Phenology of Infection on Apple Fruit by Sooty Blotch and Flyspeck Species in Iowa Apple Orchards

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Abstract

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Sooty blotch and flyspeck (SBFS) is a fungal disease complex that can cause significant economic losses to apple growers by blemishing the fruit surface with dark-colored colonies. Little is known about the phenology of host infection for this diverse group of epiphytes. In 2009 and 2010, we investigated the timing of infection of apple fruit by SBFS species in six commercial apple orchards in Iowa. Five trees in each orchard received no fungicide sprays after fruit set. Within 3 weeks after fruit set, 60 apples per tree were covered with Japanese fruit bags to minimize inoculum deposition. Subsequently, a subsample of bagged apples was exposed for a single 2-week-long period and then rebagged for the remainder of the growing season. Experimental treatments included seven consecutive 2-week-long exposure periods; control treatments were apples that were either bagged or exposed for the entire season. After apples had been stored at 2°C for 6 weeks following harvest, all SBFS colonies on the apples were identified to species using a PCR-RFLP

protocol. A total of 15 species were identified. For the seven most prevalent species, the number of infections per cm² of fruit surface was greatest on apples that had been exposed early in the season. Two SBFS species, *Peltaster fruticola* and *Colletogloeopsis*-like FG2, differed significantly from each other in time required to attain 50% of the total number of colonies per apple, and analysis of variance indicated a significant interaction of SBFS taxon with exposure period. Our findings are the first evidence of species-specific patterns in timing of SBFS inoculum deposition and infection on apple fruit, and strengthen previous observations that most SBFS infections resulting in visible colonies at harvest develop from infections that occur early in the fruit development period. By defining taxon-specific phenological patterns of fruit infection, our findings, when combined with knowledge of region-specific patterns of taxon prevalence, provide a foundation for development of more efficient and cost-effective SBFS management tactics.

Sooty blotch and flyspeck (SBFS) is a complex of fungal species that infect apples in humid climates worldwide. SBFS fungi also colonize many other fruits, including pear, grape, persimmon, mango, and plum, as well as the stems and waxy leaves of numerous woody plant species (Gleason et al. 2011). SBFS fungi are epiphytes, colonizing the epicuticular wax layer without penetrating the cuticle. SBFS colonies can reduce apple fruit quality; fruit with SBFS colonies are not acceptable for sale as fresh fruit and can reduce the market value of the crop by as much as 90% (Batzer et al. 2005; Williamson and Sutton 2000). Management of SBFS in eastern North America and western Europe typically requires application of four to 10 fungicide sprays per season; this practice is costly and can create environmental and human health hazards (Gleason et al. 2011; Trapman 2006).

Previous studies of the SBFS complex focused on species identification, inoculum sources, disease management, environmental biology, and cultivar susceptibility (Belding et al. 2000; Brown and Sutton 1995; Cooley et al. 2007; Díaz Arias et al. 2010; Spólti et al. 2011). With the advent of techniques to help delineate SBFS species using rDNA sequences (Batzer et al. 2005), a clearer picture of the SBFS species assemblage is gradually emerging. For example, Díaz Arias et al. (2010) provided evidence that many SBFS species differed in geographic distribution in the midwestern and eastern United States. Regionally important species assemblages have been delineated for Serbia, Turkey, Germany, Spain, and Norway (Batzer et al. 2013, 2015; Ivanović et al. 2010; Mayfield et al. 2012; Batzer, unpublished data).

Despite some progress in clarifying SBFS environmental biology, many ecological aspects of this epiphytic complex remain unexplored. For example, little attention has been focused on understanding phenological patterns of infection of apple fruit by individual SBFS taxa. The duration of the incubation period between SBFS infection and appearance of SBFS signs varies from a few weeks to several months (Brown and Sutton 1993; Johnson et al. 1997). The timing of infection periods, as well as the duration of wet periods, may affect the growth of SBFS fungi on apples (Johnson et al. 1997). For example, ascospores of *Schizothyrium pomi*, a major component of the SBFS complex in the northeastern United States, were shown to be released from fruiting bodies on *Rubus allegheniensis* during spring and early summer (Cooley et al. 2007). Understanding the timing of infection of SBFS fungi on apples may also have important implications for management; for example, fungicide applications can prevent SBFS blemishes during early phases of colonization but will not eradicate colonies once they have become visible (Brown and Sutton 1993).

In Iowa, Batzer et al. (2012) showed that the timing of appearance of colonies on apple fruit in late summer differed among SBFS species. These workers also presented evidence suggesting that SBFS epidemics in Iowa orchards were monocyclic; i.e., that there was no secondary spread of infections from apple to apple within the same growing season. Previous studies in Iowa and North Carolina showed that SBFS spores tended to land on apples early in the season (Barrett et al. 2003; Brown and Sutton 1993; Ocamp-Basu et al. 1988); however, neither the genera nor species of the SBFS fungi were identified. Consistent with the discovery that species differ in timing of colony appearance, we hypothesize that SBFS species also differ in the timing of infection of apple fruit.

The objectives of this study were i) to determine the timing of infection on apple fruit by the SBFS complex in Iowa, and ii) to ascertain whether there are taxon-specific patterns in the timing of infection of apple fruit.

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Materials and Methods

Sites. Trials were conducted in six central Iowa apple orchards in 2009 and four orchards in 2010 (Table 1). Orchard locations were as follows: Apple Ridge (AR; 42°31' N 93°14' W), Berry Patch (BP; 41°57' N 93°27' W), Center Grove (CG; 41°53' N 93°29' W), Deal's Orchard (DO; 42°00' N 94°26' W), Iowa State University Horticultural Research Station (HS; 42°06' N 93°35' W), and Pella Nursery (PN; 41°24' N 92°54' W). In 2010, orchards AR and HS were omitted from the study. In each of the 10 orchard-years, experimental plots consisted of five contiguous, mature, semidwarf trees (M7 rootstock; cv. Golden Delicious except for AR, which had cv. Liberty) located in a single row on the outer edge of the orchard.

