Sooty blotch and flyspeck of apple: assessment of an RFLP-based identification technique and adaptation of a warning system for the Upper Midwest

by

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ABSTRACT

Sooty blotch and flyspeck (SBFS) of apple, a disease caused by more than 30 species of fungi, reduces crop value by blemishing the fruit surface. This study investigated two research tools designed to improve identification of SBFS fungi and management of the disease.

The first objective was to validate a PCR-based method to identify SBFS pathogens. Members of the sooty blotch and flyspeck (SBFS) disease complex are challenging to identify by traditional mycological methods that rely on agar-plate isolation and morphological description. Identification using a PCR-RFLP assay was investigated as an alternative to culturing. The method involved amplification of the internal transcribed spacer region of ribosomal DNA using a Capnodiales order-specific reverse primer paired with a universal forward primer, followed by digestion using the *Hae*III restriction enzyme. When applied to 24 SBFS species from a survey in the Midwest U.S., the PCR-RFLP assay produced 14 unique band patterns, all specific to genus. The technique also identified SBFS fungi from DNA extracted directly from colonies on apples. The PCR-RFLP assay streamlined the identification process by circumventing the requirement for culturing, and should be a valuable tool for further ecological studies of the SBFS disease complex.

The second objective was to adapt a SBFS warning system for the Upper Midwest. The Sutton-Hartman warning system, developed in the Southeast U.S., uses cumulative hours of leaf wetness duration (LWD) to predict the timing of the first appearance of SBFS signs. In the Upper Midwest, however, this warning system experienced sporadic control failures. To determine if other weather variables were useful predictors of SBFS appearance, hourly LWD, rainfall, relative humidity (RH), and temperature data were collected from orchards in

IA, WI and NC. Timing of the first appearance of SBFS was determined by scouting weekly for disease signs. Receiver operating characteristic curve analysis revealed that cumulative hours of RH≥97% was a more conservative and accurate predictor than cumulative LWD for the Upper Midwest. The results suggest that the performance of the SBFS warning system in the Upper Midwest could be improved if cumulative hours of RH≥97% were substituted for cumulative hours of LWD to predict the first appearance of SBFS.

CHAPTER 1. GENERAL INTRODUCTION

Thesis organization

This thesis is composed of an abstract and four chapters. The first chapter provides background information on sooty blotch and flyspeck (SBFS) of apple, discusses the basis of SBFS identification techniques and warning system development, and presents the rationale and objectives of the research. The second chapter, a manuscript in preparation for *Plant Disease*, describes the validation of a method to identify SBFS pathogens using molecular techniques. The third chapter, a manuscript in preparation for *Plant Disease*, describes a study assessing the ability of weather variables to predict the timing of the first appearance of SBFS in order to adapt a disease warning system for the Upper Midwest. The last chapter summarizes the presented research and discusses the conclusions of the thesis.

Literature review

Sooty blotch and flyspeck (SBFS) is a fungal disease of apples that causes dark blemishes to form on the fruit epidermis. Although these blemishes do not impact fruit development or quality, they make affected apples unappealing to customers. While SBFS-blemished apples are suitable for processing, their value can be 90% lower than apples sold as fresh fruit (7,62).

The dark blemishes characteristic of SBFS are colonies of fungi living epiphytically on the apple surface. The apple cuticle provides mechanical support but apparently no nutrients for the fungi (8,9). It has been hypothesized that a primary source of nutrients for the fungi is sugar-containing exudates from the apple (9,63).

Warm and moist environments favor SBFS development. Optimal temperatures range from 20 to 25° C, with growth slowing or ceasing at temperatures below 15° C or above 30° C (13,19,34). Minimum relative humidity of 90% to 95% is required for SBFS development (19). Reported in the U.S., Europe, and Asia, the disease is present in most moist climates where apples are grown. Surveys in the U.S. have observed SBFS fungi growing on the waxy cuticle of fruit or shoots of a wide range of tree, shrub, and vine hosts (62). Other fruit crops with waxy cuticles, such as pears and plums, can also be impacted by SBFS (46).

The blemishes referred to as flyspeck superficially resemble fly frass. Flyspeck colonies consist of groups of several to hundreds of black specks that cover from a few square millimeters to the entire surface of an apple without visible mycelium between the specks (3,62). The blemishes referred to as sooty blotch are more variable in morphology than flyspeck, but typically exhibit a dark mycelial mat, either with or without black specks in the mat. Traditionally, sooty blotch fungi have been segregated into four mycelial types: rimate, ramose, punctate, and fuliginous (4,29).

SBFS taxonomy

Sooty blotch and flyspeck was first thought to be caused by a single fungus, *Dothidea pomigena* (Schw) (62). In the early 1900s, Colby (1920) reported that the disease was caused by two fungi: sooty blotch by *Gloeodes pomigena* (Schw.) Colby, and flyspeck by *Leptothyrium pomi* (Mont. & Fr.) Sacc. *Zygophiala jamaicensis* Mason, first reported as a pathogen on banana in 1945, was described on apple as the imperfect state of flyspeck in 1953 (3). After debates over fruiting body terminology, the perfect state of the flyspeck pathogen was renamed *Schizothyrium pomi* (Mont. & Fr.) v. Arx in 1959 (3).

Gloeodes pomigena was the accepted name for the causal agent of sooty blotch until the mid-1990s. Research in North Carolina then suggested that the variation in mycelial types was due not to environmental influence on a single pathogen (the previously prevailing hypothesis), but rather to multiple species of fungi causing SBFS signs (4,35,38). Using morphological data, at least three different fungi, *Peltaster fructicola*, *Leptodontium elatius*, and *Geastrumia polystigmatis*, were described as causing sooty blotch (37,38,39). In a survey of SBFS in the Midwest U.S., Batzer et al. (2005) coupled morphological evidence with phylogenetic analysis of the internal transcribed spacer and large subunit segments of ribosomal DNA, and reported that the disease is caused by at least 30 putative species of fungi. New genera found to cause sooty blotch included *Pseudocercosporella*, *Pseudocercospora*, *Colletogloeum*, *Dissoconium*, *Xenostigmina*, *Passalora*, and *Ramularia*. Three new, closely related species were classified as *Mycelia sterilia* since fruiting bodies were not observed. In some cases, several species within the same genus caused sooty blotch signs. Three new sooty blotch mycelial types were defined: compact speck, discrete speck, and ridged honeycomb. In some cases, different species within the same genus caused distinct mycelial types. Three new species flyspeck were reported including two in the genus *Zygophiala* and one in *Pseudocercospora* (6). A later study revealed that at least four species of *Zygophiala* cause flyspeck (5).

The Midwest survey demonstrated that the vast majority of SBFS species are in the order Dothideales in the phylum Ascomycota (6). Re-evaluation of the phylogeny of the Dothideomycetes has led to the reclassification of the order Dothideales as Capnodiales (53). In 2006, the first SBFS species belonging to the phylum Basidiomycota, *Wallemia sebi*, was identified in China (59).

SBFS identification

Although SBFS fungi were traditionally identified solely by morphological characterization on the apple epidermis and in pure culture, the process is often daunting. Most SBFS fungi grow extremely slowly in culture (4). Isolation is difficult due to the pathogens' sensitivity to disinfestants, which frequently leads to overgrowth by contaminants (6,38,58). Fruiting body production and sporulation can be infrequent or absent (6,35). Adding to the confusion, SBFS morphology in culture often differs greatly from that on the apple epidermis (6).

The challenge of identifying SBFS fungi efficiently has increased greatly with the recent realization of the enormous diversity in the complex. At least 30 species world-wide are now known to cause SBFS (6,20). While morphological characterization continues to be an important component of SBFS species characterization, it is too laborious and unreliable to use for most field studies. Therefore, there is need for a more rapid, reliable, and accurate method of identifying SBFS fungi.

Molecular identification techniques

Molecular techniques are widely used for identification of fungi, bacteria, and viruses (10). While there is a vast array of potential techniques for identification, many are based on the polymerase chain reaction (PCR), a method that produces many copies of a specific region of a genome. Regions often amplified for studies of fungi include mitochondrial DNA (mtDNA), ribosomal DNA (rDNA), and the β-tubulin gene. Most commonly used in classifying fungi is rDNA (10), which has three coding genes: the 28S large subunit (LSU), the 18S small subunit (SSU), and the 5.8S subunit. Ribosomal DNA also has non-coding regions including two internal transcribed spacers (ITS) and one intergenic spacer (IGS) (10).

Favorable characteristics of rDNA for identifying fungi include the presence of both variable (ITS and IGS) and conserved (LSU, SSU, and 5.8S) segments. In addition, there are hundreds to thousands of copies of the rDNA region in the genome, which can significantly increase success of PCR amplification (10,16,36). The conserved regions of rDNA have been suitable for primer design, whereas the variable regions have been useful for phylogenetic classification of fungi. Batzer et al. (2005) used sequence differences in the ITS region to classify SBFS fungi to species, and in the LSU region to classify the fungi to order and genus.

Molecular techniques are useful for visualizing variations in DNA sequences. One common technique involves PCR amplification followed by digestion of amplicons with restriction enzymes, which enzyme cut DNA at a specific recognition sequence. The digested PCR products are then visualized after gel electrophoresis. The subsequent banding pattern observed on the gel will vary depending on the size of DNA fragments in the digest. Differences in banding patterns are referred to as restriction fragment length polymorphisms (RFLP) (36).

RFLP analysis has been used to identify many different types of fungi. The technique has been used to evaluate the presence of pathogens in various field, vegetable, fruit, and tree crops (1,31,41,47,50,57). Forest mycology has embraced the technique, applying it to fruiting basidiomycetes, sapstain fungi, polypores, and mychorrizal fungi (11,22,23,28,49). Some studies utilizing RFLP analysis focus on a single genus, whereas others investigate entire communities of fungi. For example, Martin (2000) used RFLP analysis to differentiate anatomosis groups of *Rhizoctonia* spp., whereas Farmer and Sylvia (1998) differentiated

species within more than 10 genera in a survey of ectomycorrhizal fungi. Many of these fungi, like members of the SBFS complex, are difficult to isolate and identify in culture (27).

The specificity of identification of fungal taxa with RFLP analysis can be variable. Specificity is often increased by digesting PCR products with multiple enzymes. In general, digestion with two to three enzymes will result in identification to species (36).

SBFS identification with molecular techniques

Preliminary studies utilized sequence variability in the ITS region of rDNA to identify SBFS fungi (58). To increase specificity of PCR, a Capnodiales-specific primer was developed, because the majority of SBFS fungi belong to that order. Nine species-specific primers were also developed for SBFS members commonly found in the Midwest (58). The ITS primers most commonly used for amplifying fungi have the potential to amplify non-fungal DNA (36). While generally not a problem for samples in pure culture, this is an issue when attempting to amplify fungi from natural environments that are often contaminated with DNA of plants or other organisms. Designing more specific primers helps to prevent amplification of non-target DNA.

