Identification and Epidemiological Features of Important Fungal Species Causing Sooty Blotch on Apples in the Northeastern United States

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Identification and Epidemiological Features of Important Fungal Species Causing Sooty Blotch on Apples in the Northeastern United States

A Dissertation Presented

by

ANGELA M. MADEIRAS

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

FEBRUARY 2014

Plant and Soil Science
Identification and Epidemiological Features of Important Fungal Species Causing Sooty Blotch on Apples in the Northeastern United States

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ACKNOWLEDGMENTS

I would like to give special thanks to my advisor, Dr. Daniel Cooley, for his wisdom, thoughtful guidance, and all the good coffee. Thanks also to Drs. Robert Wick, Frank Caruso, David Rosenberger, and Kristen DeAngelis for serving on my committee, and for their excellent advice in the preparation of this dissertation. Thanks to Drs. Jon Hulvey and Nick Brazee, who gave me good advice on PCR, sequence editing, and the construction of phylogenetic trees. I am grateful to Dr. Geunhwa Jung and Jay Popko for their assistance with fungicide data analysis. Undergrad assistants Rina Villar and Katie Stultz were a great help to me in the lab. Arthur Tuttle and Brook Frye were wonderful companions in field work. Jon Clements and Jim Krupa provided help in the field and advice about fungicides. Thanks to Heather Faubert and Cheryl Smith for sending me blotchy apples. Thanks to Jean Batzer and Mark and Winnie Gleason for opening their homes to me during my visit to Iowa State, and to everyone in the Gleason Lab for their hospitality. I thought often of the late Dr. Thomas Boyle as I worked on this dissertation, and I am grateful for all that he taught me. Thanks finally to my husband Matt for sticking with me these 14 years. Now it’s his turn to go to grad school.
The sooty blotch and flyspeck (SBFS) complex causes blemishes on apples in humid, temperate growing regions worldwide. In contrast to flyspeck etiology, the many species of fungi causing sooty blotch (SB) have not been well studied. The first set of objectives in this study was to use PCR to identify SB species isolated from apples and selected reservoir hosts in the northeastern United States, and to identify patterns of species distribution on hosts and among sites. Results indicated that *Geastrumia polystigmatis* was the predominant species on apples, whereas *Peltaster* species were more common on reservoir hosts. Species distribution varied among sites. Phylogenetic analysis of 54 *G. polystigmatis* isolates revealed little genetic variability in the ITS region. The second set of objectives involved investigating the response of *G. polystigmatis* to changes in nutrition, temperature, heat stress, and relative humidity, and *in vitro* responses of *G. polystigmatis* and *Peltaster fructicola* to fungicides commonly used in orchards. Observation of growth on half-strength potato dextrose agar, malt extract agar, and 2% water agar revealed that mycelial growth of *G. polystigmatis* was thicker and
more melanized in the presence of readily available carbohydrates. Temperature range experiments demonstrated that the optimum temperature for growth was approximately 24°C. The fungus was able to survive exposure to 32°C for at least one week, 37°C for at least 48 hours, and 42°C for at least 8 hours. Growth was optimum at 99-100% relative humidity. Isolates of *P. fructicola* were very sensitive to thiophanate-methyl, mancozeb, cyprodinil, penthiopyrad, fenbuconazole, and trifloxystrobin. Isolates of *G. polystigmatis* were sensitive to thiophanate-methyl and cyprodinil, but significantly less sensitive to all other fungicides than *P. fructicola*. The addition of salicylhydroxamic acid to trifloxystrobin significantly reduced growth of *P. fructicola*, but not that of *G. polystigmatis*. This study represents the first in-depth investigation into the identity of species causing SB in the Northeast, the basic biology of *G. polystigmatis*, and the fungicide sensitivities of *G. polystigmatis* and *P. fructicola*.
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CHAPTER 1

REVIEW OF THE LITERATURE

Introduction

The sooty blotch and flyspeck complex (SBFS) causes dark blemishes on the surface of apple fruit. These blemishes appear in one of two general forms: round, black spots with no mycelium between them are referred to as flyspeck, and colonies consisting mainly of dark mycelium with or without spots are referred to as sooty blotch. SBFS is common in temperate, humid apple growing regions around the world. Recent or continuing research programs exist in Germany, Poland, Brazil and China, and in the American states of New York, Iowa, North Carolina, and Virginia. It is a significant issue for apple growers in New York and New England. In addition to the humid climate, the region is heavily forested and apple blocks are frequently smaller than 50 hectares, factors that place most orchards in close proximity to reservoir hosts of SBFS. Inoculum is abundant, and commercial losses from SBFS generally occur in the absence of fungicide applications.

Apples are a valuable crop in the northeastern United States. In 2010, the New England states produced an apple crop valued at over $65,000,000 and that of New York State exceeded $226,000,000 (USDA, 2010). There is generally little tolerance for blemished fruit in both wholesale and direct markets. An apple crop may be downgraded from Extra Fancy or Fancy to US Utility if SBFS is thinly scattered over more than one-tenth of the surface, or dark, heavily concentrated spots affect an area of more than one-half inch in diameter (Baxter et al., 2002; Gleason et al., 2011; USDA, 2002). Growers can face economic losses of up to 90% of crop value in this case (Gleason et al., 2011).
SBFS can also increase desiccation rates, decreasing the storage life of the fruit (Frank et al., 2010; Mirzwa-Mroz, 2012).

Conventional apple culture involves considerable pesticide use. In 2012, apples topped the influential Environmental Working Group’s “Dirty Dozen” list of crops bearing the most pesticide residue (Environmental Working Group, 2012). The primary method for SBFS control is frequent fungicide applications. As much as 40% of the fungicides applied to an apple crop in the Northeast during the growing season is aimed at preventing SBFS (Cooley and Autio, 1997). There is a strong consumer demand in this region for fresh local produce grown with a minimum of pesticides, but to date, effective methods that reduce fungicide use against SBFS have not been developed, in part because the causal fungi are not well understood.

This lack of understanding has deep roots. Nearly 200 years ago, the cause of sooty blotch (SB) was first identified on apples in the eastern United States as Dothidea pomigena (Schweinitz, 1832). For the next 88 years it was held that sooty blotch and flyspeck signs were both caused by this fungus. In the early 20th century, morphological studies revealed that flyspeck was caused by the fungus Schizothyrium pomi (Mont. & Fr.) Arx (anamorph: Zygophiala jamaicensis E. Mason) (Colby, 1920). A new genus was erected for the sooty blotch fungus, which was rechristened Gloeodes pomigena (Schwein.) Colby (Colby, 1920). Colby and others (Groves, 1933; Hickey, 1960; Sutton and Sutton, 1994) noted variation in SB colony morphology, yet Gloeodes pomigena stood as the sole cause of SB until 1997. In that year, researchers determined that SB on apples was caused by not one fungus but three, and none of these was Gloeodes pomigena; they were Peltaster fructicola Johnson, Geastrumia polystigmatis Batista & M. L. Farr, and Leptodontium elatius (G. Mangenot) De Hoog (Johnson et al., 1997).

There has been a great deal of scientific inquiry into the nature of the complex since 1997. Molecular techniques have revealed that FS signs are caused by four
species of fungi, all members of the genus *Zygophiala* (Batzer *et al*., 2008), while SB may be caused by at least 60 different species (Batzer *et al*., 2005; Diaz-Arias *et al*., 2010; Frank *et al*., 2010; Ivanovic *et al*., 2010; Li *et al*., 2011; Spolti *et al*., 2011; Li *et al*., 2012). Significant differences among SB species have been observed in several areas. These include timing of colony establishment and/or colony appearance in apple orchards (Sisson *et al*., 2007; Ismail *et al*., 2010; Batzer *et al*., 2012), species composition of the SB complex among states (Diaz-Arias *et al*., 2010) and among orchards within a state (Ismail *et al*., 2010; Batzer *et al*., 2012), physiological aspects such as temperature optima and carbon source utilization (Johnson and Sutton, 2000; Vande Voort *et al*., 2003; Tentinger, 2004; Batzer *et al*., 2010), and response to fungicides *in vitro* (Sutton *et al*., 1985; Barrett *et al*., 2002; Tarnowski *et al*., 2003). There has yet to be an in-depth investigation into the species composition of the SB complex in the northeastern United States. Once the most prevalent species in the complex are identified, investigations into their individual biology and epidemiology may be conducted. Such an investigation may provide useful information that could lead to improvement in SB control programs.

**SBFS Control**

Prior to the 1940s, growers relied on copper and sulfur to control SBFS, but these chemicals were frequently ineffective (Cooley, 2009). Currently, copper compounds are recommended only at green tip for prevention of fire blight (New England Tree Fruit Pest Management Guide, 2013). Liquid-lime sulfur can be used to control SBFS, but it can cause damage when applied under hot or slow-drying conditions, and although it controls SBFS, it tends to result in a higher incidence of summer fruit rots (Rosenberger *et al*., 2010; Rosenberger *et al*., 2011a). Elemental sulfur is easily washed from plant surfaces by rain, which may decrease its efficacy. It
also tends to be phytotoxic above 30°C (Ouimette, 2012), and when used in close
conjunction with horticultural oil (New England Tree Fruit Pest Management Guide,
2013), aspects that may further limit its use as a summer spray. Nevertheless, it has
been shown to enhance the effect of ziram on SBFS control in the Hudson Valley area of
New York State (Rosenberger et al., 1996), and can be effective against SBFS on its
own (Cox et al., 2009; Rosenberger and Meyer, 2007; Rosenberger et al., 2011). Sulfur
compounds have a multi-site mode of action (Ouimette, 2012), and resistance
development is unlikely.

The ethylenebisdithiocarbamates (EBDCs) were introduced in 1948. This is a
group of broad-spectrum fungicides that includes mancozeb. In response to concerns
about the health effects of EBDC residues, fungicide producers discontinued approval of
these fungicides on apples in 1989. After a lengthy scientific investigation, the EPA
approved the use of EBDCs on apples in 1992, restricting the amount that could be
applied to an orchard in the course of the season to 10.9 kg per acre and setting the pre-
harvest interval at 77 days (Cooley and Manning, 1995). While decreasing the amount of
EBDC residue on harvested apples, this decision also limited the availability of the
EBDCs for management of SBFS, especially later in the season. Controlling SBFS
became more difficult in the northeastern US after these changes in the mancozeb label
restricted its use in the summer months (Cooley et al., 1991).

Captan was introduced in 1949. It is a broad spectrum phthalimide compound
with multisite contact activity (Fungicide Resistance Action Committee, 2013). Captan is
often recommended in combination with thiophanate methyl or a strobilurin for control of
apple summer diseases, but its primary purpose is the inhibition of rot pathogens as it is
considered “weakly effective” against SBFS at the rates and spray timings that are
commonly used in summer (Rosenberger et al., 1991; New England Tree Fruit Pest
Management Guide, 2013). However, captan may still provide good control of SBFS
possibly because it prevents spore germination.

The systemic benzimidazoles (also called MBCs or methyl benzimidazole carbamates) were introduced in the early 1970s. This class of fungicide interferes with beta-tubulin formation, impairing mitosis. Thiophanate-methyl is now the standard for SBFS prevention in the Northeast (New England Tree Fruit Pest Management Guide, 2013; Rosenberger, 2011). While fungal resistance to the benzimidazoles is common in plant pathogens (Smith, 1988), resistance in SBFS fungi has yet to be observed.

The demethylation inhibitors, or DMIs, were introduced in the mid-1970s. This class of fungicides inhibits synthesis of ergosterol, an important component of fungal cell membranes. DMI fungicides do not affect spore germination, but act primarily by inhibiting ergosterol biosynthesis and mycelial growth (Ouimette, 2012). A popular choice for prevention of apple scab when they were first introduced, the DMIs are decreasingly recommended for scab prevention in the Northeast due to widespread resistance (Rosenberger and Cox, 2010). In the Hudson Valley region of New York, a study has shown that DMI fungicides do not control SBFS as well as strobilurins (Rosenberger et al., 2011c), and are generally rated as ineffective to fair (New England Tree Fruit Management Guide, 2013). Of the DMIs registered for use against SBFS on apples, fenarimol, flutriafol, myclobutanil and triflumizole are considered to be ineffective against SBFS, while fenbuconazole and tebuconazole are rated fair (Rosenberger et al., 1996; New England Tree Fruit Management Guide, 2013). A more recent study showed that Inspire Super, which is a combination of difenoconazole and cyprodinil, provided excellent control of SBFS (Rosenberger, et al., 2013), with the activity against SBFS presumably coming from the DMI component of this package-mix fungicide (Rosenberger et al., 2013).
Strobilurins were introduced in 1996 and were quickly adopted for use on crops of all kinds. This highly specific class of fungicides targets cytochrome B in the electron transport chain. The strobilurins are potent inhibitors of spore germination (Bartlett et al., 2002) and perform well as protectants; however, their single site mode of action carries a high risk of resistance development. Strobilurin resistance was observed in several plant pathogenic fungi within ten years of its introduction (Ouimette, 2012). For apples, a maximum of four applications per growing season was initially included as a label restriction for all strobilurin fungicides, but recent label changes now allow for more than four strobilurin applications per season so long as the fungicides are not used more than two times in succession without an intervening application of a different chemistry group. A 2001 study demonstrated that strobilurins were as effective for SBFS prevention as the standard summer treatment of captan plus thiophanate methyl (Rosenberger et al., 2002); however, it has recently been noted that trifloxystrobin appears to be less effective in the Hudson Valley than it once was (Rosenberger, 2011).

The anilinopyrimidines were introduced in the mid-1990s. Cyprodinil inhibits methionine synthesis and mycelial growth, while pyrimethanil interferes with fungal protein secretion, preventing lesion development and sporulation (Roberts and Hutson, 1999). Cyprodinil is known to be less effective at warmer temperatures, and for this reason its use is not recommended after tight cluster in the Northeast (New England Tree Fruit Pest Management Guide, 2013).

Penthiopyrad is a relatively new product. It disrupts mitochondrial respiration by targeting succinate dehydrogenase, and has been found to be effective against a broad range of fungi (Yanase et al., 2007). It inhibits spore germination, mycelial growth, and sporulation. It is registered for use against scab and some other apple diseases, but not for SBFS.
Certified organic orchards in the Northeast are rare. The humid climate, ubiquitous sources of inoculum, and abundant insect pests make organic apple culture exceedingly difficult. Yield is low and production costs are high (Moran, 2007). There are, however, a number of orchards employing low-spray programs. A number of approved organic pesticides are also available for integration into conventional spray programs (New England Tree Fruit Pest Management Guide, 2013). The efficacy of most of these compounds for SBFS control has not been well studied. Sutton et al., (2007) demonstrated that phosphites improved the efficacy of captan against SBFS in North Carolina. Attempts to control SBFS with phosphite compounds in New York State have had variable results (Cox et al., 2010; Rosenberger and Meyer, 2007; Rosenberger et al., 2008). Products containing Bacillus subtilis do not appear to control SBFS well (Rosenberger et al., 2001; Cooley et al., 2007; Cox et al., 2010; Sutton et al., 2007; Sutton et al., 2010). As mentioned previously, sulfur and liquid lime sulfur can be effective against SBFS, but certain conditions and negative side effects contraindicate their use.

It must be said that management of SBFS does not occur in a vacuum. Depending on the production region, growers must apply fungicides to manage several apple diseases. Most important among these, in New England and in much of the world, is apple scab, caused by Venturia inaequalis. Control of scab and of SBFS are inextricably linked. It is believed that regular sprays for apple scab in the spring also prevent SBFS fungi that may have overwintered on apple trees from producing inoculum (Hickey, 1960), and may prevent germination of spores that have blown onto young fruit from nearby reservoir hosts. For this to be effective, SBFS fungi must be susceptible to the fungicides used to control apple scab. To prevent SB infection, protectant fungicides that inhibit spore germination must be applied when inoculum is present. Scab control recommendations for New England offer growers a number of choices, including captan,
mancozeb, thiophanate-methyl, and strobilurins (New England Tree Fruit Management Guide, 2013). These fungicides are reasonably effective against SBFS. DMIs (with the exception of difenoconazole and fenbuconazole) and anilinopyrimidines have little effect on SBFS, but are often recommended in tank mixes with captan or mancozeb.

