

Quantification of Fungicide Resistance in *Cercospora sojina* Populations and Development of a Fungicide Application Decision Aid for Soybean in the Mid-Atlantic U.S.

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## ABSTRACT

Soybean is an important source of protein in animal feed, and growing demand for meat consumption worldwide has led to increased soybean production. Over 120 million metric tons of soybean were harvested in the United States in 2018, approximately one-third of the world production. In the Mid-Atlantic region, soybean is one of the most valuable field crops. Major foliar diseases that reduce soybean yield in the Mid-Atlantic region are frogeye leaf spot (FLS) and *Cercospora* leaf blight. In addition to crop rotation and host resistance, foliar fungicides, often with quinone outside inhibitor (QoI) active ingredients, are used to manage these soybean foliar diseases. Yield benefits of foliar fungicides have been inconsistent and this may be the result of low disease pressure, unfavorable environmental conditions for disease development, or the presence of fungal pathogen populations that have developed resistance to fungicides. The objectives of this research were 1) to develop a pyrosequencing-based assay to rapidly quantify QoI resistance frequencies in *Cercospora sojina*, the causal agent of FLS, 2) to examine the effects of fungicide application timings, disease pressure, and environmental factors on soybean yield, and 3) to develop a weather-based soybean foliar fungicide application decision aid for the Mid-Atlantic U.S. using a threshold decision rule. A pyrosequencing assay targeting the G143A mutation was designed, and a Virginia survey of *C. sojina* populations indicated that the G143A mutation conferring QoI resistance is widespread. In small plot fungicide application timing experiments, five weekly fungicide applications starting at beginning pod (R3) resulted in the greatest yield, but for single fungicide applications, R3 or 1 week after R3 resulted in the greatest yields. There was positive relationship between the cumulative number of disease favorable days

(mean daily temperature 20-30°C and  $\geq 10$  hours of relative humidity  $>90\%$ ) from planting to R3 and disease severity at the full pod stage ( $r = 0.97$ ,  $P = <0.01$ ). Higher disease severity was associated with greater yield loss ( $r^2 = 0.53$ ,  $P = 0.10$ ) suggesting foliar fungicide applications are more likely to have yield benefits as the number of disease favorable days prior to R3 increase. A disease favorable-days threshold (FDT) using the environmental parameters indicated above was evaluated in on-farm experiments throughout Virginia, Maryland, and Delaware. Based on decision rules, FDT = 8 three weeks prior to R3 was the best predictor of a yield benefit with an R3 fungicide application. The decision aid was also able to correctly predict when a fungicide application would not be profitable  $\geq 90\%$  of the time. This weather-based decision aid along with monitoring of fungicide resistance development within the region will provide soybean growers in the Mid-Atlantic U.S. with tools to maximize yields and profitability.

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**GENERAL AUDIENCE ABSTRACT**

Soybean is the third most valuable field crop in the world, ranked only behind rice and wheat in value. Over 98% of the soybean crop is used for animal feed due to its high protein content. The United States is the largest soybean producer in the world, responsible for one-third of global production. Soybean is the top cash crop in the Mid-Atlantic region. Foliar fungal diseases can reduce the soybean yield by causing lesions on the leaves that reduce photosynthesis and cause premature defoliation. Frogeye leaf spot (FLS) caused by *Cercospora sojina* is a major yield reducing soybean foliar diseases in the Mid-Atlantic region. Foliar fungicides, often with quinone outside inhibitor (QoI) active ingredients, are used to manage the disease. However, fungicide efficacy has been inconsistent. Inconsistencies may be due to low disease pressure, improper application timing, or fungicide resistance. The purpose of this research was to investigate the fungicide efficacy inconsistencies and to develop management tools to improve yield and maximize profitability. Our objectives were to 1) develop a molecular assay to quantify frequencies of the mutation conferring fungicide resistance in Virginia populations of *C. sojina*, 2) examine the effects of fungicide application timings, disease severity, and weather on soybean yield, and 3) develop a weather-based soybean foliar fungicide application decision aid for the Mid-Atlantic U.S. The *C. sojina* fungicide resistance mutation was widespread in Virginia, but overall frequencies were relatively low compared to findings from Midwest and Southern states. In fungicide timing experiments, beginning pod (R3) applications resulted in the most consistent yield benefits, and disease severity and yield loss increased as the number of weather-based disease favorable days prior to R3 increased. We used data from on-farm experiments in

Virginia, Maryland, and Delaware to develop a weather-based disease favorable-days threshold that increased the probability that a fungicide application at R3 would have a yield benefit in soybean. The results of our research have led improved fungal disease management recommendations for soybean in the Mid-Atlantic that will maximize yields and profitability.

## **DEDICATION**

I dedicate my dissertation to my father, Changhe Zhou,  
for being my source of inspiration, wisdom, support, and role model.

I would not be here if not for your love and sacrifices.

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## Chapter 1. Literature Review

Soybean (*Glycine max* (L.) Merrill) is one of the most important crops worldwide, ranked just after rice, wheat and corn in terms of value (FAOSTAT, 2013). In 2015, an estimated 118 million hectares of soybean were planted and 319 million metric tons were harvested worldwide (Office of Global Analysis, 2016). Soybean was originally domesticated in China and came to the United States in the 19<sup>th</sup> century. In the 1950s, the United States' soybean production rapidly increased. Currently, the United States is the top producer of soybean in the world, followed by Brazil and Argentina (Singh, 2010). In the 2015, the U.S. produced 107 million metric tons of soybean, more than one-third of the world's production (Office of Global Analysis, 2016). Soybeans are primarily used for animal feeds, food for human consumption, and oil. Almost 98 percent of the U.S. soybean crop is used as animal feed. Soybean is also an excellent source of plant proteins and dietary fiber along with many health benefits. Each soybean seed contains roughly 20 percent oil. The oils are used as cooking oil, ingredients in countless daily products, and as bio-fuel (Wang, 2011).

In the U.S., the upper Midwest states such as Iowa, Illinois, Minnesota, Indiana, the Dakotas, and Ohio produce more than 80 percent of the soybean in the United States. Following the Midwest regions in soybean production is the Southeast region then the Mid-Atlantic (Delaware, Maryland, New Jersey, North Carolina, Pennsylvania, and Virginia) (NASS, 2015a; USDA ERS, 2016). Virginia led the Mid-Atlantic region with 250,905 hectares of soybean harvested in 2015 valued at over \$193 million, making it the top crop in Virginia (NASS, 2015a). In the past decade, the overall price of soybean in the U.S. has dramatically increased followed by a decrease in value. Received soybean price saw a sharp increase in 2007 going from \$0.28/kg to \$0.42/kg. The price peaked in 2013 at \$0.52/kg and has since declined (USDA

ERS, 2016). The increased value of the soybean crop provided an incentive for growers to increase inputs to maximize yields such as the increased use of foliar fungicides.

Along with increased value of soybean, perceived threats from soybean rust and aggressive marketing of fungicides for “plant health” benefits also contributed to the increased use of foliar fungicides in soybean. Before the arrival of soybean rust in 2004, virtually no foliar fungicides were used in soybean production in the U.S. (NASS, 2015b). From 2002 to 2005, less than 1% of the planted soybean acreage was treated with fungicides (Gianessi and Reigner, 2005). With the discovery of soybean rust in the U.S. in 2005, there was a perceived incentive to utilize foliar fungicides in soybean to manage the potentially destructive disease (Kelly et al., 2015). The initial management strategy was applying fungicide just after flowering and followed by a second application 2 to 4 weeks later (Kelly et al., 2015). In 2007, soybean acreage treated with fungicide doubled to 4% in 19 states, with 2% treated with pyraclostrobin and 1% treated with azoxystrobin. By 2012 as estimated 11% and 26% of the soybean acreage had a foliar fungicide applied in the U.S. and Virginia, respectively (NASS, 2015b).

Prior to the arrival of soybean rust in the United States, foliar fungicides were primarily used for the control of frogeye leaf spot and *Cercospora* leaf blight in the Southern states late in the season (Kelly et al., 2015). Soybean is susceptible to many diseases caused by a diversity of pathogens. Soybean diseases can be categorized into stem/root and foliar diseases. A majority of the economic losses are caused by stem and root diseases such as nematodes, seedling diseases, and stems rots. Soybean stem and root diseases are primarily managed through crop rotation and variety resistance (Wrather and Koenning, 2009; Allen et al., 2017), whereas many foliar fungal diseases can be managed with in-season fungicide applications. From 2010 to 2014, soybean diseases caused an estimated loss of 57 million tonnes nationwide, and 9.7 million tonnes in the

southern and Mid-Atlantic states (Allen et al., 2017). Nine out of the top ten soybean diseases in the Midwest and northern states (soybean cyst nematode, sudden death syndrome, seedling diseases, *Phytophthora* root and stem rot, *Septoria* brown spot, *Sclerotinia* stem rot, brown stem rot, *Fusarium* wilt and root rot, and pod and stem blight) are root and stem diseases, causing approximately 93% of the damage in 2014 (Allen et al., 2017). In the Mid-Atlantic and southern states, however, soybean foliar diseases contributed to a much bigger portion of yield loss at 22% compared to 7% in the Midwest and northern states (Allen et al., 2017). The most yield reducing foliar diseases of soybean in the United States in descending order are *Septoria* brown spot, frogeye leaf spot, and *Cercospora* leaf blight (Wrather and Koenning, 2009; Allen et al., 2017). In the South and Mid-Atlantic, the most yield reducing foliar disease is frogeye leaf spot followed by *Cercospora* leaf blight and *Septoria* brown spot (Allen et al., 2017).

Frogeye leaf spot (FLS) is an important soybean foliar disease caused by *Cercospora sojina* K. Hara. The disease was first found in Japan in 1915 and was first discovered in the United States in 1924 (Philips, 1999). Frogeye leaf spot was not observed in Virginia until 1942 (Fenne, 1942; Rosso, 2011). *Cercospora sojina* growth is favored by warm and humid conditions (Philips, 1999). Damage and yield losses caused by FLS are mainly due to the formation of large numbers of lesions on the leaves which reduce photosynthesis and cause premature defoliation. FLS lesions can cover up to 30% of the leaf surface and reduce yield by 10 to 60% (Mian et al., 2008). From 2010 to 2014, FLS was one of the top ten soybean diseases in the United States, contributing to a total estimate yield loss of 56.8 million bushels. It is the second most damaging soybean foliar disease behind *Septoria* brown spot (Allen et al., 2017). FLS is typically found in the southern and mid-Atlantic regions of United States. FLS is present in the Midwest, however it does not cause as much damage as it does in the other regions (Allen et al., 2017). In the past

decade, distributions of FLS have been moving northward, possibly due to warmer winters and the continuous use of susceptible varieties that allow inoculum to overwinter in plant debris and soil (Mian et al., 2008).

FLS primarily causes symptoms on the soybean leaves but it can also be found on stem, pods, and seeds. The most recognizable symptom on leaves is the 1-5 mm circular to angular spots on the upper surfaces (Grau et al., 2004). The spots are reddish-brown at first, but as the lesions age, the center becomes tan surrounded by a dark maroon border. On the lower leaf surfaces, similar spots can be found with clusters of conidiophores developed in the center of each lesion. The dead tissues at the center of lesion may weather away, leaving “shot holes” in the leaves. Leaves with large number of lesions will often wilt and defoliate prematurely (Philips, 1999).

Stem infections occur when conducive conditions extend into the late season. Initial lesions are maroon in color with a dark brown to black margin. As the lesion expands, the center becomes pale gray. Lesions in the pods are reddish-brown to black in color. The lesions are often circular or elongated and appear shrunken. When rainfall and humidity are high, the fungus can penetrate through the pod wall and infect the seeds near the lesions. Infected seeds will often exhibit cracking in the seed coat and dark gray to brown blotches may develop (Philips, 1999).

*Cercospora sojina* overwinters in soybean residue or soil, and conidia produced on debris serve as the primary inoculum (Ma and Li, 1987). Conidia are more likely to infect younger leaves in the mid- to upper canopy compared to the lower canopy. After infection, lesions can form in 8 to 12 days (Philips, 1999). *Cercospora sojina* growth is favored by warm (25-30°C) and humid (>90% relative humidity) conditions (Philips, 1999; Mian et al., 2008). Under favorable conditions, sporulation occurs within 48 hours of the first visible symptoms. Secondary

infection can occur at this time when the conidia are carried to surrounding plants by wind and rain (Philips, 1999; Mian et al., 2008).

*Cercospora kikuchii* (Tak. Matsumoto & Tomoy) M.W. Gardner is another *Cercospora* species that causes disease on soybean leaves (Price et al., 2015). *Cercospora kikuchii* causes the disease Cercospora leaf blight (CLB). The pathogen also causes purple seed stain. The disease was first reported in Korea and it was found in Indiana in the United States in 1924 (Cai and Schneider, 2005). The disease can be found throughout the United States and it is becoming more common in the Midwest (Philips, 1999).

*Cercospora kikuchii* tends to infect soybeans under warm and moist conditions. Experiments suggest *C. kikuchii* grows the best between 25°C to 30°C (28°C being the optimal) with extended periods of high humidity (>95%) (Chen et al., 1979; Schuh, 1999). The fungus can survive in soybean residues in the soil or on infected seed coats (Jones, 1968). The disease usually shows symptoms during the pod filling stages. Foliar symptoms of CLB can include angular to irregular lesions form on upper surface of the leaves. Infected leaves can have a leathery appearance and a reddish- purple discoloration. The symptoms typically appear in the upper canopies (Hartman et al., 1999).

Both FLS and CLB are typically managed through the use of disease-free seed, crop rotation, cultivar resistance, and the use of foliar fungicides (Zhang, 2012; Chanda et al., 2014). Fungicides control diseases by disrupting the cellular processes of the plant fungal pathogens which results in inhibition or death. Fungicides can be classified by the specific cellular processes they inhibit; these are referred to as mode of action (MoA). There are 10 known fungicide MoA with many that still need to be determined (FRAC, 2017). For each mode of action there are sites of action. Sites of action, also known as target sites, are where the active

ingredient of the fungicide binds to a specific location on the target enzyme in the fungus. The biochemical interaction can be described as a lock and key. The key is the fungicide active ingredient and the lock is the target enzyme in the fungus. When the fungicide binds to the target site it will prevent normal fungal substrate from attaching to its proper binding site thus blocking the pathway. Among fungicides used in the management of soybean diseases, only two modes of action exist: respiration and cell membrane disrupters. The group of fungicides that have the cell membrane disrupter MoA are the DeMethylation Inhibitors (DMI). The respiration MoA group of fungicides are the Succinate dehydrogenase inhibitors (SDHI) and the Quinone outside inhibitors (QoI) (FRAC, 2017).

DMI fungicides belong to the fungicide class sterol biosynthesis inhibitors (Brent and Hollomon, 2007). A large group of DMI fungicides belong to the triazoles. The first triazole was launched by Bayer in 1973. DMI fungicides inhibit sterol C-14 $\alpha$ -demethylation of 24-methylenedihydrolanosterol during the biosynthesis of ergosterol (Karaoglanidis et al., 2000; Ma and Michailides, 2005). One of the newer Bayer triazole products, Proline (prothioconazole), was introduced in 2004 (Morton and Staub, 2008). Other DMI fungicides used for the control of soybean foliar diseases are Domark (tetraconazole), Tilt (propiconazole), and Topguard (flutriafol). DMI fungicides have a wide spectrum of activities and differ greatly in terms of efficacy (Karaoglanidis and Thanassouloupoulos, 2013).

SDHI fungicides were first used for the control of rusts and Rhizoctonia diseases in the 1960s (Avenot and Michailides, 2010). SDHI active ingredients are typically carboxamides. SDHI fungicides control disease by binding to the subunits B, C and D of succinate dehydrogenase (Complex II in the mitochondrial respiration chain) and interrupt cellular respiration (FRAC, 2017).



Strobilurins belongs to the quinone outside inhibitor (QoI) class of fungicides. It is one of the most important class of fungicides for disease management in agriculture. These fungicides are widely used on grains, corn, and soybean as well as other crops such as fruit trees, vegetables, and turf. QoI fungicides consist of the family strobilurin and two synthetic families, fenamidone and famoxadone. QoI fungicides include azoxystrobin, pyraclostrobin, trifloxystrobin, and famoxadone (Mueller, 2006). Strobilurin is derived from a secondary metabolite extracted from the fungus *Strobilurus tenacellus* (Clough et al., 1996). Strobilurin fungicides have a site specific single mode of action. They bind to the Quinol outside (Qo) binding site on cytochrome bc 1 complex in the mitochondria to inhibit cellular respiration by interrupting the electron transport chain (Zhang et al., 1998). QoI fungicides are mostly systemic. The chemicals are absorbed into the leaf tissues and move up the plant through the xylem. Most QoI fungicides have a protective period of 14 to 21 days (Mueller, 2006).

The use of foliar fungicides in soybean production has become a standard practice for some growers. However, yield benefits associated with fungicide applications are inconsistent. Foliar fungicides in soybean are typically applied at the R3 (beginning pod) stage. Over 10 years of research in Virginia indicated that soybean yield responses to foliar fungicides occur only one-third of the time. This may be due to absence of disease at the time of application and/or fungicide resistance of the disease causing pathogens. Disease development is heavily influenced by environmental conditions conducive for pathogen growth and host infection (Francl, 2001). It is unlikely one will see a yield response when applying fungicides at times when the environment is unfavorable to disease development due to the absence or low level of disease. Applying fungicide too early and too late in relation to disease onset and crop development is likely to result in little or no yield response.

Weather-based fungicide advisories and models have been developed for many crops in the United States (Table 1.1). In Virginia, weather-based disease advisories for peanuts and potatoes have been developed and implemented. The Virginia peanut advisory for *Sclerotinia* blight uses an algorithm that includes host parameters (vine growth indices and canopy closure), moisture level, and soil temperature to determine thresholds for fungicide application on a 5-day risk index. The peanut advisory is available in the forms of a phone hotline and web alert. The advisory can save growers up to three fungicide application per season, reducing input costs by optimizing the timing of applications for disease control (Langston et al., 2002).

The Virginia Potato Disease Advisory (VPDA) was developed to manage the impact of late blight on the Eastern Shore. Weather stations at six locations on the Eastern Shore record environmental data. After the collected data are tabulated and disease risks for each location are assessed, the risk values determined by the advisory and the appropriate management recommendations are distributed to the growers weekly during the potato growing season. An overwhelming majority of the growers using the advisory have found it to be helpful and some are using it “religiously” (VCE, 2014).

Another factor that may contribute to the observed inconsistency in yield response to foliar fungicide application in soybean may be the development of fungicide resistance within soybean foliar pathogen populations. Spontaneous mutation occurs constantly in all organisms. Spontaneous mutations at the fungicide target sites in a fungus can alter the conformation of the target site where the key will no longer fit the lock, and fungal genotypes with this mutation will be “resistant” to the fungicide (FRAC, 2017; Brent and Hollomon, 2007). Mutations conferring fungicide resistance occur approximately one in one billion spores in nature without exposure to fungicides. However, due to the use of fungicides in the management of crop diseases and

natural selection, fungal genotypes with mutations conferring fungicide resistance will survive and pass on the mutation, thereby causing a buildup of resistant individuals in the field (Brent and Hollomon, 2007).

QoI fungicides have a single site-specific mode of action, and fungicide resistance to this class can rapidly develop in fungal pathogen populations. The Fungicide Resistance Action Committee (2017) gave QoI fungicides (FRAC Code 11) a high risk for resistance development. Currently three mutations have been identified in the cytochrome b gene that contribute to strobilurin resistance. G143A, F129L and G137R. The G143A mutation is the most common and has complete resistance to QoI fungicides (Zeng et al., 2014). Several mutations in numerous pathogens have been discovered to confer resistance to DMI fungicides, primarily in the *cyp51/erg11* gene (FRAC, 2017; Ma and Michailides, 2005). FRAC rates DMI fungicides at a medium risk for developing resistance (FRAC, 2017). Cross resistance has been observed in *Cercospora beticola* in DMI fungicides, but due to the large size of the class and its spectrum of activities, the degree of cross resistance among the active ingredients varies (Karaoglanidis and Thanassouloupoulos, 2013). For SDHI fungicides, mutations in any of the three subunits can confer resistance. As of 2014, 15 species of fungi have developed resistance to SDHI fungicides with more than 50 subunit mutations. The FRAC rates SHDI fungicides medium to high risk of developing resistance (FRAC, 2017).

The two most important soybean foliar diseases in Virginia are frogeye leaf spot (*Cercospora sojae*) and Cercospora leaf blight (*Cercospora kikuchii*). QoI fungicides are effective in controlling these two diseases. However, in 2010, *C. sojae* isolates highly resistant to QoI fungicides were identified in Tennessee (Zeng et al., 2014). By 2013, nine other states (Table 1.2) had confirmed frogeye leaf spot resistance to QoI fungicides. In 2014, four fields in

Virginia were tested and resistant isolates were found in two fields. Though foliar fungicides provide growers with a tool to maximize yields in soybean production, their use is not always profitable. The profitability of fungicide use can be maximized by only applying fungicides when they are needed to control disease and protect yield and by using effective chemistries for target diseases.

## **Research Objectives**

The main goal of this research was to develop tools to optimize management of soybean foliar diseases and maximize yield and profitability for soybean production in the Mid-Atlantic region. The specific objectives of this work were 1) to develop a pyrosequencing-based assay to rapidly quantify QoI resistance frequencies in *Cercospora sojina*, the causal agent of FLS, 2) to examine the effects of fungicide application timings, disease pressure, and environmental factors on soybean yield, and 3) to develop a weather-based soybean foliar fungicide application decision aid for the Mid-Atlantic U.S. using a threshold decision rule.

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**Table 1.1** List of weather-based advisories and models that have been developed for crops and associated diseases.

<b>Crop</b>	<b>Disease</b>	<b>Reference</b>
Grape	Botrytis bunch rot Powdery mildew Downy mildew	UCIPM, 2014
Peanut	Early leaf spot Late leaf spot Stem Rot Sclerotinia blight	Phipps et al., 1997
Potato	Late blight	Virginia Cooperative Extension, 2014
Sugar beet	Cercospora leaf spot	Windels et al., 1998

**Table 1.2.** Confirmations of *Cercospora sojina* quinone outside inhibitor (QoI) fungicide resistance in the U.S. by state and year (Zhang et al., 2018).

<b>State</b>	<b>Year QoI resistance first confirmed</b>
Tennessee	2010
Illinois	2010
Kentucky	2010
Louisiana	2011
Missouri	2011
Alabama	2012
Arkansas	2012
Indiana	2013
Mississippi	2013
North Carolina	2013
Virginia	2014

**Chapter 2: Rapid quantification of the G143A mutation conferring fungicide resistance in Virginia populations of *Cercospora sojina* using pyrosequencing**

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T. Zhou and H. L. Mehl designed the experiments. T. Zhou conducted the experiments and wrote the manuscript. H. L. Mehl reviewed and edited the manuscript.

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**Abstract**

Quinone outside inhibitors (QoI) inhibit a wide spectrum of fungal pathogens, but there is high risk of resistance development with this class of fungicides. QoI-resistant *Cercospora sojina*, the causal agent of frogeye leaf spot in soybean, has been confirmed from 14 states, and the cytochrome b G143A mutation was confirmed as the mechanism of resistance. QoI-resistant *C. sojina* has not been well characterized in the Mid-Atlantic U.S., so objectives of this study were to (i) determine if QoI-resistant *C. sojina* is present in Virginia, (ii) develop a pyrosequencing-based method to quantify G143A frequencies, and (iii) determine frequencies and distributions of QoI-resistant *C. sojina*. Based on *in vitro* fungicide sensitivity and PCR detection of the G143A mutation, QoI-resistant *C.*

*sojina* was confirmed from nine Virginia soybean fields in 2015. A pyrosequencing assay targeting the G143A mutation was designed to detect frequencies of the mutation from mixtures of *C. sojina* DNA. A statewide survey of *C. sojina* was conducted from 2015 to 2017, and G143A frequencies within each field were quantified using pyrosequencing. G143A frequencies ranged from 1-99% (mean = 27%), with 80% of fields having a mutation frequency  $\leq 50\%$  and 40% with frequencies  $< 10\%$ . Using pyrosequencing, we monitored G143A frequencies, and thus QoI resistance, in *C. sojina* populations across locations and over time. Relatively low frequencies of QoI-resistant *C. sojina* were detected in Virginia, but fungicide resistance management practices, including monitoring fields for G143A mutation frequencies, need to be implemented to minimize increases in QoI resistance over time.

## **Keywords**

Quinone outside inhibitor; pyrosequencing; soybean; frogeye leaf spot; *Cercospora sojina*

## **Introduction**

Fungicides are an important tool for management of fungal diseases in crops, but the development of fungicide-resistant pathogens can severely reduce their efficacy (Brent and Hollomon, 2007). Quinone outside inhibitor (QoI) fungicides are widely used as they are effective in controlling a broad spectrum of fungal pathogens and slow down rates of plant senescence (Vincelli, 2002; FRAC, 2019). However, as of 2013, over 50 pathogens have been documented in the lab or field that show resistance to QoI fungicides; this includes *Cercospora sojina* Hara, the causal agent of frogeye leaf spot (FLS) in soybean (*Glycine max* (L.) Merr.) (FRAC, 2013). FLS primarily occurs in the southern and Mid-Atlantic regions of the United

States. It is the second most damaging soybean foliar disease behind *Septoria brown spot*, contributing to a total estimated loss of 1.5 million metric tons from 2010 to 2014 in the U.S. (Allen et al., 2017); the development of QoI resistance in *C. soja* populations has the potential to increase soybean yield losses due to FLS.