RFLP patterns from isolates of 14 species of SBFS fungi were identified (58). These fungi were amplified with the Capnodiales-specific primer and then restricted separately with two enzymes, *Hae*III and *Alu*I. Digestion with *Hae*III produced a greater number of unique RFLP patterns than with *Alu*I. RFLP patterns from *Hae*III digestion distinguished among seven SBFS genera (58).

To expedite ecological studies of SBFS fungi, molecular identification tools must be applicable to SBFS samples taken directly from the apple epidermis. Although most molecular identification techniques are initially applied to pure cultures, some have been

applied to fungi *in situ*. For example, *Mycosphaerella* species, which cause leaf diseases of *Eucalyptus*, were identified from mycelium taken directly from leaves (42). Additionally, *Botrytis* neck rot pathogens of onion were identified using a PCR-RFLP assay directly from onion tissue (47). In both examples, primers specific to the pathogens of interest were important in developing a successful identification technique. It is unknown whether SBFS fungi can be identified using molecular techniques without culturing.

SBFS management

Traditionally, SBFS management involves fungicide sprays every 10 to 14 days from first cover (7 to 10 days after petal fall) until shortly before harvest (26,62). Although calendar-based spray timing is generally effective, there is a need to develop more efficient approaches to controlling SBFS. Reducing chemical inputs provides many benefits to growers and consumers, including reduced input costs, prevention of mechanical damage and phytotoxicity, reduced pesticide exposure for workers and the environment, and decreased pressure for pathogens to develop fungicide resistance (12,25,30,52,64). Management concerns specific to SBFS include residues from late-season sprays (8), potential loss of affordable broad-spectrum fungicides due to the 1996 Food Quality and Protection Act (4), and evidence of fungicide resistance by at least two sooty blotch species, *P. fructicola* and *L. elatius* (37,60).

Summer pruning of orchards has been shown to increase fungicide efficacy, speed dryoff, and thereby potentially decrease number of fungicide applications required for SBFS control (18,19). Post-harvest dips in commercial disinfestants followed by mechanical brushing can partially remove SBFS signs from harvested apple, but this method has not

been adopted widely due to logistical barriers and failure to remove 100% of the signs (7,33). Another strategy to improve sustainability of SBFS management has involved developing disease-warning systems (13,51).

Disease-warning systems

Disease-warning systems are management tools that help growers assess the risk of a plant disease based on weather, pathogen, and/or host information (25,64). Development of warning systems can be either empirical (based on correlations between disease and weather records) or fundamental (based on cause-and-effect relationships determined by controlled experiments) (15,25,40). The variables used to assess disease risk can differ significantly depending on the pathosystem. The most common inputs are measurements of weather conditions. Examples of weather variables that can greatly influence disease risk include temperature, relative humidity, rainfall, leaf wetness duration, wind speed, and sun intensity (30). Non-weather-related variables that are sometimes used as inputs for warning systems include host growth stage, host resistance, fungicide efficacy, and amount and maturity of pathogen inoculum (12).

Relationships between warning system variables and disease progress are based on the underlying biology of the pathosystem (12,24,40,43). Information such as optimal temperatures and moisture requirements for infection, colonization, and sporulation is useful when developing an effective warning system. For example, the Mills model, developed to assess the risk of apple scab epidemic, relates daily periods of leaf wetness duration, and the temperature during the wet period, to the likelihood of infection by the pathogen, *Venturia inaequalis* (45,61).

Most warning systems are structured to advise growers to take an appropriate management action (often application of a fungicide) when the system estimates a sufficiently high risk of the targeted disease. By considering information about the growing environment, disease-warning systems are designed to recommend the minimum management requirements for acceptable disease control.

SBFS disease-warning system

Several aspects of the SBFS pathosystem make it appropriate for a disease-warning system. Crop diseases appropriate for warning systems should have the potential to cause significant economic damage but also should occur sporadically, with disease intensity varying annually (24,25). While at least some SBFS development is common every year in the Upper Midwest U.S., severe outbreaks are sporadic in occurrence, and SBFS can be absent during unusually dry summers (7). The high risk of crop damage by SBFS is another justification for application of a disease-warning system. Downgrading apples from fresh market sale to processing, which often is a result of SBFS blemishing, decreases growers' profit by as much as 90% (7,62). According to the National Agricultural Statistics Service, during the 2003-2004 season, growers were paid an average of 30 cents/lb for fresh-market apples and 5 cents/lb for processing apples -an 83% decline from the higher price- but losses from premium cultivars can be even higher (48).

Two warning systems have been developed for SBFS. A system developed to control flyspeck in New York is based on the residual activity of fungicides. It is assumed that the last fungicide spray to control the primary phase of apple scab, typically applied at the first-cover spray, lasts for 14 to 21 days or until accumulation of 3.5 inches of rain. After the period of residual activity ends, hours of leaf wetness duration are accumulated (with sensors

located in the orchards) until they reach a threshold of 100 h. At 100 h a fungicide spray is applied (51,62).

A more widely used warning system for SBFS was developed in North Carolina and refined in Kentucky (13,32,56). In this system, referred to as the Sutton-Hartman warning system, leaf wetness duration (LWD) measurements made beneath the canopy of an apple tree are used to predict the timing of the first appearance of SBFS signs. Accumulation of LWD begins immediately after the first-cover fungicide spray is applied. The first-cover spray is applied approximately 7 to 10 days after petal fall (14). Once LWD accumulates to a threshold, the second-cover spray is applied. Subsequent fungicide sprays are applied at 10- to 14-day intervals until shortly before harvest. Thus, this disease-warning system determines the duration of time between the first- and second-cover fungicide applications. Cumulative LWD excludes periods of less than 4 hours, which reflects the minimum wet period required for germination and/or mycelium growth of SBFS fungi (13). At least two consecutive dry hours are required to end a LWD period (4).

The cumulative LWD threshold for this warning system has been modified depending on the type of sensor used to collect the weather data. A LWD threshold of 250 hours was developed with deWit leaf wetness meters that use hemp strings as the sensing element (13). A threshold of 175 LWD hours was developed using flat-plate electronic wetness sensors, and is currently the threshold used in the warning system (56). Thresholds are conservative in order to accommodate errors in measuring LWD and time lags associated with application of a fungicide spray after the threshold is attained. For example, although SBFS signs appeared after an average of 273 hours LWD, a threshold of 200-250 hours of LWD was suggested to ensure sufficient time for growers to initiate control measures (13).

It is uncertain whether the Sutton-Hartman warning system, which was developed in the southeast U.S., is appropriate for other climates and regions. Due to variations in climate, a single warning system may not be applicable in all growing regions (15,40,52,55). For example, a warning system for potato late blight that was developed by Hyre in the northeastern U.S. was found to perform very well in that region, but not as effectively in the Midwest U.S. (40). It was determined that the important weather variables for predicting disease differed for the two regions. Hyre's system was based on daily rainfall and maximum and minimum temperatures. In the Midwest U.S., where rainfall conducive to disease development is less frequent than in the Northeast U.S., relative humidity was determined to be a more important variable for disease prediction (40). In another study, three imported fire blight models (MARYBLYT 4.3, BIS95, and Cougarblight 98C) performed poorly in Israel compared to a locally developed model. One hypothesis for the failures was that the imported models were developed in moist regions where temperature was a limiting factor for fire blight development, whereas moisture was the limiting factor in Israel (55).

The Sutton-Hartman warning system was tested in the Upper Midwest, and demonstrated the ability to save an average of two fungicide sprays per year. However, occasional control failures occurred. That is, significantly higher incidence of SBFS occurred in orchard blocks in which the warning system was used than in blocks in which the conventional schedule was used (2). Analysis of these control failures revealed no consistent association with risk factors such as inadequate pruning, low-volume spray applications, or delayed grower response to the warning system's spray advisories (2,62). Another

evaluation performed in Ohio suggested that 175 hours of LWD was too low an action threshold for well-kept orchards (21).

As the Sutton-Hartman warning system is imported to regions outside of the Southeast U.S., it is possible that weather variables other than LWD, or in addition to LWD, may be important in predicting the timing of appearance of first signs of SBFS in the new regions. If so, it may be necessary to alter the warning system to accommodate for climatic differences.

Thesis objectives

The work presented in this thesis had two objectives. The first objective was to identify members of the SBFS complex, both in pure culture and *in vivo*, using a PCR-RFLP assay. The second objective was to adapt the Sutton-Hartman warning system to the Upper Midwest by investigating the relationship between weather variables and timing of disease appearance. The overall goal was to develop tools that improve understanding and management of the SBFS disease complex.

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CHAPTER 2. AN RFLP-BASED TECHNIQUE FOR IDENTIFYING MEMBERS OF THE SOOTY BLOTCH AND FLYSPECK COMPLEX ON APPLES

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ABSTRACT

Since members of the sooty blotch and flyspeck (SBFS) disease complex of apples are difficult to identify using agar-plate isolation and morphological characterization, an RFLP-based technique was investigated as an alternative or supplement to culturing. The method involved amplification of the internal transcribed spacer region of ribosomal DNA using a Capnodiales order-specific reverse primer paired with a universal forward primer, followed by digestion using the *Hae*III restriction enzyme. When applied to isolates of 24 SBFS species, the PCR-RFLP assay produced 14 unique band patterns, all of which were genus-specific. To evaluate its performance *in vivo*, the technique was applied to DNA extracted directly from SBFS colonies on apples that were collected from three Iowa orchards. The order-specific primer prevented amplification of non-Capnodiales fungi, and the technique was robust to a non-target fungus within Capnodiales. The majority (60%) of SBFS colonies were identified to genus by RFLP analysis. The PCR-RFLP assay greatly

streamlined the identification process by circumventing the requirement for culturing, and will be a valuable tool for future ecological studies of the SBFS disease complex.

INTRODUCTION

Sooty blotch and flyspeck (SBFS) is a disease of apples (*Malus* x *domestica* Borkh.) caused by epiphytic fungi that blemish the fruit surface. SBFS appears during the mid- to late growing season in regions with moist climates. Because the dark blemishes characteristic of the disease are unappealing to customers, infested apples are downgraded from fresh market to processing use, decreasing crop value by as much as 90% (4,17).