Control of *V. inaequalis* has been further complicated by its development of insensitivity to fungicides such as dodine (Jones and Walker, 1976; Chapman *et al.*, 2011), thiophanate-methyl (Chapman *et al.*, 2011) and DMIs (Koller *et al.*, 1997; Koller *et al.*, 2005; Chapman *et al.*, 2011), and the ability to overcome some of the gene-based defenses of scab resistant cultivars (Parisi *et al.*, 1993). Many growers have since had to revert to captan and/or mancozeb as the primary fungicides for scab control (Rosenberger and Cox, 2010).

Scab-resistant cultivars do not require early season sprays for scab control, thereby decreasing overall fungicide input over the growing season. Without early season sprays, however, it may be possible for SBFS fungi that have overwintered on apple trees to produce significant inoculum early in the season, potentially setting the stage for an epidemic later (Merwin *et al.*, 1994; Prokopy, 2003). Inoculum from fungi on reservoir hosts may also be present in borders well before summer sprays begin (Sutton 1990a). For these reasons, growers may need to consider fungicide applications early in the season even on scab-resistant trees. SBFS control must be focused on apples because the causal fungi appear to be ubiquitous and removal of reservoir hosts from orchard perimeters is not practical.

Numerous field studies have examined both conventional and organic fungicide efficacy for control of SBFS, with a broad range of results (eg. Brannen *et al.*, 2010; Cromwell *et al.*, 2008; Rosenberger *et al.*, 2002; Rosenberger *et al.*, 2011a,b,c; Sutton *et al.*, 2009; Travis *et al.*, 2008). FS is generally considered to be harder to control than SB, although studies have shown that this is not always the case (Brannen *et al.*, 2010;
Hickey et al., 2001; Rosenberger et al., 2004; Rosenberger et al., 2005; Sutton et al., 2004; Sutton et al., 2009).

Because they are epiphytes and do not penetrate the fruit cuticle (Nasu and Kunoh, 1987; Belding et al., 2000), SBFS fungi must be controlled by fungicides with contact activity. Fungicides that prevent spore germination may also be helpful if they are applied when inoculum is present.

Some cultural practices can be effective for suppression of SBFS. Summer pruning contributes to SBFS control by decreasing humidity within the tree canopy and improving spray deposition (Ocamb-Basu et al., 1988; Cooley et al., 1997). Keeping ground cover mowed short can lessen SBFS on fruit in the lower canopy (Rosenberger et al., 1996). Early maturing cultivars are less prone to developing SBFS in the field than those that mature later, possibly because the apples are exposed to fewer hours of wetting and high humidity, conditions conducive to SBFS development; however, at least one study has shown differences in SBFS susceptibility even among cultivars with similar harvest dates (Biggs et al., 2010). It may be more suitable to grow early maturing cultivars in low-input orchards in areas with a history of SBFS. Fruit mummies have been shown to harbor fungi of the sooty blotch genus Peltaster (Gleason et al., 2011), so cultivars that do not retain mummies may have a lower risk of developing SBFS. One study has demonstrated that removal of fruit mummies from apple trees that retain them can improve chemical control (Rosenberger et al., 2011a). Removal of reservoir hosts of SBFS from the orchard vicinity may also be helpful (Prokopy, 2003), but no formal studies have been conducted on this topic. Integrated pest management programs have been successful in reducing pesticide input in apple orchards by employing orchard sanitation practices (Cooley, 2009).

Many growers remain reliant upon fungicide cover sprays every 2-3 weeks for SBFS prevention. Weather-based disease forecasting models are important tools in the
effort to reduce fungicide use in crops (Madden and Ellis, 1988). Despite improvements in SBFS forecasting models (Cooley et al., 2011), outbreaks and control failures occur. The reasons behind this are not always clear. Apple growers rely on a regimen of orchard sanitation, pruning, and judicious spraying of fungicides to prevent SBFS, but they are also at the mercy of the weather. Rain events may decrease the duration of protection provided by a fungicide application, or prevent application at the most crucial times of the season. It is therefore possible for even the most conscientious and careful grower to experience an outbreak of SBFS.

The SBFS fungi have been observed on a wide range of host plants and do not appear to depend on apples specifically for the completion of their life cycles. When a sooty blotch fungus is observed on apples, the SB fungus on the apples is presumably a mere fraction of that species’ population in the immediate vicinity. The fungus in the orchard may be exposed to fungicides and may develop resistance, but the population of the fungus on nearby uncultivated hosts remains unexposed and susceptible. It has therefore been hypothesized that the pressure on SBFS fungi to develop resistance may be low because so much of the population is never exposed to fungicides; however, it is still possible that fungicide use selects less sensitive strains of SBFS species in the orchard. It has been shown that SBFS species diversity is affected by fungicide regimen (Diaz-Arias et al., 2010).

Numerous studies on cultural and chemical control of SBFS have been conducted in New York and New England (Cooley et al., 1997; Cooley et al., 2007; Cromwell et al., 2008; Rosenberger et al., 1996; Rosenberger et al., 2007; Rosenberger et al., 2011a,b,c). In contrast to the active programs of control studies in the field, only three studies have examined fungicide susceptibility of SB fungi in vitro (Sutton et al., 1985; Barrett et al., 2002; Tarnowski et al., 2003). These studies revealed significant differences in susceptibility among SB isolates. There is a paucity of information on
baseline sensitivities and resistance development of SB fungi to commonly used fungicides. New information on this topic would be helpful in the development of control programs and the understanding of SB epidemiology.

Causal fungi

As recently as 1997, sooty blotch and flyspeck (SBFS) signs on apple were believed to be caused by two species of fungi. In the past 16 years, molecular techniques have enabled researchers to discover that sooty blotch and flyspeck may be caused by as many as 60 different species (Batzer et al., 2005; Diaz-Arias et al., 2010; Frank et al., 2010; Ivanovic et al., 2010; Spolti et al., 2011; Li et al., 2012), that there are at least four species of Zygophiala (Batzer et al., 2008), and that symptoms resembling flyspeck may be caused by species other than Zygophiala (Duttweiler et al., 2008; Sun et al., 2008). In a very short time, a new picture of the SBFS disease complex has begun to emerge.

There can be a considerable amount of variation in the geographical distribution of sooty blotch species. For instance, Geastrumia polystigmatis, a species found in orchards in the eastern United States, has yet to be observed west of the Mississippi (Diaz-Arias et al., 2010). Tripospermum myrti has been identified as a causal agent of sooty blotch in Germany (Noga et al., 2000) and Poland (Grabowski, 2007), but has yet to be isolated from apples in the United States. Diaz-Arias et al., (2010) observed a Stomiopeltis sp. on apples from four southern states, but not on apples from New York or New England. These three fungi make particularly interesting examples not only because of their differences in distribution, but also because of their different life cycles. Geastrumia polystigmatis has no known teleomorph, Tripospermum myrti has a known teleomorph (Trichomerium; Kirk, et al., 2008), and Stomiopeltis has no known anamorph. Because the causal fungi may differ in life cycle and susceptibility to fungicides as well
as in geographical distribution, understanding of the composition of the SB complex in a particular region may be essential for the development of effective disease management strategies.

Geographical variation can also occur on a smaller scale. For instance, significant differences were observed in the taxonomic composition of the SB complex in six Iowa orchards (Ismail, 2010; Batzer et al., 2012). This may be a reflection of differences in environmental conditions and/or fungicide use (Diaz-Arias et al., 2010; Batzer et al., 2012).

Researchers in Iowa recently investigated the geographical distribution of SBFS species on apples in the eastern half of the United States (Diaz-Arias et al., 2010). Sooty blotch species identified on apples from New York and New England included Geastrumia polystigmatis, Phialophora sessilis, Ramularia sp., Colletogloeum sp., Peltaster fructicola, and two species of Pseudocercosporella. To date, this is the only study to have explored the species makeup of the SBFS complex in the northeastern states.

In addition to a clear picture of SB species composition in a particular region, knowledge of whether different species in the complex differ in terms of epidemiological factors is also needed. Although SBFS has been a growing problem in apple orchards since the 1950s (Williamson and Sutton, 2000), it has only been 16 years since anyone looked closely enough at SB colonies to realize that they may be caused by several fungi, none of which are Gloeodes pomigena. In pursuit of effective control measures, we have based many of our assumptions about the life cycle(s) of the SB fungi on that of Z. jamaicensis. With a well-described sexual stage and distinctive conidial morphology, Z. jamaicensis has been much easier to study in vivo than the SB fungi, many of which (in contrast to the three species mentioned in the previous paragraph) lack known teleomorphs and/or produce nondescript conidia. As a result, much about the basic
biology and epidemiology of many SB species remains unknown. This is perhaps the most critical gap in our knowledge of SB fungi.

Eight studies have investigated the timing of SB infection and/or colony appearance in apple orchards (Brown and Sutton, 1993; Smigell and Hartman, 1998; Grabowski and Wrona, 2004; Sisson et al., 2007; Ismail et al., 2010; Mayr et al., 2010; Spolti et al., 2011; Batzer et al., 2012). Five of these studies do not consider differences among members of the SB complex. By collecting and incubating fruit, Brown and Sutton (1993) found that infection of apples by *Gloeodes pomigena* in North Carolina occurred within 10-21 days of petal fall. The timing of the appearance of SBFS was related to rainfall patterns in May and June. Similarly, Spolti et al., (2011) observed infection of apples in Brazil within 31 days of petal fall, a close relationship between SB appearance and rainfall, and an incubation period of at least 49 days. In an apple bagging experiment, Smigell and Hartman (1998) found that the first appearance of SBFS signs in Kentucky was correlated with the accumulation of 222 leaf wetness hours (LWH) in early July; however, the most critical time for SBFS infection was in July and August. Neither of these last two studies considered SB and FS separately. In a study of two Polish orchards over three growing seasons, Grabowski and Wrona (2004) noted that SB appeared 6-9 days earlier in the orchard that received an average 1.77mm more daily rainfall, and that the incubation period ranged from 29 to 45 days. In their studies in the Lake Constance region of southern Germany, Mayr et al., (2010) observed that SB infection could occur at any time during the growing season, but that earlier infections were more severe.

Three studies conducted in Iowa explored epidemiological differences among SB taxa. Sisson et al., (2007) were the first researchers to investigate the timing of appearance by individual SB species. Their efforts were assisted by the development of a PCR primer specific for Capnodiales and an RFLP technique designed to distinguish
SB members of that family. Apples in several orchards were observed weekly, and SBFS colonies were marked with colored pens to signify the date of appearance. After harvest, PCR studies revealed that the dominant species, sterile mycelia spp. RS1 and RS2, were the first to appear in August, but the frequency of new infections declined after the first week of September. *Dissoconium aciculare* also appeared in August, and the frequency of new infections increased significantly during September. Comparable observations of *Dissoconium* species were made by Batzer et al., (2012), who observed a similar pattern of appearance of *Microcyclosporella* species in two orchards. Ismail et al., (2010) investigated the timing of fruit inoculation by four prevalent species in six orchards. Fruit were protected by bags all season except for designated two week intervals between June 1st and September 7th. They found that inoculum of the three most common SB taxa was present during the entire experimental period. Fruit was infected by *Dissoconium* species in the four orchards where it appeared primarily during a brief period in early to mid-June, with rare infections occurring after June 15th. In the fifth orchard, *Microcyclosporella* species dominated, with the peak infection time also in early to mid-June, although infections continued at a low level for the rest of the season. The sixth orchard was dominated by *Colletogloeum* species, which infected fruit throughout June and July. Results from these studies indicate a field incubation period for both *Dissoconium* and *Microcyclosporella* of nearly three months.

To this date, there have been no studies of the timing of the appearance of SB species with distinctive conidia, such as *Tripospermum myrti* or *Geastrumia polystigmatis*. A study of the influence of environmental conditions on the timing of production and dispersal of SB inoculum would enhance efforts to streamline control programs and reduce fungicide use. Such information could be used to improve forecasting models as well.
Efforts to control SB would also benefit from a greater understanding of whether species in the complex differ in their response to fungicides. Only three studies have examined fungicide susceptibility of SBFS fungi *in vitro* (Sutton *et al.*, 1985; Barrett *et al.*, 2002; Tarnowski *et al.*, 2003). In experiments with three DMI fungicides, Sutton *et al.* (1985) observed higher EC$_{50}$ values for isolates of *Gloeodes pomigena* than for *Z. jamaicensis*. Tarnowski *et al.* (2003) observed significant differences in susceptibility to thiophanate-methyl and ziram both among and within the seven clades of fungi studied. EC$_{50}$ values ranged from 0.4 to 1.6 ppm for ziram and $<$0.1 to $>$1.0 ppm for thiophanate-methyl (Tarnowski *et al.*, 2003). Barrett *et al.*, (2002) also observed significant differences among clades in response to captan and thiophanate-methyl. It has been shown that the composition of SB taxa in an orchard may be influenced by fungicide use pattern (Diaz-Arias *et al.*, 2010); therefore, it is important not only to know which species are present, but how they may respond to fungicide treatments.

Recent research has shown that sooty blotch species can differ significantly from one another in physiological aspects such as temperature optima and carbon source utilization (Johnson and Sutton, 2000; Vande Voort *et al.*, 2003; Tentinger *et al.*, 2004; Batzer *et al.*, 2010). Johnson and Sutton (2000) demonstrated that *Leptodontium elatius* conidia germinated at 12-32°C and $\geq$97% relative humidity (optimum 32°C and 99% RH), and conidia of *Peltaster fructicola* germinated at 12-24°C and $\geq$95% relative humidity (optimum 24°C and 97-99% RH). Conidia of *P. fructicola* were also much more sensitive to drying out than those of *L. elatius*. Vande Voort (2003) observed differences in radial growth and sporulation on three types of media among six clades of SB fungi. Tentinger *et al.* (2004) observed differences in rates of conidia germination among three species of *Peltaster*. Greater germination was observed in 0.05% apple juice than in sterile deionized water for one of the three species studied. Batzer *et al.* (2010) observed that *Dissoconium aciculare* is significantly less inhibited and *Peltaster fructicola* significantly
more inhibited than three other SB species by temperatures of 10-15°C. They also demonstrated the influence of nutrient concentration on fungal morphology by growing isolates on media amended with increasing concentrations of apple juice.

The results of the aforementioned studies illustrate important differences among SB species. A greater understanding of the physiology of individual SBFS species in vitro may lead to a greater understanding of their behavior in the field.

**Causal fungi-specific genera**

Of twenty genera seen in the United States, Gleason et al. (2011) identified six genera of SB fungi that appeared in at least 9 of 39 orchards surveyed. These are *Peltaster, Geastrumia, Dissoconium, Microcyclosporella, Microcyclospora*, and “C. Colletogloeopsis-like.” The following is a review of what is known about the first five genera in relation to SB. *Mycosphaerella* has only occasionally been named as a cause of SB, but it is discussed here because of its close taxonomic relationship with *Dissoconium* and historical relationship with *Microcyclosporella* and *Microcyclospora*.

