Leptospora	-	-	-
	OTU197	-3.73	Stagonospora	-	-	-
	OTU274	-4.22	Nectriaceae_sp	-	-	-
	OTU278	-7.37	Epicoccum	-	-	-
	OTU114251	-6.41	Curvularia	-	-	-
	OTU15721	-2.33	Neoascochyta	-	-	-

Supplementary Table 4.6 (con.)

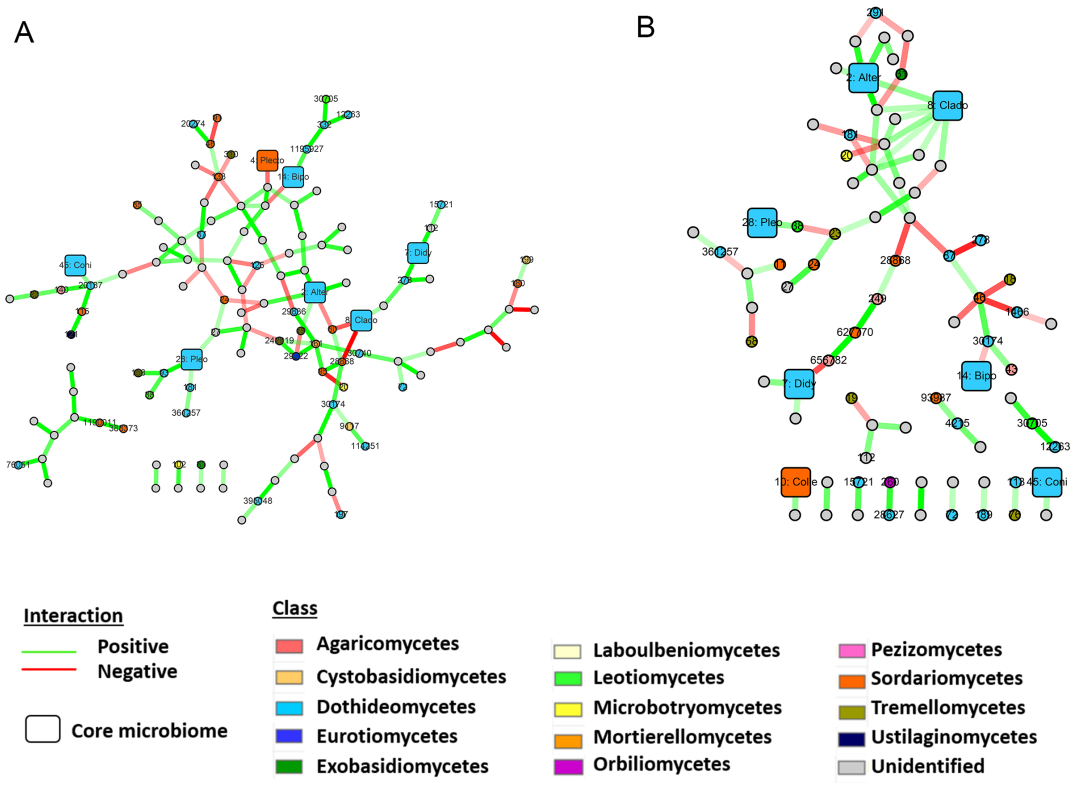
Target		ITS			LSU	
Contrast	OTU	log2FoldChange	Genus	OTU	log2FoldChange	Genus
NIC /No_Fun vs NIC /Fun	OTU1	4.08	Gibberella	OTU1	3.56	unclassified_Hypocreales
	OTU7	3.58	Didymella	OTU7	3.10	unclassified_Didymellaceae
	OTU8	-1.83	Cladosporium	OTU8	-2.40	unclassified_Davidiellaceae
	OTU14	-4.43	Bipolaris	OTU14	-5.38	Tremella
	OTU17	-2.44	Septoria	OTU17	-2.81	Septoria
	OTU18	2.37	Hannaella	-	-	-
	-	-	-	OTU20	-2.24	Leucosporidium
	OTU21	6.63	Pyxidiophora	OTU21	5.95	Pyxidiophora
	OTU23	-30.13	Tausonia	OTU23	-20.94	Guehomyces
	OTU27	3.95	Ascomycota_sp	-	-	-
	OTU28	0.80	Pleosporales_sp	-	-	-
	OTU45	2.44	Coniothyrium	-	-	-
	OTU60	5.17	Hypocreales_sp	OTU60	5.18	Niesslia
	OTU76	5.77	Papiliotrema	OTU76	3.07	Fibulobasidium
	OTU91	4.36	Fusarium	-	-	-
	-	-	-	OTU99	3.58	Gibberella
	-	-	-	OTU104	-2.44	Cladosporium_complex
	OTU112	3.14	Fungi_sp	-	-	-
	-	-	-	OTU141	3.14	unclassified_Dothideomycetes
	OTU189	2.80	Leptospora	-	-	-
	OTU197	3.11	Stagonospora	-	-	-
	OTU274	4.47	Nectriaceae_sp	-	-	-
	OTU278	5.27	Epicoccum	-	-	-
	OTU114251	6.88	Curvularia	-	-	-