QoI fungicides have a site-specific mode of action. The active ingredient binds to the Quinol oxidation (Qo) binding site on the cytochrome bc<sub>1</sub> complex in the mitochondria to inhibit cellular respiration by interrupting electron transport (Bartlett et al., 2002, Zhang et al., 1998). Due to QoI fungicides having a single site-specific model of action, nucleotide point mutations can occur, resulting in amino acid changes that alter the fungicide target site (Standish et al., 2015). Three mutations have been identified in the cytochrome b gene that confer resistance to QoI fungicides. Phenylalanine to leucine at position 129 (F129L) and glycine to arginine at position 137 (G137R) confer partial resistance or reduced sensitivity, while glycine to alanine at position 143 (G143A) confers complete resistance. The G143A mutation is the most common of the three mutations and it is the only mutation that has been to date found in *C. soja* (Zeng et al., 2015).

Fungicide resistance in the field occurs in two phases: emergence and selection. In the emergence phase, resistance conferring mutations arise naturally in pathogen individuals as small proportions in the population. When fungicide is applied, the selection phase begins. Fungicide applications create selection pressure on the pathogen population and select for the individuals resistant to the fungicide. Increased selection pressure will shift the population favoring the resistant isolates (Hobbelen et al., 2014). QoI-resistant *C. soja* was first identified in Tennessee, Illinois, and Kentucky in 2010,

and as of 2018, 14 states have confirmed QoI-resistant *C. soja* in soybean production areas (Zhang et al., 2018).

Due to the threat QoI-resistant *C. soja* poses for the management of FLS, it is important to determine the regional prevalence and distribution of QoI resistance in order to modify fungicide recommendations and develop resistance management practices. The objectives of this study were to (i) determine if QoI-resistant *C. soja* is present in Virginia, (ii) develop and validate a pyrosequencing-based method to rapidly quantify frequencies of the G143A mutation, and (iii) determine the frequencies and distributions of QoI-resistant *C. soja* in soybean fields.

## Materials and Methods

*Sample collection and fungal isolation.* Soybean leaflet samples with symptoms of frogeye leaf spot were collected from Virginia soybean fields in 2015-2017. A sample consisted of a minimum of 10 diseased leaflets arbitrarily selected from throughout the field. Samples were placed in plastic bags and stored at 4°C until pathogen isolation. A portion of each sample was stored at -20°C for future use.

Dissecting (45X) and compound (100X) microscopes were used to confirm *C. soja* based on lesion and spore morphology. Lesions on the leaflets were checked for sporulation. If no conidia were observed, leaflets with lesions were placed in a moist chamber overnight to induce sporulation. After confirming sporulation, 5 µL of sterile distilled water were pipetted into the center of the lesion and pipetted up and down to dislodge conidia. Conidial suspensions were transferred to V8 agar (5% V8 juice, 2% agar) amended with 100 mg/mL ampicillin. A sterile glass spreader was used to distribute the conidial suspension evenly on the culture plate, and plates were incubated at 25°C overnight. Five germinated conidia were selected and

transferred to separate V8 agar plates. Single-spore cultures were incubated at 25°C with 12 hours of light and 12 hours of darkness. After isolates grew to 2.5 cm diameter, agar plugs were excised from plates and stored in 40% glycerol in sterile cryogenic tubes at -80°C.

*DNA extraction from fungal cultures.* *Cercospora sojina* isolates were grown on V8 agar plates for 4 weeks prior to DNA extraction. A glass spreader was used to scrape the fungal material off culture plates. Fungal material was transferred to 2 ml Lysing Matrix A tubes (MP Biomedicals, Solon, OH) filled with 1 ml of lysis buffer (30 mM Tris, 10 mM EDTA, 1% SDS, pH 8.0). Tubes were placed in a mini-beadbeater (BioSpec Products Inc. Bartlesville, OK) for grinding (3000 RPM, 40 s). After grinding, tubes were placed in a shaking incubator (60°C, 1000 RPM) for 30 min with vortexing every 15 min. Tubes were centrifuged (14000 G, 30 min), and supernatants were transferred to 2 ml micro-centrifuge tubes and mixed with 370 µl 4M ammonium acetate and 740 µl ice-cold 100% ethanol. Tubes were incubated at -20°C for 30 min then centrifuged at maximum speed for 5 min. Supernatants were discarded and residual ethanol was removed using a pipette. DNA pellets were air dried for 30 min then re-suspended in 50 µl of sterile distilled H<sub>2</sub>O.

*PCR assay for G143A mutation.* A previously designed discriminatory PCR assay was used to screen *C. sojina* isolates for the presence of the G143A mutation (Zeng et al., 2015). Primer pair WT-Cs-For (5'-GGTTCACTATTAGGATTTTGTCTTGTA-3') and WT-Cs-Rev (5'-CTCATTA AATTAGTAATAACTGTGGCCC-3') amplified "wild-type" isolates (isolates without the G143A mutation), and primer pair G143A-For (5'-TAATACAGCTTCAGCATTTTTCTTCT-3') and G143A-Rev (5'-



CTCATTA AATTAGTAATAACTGTGGCAG-3') amplified a portion of the cytochrome b gene from isolates with the G143A mutation. PCR was carried out in Bioneer AccuPower® HotStart PCR PreMix tubes (Bioneer Corp., Munpyeongseo-ro, Daedeok-gu, Daejeon, Republic of Korea) with 1 µl of *C. soja* genomic DNA (2 ng/µl), 0.4 µl of each of the primers (0.1 µM) and 18.2 µl sterile distilled water. PCR conditions were 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 57°C (WT-Cs) or 51°C (G143A) for 30 s, 72°C for 2 min, and a final extension at 72°C for 5 min. Amplicons were visualized on a 1% agarose TBE gel stained with Gel Red (Biotium Inc., Fremont, CA). Expected amplicon sizes were 359 bp and 207 bp for the WT-Cs and G143A primers, respectively.

*Sensitivity of C. soja isolates to azoxystrobin.* Azoxystrobin ( $\geq 98\%$ , Sigma-Aldrich, Saint Louis, MO) stock solution was prepared at 100,000 ppm in acetone. The stock solution was serially diluted and added to 2% water agar to obtain final concentrations of 10, 1, 0.1, 0.01, and 0.001 ppm azoxystrobin. Non-fungicide amended water agar was included as a control, and all plates were amended with 60 µg/ml salicylhydroxamic acid (SHAM) (99%, Sigma-Aldrich, St. Louis, MO) dissolved in methanol to prevent alternative respiration in *C. soja*.

To prepare conidial suspensions for the germination assay, 4 sensitive and 7 resistant isolates confirmed by the PCR assay were grown on 5% V8 agar for 7 days at 21°C with 12 hours of light and 12 hours of dark to induce sporulation. For each isolate, 10 agar plugs with heavy sporulation were placed in a sterile 15 ml tube with 5 ml of sterile distilled water. Tubes were vortexed for 30 s to dislodge conidia. A hemocytometer was used to determine the initial conidial concentration, and final concentrations were adjusted to  $5 \times 10^4$  conidia/ml. Seventy-five microliters of conidial suspension were pipetted onto the fungicide-amended media and non-amended control plates and distributed evenly with a sterile glass spreader. After incubation at

21°C for 18 h, 100 conidia were evaluated for germination using a compound microscope. A conidium was considered germinated if the germ tube was equal to or exceeded the length of the conidium. Each azoxystrobin concentration and isolate combination was replicated 3 times in a single experiment, and the EC<sub>50</sub> value was determined for each isolate by linear interpolation using the concentrations above and below 50% inhibition (Wise et al., 2008).

*Pyrosequencing assay design.* PyroMark Assay Design 2.0 software (QIAGEN Inc., Germantown, MD) was used to design PCR and pyrosequencing primers from the complete coding sequence of the *C. sojina* cytochrome b gene (Zeng et al., 2015). The target single nucleotide polymorphism (SNP) for assay design was the G143A mutation. PCR primers FLS-F2 (5'-CTTACAAAGCACCTAGAACATTGG-3') and FLS-R2 (5'-TCCTACTCATGGTATTGCACTCA -3') were designed to amplify a 161 bp region around the G143A SNP. The 3' end of the reverse primer FLS-R2 was biotinylated and HPLC purified. The specificity of the primers was evaluated using NCBI Primer-BLAST, and the primers had 100% homology with portions of the cytochrome b gene from *Cercospora* species *C. sojina*, *C. kikuchii*, and *C. beticola*; primers did not have homology with any other DNA sequences in the database. PCR was carried out in premix tubes as described above with 3 µl of DNA (2 ng/µl), 0.4 µl of each primer (0.1 µM), and 16.2 µl sterile distilled water. A temperature gradient was performed to determine the optimal annealing temperature. PCR conditions were 94°C for 5 min followed by 45 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 10 min. Following PCR, 5 µl of the amplicons were run on a 1.5% agarose TBE gel

precast with 1X GelRed (15  $\mu$ l GelRed in 150 ml) at 100 V for 30 min to confirm adequate amplification and amplicon size.

The Vacuum Prep Tool (QIAGEN Inc., Germantown, MD) was used to prepare amplicons for pyrosequencing according to the manufacturer's instructions. Briefly, biotinylated amplicons were bound to streptavidin-coated beads, captured on filter probes, washed with 70% ethanol, denatured by submerging filter probes into 0.2 M NaOH, and washed with 10 mM Tris-Acetate, pH 7.6. To anneal the sequencing primer to the single-stranded DNA, streptavidin beads were released into a PyroMark Q24 sequencing plate containing 0.3  $\mu$ M sequencing primer (FLS-S2, 5'-TTACGGACAAATGTCTTTAT-3'), then the plate was heated at 80°C for 2 min and cooled to room temperature. Pyrosequencing was performed using the PyroMark Q24 system according to the manufacturer's instructions. Pyrogram peak heights at the target SNP (G/C) were used to detect and calculate proportions of the G143A mutation at that position. To validate the accuracy and precision of the pyrosequencing assay, DNA of *C. soja* isolates CP1A-1 (sensitive) and CP2A-5 (resistant) confirmed by PCR and fungicide sensitivity assays was mixed to obtain DNA samples with 0, 10, 25, 50, 75, 90, or 100% resistant isolate DNA. Proportions of the G143A mutation in DNA mixtures were measured as described above, and each DNA mixture was analyzed three times.

*Quantification of G143A frequencies from C. soja infected soybean.* For each field sampled, 10 leaflets were selected, and 10 FLS lesions were excised using a hand-held hole-puncher. DNA was extracted using the FastDNA™ Spin Kit (MP Biomedicals, Solon, OH) with the following modifications: Before bead-beating, the excised lesion discs were added to a 2 mL Lysing Matrix A tube with 5 to 6 medium sized beads and 1 large ceramic bead. In addition, 800  $\mu$ l of CLS-VF and 200  $\mu$ l of PPS was added to the tube. The sample was ground in the bead-

beater for 40 s at 3000 RPM. After completion of the manufacturer's protocol, DNA was eluted in 100  $\mu$ l of sterile distilled H<sub>2</sub>O. Frequencies (proportions) of the G143A mutation in each sample were quantified using the pyrosequencing assay as described above.

## Results

*Sample collection and fungal isolation.* Soybean fields were scouted for the presence of FLS, and symptomatic soybean leaflets with signs and symptoms of FLS were collected from 74 fields in 2015, 15 fields in 2016, and 40 fields in 2017 for a total of 129 field locations from 30 counties/cities (Supplemental Table 2.1). In 2015, a total of 113 *C. sojae* isolates from 24 fields in 6 counties/cities were single-spore purified.

*Screening for fungicide sensitivity.* The PCR assay was discriminatory because only one of the two primer sets (WT-Cs or G143A) was able to produce an amplicon from an isolate's DNA. The WT-Cs primer set amplified a 359-bp product indicating the G143A mutation was not present and the G143A primer set amplified a 207-bp product indicating the G143A point mutation was present in an isolate (Fig. 2.1) Of the 113 *C. sojae* isolated in 2015, DNA from 25 isolates amplified with the G143A primers in the discriminatory PCR assay and the remaining 88 isolates only amplified with the wildtype primers. Thus, the PCR assay indicated that 22% of the isolates had the G143A mutation and should be insensitive to QoI fungicides. To verify the results of the PCR assay, 4 isolates that amplified with the G143A primers and 4 isolates that amplified with the wild type primers were tested for sensitivity to azoxystrobin (Fig. 2.2, Table 2.1). For wildtype isolates, EC<sub>50</sub> values ranged from 0.0050 to 0.0056 ppm (mean = 0.0053), confirming

their sensitivity to azoxystrobin. The isolates that amplified with the G143A primers were insensitive to azoxystrobin with EC<sub>50</sub> values ranging from 5.72 to 31.60 ppm (mean= 13.93 ppm). EC<sub>50</sub> values of the isolates with the G143A mutation were significantly greater than those of the isolates that did not amplify with the G143A primers ( $P = 0.011$ ). On average, isolates with the G143A mutation were 2,630 times less sensitive to azoxystrobin than isolates lacking the mutation (range =1145 to 5640 times less sensitive). These results confirmed that isolates with the G143A mutation are resistant and isolates without the mutation are sensitive to a QoI fungicide.

*Quantification of G143A frequencies with pyrosequencing.* Primers FLS-F2 and FLS-R2 amplified a 161 bp fragment of the cytochrome b from resistant isolate CP2A-1 and sensitive isolate CP2A-5. The accuracy and precision of the pyrosequencing assay was evaluated using mixtures of DNA with known proportions of DNA from the resistant and sensitive isolates, and there was a correlation between proportions of the resistant isolate DNA in the mixture and the measured percentage of the G143A mutation with pyrosequencing ( $r^2 = 0.9948$ , Fig. 2.3). Replicate measurements were reproducible with standard deviations for each mixture ranging from 1-4%. Based on these results and the manufacturer's specifications, it was assumed that measured proportions of the G143A mutation had an error of up to 5%. Thus, for the presence of the mutation to be confirmed, a cut-off of 5% was used.

*C. sojae* DNA was isolated directly from infected soybean leaflets collected from 129 fields in 2015-2017, and percentages of the G143A mutation within each sample were determined (Supplemental Table 2.1). The mean frequency of the G143A mutation across all years and locations was relatively low at 27.2%, but percentages within individual fields ranged from 1 to 99% (Table 2.2). Using the threshold of 5% error measured with the pyrosequencing

assay to confirm presence of the QoI-resistance conferring mutation, the G143A mutation was detected from 25 of the 30 counties/cities from which samples were collected (Table 2.2). The counties/cities were grouped into soybean production areas assigned by the Virginia Soybean Board based on planted acreage. Aside from the Eastern Shore and Blackwater regions, the different areas had similar average estimated frequencies of G143A mutation that ranged from 22 to 34% with standard deviations ranged from 22 to 29%. In the counties/cities within a production area, the G143A frequencies were highly variable. For example, in Brunswick County, 15 fields were sampled and G143A frequencies ranged from 1 to 73% (Table 2.2). Across all years and locations, 38% of fields (N=49) had G143A mutation frequencies less than 10% whereas only 2% of fields (N=3) had frequencies greater than 90%. A majority of fields (103/129) had a frequency of less than 50% and 119 out of 129 field locations had a G143A mutation frequencies of less than 75%, indicating high level of QoI resistance are rare in Virginia soybean fields (Table 2.3).

## **Discussion**

In the current study, the presence of QoI-resistant *C. soja* was documented in Virginia, and a pyrosequencing method for rapid quantification of frequencies of the QoI-resistance conferring G143A mutation was developed. This is the first widespread survey of fungicide resistance within *C. soja* populations in the Mid-Atlantic U.S., and the results have important implications for fungicide recommendations and fungicide resistance management in the region. Though its occurrence was widespread, overall frequencies of the G143A mutation in populations of *C. soja* from sampled Virginia

soybean fields were low to moderate (mean = 27.2%) and lower than the frequencies detected in isolates collected and screened in Illinois (64%), Tennessee (79%) and Mississippi (93%) from 2015 to 2017 (Standish et al., 2015; Zeng et al., 2015; Shreshtha et al., 2017). Our survey was only able to sample from a portion of the soybean growing regions in Virginia. We could not obtain samples from all the counties in the Northern Neck, Middle and Virginia Peninsula, and parts of the Blackwater regions where a large proportion of the soybeans are grown. In addition, samples from at least 10 locations were collected after the soybeans were treated with fungicide. The fungicide application selects for individuals with G143A mutation. Due to the polycyclic nature of *C. soja*, the pathogen population may have shifted to favor QoI-resistance. Fungicide treated fields may have a greater frequencies of the G143A mutation than from untreated fields. The true frequencies of the G143A mutation in *C. soja* population in Virginia may be greater or less than the estimated 27%. Additional sampling in the un-sampled soybean growing regions will provide a more accurate estimate of frequencies of the G143A mutation.

The sensitivity of the Virginia *C. soja* isolates to azoxystrobin were comparable to findings from other states. The baseline (isolates have never been exposed to QoI fungicides) EC<sub>50</sub> for isolates collected between 2007 to 2009 from Illinois and Missouri ranged from 0.00297 to 0.03241 and from Mississippi was 0.0002 (Standish et al., 2015; Zhang et al., 2012a). Wildtype isolates collected in Virginia had similar but slightly greater EC<sub>50</sub> values ranging from 0.0050 to 0.0056, but these were not true baseline isolates since they were from areas where QoI fungicides have been applied. The first report of QoI-resistant *C. soja* in North America was from Tennessee in 2010 where isolates were 249 to 7,144 times less sensitive to QoI fungicides compared to baseline isolates (Zhang et al., 2012b). For a group of *C. soja* isolates collected in Mississippi from 2013-2015, resistant isolates were 192 to 11,400 times less sensitive to

azoxystrobin (Standish et al., 2015). In the current study, baseline isolates were not included, but on average, Virginia isolates of *C. soja* with the G143A mutation were 2,630 times less sensitive to azoxystrobin than isolates without the mutation; this is within the range of levels of reduced sensitivity reported in previous studies that included baseline isolates. Thus, even though overall frequencies of QoI-resistant *C. soja* in Virginia are lower compared to those reported from other states, when QoI resistance does occur, the magnitude of reduced sensitivity to fungicides is similar among regions.

The current study documents that QoI resistance is present within *C. soja* populations in Virginia, but the relatively low frequency of the G143A mutation within populations indicates QoI-based fungicides should still have some efficacy in controlling FLS in Virginia soybean fields. However, to maintain the utility of QoI fungicides, fungicide use practices should be implemented to reduce the selection pressure on the QoI-resistant isolates and avoid the risk of increasing the level of resistance. Non-chemical management practices such as planting FLS-resistant soybean varieties and rotation to non-host crops will play an important role in fungicide resistance management. Planting of consecutive soybean crops is a common practice in some areas, but there must be at least 2 years in between planting soybean to reduce the survival of overwintering *C. soja* in the soil or plant debris that can serve as inoculum (Zhang and Bradley, 2004). In areas where only soybean and corn are grown and the addition of other rotation crops is not economical, short rotations out of soybean may result in greater disease pressure and levels of fungicide use (Zeng et al., 2015). In Virginia, soybean is usually in rotation with corn and small-grains, but in some parts of the state, tobacco, sorghum, peanut, and cotton are also used in the rotation. Overall, the greater diversity of



crops and longer rotations out of soybean in Virginia may be an explanation of the lower disease pressure, lower rates of fungicide use, and lower frequencies of the G143A mutation in Virginia populations of *C. soja* compared to some other states (Standish et al., 2015; Zhang et al., 2012b). For example, estimated soybean disease losses in 2010-2014 were 9% in Virginia, but in Tennessee and Mississippi where high rates of QoI-resistant *C. soja* have been reported, disease loss was estimated at 16% and 14%, respectively (Allen et al., 2017). According to the USDA National Agricultural Statistics Service, soybean acreage treated with a QoI fungicide in 2012 was 10% in Virginia 14% in Tennessee, and 31% in Mississippi. These data suggest that in areas with greater disease pressure and rates of fungicide use, there is a greater risk of fungicide resistance emerging within *C. soja* populations. When disease pressure is high and chemical management is warranted, the use of alternative fungicide classes such as demethylation inhibitors (DMI) and succinate dehydrogenase inhibitors (SDHI) may reduce the selection pressure on QoI-resistant isolates, but modes of action should be rotated to minimize the development of fungicide resistance by *C. soja* towards DMI and SDHI fungicides.

Frequencies of the G143A mutation in *C. soja* populations in Virginia and the surrounding Mid-Atlantic region need to be continually monitored to determine if levels of resistance are increasing over time. Molecular methods such as PCR, real-time PCR, and PCR-RFLP have been developed previously to detect the G143A mutation in *C. soja* (Standish et al., 2015; Zeng et al., 2015). These methods are adequate for determining whether or not an isolate has the resistance conferring G143A mutation, but for determining frequencies of the G143A mutation within populations, these methods require significant time and resources for pathogen isolation and culture maintenance. In contrast to determining the presence of the G143A mutation from individual isolates, the pyrosequencing assay developed as part of this study

allowed for quantification of G143A frequencies from multiple genotypes within plant tissues collected from large portions of fields. Pyrosequencing has many applications in the detection and quantification of single nucleotide polymorphisms (SNP). The method has been previously utilized to quantify strain-specific SNPs in *Aspergillus flavus* (Das et al., 2008; Mehl and Cotty, 2010) and to detect multi-drug resistant *Mycobacterium tuberculosis* strains by targeting known mutations associated with resistance development to antibiotics (Lacoma et al., 2015). Similar to the assay for detection of the G143A mutation in *C. sojae*, a pyrosequencing-based method was developed to quantify the mutations associated with resistance to succinate dehydrogenase inhibitor fungicides in *Botrytis* spp. (Gobeil-Richard et al., 2015). The results of the current and previous studies demonstrate the utility of pyrosequencing for detection and quantification of fungicide-resistance conferring point mutations in fungal pathogens, and this approach can be used to monitor for the presence of these mutations in fungal populations over time.

## **Conclusion**

In summary, we have developed a pyrosequencing-based method that can rapidly detect and quantify the QoI-resistance conferring G143A mutation within *C. sojae* populations without the need to culture the pathogen. This method can be applied to the study of fungicide resistance in other fungal pathogens. Using this method, we monitored the G143A frequencies in *C. sojae* populations across locations and over time. Though the presence of the G143A mutation was detected in *C. sojae* populations from soybean fields throughout Virginia, frequencies of the mutation were relatively low compared to findings from other states. G143A mutation frequencies in *C. sojae* populations should

be monitored and fungicide resistance management practices should be implemented to minimize future increases in QoI resistance within the region.

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**Table 2.1.** Sensitivity of *Cercospora sojina* isolates with and without the G143A mutation conferring resistance to quinone outside inhibitor fungicide azoxystrobin.

Isolate ID	County	G143A mutation <sup>a</sup>	EC <sub>50</sub> (ppm) <sup>b</sup>
CP2A-5	Culpeper	No	0.005
FQ1C-5	Fauquier	No	0.005
CP1A-1	Culpeper	No	0.006
SX1-1	Sussex	No	0.006
CP2A-1	Culpeper	Yes	5.725
FQ1A-2	Fauquier	Yes	8.333
CP2D-6	Culpeper	Yes	10.363
CP2F-2	Culpeper	Yes	12.160
FQ1B-7	Fauquier	Yes	13.000
FQ1D-7	Fauquier	Yes	16.353
NK1-1	New Kent	Yes	31.600

<sup>a</sup>Presence or absence of the G143A mutation in the cytochrome b gene was confirmed with a discriminatory PCR assay. <sup>b</sup>Effective concentration at which 50% of conidial germination was inhibited by azoxystrobin.

**Table 2.2.** Frequencies of the G143A mutation conferring quinone outside inhibitor fungicide-resistance in *Cercospora soja* populations sampled from 2015-2017.