In the early 1800s, SBFS was attributed to a single fungus, *Dothidea pomigena* Schwein (13). By the 1920s, sooty blotch and flyspeck were determined to have distinct causal agents, *Gleoedes pomigena* (Schwein.) Colby and *Schizothyrium pomi* (Mont. & Fr.) Arx, respectively (5,17). At this time, distinct sooty blotch mycelial types were recognized (5). In the 1990s, Sutton and co-workers determined that sooty blotch was caused by at least three different fungi: *Peltaster fructicola* Johnson, Sutton and Hodges, *Leptodontium elatius* de Hoog, and *Geastrumia polystigmatis* Batista & M.L. Farr (10).

Recent research has revealed a surprisingly high level of diversity within the SBFS disease complex. By coupling morphological description with ribosomal DNA (rDNA) analysis, Batzer et al. (2005) determined that SBFS is caused by at least 30 species of fungi in 11 genera. This study also redefined SBFS mycelial types to include discrete speck, compact speck, punctate, ramose, ridged honeycomb, and fuliginous (3). The number of species in the disease complex continues to increase as SBFS fungi from Europe and the eastern U.S. are surveyed (2,6).

A major reason for the convoluted history and slow progress of SBFS taxonomy is that these fungi are challenging to culture and identify by traditional mycological techniques. Most SBFS fungi grow slowly in culture and are highly sensitive to surface disinfestants, so they are often overgrown by contaminants. Morphology of SBFS fungi in culture often differs radically from that on apples, further complicating identification. In addition, most SBFS fungi sporulate sporadically, or not at all, in culture. These limitations, coupled with the immense diversity of the complex, mean that alternative techniques are needed to facilitate rapid and reliable identification.

Current PCR-based identification methods for SBFS fungi assess variation in the large subunit (LSU) and internal transcribed spacer (ITS) regions of rDNA (3,14). However, species identification by sequence homology demands substantial time and expense. Species-specific primers could aid the identification process, but may be laborious to develop for all the members of the disease complex, and it may be uncertain which primers to use for identification of a particular unknown SBFS colony or isolate.

An alternative approach involves the use of restriction fragment length polymorphism (RFLP) of PCR products to aid in SBFS identification. Sun et al. (2004) amplified the ITS region of isolates of SBFS species using a reverse primer specific to the order Capnodiales (the order including the vast majority of SBFS fungi) paired with a universal forward primer, and then observed RFLP banding patterns potentially useful for identification after digestion with the *Hae*III and *Alu*I restriction enzymes. However, this assay was performed on a limited number of species and used pure cultures. Identification by RFLP analysis of SBFS colonies sampled directly from apples would greatly facilitate ecological studies of the

disease complex by circumventing the need for agar-plate isolation. Several other plant pathogenic fungi have been identified by PCR-RFLP analysis from *in vivo* samples (7,8,11).

The objective of the study was to determine the ability of this PCR-RFLP assay to distinguish members of the SBFS complex in culture and *in vivo*.

MATERIALS AND METHODS

SBFS isolates. SBFS fungi isolated from apples in 2000 from nine orchards in Illinois, Iowa, Missouri, and Wisconsin, and previously identified by morphological characters and rDNA sequence analysis (Table 1), were used in testing of the RFLP technique. The isolates, stored in glycerol at -80° C, were grown on potato dextrose agar (Difco, Detroit, Michigan) at room temperature prior to DNA extraction.

Polymerase chain reaction and restriction enzyme digestion. For PCR assays, DNA of SBFS isolates used for PCR was extracted from 1- to 6-wk-old mycelium using the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, California). The complete ITS region along with a portion of the LSU of rDNA was amplified using the ITS1-F (16) and Myc1-R primers (14). Myc1-R (ACTCGTCGAAGGAGCTACG) is a reverse primer located in the LSU, designed specifically to amplify fungi in the order Capnodiales to which most known SBFS species belong (3). The 50-µl PCR reaction mixture included 31.25 µl of distilled water, 8 µl $MgCl_2$, 5 µl of 10X PCR buffer, 5 µl of 10X dNTPs, 0.25 µl of each primer, 0.25 µl of *Taq* polymerase (Promega, Madison, Wisconsin), and 2 µl of DNA extract. To increase amplification product for some isolates, 5% DMSO was included in the PCR mixture and/or the DNA extract was diluted 10-fold. Cycling conditions for PCR (Model PCT-100, MJ Research Inc., Waltham, Massachusetts) included: hot start at 85°C; initial denaturation at 95°C for 95 s; 35 cycles of denaturation at 95°C for

60 s, annealing at 58°C for 60 s, and extension at 72°C for 60 s; followed by final annealing at 58°C for 60 s and final extension at 72°C for 5 min. Amplification was verified by running 10 µl of the PCR product on a 1x Tris-borate EDTA (TBE) 2% agarose gel (BioRad, Hercules, California), stained with ethidium bromide and visualized with ultraviolet light.

Amplified DNA was digested with the restriction enzyme *Hae*III (Invitrogen, Carlsbad, California). Three units of *Hae*III were added directly to 20 µl of PCR product, which was then incubated at 37° C for 2 h. Digested PCR products were observed on a 1x TBE 2% agarose gel, run for 2 h at 150 volts in 1x TBE buffer, stained with ethidium bromide, and visualized with ultraviolet light. Band size was determined against a 1 kb plus ladder using the band-matching feature of the Quantity One software (BioRad, Hercules, California) coupled with sequence analysis of the rDNA segment amplified. Non-restricted samples were sequenced at the Iowa State University DNA Sequencing and Synthesis Facility after purification of PCR product using a QIAquick DNA Purification Kit (QIAgen, Valencia, California) and quantification of DNA using a Hoefer DyNA Quant 200 Fluorometer (Amersham Pharmacia Biotech, Piscataway, New Jersey). Sequences were edited using Sequence Navigator (Applied Biosystems, Foster City, California). The PCR-RFLP analysis was performed at least twice for all isolates listed in Table 1.

Sampling SBFS colonies on apples. SBFS colonies were collected from apples in fall 2003 from three orchards near Fort Dodge, Gilbert, and Jefferson, Iowa. After excising the SBFS sample with supporting apple epidermis and pressing it between sheets of paper until dry, colonies were classified by mycelial type (including discrete speck, flyspeck, fuliginous, punctate, ramose, and ridged honeycomb) and photographed. Each of the 318 preserved samples appeared to consist of a single SBFS colony. Colony DNA was extracted

by subjecting mycelium scraped from the apple cuticle with a scalpel to the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, California). Conditions for PCR and enzymatic digestion were carried out as described above. Resulting RFLP patterns were compared to the patterns observed from isolates of previously identified SBFS species and also correlated with mycelial type. Samples that resulted in previously unidentified RFLP patterns were sequenced as previously described and subjected to a BLAST search (NCBI, Bethesda, MD). To check accuracy and reliability of resulting RFLP patterns, the ITS products of a sub-sample of the unidentified colonies that had previously identified band patterns were sequenced, edited, and subjected to a BLAST search as described previously.

The DNA of SBFS samples that produced weak or no PCR product with the ITS1-F/Myc1-R primer set was subjected to a second attempt to amplify the ITS region, replacing the Capnodiales-specific primer with a universal reverse primer, ITS4 (16). Other than replacement of the reverse primer, the PCR protocol was performed as described above. The DNA of SBFS samples that did not amplify with either ITS primer set was subjected to another round of PCR in an attempt to amplify the LSU region. The PCR protocol was performed as described above, except that the primer set was LROR/LR5 (15), 5% DMSO was always included, and the annealing temperature was 49°C. Amplified samples were then sequenced and analyzed as described above, and subjected to a BLAST search.

Repeatability of *in vivo* assay. To test repeatability of the technique, we evaluated whether sub-samples from single colonies on apple gave consistent results. Seven SBFS colonies, including six different mycelial types, collected from SBFS infected apples in fall 2004 from three orchards in Iowa and preserved on apple peels, were each divided into four

quadrants. Three of the four quadrants from each colony were individually subjected to the RFLP assay as described above.

RESULTS

The Capnodiales-specific primer set amplified each of the 59 previously identified SBFS isolates included in the study (Table 1). The amplification products ranged from 784 to 834 base pairs based on sequence analysis, except that two of the three *Peltaster* sp. P2.1 isolates and all of the *Peltaster* sp. P2.2 isolates produced amplicons of approximately 1400 base pairs due to a large insert in the ITS1 region.

Digestion of the SBFS isolates with *Hae*III yielded 14 distinct RFLP patterns (Table 1, Figure 1). All of the RFLP patterns were unique to genus, three were unique to species, and one was unique to isolate. More than one RFLP pattern was observed for four of the nine genera tested. Only two band patterns, those of *Dissoconium* sp. FG4 and *Pseudocercospora* spp., appeared to be potentially difficult to visually differentiate. Intraspecific variability was observed for only two species, *Zygophiala jamaicensis* and *Peltaster* sp. P2.1. Each RFLP pattern corresponded to a single SBFS mycelial type, but a mycelial type sometimes corresponded to more than one RFLP pattern.

Validation of RFLP identification method. Of 318 unidentified SBFS colonies sampled from apple peels, 223 (70%) were amplified using the Capnodiales-specific primer and 191 (60% of all colonies, constituting 85% of those that amplified) produced RFLP patterns matching those of previously identified SBFS fungi. Eight of the 14 previously identified SBFS RFLP patterns were observed. For each of the observed SBFS RFLP patterns, the ITS region of two to nine colonies was sequenced and subjected to a BLAST search. For seven of the eight patterns, the sequences aligned with SBFS species as predicted

by RFLP patterns. The only unexpected outcome occurred when the sequences of three SBFS colonies exhibiting the RFLP band pattern of Sterile mycelia sp. RS2 aligned more closely to Sterile mycelia sp. RS1. Direct comparison of the sequences showed that the majority of the sequences matched Sterile mycelia sp. RS1, and that the variation in RFLP pattern was due to the presence of an extra *Hae*III restriction site found in Sterile mycelia sp. RS2 but not in Sterile mycelia sp. RS1 isolates previously observed. This observation showed that while the Sterile mycelia sp. RS1 and RS2 initially appeared to have species-specific RFLP patterns, the patterns were instead unique at the genus level.

Nineteen of the SBFS colonies (5% of all colonies, 9% of the samples that amplified) did not produce RFLP patterns that matched those of SBFS fungi. Of the 19 samples, six produced a fragment of approximately 800 base pairs, and 13 instances had the 800-base pair fragment as well as either one or two smaller fragments (approximately 710 and 620 base pairs). The ITS region of five of those samples were sequenced and aligned with *Cladosporium* sp. (NCBI accession number EF432298.1). When the ITS region of a pure culture of *Cladosporium* sp. (previously isolated from an apple epidermis) was amplified using the ITS1-F/Myc1-R primer set, the amplicon was approximately 800 base pairs. Observation of equivalent fragment sizes before and after digestion indicated that at least a portion of the amplified *Cladosporium* sample remained uncut. The smaller bands range may be due to incomplete digestion or to the presence of some other organism(s) not yet identified. Amplification of *Cladosporium* sp. was not surprising as it belongs to the order Capnodiales.