**Peltaster species**

In 1996, Johnson et al. identified certain colonies of sooty blotch as *Peltaster fructicola*. *Peltaster* has since become one of the most commonly identified sooty blotch genera. It has been isolated from apples in the United States (Duttweiler et al., 2008), Brazil (Spolti et al., 2008), Poland (Grabowski et al., 2007; Mirzwa-Mroz and Winska-Krysiak, 2011), Serbia and Montenegro (Ivanovic et al., 2010), and Turkey (Blaser et al., 2010; Mayfield et al., 2013). In addition, it has been found on several other hosts, including blackberry in North Carolina (Johnson and Sutton, 1994) hawthorn in China (Li et al., 2009), pawpaw in Iowa (Hemnani et al., 2008), and avocado, banana, mango, and carambola in Florida (Ploetz et al., 2000; Perez-Martinez et al., 2009).
Colonies of *Peltaster fructicola* are described as ramose. Colonies exhibit superficial asexual fruiting bodies called pycnothryia. When conidia are mature, the shield-like coverings of the pycnothryia rupture and spores are released.

The Catalog of Life (www.catalogueoflife.org) places the genus *Peltaster* in the class Dothideomycetes, but its order and family are *incertae sedis* (of uncertain placement). The International Mycological Association (www.mycobank.org) lists 13 recognized species of *Peltaster*, but virtually nothing is known about any species besides *Peltaster fructicola*. *Peltaster hedyotidis*, described by Sydow and Sydow (1917), is the type species of the genus. *P. fructicola* is frequently named as a cause of sooty blotch. Other species of the genus have been isolated from apples and identified through genetic studies (Batzer *et al.*, 2005; Diaz-Arias *et al.*, 2010), but none have been formally described or named to date.

*P. fructicola* is perhaps the best studied species of SB *in vitro*. Experiments by Belding *et al.* (2000) demonstrated that *P. fructicola* does not metabolize the cuticular waxes of fruit. This is believed to be true of all SB fungi, and that these fungi are dependent on exudates leaching through the fruit cuticle, although it is possible that leaf exudates falling onto fruit also play a role. Experiments by Frank *et al.* (2011) with an unidentified species of *Peltaster* isolated from apples in Slovenia produced results similar to those of Belding *et al.* Wrona and Grabowski (2004) found that fructose and glucose have a profound effect on germination of *P. fructicola* conidia. Wrona and Gleason (2005) demonstrated that conidial germination is affected by glucose concentration, but not by amino acids found on the surface of Golden Delicious apples, although amino acids appeared to be an important source of nitrogen for germ tube and mycelial growth. These results lend credence to the theory that *P. fructicola* and other SB fungi rely upon fruit exudates for nutrition. Johnson and Sutton (2000) identified optimum conditions for germination of *P. fructicola* conidia as 24°C and >95% RH, and
the optimum temperature range for mycelial growth as 12-24°C. Mycelial growth of *P. fructicola* is significantly inhibited at 10°C and 30°C, markedly more so than that of an unidentified *Peltaster* species used in the same study (Batzer et al., 2010).

**Geastrumia polystigmatis**

*Geastrumia polystigmatis* was first described in 1960 by Batista et al. as the sole member of its genus. Like *Peltaster*, *G. polystigmatis* forms ramose colonies with pycnothyria. The name *Geastrumia* was derived from the way in which the pycnothyria of the fungus tear when the conidia mature (Batista et al., 1960). The remnants of tissue that covered the pycnothyrium appear petal-like and frame the base of the fruiting body, producing a structure reminiscent of the basidiocarp of *Geastrum* species. *Geastrumia* was originally assigned to the family Discellacea, but now its taxonomy is in flux. Uniprot.org, Encyclopedia of Life, and National Center for Biotechnology Information (NCBI) list this genus as belonging to the order Capnodiales and the family Mycosphaerellaceae, but cite no references. Species Fungorum and Catalogue of Life list its class, order, and family as *incertae sedis*. Kirk et al. (2008) describe it as “anamorphic Pezizomycotina.” A phylogeny by Diaz Arias et al. (2010) places it within the Dothidiomycetes, but outside the subclass Dothideomycetidae. Dothidiomycetidae contains the order Capnodiales, which includes most, but not all, sooty blotch fungi identified to date.

Until the modern era of SBFS research, *G. polystigmatis* was seldom noticed. It was described by K.A. Pirozynski on *Hymenocardia acida* and *Costus afer* from Tanzania and *Andira jamaicensis* from the Dominican Republic (1971). Its distinctive conidia were found in rainwater pools at the bases of pine trees in Japan (Ando and Tsubaki, 1984) and in rivers in southern Spain (Roldan et al., 1987). In Brazil, *G. polystigmatis* and a second putative species of *Geastrumia* have been isolated from
leaves of the medicinal plant *Salacia crassifolia* (a close relative of *Celastrus*, a reservoir host species for SB in North America) (Dos Santos, 2011). *G. polystigmatis* is also associated with *Dimorphandra wilsonii* in Brazil (da Silva, 2012). It is reasonable to believe that *G. polystigmatis* may be found on various plant hosts worldwide. In all instances it has been described as an epiphyte.

The name *Geastrumia polystigmatis* is frequently mentioned in articles on sooty blotch because it was one of the species identified by Johnson *et al.*, (1997) in their landmark investigation into the true identity of *Gloeodes pomigena*. This fungus has received very little attention since Johnson *et al.*, and little is known of its physiology and life cycle. It has been associated with SB on apples in several eastern states (Johnson *et al.*, 1997; Diaz Arias *et al.*, 2010) and blackberry stems (Johnson and Sutton, 1994), but has yet to be identified as a causal agent of SB west of the Mississippi or outside the United States.

**Dissoconium species**

*Dissoconium aciculare*, *D. commune*, *D. dekkeri*, *D. mali*, *D. luensis*, *D. proteae*, and four unnamed *Dissoconium* species have been associated with SBFS on apples in the US and China (Gleason *et al.*, 2011; Zhang *et al.*, 2012), pawpaw in Iowa (Hemnani *et al.*, 2008), and on persimmon in Korea (Kwon *et al.*, 2012) and China (Sun *et al.*, 2008). *D. aciculare* is the type species of the genus. A good deal of taxonomic work has been done on this genus in recent years. An extensive phylogenetic study of the Capnodiales led to the establishment of the family Dissoconiaceae, which includes the genus *Dissoconium* and the SB fungi *Ramichloridium apiculatum*, *Pseudoveronaea ellipsoidea* and *Pseudoveronaea obclavata* (Crous *et al.*, 2009; Li *et al.*, 2012). Recently, the genus *Dissoconium* has been divided into *Dissoconium*, which contains *D. aciculare* and *D. mali*, and *Uwebraunia*, which includes the erstwhile *D. commune* and *D. dekkeri*.
(Li et al., 2012). This separation is supported by phylogenetic studies, morphological differences in culture, and the association of Uwebraunia with teleomorphs that resemble Mycosphaerella. Little is known about the ecology of either genus (Li et al., 2012).

*D. aciculare* has been reported to cause sooty blotch on apples in the US (Diaz-Arias et al., 2010; Batzer et al., 2012; Li et al., 2012), and on pawpaw in Iowa (Hemnani et al., 2008). The fungus was once reported to have an antagonistic effect on some powdery mildew fungi (Heiwegen and Buchenauer, 1984), but this subject was not investigated further. Batzer et al. (2010) observed that mycelial growth of *D. aciculare* was significantly less inhibited by temperatures of 10 and 15°C than other SB fungi in the study. This cold tolerance is reflected in the observation that *D. aciculare* is more abundant on apples late in the season and after a period of cold storage (Sisson, 2009; Batzer et al., 2012). This is very interesting in light of observations that Dissoconium species infect apples rather early in the growing season (Ismail et al., 2010). If this data reflects a typical population of *D. aciculare*, then colonies must establish themselves on the fruit cuticle and remain in a period of relative stasis for two to three months while summer temperatures and/or fungicide residues make conditions unfavorable for growth. Alternatively, if this data is derived from a population dominated by Dissoconium species other than *D. aciculare*, it may imply that warmer temperatures are more favorable for growth of these other species.

**Mycosphaerella species**

*Mycosphaerella*, a group of closely related teleomorphs, has long been one of the largest genera of fungi. Since it was shown to be polyphyletic (Hunter et al., 2006; Crous et al., 2007), the genus has undergone a taxonomic overhaul. It is now recommended that *Mycosphaerella sensu stricto* be limited to species with *Ramularia*
anamorphs (Verkley et al., 2004; Crous et al., 2009; Koike et al., 2011), while
designating other related forms as ‘Mycosphaerella’ until their true taxonomic placement
is resolved. Mycosphaerella belongs to the family Mycosphaerellaceae, which is also
home to the SB genera Pseudocercospora, Pseudocercosporella, Microcyclosporella,
Passalora, and Zasmidium.

The genus Teratosphaeria now contains several species formerly ascribed to
Mycosphaerella (Crous et al., 2007), and resides in a new family, the
Teratosphaeriaceae (Crous et al., 2012). Teratosphaeria includes taxa with Kirramyces
and Colletogloeopsis anamorphs. The Teratosphaeriaceae also includes the SB genera
Devriesia, Microcyclospora, and Tripospermum.

Mycosphaerella madeireae causing SB has been isolated from apples from the
US and Germany (Tatalovic, 2009), and an undetermined Mycosphaerella species from
hawthorn in China (Li et al., 2009). Ramularia species are frequently isolated from SB
colonies on apples in the US (Batzer et al., 2005; Duttweiler et al., 2008; Sisson, 2009;
Diaz-Arias et al., 2010; Batzer, 2012), and have also appeared in Poland (Grabowski,
2007), and China (Sun et al., 2008). To date, none of these isolates have been identified
to species.

Uwebraunia dekkeri (formerly known as U. lateralis), has been isolated from
apples in the US (Li et al., 2012). Its teleomorph, Mycosphaerella lateralis, is frequently
isolated in studies of Mycosphaerella leaf disease of eucalyptus, which is associated
with a complex of species (Jackson et al., 2008; Perez et al., 2009; Teodoro et al., 2012).
M. lateralis will infect detached eucalyptus leaves through stomata (Jackson et al., 2004).
This infection process is similar to that of M. nubilosa, a common cause of
Mycosphaerella leaf disease on eucalyptus; however, it is not known whether U. dekkeri
dwells in living leaves as a pathogen or as an endophyte. Mycosphaerella communis
(anamorph, Uwebraunia commune) is also associated with leaf spots on eucalyptus in
Spain and *Protea* in Australia (Crous *et al.*, 2008), as well as appearing on banana in Trinidad (Arzanlou *et al.*, 2008). It is not known whether *M. communis* is pathogenic or merely opportunistic in any of these cases. It would be interesting to compare isolates of *U. dekkeri* and *U. commune* from apples with those found on eucalyptus and other hosts.

*Mycosphaerella pomi* causes Brooks fruit spot of apples. Ascospores infect apples at the lenticels, but the fungus is primarily a pathogen of leaves and completes its life cycle there (Sutton *et al.*, 1987). *M. pomi* has not been associated with sooty blotch.

**Microcyclospora and Microcyclosporella**

These two species were once regarded as *Pseudocercospora* and *Pseudocercosporella* respectively. *Pseudocercospora* was long treated as one of the 30 genera of anamorphs associated with *Mycosphaerella*, but it is now recognized as a single name genus of its own (Hawksworth *et al.*, 2011; Crous *et al.*, 2012). Some members of the genus do have *Mycosphaerella*-like teleomorphs, but the name *Mycosphaerella* is now reserved for species with *Ramularia* anamorphs (Verkley *et al.*, 2004; Crous *et al.*, 2009; Koike *et al.*, 2011).

A recent investigation of *Pseudocercospora* isolates from SB on apples from Germany and Slovenia revealed that this genus contained two distinct taxonomic groups (Frank *et al.*, 2010). The genus *Microcyclospora* was established to accommodate genetic and morphological differences among the *Pseudocercospora*. Three species of *Microcyclospora* were described: *M. malicola*, *M. pomicola*, and *M. tardicrescens*. These species have since been transferred to the Teratosphaeriaceae (Crous *et al.*, 2012). It is likely that some SB isolates originally identified as *Pseudocercospora* are actually species of *Microcyclospora*. A BLAST search revealed six accessions of *Pseudocercospora* with a high degree of identity to *M. malicola* (Crous *et al.*, 2012). *Pseudocercospora* isolates causing SB on apples have been identified in the US (Batzer
et al., 2005; Duttweiler et al., 2008; Diaz-Arias et al., 2010), Serbia and Montenegro (Ivanovic et al., 2010), and Poland (Mirzwa-Mroz et al., 2008). Microcyclospora isolates causing SB on apples have been identified in Poland (Mirzwa-Mroz and Winska-Krysiak, 2011), Turkey (Mayfield et al., 2013), Germany and Slovenia (Frank et al., 2010). It has not yet been definitively resolved which isolates, if any, are truly Pseudocercospora and which are Microcyclospora.

In the same investigation, the genus Pseudocercosporella was also found to contain two distinct taxa, and this led to the establishment of the genus Microcyclosporella (Frank et al., 2010). This genus currently contains one described species, M. mali. Microcyclosporella is polyphyletic within the Mycosphaerellaceae (Crous et al., 2009; Frank et al., 2010), and the taxonomy of this group is likely to remain in flux for some time. Some GenBank accessions listed as Pseudocercosporella are likely to be Microcyclosporella mali or an undescribed species of Microcyclosporella (Frank et al., 2010). Pseudocercosporella isolates causing SB on apples have been identified in the US (Batzer et al., 2005; Duttweiler et al., 2008; Tatalovic et al., 2008; Sisson, 2009; Tatalovic et al., 2009; Batzer et al., 2010; Diaz-Arias et al., 2010), Turkey (Blaser et al., 2010; Mayfield et al., 2013), Serbia and Montenegro (Ivanovic et al., 2010), Germany (Tatalovic et al., 2009) and Poland (Grabowski, 2007; Mirzwa-Mroz et al., 2008). It has also been found on wild plum in Iowa (Latinovic et al., 2007) and blackberry, multiflora rose, smooth sumac, kiwi, and honeysuckle in the midwestern US (Hemnani et al., 2007). Microcyclosporella has been identified on apples in Germany and Slovenia (Frank et al., 2010) and on apples (Mirzwa-Mroz and Winska-Krysiak, 2011) as well as domesticated plums in Poland (Mirzwa-Mroz et al., 2011). As with the previous group, it has not yet been definitively resolved which isolates, if any, are truly Pseudocercosporella and which are Microcyclosporella. It is interesting to note that
these fungi appear to be more commonly associated with SB than those of the *Pseudocercospora*-Microcyclospora group.

**Growth on the fruit cuticle**

SBFS fungi inhabit the waxy cuticles of their host plants and do not penetrate the underlying epidermis (Nasu and Kunoh, 1987; Belding *et al.*, 2000). The fungi of the SBFS complex are therefore best described as epiphytes. They do not appear to metabolize epicuticular waxes, and so it is hypothesized that SBFS fungi obtain nutrients from fruit exudates (Belding *et al.*, 2000; Wrona and Grabowski, 2004). The observation that sooty blotch fungi do not grow on russeted apple tissue, which is impermeable to water and therefore to fruit exudates, also supports this hypothesis (Belding *et al.*, 2000).

Because of their epiphytic nature, sooty blotch fungi must be adapted to endure the forces of desiccation, high surface temperatures, UV radiation, and competition among microbes that are facets of existence on the plant cuticle. The SB fungi differ in this way from most plant pathogenic fungi, which penetrate host tissues and enjoy some protection from the environment once infection has been established. The plant cuticle is subject to frequent, sudden changes in environmental conditions, and therefore may be considered to be an extreme environment (Fonseca and Inacio, 2006; Vorholt, 2012). Because nutrients are limited and the focus of competition amongst epiphytic bacteria, yeasts, and fungi, the plant cuticle is also considered to be an oligotrophic environment (Vorholt, 2012). This also sets the SB fungi apart from most plant pathogenic fungi, which find abundant nutrients within host tissues.