Region <sup>a</sup>	County/city	Year(s) sampled	# fields <sup>b</sup>	% G143A <sup>c</sup>			
				Mean	Min	Max	SD
<b>Eastern shore</b>	Accomack	2016	2	94	89	99	5
	<b>Region total</b>	<b>2016</b>	<b>2</b>	<b>94</b>	<b>89</b>	<b>99</b>	<b>5</b>
<b>Northern Neck (eastern)</b>	Caroline	2017	4	9	5	16	5
	Lancaster	2016	3	28	3	44	22
	Richmond	2015, 2017	5	55	9	80	28
	<b>Region total</b>	<b>2015-2017</b>	<b>12</b>	<b>33</b>	<b>3</b>	<b>80</b>	<b>27</b>
<b>Middle and Virginia Peninsula (eastern)</b>	Charles City	2015, 2017	2	3	1	4	2
	Middlesex	2015, 2016	7	30	3	81	27
	New Kent	2015	4	24	7	51	19
	<b>Region total</b>	<b>2015-2017</b>	<b>13</b>	<b>24</b>	<b>1</b>	<b>81</b>	<b>22</b>
<b>Mountain and Valley (central)</b>	Appomattox	2015	1	41	---	---	---
	Bedford	2015	1	14	---	---	---
	Chesterfield	2015	2	3	2	3	0.7
	Culpeper	2015	10	13	1	34	10
	Cumberland	2015	2	55	38	71	23
	Fauquier	2015	6	52	16	81	27
	Goochland	2015	2	43	38	47	6
	Madison	2015	5	29	1	91	45
	Nelson	2015	1	11	---	---	---
	Orange	2015	3	39	32	50	10
	Powhatan	2015	2	2	1	3	1
	Spotsylvania	2015	2	2	1	2	0.7
	<b>Region total</b>	<b>2015</b>	<b>37</b>	<b>27</b>	<b>1</b>	<b>91</b>	<b>27</b>
<b>South Hampton Roads (southeastern)</b>	Chesapeake	2015, 2016	5	70	52	88	17
	Isle of Wight	2015	1	39	---	---	---
	Suffolk	2015, 2017	13	22	1	93	28
	Virginia Beach	2015, 2016	6	32	4	60	20
	<b>Region total</b>	<b>2015-2017</b>	<b>25</b>	<b>34</b>	<b>1</b>	<b>93</b>	<b>29</b>
<b>Blackwater (southeastern)</b>	Prince George	2015, 2017	8	14	2	48	16
	Sussex	2015	3	4	1	8	4
	<b>Region total</b>	<b>2015, 2017</b>	<b>11</b>	<b>11</b>	<b>1</b>	<b>48</b>	<b>13</b>
<b>Southside (southern)</b>	Brunswick	2017	15	24	1	73	22
	Dinwiddie	2016	1	57	---	---	---
	Lunenburg	2016, 2017	3	34	1	67	33
	Mecklenburg	2015, 2017	8	12	3	47	19
	Nottoway	2015	2	3	3	3	0
	<b>Region total</b>	<b>2015-2017</b>	<b>29</b>	<b>22</b>	<b>1</b>	<b>73</b>	<b>23</b>

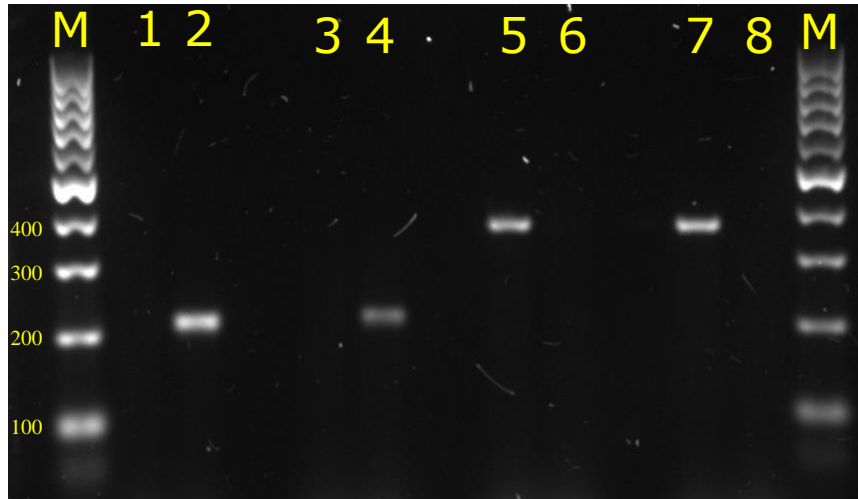
<sup>a</sup>Virginia soybean production regions assigned by the Virginia Soybean Board based on soybean acreage. <sup>b</sup>Total number of fields sampled in the indicated county/city or soybean production region (bold). <sup>c</sup>Mean, minimum (Min), maximum (Max), and standard deviation (SD) of the percentage of *C. soja* population with the G143A mutation quantified by pyrosequencing.



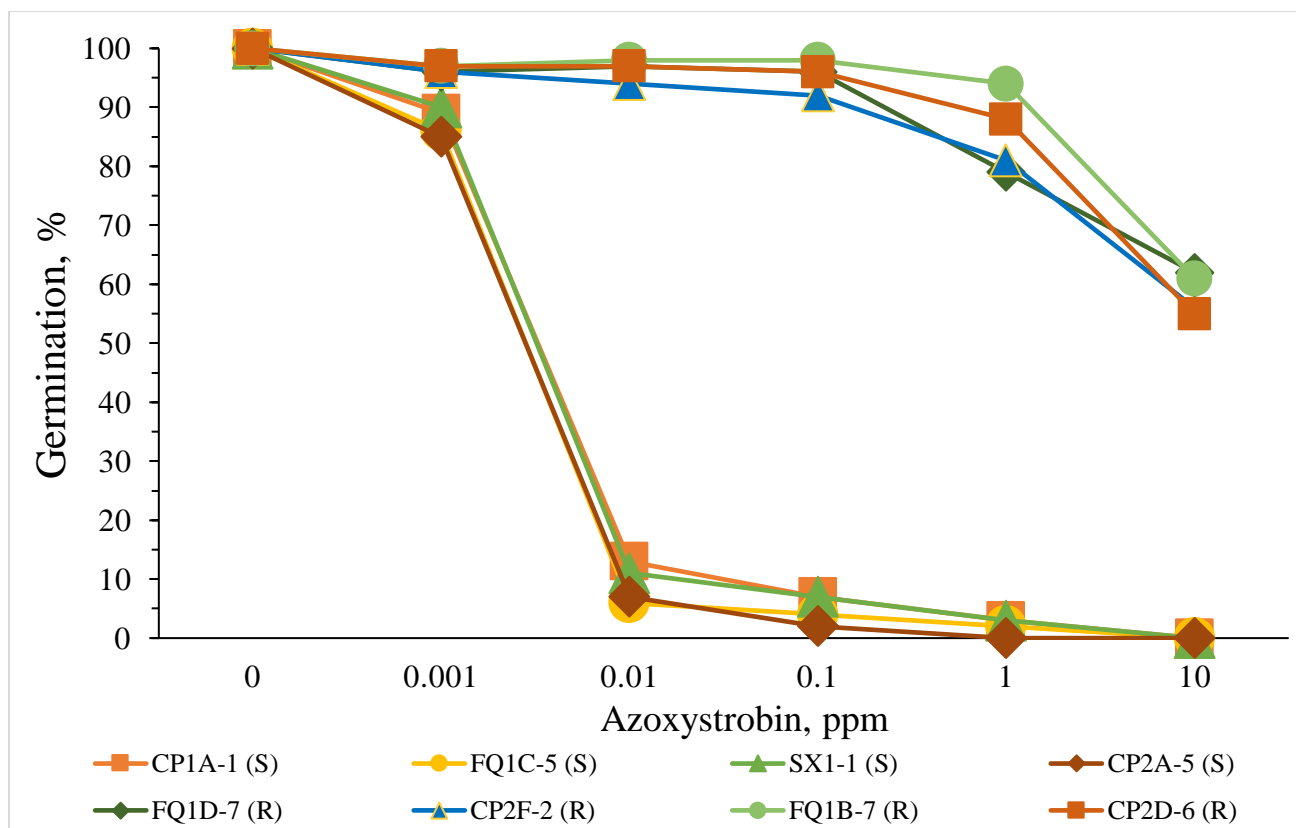
**Table 2.3.** Frequencies of the G143A mutation conferring quinone outside inhibitor fungicide resistance in *Cercospora sojina* populations from fields sampled in Virginia from 2015-2017.

Year	# fields	%G143A mutation <sup>a</sup>			# fields with indicated percentage of the G143A mutation (% fields)					
		Min	Max	Mean	<10%	10-25%	26-50%	51-75%	76-90%	>90%
2015	74	1	93	26	28 (38%)	16 (22%)	16 (22%)	10 (14%)	2 (3%)	2 (3%)
2016	15	3	99	57	2 (13%)	0 (0%)	4 (27%)	3 (20%)	5 (33%)	1 (7%)
2017	40	1	73	18	19 (48%)	11 (28%)	7 (18%)	3 (8%)	0 (0%)	0 (0%)
<b>Total</b>	<b>129</b>	<b>1</b>	<b>99</b>	<b>27</b>	<b>49 (38%)</b>	<b>27 (21%)</b>	<b>27 (21%)</b>	<b>16 (12%)</b>	<b>7 (5%)</b>	<b>3 (2%)</b>

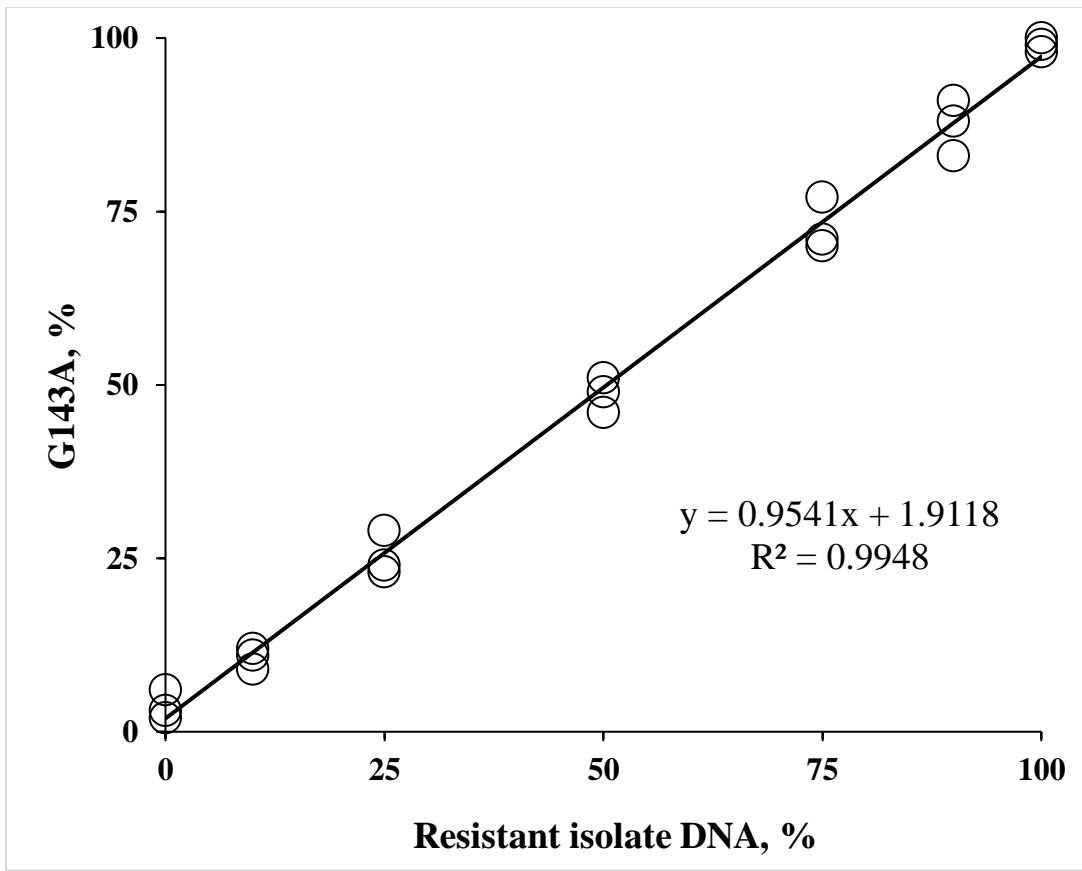
<sup>a</sup> Minimum, maximum, and mean percentages of *C. sojina* population with the G143A mutation quantified by pyrosequencing.



**Figure 2.1.** PCR products amplified from DNA of four *Cercospora sojina* isolates using wildtype (WT) and G143A primer sets to determine if isolates have the G143A point mutation in the cytochrome b gene. The expected amplicon sizes for the WT and G143A primers are 359 bp and 207 bp, respectively. M: 100-bp DNA ladder, 1: Isolate 1 – WT primers, 2: isolate 1 – G143A primers, 3: isolate 2 – WT primers, 4: isolate 2 – G143A primers, 5: isolate 3 – WT primers, 6: isolate 3 – G143A primers, 7: isolate 4 – WT primers, 8: isolate 4 – G143A primers. Based on the assay, isolates 1 and 2 have the G143A mutation while isolates 3 and 4 do not.



**Fig. 2.2.** Inhibition of *Cercospora soja* conidial germination on 2% water agar media plates amended with the quinone outside inhibitor (QoI) fungicide azoxystrobin. *C. soja* conidia of 4 isolates with (R) and 4 isolates without (S) the QoI-resistance conferring G143A mutation in the cytochrome b gene were exposed to five concentrations of azoxystrobin for 18 hours prior to evaluation of percent germination.



**Fig. 2.3.** Proportions of the G143A mutation in the cytochrome b gene measured with pyrosequencing from mixtures of DNA isolated from quinone outside inhibitor (QoI) sensitive and resistant isolates of *Cercospora sojina*. DNA from QoI resistant and sensitive isolates was mixed in ratios of 0:100, 10:90, 25:75, 50:50, 75:25, 90:10 and 100:0, and the proportion of the G143A mutation within DNA mixtures was measured in triplicate.

**Supplemental Table 2.1.** List of frogeye leaf spot infected soybean samples collected in Virginia from 2015 to 2017 and the percentage of the *Cercospora sojina* population from each field with the quinone outside inhibitor fungicide-resistance conferring G143A mutation.

County/city	Longitude	Latitude	Year	Date collected	Sample ID	Alternate ID	G143A, %	Fungicide (Y/N) <sup>a</sup>
Accomack	---	---	2016	10/6	AK16.01	AK1	99	N
Accomack	---	---	2016	10/6	AK16.02	AK2	89	N
Appomattox	N 37.340193	W 78.835914	2015	8/4	AX15	AX1	41	N
Bedford	N 37.30035	W 79.36313	2015	8/6	BF15	BF1	14	N
Brunswick	N 36.787779	W 77.807715	2017	9/28	BK17.01	BK1	3	N
Brunswick	N 36.649207	W 77.883186	2017	9/28	BK17.10	BK10	42	N
Brunswick	N 36.649638	W 77.883371	2017	9/28	BK17.11	BK11	28	N
Brunswick	N 36.649319	W 77.883384	2017	9/28	BK17.12	BK12	73	N
Brunswick	N 36.649556	W 77.882641	2017	9/28	BK17.13	BK13	8	N
Brunswick	N 36.649780	W 77.882108	2017	9/28	BK17.14	BK14	23	N
Brunswick	N 36.649104	W 77.882083	2017	9/28	BK17.15	BK15	2	N
Brunswick	N 36.786834	W 77.805988	2017	9/28	BK170.2	BK2	29	N
Brunswick	N 36.653879	W 77.799079	2017	9/28	BK17.03	BK3	26	N
Brunswick	N 36.652881	W 77.797587	2017	9/28	BK17.04	BK4	1	N
Brunswick	N 36.653096	W 77.797942	2017	9/28	BK17.05	BK5	68	N
Brunswick	N 36.654507	W 77.798145	2017	9/28	BK17.06	BK6	25	N
Brunswick	N 36.650342	W 77.906236	2017	9/28	BK17.07	BK7	14	N
Brunswick	N 36.650832	W 77.907673	2017	9/28	BK17.08	BK8	3	N
Brunswick	N 36.651297	W 77.909905	2017	9/28	BK17.09	BK9	15	N
Charles City	N 37.42577	W 76.96196	2015	8/3	CC15	CC1	4	N
Charles City	N 37.247768	W 76.950198	2017	9/29	CC17.01	CC17-1	1	N
Chesterfield	N 37.365241	W 77.819316	2015	8/13	CF15.01	CF1-1	3	N
Chesterfield	N 37.365241	W 77.819316	2015	8/13	CF15.02	CF1-2	2	N
Cumberland	N 37.82216	W 78.373268	2015	8/17	CL15.01	CL1-1	71	---
Cumberland	N 37.82216	W 78.373268	2015	8/17	CL15.02	CL1-2	38	---
Culpeper	---	---	2015	7/20	CP1A	CP1A	8	N

County/city	Longitude	Latitude	Year	Date collected	Sample ID	Alternate ID	G143A, %	Fungicide (Y/N) <sup>a</sup>
Culpeper	---	---	2015	7/20	CP1B	CP1B	1	N
Culpeper	---	---	2015	7/20	CP1C	CP1C	1	N
Culpeper	---	---	2015	7/20	CP1D	CP1D	5	N
Culpeper	---	---	2015	7/20	CP2A	CP2A	12	N
Culpeper	---	---	2015	7/20	CP2B	CP2B	24	N
Culpeper	---	---	2015	7/20	CP2C	CP2C	14	N
Culpeper	---	---	2015	7/20	CP2D	CP2D	18	N
Culpeper	---	---	2015	7/20	CP2E	CP2E	34	N
Culpeper	---	---	2015	7/20	CP2F	CP2F	14	N
Caroline	N 38.006989	W 77.286438	2017	9/29	CR17.01	CR17-1	16	N
Caroline	N 38.006782	W 77.286621	2017	9/29	CR17.02	CR17-2	5	N
Caroline	N 38.006376	W 77.287179	2017	9/29	CR17.03	CR17-3	7	N
Caroline	N 38.005911	W 77.287608	2017	9/29	CR17.04	CR17-4	7	N
Chesapeake	---	---	2015	9/25	CH15.01	CSPK NTC2	52	N
Chesapeake	---	---	2015	9/25	CH15.02	CSPK NTC3	52	N
Chesapeake	N 36.63958	W 76.16347	2016	8/25	CH15.01	CSPK1A	88	Y
Chesapeake	N 36.63973	W 76.16404	2016	8/25	CH16.01	CSPK1B	82	Y
Chesapeake	---	---	2016	8/25	CH16.02	CSPK1C	77	Y
Dinwiddie	---	---	2016	8/20	DW16	DW1	57	N
Fauquier	---	---	2015	7/20	FQ1A	FQ1A	71	N
Fauquier	---	---	2015	7/20	FQ1B	FQ1B	43	N
Fauquier	---	---	2015	7/20	FQ1C	FQ1C	81	N
Fauquier	---	---	2015	7/20	FQ1D	FQ1D	73	N
Fauquier	---	---	2015	7/20	FQ1E	FQ1E	16	N
Fauquier	---	---	2015	7/20	FQ1F	FQ1F	29	N
Goochland	N 37.73814	W 78.106267	2015	8/14	GL15.01	GL1-1	47	---
Goochland	N 37.73814	W 78.106267	2015	8/14	GL15.02	GL1-2	38	---
Isle of Wight	---	---	2015		IW15	IOW1	39	---

County/city	Longitude	Latitude	Year	Date collected	Sample ID	Alternate ID	G143A, %	Fungicide (Y/N) <sup>a</sup>
Lancaster	---	---	2016	8/24	LC16.01	LC1-1	3	---
Lancaster	N 37.746863	W 76.461886	2016	10/4	LC16.02	LC1-2	44	N
Lancaster	N 37.747037	W 76.462460	2016	10/4	LC16.03	LC1-3	36	N
Lunenburg	---	---	2016	9/6	LN16.01	LN1	67	N
Lunenburg	---	---	2017	9/28	LN17.01	LN3	1	N
Lunenburg	---	---	2016	9/6	LN16.02	LN2	35	N
Madison	N 38.298455	W 78.1443389	2015	8/12	MA15.01	MA1	75	N
Madison	N 38.288402	W 78.141075	2015	8/12	MA15.02	MA2-1	91	N
Madison	N 38.288402	W 78.141075	2015	8/12	MA15.03	MA2-2	1	N
Madison	N 38.288402	W 78.141075	2015	8/12	MA15.04	MA2-3	1	N
Madison	N 38.300217	W 78.137394	2015	9/14	MA15.03	MA3	4	---
Mecklenburg	---	---	2015	8/11	MB15.01	MB1	3	N
Mecklenburg	---	---	2015	8/11	MB15.02	MB2	3	N
Mecklenburg	---	---	2015	8/11	MB15.03	MB3	5	N
Mecklenburg	N 36.812120	W 77.786989	2017	9/28	MK17.01	MK17-1	37	N
Mecklenburg	N 36.812060	W 77.788276	2017	9/28	MK17.02	MK17-2	1	N
Mecklenburg	N 36.812094	W 77.789413	2017	9/28	MK17.03	MK17-3	2	N
Mecklenburg	N 36.813829	W 77.788759	2017	9/28	MK17.04	MK17-4	47	N
Mecklenburg	N 36.816690	W 77.789209	2017	9/28	MK17.05	MK17-5	1	N
Middlesex	---	---	2015	9/9	MS15.01	MS1	42	---
Middlesex	---	---	2016	10/4	MS15.07	MS7	81	---
Middlesex	---	---	2015	9/9	MS15.02	MS2	16	---
Middlesex	---	---	2015	9/9	MS15.03	MS3	14	---
Middlesex	---	---	2015	9/9	MS15.04	MS4	3	---
Middlesex	---	---	2015	9/9	MS15.05	MS5	13	---
Middlesex	---	---	2015	9/9	MS15.06	MS6	38	---
New Kent	N 37.61430	W 77.13537	2015	8/4	NK15.01	NK1`	14	N
New Kent	N 37.61201	W 77.12520	2015	8/10	NK15.02	NK2	7	N

<b>County/city</b>	<b>Longitude</b>	<b>Latitude</b>	<b>Year</b>	<b>Date collected</b>	<b>Sample ID</b>	<b>Alternate ID</b>	<b>G143A, %</b>	<b>Fungicide (Y/N)<sup>a</sup></b>
New Kent	N 37.61436	W 77.13535	2015	8/24	NK15.03	NK3-1	25	---
New Kent	N 37.61436	W 77.13535	2015	8/24	NK15.04	NK3-2	51	---
Nelson	N 37.63647	W 78.72012	2015	8/20	MM15	NN1	11	---
Nottoway	N 37.261851	W 78.131205	2015	8/13	NW15.01	NW1-1	3	N
Nottoway	N 37.261851	W 78.131205	2015	8/13	NW15.02	NW1-2	3	N
Orange	N 38.27632	W 77.7964472	2015	8/12	OG15.01	OG1-1	32	---
Orange	N 38.27632	W 77.7964472	2015	8/12	OG15.02	OG1-2	50	---
Orange	N 38.260197	W 78.12166	2015	9/14	OG15.03	OG2	35	---
Prince George	---	---	2015	8/11	PG15	PG1	48	N
Prince George	N 37.132383	W 77.364494	2017	9/29	PG17.01	PGCR1	15	N
Prince George	N 37.132297	W 77.364301	2017	9/29	PG17.02	PGCR2	3	N
Prince George	N 37.132733	W 77.363609	2017	9/29	PG17.03	PGCR3	2	N
Prince George	N 37.132549	W 77.363266	2017	9/29	PG17.04	PGCR4	3	N
Prince George	N 37.132344	W 77.362997	2017	9/29	PG17.05	PGCR5	19	N
Prince George	---	---	2017	9/29	PG17.06	PGER1	3	N
Prince George	---	---	2017	9/29	PG17.08	PGER2	17	N
Powhatan	N 37.583685	W 77.934204	2015	8/6	PW15.01	PW1-1	1	---
Powhatan	N 37.583685	W 77.934204	2015	8/6	PW15.02	PW1-2	3	---
Richmond	---	---	2015	8/10	RM15.01	RMWAR1	59	N
Richmond	---	---	2015	8/10	RM15.02	RMWAR2	80	N
Richmond	---	---	2015	8/10	RM15.04	RMWAR4	53	N
Richmond	---	---	2017		RM17	RM5	9	N
Richmond	---	---	2015	8/10	RM15.03	RMWAR3	74	N
Suffolk	---	---	2015	7/24	SF15.01	SF3A	2	N
Suffolk	---	---	2015	7/16	SF15.02	SFF215 201-2	15	N
Suffolk	---	---	2015	7/16	SF15.03	SFF215 204-7	5	N
Suffolk	---	---	2015	7/16	SF15.04	SFF215 206-6	9	N
Suffolk	---	---	2015	7/16	SF15.05	SFF215 303-4	11	N



County/city	Longitude	Latitude	Year	Date collected	Sample ID	Alternate ID	G143A, %	Fungicide (Y/N) <sup>a</sup>
Suffolk	---	---	2015	7/16	SF15.06	SFF215 401-1	93	N
Suffolk	---	---	2015	7/16	SF15.07	SFF215 406-5	1	N
Suffolk	N 36.683078	W 76.765279	2017	9/20	SF17.01	SFK17-1	11	Y
Suffolk	N 36.682828	W 76.766054	2017	9/20	SF17.02	SFK17-2	4	Y
Suffolk	N 36.676818	W 76.754617	2017	9/20	SF17.03	SFK17-3	13	Y
Suffolk	N 36.676280	W 76.753573	2017	9/20	SF17.04	SFK17-4	13	Y
Suffolk	N 36.676966	W 76.753329	2017	9/20	SF17.05	SFK17-5	67	Y
Suffolk	N 36.675682	W 76.753858	2017	9/20	SF17.06	SFK17-6	37	Y
Spotsylvania	N 38.313322	W 77.6714805	2015	8/12	SP15.01	SP1-1	1	N
Spotsylvania	N 38.313322	W 77.6714805	2015	8/12	SP15.02	SP1-2	2	N
Sussex	---	---	2015	7/23	SX15.01	SX1	8	N
Sussex	---	---	2015	7/23	SX15.02	SX2	1	N
Sussex	---	---	2015	7/23	SX15.03	SX3	2	N
Virginia Beach	---	---	2015	8/10	VB15.01	VB1	47	N
Virginia Beach	N 36.745871	W 76.087528	2016	8/12	VB16.01	VB2A	60	---
Virginia Beach	N 36.746434	W 76.087426	2016	8/12	VB16.02	VB2B	4	---
Virginia Beach	N 36.746758	W 76.087405	2016	8/12	VB16.03	VB2C	28	---
Virginia Beach	---	---	2015	8/10	VB15.02	VB2	19	N
Virginia Beach	---	---	2015	8/10	VB15.03	VB3	33	N

<sup>a</sup> Y = yes, samples were collected after the soybeans were treated with fungicide; N = no, samples were collected from soybeans not treated with fungicide; --- indicates information is not known.