Seventy-one (37%) of all the SBFS RFLP patterns observed also included fragment patterns of *Cladosporium* sp. as described above, and therefore showed evidence that these

samples including both SBFS species and *Cladosporium* sp. All of the 11 SBFS RFLP patterns observed in the *in vivo* survey were clearly distinguishable from the *Cladosporium* fragments (Figure 2).

Three SBFS colonies produced RFLP band patterns that had not been observed previously. Their sequences most closely aligned with *Pseudocercosporella* sp. 4ra (NCBI accession number DQ363411), *Pseudocercospora* sp. 43.1a (NCBI accession number DQ363419), and unnamed fungal endophyte sp. (NCBI accession number AM262372), all of which have been observed to cause SBFS in regions other than the Midwest (2,6).

One hundred twelve (35%) of the SBFS colonies either did not amplify with the Capnodiales primer or did not produce discernible RFLP patterns. While all 112 samples were subjected to PCR using the general ITS and/or LSU primers, the LSU region of only 20 samples and the ITS region of only nine samples were successfully sequenced and subjected to a BLAST search. The majority of the sequences (including both ITS and LSU regions) matched non-SBFS, non-Capnodiales fungi including *Penicillium* sp. (12 samples), *Athelia* sp. (5 samples), *Tilletiopsis* sp. (4 samples), *Exophiala* sp., *Stropharia* sp., *Acremonium* sp., *Bulleromyces* sp., and *Aureobasidium* sp. The sequences of five samples matched those of known SBFS species *Colletogloeum* sp. FG2.3 (2 samples), Sterile mycelia sp. RS1 (2 samples), and *Xenostigmina* sp, and one sequence matched *Cladosporium* sp.

Repeatability. In each of the seven SBFS colonies assayed, all three of the quadrants sampled from the same colony produced identical RFLP patterns.

DISCUSSION

This report provides strong evidence that the RFLP assay can make identification of SBFS fungi substantially faster, simpler and more reliable. The RFLP assay rapidly and

accurately identified the genera of SBFS fungi found in the Midwest, U.S. The assay also identified 60% of SBFS colonies that were sampled directly from the apple epidermis. As indicated by sequence analysis of a sub-sample of *in vivo* SBFS colonies, the technique was consistently accurate. RFLP patterns corresponded to SBFS morphology and were useful for discriminating difficult to differentiate mycelial types (3). By circumventing agar-plate isolation, this *in vivo* assay reduced the time needed for identification from weeks or months to hours. In addition, the identification rate was increased from less than 35% using agar-plate isolation (3) to at least 60% using the RFLP assay.

Traditional identification methods, including description of growth rate, morphology, and sporulation *in vivo* and *in vitro*, led to misidentification of SBFS for over a decade (1,5). RFLP band patterns are consistent and unaffected by environmental factors. Therefore, the RFLP-based identification method is much simpler, faster, and easier than traditional methods, which require extensive training in recognizing subtle morphological differences.

The RFLP assay offers a valuable first step toward species identification. For the vast majority of SBFS fungi, it is impossible to identify to the genus level (let alone species level) based on morphology *in vivo*, and can be done *in vitro* only when sporulation occurs (a rare event in many species) or if the rDNA of samples is sequenced (3). The initial RFLP assay could be followed up by use of additional restriction enzyme(s) to reveal species-specific banding patterns. For example, some colonies of Sterile mycelia spp. RS1 and RS2 had the same banding patterns after *Hae*III digestion, but were clearly differentiated after digestion with the restriction enzyme *Alu*I (14,Duttweiler, *unpublished data*). Also, the RFLP technique would be an essential first step if coupled with specific primers developed to confirm species identity. Knowing the genus of a SBFS fungus would enable efficient

selection of species-specific primers, and thereby streamline DNA-based species identification.

For identification of SBFS fungi directly from the apple epidermis, the Capnodiales-specific primer proved to be a valuable tool that can avoid the frustrations and failures that often accompany attempts at agar-plate isolation of these fungi. The technique was robust to non-Capnodiales fungi that colonize the apple fruit surface, as observed after sequencing the ITS or LSU regions of the *in vivo* samples that did not amplify with the Capnodiales-specific primer. Of the samples sequenced, 85% belonged to an order other than Capnodiales, indicating that the order-specific primer did not amplify the many non-Capnodiales fungal epiphytes. The remaining 15% of samples were SBFS fungi; however, the Capnodiales primer did not appear to selectively exclude any SBFS species since the sequences matched SBFS fungi with a diverse range of genera and mycelial types.

Although 37% of the samples identified using RFLP analysis exhibited both SBFS and non-SBFS fragment patterns, the assay consistently distinguished SBFS fungi from non-SBFS fungi. Therefore, the RFLP assay is robust to non-target organisms, whereas pure SBFS cultures are required for identification using agar-plate isolation (3,9). Future surveys, however, may find that other non-SBFS fungi in the order Capnodiales are common apple epiphytes. If so, it would be necessary to determine their RFLP patterns in order to exclude them from SBFS surveys.

Three new SBFS RFLP patterns were identified from the 2003 survey. BLAST searches of the ITS sequences of the three samples indicated that each was a previously identified SBFS species that had not yet been assayed using the RFLP technique. This finding indicates that the RFLP assay is likely to be useful for identification of SBFS fungi

not included in the present study. The RFLP library could thus be expanded to include the more than 30 members of the SBFS complex.

As the RFLP library expands to SBFS members in regions other than the Upper Midwest, the banding patterns may become more difficult to differentiate. For example, it may be difficult to distinguish between *Dissoconium* sp. FG4 and *Pseudocercospora* sp. FG1.1 and FG4 banding patterns unless run adjacently in the same gel. It is also possible that additional RFLP patterns of SBFS fungi may resemble those of *Cladosporium* sp. or other non-SBFS epiphytes. Therefore, it may be helpful to investigate use of additional restriction enzymes in order to increase differentiation of RFLP patterns.

The RFLP assay has the potential to minimize the misidentification of SBFS species that has previously impeded research on this pathosystem. The challenge of identifying multiple pathogens that produce the same disease signs is more easily managed using this assay. In phylogenetic studies, RFLP band patterns could be additional distinguishing features for classifying SBFS fungi (8). It could also be used for rapid identification of samples taken directly from field studies and to expedite identification of pure cultures. The RFLP-based identification technique, therefore, can become a valuable tool for improving the ecological understanding of the relatively unexplored SBFS complex.

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Table 1. *Hae*III restriction fragments of amplified ITS region of rDNA and origins of isolates of sooty blotch and flyspeck fungi (3).

Species	Mycelial type	Isolate	State of origin	Fragment sizes (bp)	RFLP type
Zygophiala jamaicensis (FS1)	Flyspeck	GTA2a	Illinois	262, 258, 91, 81	A
		MSTA1a	Wisconsin	259, 205, 91, 82	B
		MSTA2a	Wisconsin	262, 258, 91, 81	A
		UMA7b	Missouri	262, 258, 91, 81	A
Zygophiala sp. FS2	Flyspeck	FVA2a	Iowa	261, 258, 90, 82	A
		MWA6a	Iowa	261, 258, 90, 82	A
		MWA8b	Iowa	261, 258, 90, 82	A
Zygophiala sp. FS3.1	Flyspeck	GTA5c	Illinois	259, 205, 91, 82	B
		GTA8c	Illinois	259, 205, 91, 82	B
		MSTA8a	Wisconsin	259, 205, 91, 82	B
Zygophiala sp. FS3.2	Flyspeck	MWA1a	Iowa	261, 259, 91, 82	A
		MWA1d	Iowa	261, 259, 91, 82	A
Dissoconium sp. DS1.1	Discrete speck	CUB2c	Illinois	552, 91, 86, 80	C
		MSTB5b	Wisconsin	552, 91, 86, 80	C
		MWB7b	Iowa	552, 91, 86, 80	C
Dissoconium sp. DS1.2	Discrete speck	UMB2a	Missouri	552, 91, 86, 80	C
		UMB4b	Missouri	552, 91, 86, 80	C
Dissoconium sp. FG4	Fuliginous	MSTF2	Wisconsin	247, 292, 99	D
		MSTF3b	Wisconsin	247, 292, 99	D
Dissoconium sp. FG5	Fuliginous	UIF3a	Illinois	555, 88	C
Pseudocercospora sp. FG1.1	Fuliginous	MWF7a	Iowa	285, 251, 91, 90	E
		MSTF5b	Wisconsin	285, 251, 91, 90	E
Pseudocercospora sp. FS4	Flyspeck	MWA4b	Iowa	285, 251, 91, 81	E
Pseudocercosporella sp. RH1	Ridged honeycomb	AHD1a	Missouri	357, 193, 90, 80	F
		GTD2a	Illinois	357, 193, 90, 80	F
		MSTD1a	Wisconsin	357, 193, 90, 80	F
		UMD7a	Missouri	357, 193, 90, 80	F
Pseudocercosporella sp. RH2.1	Ridged honeycomb	AHE9a	Missouri	359, 195, 91, 81	F
		GTE5b	Illinois	359, 195, 91, 81	F
		UMD1a	Missouri	359, 195, 91, 81	F
Pseudocercosporella sp. RH2.2	Ridged honeycomb	GTC1a	Illinois	357, 194, 91, 81	F
		GTC4a	Illinois	357, 194, 91, 81	F
		UMD8b	Missouri	357, 194, 91, 81	F
Colletogloeum sp. FG2.1	Fuliginous	AHF3a	Missouri	331, 193, 168, 90	G
		AHF4a	Missouri	331, 193, 168, 90	G
		UMF4a	Missouri	331, 193, 168, 90	G
Colletogloeum sp. FG2.2	Fuliginous	CUF3c	Illinois	329, 193, 168, 91	G
		MWF1a	Iowa	329, 193, 168, 91	G
		UMF2a	Missouri	329, 193, 168, 91	G
Colletogloeum sp. FG2.3	Fuliginous	CUF2d	Illinois	329, 193, 168, 90	G
		UIF1	Illinois	329, 193, 168, 90	G
Peltaster fructicola (P1)	Punctate	GTE1a	Illinois	713, 72	H
		MSTE10b	Wisconsin	713, 72	H
		UME4a	Missouri	713, 72	H
Peltaster sp. P2.1	Punctate	GTE5a	Illinois	367, 261, 163	I
		GTE6d	Illinois	367, 258, 228, 179, 146	J
		GTE6a	Illinois	367, 258, 228, 179, 146	J
		AHE5d	Missouri	367, 258, 228, 179, 146	J
Peltaster sp. P2.2	Punctate	CUE2b	Illinois	367, 258, 228, 179, 146	J
Xenostigmina sp. P3	Punctate	AHE3a	Missouri	282, 173, 146	K
		AHE7a	Missouri	282, 173, 146	K
Xenostigmina sp. P4	Punctate	UIE3a	Illinois	282, 173, 145	K
Sterile mycelia sp. RS1	Ramose	PEC6a	Iowa	281, 174, 146, 91	L
		UMC4	Missouri	281, 174, 146, 91	L
Sterile mycelia sp. RS2	Ramose	AHC1a	Missouri	280, 145, 92, 90	M
		AHC4b	Missouri	280, 145, 92, 90	M
		UMC2	Missouri	280, 145, 92, 90	M
		UMC7a	Missouri	280, 145, 92, 90	M
Ramularia sp. P5	Punctate	UME2a	Missouri	417, 249, 83	N