SB fungi appear to have more in common with the so-called black yeasts and lichenous fungi than they do with most plant pathogens. It is not surprising that species of some of the genera associated with SBFS (*Mycosphaerella*, *Teratosphaeria*, *Phialophora*, *Tripospermum*, and *Devriesia*) are also known to inhabit rock surfaces,
another oligotrophic habitat subject to environmental extremes (Caretta et al., 2006; Ruibal et al., 2009; Gostincar et al., 2012). Copious production of melanin and a slow growth rate are two survival tactics shared by both SBFS fungi and their lithobiont cousins. Melanin is known to play a role in protecting fungi from UV radiation, osmotic stress, desiccation, enzymatic lysis, metal ions, and reactive oxygen and nitrogen species (Butler and Day, 1998; Fogarty and Tobin, 1996; Gadd and deRome, 1998; Langfelder, 2003; Sterflinger, 2006). A slow growth rate may be associated with life in an oligotrophic environment. It may also be associated with the considerable energy demands of melanin production, or with trehalose accumulation, a pre-requisite for dessication tolerance in some lithobiont fungi (Sterflinger, 1998; Sterflinger, 2006); however, trehalose production in SBFS fungi has not been investigated.

Since they survive on exudates that trickle out to plant surfaces, it may be said that the sooty blotch fungi occupy a nutritional niche between fungi such as the black yeasts, which survive on very little nutrition and have remarkably slow rates of growth and reproduction, and the sooty molds, which take advantage of a rich nutrient source (insect honeydew) to grow rapidly and produce abundant conidia in a short time.

There is some evidence of differences in susceptibility to SBFS among apple cultivars (Biggs et al., 2010). The morphological and chemical nature of the fruit cuticle may differ among cultivars (Belding et al., 1998; Verdaro et al., 2003) and in response to environmental conditions such as plant nutrition (Richmond and Martin, 1959), although any direct influence of these factors on SBFS susceptibility has yet to be investigated.

**Reservoir hosts**

It is believed that orchard borders are a haven for SBFS species, and that outbreaks in orchards are the result of inoculum produced on nearby reservoir hosts (Gleason et al., 2011). SB fungi have been isolated from several perennial plants that
may be found in orchard borders (Colby, 1920; Zaring, 1929; Gardner and Baines, 1931; Hickey, 1960; Johnson et al., 1997; Hemnani et al., 2007). Gardner and Baines (1931) isolated Gloeodes pomigena from 24 reservoir hosts; isolates from 15 of these were found to infect apples under laboratory conditions. Hickey isolated G. pomigena from eight reservoir host species and succeeded in infecting apples with these isolates.

Since the single species theory of SB has been debunked, very little attention has been devoted to examining the host range of specific SB species. Geastrumia polystigmatis (Johnson and Sutton, 1994), and Peltaster fructicola (Johnson et al., 1997) are known to infect blackberry (Rubus alleghaniensis) in North Carolina. Pseudocercosporella and Stomiopeltis species have been detected on several reservoir hosts in Iowa and Illinois (Hemnani et al., 2007). Further investigation of SB species prevalent on reservoir hosts may reveal information about the life cycles of the fungi, thereby enhancing our understanding of SBFS epidemiology.

A single SB species may have different colonial morphology on one host than it does on another (Hemnani et al., 2008). This may be the result of differences in the composition of epicuticular waxes or of plant exudates among species. Host preferences among SB species have not been investigated.

The presence of reservoir hosts in orchard borders is a potential problem for growers, particularly those who experience frequent SBFS epidemics. Removal of reservoir hosts may not be a practical solution, especially in regions such as the Northeast where orchards may be bordered by large woodland areas. Because of the abundance of reservoir hosts and ubiquitous nature of SBFS inoculum, control efforts must focus on orchards.
Conclusion

The next step in developing more effective and efficient management of the SBFS complex on apples is to determine the major fungal species contributing to SB signs on fruit. The major contributor to flyspeck signs in the Northeast, *Schizothyrium pomi*, has been identified and well characterized in terms of its life cycle, but little is known about which of many fungi that can cause SB predominate in the region. Once the key players causing SB are identified, further study of their biology is needed to provide useful management information. Determination of nutrition, temperature range, host range, and fungicide susceptibility for these species should enhance our basic understanding of these interesting epiphytic fungi, and potentially lead to the development of improved control methods for the SBFS complex.

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CHAPTER 2

GEASTRUMIA POLYSTIGMATUS AND OTHER IMPORTANT SOOTY BLOTCH SPECIES ON APPLES AND RESERVOIR HOSTS IN THE NORTHEASTERN UNITED STATES

Abstract

The sooty blotch and flyspeck complex (SBFS) causes blemishes on apples in humid, temperate growing regions worldwide. For many years, only two fungi were believed to cause the blemishes, and management practices focused on flyspeck. More recently the number of recognized causal agents has grown tremendously, and sooty blotch signs have become more troublesome in commercial apple production. In contrast to flyspeck etiology, the many species of fungi causing sooty blotch (SB) have not been well studied. Sooty blotch species are known to reside on reservoir hosts in orchard borders, and reservoir hosts are believed to be the major source of SB inoculum for commercial orchards. SB fungi on apples and selected reservoir hosts were collected from orchards in the northeastern United States and morphological observations combined with PCR amplification and sequencing of the ITS region were used to identify samples. Geastrumia polystigmati was the species most often found on apples, while species of Peltaster were more common on reservoir hosts. A genetic study of 54 G. polystigmati isolates revealed little genetic variability among isolates from different hosts and different regions. This is the first study to investigate the species composition of the sooty blotch complex in the northeastern United States and the genetic diversity among isolates of G. polystigmati.
Introduction

The sooty blotch and flyspeck (SBFS) complex causes blemishes on apples in humid, temperate growing regions worldwide. As recently as 1997, sooty blotch and flyspeck (SBFS) diseases of apple were believed to be caused by two species of fungi: sooty blotch (SB) by Gloeodes pomigena (Schwein.) Colby, and flyspeck (FS) by Schizothyrium pomi (Mont. & Fr.) Arx (anamorph: Zygophiala jamaicensis E. Mason)(Williamson and Sutton, 2000). In the past 16 years, molecular techniques have enabled researchers to discover that there are at least four species of Zygophiala (Batzer et al., 2008) that can cause FS signs, that signs resembling FS may be caused by species other than Zygophiala (Duttweiler et al., 2008; Batzer et al., 2005), and that SB may be caused by at least 60 different species (Batzer et al., 2005; Diaz-Arias et al., 2010; Frank et al., 2010; Ivanovic et al., 2010; Li et al., 2011; Spolti et al., 2011; Li et al., 2012). In a very short time, a new picture of the SBFS complex has emerged.

SBFS is an economically important issue for apple growers in temperate, humid regions around the world, where the complex can cause substantial economic losses when the harvest is downgraded from Extra Fancy or Fancy to US Utility (Batzer et al, 2002; Gleason et al, 2011). Many assumptions about the life cycles of SB fungi have been based on that of the flyspeck fungus Zygophiala jamaicensis. With a well-described sexual stage and distinctive conidial morphology, Z. jamaicensis has been much easier to study in vivo than the SB fungi, many of which lack known teleomorphs and/or produce nondescript conidia. In addition, the study of Z. jamaicensis made sense from the perspective of applied agriculture because FS has traditionally been considered more difficult to control than SB; however, recent studies have shown that this is not always the case (Brannen et al., 2010; Hickey et al., 2001; Rosenberger et al., 2004; Rosenberger et al., 2005; Sutton et al., 2004; Sutton et al., 2009). Much about the life cycles of the SB fungi remains unknown, and even the composition of communities of
SB fungi in specific regions is not clear. This lack of knowledge hinders efforts to improve SBFS management programs.

Recent research has shown that species of SB fungi can differ significantly from one another in terms of geographic distribution, important epidemiological factors, and physiological responses to the environment. Species composition of the SB complex varies among states (Diaz-Arias et al., 2010) and among orchards within a state (Ismail et al., 2010; Batzer et al., 2012). The timing of colony establishment and/or colony appearance in apple orchards differs between species (Sisson et al., 2007; Ismail et al., 2010; Batzer et al., 2012). Different SB fungi have distinct temperature optima and modes for carbon source utilization (Johnson and Sutton, 2000; Vande Voort et al., 2003; Tentinger, 2004; Batzer et al., 2010). Perhaps most important from an applied management perspective, they have disparate responses to fungicides in vitro (Sutton et al., 1985; Barrett et al., 2002; Tarnowski et al., 2003). To optimize management strategies, a better understanding of the composition of the SB complex in a particular region is clearly essential.

It is believed that orchard borders are a haven for SB species, and that SB outbreaks in orchards are the result of inoculum produced on nearby reservoir hosts (Gleason et al., 2011). SB fungi have been isolated from several perennial plants that are frequently found in orchard borders (Colby, 1920; Zaring, 1929; Gardner and Baines, 1931; Hickey, 1960; Johnson et al., 1997; Hemnani et al., 2007). Baines and Gardner (1931) isolated Gloeodes pomigena from 24 reservoir hosts; isolates from 15 of these were found to infect apples under laboratory conditions. Hickey (1960) isolated G. pomigena from eight reservoir host species and succeeded in infecting apples with these isolates. Geastrumia polystigmatis (Johnson and Sutton, 1994) and Peltaster fructicola (Johnson et al., 1997) are known to infect blackberry (Rubus alleghaniensis) in North Carolina. Pseudocercosporella and Stomiopeltis species have been detected on several
reservoir hosts in Iowa and Illinois (Hemnani et al., 2007). Further investigation of SB species prevalent on reservoir hosts may reveal information about the life cycles of the fungi, thereby enhancing our understanding of SB epidemiology. Host preferences among SB species have not been investigated.

PCR technology has facilitated the identification of many SBFS species, with samples coming from scrapings directly from infested plant cuticles (Duttweiler et al., 2008) and from cultures (Batzer et al., 2005; Sun et al., 2004). Molecular methods were used by Diaz-Arias et al. (2010) to identify SBFS species on apples from eastern and midwestern states. To date, there has not been a comprehensive investigation into the SB species complex on apples or reservoir hosts in the northeastern United States. The objectives of this research were to use PCR to identify species of fungi causing SB on apples and reservoir hosts in the northeastern US, to compare the composition of the SB complex on apples with that on selected reservoir hosts, and to investigate genetic variability in one of the most prevalent species found on apples, *Geastrumia polystigmatis*.

**Materials and Methods**

*Isolate collection.* Reservoir host tissues (stems) and apple fruit were collected biweekly from June 15 to September 30, 2012 at sites in Leominster, Harvard, and Belchertown, MA, while plant material was sampled once at the other sites (Figure 1). Reservoir host species varied among sites: common blackberry (*Rubus allegheniensis*), Oriental bittersweet (*Celastrus orbiculatus*), North American sassafras (*Sassafras albidium*), wild grape (*Vitis labrusca*), and multiflora rose (*Rosa multiflora*) were sampled. Plant samples were transported to the laboratory where they were rinsed in running tap water for 20-30 minutes and dried in a laminar flow hood. Up to 12 randomly selected colonies were excised from the cuticle of each apple or reservoir host stem and
photographed under a stereoscope (Figure 2). A small amount of mycelium was taken from each colony and cultured on acidified 2% water agar. Excised colonies were preserved by pressing between sheets of paper towel (Duttweiler et al., 2008). Dark, slow growing fungi were considered possible SB species. Pure cultures were established either by removal of hyphal tips or by streaking mycelium on 2% water agar and subculturing distinct colonies on quarter-strength PDA (25% PDA: 4.875g PDA and 4g agar per 500ml distilled deionized water).

**Species identification by PCR.** Approximately 75-125 mg of mycelium from each culture was placed directly into 50 µl of Prepman Ultra extraction reagent (Applied Biosystems, Foster City, CA) and processed for DNA extraction according to manufacturer instructions. For PCR, sample DNA was diluted 1:10 with DNA-free water and amplified using ITS-1F and ITS-4 primers. Each 50µl PCR reaction contained 1x buffer, 0.8mM dNTPs, 3.5mM MgCl₂, 0.5mM of each primer, 1 µl DMSO, and 1.25 U Taq polymerase. The thermocycler (Eppendorf AG, Hamburg, Germany) program consisted of a 2 minute hot start at 94°C, then 40 cycles of denaturation at 94°C for 15s, annealing at 58°C for 15s, and extension at 72°C for 60s, followed by a final period of 72°C for 10 min. Amplification product was cleaned with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) according to manufacturer instructions. Amplification was verified by electrophoresis of 5µl PCR product in a 2% agarose gel and staining with ethidium bromide. DNA extracts that did not amplify were diluted 1:20 and subject to PCR a second time. Amplified isolates were sequenced in a 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were identified by BLAST search in GenBank. Only matches with E values of 0.0 were considered. Isolates belonging to fungal genera known to cause SB on apples were included in the final analysis. Due to the unresolved nature of species in the genus, isolates identified as *Peltaster fructicola*
and those identified as *Peltaster* species were included in the general category ‘*Peltaster* species’.

*Frequency of appearance of species within SB communities.* Experimental sites were assigned to one of three regions. Eastern and Western New England are divided by 72W longitude (Figure. 1). The Hudson Valley region included all sites in eastern New York State. The sites were divided into these three regions based on shared climatic characteristics. The number of isolates of each species of SB fungus identified was listed by region and by host species. Experimental sites were considered by geographic area and as a group. SB species profiles for apples were compared to those of reservoir host species. Chi square analyses \( p \leq 0.05 \) were used to determine significant differences among regions and host species.

*Genetics of Geastrumia polystigmatis.* The most commonly identified SB species was *G. polystigmatis*. There is little information on the genetics of this fungus, and therefore the isolates in this study were analyzed further. Sequences from 54 isolates identified as *G. polystigmatis* were manually edited using MEGA 5.1 software, aligned by Muscle, and compiled in a neighbor-joining phylogenetic tree with 1,000 bootstrap replicates. The single *Geastrumia polystigmatis* sequence available from GenBank was included in the analysis. A sequence of *Libertella* sp., another species closely resembling *G. polystigmatis*, was included as an outlier.

**Results**

A total of 168 cultures were obtained from apples (Table 1). Of these cultures, 77 (45.8%) yielded sequences. One sequence could not be identified to genus and was classified as unidentified along with the isolates that yielded no PCR product. Of the remaining 76 sequences, 46 (60.5%) were identified as *Geastrumia polystigmatis*, and 18 (23.7%) were identified as either *Peltaster fructicola* or *Peltaster* species. The
remaining 12 isolates included members of the genera *Microcyclospora*, *Microcyclosporella*, *Mycosphaerella/Teratosphaeria*, and *Dissoconium*.

A total of 195 cultures were obtained from reservoir hosts (Table 1). Of these cultures, 107 (54.9%) yielded sequences. Fifteen sequences could not be identified to genus and are classified as unidentified along with the isolates that yielded no PCR product. Of the remaining 92 sequences, 23 (21.5%) were identified as *G. polystigmatis*, and 30 (28%) were identified as either *Peltaster fructicola* or *Peltaster* species. The 39 remaining isolates included eight *Microcyclospora*, seven *Tripospermum*, and five or fewer isolates from each of the following genera: *Microcyclosporella*, *Ramichloridium*, *Dissoconium*, *Diatractium*, *Devriesia*, *Mycosphaerella/Teratosphaeria*, and *Strelitziana*.

Results were tabulated by host species. *G. polystigmatis* was the species most frequently isolated from apples, while *Peltaster fructicola* and *Peltaster* spp. were more frequently isolated from reservoir hosts. Overall, these two groups of fungi were far more common than any other. Species of fungi other than *G. polystigmatis* and *Peltaster* spp. were more common on reservoir hosts than on apples. Chi square analyses indicated that these differences are significant (*p < 0.001*) when reservoir host species are divided into three categories, and also when all reservoir host species are treated as one category.