### **Chapter 3: Effects of fungicide application timing and weather variables on disease severity and yield loss in soybean**

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#### **Abstract**

Previous studies have demonstrated that in the absence of disease pressure, yield loss to disease does not occur and foliar fungicide applications are not profitable in soybean production. Thus, the timing of foliar fungicide applications should coincide with periods of risk for foliar disease and yield loss. The objectives of this study were to 1) compare different fungicide application timings for disease control and yield protection, 2) determine if weather-based disease-favorable days can be a better predictor of fungicide timing than developmental stages in terms of disease control and yield protection, and 3) determine if higher disease pressure is associated with greater yield loss and predictive of the need for fungicides to protect yield. Six experiments were conducted at two locations in Virginia from 2014 to 2016. Fungicide was applied at either beginning pod (R3), 1 to 4 weeks after R3, or weekly applications between R3 and full pod (R5). Foliar disease was rated and yield was measured at harvest. Temperature and relative humidity were monitored for each experiment, and a day was considered favorable for disease if the daily average temperatures were between 20 and 30°C and there were 10 or more

hours of relative humidity  $\geq 90\%$  within a 24-hour period from noon to noon. Foliar disease was observed late in the season, but fungicide applications reduced disease severity for only a single experiment. Yield decreased as fungicide applications were made later and later after R3 stage ( $r^2 = 0.80$ ,  $P = 0.01$ ). Weekly fungicide application resulted in the greatest yield, however it was not statistically different from the single application timings except four weeks after R3 which did not differ from non-treated control. Higher numbers of cumulative disease favorable days between planting and R3 were associated with greater late season disease severity ( $r^2 = 0.94$ ,  $P = <0.01$ ). Correlation between disease severity and yield loss ( $r^2 = 0.53$ ,  $P = 0.10$ ) suggests a foliar fungicide application is more likely to be beneficial as disease favorable days prior to R3 increase. Thus, prior to deciding whether a foliar fungicide is needed for soybean, the crop's developmental stage, disease pressure, and environmental parameters need to be taken into consideration.

## **Keywords**

Soybean; foliar fungicide application timing; soybean foliar diseases

## **Introduction**

Foliar fungicides are used to control fungal diseases in a variety of crops, but they were used minimally in the management of soybean foliar diseases in the United States until the confirmation of soybean rust (*Phakopsora pachyrhizi*) in 2004. Soybean rust is a potentially devastating foliar disease of soybean that can cause between 55 and 80% yield loss if unmanaged (Hartman et al., 1991; Miles et al., 2007; Mueller et al., 2009, Hartman et al. 2011). Prior to 2004, less than 1% of soybean was treated with fungicides, but even though soybean rust was not

as devastating to the U.S. soybean crop as anticipated, the percentage of the crop treated with fungicide increased incrementally to 2% in 2005, 4% in 2006, 11% in 2012, and 14% in 2017 (USDA NASS, 2019). In addition to the potential threat of soybean rust, the increased use of foliar fungicides may be a result of the increased grain value, larger profit margins that make it more economical for growers to apply foliar fungicides, and plant health benefits marketed by industry (Henry et al., 2011). Since 2014, soybean prices have been on the decline, and with rising fungicide costs, the profit margins have narrowed. Thus, it has become increasingly important for growers to consider whether or not a foliar fungicide will be profitable prior to making an application.

Fungicide application timing is an important factor in determining the effectiveness of disease control and yield protection in soybean (Mueller et al., 2009; MacLean et al., 2018). Research has been conducted to determine the optimal fungicide application timing for soybean, and previous studies have shown that applications made between R1 (beginning flowering) and R3 (beginning pod) provide the best disease control and yield protection (Mueller et al., 2009; Akem, 1995; Boyer et al., 2017). Fungicide application made before R1 or after R6 (full seed) are not recommended (Board et al., 2010). In the Mid-Atlantic region, a single foliar fungicide application is typically made at the R3 developmental stage for soybean. The initial disease onset of soybean foliar diseases usually occurs between R1 and R3 stage. Loss of photosynthetic leaf area during pod development, which can be caused by insect damage and diseases, is the major cause of yield loss during this period. A defoliation rate of 50% during these stages can result in 6% yield loss (Hodgson et al., 2012). Pod development and filling occurs between R3 and R6 stage. This period is crucial for yield setting in soybean (Parvej and Holshouser, 2017). Fungicide applied at R3 protects the photosynthetic leaf area of the crop from fungal infection

and reduces stress during the pod filling stages. However, yield benefits associated with R3 fungicide applications are inconsistent (Koenning and Wrather, 2010; Dorrance et al., 2010). Fungicide trials conducted between 2006 and 2013 at the Virginia Tech Tidewater Agricultural Research and Extension Center in Suffolk, Virginia resulted in a significant difference in yield between fungicide treated and non-treated soybean approximately one-third of the time. The lack of significant yield differences may be due to improper timing, low disease pressure, and/or unfavorable environmental conditions for disease development.

Little research has been conducted to determine the optimal foliar fungicide timing for soybean in the Mid-Atlantic U.S., but a pathogen requires a susceptible host and favorable environmental conditions in order for it to infect a crop and cause disease (Hughes et al., 1999; Francl, 2001). In Virginia, frogeye leaf spot (*Cercospora sojina* K. Hara) and Cercospora leaf blight (*Cercospora kikuchii* (Matsumoto & Tomoyasu) M.W. Gardner) are the primary foliar diseases that cause yield losses in soybean (Allen et al., 2017). These foliar diseases are favored by warm (20 to 30°C) and wet (relative humidity of  $\geq 90\%$ ) conditions (Mian et al., 2008; Cruz and Dorrance, 2009; Schuh, 1991; Schuh and Adamowicz, 1993). A risk scoring system was developed in Argentina to better time foliar fungicides to control late season soybean diseases (LSD) such as Septoria brown spot and Cercospora leaf blight. Rainfall amount and intensity between R3 and R5 were the two environmental factors most closely associated with LSD epidemics (Carmona et al., 2015). Rainfall, in addition to providing moisture needed for the pathogen's development, can splash fungal spores upwards and spreads disease within the leaf canopy (Cruz et al., 2010). Conducive conditions for disease development increase the risk of yield loss and may indicate that a foliar fungicide application is needed to maximize crop yield.

The “plant disease triangle” has three components: susceptible host, presence of a pathogen, and conducive environment for disease development (Yuen and Hughes, 2002). Disease cannot occur if any component of the triangle is absent. Application of fungicides in the absence of disease or when disease pressure is low is unlikely to be profitable. Field trials in the Texas Gulf Coast reported only 13% of the fungicide applications had a net increase in profit when foliar diseases were at low levels (Grichar, 2013).

Crop susceptibility to disease, crop developmental stage, disease pressure, and environmental conditions all contribute to risk of disease and crop yield loss, and these risk factors determine the extent to which a foliar fungicide application will be economical. Therefore, the objectives of this study were to 1) compare different fungicide application timings for disease control and yield protection, 2) determine if weather-based disease-favorable days can be a better predictor of fungicide timing than developmental stages in terms of disease control and yield protection, and 3) determine if higher disease pressure is associated with greater yield loss and predictive of the need for fungicides to protect yield.

## **Material and methods**

*Evaluation of fungicide application timings.* Field experiments were conducted in 2014 (n=2), 2015 (n=3), and 2016 (n=1) at the Virginia Tech Tidewater Agricultural Research and Extension Center (AREC) in Suffolk, Virginia and the Eastern Virginia AREC in Warsaw, Virginia (Table 3.1). Experimental design was four randomized complete blocks with 2.44 m alleys between blocks. Each plot was 3.66 m wide planted to four 9.14-m rows spaced 0.20 m apart. Though soybean variety planted varied among experiments, all were Asgrow varieties that

are susceptible to frogeye leaf spot and *Cercospora* leaf blight. Pyraclostrobin + fluxapyroxad was applied at 0.29 L/ha (Priaxor 4.17SC, BASF Corporation, Research Triangle Park, North Carolina) in 2014 and 2015. Strobilurin-resistant frogeye leaf spot was confirmed in Virginia in 2015, so in 2016, pyraclostrobin + fluxapyroxad was applied but it was mixed with tetraconazole at 0.29 L/ha (Domark, Isagro USA, Inc., Morrisville, North Carolina) for fungicide resistance management (Table 3.1). For trials at Suffolk, VA, fungicide was applied with non-ionic surfactant at 0.47 L/ha (Induce, Helena Agri-Enterprises, LLC, Collierville, Tennessee) using a Lee Spider sprayer with eight 8002VS nozzles spaced 0.46 m apart. The volume of fungicide delivered was 186 L/ha. For trials at Warsaw, VA, fungicide was applied with non-ionic surfactant at 0.47 L/ha (Induce, Helena Agri-Enterprises, LLC, Collierville, Tennessee) using a backpack sprayer with two 8002VS nozzles 47 cm apart delivering 182 L/ha. Seven treatments were applied starting at the beginning pod (R3) developmental stage of the soybean crop. Treatments included fungicide applied at R3, 1 week after R3, 2 weeks after R3, 3 weeks after R3, 4 weeks after R3, five weekly applications beginning at R3, and a non-treated control.

*Disease ratings and yield.* Disease ratings were taken bi-weekly from the center 2 treatment rows. The overall disease severity for each plot was rated on a 0 to 10 categorical scale where 0 = no symptoms, 1 = lesions covering 1 to 10% of the leaf surface on one or a few plants, 2 = lesions covering 11 to 30% of the leaf surface on one or a few plants, 3 = lesions covering 1 to 10% of the leaf surface on most (>50%) of the plants, 4 = lesions covering 11 to 30% of the leaf surface on most (>50%) of the plants, 5 = all plants infected with spots covering 1 to 10% leaf surface on all plants, 6 = lesions covering 11 to 30% of the leaf surface on all plants, 7 = lesions covering 31 to 50% of the leaf surface on all plants, 8 = lesions covering 51 to 70% of the leaf surface on all plants, 9 = lesions covering 71 to 80% of the leaf surface on all plants, and 10

= lesions covering >80% of the leaf surface on all plants. Soybeans were harvested at maturity and weighed. Percent moisture of soybeans harvested from each plot was measured using a Dickey-John GAC2000 Moisture Tester (Dickey-John, Auburn, IL, USA). Yield of soybean from each plot was calculated using measured weight of soybean from harvested plot, percent moisture, and plot dimensions.

*Comparison of fungicide application timings.* Analysis of variance (ANOVA) was used to compare different fungicide application timing treatments for yield and disease control. Experiment and experiment by treatment interactions were included in the model. Fisher's Protected LSD test was used to separate the means post-ANOVA for comparison. The relationships between disease, yield, and fungicide application timing relative to R3 were determined using linear regression. Application timing was converted to a continuous variable as follows: 0 = R3, 1 = 1 week after R3, 2 = 2 weeks after R3, 3 = 3 weeks after R3, and 4 = 4 weeks after R3. Since weekly applications were expected to provide greater control than R3 applications, -1 was used to represent this treatment in the analysis. All data analysis was done in JMP Pro 13.0.0, SAS Institution Inc., Cary, NC.

*Weather data and determination of disease favorable days.* For the Suffolk experimental location, daily average temperature and hourly relative humidity were obtained from a weather station at the Tidewater AREC. For the Warsaw location, weather data from Lottsburg, VA were used for analyses. Lottsburg weather data were obtained from National Oceanic and Atmospheric Administration (NOAA) National Weather Service at Wakefield, VA. Disease favorable days were determined based on environmental variables favorable for sporulation, infection, and growth of important foliar fungal pathogens of soybean. A 24 h period (noon to noon) was considered a favorable day if mean temperatures were between 20 and 30°C and there



were 10 or more hours of relative humidity  $\geq 90\%$ . Cumulative favorable days were calculated from the date of planting to harvest for each experiment.

*Relationship between disease favorable days, disease severity, and yield loss.* Linear regression was used to examine the relationships between disease favorable days, disease severity, and yield loss. Disease severity was represented by the disease ratings taken between late R5 and early R6 from the non-treated controls in each experiment. Yield loss was calculated as the difference in yield between weekly sprays and the non-treated controls for each experiment. Disease favorable days were determined for the following intervals for each experiment: from planting to R3, from three weeks before R3 to R3, from two weeks before R3 to R3, from R3 to R6, and from planting to R6.

## **Results**

*Comparison of fungicide application timings.* Disease severity at the late R5/early R6 developmental stage of the soybean crop varied among experiments ( $P < 0.01$ ), application timings ( $P < 0.01$ ), and there was an interaction between experiment and application timing ( $P < 0.01$ ). Disease severity ratings in non-treated controls ranged from 4.5 to 6.5, but only one of the experiments had significant differences in disease severity between fungicide treatments and the non-treated control (Table 3.2). When disease severity versus fungicide application timing relative to R3 was analyzed, there was a weak but significant relationship between the two variables for Suffolk-2014 ( $r^2 = 0.29$ ,  $P < 0.01$ ) and Warsaw-2015 ( $r^2 = 0.22$ ,  $P = 0.01$ ) with greater disease severity as the number of weeks after R3 increased for the application timing.

Yield varied among experiments ( $P < 0.01$ ) and application timings ( $P < 0.01$ ), but there was not an experiment by application timing interaction ( $P = 0.94$ ). Among experiments, yield of

the non-treated controls (NTC) ranged from 3166 to 4165 kg/ha with lowest yields in experiments conducted in 2015 (Table 3.3). Though individual experiments had similar trends, only Warsaw-2014 had significant differences among treatments (Table 3.3). The combined analysis of variance had the same relative differences among treatments as the Warsaw-2014 experiment; only weekly fungicide applications resulted in greater yield compared to the non-treated, but yield for this treatment was not statistically different from any of the other fungicide application treatments with the exception of four weeks after R3 (Table 3.3). Still, the overall trend was that yield progressively decreased (and yield loss increased) as fungicide application was made later and later after the R3 stage ( $r^2 = 0.80$ ,  $P = 0.01$ ).

*Relationship between disease favorable days, disease severity, and yield loss.*

Accumulation of disease favorable days varied among experiments, with the total number of disease favorable days between planting and harvest ranging from 15 to 30 (Fig. 3.1). Number of disease favorable prior to each fungicide application timing was determined for each experiment, but no relationship was found between disease favorable days and yield relative to the untreated control ( $r^2 = 0.09$ ,  $P = 0.08$ ). There was a weak but significant relationship between disease favorable days prior to fungicide application and disease severity at the late R5/early R6 stage ( $r^2 = 0.11$ ,  $P = 0.04$ ) with disease severity increasing as the number of disease favorable days prior to the fungicide application increased (Fig. 3.2). Cumulative favorable days for different intervals were determined and correlated with disease severity and yield loss (difference in yield between weekly sprays and non-treated). There were weak correlations between number of disease favorable days and yield loss for all intervals prior to R3 except 2 weeks prior to R3 ( $r^2 = 0.42$ ) which had moderate predictive values (Table 3.4, Fig. 3.3). There was a positive correlation between disease favorable days and disease severity in non-treated controls at late

R5/early R6 (Table 3.4). Among the intervals tested, the number of favorable days between planting and R3 had the strongest relationship with late season disease severity ( $r^2 = 0.94$ ) (Table 3.3), but number of favorable days 2 weeks prior to R3 also showed moderate predictive value for disease ( $R^2 = 0.423$ ) (Table. 3.4).

*Relationship between disease pressure and yield loss.* Types and onset of diseases varied among experiments, but the dominant disease in each field was Cercospora leaf blight (CLB) and/or frogeye leaf spot (FLS). CLB was the primary disease in Suffolk experiments in all years, and FLS was the dominant disease for Warsaw experiments. First symptoms of FLS were found near the R3 stage in small clusters in the field, and as the season progressed, spots became more uniform on the leaves in the upper canopy. Initial symptoms of CLB begin to appear on the upper leaves of plants at the R5 stage, and it was the dominant disease at R6. Disease severity ratings of non-treated controls were plotted against yield loss (yield of weekly application minus yield of non-treated) to determine if greater disease pressure resulted in greater yield loss. Disease severity at late R5/early R6 explained 53% of the variation in yield loss ( $P=0.10$ ) with greater disease severity being associated with greater yield loss (Fig. 3.4).

## **Discussion**

Disease severity and associated yield losses in crops are driven by interactions among the plant host, environment, and pathogen. The overall objective of the current study was to evaluate foliar fungicide application timing for soybean as it relates to the developmental stage of the crop, weather conditions conducive for disease, and the presence of fungal pathogens. No single factor was highly predictive of disease severity or yield loss to disease. The analysis that

examined the relationship between favorable days, disease, and yield supports the notion that all three components of the plant disease triangle must be evaluated to determine if and when fungicide applications will be optimal to protect crop yield.

Results of the current study provide support for the beginning pod stage (R3) of the soybean crop as the optimum time to apply a foliar fungicide. Though weekly fungicide applications resulted in the highest yield, among single applications the R3 fungicide timing had the numerically highest yield across the six experiments. Weekly applications during pod development are not practical due to the cost of fungicides, and in most situations even two fungicide applications would not be economical. Foliar fungicide for soybean is usually applied between R3 and R5. The relationship between fungicide application timing and both yield and disease in this study suggest that applications during early soybean pod development are more likely to result in greater yield and lower disease severity than fungicides applied at R4 or R5 developmental stages.

Frogeye leaf spot was found at all locations in all years. In Suffolk, Virginia, the dominant foliar disease between R3 and R5 was frogeye leaf spot. After R5, *Cercospora* leaf blight was the dominant foliar disease. Frogeye leaf spot is a polycyclic disease, meaning multiple infection cycles can occur in a single crop cycle (Arneson, 2001; Kim et al., 2013). Severity of polycyclic disease is partially dependent on the initial inoculum levels (Fry, 1982). Applying fungicide in the early reproductive stages can reduce and delay secondary infections to slow down disease development. Older and full-grown soybean leaves reportedly were more resistant to *C. sojina* infection (Mian et al., 2008). Though in some experiments fungicide applications reduced disease severity, yield differences between fungicide treated and non-treated plots were small. This may be in part due to the low disease pressure observed across the

two experimental locations and three years of the study. The highest categorical disease rating taken at R6 for a plot was 6.5 (equivalent to 20% of leaf surface was covered with lesions on all plants in the plot) and the average disease rating at R6 was 5.7 (equivalent to lesions covering 1 to 10% of all plants' leaf surface).

Conducive environmental conditions are an important component of disease development. In this study we used “disease favorable days” based on daily average temperature and relative humidity to quantify favorable conditions for the development of soybean foliar diseases including frogeye leaf spot and *Cercospora* leaf blight. We used least-square regression to determine if favorable days can be a better predictor of fungicide timing than developmental stages for disease control and yield protection. Higher numbers of cumulative disease favorable days between planting and R3 were associated with greater late season disease severity. Correlation between disease severity and yield loss suggests a foliar fungicide application is more likely to be beneficial as disease favorable days prior to R3 increase. For frogeye leaf spot, the initial infection and first symptoms usually occur between R1 to R3, when the soybean leaves are young (Lin and Kelly, 2018). R1 to R3 is one of the critical stages for determining soybean yield as vegetative, flower, and pod development occurs simultaneously during this period. However, the effect of stress during this period on yield loss may not be significant as soybean can compensate some loss by producing more seeds per pod or increasing the seed size. Extended favorable conditions during this critical early pod development stage can promote conidia sporulation and secondary infection of expanding leaves (Lin and Kelly, 2018). Number of favorable days within 2 weeks before R3 shows moderate predictive value for yield loss (Fig. 3.3) and disease ( $R^2 = 0.423$ ), and this corresponds to the early reproductive stages of the soybean crop.

## **Conclusions**

Environmental conditions influence fungal disease development and can be used to help predict whether or not making a foliar fungicide application in soybean will be profitable. The beginning pod (R3) to full pod (R5) stages of the soybean crop are critical for yield development. The decision of whether or not to make a foliar fungicide application in soybean needs to take into account crop developmental stage, disease pressure, and environmental conditions in order to increase the likelihood that the foliar fungicide application will result in higher yield and a positive return on investment.

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**Table 3.1.** Location, soybean variety, fungicide, and dates of planting, beginning pod (R3), and harvest for field experiments conducted in Virginia from 2014-2016.

<b>Year</b>	<b>Location</b>	<b>Variety<sup>a</sup></b>	<b>Fungicide<sup>b</sup></b>	<b>Planting date</b>	<b>R3 date<sup>c</sup></b>	<b>Disease rating date<sup>d</sup></b>	<b>Harvest date</b>
2014	Suffolk	AG5332	Priaxor SC	06 Jun	10 Aug	23 Sep	19 Oct
2014	Warsaw	AG4934	Priaxor SC	27 May	25 Jul	23 Sep	09 Oct
2015	Suffolk	AG5732	Priaxor SC	21 May	31 Jul	3 Sep	30 Oct
2015	Suffolk	AG5732	Priaxor SC	05 Jun	26 Aug	24 Sep	30 Oct
2015	Warsaw	AG4934	Priaxor SC	01 Jun	30 Jul	4 Sep	15 Oct
2016	Suffolk	AG4934	Priaxor SC + Domark ME	09 Jun	10 Aug	15 Sep	07 Nov

<sup>a</sup> Three different Asgrow (AG) soybean varieties were used, but all are susceptible to frog-eye leaf spot (FLS) and Cercospora leaf blight.

<sup>b</sup> Indicated fungicides were each applied at a rate of 0.29 L/ha. Priaxor is a combination of a strobilurin (pyraclostrobin) and SDHI (fluxapyroxad) fungicide and Domark is a triazole fungicide (tetraconazole). Domark was mixed with Priaxor in 2016 after strobilurin-resistant FLS was confirmed in Virginia.

<sup>c</sup> R3 is the beginning pod stage of the soybean crop, and the first fungicide application was made at R3.

<sup>d</sup> Disease rating used for analysis was taken between late R5 and early R6 developmental stage.

**Table 3.2.** Disease severity ratings for soybean from different fungicide application timing treatments for six experiments conducted in Virginia from 2014-2015.

Fungicide timing <sup>a</sup>	Disease severity rating <sup>b,c</sup>					
	SF14	WS14	SF15-1	SF15-2	WS15	SF16
Weekly	6.0 ab	6.5 a	5.0 a	5.0 a	5.8 a	4.5 a
R3	6.0 ab	6.5 a	5.0 a	5.0 a	6.1 a	4.5 a
R3+1wk	5.0 b	6.5 a	5.0 a	5.0 a	5.9 a	4.5 a
R3+2wk	6.0 ab	6.5 a	5.1 a	5.0 a	5.9 a	4.5 a
R3+3wk	6.0 ab	6.5 a	5.0 a	5.0 a	5.9 a	4.5 a
R3+4wk	6.5 a	6.5 a	5.0 a	5.0 a	5.8 a	4.5 a
NTC	6.5 a	6.5 a	5.0 a	5.0 a	6.5 a	4.5 a
<b>P-value</b>	<0.01	---	0.86	---	0.57	---
<b>LSD</b>	0.28	---	0.42	---	0.81	---

<sup>a</sup> Single fungicide applications were made at beginning pod (R3) or one to four weeks after beginning pod (R3 + numbers of weeks after). The weekly treatment included five weekly fungicide applications with the first application at R3 and the final application four weeks after R3 (R + 4 wk). NTC = non-treated control.

<sup>b</sup> Foliar disease was rated between the late R5 and early R6 stage on a 0 to 10 scale where 0 = no disease and 10 = all plants severely infected with loss of more than 80% of photosynthetic area. The major disease in Suffolk pre-R6 was frogeye leaf spot (FLS) and post-R6 was Cercospora leaf blight (CLB). The major disease in Warsaw was FLS.

<sup>c</sup> SF = Suffolk, WS = Warsaw. The number following the two-letter location code indicates the year. SF15-1 and SF16-1 were the first and second planting date in Suffolk 2015, respectively. Within a column, values followed by the same letter are not significantly different ( $P>0.05$ ) according to Fisher's Protected LSD.

**Table 3.3.** Soybean yield from different fungicide application timing treatments for six experiments conducted in Virginia from 2014-2016.