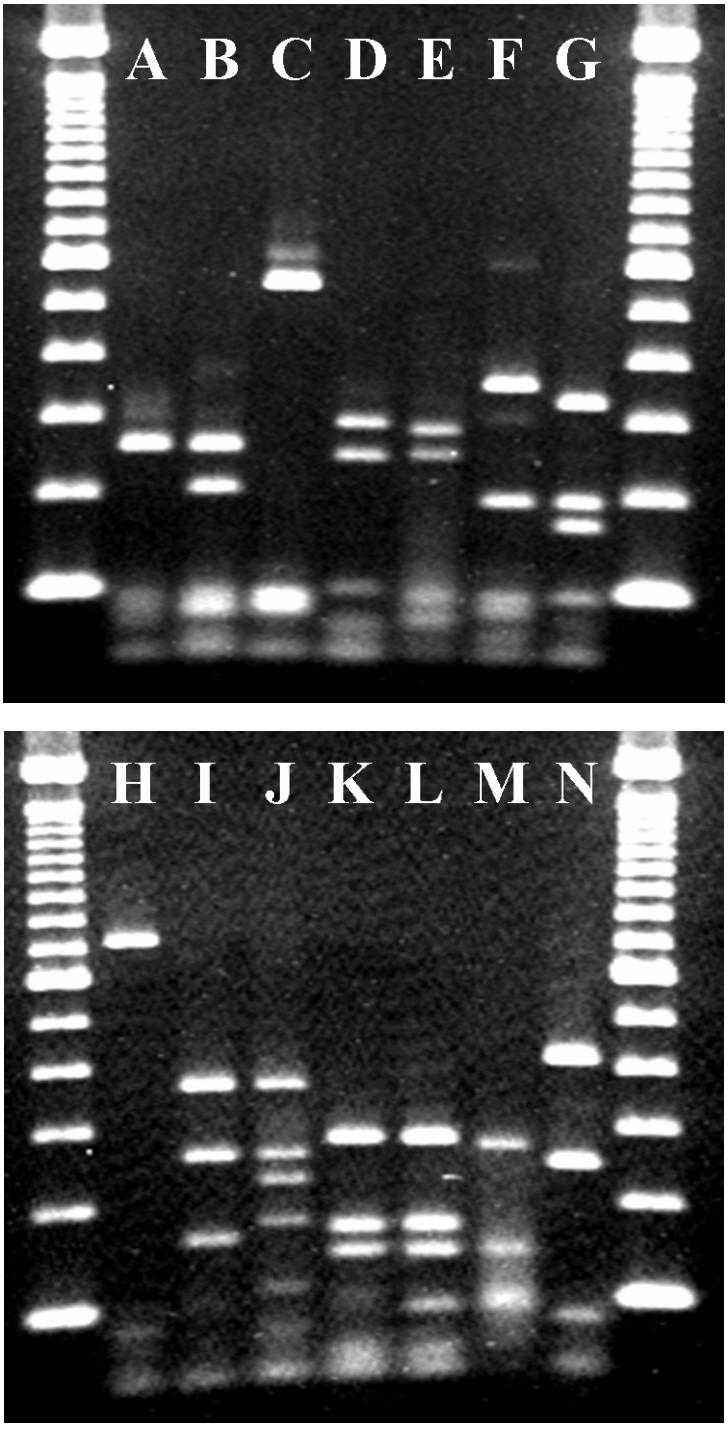

Figure 1. The 14 distinct RFLP patterns from SBFS fungi. Lanes are labeled with letters that correspond to RFLP type (Table 1). Unmarked lanes are 100 base pair ladders.

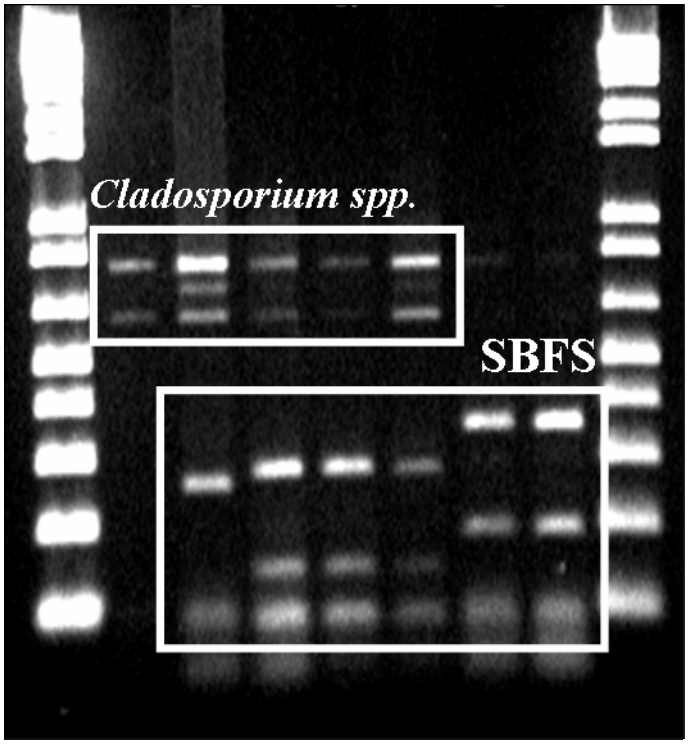

Figure 2. Examples of *in vivo* samples including RFLP patterns of both SBFS fungi and *Cladosporium* sp. ran with 1 kb plus ladders.

CHAPTER 3. ADAPTATION OF A SOOTY BLOTCH AND FLYSPECK WARNING SYSTEM FOR THE UPPER MIDWEST

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ABSTRACT

A disease-warning system for sooty blotch and flyspeck (SBFS) of apple, developed in the Southeast U.S., uses cumulative hours of leaf wetness duration (LWD) to predict the timing of the first appearance of signs. In the Upper Midwest U.S., however, trials of this warning system have resulted in sporadic control failures. To determine whether the warning system's algorithm should be modified to provide more reliable assessment of SBFS risk, hourly LWD, rainfall, relative humidity (RH), and temperature data were collected from orchards in IA, WI and NC. Timing of the first appearance of SBFS was determined by scouting weekly for disease signs. Preliminary analysis using scatterplots and boxplots suggested that cumulative hours of RH≥97% could be a useful predictor of SBFS appearance. Receiver operating characteristic curve analysis was used to compare the

predictive performance of cumulative LWD and cumulative hours of RH≥97%. Cumulative hours of RH≥97% was a more conservative and accurate predictor than cumulative LWD for sites in the Upper Midwest but not for sites in North Carolina. Performance of the SBFS warning system in the Upper Midwest may be improved if cumulative hours of RH≥97% is substituted for cumulative LWD to predict the first appearance of SBFS.

INTRODUCTION

Sooty blotch and flyspeck (SBFS) is a disease of apple (*Malus* x *domestica* Borkh.) caused by more than 30 species of fungi in humid production regions worldwide (2,10). Apples infested with SBFS develop dark blemishes that prevent their fresh market sale, resulting in revenue loss as high as 90% (5,31). Colonies of SBFS fungi begin to appear during the mid- to late growing season, at least partly in response to weather conditions. SBFS incidence and severity are greater, and signs appear earlier, in wetter than average growing seasons (31). The conventional calendar-based fungicide spray program, consisting of fungicide applications every 10 to 14 days from fruit appearance until harvest (11), does not consider the impact of environmental conditions on disease risk, and therefore may sometimes result in over-application of fungicides (6).

A SBFS warning system, the Sutton-Hartman warning system, was developed to help growers control the disease in a more cost-effective and environmentally safe manner. Developed in North Carolina and Kentucky, the Sutton-Hartman warning system predicts timing of the first appearance of SBFS based on accumulated hours of leaf wetness duration (LWD) (6). The warning system is designed to delay the second-cover fungicide spray until

175 h of LWD have accumulated since the first-cover fungicide spray (applied 7 to 10 days after petal fall) (14).

In field experiments and on-farm trials, the warning system saved an average of two fungicide sprays compared to the conventional schedule (1,26). However, field trials in the Upper Midwest (IA, IL and WI) resulted in sporadic control failures; i.e., significantly higher incidence of SBFS in orchard blocks in which the warning system was used than in blocks in which the conventional schedule was used (1). Growers are unlikely to adopt the warning system if there is a substantial risk of increased disease, since SBFS damage to a crop can cost far more than the savings associated with reduced fungicide spraying. Analysis of the control failures revealed no consistent association with risk factors such as inadequate pruning, low-volume spray applications, or delayed response to the warning system's spray advisories (9,31).

Another possible cause for these failures could be climatic differences between the Southeast U.S., where the system was developed, and the Upper Midwest. When warning systems developed in one climatic region are imported to other regions, adaptation of the system's algorithms to better fit the climate of the new region is often needed (19,23,25). The objectives of this study were to determine the ability of weather variables to predict timing of the appearance of SBFS in the Upper Midwest and to investigate if modification of the existing LWD-based warning system could increase its reliability in this region.

MATERIALS AND METHODS

Locations. Sites for weather and disease monitoring were located in apple orchards with a history of SBFS in Iowa, North Carolina, and Wisconsin during the 2005 and 2006 growing seasons (Table 1). Cultural management, block age, geographic features, and

vegetative surroundings varied among orchard sites. Study plots, consisting of three to five trees in a single row in each orchard, were primarily cv. Golden Delicious, but some plots included other light-colored cultivars including Goldrush, Jonathan, and Jonee. Fungicides without activity against SBFS were used to control springtime diseases (primarily apple scab and cedar-apple rust), and additional fungicide applications were withheld after the scheduled first-cover spray (approximately 10 days after petal fall).