Results were also tabulated by region (Tables 2). In western New England and the Hudson Valley, *G. polystigmatis* was more frequently encountered than *Peltaster* species. The opposite was true in eastern New England, where *Peltaster* species were more common. Chi square results indicate that species composition of the isolates identified is significantly (*p< 0.001*) different among the three regions surveyed.

The final alignment of *G. polystigmatis* isolates was 384 bp in length. There appears to be little genetic variation among isolates (Figure 3.) Of the 54 sequences, 32 were identical to the *G. polystigmatis* sequence found in Genbank. Of the 22 isolates
that demonstrated differences, 8 differed by a single base, 10 by 2 bases, 4 by 3 bases, and 1 by 4 bases (Table 3).

There was some variation in morphology of G. polystigmas cultures (Figure 4). Most were dark brown to olive green, sometimes with buff-colored mycelium. Sectoring was common. No conidia were observed during microscopic observations.

**Discussion**

This study represents the first investigation into the species composition of SB on apples and reservoir hosts in the northeastern US. Although it is a small area relative to other apple producing regions, our results indicate that there are differences in the SB communities found in the three regions studied. There are several possible reasons for these differences. Cultural practices may have influenced the species found on apples. The apples in this study came from sites that were subject to fungicide regimes ranging from organic to low-spray to traditional calendar based schedules. The three regions may also differ in average rainfall, relative humidity, and temperature. Similar patterns of large differences among sites in small areas have been noted by researchers in Iowa (Ismail et al. 2010; Batzer et al., 2012).

Our results indicate that there may be significant differences in SB species found on the reservoir hosts compared with those found on apples. Fungicide treatments may differentially suppress each SB species, and thereby account for differences in the SB species found on apples versus those found on reservoir hosts. If fungicides do suppress growth of some species, more diversity of SB species would be expected on reservoir hosts. Diaz-Arias et al. (2010) reported that there were fewer SBFS species found on apples from orchards using conventional fungicide programs than on apples from non-sprayed orchards. It is noteworthy that fewer isolates of species other than G. polystigmas and Peltaster spp. were found on apples than on reservoir hosts.
Individual SB species may also have differing relative humidity and temperature optima (Johnson and Sutton, 2000). It is reasonable to believe that SB species requiring higher humidity may be more likely to be found in humid, unkempt areas of low-growing border vegetation than in the canopies of well-pruned apple trees (Ocamb-Basu, 1988; Cooley et al., 1997).

Very little genetic variability was observed among the 54 isolates of *G. polystigmatis* in this study, but only a small portion of the fungal genome was analyzed. Based on this trend, it appears that there is little variability in this fungus, the most common SB species found on apple fruit in this study. This may indicate that future epidemiological work on SB should focus on *G. polystigmatis*; however, further exploration of this organism’s genome will be necessary to reveal the full extent of its variability and to determine if it is indeed one species or polyphyletic. It also should be noted that this study identified approximately 47% of the 363 isolates collected. It is possible that the isolates that were not identified may include other species that are important to SBFS epidemiology and management, though the prevalence of *G. polystigmatis* and *Peltaster* spp. found here indicate that future work on SB management must include management of these fungi.

Understanding the species common to a particular region may be helpful in the development of improved SBFS control programs. For instance, both *G. polystigmatis* and *P. fructicola* are believed to be polycyclic on apples (Johnson *et al.*, 1997; Williamson and Sutton, 2000). This means that the epidemiology of SBFS is different in the Northeast than it is in a place such as Iowa, where the predominant species are monocyclic (Batzer *et al.*, 2010; Batzer *et al.*, 2012; Rosenberger *et al.*, 1993). This is an important distinction, as it means control strategies in the two regions would best be tailored to fungi with different epidemiology.
References


Table 2.1. Total numbers and proportions of fungal isolates recovered from sooty blotch colonies on different host species compared to the number of isolates that were identified by PCR.

<table>
<thead>
<tr>
<th>Host group</th>
<th>Total isolates recovered</th>
<th>Numbers and proportions of total isolates that were:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unidentified</td>
</tr>
<tr>
<td>Malus</td>
<td>168</td>
<td>92 (54.8%)</td>
</tr>
<tr>
<td>All reservoir hosts</td>
<td>195</td>
<td>103 (52.8%)</td>
</tr>
<tr>
<td>Rubus</td>
<td>73</td>
<td>32 (43.8%)</td>
</tr>
<tr>
<td>Celastrus</td>
<td>79</td>
<td>46 (58.2%)</td>
</tr>
<tr>
<td>Other(^x)</td>
<td>43</td>
<td>25 (58.1%)</td>
</tr>
</tbody>
</table>

\(^2\)χ\(^2\) value p<0.0001 for differences between Malus and all reservoir hosts, and for differences among Rubus, Celastrus, and Other
\(^y\)Includes 5 or fewer isolates from each of the following genera: Microcyclospora, Microcyclosporella, Ramichloridium, Mycosphaerella/Teratosphaeria, Tripospermum, Devriesia, Dissoconium, Diatractium, Strelitziana

\(^x\)Sassafras, Vitis, and Rosa species
Table 2.2. Total numbers and proportions of fungal isolates recovered from sooty blotch colonies from different geographical regions compared to the number of isolates that were identified by PCR.

<table>
<thead>
<tr>
<th>Region</th>
<th>Total isolates recovered</th>
<th>Numbers and proportions of total isolates that were:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unidentified</td>
</tr>
<tr>
<td>Eastern NE(^y)</td>
<td>144</td>
<td>90 (62.5%)</td>
</tr>
<tr>
<td>Western NE(^x)</td>
<td>137</td>
<td>64 (46.7%)</td>
</tr>
<tr>
<td>Hudson Valley(^w)</td>
<td>82</td>
<td>41 (50.0%)</td>
</tr>
</tbody>
</table>

\(^y\)Includes 5 or fewer isolates from each of the following genera: Microcyclospora, Microcyclosporella, Ramichloridium, Mycosphaerella/Teratosphaeria, Tripospermum, Devriesia, Dissoconium, Diatractium, Strelitziana

\(^x\)Includes sites east of 72W longitude in Massachusetts, New Hampshire, and Rhode Island

\(^w\)Includes sites west of 72W longitude in Massachusetts, New Hampshire, Vermont, and Connecticut

\(^w\)Includes sites in New York State
Table 2.3. Differences among 22 isolates of *G. polystigmatis* within a 384bp segment of the ITS region.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>State</th>
<th>Location</th>
<th>Host</th>
<th>Substituted Positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>N7</td>
<td>NY</td>
<td>HI</td>
<td><em>Celastrus</em></td>
<td>92, 93, 361</td>
</tr>
<tr>
<td>N8</td>
<td>NY</td>
<td>HI</td>
<td><em>Celastrus</em></td>
<td>92, 93, 361</td>
</tr>
<tr>
<td>E15</td>
<td>MA</td>
<td>CSO</td>
<td><em>Celastrus</em></td>
<td>33, 300</td>
</tr>
<tr>
<td>E17</td>
<td>MA</td>
<td>CSO</td>
<td><em>Celastrus</em></td>
<td>33, 300</td>
</tr>
<tr>
<td>E19</td>
<td>MA</td>
<td>CSO</td>
<td><em>Celastrus</em></td>
<td>91</td>
</tr>
<tr>
<td>J14</td>
<td>MA</td>
<td>SH</td>
<td><em>Celastrus</em></td>
<td>12, 90, 300</td>
</tr>
<tr>
<td>C16</td>
<td>MA</td>
<td>HV</td>
<td><em>Rubus</em></td>
<td>181</td>
</tr>
<tr>
<td>M24</td>
<td>NY</td>
<td>MD</td>
<td><em>Sassafras</em></td>
<td>300, 371</td>
</tr>
<tr>
<td>L10</td>
<td>NY</td>
<td>NY</td>
<td>apple</td>
<td>4</td>
</tr>
<tr>
<td>M9</td>
<td>NY</td>
<td>MD</td>
<td>apple</td>
<td>2</td>
</tr>
<tr>
<td>M18</td>
<td>NY</td>
<td>MD</td>
<td>apple</td>
<td>12, 19</td>
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<td>P20</td>
<td>NY</td>
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<td>92</td>
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<tr>
<td>N11</td>
<td>MA</td>
<td>HV</td>
<td>apple</td>
<td>12, 19</td>
</tr>
<tr>
<td>N12</td>
<td>MA</td>
<td>HV</td>
<td>apple</td>
<td>33, 53, 300</td>
</tr>
<tr>
<td>N13</td>
<td>MA</td>
<td>HV</td>
<td>apple</td>
<td>12, 90, 258, 300</td>
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<tr>
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<td>MA</td>
<td>HV</td>
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<td>75, 317</td>
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<tr>
<td>N15</td>
<td>MA</td>
<td>HV</td>
<td>apple</td>
<td>12, 19</td>
</tr>
<tr>
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<td>MA</td>
<td>HV</td>
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<td>12, 19</td>
</tr>
<tr>
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<td>MA</td>
<td>HV</td>
<td>apple</td>
<td>12, 300</td>
</tr>
<tr>
<td>F23</td>
<td>MA</td>
<td>CSO</td>
<td>apple</td>
<td>12, 19</td>
</tr>
<tr>
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<td>RI</td>
<td>NG</td>
<td>apple</td>
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<td>L24</td>
<td>RI</td>
<td>NG</td>
<td>apple</td>
<td>91</td>
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</tbody>
</table>
Figure 2.1. Location of observation sites. The blue line represents 72W longitude, dividing the regions of Eastern and Western New England. The Hudson Valley Region includes sites in New York State.
Figure 2.2. *Geastrumia polystigmatis* colonies on apples and reservoir hosts. A. Gold Rush apple; B. Rome apple; C. Sassafras; D. *Rubus*; E. and F. *Celastrus*.
Figure 2.3. Neighbor-joining phylogenetic tree depicting relationships among 54 isolates of *Geastrumia polystigmatis* collected from apples and reservoir hosts in the northeastern United States. Genbank sequences of *G. polystigmatis* and *Libertella* sp. are included.
Figure 2.4. Three-week old colonies of *Geastrumia polystigmatis* on half-strength PDA. Isolates are from apples (A-C) and *Sassafras* (D-F).
CHAPTER 3

EFFECTS OF CARBOHYDRATE SOURCE, TEMPERATURE, AND RELATIVE HUMIDITY ON IN VITRO GROWTH OF GEASTRUMIA POLYSTIGMATIS, A CAUSAL AGENT OF SOOTY BLOTCH ON APPLES

Abstract

Little is known of the biology and epidemiology of Geastrumia polystigmatis, a common cause of sooty blotch on apples in the northeastern United States. The objective of this research was to study the effects of carbohydrate source, temperature, and relative humidity (RH) on in vitro mycelial growth of G. polystigmatis. Colony growth was greater on half-strength potato dextrose agar (50% PDA) than on malt extract agar (MEA) or 2% water agar (WA). Mycelia on 50% PDA and MEA appeared thicker and more melanized than those on WA. All isolates demonstrated growth after three weeks incubation at constant temperatures of 8, 16, and 24 ºC, but not at 32 ºC. The optimum temperature for growth in this study was 24 ºC. Heat stress experiments demonstrated that the fungus can survive 32 ºC exposure for at least one week. The fungus also survived exposure to 37 ºC for 48 h and 42 ºC for 8 h. Mycelia grew relatively slowly at 95% RH, but did not grow at 92% RH. The most growth occurred at 99 and 100% RH. This study is the first to investigate the biology of G. polystigmatis, and indicates that in terms of its response to temperature, relative humidity and carbohydrates, it behaves much like other sooty blotch fungi that have been studied.

Introduction

Batista et al. first described the epiphytic fungus Geastrumia polystigmatis in 1960 as the sole member of its genus. K.A. Pirozynski (1971) reported it on
*Hymenocardia acida* and *Costus afer* from Tanzania and *Andira jamaicensis* from the Dominican Republic. Its distinctive conidia were found in rainwater pools at the bases of pine trees in Japan (Ando and Tsubaki, 1984) and in rivers in southern Spain (Roldan et al., 1987). In Brazil, *G. polystigmatis* and a second putative species of *Geastrumia* were isolated from leaves of the medicinal plant *Salacia crassifolia* (Dos Santos, 2011). *G. polystigmatis* is also associated with *Dimorphandra wilsonii* in Brazil (da Silva, 2012). In the United States, the fungus has been found on common blackberry (Johnson and Sutton, 1994) and on members of the genera *Celastrus*, *Vitis*, *Rosa*, *Rubus*, and *Sassafras* (see Chapter 2). Given the range of hosts and locales identified to date, it is reasonable to believe that *G. polystigmatis* can be found on a variety of plants worldwide (Williamson and Sutton, 2000).

The name *Geastrumia polystigmatis* is frequently mentioned in articles on sooty blotch and fliespeck of apples (SBFS) because it was one of the species identified by Johnson et al. (1997) in their landmark investigation into the true identity of *Gloeodes pomigena*. The blemishes on apple fruit caused by the SBFS complex are varied, and as the name implies, they fall into two general categories: sooty blotch (SB), a relatively diffuse, irregularly-shaped, dark blotchiness; and fliespeck (FS), groups of distinct dark, shiny round dots less than 1 mm in diameter (Williamson & Sutton, 2000). Originally, SBFS signs were thought to be caused by different forms of one fungus, *Leptothyrium pomii*, then by two fungi, with *Gloeodes pomigena* causing SB (Colby, 1920). Johnson et al. (1997) identified three fungi causing SB signs, *Peltaster fructicola*, *Leptodontium elatus*, and *G. polystigmatis*. More recently, the number of fungi causing SB on apple has ballooned, with at least 60 different species identified to date (Gleason et al, 2011).

Since Johnson et al. (1997) identified *G. polystigmatis* as one of the causal agents of SB, the fungus has received scant attention, and little is known of its physiology, epidemiology, or life cycle. In addition to its presence on blackberry in
orchard borders in North Carolina (Johnson and Sutton, 1994), the fungus has been associated with ramose colonies on apples in several eastern states (Johnson et al., 1997; Diaz Arias et al., 2010). Recently, G. polystigmatis was revealed to be one of the most common species causing sooty blotch signs on apples in the northeastern U. S. (see Chapter 2). Presently, management of SBFS focuses on FS, and most information regarding epidemiology of the complex is based on studies of a single FS fungus, Zygiophiala jamaicensis (Cooley et al., 2011). While empirical correlative studies indicate SB and FS fungi respond similarly to environmental factors, little specific information on the response of SB fungi to important environmental parameters exists. Our lack of understanding of G. polystigmatis therefore represents a critical gap in our knowledge of SB fungi. In addition to enhancing our understanding of this interesting epiphytic fungus, greater knowledge of its biology may facilitate development of more effective SBFS control methods that reduce fungicide use.

The objective of our research was to gather basic physiological information about the fungus, specifically the effects of media, temperature, and relative humidity (RH) on in vitro growth of G. polystigmatis.

**Materials and Methods**

*Isolate collection.* Apples (*Malus x domestica*) and stems of sassafras (*Sassafras albidium*) bearing signs of sooty blotch were collected from orchards and orchard borders in Massachusetts, New York, and Rhode Island. Sassafras stems and apple fruit were rinsed in running tap water for 20-30 minutes and dried in a laminar flow hood. Sooty blotch colonies were excised on small pieces of host tissue and placed beneath a stereoscope. A small amount of mycelium was removed from each colony with a sterile needle and cultured on 2% acidified water agar. Plates were incubated for up to 42 days. Dark, slow growing colonies were considered putative sooty blotch species. Pure
cultures were established either by removal of hyphal tips or by streaking mycelium on 2% water agar and subculturing isolated colonies on quarter-strength PDA (25% PDA: 4.875 g PDA and 4 g agar per 500 ml distilled deionized water), which has proven to be an effective and economical medium on which to culture sooty blotch fungi.