Fungicide timing <sup>a</sup>	Yield, kg/ha <sup>b</sup>						
	SF14	WS14	SF15-1	SF15-2	WS15	SF16	Combined
Weekly	4633 a	4543 a	3700 a	3421 a	3547 a	3759 a	4008 a
R3	4503 a	4121 ab	3505 a	3027 a	3287 a	3857 a	3796 ab
R3 + 1 wk	4282 a	4318 ab	3525 a	2812 a	3457 a	4002 a	3787 ab
R3 + 2 wk	4450 a	4080 ab	3329 a	3039 a	3093 a	3957 a	3741 ab
R3 + 3 wk	4477 a	4201 ab	3398 a	3054 a	3400 a	3960 a	3793 ab
R3 + 4 wk	4180 a	3944 b	3166 a	3282 a	3022 a	3669 a	3606 b
NTC	4165 a	3764 b	3355 a	3166 a	3503 a	3581 a	3595 b
<b>P-value</b>	0.10	0.01	0.74	0.47	0.30	0.78	0.01
<b>LSD</b>	539	402	1989	1790	1603	1989	314

<sup>a</sup> Single fungicide applications were made at beginning pod (R3) or one to four weeks after beginning pod (R3 + numbers of weeks after). The weekly treatment included five weekly fungicide applications with the first application at R3 and the final application four weeks after R3 (R + 4 wk). NTC = non-treated control.

<sup>b</sup> SF = Suffolk, WS = Warsaw. The number following the two-letter location code indicates the year. SF15-1 and SF16-1 were the first and second planting date in Suffolk 2015, respectively. Within a column, values followed by the same letter are not significantly different ( $P>0.05$ ) according to Fisher's Protected LSD.

**Table 3.4.** Coefficient of determination ( $r^2$ ) and  $P$ -values for the relationship between disease favorable days (DFD)<sup>a</sup> during selected intervals and disease severity or yield loss in soybean foliar fungicide experiments conducted in Virginia from 2014-2016.

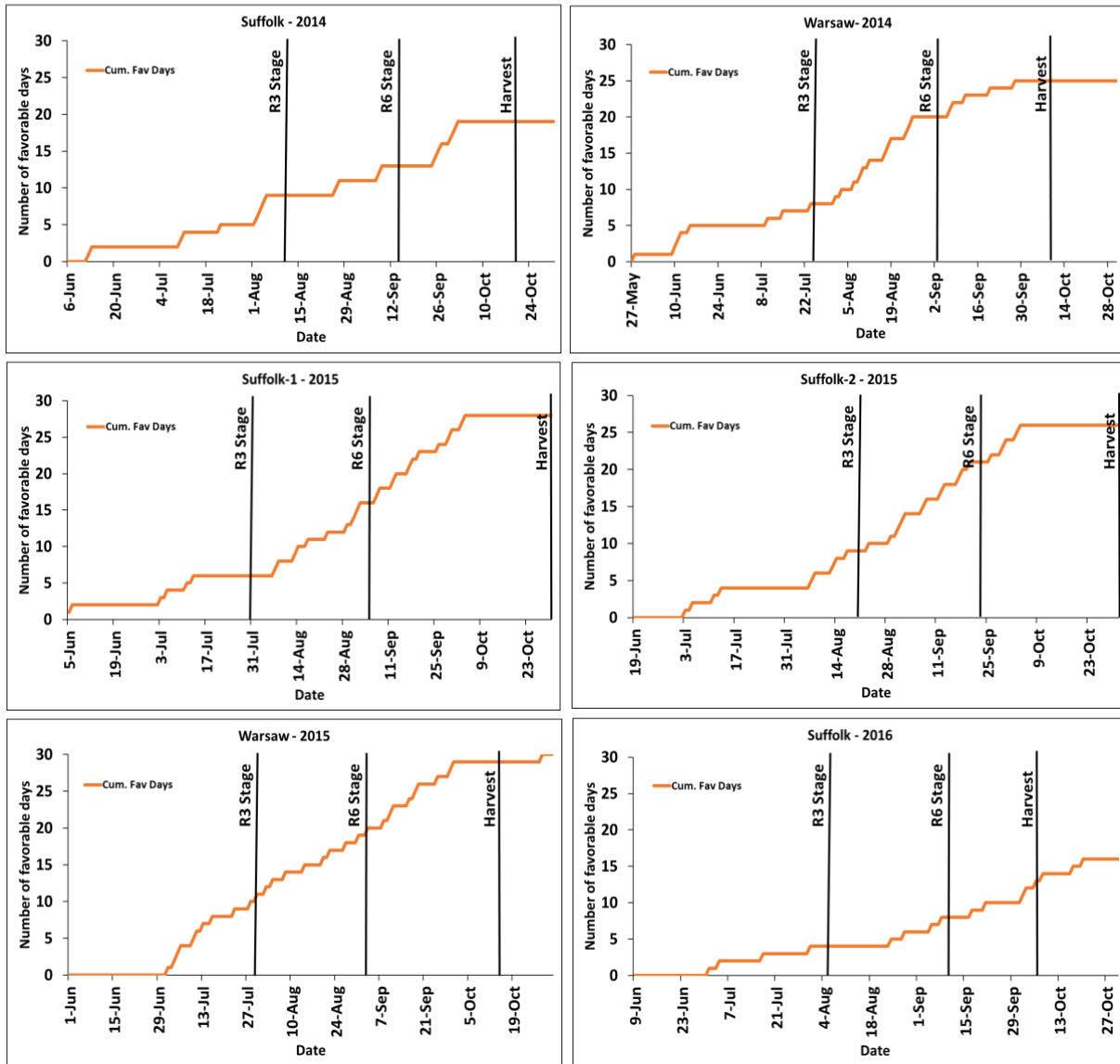
Interval <sup>b</sup>	DFD vs disease <sup>c</sup>		DFD vs yield loss <sup>d</sup>	
	$r^2$	$P$	$r^2$	$P$
<b>Planting to R3</b>	0.94	<0.01	0.37	0.20
<b>3 wks before R3</b>	0.33	<0.01	-0.003	0.85
<b>2 wks before R3</b>	0.42	<0.01	0.42	0.18
<b>R3 to R6</b>	0.18	0.05	0.22	0.31
<b>Planting to R6</b>	0.66	<0.01	0.42	0.15

<sup>a</sup>Disease favorable day (DFD): average temperature of 20 to 30°C with 10 or more hours of relative humidity  $\geq$  90% in a 24 h period from noon to noon.

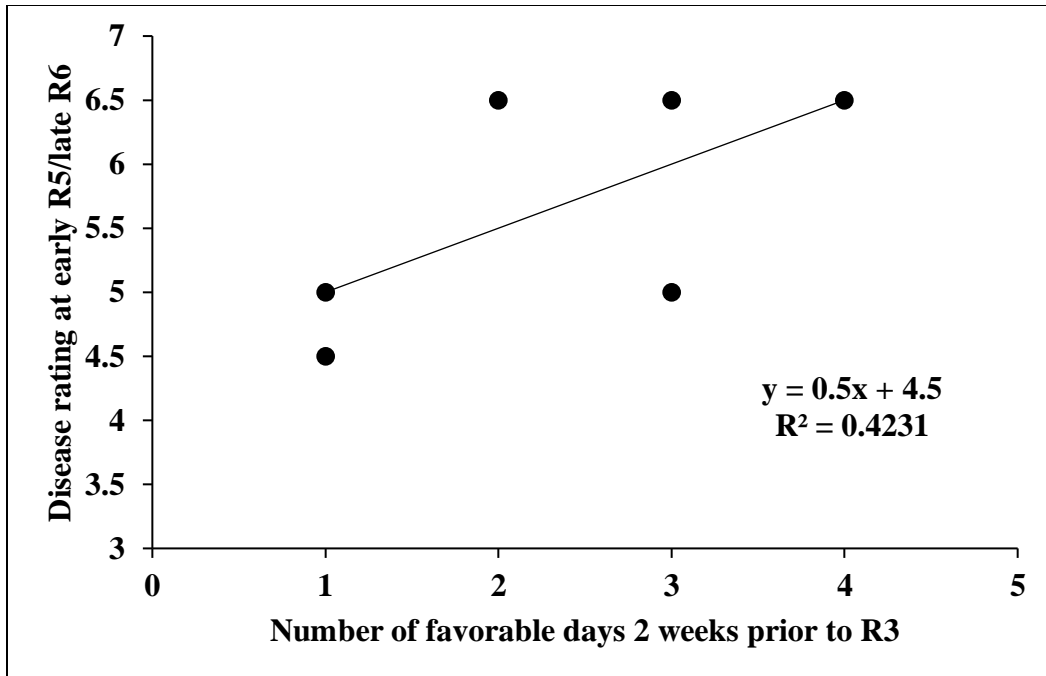
<sup>b</sup>Planting to R3: days between planting and beginning pod (R3); 3 wks before R3: days the three weeks prior to R3; 2 wks before R3: days the two weeks prior to R3; R3 to R6: days between R3 and full pod (R6); Planting to R6: days from planting to R6.

<sup>c</sup>Disease: disease severity in non-treated plots at R6. Disease severity was rated on a 0 to 10 scale where 0 = no disease and 10 = all plants severely infected with loss of more than 80% of photosynthetic area.

<sup>d</sup>Yield loss = the yield difference between plots with weekly fungicide applications and non-treated control.

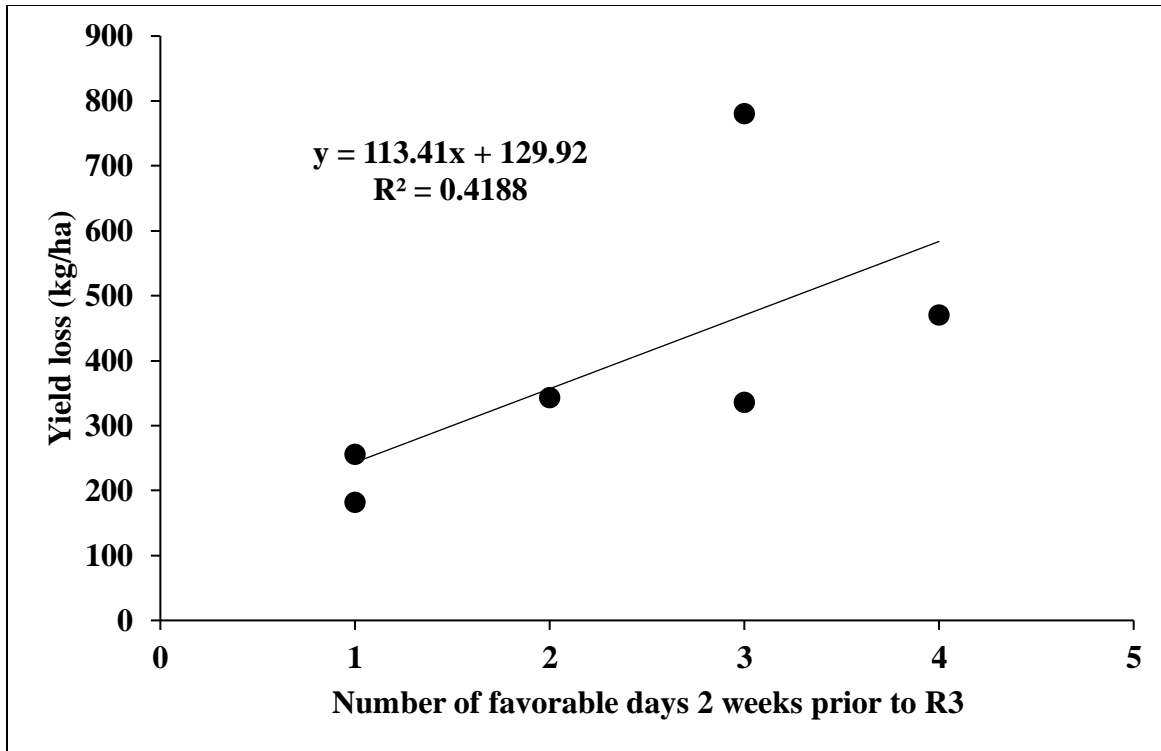


**Fig. 3.1.** Accumulation of disease favorable days from soybean planting through harvest in experiments conducted in Virginia from 2014-2016. A disease favorable day is average temperature of 20 to 30°C with 10 or more hours of relative humidity  $\geq 90\%$  in a 24-hour period from noon to noon.

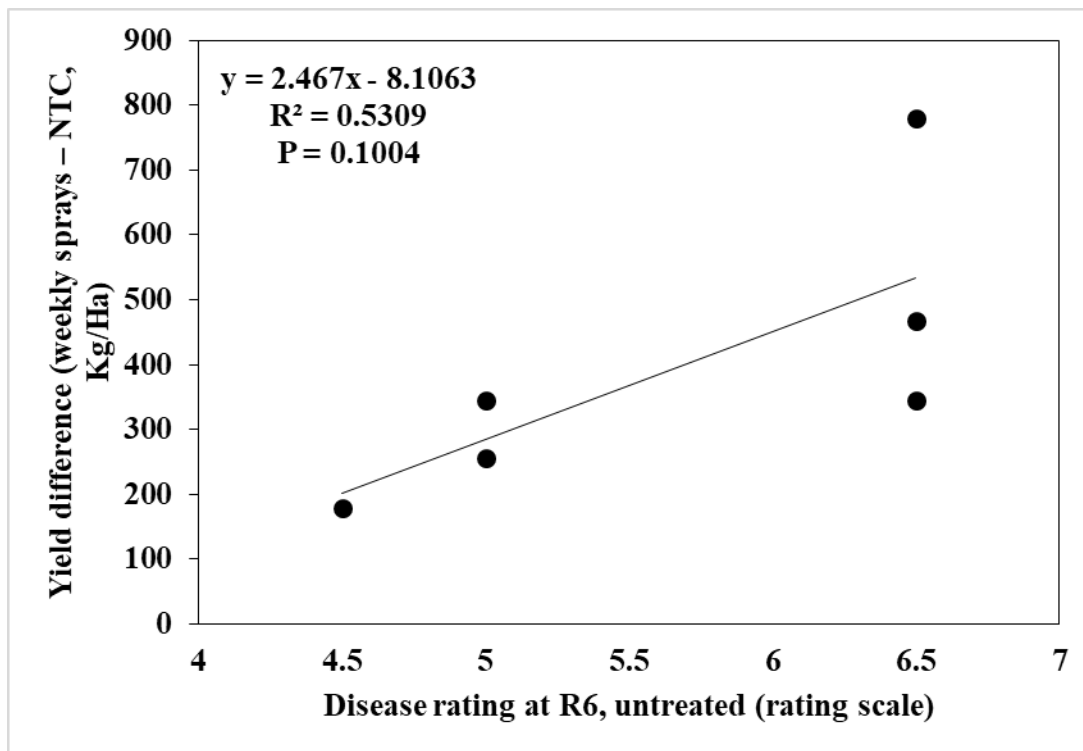


**Fig. 3.2.** Relationship between number of disease favorable days 2 weeks prior to R3 and disease severity at late R5/early R6 stage for six experiments conducted in Virginia from 2014-2016. A disease favorable day is daily average temperature of 20 to 30°C with 10 or more hours of relative humidity  $\geq 90\%$  in a 24 h period from noon to noon. Disease severity was rated in non-treated plots at early maturity of the soybean crop on a 0 to 10 scale where 0 = no disease and 10 = all plants severely infected with loss of more than 80% of photosynthetic area.





**Fig. 3.3.** Relationship between number of disease favorable days 2 weeks prior to R3 and yield loss measured as the yield of plots that received weekly fungicide applications (application beginning at R3 to 4 weeks after R3) minus the yield of non-treated controls. A favorable day is daily average temperature of 20 to 30°C with 10 or more hours of relative humidity  $\geq 90\%$  in a 24 h period from noon to noon.



**Fig. 3.4.** Relationship between foliar disease severity and yield loss in soybean for six experiments conducted in Virginia from 2014-2016. Disease severity was rated in non-treated plots at early maturity of the soybean crop on a 0 to 10 scale where 0 = no disease and 10 = all plants severely infected with loss of more than 80% of photosynthetic area. Yield loss was estimated based on the difference in yield between weekly fungicide applications between beginning pod (R3) and four weeks after R3 (five applications total) and non-treated controls (NTC).

## **Chapter 4: A weather-based foliar fungicide application decision aid for soybean using a threshold decision rule**

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T. Zhou and H. L. Mehl designed and conducted the experiments as well as coordinated on statistical analysis and writing of the manuscript. D. Holshouser assisted with the experimental designs, coordination of on-farm experiment sites, data collection, and review of the manuscript.

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### **Abstract**

Fungicides are commonly used by soybean growers to manage foliar diseases; however, the yield benefits associated with their use are inconsistent. Fungicide efficacy is in part dependent on the presence of foliar fungal pathogens and favorable environmental conditions for disease development. The objectives of this study were to i) compare developmental stage-based fungicide applications to weather-based fungicide applications for foliar disease control and yield in soybean; and ii) develop a weather-based fungicide decision aid for control of soybean foliar diseases and yield protection in the Mid-Atlantic U.S. On-farm strip plot experiments were conducted at 21 locations in Virginia, Maryland, and Delaware from 2014 to 2016. Treatments were non-treated control (NTC), application at beginning pod (R3) stage, or application when two consecutive weather-based disease favorable days (FD) occurred. A day was considered

favorable for disease when daily average temperatures of 20-30°C occurred with  $\geq 90\%$  relative humidity for  $\geq 10$  hours. Foliar disease ratings were taken bi-weekly and yields were measured at harvest. Weather-based sprays were applied at 20 of the 21 locations. However, significantly greater and numerically greater yield in fungicide treated compared to non-treated plots were only detected for 7 and 12 experiments, respectively. Thus, numbers of FD one, two, and three weeks before R3 stage were examined to determine if a favorable-days threshold (FDT) could be identified to predict yield benefits of an R3 foliar fungicide application. Sensitivity, specificity, percent predicted correct, percent sprayed correct, percent not sprayed correct, and odds ratios were calculated to evaluate and compare the predictive values of the different thresholds. Thresholds obtained from on-farm experiments were validated with data from small plot soybean fungicide experiments conducted from 2006 to 2012. When there were eight favorable days within three weeks prior to R3, a yield difference between fungicide treated and non-treated soybean was more likely than if a fungicide was applied regardless of weather conditions and disease risk. This threshold increases the likelihood that a fungicide at R3 will result in a yield benefit that covers application costs. The threshold also predicts when a fungicide application will not result in a yield benefit. This weather-based decision aid will provide soybean growers in the Mid-Atlantic U.S. with a tool to improve the profitability of foliar fungicide applications.

### **Keywords**

Soybean; integrated disease management; foliar fungicide application timing; decision rule; *Cercospora sojina*; *Cercospora kikuchii*

### **Introduction**

Increased global demand for meat consumption has led to the rapid expansion in soybean production around the world. Soybean is a major source of protein in both animal and human diets (Grau et al., 2005). In terms of total farm income, soybean (*Glycine max* (L.) Merrill) is one of the most valuable crops worldwide, ranked just after rice, wheat and corn (FAOSTAT, 2013). An estimated 339 million metric tons of soybean were harvested in 2017/2018 worldwide (USDA, 2018). The U.S. is the top producer of soybean in the world, followed by Brazil then Argentina (USDA FAS, 2019). In 2017/2018, the U.S. harvested 120 million metric tons, roughly 35% of the world production (USDA, 2018). The upper Midwest states of Illinois, Iowa, Minnesota, Indiana, the Dakotas, and Nebraska produced more than 80% of the soybeans in the U.S. Ranking after the midwestern region in soybean production is the Southern region (Arkansas, Mississippi, Tennessee, Louisiana, etc.), followed by the Mid-Atlantic (Delaware, Maryland, New Jersey, North Carolina, Pennsylvania, and Virginia) (USDA NASS, 2015a). Compared to the other regions, agriculture in the Mid-Atlantic region is characterized by smaller scale farms with a wide range of crops and livestock (Abler and Shortle, 2010). Soybean is one of the most valued field crops in the Mid-Atlantic, second only to corn (USDA NASS, 2018). Since 2006, the overall price of soybean in the U.S. has increased, peaking in 2012 at \$0.60/kg (USDA NASS, 2019). The increased value of the soybean crop provided an incentive for growers to increase inputs including foliar fungicides in order to maximize yields. However, in June 2018, the price of the soybean dropped below \$0.33/kg for the first time since 2015 (NASDAQ, 2019) which decreases return on investment from the added costs of fungicide applications.

Along with the increased value of soybean, perceived threats from soybean rust and marketing of fungicides for “plant health” benefits have also contributed to the increased use of

foliar fungicides in soybean and other agronomic crops (Wise et al. 2011). Before the arrival of soybean rust in 2004, virtually no foliar fungicides were applied to soybean in the U.S. (USDA NASS, 2015b). From 2002 to 2005, less than 1% of the planted soybean acreage was treated with fungicides (Gianessi and Reigner, 2005). Soybean rust was first reported in the United States in 2005, and there was concern that this invasive disease could potentially devastate the soybean crop without aggressive management that included applying a fungicide just after flowering and a follow up application two to four weeks later (Kelly et al., 2015). In 2007, soybean acreage treated with fungicide doubled to 4% in 19 states, with 2% treated with pyraclostrobin and 1% treated with azoxystrobin. By 2012 an estimated 11% of the soybean acreage had a fungicide applied in the U.S. (USDA NASS, 2015b).

Even though the arrival of soybean rust in the U.S. prompted increased fungicide use due to the potential yield loss associated with this disease, overall, soybean rust has had little impact on the U.S. soybean crop. However, from 2010 to 2014, soybean foliar diseases caused an estimated loss of 6 million tonnes nationwide (Allen et al., 2017). Among the top ten most destructive soybean diseases, frogeye leaf spot (*Cercospora sojina* K. Hara), *Cercospora* leaf blight/purple seed stain (*Cercospora kikuchii* (Matsumoto & Tomoyasu) M.W. Gardner), and *Septoria* brown spot (*Septoria glycines* Hemmi) were the most damaging foliar fungal diseases (Allen et al., 2017). Foliar diseases reduce yield and seed quality by producing lesions that decrease photosynthetic area and efficiency of the leaves. When disease is severe, premature defoliation can occur. Reduced photosynthesis leads to inhibition of seed filling resulting in smaller seeds (Shtienberg, 1992). Several fungicides are labelled for foliar application to control these diseases, but the yield benefits of fungicide applications in soybean are inconsistent (Koenning and Wrather, 2010).

Even though yield benefits of fungicide applications are variable, the use of foliar fungicides in soybean production has become a standard practice for some growers. Numerous studies have been conducted to determine the optimal foliar fungicide timing or to develop a spray program to control soybean foliar diseases (Mueller et al., 2009; Akem, 1995; Boyer et al., 2017). These studies concluded that fungicide applications made at beginning flowering (R1) and beginning pod (R3) stages resulted in most consistent disease control and highest yield. However, the yield response to fungicide application is inconsistent across all soybean producing regions in the United States (Koenning and Wrather, 2010). In the Mid-Atlantic region, a single foliar fungicide application is typically applied at the R3 stage for soybean. Field trials conducted between 2006 and 2013 at Tidewater Agriculture Research and Extension Center in Suffolk, VA showed greater yields in fungicide treated plots compared to untreated controls only one-third of the time. This may have been due to absence of disease, the absence of environmental conditions conducive for disease development, improper timing of application, and/or fungicide resistance of the disease-causing pathogens. Disease development is heavily influenced by environmental conditions conducive for pathogen growth and host infection (Francl, 2001), and fungicides are unlikely to provide a yield benefit if applied when the environment is unfavorable for disease development and the pathogen is absent or at low inoculum levels.

Disease management decision support systems (DSS) use risk algorithms and predetermined thresholds to assess whether or not disease control measures need to be implemented (Hughes et al., 2002; Pfender et al., 2009). Risk algorithms are largely based on parts of or the entire “disease triangle” – susceptible host, presence of a pathogen, and conducive environment for disease development (Hughes et al., 2002). Risk algorithms can be evaluated

using receiver operating characteristic (ROC) analysis to estimate the probability of a correct decision (Hughes et al., 2002). The use of DSS can optimize management decisions by reducing disease-related input costs such as unnecessary fungicide applications (Pfender et al., 2011). Many fungicide application decision aids have been developed based on simple or complex algorithms of weather data collected over a short period of time. Examples include fungicide decision aids for *Sclerotinia* blight of peanuts (Langston et al., 2002), anthracnose and *Botrytis* fruit rot of strawberry (Pavan et al., 2012), white rust in spinach (Sullivan et al., 2003), and *Fusarium* head blight of barley (Bondalapati et al., 2012). A fungicide application timing decision support tool was developed in Argentina for management of late soybean disease complexes based on both agronomic and weather factors identified through correlation with yield in fungicide treated compared to non-treated soybean (Carmona et al., 2011; 2015; and 2018). The decision tool uses a scoring system based on the identified factors, weighed by their impact on yield, to determine if there is a high risk for late soybean disease epidemic and will recommend whether a fungicide application is needed based on probability of positive economic return.