Data collection. Air temperature, relative humidity (RH), rainfall, and leaf wetness duration (LWD) were recorded hourly with WatchDog Plant Disease Weather Stations (Spectrum Technologies, Plainfield, IL). Leaf wetness sensors were placed at approximately 1.5 m height under the tree canopy facing north, with an inclination angle of 45° to horizontal. For six of the site years, weather data were collected from the date of scheduled first-cover fungicide spray until the first appearance of SBFS. For the remaining 13 site years, weather data collection extended from the date of the first-cover spray until 1 to 6 weeks after SBFS appeared.

Missing temperature, RH, or rainfall data due to sensor failure was estimated from nearby weather stations. In North Carolina, missing LWD data was estimated with deWit leaf wetness meters (Instrumentenfabrick IFG deWit, Rhoden, The Netherlands) located within the same orchards as the study plots. In IA and WI, missing LWD data was estimated using an algorithm based on dew point deficit (12). Briefly, dew point deficit estimates a wetness event based on the difference between ambient temperature and the temperatures at dew onset and dew dryoff (12). Leaf wetness duration measurements were used to empirically fit average temperature thresholds that corresponded to dew onset and dryoff in apple orchards. The most accurate correlations were obtained with thresholds of 1.0º C for

dew onset and 2.7º C for dew dryoff. Therefore, hours when the dew point deficit was ≤ 1.0º C were considered wet, and subsequent hours were considered wet until the dew point deficit was ≥ 2.7 º C. These thresholds were used to estimate and replace missing LWD data. In total, approximately 12% of the wetness data was estimated rather than measured with Spectrum sensors over the 19 site-years.

Observations of disease. The date of first appearance of SBFS was determined by scouting weekly for SBFS signs. Scouting involved visually noting the presence or absence of SBFS colonies on approximately 40 apples arbitrarily chosen from three trees in the monitoring plots at each study site.

Scatterplot and boxplot analysis. Scatterplots were used to assess relationships between weather variables and the time of appearance of first SBFS signs. Weather variables tested included maximum, minimum and average daily temperature, cumulative degree days (minimum and maximum threshold of 15° C and 30° C, respectively), rainfall frequency and amount, cumulative hours of LWD and of high RH (thresholds of 80, 85, 87, 90, 95, and 97%), and combinations of rainfall, LWD, and RH. Cumulative LWD excluded periods of less than 4 h, which reflects the minimum wet period required for germination and/or mycelial growth of some SBFS fungi (6). At least two consecutive dry hours were required to end a LWD period (4), whereas single hours that were measured as dry but occurred between at least three consecutive wet hours were scored as wet. Hours of high RH were accumulated using the same decision rules as for LWD, except that the hours accumulated were those greater than a threshold RH value. The number of days from application of the first-cover fungicide spray until the first appearance of SBFS was graphed as a function of weather variables. Weather variables exhibiting clustering on scatterplots were identified as

potential predictors. An ideal predictor would appear as a vertical line, indicating that the risk of SBFS appearance was related to a single value of the variable that could be used subsequently as a threshold to trigger a fungicide spray recommendation.

In order to be a useful predictor, an algorithm must be able to warn growers when the appearance of first signs of SBFS is imminent. Therefore, weather inputs must be able to predict the risk of SBFS for each day of the growing season. Accordingly, each day of each orchard year was assigned to one of two categories depending on whether SBFS was present or absent. Boxplots were generated for weather variables with predictive potential as indicated by scatterplots, and used to investigate the degree of separation between the two categories for each weather variable.

Receiver operating characteristic curve analysis. Predictive ability of weather variables was compared using receiver operating characteristic (ROC) curve analysis. ROC curve analysis is a statistic method that evaluates and compares the performance of diagnostic tests (17,29). The accuracy of a predictor is based on the four possible test outcomes: true positive (test predicts disease and disease is actually present); true negative (test predicts no disease and disease is actually absent); false positive (test predicts disease and disease is actually absent); and false negative (test predicts no disease and disease is actually present). A ROC curve graphs the true positive rate as a function of the false positive rate at all possible decision thresholds of the predictor variable (32). Area under the ROC curve (AUROC) is used to compare the performance of different predictors; the accuracy of a predictor increases as the value of AUROC approaches one (29,30).

In this study, in order to provide sufficient time for a grower to apply fungicides and to account for variability in the exact date of SBFS appearance, weather variables were

evaluated for their ability to predict 7 days before first appearance of SBFS signs. Therefore, ROC curve analysis was performed to indicate how accurately a weather variable classified days as occurring either before or after the threshold of 7 days prior to first recorded appearance of SBFS signs. ROC curves plotted the proportion of days correctly classified to occur after the threshold (true positives) against the proportion of days incorrectly classified to occur after the threshold (false positives) for all values of the weather variable. AUROCs were used to compare the predictive performance of weather variables. ROC analysis was performed using SAS (SAS Institute, Cary, NC) and Excel (Microsoft, Redmond, WA). To investigate differences due to geographic region, the NC datasets were analyzed independently from the IA and WI datasets.

Since the cumulative variables used to develop the models were highly interdependent, bootstrapping was used to determine p-values for comparing ROC areas. Bootstrapping was performed 10,000 times with resampling and a paired t-test was used to calculate the p-value from the simulation. Bootstrapping of ROC curves and paired t-tests were performed in R (R Foundation for Statistical Computing, Vienna, Austria).

Predictor threshold. An action threshold value was determined for the weather variables that had predictive potential. Thresholds that minimized false positives were considered to be less conservative, whereas those that minimized false negatives were considered to be more conservative. It was determined that the threshold must be conservative since the cost of false-negative outcomes (loss of crop value for fresh market sale) can greatly exceed the cost of false-positive outcomes (increased fungicide sprays). The threshold in each site-year was determined by minimizing false positives, while allowing relatively few false negatives. It was decided that when determining the action threshold, the

incidence of false negatives would be allowed to occur in only one site year for Upper Midwest orchards.

Predictor evaluation. Performance of predictors was evaluated by comparing the accuracy and percentages of true positives, true negatives, false positives, and false negatives. Performance of predictors for each site year was also evaluated. The difference was calculated between the observed threshold day (7 days before first appearance of SBFS signs) and the threshold day predicted by weather variables. Optimal predictions were defined to occur within 1 wk before the observed threshold day. Predictions prior to 1 wk before the actual threshold were scored as false positives, whereas those occurring later were scored as false negatives. The number of extra fungicide sprays entailed by each false positive outcome was estimated for each orchard year, under the assumption that a conventional calendar-based spray program would specify making applications every 2 wk after the first-cover spray (10). As defined, optimal predictions never led to excessive fungicide sprays.

RESULTS

For the Upper Midwest (Iowa and Wisconsin), scatterplots indicated that cumulative hours of high RH had the greatest potential as a predictor of the timing of first appearance of SBFS signs (Figure 1). Scatterplots also indicated that LWD had greater potential as a predictor than any of the rainfall and temperature variables, but less potential than RH. The greatest clustering of independent values was observed when a threshold of RH≥97% was used. Boxplots also indicated that cumulative hours of RH≥97% resulted in a greater degree of separation of non-SBFS and SBFS groups than LWD (Figure 2). Therefore, cumulative

hours of RH≥97% was determined to have the greatest prediction potential for the Upper Midwest.

The AUROC was significantly greater ($p < 0.0001$) for cumulative hours of RH≥97% (0.98) than LWD (0.93) (Figure 3). An action threshold of 192 h of RH≥97% was determined to predict 7 days before the time SBFS signs first appeared.

Table 2 and Figure 4 compare the predictive performance of LWD and hours of RH≥97%. Accuracy, defined as the total number of true positives and true negatives divided by the total number of outcomes, was greater and fewer false negatives and false positives were observed for cumulative hours of RH≥97% than LWD. Among the 15 Upper Midwest site years, there were three instances of false negatives using LWD compared to one using cumulative hours of RH≥97%. Optimal predictions (defined as occurring within 1 wk before the threshold of 7 days prior to appearance of SBFS signs) occurred in two and five Upper Midwest orchards for LWD and RH≥97%, respectively. Estimated number of extra fungicide sprays per Upper Midwest orchard averaged one fewer for cumulative hours of RH≥97% than LWD.

When analyses were applied to all 19 site-years, rather than only the 15 Upper Midwest site years, differences in predictive potential among the weather variables were reduced. Boxplots were more difficult to differentiate (Figure 2), and there was no significant difference ($p = 0.5868$) between the AUROCs of cumulative LWD (0.93) and cumulative hours of RH≥97% (0.92) (Figure 3). While the overall prediction accuracy of the weather variables differed by only one percent, cumulative hours of RH≥97% appeared to be a less conservative predictor (with higher incidence of false negatives and fewer incidence of false positives) than LWD (Table 2).

DISCUSSION

The results provide evidence that the Sutton-Hartman warning system may need to be changed fundamentally for adaptation to the Upper Midwest U.S. In Iowa and Wisconsin, hourly accumulation of RH≥97% was both a more accurate and conservative predictor than LWD for the timing of the first appearance of SBFS on apples.

There is a biometeorological rationale for the differential performance of the LWD-based warning system in the Upper Midwest compared to the Southeast U.S., where the Sutton-Hartman system was developed. In the Midwest, substantial spatial heterogeneity of LWD measurements in apple canopies has been documented (3,24). Leaf wetness duration measurements varied significantly even among sensors placed at 1.5 m height within the same canopy (3). In Iowa orchards of mature semi-dwarf trees, this spatial variability was much greater for days when dew was the sole source of wetness than on days when rainfall occurred (4). Approximately two-thirds of wet hours are caused by dew in the Upper Midwest (18). In the apple-growing region of western NC, in contrast, approximately 70% of wet hours are associated with rainfall (Sutton and Duttweiler, *unpublished data*). Therefore, spatial heterogeneity of within-canopy LWD measurements, such as those used as inputs to the Sutton-Hartman warning system, is likely to be much more pronounced in the Upper Midwest than in the Southeast.

In order to accurately measure LWD in apple orchards or blocks, it has been recommended to place several wetness sensors within the canopy (16). However, this is frequently impractical for growers due to cost and labor limitations. Therefore, LWD of an orchard or block is commonly measured by a single sensor, which can lead to substantial over- or underestimation of LWD in the Upper Midwest (3). In contrast, RH within the

canopy of an orchard can be measured confidently with a single sensor, since it is far less subject to within-canopy variability than LWD (16). Unlike LWD, which is a measurement of liquid water, RH is a measurement of water vapor. The physical nature of water in the form of vapor is less influenced by leaf area, plant architecture, arrangement of plants in the field, and crop height than LWD. The continuous movement of air homogenizes the gaseous water throughout an apple orchard, thereby leading to similar measurements of RH regardless of sensor placement (27). Additional challenges of LWD measurement include the lack of an accepted measurement standard and the fact that results that can vary considerably depending on sensor type, deployment angle, compass orientation, and whether sensors are painted (13,16,20).