To identify *Geastrumia* and other cultured fungi, genetic sequences of the ITS region were obtained and compared to similar regions from identified fungi using the following procedure. Approximately 75-125 mg of mycelium from each culture was placed directly into 50 µl of Prepman Ultra extraction reagent (Applied Biosystems, Foster City, CA) and processed for DNA extraction according to manufacturer instructions. For PCR amplification, sample DNA was diluted 1:10 with DNA-free water and amplified using ITS-1F and ITS-4 primers. Each 50 µl PCR reaction contained 1x buffer, 0.8 mM dNTPs, 3.5 mM MgCl₂, 0.5 mM of each primer, 1 µl DMSO, and 1.25 U Taq polymerase. The thermocycler (Eppendorf AG, Hamburg, Germany) program consisted of a 2 minute hot start at 94°C, then 40 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 60 s, followed by a final period of 72°C for 10 min. Amplification product was cleaned with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) according to manufacturer instructions. Amplification was verified by electrophoresis of 5 µl PCR product in a 2% agarose gel and staining with ethidium bromide. Isolates that did not amplify were diluted 1:20 and subject to PCR a second time. Amplified isolates were sequenced in a 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were identified by BLAST search in GenBank on the National Center for Biotechnology (NCBI) website. Isolates were identified as *G. polystigmatis* based on an E value of zero. Of these isolates, 5 from *Sassafras* and 10 from apples were randomly selected for use in the following experiments (Table 1).
Fungi were grown on 60 mm plates. Plates in all experiments were inoculated with 4 mm agar plugs taken from the growing edge of 21-28 day old colonies on quarter-strength PDA. All plates were incubated in darkness. Colony diameter was assessed by averaging two perpendicular measurements taken with a ruler. All experiments were repeated once.

*Carbohydrate source experiments.* Malt extract agar (MEA), half-strength PDA (50% PDA; 9.75 g PDA and 2 g agar per 500 ml distilled deionized water), and 2% water agar (WA) were chosen for this experiment based on their different carbohydrate content (Table 2). At half strength, PDA (Becton-Dickinson, Franklin Lakes, New Jersey, USA) contains 2 g potato starch and 10 g dextrose, a monosaccharide, per liter of prepared medium. At full strength, MEA (Fluka Analytical, Buchs, Switzerland) contains 30 g malt extract/L. Malt extract contains 8-12% monosaccharides (Hickenbottom, 2013), for a total of 2.4-3.6 g/L. MEA also contains 5 g/L peptone. Water agar contains no monosaccharides or peptone.

Five isolates of *G. polystigmatis* from sassafras and five from apples were randomly selected. Three plates each of MEA, 50% PDA, and 2% water agar (WA) were inoculated with plugs of each isolate. Plates were incubated in growth chambers (Thermo Fisher Scientific, Waltham, MA, USA) in darkness at 24ºC. Colony diameter was measured at 14, 28, and 42 days.

*Temperature range experiments.* Ten isolates of *G. polystigmatis* from apples were selected, including those from the media experiment. Quarter-strength PDA plates were inoculated as described above. Three plates of each isolate were incubated at 8, 16, 24, or 32ºC. Colony diameter was measured at 14, 28, and 42 days.

*Heat stress experiments.* A subset of five isolates was randomly selected from those used in the temperature range experiments. Plates were inoculated and placed in growth chambers set at 32, 37, or 42ºC. For one week, two plates of each isolate were
removed from the 32 and 37°C growth chambers at 24-hour intervals and placed in a 24°C growth chamber. Plates incubated at 42°C were removed after 8, 16, or 24 h and placed in a 24°C growth chamber. Colony diameter was measured after 7 and 14 days incubation at 24°C.

**Relative Humidity (RH) experiments.** This experiment employed the same isolates as the temperature range experiments and an experimental design adapted from that used by Ocamb-Basu *et al.* with *S. pomi* (1988). Humidity chambers were made according to the isopiestic equilibration method developed from the work of Lang (1967) by Harris *et al.* (1970), modified by Alderman and Beute (1986), and employed by Arauz and Sutton (1989). Water agar (1.5%) made with sodium chloride solutions of 0, 0.3, 0.6, 1.5, 2.2, and 3.1 m were poured into plastic petri dishes. Once cooled and sealed with Parafilm, the RH inside of the petri dishes was 100, 99, 98, 95, 92, and 88.5% respectively. Filter paper disks (7mm) were autoclaved in V8 juice and dried in a laminar flow hood for 4-5 h. One filter paper disk was placed inside the lid of each petri dish. Filter paper disks were inoculated with agar plugs as described above. The bottom of the petri dish was placed over the lid, suspending the agar above the inoculated filter paper, and the petri dish was sealed with Parafilm. Colony diameter on the filter paper was measured after 21 days incubation at 24°C.

**Data analysis.** Data from the two repetitions of each experiment were pooled for analysis. Data collected after different incubation periods were analyzed separately within each experiment. ANOVA was used to assess significance of main effects. Mean separation in the media and RH experiments was done by Tukey’s HSD, and in the temperature experiments by polynomial regression.
Results

Carbohydrate source experiments. Type of medium had a significant effect ($p<0.0001$) on colony diameter within each of the four measurement periods (Figure 1). Average growth was greater on 50% PDA than on MEA and WA. Growth on MEA did not differ significantly from that on WA when measured after 7 and 14 days, but growth was significantly greater on WA than on MEA when measured after 21 and 28 days. Significant differences ($p<0.0001$) in mean colony diameter were also observed among isolates on all three media (Table 3).

A great deal of phenotypic variation was observed among isolates (Figure 2). Sectoring was common. Colony growth was dense and tightly appressed to the surface of the agar in MEA and 50% PDA plates. Growth on WA was comparatively sparse and less melanized than that on 50% PDA or MEA.

Temperature range experiments. Temperature had a significant effect ($p<0.0001$) on colony diameter in all three measurement periods (Figure 3). Optimum growth was observed at 24°C for all isolates. All isolates grew very little at 8°C, and not at all at 32°C. A cubic fit best represented the data, with $r^2$ values $>0.90$ for each of the three measurement periods. Growth rates among the isolates also varied. Significant ($p<0.0001$) differences in growth were observed among isolates grown for 42 days at 8, 16, and 24°C (Table 3). Some isolates (e.g. MDgdM15 and HVrmN16) grew more rapidly relative to others at cooler temperatures. Some isolates, (e.g. HIrmP22 and NGapL24) grew more rapidly relative to others at warmer temperatures. Some isolates, (e.g. CSrdB13 and NGapL23) grew at a rate that was relatively consistent across all three temperatures.

Heat stress experiments. The duration of exposure to high temperatures significantly affected colony growth in all three experiments. Mean separation of isolates
exposed to 37°C (Table 5) is representative of the trend observed in results from all three experiments.

All ten isolates survived up to 168 h exposure to 32°C, but colony growth at both 7 and 14 days was significantly affected ($p<0.0002$, $p<0.0001$) by the duration of exposure (Figure 4). There was a significant ($p<0.01$) effect of isolate in all durations of exposure to 32°C in the 14 day measurement period (data not shown).

After 24 h exposure to 37°C followed by incubation at 24°C, growth was observed within 7 days. After 48 h exposure, no growth was observed after 7 days incubation at 24°C, but growth was evident in all isolates after 14 days incubation (Figure 5). No isolates grew within 14 days of incubation at 24°C following 72 h exposure to 37°C. There was a significant ($p<0.0001$) effect of isolate within the 0 and 24h durations of exposure to 37°C in the 14 day measurement period, but not within the 48h exposure (Table 5).

A similar pattern was noted after 8 h exposure to 42°C. No growth was observed within 7 days incubation at 24°C, but growth was evident in all isolates after 14 days of incubation at 24°C (Figure 6). No isolates grew within 14 days of incubation at 24°C after 16 h exposure to 42°C. There was a significant ($p<0.037$) effect of isolate within the 8 hour duration of exposure to 42°C in the 14 day measurement period (data not shown).

**RH experiments.** Relative humidity had a significant effect ($p<0.0001$) on mycelial growth, with greater growth at higher RH (Figure 7). No growth occurred at 88.5 or 92% RH. Little growth occurred in all isolates at 95% RH. Colonies grew well at 98% RH, and significantly larger at 99 and 100% RH. There was a significant ($p<0.02$) effect of isolate in all RH levels where growth occurred (Table 6).
Discussion

The growth of *G. polystigmas* colonies on both 50% PDA and MEA was very dense and melanized, while that on WA was comparatively sparse and less melanized. Colony diameter on 50% PDA was significantly greater than that on MEA or WA, though the diameter of colonies on WA was greater than that on MEA. These differences in colony morphology are probably explained by the ready availability of monosaccharides in 50% PDA and MEA, and their absence in WA. Malt extract as used in this study contained approximately one third the concentration of monosaccharides as 50% PDA. Wrona and Grabowski (2004) showed that the first appearance of SBFS signs on apple fruit coincided with increased production of fructose and glucose. It is believed that SBFS fungi take advantage of sugars in fruit exudates, and it has been noted that increasing concentration of apple juice, which increases sugar concentration, also increases the melanization of some colonies of sooty blotch species (Bazer *et al*., 2010). Vande Voort *et al*. (2003) observed variability among isolates from six different, unnamed sooty blotch clades grown on different media, which they interpreted to mean that the clades differed at the genus and species level. In this study, alignment of a 348 bp section of the ITS region of the isolates used revealed that nine isolates were identical to the single *G. polystigmas* isolate in GenBank; the remaining isolate, NGapL24, differed by a single base (data not shown). This indicates that at least nine isolates belong to the same clade and species, *G. polystigmas*; however, there was significant variability in colony morphology among the different isolates examined. Assuming the ITS data indicating these isolates belong to the same species is valid, then variability in colony morphology on the same or different media do not necessarily indicate a difference in taxonomic classification; however, it is possible that there is more than one species in the genus (Dos Santos, 2011).
Results indicate that the optimum temperature for mycelial growth of *G. polystigmas* is approximately 24°C, with slower growth at 8 to 16°C and no growth at 32°C. In a study of six SBFS species, not including *G. polystigmas*, Batzer *et al.* (2010) also found that optimal growth of those species occurred at 20 to 25°C, with slower growth at 10 and 15°C and little to no growth at 30 or 35°C. The same study showed that those species differed in their responses to temperatures above and below the optimal range. A similar pattern was observed among isolates of *G. polystigmas* in the current study. More information is needed to determine whether this is due to genetic variability within the species or indicative of the existence of more than one species in the genus.

It is noteworthy that some growth of *G. polystigmas* occurred at 8°C, as this may indicate that the fungus can continue to grow well into the autumn harvest season, and perhaps on fruit under refrigeration. This may have implications for fruit in cold storage, as SBFS fungi are known to increase desiccation rates, decreasing the storage life of fruit (Frank *et al.*, 2010; Mirzwa-Mroz *et al.*, 2012).

Conversely, survival after 48 h exposure to 37°C and 8 h exposure to 42°C implies that *G. polystigmas* is equipped to endure high temperatures such as those that can occur during the daytime on plant surfaces directly exposed to the sun. For example, the surface temperature of apple fruit in an orchard may reach as high as 42.3°C (Glenn *et al.*, 2002). Heat tolerance is an important quality of survival for epiphytic fungi, which, unlike most plant pathogens, do not benefit from the shelter to be found inside plant tissues. The heat stress apparently stops or slows fungal growth, and the more extreme the temperature and longer the exposure to it, the more pronounced the impact. While *G. polystigmas* did not grow at 24°C following sufficiently long exposure to temperatures of 37°C or greater, it is possible that longer incubation at 24°C might have demonstrated that the fungus could survive and grow.
Researchers have long observed a prolonged period of apparently cryptic growth or stasis in SBFS fungi from the time spores land on the fruit to the time signs are first visible (Brown and Sutton, 1993; Rosenberger and Meyer, 2007; Cooley et al., 2011; Batzer et al., 2012). Our data indicate that *G. polystigmas* can survive relatively long periods of prolonged heat stress. Our data, as well as other’s (Batzer et al., 2010; Wrona and Grabowski, 2004), also show that in the absence of sugars, growth of SB fungi is less melanized, which would make them less visible on fruit surfaces. This supports the hypothesis that SBFS fungi can and do grow on fruit surfaces, surviving stress periods, and while it may appear that SBFS signs reflect a sudden and rapid growth of these fungi, it more likely reflects a sudden melanization of fungal tissue in response to exudates from fruit and/or with maturation of the colonies.

Researchers have also observed that moisture and very high relative humidity have a large effect on the timing of the first appearance of SBFS signs, and have attempted to forecast and treat SBFS using measurements of leaf wetness (Cooley et al., 2011). In this study, relative humidity had a significant effect on mycelial growth. Mycelia could grow, albeit relatively slowly, at 95% RH, but did not grow at 92% RH. The most growth occurred at 99 and 100% RH. Small differences in relative humidity in apple canopies in the range of 90 to 100% RH can have a significant impact on the development of SBFS fungi on fruit (Cooley et al., 2007). The response of *G. polystigmas* to RH and temperature in this study is similar to those observed for *Peltaster fructicola* and *Leptodontium elatius* by Johnson and Sutton (2000), in that growth optima for RH were above 95% and for temperature occurred between 20 and 28°C. While it is clear that SBFS fungi require high RH to grow, it is not clear that high RH impacts melanization, which would make them visible on fruit. This may account in part for the occasional failures of leaf wetness based forecast models for SBFS (Cooley et al., 2011).
Desiccation tolerance is also an important survival strategy for epiphytes. One might conclude from this study that mycelial growth may not occur at less than 92% RH in an orchard; however, it should be noted that RH inside the tree canopy is generally much higher than it is outside the canopy, and in some climates RH may vary significantly within different parts of the canopy (Cooley et al., 1997; Duttweiler et al., 2008). As typical weather stations record conditions outside the canopy, the RH reported for a site may be considerably lower than the RH at the fruit surface, and such discrepancies need to be considered in developing risk forecast models for SBFS based on RH.

No production of conidia or ascospores was observed directly in the course of these experiments, although the appearance of secondary colonies on filter paper and lids of petri dishes incubated at 100% RH indicate that spore production may indeed have occurred. It is believed that G. polystigmatis colonies on apples produce conidia (Williamson and Sutton, 2000), thereby initiating polycyclic infections. Many fungi produce conidia that rely upon water for dispersal, and in fact, the highly branched composite conidia of G. polystigmatis (described by Pirozynski, 1971) bear some resemblance to the tetraradiate spores of some aquatic species. Repetition of the temperature and RH experiments with frequent microscopic observations of the colonies may confirm or rule out spore production under certain conditions. Culturing the fungus in liquid media might also stimulate spore production. Greater knowledge of the influence of temperature and relative humidity on the production and germination of spores in G. polystigmatis would further enhance understanding of the epidemiology of this important sooty blotch fungus.
References


Table 3.1. Origins of isolates of *Geastrumia polystigmatis* used in experiments.

<table>
<thead>
<tr>
<th>Name</th>
<th>Location</th>
<th>Name</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIrmP22</td>
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<td>HVssC1</td>
<td>Harvard, MA</td>
</tr>
<tr>
<td>MDgdM15*</td>
<td>Modena, NY</td>
<td>HVssA23</td>
<td>Harvard, MA</td>
</tr>
<tr>
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<td>MDssM4</td>
<td>Modena, NY</td>
</tr>
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</tr>
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<td>HVrmN19*</td>
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<tr>
<td>HVrmN15*</td>
<td>Harvard, MA</td>
<td></td>
<td></td>
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<tr>
<td>NGapL23*</td>
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</tr>
<tr>
<td>NGapL24</td>
<td>North Kingstown, RI</td>
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</table>

* isolates used in heat stress experiments
Table 3.2. Amount of available monosaccharides and polysaccharides in 1L of half-strength potato dextrose agar (50% PDA), malt extract agar (MEA), and 2% water agar (WA).