It should be possible to develop a weather-based foliar fungicide decision aid based on conditions conducive for disease development such as germination of conidia and host infection by a fungal pathogen. Previous studies have determined optimal environmental conditions for infection, sporulation and germination of *C. sojina*, *C. kikuchii*, and *S. glycines*, the causal agents of the most common foliar diseases of soybean in the Mid-Atlantic region. *Cercospora sojina* conidia develop within 48 hours of first visible symptoms under favorable conditions, and secondary infection can occur at this time with the presence of rain or wind. Growth is favored by warm temperatures ranging from 25 to 30°C (optimum being 25°C) with relative humidity of



$\geq 90\%$  (Philips, 1999, Mian et al., 2008; Cruz and Dorrance, 2009). *Cercospora kikuchii* grows at temperatures ranging from 15 to 30°C (optimum being 25°C), and in vitro germination of *C. kikuchii* conidia requires a minimum of 92% relative humidity (Chen, 1979; Schuh, 1991). *Septoria glycines* requires six or more hours of leaf wetness period for infection, and the optimal conditions for development of Septoria brown spot are 15 to 30°C with high relative humidity (Schuh and Adamowicz, 1993). Thus, all three of the pathogens and their associated foliar diseases are favored by moderate temperatures (20-30 °C) and extended periods of high (>90%) relative humidity. Based on these parameters, the objectives of this study were to i) compare crop developmental stage-based fungicide applications to weather-based fungicide applications for foliar disease control and yield in soybean; and ii) develop a weather-based fungicide decision aid for control of soybean foliar diseases and yield protection in the Mid-Atlantic U.S.

## **Materials and Methods**

*Weather monitoring.* Based on optimum temperature ranges for target foliar fungal pathogens to disperse, infect, and colonize the soybean crop as described above, temperatures from 20 to 30°C were considered favorable for disease development. Since foliar fungal pathogens require extended periods of moisture for germination and growth, the aforementioned temperatures and relative humidity  $\geq 90\%$  for ten or more hours within a 24 h period were used as the criteria for determining disease favorable days. Data from the nearest weather station within a 16.1 km radius of each field in the study were used to determine disease favorable days. Location-specific daily temperatures (°C) and relative humidity (%) data were obtained from the National Oceanic and Atmospheric Administration (NOAA) National Weather Service at Wakefield, Virginia (Supplemental Tables S4.1-S4.2).

*On-farm experiments.* To compare the efficacy of soybean developmental stage-based fungicide applications to weather-based applications, on-farm experiments were conducted over 21 site-years in Virginia, Maryland, and Delaware in 2014, 2015 and 2016 (Table 4.1). The soybean variety and crop management practices besides fungicide applications were at the discretion of the cooperating growers. Each strip plot was the width of the grower's spray boom and the length of the entire field. Treatments were replicated three times in randomized strips across the field. The treatments included a non-treated control (NTC), beginning pod (R3) fungicide application, and weather-based (WB) fungicide applications. Weather data were collected as described above, and the presence or absence of a disease favorable day was determined at the end of each 24-hour period. When two consecutive disease favorable days occurred one week prior to or after the onset of the R3 developmental stage of the crop, the weather-based fungicide applications were made. The fungicide applied was at the discretion of the grower, but a pre-mix fungicide including a quinone outside inhibitor (QoI) (FRAC code 11) with either a demethylation inhibitor (DMI) (FRAC code 3) or succinate-dehydrogenase inhibitor (SDHI) (FRAC code 7) was used at all locations (Table 4.1). Each strip plot was harvested separately with a combine, and the yield was measured with a weigh wagon. The length of each harvested strip was measured with a measuring wheel. A sub-sample of the grain harvested from each field location was used to measure moisture content, and yield was converted to kg/ha.

*Disease ratings.* Disease ratings were taken biweekly using a 1-10 scale where 0 = no symptoms, 1 = lesions covering 1 -10% of the leaf surface on one or a few plants, 2 = lesions covering 11 to 30% of the leaf surface on one or a few plants, 3 = lesions covering 1 to 10% of the leaf surface on most (>50%) of the plants, 4 = lesions covering 11 – 30% of the leaf surface

on most (>50%) of the plants, 5 = lesions covering 1 – 10% of the leaf surface on all plants, 6 = lesions covering 11 – 30% of the leaf surface on all plants , 7 = lesions covering 31 – 50% of the leaf surface on all plants, 8 = lesions covering 51 – 70% of the leaf surface on all plants , 9 = lesions covering 71 – 80% of the leaf surface on all plants , and 10 = lesions covering >80% of the leaf surface on all plants. Ratings were taken from the center rows of each plot starting 7.62 meters from the edge of the plot and at 5 to 8 GPS-referenced location spots (dependent on length of field) spaced at least 7.62 m apart. Fifty leaf samples were collected from each location within the field that was rated to assess the types of diseases present in the field. For disease identification, leaves were incubated in a moist chamber at room temperature for 2-3 days to induce sporulation within lesions, and fungal pathogens were identified based on spore morphology observed under the dissecting microscope and compound microscope. Disease ratings were converted into area under the disease progress curve AUDPC for analysis. ANOVA was used to compare the yield and disease ratings for the treatments (NTC, R3, and WB). Tukey's HSD test was used post-ANOVA for separation of means. Analyses were conducted using JMP Pro 13.0.0 (SAS Institution Inc., Cary, NC).

*Decision aid favorable-days threshold.* In the current and previous studies, R3 fungicide applications resulted in the most consistent yield benefits for soybean (Akem, 1995; Mueller et al., 2009; Scherm et al., 2009 Boyer et al., 2017). Thus, in addition to evaluating the weather-based fungicide applications as described above, data were examined to determine if disease favorable days prior to R3 could be used to predict whether or not an R3 fungicide application will result in higher yield compared to the non-treated plots. In the on-farm experiments, two consecutive disease favorable days at or following R3 were used to determine the timing of weather-based fungicide applications, but different thresholds for disease favorable days prior to

R3 were also evaluated. Cumulative favorable days were calculated for one, two and three weeks before the R3 stage application for each trial. The thresholds evaluated were 1-5 days one week before R3 application, 1-7 days two weeks before R3 application, and 1-14 days three weeks before R3 application. Each experiment was categorized as 1) “yes” or “no” for actual yield response to fungicide application and 2) “yes” or “no” for predicted yield response based on whether or not number of favorable days met or exceeded the favorable-days threshold (FDT) being tested. Two different criteria were used to categorize an experiment as a “yes” for actual yield response: 1) significantly greater yield ( $P < 0.05$ ) in the R3 fungicide treatment compared to the non-treated control (NTC) and 2) numerically greater yield in the R3 compared to NTC that would, in many situations, offset the cost of the fungicide application (yield difference  $\geq 134.5$  kg/ha). Two sets of analyses were conducted to compare these two methods for determining yield benefits of fungicide applications. A “correct” outcome was designated for the trial if the actual and predicted yield response were both categorized as “yes” (A) or both categorized as “no” (D). An “incorrect” outcome was designated for the trial if the actual yield response was “no” and the predicted yield response was “yes” (C) or if the actual yield response was “yes” and the predicted yield response was “no” (B). For each threshold, the following were calculated using methods described previously: sensitivity, specificity, percent predicted correctly, percent sprayed correct, percent not sprayed correct, and odds ratio (Yuen, 2006; Yuen and Hughes, 2002; Hughes et al., 1999; Supplemental Table S4.3). If zeros were encountered when calculating the odds ratio, 0.5 was added to all input values to estimate the odds ratio (Deeks and Higgins, 2010). Sensitivity or true positive proportion (TPP) and specificity or true negative proportion (TNP) was calculated for each evaluated threshold as indicated in Supplemental Table S4.4. Sensitivity is the ability of the decision aid to predict a true positive result. Specificity is

the ability of the decision aid to correctly predict a true negative result (Yuen, 2006; Yuen and Hughes, 2002; Hughes et al., 1999). The true positive proportion (TPP) or sensitivity and false positive proportion (FPP) or  $1 - \text{specificity}$  was calculated for each threshold and plotted as receiver operating characteristic (ROC) curve ( $\frac{\text{TPP}}{\text{FPP}}$ ). The area under the receiver operating characteristic curve (AUROC) was also estimated. The positive ( $\frac{\text{Sensitivity}}{1-\text{specificity}}$ ) and negative ( $\frac{1-\text{Sensitivity}}{\text{Specificity}}$ ) likelihood ratios (LR+/-) along with Youden's Index ( $\text{Sensitivity} + \text{Specificity} - 1$ ) were used to determine the optimal threshold (Yuen and Hughes, 2002; Choudhury et al., 2016). A LR+ of greater than 1 indicates the decision aid is more likely than not to correctly predict the yield response to a fungicide application. A LR- of less than 1 indicates that the decision aid recommends not to spray, and that it is less likely there will be a significant difference between sprayed and not sprayed. A likelihood ratio of 1 indicates no predictive value. Youden's Index equally weighs false positive and false negative rates with a value ranged from 0 to 1. A value of 0 indicates FDT has no predictive value. A value of 1 indicates the threshold has perfect accuracy providing no false positives or negatives (Youden, 1950).

*Favorable-days threshold validation.* A total of 23 small-plot soybean experiments conducted at the Virginia Tech Tidewater Agricultural Research and Extension Center at Suffolk, VA from 2006 to 2012 were used to validate the FDT determined from on-farm experiments. For each experiment, only data from the R3 treatments were used for validation. Yield, yield response (fungicide treated yield – untreated yield) and number of favorable days were calculated for three weeks before the R3 developmental stage fungicide application. The predictors were “spray” or “no spray”. The outcomes were “correct” or “not correct” and were designated as described above. Sensitivity, specificity, percent predicted correctly, percent sprays

correct, percent no sprays correct and odds ratio were calculated as described in the previous section. LR+, LR-, and Youden's Index were used to evaluate and validate the tested threshold.

## Results

*Foliar disease severity and impact of fungicides.* Septoria brown spot, Cercospora leaf blight, and frogeye leaf spot were the most common diseases observed in the on-farm experiments. In 2014, frogeye leaf spot was the dominant disease in Orange, and a combination of frogeye leaf spot and Cercospora leaf blight was dominant in Amelia. Cercospora leaf blight was the dominant disease in Culpeper, Nottoway, Sussex, and Accomack while Septoria brown spot was the dominant foliar disease in Stafford. In 2015, Cercospora leaf blight was the dominant disease at Sussex (DE), Stafford, Culpeper and Talbot. Frogeye leaf spot was the dominant disease at Chesapeake, Sussex (VA), and Middlesex. Septoria brown spot was found in small quantities in different counties and it was the major disease in Orange. Frogeye leaf spot and Cercospora leaf blight were the major diseases detected at all locations in 2016. Frogeye leaf spot was the dominant disease from R3 to R5 (beginning seed). The first symptoms of Cercospora leaf blight were detected between R5 and R6 stage and it was the dominant disease after the R5 stage.

Disease severity varied among the 21 on-farm experiments (Table 4.1). In 2014, disease severity at the R6 stage of the soybean crop ranged from 2 to 24% of the leaf area in the non-treated control plots. Ratings of disease at R6 ranged from 3.0 to 7.3 in 2015 and 5.0 to 6.8 in 2016 where a rating of 3.0 corresponds to lesions covering 1 to 10% of the leaf surface on >50% of the plants and 7.0 corresponds to lesions covering 31 – 50% of the leaf surface on all plants.

Disease ratings at R6 did not differ among treatments in 2015 and 2016, but fungicide treatments slowed down disease development (reducing AUDPC) at some locations. Foliar disease was reduced by R3 and/or weather-based fungicide applications at 4 of 7 locations in 2014, 2 of 8 locations in 2015, and 3 of 6 locations in 2016 (Table 4.1).

*Impact of R3 and weather-based fungicide application timings on yield.* The weather-based spray threshold of two consecutive disease favorable-days indicated a fungicide should be applied at 20 of the 21 on-farm experiments in 2014-2016, and the number of days between the R3 application and weather-based application ranged from 1 to 26 days (Table 4.1). However, there were significant differences in yield among treatments in only 7 experiments ( $P < 0.05$ ) and numerically higher yield in the fungicide treatments compared to non-treated in only 12 experiments. Over all experiments, the difference in yield between fungicide-treated plots and non-treated controls ranged from -551 to 451 kg/ha. When fungicide applications resulted in a greater yield compared to the NTC, the yields of R3 fungicide treatments were equal to weather-based treatment at 3 of 7 locations and were greater at 4 of 7 locations. Reductions in disease were only observed in 4 of the 7 experiments with significantly greater yield in the fungicide treated plots. Six experiments with disease reduction did not have greater yields in the fungicide treatments (Table 4.1).

*Decision aid favorable-days threshold (FDT) and validation.* On-farm experiments indicated that the R3 fungicide application timing was more likely to result in greater yields compared to weather-based fungicide applications that occurred after R3; therefore, different FDT for one, two, and three weeks prior to R3 stage of the crop were evaluated. With the exception of the Virginia Beach experiment in 2016 where average daily temperatures frequently exceeded 30°C the three weeks prior to R3, a majority of the days during this period fell within

the range of 20-30°C (Supplemental Table S4.1). Thus, relative humidity had a greater role in determining favorable days than temperature (Supplemental Table S4.2). In an initial exploratory data analysis, values ranging from 1-24 hours of relative humidity  $\geq 90\%$  were evaluated, and the 10 hours interval was determined to be the most predictive of yield benefits with a fungicide application at R3 (Supplemental Tables S4.5-S4.9).

Across all experiments, the R3 fungicide applications resulted in yields significantly greater than the NTC 33% of the time and numerically greater than the NTC 57% of the time (Table 4.1). The trend observed for evaluated thresholds was that as the FDT increased, the decision aid shifted from recommending fungicide applications to not recommending applications (Supplemental Tables S4.10- S4.17). The sensitivity of the decision aid decreased while the specificity increased as the FDT increased. The trade-off between sensitivity and specificity can be observed in the ROC curves in Figure 4.1. Thresholds three weeks before R3 were more predictive and consistent than thresholds two weeks before R3. AUROC for thresholds two weeks before R3 ranged from 0.282 to 0.821 while AUROC for thresholds three weeks before R3 ranged from 0.718 to 0.823 (Figure 4.1). On-farm and validation trials all had AUROC above 0.7 for thresholds three weeks before R3 using both significant yield and numerically higher yield as criteria for categorizing a spray as “correct” (Table 4.2). Thresholds of FDT=7, 8, and 9 within 3 weeks of R3 consistently had the best predictive values for all evaluated thresholds (Table 4.2, Supplemental Tables S4.10-4.17). FDT=8 and 9 had the same predictive values for on-farm trials, however FDT=8 was the best threshold with the highest percent sprayed correct, percent predicted correct, odds ratio, and Youden’s index (Table 4.2). Significant yield and positive yield performed similarly as the criteria to determine if the application decision was correct for both on-farm and validation trials, with significant yield



performing marginally better than positive yield (Fig 4.1, Table 4.2). Using positive yield as the criterion allowed more fungicide applications to be counted as “correct” than significant yield, thus resulting in more percent sprayed correct and greater specificity than using significant yield. Using significant yield as the criterion favored no fungicide application, resulted in more percent not sprayed correct and higher sensitivity.

The yield difference between the R3 fungicide treated and non-treated control for experiments that met or exceeded the FDT was calculated for FDT ranging from 1-14 (Table 4.3). The mean yield difference between R3 and NTC for all experiments was 74 and 235 kg/ha for on-farm and validations experiments, respectively. As the FDT was increased, the mean yield difference increased up to FDT = 7 for on-farm experiments and FDT = 9 for validation experiments.

## **Discussion**

In the Mid-Atlantic region, one foliar fungicide application is typically made at the R3 (beginning pod) stage for soybean. On-farm experiments in this study demonstrated that both R3 stage applications and weather-based fungicide applications inconsistently result in greater yields compared to soybean that had not received a foliar fungicide application. When fungicide applications did result in greater yields compared to non-treated, yield for weather-based fungicide applications were equal to or less than R3 stage applications, supporting that R3 stage is the optimal time to apply foliar fungicide. R3 is a vital developmental stage for soybean as stages between R3 to R6 are most crucial for setting the yield for soybean (Parvej and Holshouser, 2017). Stress caused by foliar diseases at these stages can interrupt photosynthesis

and reduce yield by causing pod and seed abortion, total seed number and seed size (Pedersen and Licht, 2014). Foliar fungicides typically have a protection period of 14 to 21 days, so R3 applications are likely to protect the crop through pod development (R3 to R5 stage takes approximately 20 to 25 days) (Parvej and Holshouser, 2017). It is important to protect the crop from disease during critical periods of yield development, but our data indicate that using developmental stage alone as an indicator to apply fungicide is unreliable and other factors, including risk of disease and yield loss, need to be considered.

Disease development requires the presence of a pathogen, a susceptible host, and a conducive environment for infection and growth. For a foliar fungicide application to be effective and profitable, the grower needs to consider price of soybean, fungicide application costs, seed costs, and foliar disease severity (Paul et al., 2011). Overall disease pressure in Virginia for 2014, 2015, and 2016 was low. The majority of disease ratings at the R6 stage were at or below 5 (all plants in the field infected with diseased spots covering 1 to 10% of the photosynthetic areas on the leaves). Only three locations had disease ratings  $\geq 7$  at the R6 stage (Amelia 2014, Lancaster 2016, and Talbot 2016), and there was a yield benefit with a fungicide application for all three of these experiments. Furthermore, high disease severity of frogeye leaf spot and *Cercospora* leaf blight were present in the fields at Amelia and Lancaster County. It is likely the observed yield benefits from fungicide application were due to the high levels of these two diseases and yield protection by the foliar fungicide applications. Conditions were dry in 2015 during the pod developmental period, (July to mid-September) which may have contributed to low disease severity and yield potential and explain the lack of yield benefits from foliar fungicide applications in 2015.

The soybean foliar fungicide application decision aid we developed not only takes into consideration the developmental stage of the crop but also conducive environmental parameters (temperature and relative humidity) associated with the development of the targeted diseases. Results from our study indicate applications made at the R3 stage are more likely to have a yield benefit when environmental parameters conducive for disease development occur within three weeks before the R3 stage which approximately corresponds to the R1 (beginning flower) stage. Favorable conditions for fungal pathogen growth during the period between R1 and R3 promote germination of conidia and initial infection. In Virginia, symptoms of frogeye leaf spot and Septoria brown spot can be first observed near the R3 stage. Foliar fungicide application at R3 can reduce secondary infection and delay disease progress, which may explain why fungicide applied at R3 was more likely to result in a yield benefit when there were  $\geq 8$  disease favorable-days the three weeks prior to R3. Though the decision aid increased the probability of a fungicide application resulting in a yield benefit, another purpose of the decision aid is to predict when an application will not result in a yield benefit in order to eliminate unnecessary input costs and selection pressure toward fungicide resistance in pathogens. Our FDT-based decision aid was able to correctly predict that a foliar fungicide should not be applied  $\geq 90\%$  of the time when significantly greater yield was used as the criterion and 55% of the time when numerically greater yield was used as the criterion (Table 4.2). This suggests the greatest utility of the decision aid may be for predicting when a fungicide application is not needed, thereby saving growers the cost of an unnecessary fungicide.

The yield benefits of foliar fungicides in soybean are highly variable and are sometimes difficult to quantify due to variations in yield across a field that are not attributable to disease control and yield protection. We cannot be certain that yield differences between fungicide

treated and non-treated plots were due to fungicide treatment effects in experiments that lacked statistical differences ( $P>0.05$ ), but in some experiments, the numerically greater yield in the fungicide treated plots would cover the cost of a fungicide application. In our analyses for “positive yield,” we used 134.5 kg/ha as the “break even” yield needed since this would allow for a mid-range fungicide application cost even at relatively low soybean prices (Table 4.4). Sensitivity and specificity of the tested favorable-days thresholds differed between “significant yield” and “positive yield” criteria for a “correct” fungicide spray. However, the percent predicted correct outcomes was similar for significant and numerically greater yield at FDT=8, the threshold determined to be the overall most predictive (Table 4.2).

In terms of profitability, a weather-based fungicide decision aid can help to predict when a fungicide application is likely to result in a yield benefit that has a value exceeding the cost of the fungicide application. Fungicide application costs for soybean in the U.S. range from \$37.07/ha to \$74.13/ha depending on the specific fungicide product and mode of application (ground or air). Growers are more likely to apply foliar fungicides when soybean prices are high since even relatively small yield increases can cover the cost of application and provide a return on investment. The price of soybean between 2007 and 2014 ranged from \$0.37/kg (\$10.1/bu) to \$0.53/kg (\$14.4/bu), but in 2018 the price fell below \$0.33/kg (\$9/bu) (USDA NASS, 2018). In the on-farm experiments, the average yield benefit of R3 stage application without the decision aid was 74 kg/ha while the average yield benefit of R3 stage applications when FDT=8 for the decision aid was met was 129 kg/ha (Table 4.3). Based on break-even yields for different fungicide application cost/soybean price received combinations, R3 fungicide applications applied regardless of disease risk would not, on average, result in a positive return on investment

(Table 4.4). However, using the weather-based decision aid, fungicide applications would be profitable even when the price received for soybean is low.

Additional field experiments in the mid-Atlantic soybean growing region are needed in the future for further validation of the FDT determined in the current study. The ultimate goal of this research is to develop a smartphone app for growers, extension agents, and consultants that can be used as a site-specific soybean fungicide decision aid. Research is also needed to identify other environmental parameters or agronomic factors associated with greater risk of disease and associated yield loss in soybean. Such factors could be incorporated into the current decision aid to improve the accuracy and increase the probability that foliar fungicide applications will be profitable.

## **Conclusions**

The use of a weather-based disease favorable-days threshold increases the probability that a foliar fungicide application will be profitable in soybean when compared to calendar or developmental stage-based fungicide applications that do not take environmental conditions into consideration. The decision aid developed during this study will be a useful tool for growers when soybean prices are low and a greater yield benefit is needed to cover the cost of fungicide applications. Although this study focused on soybean production areas of the Mid-Atlantic U.S., this decision aid based on derived decision rules can be adopted in other soybean growing regions.

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**Table 4.1.** Fungicide and rates fungicide application date, disease rating, and yield for on-farm experiments conducted between 2014 and 2016 to compare weather-based foliar fungicide applications to developmental stage-based applications for soybean.

Year	Location	Fungicide, L/ha <sup>a</sup>	Appl. date		Disease (R6 or AUDPC) <sup>b,c</sup>			Yield, kg/ha <sup>c</sup>			Fungicide efficacy <sup>d</sup>	
			R3 <sup>e</sup>	WB <sup>f</sup>	NTC <sup>g</sup>	R3 <sup>e</sup>	WB <sup>f</sup>	NTC <sup>g</sup>	R3 <sup>e</sup>	WB <sup>f</sup>	Disease	Yield
2014	Orange, VA	Priaxor, 0.28	29 Jul	4 Aug	9.5 a	11.0 a	9.0 a	3188 b	3537 a	3497 a	-	+
	Culpeper, VA	Priaxor, 0.28	11 Aug	20 Aug	14.6 a	14.0 a	12.4 a	4102 a	4015 a	3847 a	-	-
	Stafford, VA	Quadris Top SB, 0.25	7 Aug	16 Aug	24.0 a	28.0 a	14.0 b	4560 a	4687 a	4721 a	+	-
	Amelia, VA	Quadris Top SB, 0.70	15 Aug	20 Aug	23.0 a	16.4 b	12.7 b	2784 c	3208 a	3100 b	+	+
	Nottoway, VA	N/A	16 Sep	26 Sep	2.0 a	1.3 a	1.0 a	3053 b	3141 a	3141 a	-	+
	Sussex, VA	Stratego YLD, 0.42	3 Sep	16 Sep	8.0 a	6.3 a	5.3 b	2018 b	2300 a	2199 a	+	+
	Accomack, VA	Stratego YLD, 0.28	11 Sep	15 Sep	8.7 a	3.3 b	3.7 b	3679 a	3605 a	3282 a	+	-
2015	Orange, VA	Priaxor, 0.28	23 Jul	11 Aug	173 a	173 a	167 a	4728 a	4540 a	4600 a	-	-
	Culpeper, VA	Priaxor, 0.28	27 Jul	11 Aug	247 ab	262 a	222 b	4600 b	4856 a	4627 b	+	+
	Stafford, VA	Quadris Top SB, 0.56	8 Aug	None	76 a	86 a	None	3941 a	4183 a	None	-	-
	Chesapeake, VA	Priaxor, 0.28	13 Aug	22 Aug	210 a	182 c	193 b	2852 a	2966 a	3107 a	+	-
	Middlesex, VA	Aproach Prima, 0.48	17 Aug	9 Sep	160 a	158 a	158 a	2973 a	2905 a	3154 a	-	-
	Sussex, VA	N/A	4 Sep	8 Sep	106 a	116 a	95 a	2495 a	2169 a	2044 a	-	-
	Talbot, MD	Quadris Top SB, 0.70	5 Aug	14 Aug	94 a	120 a	119 a	4768 a	4607 a	4694 a	-	-
	Sussex, DE	Quadris Top SB, 0.70	29 Aug	9 Sep	120 a	109 a	83 b	3369 a	3349 a	3443 a	+	-
2016	Culpeper, VA	Quadris Top SB, 0.49	2 Aug	20 Aug	157 a	125 b	137 a	3732 a	3847 a	3813 a	+	-
	Lancaster, VA	N/A	20 Aug	7 Sep	202 a	121 b	207 a	2313 c	2764 a	2549 b	+	+
	Middlesex, VA	Aproach Prima, 0.42	2 Sep	6 Sep	230 a	206 a	227 a	2885 a	2724 a	2919 a	-	-
	VA Beach, VA	Quadris Top SB, 0.70	12 Aug	7 Sep	181 a	154 b	178 ab	3981 a	3753 ab	3430 b	+	-
	Accomack, VA	N/A	6 Sep	7 Sep	81 a	63 a	67 a	3867 a	4028 a	4048 a	-	-
	Talbot, MD	Quadris Top SB, 0.84	18 Aug	6 Sep	111 a	86 a	98 a	3867 b	4116 a	3988 ab	-	+

<sup>a</sup> N/A = information on fungicide used was not available. <sup>b</sup> In 2014, disease severity was rated (1-10 scale) at the full seed (R6) stage. In 2015 and 2016, disease was rated biweekly following R3 fungicide applications, and area under the disease progress curve (AUDPC) was calculated.