Twelve of 28 previous field trials with the Sutton-Hartman warning system in commercial orchards in the Upper Midwest (Iowa, Illinois, and Wisconsin) resulted in higher incidence of SBFS than observed when using the conventional system of biweekly fungicide sprays (1). Similarly, in two site-years in Upper Midwest orchards during the present study, the first signs of SBFS appeared before LWD had reached the 175-h threshold. These observations suggest that the control failures in the Upper Midwest may have resulted from inability of LWD to provide a consistently reliable action threshold for disease suppression.

Empirically derived disease-warning systems are often optimized to the region where they were developed, and therefore may not be appropriate for adoption in other regions. For example, three imported fire blight models (MARYBLYT 4.3 and Cougarblight 98C developed in the U.S., and BIS95 developed in England) performed poorly when tested in Israel compared to a locally developed model (25). A hypothesized reason for the failures

was that the imported models were developed in moist regions where temperature was a limiting factor for fire blight development, whereas moisture was the limiting factor in Israel.

Brown and Sutton (1995) suggested that temperature might need to be considered if the LWD-based SBFS warning system were to be applied successfully in cooler climates such as the Midwest and Northeast. In Massachusetts, maturation of flyspeck fruiting bodies was shown to be related to degree-days (9). In contrast, temperatures in our study fell within the optimum range for mycelial growth of several common SBFS species throughout the growing season in Iowa and Wisconsin (15). Moisture, although measured as RH rather than as LWD, still appeared to be the limiting factor for appearance of SBFS signs in the Upper Midwest.

Regional variation of SBFS species may also influence performance of weather variables as predictors of disease. Unlike the majority of warning systems, which target a single pathogen species (7,19,23), the SBFS warning system encompasses a disease complex including many fungi. The SBFS species that dominate in the Southeast U.S. are different from those that dominate in the Upper Midwest (2,10). Laboratory studies have shown that optimal temperature, RH, nutritional requirements, and fungicide sensitivity can vary substantially among SBFS species (15,21,28,31). Therefore, the ability of weather variables to predict disease could vary regionally with differences in the predominant SBFS species.

The ROC curve technique proved to be a valuable method for assessing performance of disease warning systems. The technique provides an easy method to quantify both false positive and false negative errors that can be used by pathologists, IPM specialists, and growers to evaluate the tradeoffs associated with a warning system and its threshold. Since economic consequences of each type of error are likely to differ substantially, the economic

risks associated with warning systems can be assessed readily (29). ROC curve analysis is particularly useful for assessing the risk of binary events such as the absence or presence of disease signs, which is often inappropriate for commonly used modeling approaches such as regression (22).

By re-evaluating the underlying mechanism of the existing SBFS warning system, the present study provided insight into the observed failures in implementing the system in the Upper Midwest. The results identified cumulative hours of RH≥97% as a preferable variable for the warning system in the Upper Midwest and further validated the use of LWD in the Southeast. However, before the SBFS warning system based on cumulative hours of RH≥97% can be recommended for grower use in the Upper Midwest, it must be validated in field trials over numerous sites and years.

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Table 1. Location of orchards, apple cultivar(s) and growing season year(s) for which data were collected.

State	City	Coordinates	Cultivar(s)	Year(s)
IA	Adel	41°34'N 94°05'W	Goldrush	2005, 2006
IA	Cambridge	41°52'N 93°28'W	Golden Delicious	2005, 2006
IA	Fort Dodge	42°33'N 94°11'W	Golden Delicious	2005, 2006
IA	Gilbert	42°06'N 93°35'W	Golden Delicious, Jonathan	2006
IA	Iowa Falls	42°31'N 93°12'W	Golden Delicious	2005, 2006
IA	Jefferson	41°59'N 94°24'W	Golden Delicious	2005
IA	Nevada	41°55'N 93°27'W	Golden Delicious	2005, 2006
WI	Fitchburg	42°57'N 89°28'W	Jonee	2006
WI	Madison	43°04'N 89°24'W	Golden Delicious	2006
WI	Oakwood	43°20'N 90°23'W	Golden Delicious	2005
NC	Clayton	35°39'N 78°27'W	Golden Delicious	2005, 2006
NC	Fletcher	35°25'N 82°30'W	Golden Delicious	2005, 2006

Table 2. Performance of cumulative hours of LWD and RH≥97% as predictors of a threshold occurring 7 days before first SBFS signs appear, evaluated separately for all 19 site years (NC, IA and WI) and the 15 site years in Upper Midwest (IA and WI) orchards. Each day in an orchard year represents an observation; there were a total of 1,877 days for all 19 site years and 1,531 days for the 15 Upper Midwest site years.

	Predictor performance[a]			
	All site years		**IA and WI site years**	
	LWD	**RH≥97%**	**LWD**	**RH≥97%**
Accuracy[b]	81	82	81	87
True positives	97	84	96	98
True negatives	76	82	76	83
False positives	24	18	24	17
False negatives	3	16	4	2

[a] Performance of predictors is calculated as percent of observations.
[b] Accuracy = total of true positives and true negatives divided by total number of observations.

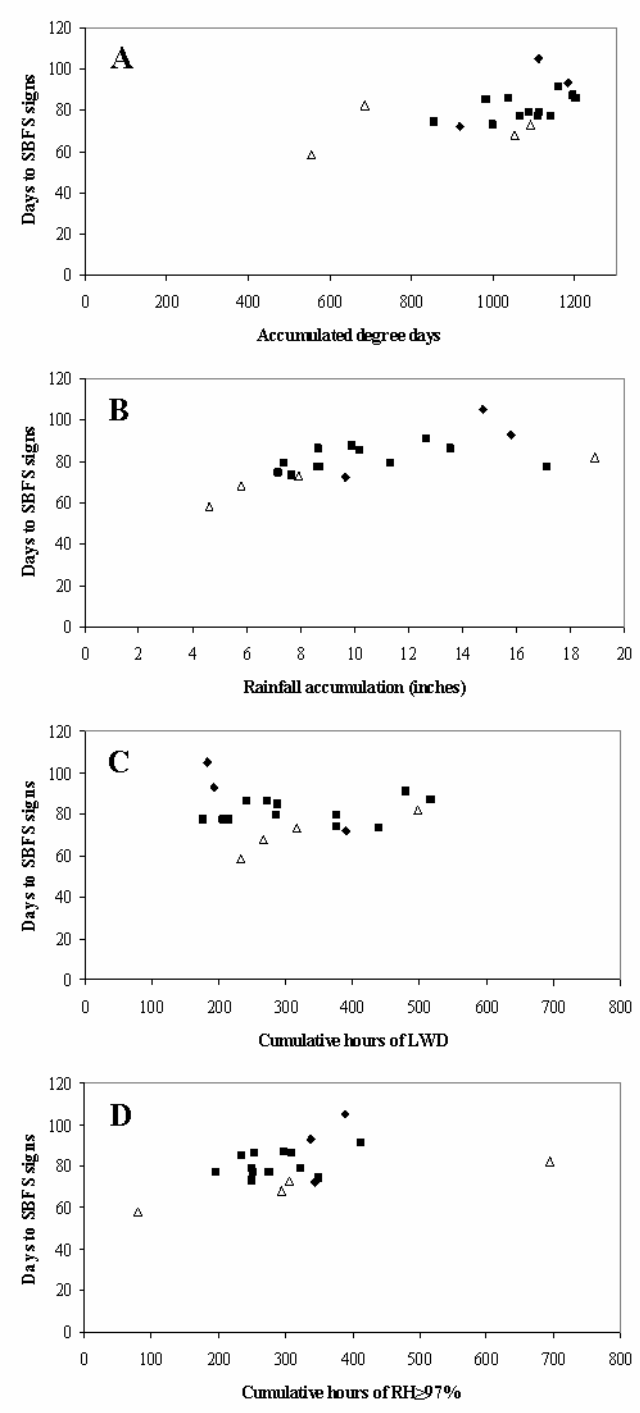

Figure 1. Scatterplots of time (days) from the first-cover fungicide spray until SBFS signs first appeared, as a function of temperature (A), rainfall (B), leaf wetness duration (C), and relative humidity (D). The symbol ■ represent orchards in Iowa, ◆ represent orchards in Wisconsin, and △ represent orchards in North Carolina. The degree of lateral clustering was assumed to be proportional to the predictive potential of weather variables.