<table>
<thead>
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<th>Medium</th>
<th>Carbohydrate (g/L)</th>
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<tbody>
<tr>
<td></td>
<td>Monosaccharides</td>
<td>Polysaccharides</td>
<td></td>
</tr>
<tr>
<td>50% PDA</td>
<td>10</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>MEA²</td>
<td>2.4-3.6</td>
<td>26.4-27.6</td>
<td></td>
</tr>
<tr>
<td>WA</td>
<td>0</td>
<td>0</td>
<td></td>
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</table>

²Based on information from Malt Products Corporation, Saddle Brook, NJ
Table 3.3. Mean colony diameter (mm) of ten *Geastrumia polystigmatitis* isolates grown on half-strength potato dextrose agar (50% PDA), maltose extract agar (MEA), and 2% water agar (WA) at 24ºC for 28 days. The effect of isolate was significant (*p* < 0.0001) on all three media. Mean separation by Tukey’s HSD at *p* = 0.05.

<table>
<thead>
<tr>
<th>Medium</th>
<th>50% PDA</th>
<th>MEA</th>
<th>WA</th>
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<tbody>
<tr>
<td>Isolate</td>
<td>Mean</td>
<td>CI 95%</td>
<td>Isolate</td>
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<tr>
<td>CSrdF9</td>
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<td>20.5 a ± 1.7</td>
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<td>MDgdM15</td>
<td>19.8 a ± 1.7</td>
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<tr>
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<td>HVrmN15</td>
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<tr>
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<td>17.8 ab ± 1.7</td>
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<tr>
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<td>NGapN23</td>
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<td>CSrdB13</td>
<td>10.5 d ± 1.7</td>
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Table 3.4. Mean colony diameter (mm) of ten *Geastrumia polystigmatis* isolates grown at 8, 16, and 24°C for 42 days. The effect of isolate was highly significant ($p < 0.0001$) at all three temperatures. Mean separation by Tukey’s HSD at $p = 0.05$.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>8</th>
<th>16</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>CI 95%</td>
<td>Mean</td>
</tr>
<tr>
<td><strong>Isolate</strong></td>
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<td></td>
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<td></td>
<td>HVrnN15</td>
</tr>
<tr>
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</tr>
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<td>7.0 b ± 0.4</td>
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<tr>
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<td>4.5 c ± 0.4</td>
<td></td>
<td>HVrnN19</td>
</tr>
<tr>
<td>HVrnN19</td>
<td>4.3 c ± 0.4</td>
<td></td>
<td>NGapL23</td>
</tr>
<tr>
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<td>4.1 c ± 0.4</td>
<td></td>
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<td>3.7 c ± 0.4</td>
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Table 3.5. Mean colony diameter (mm) of five *Geastrum polystigmatis* isolates after 0, 24, and 48 hours exposure to 37°C. Growth was measured after 14 days incubation at 24°C. Analysis of variance showed significant differences among isolates for the controls (0 hr of exposure) and after 24 hr exposure, but not following 48 hr exposure (p < 0.0001). Mean separation by Tukey’s HSD at p = 0.05.

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<td>CI 95%</td>
<td>Isolate</td>
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<td>± 0.5</td>
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<td>16.8 ab</td>
<td>± 0.5</td>
<td>HVrmN15</td>
</tr>
<tr>
<td>NGapL23</td>
<td>16.1 bc</td>
<td>± 0.5</td>
<td>NGapL23</td>
</tr>
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<td>MDgdM15</td>
<td>15.9 bc</td>
<td>± 0.5</td>
<td>HVrmN19</td>
</tr>
<tr>
<td>HVrmN19</td>
<td>15.3 c</td>
<td>± 0.5</td>
<td>MDgdM15</td>
</tr>
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</table>
Table 3.6. Mean colony diameter (mm) of five Geastrumia polystigmatis isolates grown at 100, 99, 98, and 95% relative humidity (RH) for 21 days. Analysis of variance showed significant differences among isolates for all levels of RH (p <0.05) by Tukey’s HSD at p= 0.05.

<table>
<thead>
<tr>
<th>Isolate</th>
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<th>98</th>
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<tbody>
<tr>
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<td>CI</td>
<td>Mean</td>
<td>CI</td>
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<td>CI</td>
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<td>± 2.4</td>
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<td>± 1.6</td>
<td>16.6 a</td>
<td>± 2.0</td>
</tr>
<tr>
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<td>± 2.2</td>
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<td>11.6 b</td>
<td>± 2.0</td>
</tr>
<tr>
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<td>± 2.2</td>
<td>14.0 ab</td>
<td>± 1.6</td>
<td>8.7 b</td>
<td>± 2.0</td>
</tr>
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<td>NGapL23</td>
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<td>13.1 ab</td>
<td>± 1.9</td>
<td>8.6 b</td>
<td>± 2.0</td>
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<td>8.5 b</td>
<td>± 2.0</td>
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Figure 3.1. Mean growth (mm) of ten Geastrumia polystigmati is isolates on half-strength potato dextrose agar (50% PDA), maltose extract agar (MEA), and 2% water agar (WA) at 24°C. Growth was measured every 7 days for 28 days.
Figure 3.2. Growth of five Geastrumia polystigmatis isolates on 66 mm diameter plates containing half-strength potato dextrose agar (50% PDA, left), malt extract agar (MEA, center), and 2% water agar (WA, right). Photographed after 32 days growth at 24°C.
Figure 3.3. Mean growth of ten Geastrumia polystigmatis isolates grown on quarter-strength potato dextrose agar at 8, 16, 24, and 32°C. For each measurement period, a cubic regression of growth versus temperature was highly significant (p < 0.0001). Regression equations for the 14, 28 and 42 day measurement periods were respectively:

\[ y = -9.360 + 0.942T - 0.064(T-20.118)^2 - 0.007(T-20.118)^3 \quad (r^2 = 91.8); \]
\[ y = -19.406 + 2.0390T - 0.137(T-20)^2 - 0.0151(T-20)^3 \quad (r^2 = 94.0); \]
\[ y = -15.509 + 2.278T - 0.192(T-20.017)^2 - 0.017(T-20.017)^3 \quad (r^2 = 94.4). \]
Figure 3.4. Mean growth of five Geastrumia polystigmatis isolates incubated at 24°C after exposure to 32°C for intervals of 0, 48, 72, 96, 120, 144, and 168 h. Growth was measured at 7 and 14 days post-exposure. For each measurement period, a cubic regression of growth versus duration of exposure was highly significant ($p < 0.0001$). Regression equations for each measurement period were respectively:

$y = 4.815 - 0.005D - 2.453e^{-5}(D-89.771)^2 - 4.858e^{-7}(D-89.771)^3$ ($r^2 = 15.9$, $p < 0.0002$);

$y = 12.272 - 0.013D + 1.714e^{-5}(D-90.870)^2 - 3.845e^{-7}(D-90.870)^3$ ($r^2 = 14.1$, $p < 0.0001$).
Figure 3.5. Mean growth of five *Geastrumia polystigmatis* isolates at 24C after exposure to 37°C for 24 to 72 h. Growth was measured at 7 and 14 days post-exposure. For each measurement period, a cubic regression of growth versus duration of exposure was highly significant ($p= <0.0001$). Regression equations for each measurement period were respectively:

\[ y = 0.775 - 0.017D + 0.002(D-36.3)^2 - 3.683e-5(D-36.3)^3 \quad (r^2= 90.7); \]
\[ y = 9.439 - 0.185D + 0.003(D-36.152)^2 + 1.1065e-5(D-36.152)^3 \quad (r^2= 88.7) \]
Figure 3.6. Mean growth of five Geastrumia polystigmatis isolates at 24C after exposure to 42C for 0, 8 or 16 h. Growth was measured at 7 and 14 days post-exposure. For each measurement period, a cubic regression of growth versus duration of exposure was highly significant (p = <0.0001). Regression equations for each measurement period were respectively:

\[ y = 2.413 - 0.302D + 0.038(D-8)^2 + 0(D-8)^3 \quad (r^2 = 95.2); \]
\[ y = 9.569 - 0.771D + 0.0433(D-8)^2 + 0(D-8)^3 \quad (r^2 = 93.7). \]
Figure 3.7. Mean growth of five *Geastrumia polystigmatis* isolates exposed to 100, 99, 98, 95, 92, or 88.5% relative humidity (RH) after 3 weeks at 24°C. Mean separation by Tukey’s HSD. Columns with the same letter are not significantly different from one another. Error bars represent 1 standard error from the mean.
CHAPTER 4

IN VITRO SENSITIVITY OF SOOTY BLOTCH FUNGI GEASTRUMIA POLYSTIGMATIS AND PELTASTER FRUCTICOLA TO SELECTED FUNGICIDES

Abstract

Control of the sooty blotch and flyspeck complex (SBFS) is an important issue for apple growers in the northeastern U.S., and the primary method for SBFS control in this region is frequent fungicide applications. Numerous field studies have examined fungicide efficacy for control of SBFS, but few have examined fungicide sensitivity of SBFS fungi in vitro. The objective of this research was to investigate the growth responses of Geastrumia polystigmatis and Peltaster fructicola, two common agents of SBFS in the northeastern United States, to trifloxystrobin, fenbuconazole, captan, mancozeb, thiophanate-methyl, penthiopyrad, and cyprodinil. Fungi were grown on quarter-strength PDA amended with fungicides for final concentrations of 0, 0.01, 0.1, 1, and 10 µl a.i. ml⁻¹. EC₅₀ values could not be calculated for captan due to irregular growth, and the data was excluded from analyses. P. fructicola was highly sensitive to all other fungicides tested, with mean EC₅₀ values ≤3.2 ppm. Mean EC₅₀ values for G. polystigmatis were <10 ppm for fenbuconazole, cyprodinil, and thiophanate-methyl, and >10 ppm for all other fungicides. The difference between mean EC₅₀ values of the two fungal species was statistically significant for all fungicides except cyprodinil and thiophanate-methyl. The addition of salicylhydroxamic acid (SHAM) to media amended with trifloxystrobin significantly decreased EC₅₀ values of P. fructicola versus trifloxystrobin alone, but SHAM had no significant effect on EC₅₀ values of G. polystigmatis. Information on fungicide sensitivity of these fungi may contribute to the improvement of SBFS control programs in the Northeast.
Introduction

Control of the sooty blotch and flyspeck complex (SBFS) is an important issue for apple growers in the northeastern U.S., and the primary method for SBFS control in this region is frequent fungicide applications. As much as 40% of the fungicides applied to an apple crop in the Northeast during the growing season is aimed at preventing SBFS (Cooley and Autio, 1997). SBFS forecast models have been developed and in some cases these have reduced fungicide use, though their performance has not been consistent (Cooley et al., 2011). This is due in part to the fact that most of the fungi in the SBFS complex- currently >60 species (Gleason et al., 2011)- are not well understood.

Numerous field studies have examined both conventional and organic fungicide efficacy for control of SBFS, with a broad range of results (e.g. Brannen et al., 2010; Cromwell et al., 2008; Rosenberger et al., 2002; Rosenberger et al., 2011a,b,c; Sutton et al., 2009; Travis et al., 2008). Presently, management of SBFS focuses on flyspeck (FS), and most information regarding epidemiology of the disease complex is based on studies of a single FS fungus, Zygiophiala jamaicensis (Rosenberger and Meyer, 2007; Cooley et al., 2011). FS is generally considered to be harder to control than sooty blotch (SB), although studies have shown that this is not always the case (Brannen et al., 2010; Hickey et al., 2001; Rosenberger et al., 2004; Rosenberger et al., 2005; Sutton et al., 2004; Sutton et al., 2009). While empirical correlative studies indicate SB and FS fungi respond similarly to environmental factors, little specific information on the response of SBFS fungi to fungicides exists.

In contrast to the active programs of control studies in the field, only three studies have examined fungicide susceptibility of SBFS fungi in vitro (Sutton et al., 1985; Barrett et al., 2002; Tarnowski et al., 2003). These studies revealed significant differences in fungicide sensitivity among SBFS clades. There is a paucity of information on sensitivity
and resistance development of specific, commonly occurring SBFS fungi to commonly used fungicides. New information on this topic would be helpful in the development of SBFS control programs.

It is generally believed that early season SBFS infestation is controlled by fungicides applied to control primary infections of the disease apple scab (causal agent *Venturia inaequalis*) (Rosenberger and Meyer, 2007). Some fungicides used for scab control, such as penthiopyrad and products containing cyprodinil as the sole active ingredient, are not labeled for use against SBFS, and therefore there is a lack of information about the effectiveness of these fungicides specifically for SBFS control.

The objective of this research was to investigate the growth responses of *Geastrumia polystigmatis* and *Peltaster fructicola*, two common agents of SB in the northeastern United States (see Chapter 2), to fungicides commonly used in apple scab and SBFS control programs.

**Materials and Methods**

Fungal isolates were obtained in 2012 from apple fruit and identified as previously described (see Chapter 2). Ten isolates of *G. polystigmatis* and eight isolates of *P. fructicola* were used in the present study (Table 1).

Seven fungicides commonly used in apple orchards were chosen: trifloxystrobin, fenbuconazole, captan, mancozeb, thiophanate-methyl, penthiopyrad, and cyprodinil (Table 2). These fungicides were chosen because they have different specific modes of action and represent different classes of fungicide; that is, each fungicide represents a major fungicide class and differs in terms of the physiological process(es) it affects in fungi. Except for penthiopyrad, commercial formulations of these active ingredients were used in this study.
All fungicides were dissolved in water-based stock solutions. Media were amended by adding measured amounts of stock solutions to quarter strength potato dextrose agar (25% PDA) that had been autoclaved and cooled to approximately 55°C. Final fungicide concentrations were 0.01, 0.1, 1 and 10 µl active ingredient (a.i.) ml\(^{-1}\). Media were poured into 60 mm plastic petri plates. To test strobilurin sensitivity in the presence of alternative oxidase inhibition, salicylhydroxamic acid (SHAM) was dissolved in 1:1 methanol: acetone and added to agar amended with 0, 0.01, 0.1, 1 and 10 µl a.i. ml\(^{-1}\) trifloxystrobin. Growth of fungi on 25% PDA amended with the solvent alone did not differ significantly from growth on unamended 25% PDA. The final SHAM concentration in all plates was 100 \(\mu g/ml\). Colony diameter of \textit{G. polystigmatis} on 25% PDA amended with SHAM alone was reduced by an average of 11% compared with growth on unamended 25% PDA, and colony diameter of \textit{P. fructicola} was reduced by an average of 40%.

In a separate experiment, 25% PDA was amended with much higher rates of trifloxystrobin for final fungicide concentrations of 15, 30, 45, and 60 µl a.i. ml\(^{-1}\). These plates were inoculated with \textit{G. polystigmatis}, incubated, and assessed as described above.

For \textit{G. polystigmatis}, a 4mm cork borer was used to remove plugs from the margins of 3-4 week old cultures grown on 25% PDA at 24°C. Due to the leathery nature of the colony thallus, plugs were taken from “lawns” of \textit{P. fructicola} created by scraping mycelium into sterile water blanks, vortexing for 20-30 seconds, spreading the resulting slurry over the surface of 25% PDA, and incubating 2-3 weeks at 24°C. Plugs were placed mycelium-side down in the center of fungicide-amended plates. Plates were sealed with Parafilm, placed in a growth chamber (Thermo Fisher Scientific, Waltham, MA, USA) in a completely randomized fashion, and incubated in darkness at 24°C for 21 days. Each treatment was replicated three times, and the experiment was repeated once.
After incubation, two perpendicular measurements of each colony were made with a ruler and the average diameter was recorded.