<sup>c</sup> Within a row, values followed by the same letter are not significantly different according to Tukey's HSD ( $p > 0.05$ ). <sup>d</sup> - = NTC not significantly different from fungicide treatments, + = NTC significantly different from fungicide applications ( $p = 0.05$ ). <sup>e</sup> R3 = beginning pod stage fungicide application. <sup>f</sup> WB = weather-based fungicide application. <sup>g</sup> NTC = non-treated control.

**Table 4.2.** Statistics summary of on-farm and validation experiments for significant and positive yield using different favorable-days thresholds (FDT).

	Sig. yld. on-farm <sup>a</sup>			Sig. yld. valid. <sup>b</sup>			Pos. yld. on-farm <sup>c</sup>			Pos. yld. valid. <sup>d</sup>		
	FDT=7	FDT=8	FDT=9	FDT=7	FDT=8	FDT=9	FDT=7	FDT=8	FDT=9	FDT=7	FDT=8	FDT=9
Sprayed correct, %	50	60	60	36	38	40	75	70	70	93	100	100
Not sprayed correct, %	89	91	91	100	100	93	67	55	55	55	58	47
Predicted correct, %	67	76	76	64	68	72	75	70	70	76	80	68
Mean yield difference, kg/ha	251	260	260	355	406	409	251	260	260	355	409	445
Sensitivity, %	86	86	86	100	100	80	75	58	58	72	72	56
Specificity, %	57	71	71	55	60	70	67	67	67	86	100	100
Likelihood Ratio +	2.00	3.00	3.00	2.22	2.50	2.67	2.25	1.75	1.75	5.06	---	---
Likelihood Ratio -	0.25	0.20	0.20	0.00	0.00	0.29	0.38	0.62	0.62	0.32	0.28	0.44
Odds ratio	8.00	15.00	15.00	13.30	16.18	9.33	6.00	2.80	2.80	15.60	36.82	18.53
P-value	0.085	0.028	0.028	0.09	0.07	0.45	0.07	0.26	0.26	0.02	0.02	0.06
Youden's Index	0.43	0.57	0.57	0.55	0.60	0.50	0.42	0.25	0.25	0.58	0.72	0.56
AUROC		0.777			0.823			0.718			0.813	

<sup>a</sup> On-farm experiments using significant yield (fungicide treated yield significantly greater than non-treated at  $P = 0.05$ ) as the criterion to determine whether or not the application decision was correct.

<sup>b</sup> Validation experiments using significant yield (fungicide treated yield significantly greater than non-treated at  $P = 0.05$ ) as the criterion to determine whether or not the application decision was correct.

<sup>c</sup> On-farm experiments using positive yield (yield difference  $\geq 134.5$  kg/ha) as the criterion to determine whether or not the application decision was correct.

<sup>d</sup> Validation experiments using positive yield (yield difference  $\geq 134.5$  kg/ha) as the criterion to determine whether or not the application decision was correct.

**Table 4.3.** Favorable-days thresholds (FDT) and yield differences between fungicide treated and non-treated plots for on-farm and validation experiments.

FDT <sup>c</sup>	On-farm experiments <sup>a</sup>			Validation <sup>b</sup>		
	No. above threshold <sup>d</sup>	Yield diff above FDT, kg/ha <sup>e</sup>	Yield diff below FDT, kg/ha <sup>f</sup>	No. above threshold	Yield diff above FDT, kg/ha	Yield diff below FDT, kg/ha
0	21	74	0	26	235	0
1	20	89	-228	26	235	0
2	20	89	-228	24	244	141
3	20	89	-228	23	248	143
4	19	97	-151	22	258	118
5	18	96	-63	18	258	144
6	16	120	-74	15	288	123
7	12	141	-16	14	355	84
8	10	129	23	13	409	48
9	10	129	23	10	445	96
10	9	105	50	10	445	96
11	9	105	50	10	445	96
12	7	141	40	9	432	125
13	7	141	40	6	500	152
14	7	141	40	0	0	235

<sup>a</sup> On-farm experiments conducted from 2014 to 2016 at field sites in Virginia, Maryland, and Delaware.

<sup>b</sup> Small-plot soybean experiments conducted from 2006 to 2012 at the Tidewater AREC in Suffolk, Virginia were used to validate disease favorable-days thresholds.

<sup>c</sup> Favorable-days thresholds from 0 to 14 FDT.

<sup>d</sup> Number of experiments where the cumulative number of favorable days within 3 weeks before R3 met or exceeded the designated FDT.

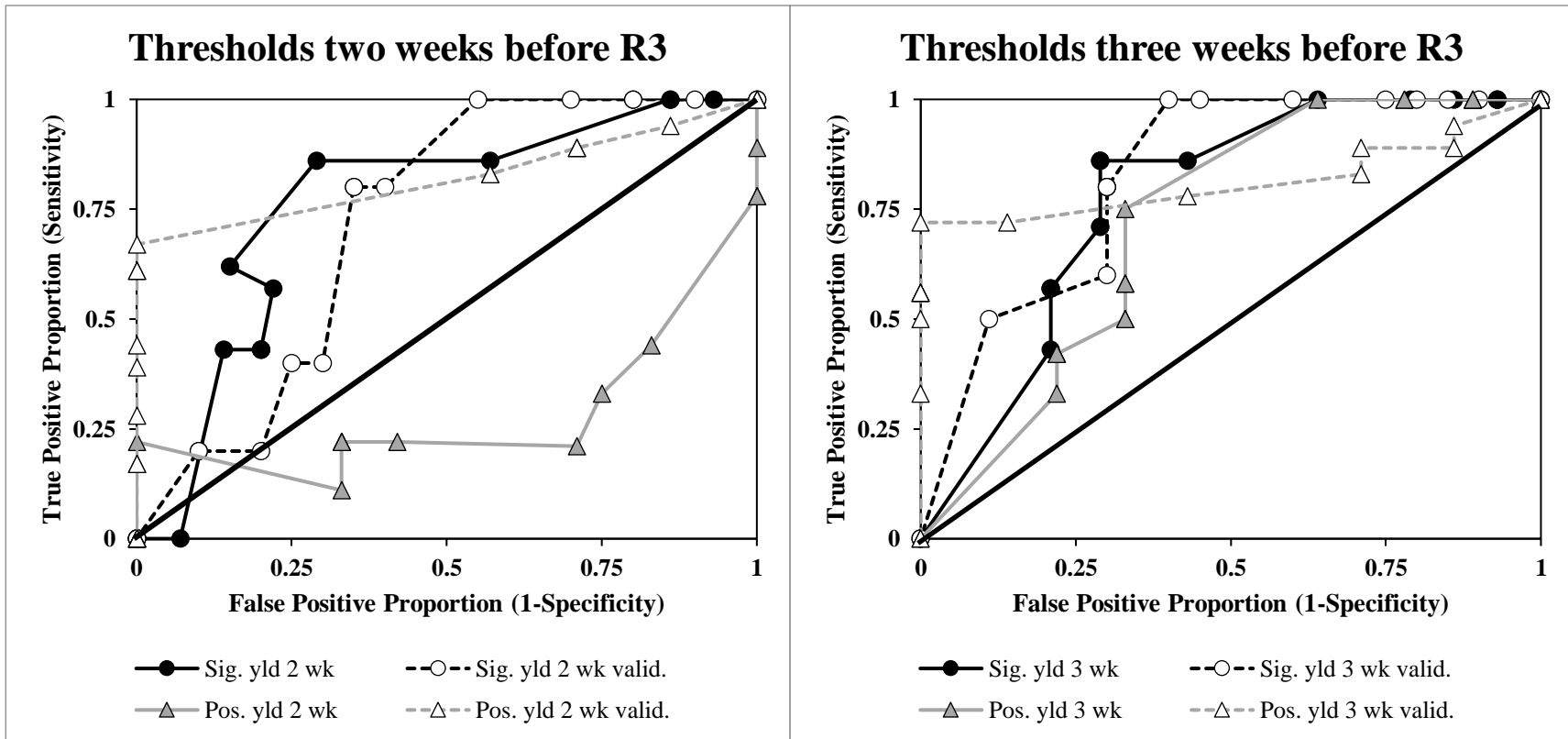
<sup>e</sup> Average yield differences between treated and non-treated control plots in kilograms per hectare when the number of favorable days met or exceeded the FDT.

<sup>f</sup> Average yield difference between treated and non-treated control plots in kilograms per hectare when number of favorable days was below the FDT.

**Table 4.4.** Yield protection break-even points in kg/ha to cover the cost of a single foliar fungicide application based on price received for the soybean crop.

Fungicide application cost (\$/ha) <sup>a</sup>	Soybean price (\$/kg)				
	0.33	0.37	0.40	0.44	0.48
37.07	112	100	93	84	77
39.54	119	107	99	90	82
42.01	127	114	105	96	88
44.48	135	120	111	101	93
46.95	142	127	118	107	98
49.42	149	134	124	112	103
51.89	157	140	130	118	108
54.36	165	147	136	124	113
56.83	172	154	142	129	118
59.31	180	160	148	135	124
61.78	187	167	155	140	129
64.25	195	174	161	146	134
66.72	202	180	167	152	139
69.19	210	187	173	157	144
71.66	217	194	179	163	149
74.13	225	200	185	169	154

<sup>a</sup> Fungicide application cost includes the cost of the fungicide plus the cost of the application method (ground or air).



**Fig. 4.1.** Receiver operating characteristic (ROC) curves for favorable-days thresholds two weeks and three weeks before R3. ROC curves are compared for on-farm and validation (valid.) experiments using either positive yield (yield difference between fungicide treated and non-treated  $\geq 134.5$  kg/ha) or significant yield (yield of fungicide treated significantly greater than non-treated at  $P = 0.05$ ) as the criterion for determining a “correct” fungicide application. Area under the receiver operating characteristic curves (AUROC) were calculated and compared to determine the thresholds with the highest probability of correctly recommending a fungicide application in soybean. The diagonal line is the line of no discrimination. AUROC value for thresholds two weeks before R3 were 0.723, 0.282, 0.720, and 0.821, respectively, for on-farm experiments significant yield, on-farm experiments positive yield, validation experiments significant yield, and validation experiments positive yield. For thresholds three weeks before R3, the AUROC values were 0.777, 0.718, 0.8225, and 0.813, respectively, for on-farm experiments significant yield, on-farm experiments positive yield, validation experiments significant yield, and validation experiments positive yield. AUROC value of 1 indicates perfect predictive performance while 0 indicates no predictive value. A value of 0.5 means the model lacks class separation capacity.



**Supplemental Table S4.1.** Minimum, maximum, and average daily temperatures for three weeks prior to the beginning pod (R3) stage of the soybean crop for on-farm experiments conducted from 2014 to 2016 in Virginia, Maryland, and Delaware.

Experiment <sup>a</sup>	Value <sup>b</sup>	Days before R3, temperature (°C)																				Yield diff. <sup>c</sup>	Sig. diff. <sup>d</sup>	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20			21
SussexVA15	Min	21	19	21	21	22	16	14	15	18	19	21	19	15	18	22	12	21	21	18	12	14	-326	N
	Max	34	34	33	26	31	30	29	27	27	31	32	31	30	30	31	27	31	34	32	31	30		
	Ave	26	26	26	23	24	23	22	21	23	24	25	24	22	24	28	25	24	27	24	17	22		
VaBeach16	Min	27	27	27	26	26	28	27	24	26	26	27	27	26	26	29	29	29	29	28	28	27	-228	N
	Max	34	34	32	29	33	35	31	30	30	31	35	33	28	33	38	38	37	38	37	37	35		
	Ave	31	31	29	27	28	31	29	27	28	28	31	28	28	30	32	32	31	33	33	33	31		
Orange15	Min	17	18	23	23	22	21	17	16	20	21	21	17	20	23	23	23	21	21	20	20	17	-188	N
	Max	30	30	33	35	32	29	27	29	31	27	28	28	30	33	32	31	30	28	29	26	25		
	Ave	23	24	28	29	27	24	22	22	24	12	23	22	12	28	26	26	24	24	22	22	26		
Talbot15	Min	21	26	20	20	23	22	26	23	23	22	22	18	17	20	22	27	27	24	25	18	19	-161	N
	Max	33	33	32	32	32	32	32	33	30	32	32	30	29	30	32	34	34	33	29	28	27		
	Ave	27	29	28	28	29	25	28	27	27	28	28	27	23	24	27	30	31	29	28	23	23		
Middlesex16	Min	20	23	19	19	18	19	12	22	19	16	17	18	23	20	21	23	25	12	22	26	26	-161	N
	Max	27	33	33	32	32	33	35	33	29	29	28	32	32	32	30	34	34	35	36	35	33		
	Ave	12	27	26	26	24	25	28	27	24	22	22	25	27	26	26	27	29	28	29	31	29		
Culpeper14	Min	14	16	16	15	17	18	16	17	19	17	18	13	9	11	18	20	19	13	16	22	21	-87	N
	Max	28	29	28	27	29	29	31	29	28	26	25	29	26	12	27	29	31	28	26	34	29		
	Ave	21	23	22	21	23	12	12	12	24	22	22	21	18	17	23	24	25	21	21	28	25		
Accomack14	Min	18	20	20	19	20	23	21	20	23	21	22	16	16	16	25	22	15	16	18	20	21	-74	N
	Max	27	25	22	23	32	32	32	29	33	31	31	28	26	27	27	26	25	24	26	27	29		
	Ave	22	22	21	22	26	27	26	29	29	26	27	12	20	22	27	25	21	20	22	23	24		
Middlesex15	Min	17	16	15	13	17	19	22	21	16	21	22	22	22	24	18	20	18	21	23	22	21	-68	N
	Max	32	30	29	28	28	31	26	29	27	24	29	32	33	32	30	32	31	33	31	32	31		
	Ave	24	24	22	21	22	12	12	12	21	22	24	26	27	27	24	26	24	26	27	25	25		

Experiment <sup>a</sup>	Value <sup>b</sup>	Days before R3, temperature (°C)																				Yield diff. <sup>c</sup>	Sig. diff. <sup>d</sup>	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20			21
SussexDE15	Min	16	13	53	13	14	17	16	13	16	22	22	21	18	16	15	14	16	18	20	17	16	-20	N
	Max	29	28	27	28	29	31	28	28	29	29	29	31	32	32	31	29	28	28	29	24	29		
	Ave	21	20	19	21	24	12	21	23	25	25	25	24	24	23	22	22	22	12	12	21	22		
Nottoway14	Min	12	18	21	18	19	19	17	18	17	18	18	17	18	20	13	16	17	22	22	18	16	88	Y
	Max	27	28	27	27	26	27	29	31	31	30	28	25	23	22	28	26	30	29	32	29	29		
	Ave	21	12	24	23	21	22	12	22	24	24	22	21	20	12	21	20	22	26	26	12	21		
Chesapeake15	Min	21	22	21	22	23	23	23	25	24	23	12	22	12	12	12	12	24	19	20	22	22	114	N
	Max	28	29	28	27	26	27	29	34	34	33	30	33	31	36	33	32	31	32	30	29	29		
	Ave	25	26	24	24	24	25	26	29	29	28	27	27	27	29	28	28	28	26	25	26	26		
Culpeper16	Min	22	22	21	22	22	23	23	24	24	23	21	19	16	19	20	21	17	19	21	21	22	115	N
	Max	31	32	30	31	33	33	34	36	35	35	33	31	30	30	33	33	33	31	34	33	29		
	Ave	26	26	24	26	26	27	27	29	29	29	27	25	12	12	24	27	24	26	27	27	26		
Stafford14	Min	18	16	17	19	17	18	13	9	11	35	20	19	13	16	22	21	21	18	19	14	13	127	N
	Max	29	31	29	28	26	25	29	26	12	27	29	31	28	26	28	29	28	27	27	28	27		
	Ave	12	12	12	24	22	22	21	18	17	23	24	25	21	21	28	25	24	23	23	22	20		
Accomack16	Min	21	18	19	21	19	23	19	20	18	22	22	12	17	14	18	23	21	21	22	12	27	161	N
	Max	28	24	12	27	28	29	31	31	29	32	33	31	29	27	28	32	31	32	31	34	34		
	Ave	12	21	21	12	24	26	26	25	12	26	27	27	23	21	23	26	26	26	26	28	29		
Stafford15	Min	22	21	22	22	24	19	19	19	19	24	21	22	12	20	18	19	19	22	26	24	12	242	N
	Max	26	28	33	35	34	31	33	32	34	32	32	32	33	32	31	29	31	32	34	35	33		
	Ave	12	24	27	28	29	25	26	26	26	28	26	26	28	27	24	24	24	26	30	30	28		
Talbot16	Min	24	27	12	24	28	28	28	27	25	19	21	23	24	19	18	19	23	25	24	24	24	249	Y
	Max	34	34	34	36	35	35	34	33	29	32	32	32	31	29	27	30	32	32	31	30	32		
	Ave	28	32	31	30	32	30	30	29	27	26	27	27	27	23	22	26	27	29	27	27	26		
Culpeper15	Min	21	18	15	16	16	20	23	22	21	21	15	13	18	20	19	15	19	22	21	22	21	256	Y
	Max	31	29	28	28	28	29	32	33	31	27	26	29	29	26	28	28	28	32	30	31	29		
	Ave	26	24	22	22	22	24	27	28	26	24	21	22	24	23	22	22	12	27	25	26	24		

Experiment <sup>a</sup>	Value <sup>b</sup>	Days before R3, temperature (°C)																					Yield diff. <sup>c</sup>	Sig. diff. <sup>d</sup>
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
SussexVA14	Min	22	22	15	19	19	17	20	22	20	21	21	18	21	17	17	17	25	12	18	20	23	282	Y
	Max	31	29	29	27	28	30	32	31	29	26	23	22	29	27	27	31	29	31	29	27	33		
	Ave	26	25	21	22	12	23	24	25	24	23	22	19	24	22	21	12	26	26	12	22	28		
Orange14	Min	12	21	20	18	14	21	21	22	21	18	18	17	17	20	21	23	21	20	17	20	20	349	Y
	Max	28	28	30	28	25	35	32	27	27	27	28	26	26	31	34	33	32	30	27	30	33		
	Ave	21	25	24	22	19	26	25	24	12	22	23	22	22	24	26	27	26	24	23	24	24		
Amelia14	Min	12	18	21	18	19	19	17	18	17	18	18	17	18	20	13	16	17	22	22	18	16	424	Y
	Max	27	28	27	27	26	27	29	31	31	30	28	25	23	22	28	26	30	29	32	29	29		
	Ave	21	12	24	23	21	22	12	22	24	24	22	21	20	12	21	20	22	26	26	12	21		
Lancaster16	Min	20	21	12	26	24	12	27	27	25	24	23	19	19	23	24	20	19	22	23	23	22	451	Y
	Max	33	31	34	35	34	36	35	33	33	32	31	26	31	32	29	29	28	28	31	32	31		
	Ave	26	26	28	30	28	29	31	30	29	28	26	21	24	27	26	24	12	25	27	27	25		

<sup>a</sup> On-farm trial ID with location name followed by the year the experiment was conducted.

<sup>b</sup> Min = low temperature, Max = high temperature, and Ave = average temperature in degrees Celsius in a 24-hour period from noon to noon.

<sup>c</sup> Yield difference between R3 stage application and non-treated control in kg/ha. Experiments are ordered from smallest to greatest yield increase with a fungicide application compared to the non-treated.

<sup>d</sup> Sig. diff. = difference in yield between the fungicide treated and non-treated control was significantly different (Y = yes, p<0.05) or not (N = no, p>0.05) at the 95% confidence level.

**Supplemental Table S4.2.** Number of hours with 90% or greater relative humidity for each day within three weeks prior to the beginning pod (R3) stage of the soybean crop for on-farm experiments conducted from 2014 to 2016 in Virginia, Maryland, and Delaware.

Experiment <sup>a</sup>	Days before R3, number of hours RH $\geq$ 90%																					Yield diff. <sup>b</sup>	Sig. diff. <sup>c</sup>
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
SussexVA15	12	4	11	16	0	9	8	10	0	8	6	1	11	2	1	17	6	10	10	10	10	-326	N
VaBeach16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-228	N
Orange15	10	3	11	4	12	12	11	0	11	14	12	11	14	1	7	8	12	12	17	14	14	-188	N
Talbot15	11	1	3	3	0	11	2	12	11	6	2	2	6	0	0	10	5	4	0	8	3	-161	N
Middlesex16	12	10	12	11	13	12	12	10	9	11	12	6	14	14	14	12	3	15	8	7	10	-161	N
Culpeper14	19	0	2	0	0	2	1	2	13	0	0	0	0	0	2	2	0	0	3	4	12	-87	N
Accomack14	3	3	5	2	4	2	12	0	1	0	0	0	0	0	0	5	0	1	10	10	-74	N	
Middlesex15	5	4	10	7	7	7	3	2	11	0	7	5	10	0	10	1	7	10	5	16	4	-68	N
SussexDE15	0	0	0	0	6	0	0	0	15	12	13	9	2	1	0	0	0	10	14	0	0	-20	N
Nottoway14	6	15	13	7	14	14	7	16	11	12	14	17	21	11	10	8	0	6	10	10	17	88	Y
Chesapeake15	0	1	0	0	7	11	2	2	3	7	5	0	4	0	0	0	0	0	0	0	0	114	N
Culpeper16	13	15	17	13	13	11	14	11	10	7	10	13	11	12	13	8	15	11	8	13	12	115	N
Stafford14	2	0	10	14	0	3	0	0	0	5	5	0	0	3	4	11	3	0	0	0	0	127	N
Accomack16	0	0	0	0	2	3	10	5	11	10	10	0	10	7	0	5	10	7	10	6	0	161	N
Stafford15	3	10	0	4	0	8	0	6	15	10	17	11	2	1	7	10	5	7	8	6	10	242	N
Talbot16	6	4	3	6	5	8	10	8	8	3	4	3	10	10	10	4	6	2	9	13	16	249	Y
Culpeper15	3	10	10	10	10	0	13	10	12	12	12	5	7	16	15	10	14	0	11	10	13	256	Y
SussexVA14	0	3	4	10	0	2	7	15	13	11	3	12	5	11	12	16	10	12	10	0	4	282	Y
Orange14	3	4	11	11	14	3	11	12	5	0	5	6	3	8	12	2	1	10	6	14	13	349	Y
Amelia14	6	15	13	7	14	14	7	16	11	12	14	17	21	11	10	8	0	6	10	10	17	424	Y
Lancaster16	12	11	5	0	10	8	0	3	3	11	12	19	11	8	11	10	10	11	11	11	15	451	Y

<sup>a</sup> On-farm trial ID with location name followed by the year the experiment was conducted.

<sup>b</sup> Yield difference between R3 stage application and non-treated control in kg/ha. Experiments are ordered from smallest to greatest yield increase with a fungicide application compared to the non-treated.

<sup>c</sup> Sig. diff. = difference in yield between the fungicide treated and non-treated control was significantly different (Y = yes,  $p < 0.05$ ) or not (N = no,  $p > 0.05$ ) at the 95% confidence level.

**Supplemental Table S4.3.** Equations for calculating predictive values to assess different favorable-days thresholds.

<b>Name</b>	<b>Equation</b>
Percent predicted	$\frac{A + D}{A + B + C + D}$
Percent sprayed correct (Positive predictive value)	$\frac{A}{A + C}$
Percent no spray correct (Negative predictive value)	$\frac{D}{B + D}$
Odds ratio	$\frac{(A)(D)}{(B)(C)}$

**Supplemental Table S4.4.** Equations for calculating sensitivity and specificity of favorable-days thresholds.

Name	Equation
Sensitivity	$\left(\frac{A}{A+B}\right) \times 100$
Specificity	$\left(\frac{D}{D+C}\right) \times 100$

**Supplemental Table S4.5.** Number of days three weeks prior to beginning pod (R3) stage of the soybean crop that met or exceeded the indicated favorable-hours threshold for on-farm experiments conducted from 2014 to 2016 in Virginia, Maryland, and Delaware.