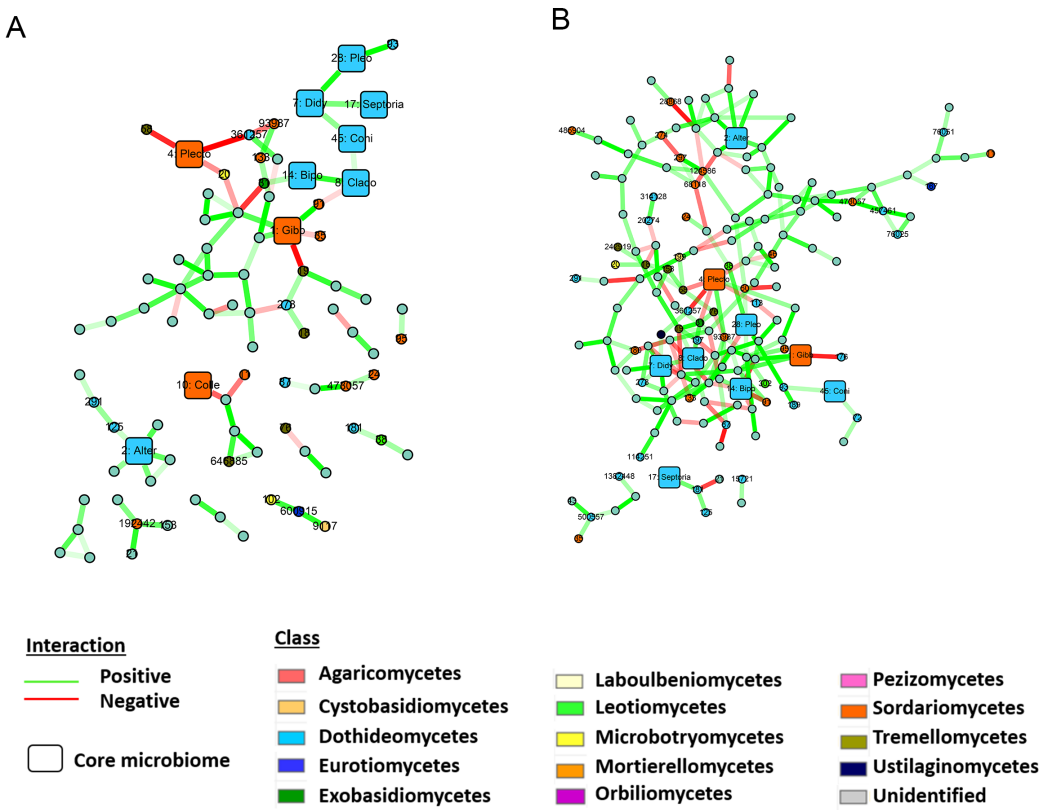
Supplementary Figure 4.1 The relative abundance of core microbiomes at V4, R3, and R5 stage. At V4 and R3 stage, each box consisted of three replicates for W(illmas), LD12(-8677), and LD13(-14071R2). At R5 stage, the cultivars were merged for analysis since there was no significant difference between cultivars, and each box consisted of 6 replicates. The Wilcoxon rank-sum tests were performed for treatments (Inoc vs NIC, Fun vs No_Fun).(*p<0.1, **p<0.05, ***p<0.005)