*Data analysis.* Data for each fungal species were analyzed separately. The effective concentration that reduced mycelial growth by 50% (EC$_{50}$) was calculated for each of the three replications of each isolate/fungicide/concentration by first calculating percent inhibition using the following equation:

\[
\frac{(\text{Diameter on unamended agar}) - (\text{Diameter on fungicide amended agar})}{\text{Diameter on unamended agar}}
\]

Percent inhibition was then probit transformed by and regressed against the log$_{10}$ concentrations of the fungicides.

There were no significant differences between repetitions of the experiment, so EC$_{50}$ values from the two repetitions were pooled for analysis, giving a total of 6 samples per EC50 calculation for each isolate. Mean EC$_{50}$ values were calculated for each fungicide. ANOVA was used to determine significance of main effects. Because the highest fungicide concentration tested was 10 ppm, EC$_{50}$ values greater than 10 were entered for data analysis as 10.1 ppm and are reported in the results as >10 ppm. Mean separation was done by Tukey’s HSD. Differences between species were determined for each fungicide by t-test. The results from the experiment with higher concentrations of trifloxystrobin were analyzed separately. Because the highest fungicide concentration tested in this experiment was 60 ppm, EC$_{50}$ values greater than 60 are reported as >60 ppm.

**Results**

Captan had a negligible effect on colony diameter of either species; however, mycelia were sparse and grew directly from the 25% PDA plug used to inoculate plates, with little growth on the amended medium. Colony diameter data were therefore
considered to misrepresent a truly resistant reaction to the fungicide. Calculation of a meaningful EC$_{50}$ was not possible, and captan was excluded from analyses.

Type of fungicide had a significant effect ($p<0.0001$) on colony diameter of *G. polystigmatis* (Figure 2). Mean EC$_{50}$ was $>10$ ppm for penthiopyrad, trifloxystrobin, trifloxystrobin + SHAM, and mancozeb, and $<10$ ppm for fenbuconazole, cyprodinil, and thiophanate-methyl.

Type of fungicide had a significant effect ($p<0.0001$) on *P. fructicola* colony diameter (Figure 3). Mean EC$_{50}$ was $<2$ ppm for all treatments except cyprodinil and mancozeb, for which the mean EC$_{50}$ values were 3.2 and 2.8 ppm, respectively.

Significant differences ($p<0.0001$) between the EC$_{50}$ values of *G. polystigmatis* and *P. fructicola* were observed in the fenbuconazole, mancozeb, penthiopyrad, trifloxystrobin, and trifloxystrobin + SHAM treatments (Table 3).

The addition of SHAM to media containing trifloxystrobin significantly ($p<0.0001$) reduced the mean EC$_{50}$ of *P. fructicola* versus trifloxystrobin alone, but had no significant effect on the mean EC$_{50}$ of *G. polystigmatis*. *P. fructicola* was more sensitive to trifloxystrobin alone than *G. polystigmatis*.

The mean EC$_{50}$ of *G. polystigmatis* isolates grown on higher concentrations of trifloxystrobin was $>60$ ppm (data not shown).

**Discussion**

The two species used in this study demonstrated statistically significant differences in their fungicide sensitivity spectra. *P. fructicola* was highly sensitive to all fungicides tested, with mean EC$_{50}$ values $\leq 3.2$ ppm. Mean EC$_{50}$ values for *G. polystigmatis* were $<10$ ppm for fenbuconazole, cyprodinil, and thiophanate-methyl, and $>10$ ppm for all other fungicides. Mean EC$_{50}$ values were significantly different for all fungicides except cyprodinil and thiophanate-methyl. These results parallel the findings
of previous authors that SBFS clades may vary in fungicide sensitivity (Sutton et al., 1985; Barrett et al., 2002; Tarnowski et al., 2003).

Both *G. polystigmas* and *P. fructicola* were sensitive to thiophanate-methyl (EC$_{50}$ 0.4 and 0.6 ppm, respectively) and to fenbuconazole (EC$_{50}$ 5.01 and 0.2 ppm, respectively), and both materials are recommended for use against both SBFS and apple scab in the Northeast (New England Tree Fruit Management Guide, 2013).

Thiophanate-methyl is rated as “excellent” for SBFS control in the field, while fenbuconazole is rated only fair (Table 2). Both fungal species were also sensitive to mancozeb, though the EC$_{50}$ for *G. polystigmas* was >10 ppm and that for *P. fructicola* was 2.80 ppm. Mancozeb is also recommended for use against apple scab, but its use must be discontinued 77 days prior to harvest of fruit, so while it is rated as highly effective against SBFS in the field (Table 2), it cannot be applied to fruit during the latter part of the growing season when SBFS is most active, and hence it is not useful as an SBFS treatment during summer.

Both fungi were also sensitive to cyprodinil (EC$_{50}$ 3.2 and 3.8 ppm, respectively), an active ingredient that is recommended in tank mixes for prevention of apple scab and powdery mildew, but is not labeled for use against SBFS. Efficacy of cyprodinil against SBFS is rated as “none” in the field (Table 2). However, the pre-mixed combination of difenoconazole (a DMI) plus cyprodinil (marketed as Inspire Super) is rated as highly effective against SBFS (New England Tree Fruit Management Guide, 2013). Since DMI fungicides in general are rated as having at best fair efficacy in the field, it may be worth re-examining the value of cyprodinil against SBFS *in vivo*.

Penthiopyrad is labeled for use against apple scab and powdery mildew, but not SBFS. Field efficacy for the fungicide has not been determined (Table 1). The results of this study indicate that *P. fructicola* is very sensitive to penthiopyrad, but *G. polystigmas*
is less so. Field studies would yield useful information about the efficacy of penthiopyrad for SBFS control.

$\text{EC}_{50}$ values from \textit{in vitro} studies cannot be directly interpreted as indications of susceptibility or resistance \textit{in vivo}, as there are many factors that may change the toxicity of chemicals in a “poison plate” assay relative to toxicity on to fungi on plant surfaces (Neely, 1969). However, relative efficacy of fungicides \textit{in vitro} at least provides a baseline of initial data on direct toxicity of fungicides to specific organisms. Under field conditions, the relative toxicity observed in lab studies may be altered by the fungicides’ abilities to redistribute on the plant tissue during subsequent rains, by it’s propensity to resist wash-off during rains, and by its stability during extended exposure to sunlight and moisture.

Resistance to strobilurin fungicides may be achieved either through the G143A mutation of the cytochrome b target site or by the use of an alternative oxidase pathway, which circumvents the action of strobilurins on cytochrome b (Wood and Holloman, 2003). SHAM inhibits the alternative oxidase pathway; significant growth in the presence of both trifloxystrobin and SHAM indicates that the fungus does not rely on the alternative oxidase pathway. In the current study, \textit{P. fructicola} was very sensitive to trifloxystrobin alone, while \textit{G. polystigmatis} was less sensitive. The addition of SHAM to media containing trifloxystrobin significantly reduced the $\text{EC}_{50}$ of \textit{P. fructicola}, but had no significant effect on that of \textit{G. polystigmatis}. These observations may indicate that some of the \textit{G. polystigmatis} isolates used in this study have either the G143A mutation or another form of resistance to trifloxystrobin. Investigation into the genetics of the Cyt b gene is necessary to determine whether resistance in conferred by the G143A mutation. As with the other fungicides, field studies will be necessary to determine the efficacy of strobilurins for SBFS control.
Results of the tests on captan were difficult to interpret. Although the mean diameter of colonies on plates amended with captan was similar to that on the control plates, colonies on captan-amended plates had more aerial hyphae than the control cultures, and the mycelia did not seem to penetrate the agar. It is possible that the fungus utilized the nutrients in the 25% PDA plug to continue growing, but this type of growth response was different from that seen with any other fungicide tested; it appeared that the fungus was attempting to avoid the fungicide as well as it could. This is interesting in light of the fact that captan is considered “good” against SBFS in the field (Table 2). It may be that captan is not truly fungicidal, but fungistatic, in that at least SB fungi do not grow in the presence of captan but captan is not toxic to the fungi. It may also be that captan suppresses conidial germination but is less effective for inhibiting mycelial growth than the other fungicides tested. Testing the ability of fungicides to inhibit spore germination may yield definitive results.

Differences in water solubility among the fungicides used in this study may have influenced fungal growth. Water-based stock solutions were used in an attempt to simulate the conditions in which fungi encounter fungicides in the field.

It should be noted that the current study used only ten isolates of *G. polystigmatis* and eight of *P. fructicola*. These isolates were gathered from several sites that were subject to a variety of fungicide regimens. An investigation utilizing more numerous isolates from each location would be necessary to determine whether or not any of the EC$_{50}$ values generated in the current study correspond to control failures in the field. A study with a larger number of isolates would also yield useful information about baseline sensitivities and the response of fungal populations to fungicide exposure.

The differences between the EC$_{50}$ values of these two species highlight the importance of understanding the species that comprise the SBFS complex in a particular region. It cannot be assumed that all species in the complex will respond to a fungicide
in the same way. This study provides some preliminary information about the sensitivity of *G. polystigmatis* and *P. fructicola* to fungicides commonly used in apple orchards in the Northeast. Further investigations may yield useful information for the development of improved SBFS control programs for that region.


Table 4.1. Origins of isolates of *Geastrumia polystigmatis* and *Peltaster fructicola* used in experiments.

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<th>Peltaster fructicola</th>
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Table 4.2. Fungicides used in this study.

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<th>Active ingredient (trade name for product in apples)</th>
<th>Manufacturer</th>
<th>Fungicide Class</th>
<th>Recommended Rate per 100 Gallons&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Field Efficacy Rating SBFS&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>captan (Captan 80 WDG)</td>
<td>Arysta Life Science</td>
<td>phthalimide</td>
<td>5/8 lb.</td>
<td>good</td>
</tr>
<tr>
<td>cyprophalin (Vanguard 75 WG)</td>
<td>Syngenta</td>
<td>anilinopyrimidine (AP)</td>
<td>3-5 oz.</td>
<td>none</td>
</tr>
<tr>
<td>fenbuconazole (Indar 2F)</td>
<td>Dow Agrosciences</td>
<td>demethylation inhibitor (DMI)</td>
<td>6-8 fl. oz.</td>
<td>fair</td>
</tr>
<tr>
<td>mancozeb (Manzate 75 DF)</td>
<td>Griffin</td>
<td>ethylene bis dithiocarbamates (EBDC)</td>
<td>1lb.</td>
<td>excellent</td>
</tr>
<tr>
<td>penthiopyrad (Fontelis)</td>
<td>DuPont</td>
<td>succinate dehydrogenase inhibitor (SDHI)</td>
<td>5.3-6.7 fl. oz.</td>
<td>unknown</td>
</tr>
<tr>
<td>thiopanate methyl (Topsin M 70WP)</td>
<td>Cerexagri-Nisso LLC</td>
<td>benzimidazole</td>
<td>4-6 oz.</td>
<td>excellent</td>
</tr>
<tr>
<td>trifloxystrobin (Flint)</td>
<td>BASF</td>
<td>quinone outside inhibitor (QoI)</td>
<td>0.67-0.8 oz</td>
<td>excellent</td>
</tr>
</tbody>
</table>

Table 4.3. Separation of means of EC$_{50}$ values (ppm) for *G. polystigmas* and *P. fructicola* within each fungicide by t test.

<table>
<thead>
<tr>
<th>Active Ingredient (a.i.)</th>
<th>Mean EC$_{50}$ $^z$ (ppm)</th>
<th>Mean EC$_{50}$ ppm</th>
<th>$p^w$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>G. polystigmas</em> $^\gamma$</td>
<td><em>P. fructicola</em> $^\times$</td>
<td></td>
</tr>
<tr>
<td>trifloxystrobin</td>
<td>&gt;10</td>
<td>0.60</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>trifloxystrobin + SHAM $^v$</td>
<td>&gt;10</td>
<td>0.05</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>mancozeb</td>
<td>&gt;10</td>
<td>2.80</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>pencythiopyrad</td>
<td>&gt;10</td>
<td>0.10</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>fenbuconazole</td>
<td>5.01</td>
<td>0.20</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>cyprodinil</td>
<td>3.8</td>
<td>3.2</td>
<td>0.1126</td>
</tr>
<tr>
<td>thiofanate-methyl</td>
<td>0.6</td>
<td>0.4</td>
<td>0.1618</td>
</tr>
</tbody>
</table>

$^z$EC$_{50}$ values calculated by probit analysis

$^\gamma$10 isolates, 6 replications per isolate

$^\times$8 isolates, 6 replications per isolate

$^w$Differences between values in rows with $p$ values <0.01 are significant

$^v$Salicylhydroxamic acid
Figure 4.1. Mean EC$_{50}$ values (ppm) for *Geastrumia polystigmatis*. Columns marked with asterisks represent values $>$10ppm, the highest concentration used in the plate assays, and are excluded from mean separation. Mean separation of values $<$10ppm by Tukey’s HSD.
Figure 4.2. Mean EC$_{50}$ values (ppm) for *Peltaster fructicola*. Mean separation by Tukey's HSD.
CHAPTER 5
CONCLUSION

The current study represents the first in-depth investigation into the identity of common sooty blotch (SB) fungi on apples and reservoir hosts in the Northeast. The results indicate that *Geastrumia polystigmatis* is the predominant cause of SB on apples in the region, while *Peltaster* species were more common on reservoir hosts. The species composition of the sooty blotch and flyspeck complex (SBFS) can differ widely among locations (Diaz-Arias *et al*., 2010; Ismail *et al*., 2010). Understanding the species common to a particular region may be helpful in the development of improved SBFS control programs. For instance, both *G. polystigmatis* and *P. fructicola* are believed to be polycyclic on apples (Johnson *et al*., 1997; Williamson and Sutton, 2000). This means that the epidemiology of SBFS is different in the Northeast than it is in a place such as Iowa, where the predominant species are monocyclic (Batzer *et al*., 2010; Batzer *et al*., 2012; Rosenberger *et al*., 1993). This is an important distinction, as it means control strategies in the two regions would best be tailored to fungi with different epidemiology.

Comparison of ITS sequences from 54 isolates of *G. polystigmatis* collected in this study revealed relatively little genetic variation in this region of the genome. ITS has been proposed for use as a universal barcode to distinguish among species of fungi (Schoch *et al*., 2012); however, this idea has never become widely accepted in the mycological community, and the concept of multi-gene phylogeny appears to be gaining favor (Rintoul *et al*., 2012). Only one sequence for *G. polystigmatis* is currently available in GenBank. Further exploration of this organism’s genome will be necessary to reveal the full extent of its variability and to determine if it is indeed one species or polyphyletic.

SBFS fungi are known to have long periods of “cryptic growth,” taking as long as three months to grow from germinated spores to visible colonies (Brown and Sutton,
In an apple orchard, this means that the fungi must be able to survive daytime surface temperatures of up to 42.3°C (Glenn et al., 2002). The results of this study demonstrated that G. polystigmatis is tolerant of heat stress, surviving exposure to 42°C for at least eight hours. Long-term survival during the growing season in many orchards would also involve exposure to fungicides. The results of this study showed that G. polystigmatis was much less sensitive than Peltaster fructicola to many of the fungicides tested. Coupled with the finding that G. polystigmatis was the dominant species on apple, this result indicates that reduced fungicide sensitivity may give G. polystigmatis a competitive edge over some other SB species in the field. A comprehensive investigation of SB species from orchards subject to different fungicide regimes may confirm this finding.

Further investigation into the epidemiology of G. polystigmatis is warranted. Spore trapping in orchards may reveal the timing of inoculum production in spring. More useful information may also be provided by studies of the effects of temperature and RH on spore production and germination, and the efficacy of fungicides for inhibition of spore germination.

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