Favorable-hours threshold (in 24 h) <sup>a</sup>	Number of days three weeks prior to R3 that met or exceeded indicated threshold																				
	SussexVA15	VaBeach16	Orange15	Talbot15	Middlesex16	Culpeper14	Accomack14	Middlesex15	SussexDE15	Nottoway14	Chesapeake15	Culpeper16	Stafford14	Accomack16	Stafford15	Talbot16	Culpeper15	SussexVA14	Orange14	Amelia14	Lancaster16
1	19	0	20	17	21	11	12	19	9	20	9	21	10	14	18	21	19	18	20	20	19
2	17	0	19	16	21	10	10	18	8	20	8	21	10	14	17	21	19	18	19	20	19
3	16	0	19	13	21	5	8	17	7	20	6	21	9	13	16	20	19	17	18	20	19
4	16	0	18	10	20	4	6	16	7	20	5	21	6	12	15	17	18	15	15	20	17
5	15	0	17	9	20	3	5	14	7	20	4	21	5	12	14	14	18	13	14	20	17
6	15	0	17	8	20	3	3	11	7	20	3	21	3	10	13	13	17	12	12	20	16
7	13	0	17	6	19	3	4	12	7	19	4	21	4	10	12	11	18	13	11	19	16
8	13	0	16	6	18	3	3	6	6	16	1	20	3	7	9	10	16	11	10	16	16
9	11	0	15	5	17	3	3	6	6	15	1	18	3	7	7	7	16	11	9	15	14
10	10	0	15	5	16	3	3	6	5	15	1	18	3	7	7	6	16	11	9	15	14
11	5	0	14	4	13	3	1	2	4	12	1	16	2	1	3	2	9	8	8	12	11
12	3	0	10	1	11	3	1	1	4	10	0	12	1	0	2	2	8	6	5	10	4
13	2	0	5	0	5	2	0	1	3	9	0	10	1	0	2	2	5	3	3	9	2
14	2	0	5	0	4	1	0	1	2	8	0	4	1	0	2	1	3	2	2	8	2
15	2	0	1	0	1	1	0	1	1	5	0	3	0	0	2	1	2	2	0	5	2
16	2	0	1	0	0	1	0	1	0	4	0	1	0	0	1	1	1	1	0	4	1
17	1	0	1	0	0	1	0	0	0	3	0	1	0	0	1	0	0	0	0	3	1
18	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1
19	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1
20	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
21	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Yield diff. <sup>b</sup>	-326	-228	-188	-161	-161	-87	-74	-68	-20	88	114	115	127	161	242	249	256	282	349	424	451
Sig. diff. <sup>c</sup>	N	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	Y	Y	Y	Y	Y	Y

<sup>a</sup> To meet the threshold, a 24 h period from noon to noon must meet or exceed the indicated number of favorable (relative humidity  $\geq$  90%) hours. <sup>b</sup> Yield difference between R3 application and non-treated control in kg/ha. Experiments are ordered from smallest to greatest yield increase with a fungicide application compared to the non-treated. <sup>c</sup> Sig. diff. = difference in yield between the fungicide treated and non-treated control was significantly different (Y = yes,  $p < 0.05$ ) or not (N = no,  $p > 0.05$ ) at the 95% confidence level.

**Supplemental Table S4.6.** Statistics for different favorable-hours thresholds (hours with  $\geq 90\%$  RH) in a 24-hour period from noon to noon for predicting a need for a foliar fungicide application in soybean using data from on-farm experiments conducted from 2014 to 2016 in Virginia, Maryland, and Delaware.

	<b>Favorable-hours threshold (in 24 hours)</b>						
	<b>FHT=7</b>	<b>FHT=8</b>	<b>FHT=9</b>	<b>FHT=10</b>	<b>FHT=11</b>	<b>FHT=12</b>	<b>FHT=13</b>
Predicted correct	67%	76%	76%	76%	71%	62%	67%
Sprayed correct	50%	58%	60%	60%	57%	40%	50%
Not sprayed correct	100%	100%	91%	91%	79%	69%	71%
Odds ratio	15.00	25.90	15.00	15.00	4.89	1.47	2.40
P-value	0.08	0.04	0.03	0.03	0.11	0.72	0.44
Sensitivity	100%	100%	86%	86%	57%	29%	29%
Specificity	50%	64%	71%	71%	79%	79%	86%
Likelihood ratio +	2.00	2.80	3.00	3.00	2.67	1.33	2.00
Likelihood ratio -	0.00	0.00	0.20	0.20	0.55	0.91	0.83



**Supplemental Table S4.7.** Number of hours with 90% or greater relative humidity for each day within three weeks prior to the beginning pod (R3) stage of the soybean crop for small-plot fungicide trials conducted at the Tidewater AREC in Suffolk, Virginia from 2007 to 2012.

# of days before R3	2007			2008			2009			2010			2011		2012	
	SOYRUST107	SOYRUST207	SOYRUST307	SOYRUST108	SOYRUST208	SOYRUST308	SOYRUST109	SOYRUST309	SOYRUST409	SOYRUST110	SOYRUST210	SOYRUST310	SOYRUST111	SOYRUST211	SOYRUST112	SOYRUST212
1	13	12	11	6	6	10	3	12	8	17	17	8	3	3	12	18
2	12	13	12	9	9	7	8	12	12	4	4	6	6	6	12	9
3	0	12	13	7	7	5	12	20	12	7	7	16	7	7	10	13
4	6	0	12	9	9	8	12	16	20	6	6	15	10	10	13	12
5	3	6	0	15	15	7	20	19	16	9	9	8	12	12	13	16
6	10	3	6	7	7	6	16	12	19	7	7	9	9	9	18	18
7	8	10	3	11	11	5	19	6	12	15	15	12	5	5	9	7
8	8	8	10	11	11	10	12	12	6	10	10	3	11	11	13	10
9	7	8	8	10	10	9	6	1	12	0	0	3	11	11	12	9
10	7	7	8	7	7	3	12	13	1	9	9	8	18	18	16	16
11	10	7	7	5	5	4	1	16	13	11	11	9	8	8	18	12
12	15	10	7	8	8	9	13	15	16	12	12	0	10	10	7	8
13	8	15	10	7	7	2	16	10	15	9	9	0	9	9	10	12
14	3	8	15	6	6	5	15	10	10	12	12	6	6	6	9	10
15	4	3	8	5	5	8	10	14	10	8	8	3	2	2	16	3
16	10	4	3	10	10	12	10	12	14	6	6	10	13	13	12	11
17	10	10	4	9	9	7	14	9	12	16	16	3	12	12	8	4
18	1	10	10	3	3	11	12	8	9	15	15	0	0	0	12	5
19	7	1	10	4	4	11	9	9	8	8	8	6	0	0	10	5
20	8	7	1	9	9	17	8	8	9	9	9	0	0	0	3	4
21	9	8	7	2	2	3	9	14	8	12	12	9	6	6	11	12

**Supplemental Table S4.8.** Number of days three weeks prior to beginning pod (R3) stage of the soybean crop that met or exceeded the indicated favorable-hours threshold for small-plot trials conducted at Tidewater AREC in Suffolk, VA from 2007 to 2012.

Favorable-hours threshold (in 24 h) <sup>a</sup>	2007			2008			2009			2010			2011		2012	
	SOYRUST107	SOYRUST207	SOYRUST307	SOYRUST108	SOYRUST208	SOYRUST308	SOYRUST109	SOYRUST309	SOYRUST409	SOYRUST110	SOYRUST210	SOYRUST310	SOYRUST111	SOYRUST211	SOYRUST112	SOYRUST212
1	20	20	20	21	21	21	21	21	21	20	20	17	18	18	21	21
2	19	19	19	21	21	21	20	20	20	20	20	17	18	18	21	21
3	19	19	19	20	20	20	20	20	20	20	20	17	17	17	21	21
4	17	17	17	19	19	18	19	20	20	20	20	13	16	16	20	20
5	16	16	16	18	18	17	19	20	20	19	19	13	16	16	20	18
6	16	16	16	16	16	14	19	20	20	19	19	13	15	15	20	16
7	15	15	15	14	14	13	18	19	19	17	17	10	12	12	20	16
8	12	12	12	10	10	10	18	19	19	15	15	10	11	11	19	15
9	8	8	9	9	9	8	16	17	16	13	13	7	10	10	18	14
10	7	8	9	5	5	6	14	15	14	9	9	4	8	8	16	12
11	3	4	5	3	3	4	12	13	12	8	8	3	6	6	13	10
12	3	4	4	1	1	2	12	13	12	7	7	3	4	4	12	9
13	2	2	2	1	1	1	7	8	7	4	4	2	2	2	7	5
14	1	1	1	1	1	1	6	7	6	4	4	2	1	1	4	4
15	1	1	1	1	1	1	5	5	5	4	4	2	1	1	4	4
16	0	0	0	0	0	1	4	4	4	2	2	1	1	1	4	4
17	0	0	0	0	0	1	2	2	2	1	1	0	1	1	2	2
18	0	0	0	0	0	0	2	2	2	0	0	0	1	1	2	2
19	0	0	0	0	0	0	2	2	2	0	0	0	0	0	0	0
20	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

<sup>a</sup> To meet the threshold, a 24 h period from noon to noon must meet or exceed the indicated number of favorable (relative humidity  $\geq$  90%) hours.

**Supplemental Table S4.9.** Statistics for different favorable-hours thresholds (hours with  $\geq 90\%$  RH) in a 24-hour period from noon to noon for predicting a need for a foliar fungicide application in soybean using data from small-plot fungicide trials conducted at Tidewater AREC in Suffolk, VA from 2007 to 2012.

	<b>Favorable-hours threshold (in 24 hours)</b>		
	FHT=8	FHT=9	FHT=10
Predicted correct	25%	50%	75%
Sprayed correct	25%	33%	50%
Not sprayed correct	---	100%	100%
Odds ratio	0.36	4.76	17.00
P-value	0.62	0.33	0.08
Sensitivity	100%	100%	100%
Specificity	0%	33%	67%
Likelihood ratio +	1.0	1.5	3.0
Likelihood ratio -	---	0	0

**Supplemental Table S4.10.** Threshold decision rule statistics for application of fungicides in soybean using disease favorable-days thresholds (FDT) ranging from 1 to 14 for the 2 weeks prior to the beginning pod (R3) stage of the crop to evaluate positive yield as the criterion when determining if spray or no spray is correct for on-farm experiments conducted between 2014 and 2016.

	Favorable-days threshold <sup>a</sup>													
	FDT=1	FDT=2	FDT=3	FDT=4	FDT=5	FDT=6	FDT=7	FDT=8	FDT=9	FDT=10	FDT=11	FDT=12	FDT=13	FDT=14
Sensitivity, %	100	100	100	83	75	71	42	33	33	33	33	0	0	0
Specificity, %	11	22	22	56	67	79	78	78	78	78	89	78	100	100
Likelihood Ratio +	1.12	1.29	1.29	1.88	2.25	3.33	1.88	1.50	1.50	1.50	3.00	0.00	0.00	0.00
Likelihood Ratio -	0.00	0.00	0.00	0.30	0.38	0.36	0.75	0.86	0.86	0.86	0.75	1.12	1.00	1.00
Sprayed correct, %	60	63	63	71	75	63	71	67	67	67	80	0	0	0
Not sprayed correct, %	100	100	100	71	67	85	50	47	47	47	50	40	43	43
Predicted correct, %	62	67	67	71	71	76	57	52	52	52	57	38	43	43
Odds ratio	4.41	8.33	8.33	6.25	6.00	9.17	2.50	1.75	1.75	1.75	4.00	0.23	0.76	0.76
P-value	0.381	0.190	0.190	0.074	0.065	0.037	0.356	0.579	0.579	0.579	0.258	0.381	0.893	0.893
Mean yield difference, kg/ha	85	93	93	211	251	250	330	309	309	309	409	-161	--- <sup>b</sup>	---
Youden's Index	0.11	0.22	0.22	0.39	0.42	0.50	0.20	0.11	0.11	0.11	0.22	0.00	0.00	0.00

<sup>a</sup> Favorable-days threshold examined for predictive value.

<sup>b</sup> --- Indicates calculated value does not apply.

**Supplemental Table S4.11.** Threshold decision rule statistics for application of fungicides in soybean using disease favorable-days thresholds (FDT) ranging from 1 to 14 for the 2 weeks prior to the beginning pod (R3) stage of the crop to evaluate significant yield as the criterion when determining if spray or no spray is correct for on-farm experiments conducted between 2014 and 2016.

	Favorable-days threshold <sup>a</sup>													
	FDT =1	FDT=2	FDT=3	FDT=4	FDT=5	FDT=6	FDT=7	FDT=8	FDT= 9	FDT=10	FDT=11	FDT=12	FDT=13	FDT=14
Sensitivity, %	100	100	100	86	86	62	57	43	43	43	43	0	0	0
Specificity, %	7	14	14	43	71	85	78	80	80	80	86	93	100	100
Likelihood Ratio +	1.08	1.17	1.17	1.50	3.00	4.06	2.67	2.14	2.14	2.14	3.00	0.00	--- <sup>b</sup>	---
Likelihood Ratio -	0.00	0.00	0.00	0.33	0.20	0.44	0.55	0.71	0.71	0.71	0.67	1.08	1.00	1.00
Sprayed correct, %	35	37	37	43	60	62	57	50	50	50	60	0	---	---
Not sprayed correct, %	100	100	100	86	91	85	79	73	73	73	75	65	67	67
Predicted correct, %	38	43	43	67	76	76	71	67	67	67	71	62	67	67
Odds ratio	1.67	3.00	3	4.50	15.00	9.17	4.89	2.75	2.75	2.75	4.50	0.60	1.93	1.93
P-value	0.763	0.497	0.500	0.212	0.028	0.037	0.114	0.314	0.314	0.314	0.164	0.763	0.748	0.748
Mean yield difference, kg/ha	85	93	93	208	310	324	329	309	309	309	409	-161	---	---
Youden's Index	0.07	0.14	0.14	0.29	0.57	0.5	0.36	0.23	0.23	0.23	0.29	-0.07	0	0

<sup>a</sup> Favorable-days threshold examined for predictive value.

<sup>b</sup> --- Indicates calculated value does not apply.

**Supplemental Table S4.12.** Threshold decision rule statistics for application of fungicides in soybean using disease favorable-days thresholds (FDT) ranging from 1 to 14 for the 2 weeks prior to the beginning pod (R3) stage of the crop to evaluate positive yield as the criterion when determining if spray or no spray is correct for validation experiments conducted between 2006 to 2012.

	Favorable-days threshold <sup>a</sup>													
	FDT =1	FDT=2	FDT=3	FDT=4	FDT=5	FDT=6	FDT=7	FDT=8	FDT= 9	FDT=10	FDT=11	FDT=12	FDT=13	FDT=14
Sensitivity, %	94	89	89	83	83	67	61	61	44	39	28	17	17	0
Specificity, %	14	29	29	43	43	100	100	100	100	100	100	100	100	100
Likelihood Ratio +	1.10	1.24	1.24	1.46	1.46	--- <sup>b</sup>	---	---	---	---	---	---	---	---
Likelihood Ratio -	0.39	0.39	0.39	0.39	0.39	0.33	0.39	0.39	0.56	0.61	0.72	0.83	0.83	1.00
Sprayed correct, %	74	76	76	79	79	100	100	100	100	100	100	100	100	---
Not sprayed correct, %	50	50	50	50	50	54	50	50	41	39	35	32	32	28
Predicted correct, %	72	72	72	72	72	76	72	72	60	56	48	40	40	28
Odds ratio	2.83	3.20	3.20	3.75	3.75	28.85	23.00	23.00	12.14	9.78	6.11	3.39	3.39	0.41
P-value	0.49	0.30	0.30	0.18	0.18	0.03	0.04	0.04	0.10	0.14	0.24	0.44	0.44	0.66
Mean yield difference, kg/ha	245	279	279	293	372	408	408	408	325	343	308	321	321	0
Youden's Index	0.08	0.18	0.18	0.26	0.26	0.67	0.61	0.61	0.44	0.39	0.28	0.17	0.17	0.00

<sup>a</sup> Favorable-days threshold examined for predictive value.

<sup>b</sup> --- Indicates calculated value does not apply.

**Supplemental Table S4.13.** Threshold decision rule statistics for application of fungicides in soybean using disease favorable-days thresholds (FDT) ranging from 1 to 14 for the 2 weeks prior to the beginning pod (R3) stage of the crop to evaluate significant yield as the criterion when determining if spray or no spray is correct for validation experiments conducted between 2006 to 2012.

	Favorable-days threshold <sup>a</sup>													
	FDT =1	FDT=2	FDT=3	FDT=4	FDT=5	FDT=6	FDT=7	FDT=8	FDT= 9	FDT=10	FDT=11	FDT=12	FDT=13	FDT=14
Sensitivity, %	100	100	100	100	100	80	80	80	40	40	20	20	20	--- <sup>b</sup>
Specificity, %	10	20	20	30	45	60	65	65	70	75	80	90	90	80
Likelihood Ratio +	1.11	1.25	1.25	1.43	1.82	2.00	2.29	2.29	1.33	1.60	1.00	2.00	2.00	0.00
Likelihood Ratio -	0.00	0.00	0.00	0.00	0.00	0.33	0.31	0.31	0.86	0.80	1.00	0.89	0.89	1.25
Sprayed correct, %	22	24	24	26	31	33	36	36	25	29	20	33	33	0
Not sprayed correct, %	100	100	100	100	100	92	93	93	82	83	80	82	82	100
Predicted correct, %	28	36	36	44	56	64	68	68	64	68	68	76	76	80
Odds ratio	1.49	3.00	3.00	4.93	9.09	6.00	7.43	7.43	1.56	2.00	1.00	2.25	2.25	3.73
P-value	0.81	0.48	0.48	0.30	0.15	0.14	0.10	0.10	0.67	0.51	1.00	0.55	0.55	0.52
Mean yield difference, kg/ha	245	279	279	293	372	413	413	429	333	352	328	318	318	0
Youden's Index	0.10	0.20	0.20	0.30	0.45	0.40	0.45	0.45	0.10	0.15	0.00	0.10	0.10	---

<sup>a</sup> Favorable-days threshold examined for predictive value.

<sup>b</sup> --- Indicates calculated value does not apply.

**Supplemental Table S4.14.** Threshold decision rule statistics for application of fungicides in soybean using disease favorable-days thresholds (FDT) ranging from 1 to 14 for the 3 weeks prior to the beginning pod (R3) stage of the crop to evaluate positive yield as the criterion when determining if spray or no spray is correct for on-farm experiments conducted between 2014 to 2016.

	Favorable-days threshold <sup>a</sup>													
	FDT =1	FDT=2	FDT=3	FDT=4	FDT=5	FDT=6	FDT=7	FDT=8	FDT= 9	FDT=10	FDT=11	FDT=12	FDT=13	FDT=14
Sensitivity, %	100	100	100	100	100	100	75	58	58	50	50	42	42	42
Specificity, %	11	11	11	22	22	36	67	67	67	67	67	78	78	78
Likelihood Ratio +	1.12	1.12	1.12	1.29	1.18	1.56	2.25	1.75	1.75	2.50	2.50	1.88	1.88	1.88
Likelihood Ratio -	0.00	0.00	0.00	0.00	0.38	0.00	0.38	0.62	0.62	0.40	0.40	0.75	0.75	0.75
Sprayed correct, %	60	60	60	63	61	69	75	70	70	67	67	71	71	71
Not sprayed correct, %	100	100	100	100	67	80	67	55	55	50	50	50	50	50
Predicted correct, %	62	62	62	63	61	69	75	70	70	67	67	71	71	71
Odds ratio	4.41	4.41	4.41	8.33	3.14	8.80	6.00	2.80	2.80	2.00	2.00	2.50	2.50	2.50
P-value	0.381	0.381	0.381	0.19	0.39	0.08	0.07	0.26	0.26	0.45	0.45	0.36	0.36	0.36
Mean yield difference, kg/ha	85	85	85	93	92	201	251	260	260	250	250	330	330	330
Youden's Index	0.11	0.11	0.11	0.22	0.22	0.36	0.42	0.25	0.25	0.17	0.17	0.20	0.20	--- <sup>b</sup>

<sup>a</sup> Favorable-days threshold examined for predictive value.

<sup>b</sup> --- Indicates calculated value does not apply.



**Supplemental Table S4.15.** Threshold decision rule statistics for application of fungicides in soybean using disease favorable-days thresholds (FDT) ranging from 1 to 14 for the 3 weeks prior to the beginning pod (R3) stage of the crop to evaluate significant yield as the criterion when determining if spray or no spray is correct for on-farm experiments conducted between 2014 to 2016.

	Favorable-days threshold <sup>a</sup>													
	FDT =1	FDT=2	FDT=3	FDT=4	FDT=5	FDT=6	FDT=7	FDT=8	FDT= 9	FDT=10	FDT=11	FDT=12	FDT=13	FDT=14
Sensitivity, %	100	100	100	100	100	100	86	86	86	71	71	57	57	57
Specificity, %	7	7	7	14	21	36	57	71	71	71	71	79	79	79
Likelihood Ratio +	1.08	1.08	1.08	1.17	1.27	1.56	2.00	3.00	3.00	2.50	2.50	2.67	2.67	2.67
Likelihood Ratio -	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.20	0.20	0.40	0.40	0.55	0.55	0.55
Sprayed correct, %	35	35	35	37	39	44	50	60	60	56	56	57	57	57
Not sprayed correct, %	100	100	100	100	100	100	89	91	91	83	83	79	79	79
Predicted correct, %	38	38	38	43	48	57	67	76	76	71	71	71	71	71
Odds ratio	1.67	1.67	1.67	3.00	4.57	8.68	8.00	15.00	15.00	6.25	6.25	4.89	4.89	4.89
P-value	0.301	0.301	0.301	0.497	0.338	0.165	0.085	0.028	0.028	0.012	0.012	0.114	0.114	0.114
Mean yield difference, kg/ha	85	85	85	93	33	201	251	260	260	250	250	329	329	329
Youden's Index	0.07	0.07	0.07	0.14	0.21	0.36	0.43	0.57	0.57	0.42	0.42	0.36	0.36	0.36

<sup>a</sup> Favorable-days threshold examined for predictive value.

**Supplemental Table S4.16.** Threshold decision rule statistics for application of fungicides in soybean using disease favorable-days thresholds (FDT) ranging from 1 to 14 for the 3 weeks prior to the beginning pod (R3) stage of the crop to evaluate positive yield as the criterion when determining if spray or no spray is correct for validation experiments conducted between 2006 to 2012.

	Favorable-days threshold <sup>a</sup>													
	FDT =1	FDT=2	FDT=3	FDT=4	FDT=5	FDT=6	FDT=7	FDT=8	FDT= 9	FDT=10	FDT=11	FDT=12	FDT=13	FDT=14
Sensitivity, %	100	94	89	89	83	78	72	72	56	56	56	50	33	0
Specificity, %	0	14	14	29	29	57	86	100	100	100	100	100	100	100
Likelihood Ratio +	1.00	1.10	1.04	1.24	1.17	1.81	5.06	---	---	---	---	---	---	---
Likelihood Ratio -	--- <sup>b</sup>	0.39	0.78	0.39	0.58	0.39	0.32	0.28	0.44	0.44	0.44	0.50	0.67	1.00
Sprayed correct, %	72	74	73	76	75	82	93	100	100	100	100	100	100	---
Not sprayed correct, %	---	50	33	50	40	50	55	58	47	47	47	44	37	28
Predicted correct, %	72	72	68	72	68	72	76	80	68	68	68	64	52	28
Odds ratio	2.47	2.83	1.33	3.20	2.00	4.67	15.60	36.82	18.53	18.53	18.53	15.00	7.80	0.41
P-value	0.66	0.49	0.82	0.30	0.51	0.11	0.02	0.02	0.06	0.06	0.06	0.08	0.18	0.66
Mean yield difference, kg/ha	235	244	248	258	258	288	355	409	445	445	445	432	500	0
Youden's Index	0.00	0.08	0.03	0.17	0.12	0.35	0.58	0.72	0.56	0.56	0.56	0.50	0.33	---

<sup>a</sup> Favorable-days threshold examined for predictive value.

<sup>b</sup> --- Indicates calculated value does not apply.

**Supplemental Table S4.17.** Threshold decision rule statistics for application of fungicides in soybean using disease favorable-days thresholds (FDT) ranging from 1 to 14 for the 3 weeks prior to the beginning pod (R3) stage of the crop to evaluate significant yield as the criterion when determining if spray or no spray is correct for validation experiments conducted between 2006 to 2012.

	Favorable-days threshold <sup>a</sup>													
	FDT =1	FDT=2	FDT=3	FDT=4	FDT=5	FDT=6	FDT=7	FDT=8	FDT= 9	FDT=10	FDT=11	FDT=12	FDT=13	FDT=14
Sensitivity, %	100	100	100	100	100	100	100	100	80	80	80	60	50	---
Specificity, %	0	10	15	20	25	40	55	60	70	70	70	70	89	80
Likelihood Ratio +	1.00	1.11	1.18	1.25	1.33	1.67	2.22	2.50	2.67	2.67	2.67	2.00	4.75	0.00
Likelihood Ratio -	--- <sup>b</sup>	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.29	0.29	0.29	0.57	0.56	1.25
Sprayed correct, %	20	22	23	24	25	29	36	38	40	40	40	33	60	0
Not sprayed correct, %	---	100	100	100	100	100	100	100	93	93	93	88	85	100
Predicted correct, %	20	28	32	36	40	52	64	68	72	72	72	68	80	80
Odds ratio	0.27	1.49	2.20	3.00	3.90	7.48	13.30	16.18	9.33	2.67	2.67	3.50	8.50	3.73
P-value	0.52	0.81	0.62	0.48	0.38	0.19	0.09	0.07	0.45	0.45	0.45	0.23	0.05	0.52
Mean yield difference, kg/ha	235	244	248	258	258	288	355	406	409	445	445	432	500	0
Youden's Index	0.00	0.10	0.15	0.20	0.25	0.40	0.55	0.60	0.50	0.50	0.50	0.30	0.39	---

<sup>a</sup> Favorable-days threshold examined for predictive value.

<sup>b</sup> --- Indicates calculated value does not apply.