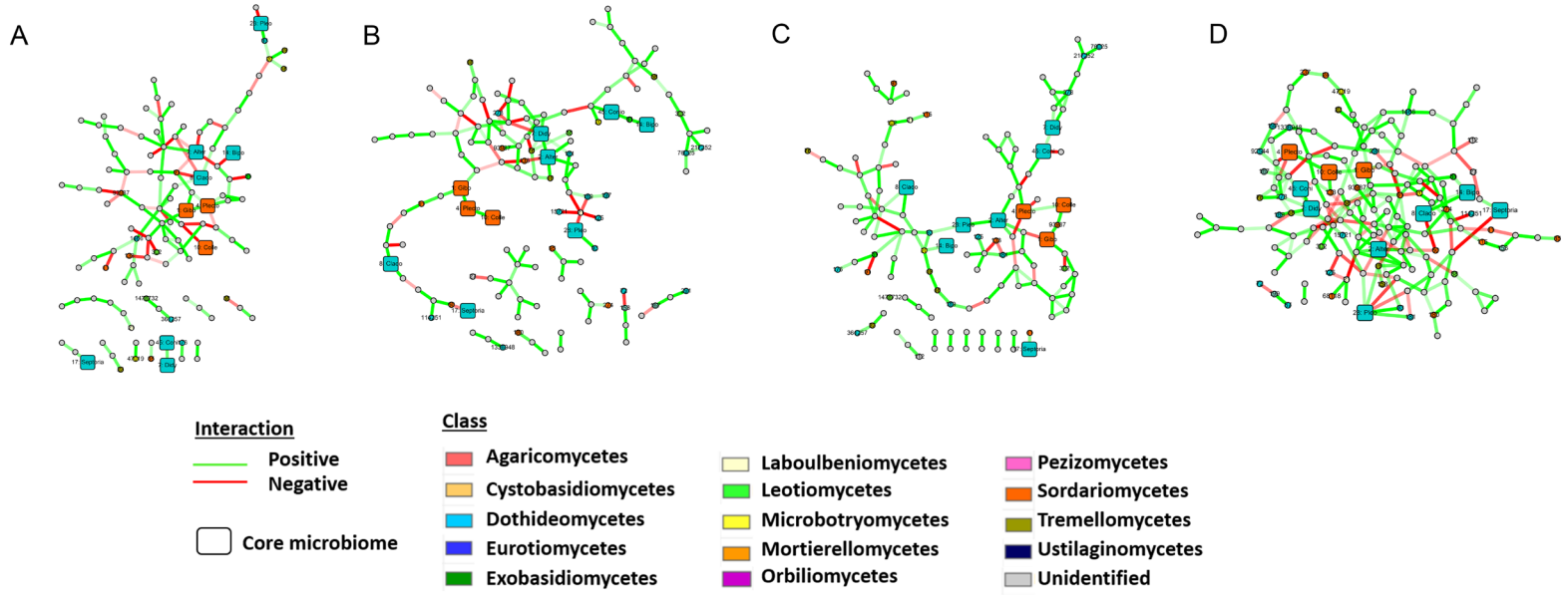
Supplementary Figure 4.2 Networks for (A) V4, (B) R3 and (C) R5 data. The squared node represents that the OTUs were identified as core microbiome from the core microbiome analysis. The node color corresponds to the OTUs at the Class level. Edges color corresponds to potential positive (green) and negative (red) interactions between OTUs.



Supplementary Figure 4.3 Networks for V4 stage data (A) Inoculated and (B) non-inoculated. The squared node represents that the OTUs were identified as core microbiome from the core microbiome analysis. The node color corresponds to the OTUs at the Class level. Edges color corresponds to potential positive (green) and negative (red) interactions between OTUs.



Supplementary Figure 4.4 Networks for R3 stage data (A) Inoculated and (B) non-inoculated. The squared node represents that the OTUs were identified as core microbiome from the core microbiome analysis. The node color corresponds to the OTUs at the Class level. Edges color corresponds to potential positive (green) and negative (red) interactions between OTUs.



Supplementary Figure 4.5 Networks for R5 stage data (A) R5_Fun (B) R5_noFun (C) R5_Inoc, and (D) R5_NIC . The squared node represents that the OTUs were identified as core microbiome from the core microbiome analysis. The node color corresponds to the OTUs at the Class level. Edges color corresponds to potential positive (green) and negative (red) interactions between OUT.

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