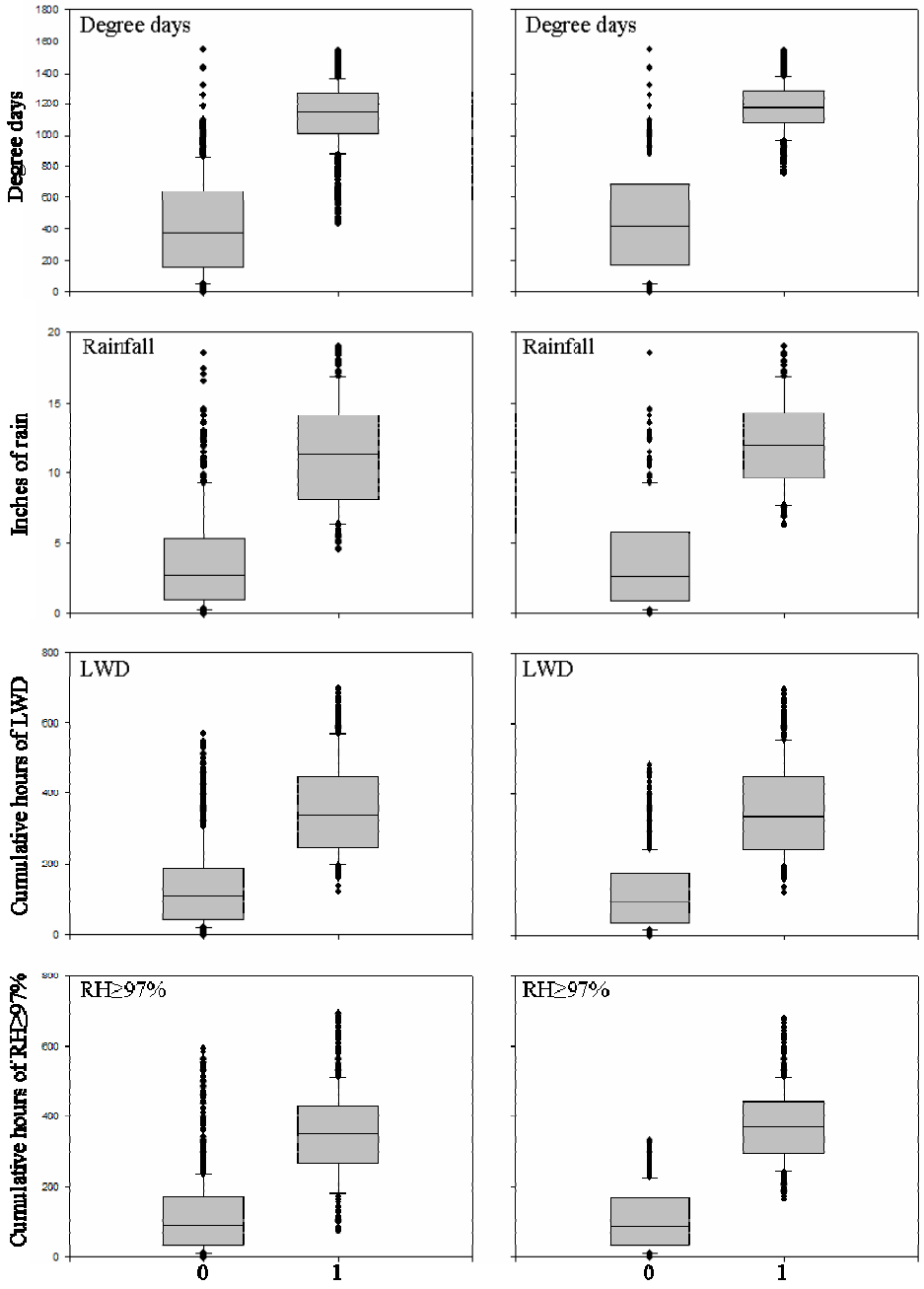

Figure 2. Boxplots of four weather variables tested as potential predictors of the timing of appearance of SBFS signs. Boxplots on the right include all 19 sites years (including four from NC), whereas boxplots on the left include only the 15 Upper Midwest site years. Each box represents the distribution of weather variables on days before (0) and after (1) SBFS appeared. The extent of vertical separation of the two shaded boxes was assumed to be proportional to the potential of the weather variable to differentiate between no-risk and risk periods for SBFS.

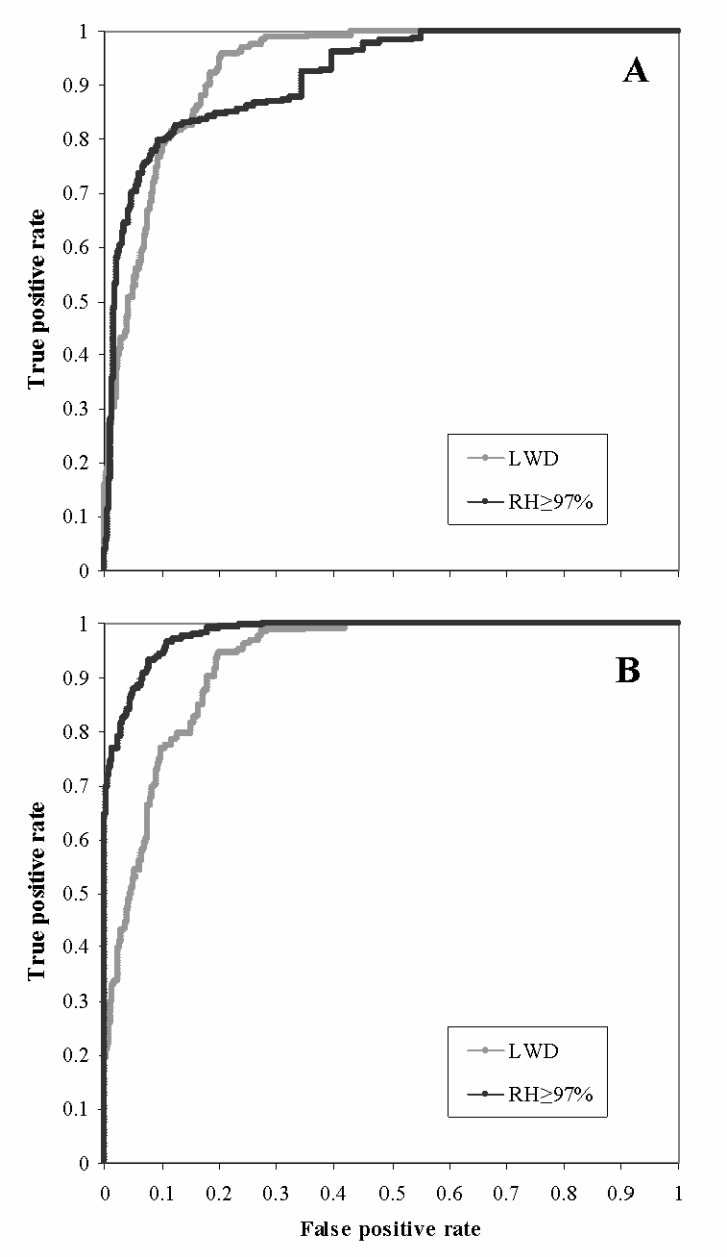

Figure 3. Receiver operating characteristic (ROC) curves of cumulative hours of LWD and RH≥97% as predictors of the first appearance of SBFS. Graph A. represents the ROC curve of all 19 orchard site years; the area under the ROC curve (AUROC) for cumulative hours of LWD and RH≥97% are 0.93 and 0.92 respectively. Graph B. represents the ROC curve for the 15 Upper Midwest site years; the AUROC for cumulative hours of LWD and RH≥97% are 0.93 and 0.98 respectively.

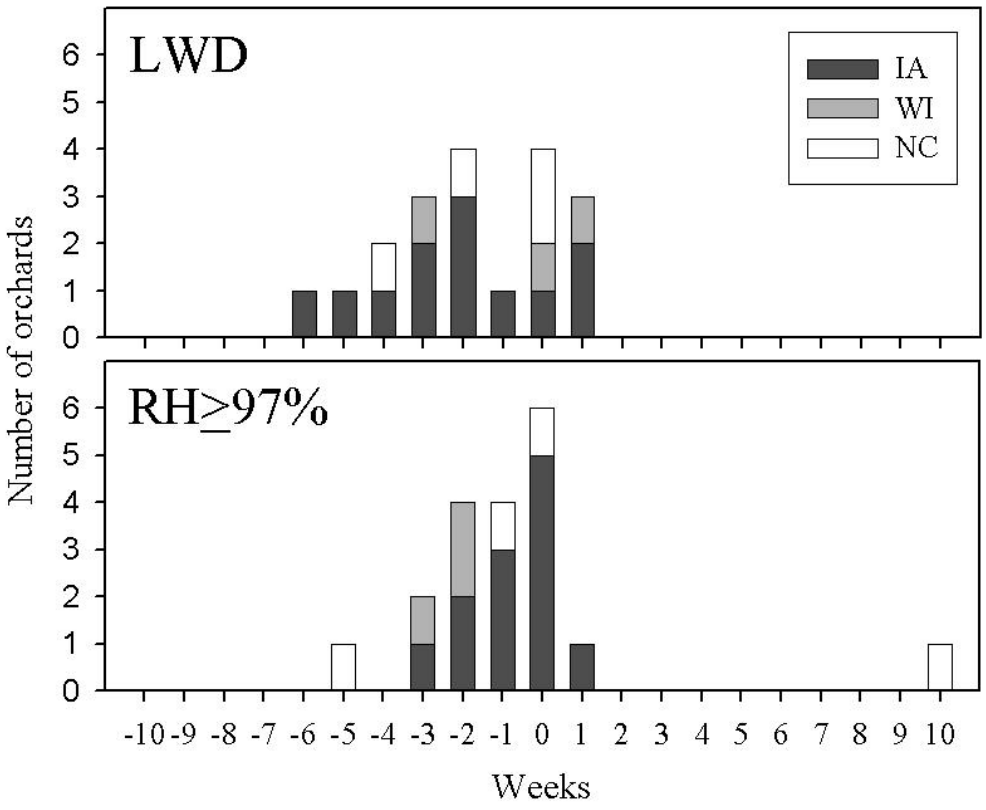

Figure 4. Difference between the actual SBFS threshold day (7 days before the first appearance of SBFS) and the threshold day predicted by cumulative hours of LWD and RH≥97% for each site year. Week 0 represents ideal predictions (occurring within 1 wk before the actual threshold). Negative values represent false positives, whereas positive values represent false negatives.

CHAPTER 4. GENERAL CONCLUSIONS

Sooty blotch and flyspeck (SBFS) is an important disease impacting apples in all humid growing regions of the world. SBFS signs, comprised of fungi growing epiphytically on the fruit surface, significantly decrease crop value because blemished apples are unacceptable for fresh market sale. The objective of the presented research was to develop techniques to help improve the identification and management of SBFS of apple.

Little is known about the SBFS pathogens as it was only recently discovered that the disease complex includes more than 30 species world wide. One explanation for the limited knowledge of the disease is that few researchers have surmounted the challenges of culturing SBFS fungi. The present research presents an alternative identification method based on molecular techniques rather than agar-plate isolation. The method, which involves amplification of the ITS region utilizing a Capnodiales-specific primer and subsequent digestion with the *Hae*III restriction enzyme, correctly identified to genus all 24 species tested from pure culture. SBFS colonies taken directly from the apple surface were also identified accurately and reliably to genus using the same technique. Identification using the PCR-RFLP assay takes hours compared to the weeks or months needed for agar-plate culturing, and does not require the expertise of an experienced mycologist. To complete the SBFS RFLP library, the banding patterns of all the members of the disease complex should be determined. Further development should involve applying similar techniques, such as digestion with other restriction enzymes or development of species-specific primers, in order to increase specificity of the method.

The Sutton-Hartman warning system was designed to gauge the risk of appearance of SBFS signs based on cumulative leaf wetness duration (LWD). However, this system has

experienced control failures when applied in the Upper Midwest. It has been suggested that the Sutton-Hartman warning system may require modification to account for regional climatic differences between the Southeast and Upper Midwest. By evaluating the potential of four weather variables (including temperature, RH, rainfall, and LWD) to predict the timing of the first appearance of SBFS, it was determined that cumulative hours of RH≥97% was a more accurate and conservative predictor than cumulative hours of LWD in the Upper Midwest. These results indicate that the warning system performance in the Upper Midwest may be improved if cumulative hours of RH≥97% was substituted for LWD. Field trials throughout the Upper Midwest over several years must be conducted to validate the SBFS warning system based on cumulative hours of RH≥97%.

This thesis has presented two tools with the potential to help improve the understanding of and management of the SBFS disease complex. Identification using the PCR-RFLP assay will expedite ecological research both *in vitro* and *in vivo*. Adaptation of the disease warning system to the location of implementation will ensure growers are able to efficiently and effectively prevent SBFS.

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