These trees received no fungicides after the first-cover spray, which was applied 7 to 10 days after petal fall (Wienzierl et al. 2010); petal fall is the stage of apple phenology when all blossom petals have fallen from the trees. First-cover spray dates in 2009 were 25 May (AR, BP, CG, and DO), 28 May (PN), and 3 Jun (HS); first-cover spray dates in 2010 were 9 (PN), 15 (CG), 16 (BP), and 17 May (DO). Harvest dates were 14 (AR), 15 (DO), 16 (PN), and

17 Sep (BP, CG, and HS) in 2009, and 3 (PN), 8 (DO), and 13 Sep (BP, CG) in 2010. From green tip (leaf-bud break) through first cover, trees in experimental plots were sprayed with demethylation inhibitor (DMI) fungicides in order to suppress apple scab (caused by *Venturia inaequalis*), rust diseases (*Gymnosporangium* spp.), and powdery mildew (*Podosphaera leucotricha*). DMI fungicides were selected because their impact on SBFS fungi is less than that of other fungicide classes registered for use on apples in the Midwest United States (Wienzierl et al. 2010). A protectant program of insecticide sprays (Wienzierl et al. 2010) was applied throughout the season to control arthropod pests.

Treatments. Twelve to 21 days after petal fall, 60 fruit clusters per tree were thinned manually to one fruit per cluster; these fruit were then covered with two-layer paper fruit bags (Kobayashi Bag Manufacturing Co., Iida, Japan). These so-called Japanese fruit bags are used commercially in several countries to exclude fungi and insect pests (Kitagawa et al. 1992). The start date of the first exposure period differed among site-years by up to 9 days in order to accommodate differences in the date of application of the "first-cover" fungicide and insecticide spray (typically 7 to 10 days after petal fall). At

Table 1. Exposure periods, number of apples collected, mean daily temperature, and leaf wetness duration (LWD) during each 2-week-long exposure period for six orchards in central Iowa in 2009 and 2010

Exposure period ^a		2009						2010			
		AR ^b	BP	CG	DO	HS	PN	BP	CG	DO	PN
1	Start date ^c	1 Jun	5 Jun	5 Jun	4 Jun	5 Jun	3 Jun	24 May	19 May	21 May	14 May
	End date	15 Jun	19 Jun	19 Jun	18 Jun	19 Jun	17 Jun	7 Jun	2 Jun	4 Jun	28 May
	No. of apples	10	26	23	31	62	34	29	26	33	25
	Mean temperature ^d	ND ^f	19.2	19.4	18.7	20.6	19.2	21.3	19.8	20.4	ND
	LWD ^e	ND	56	70	105	22	108	93	103	101	58
2	Start date	15 Jun	19 Jun	19 Jun	18 Jun	19 Jun	17 Jun	7 Jun	2 Jun	4 Jun	28 May
	End date	29 Jun	3 Jul	3 Jul	2 Jul	3 Jul	11 Jul	21 Jun	16 Jun	18 Jun	11 Jun
	No. of apples	9	20	22	21	17	22	22	19	20	24
	Mean temperature	27.7	23.3	23.4	23.8	23.4	23.7	21.7	21.4	21.2	22
	LWD	56	56	45	132	27	123	121	115	119	104
3	Start date	29 Jun	3 Jul	3 Jul	2 Jul	3 Jul	11 Jul	21 Jun	16 Jun	18 Jun	11 Jun
	End date	13 Jul	17 Jul	17 Jul	16 Jul	17 Jul	15 Jul	5 Jul	30 Jun	2 Jul	25 Jun
	No. of apples	5	19	23	15	17	21	25	25	17	21
	Mean temperature	19.6	21.1	19.5	21.8	20.7	ND	23.2	23.3	22.4	23.7
	LWD	69	89	72	112	29	ND	95	134	123	123
4	Start date	13 Jul	17 Jul	17 Jul	16 Jul	17 Jul	15 Jul	5 Jul	30 Jun	2 Jul	25 Jun
	End date	27 Jul	31 Jul	31 Jul	30 Jul	31 Jul	29 Jul	19 Jul	14 Jul	16 Jul	9 Jul
	No. of apples	5	17	22	20	14	20	23	21	19	19
	Mean temperature	19	24.7	19.7	19.9	19.8	ND	24.2	23.8	23.5	23.1
	LWD	38	150	29	88	21	ND	115	105	137	131
5	Start date	27 Jul	31 Jul	31 Jul	30 Jul	31 Jul	29 Jul	19 Jul	14 Jul	16 Jul	9 Jul
	End date	10 Aug	14 Aug	14 Aug	13 Aug	14 Aug	12 Aug	2 Aug	28 Jul	30 Jul	23 Jul
	No. of apples	5	18	17	15	14	22	21	20	21	23
	Mean temperature	20.3	22.3	22.4	22.2	22.3	ND	24.4	25.5	24.2	24.9
	LWD	20	22	72	99	27	ND	109	ND	117	140
6	Start date ^c	10 Aug	14 Aug	14 Aug	13 Aug	14 Aug	12 Aug	2 Aug	28 Jul	30 Jul	23 Jul
	End date	24 Aug	28 Aug	28 Aug	27 Aug	28 Aug	26 Aug	16 Aug	11 Aug	13 Aug	7 Aug
	No. of apples	11	17	18	16	9	20	20	18	20	22
	Mean temperature ^d	20.2	19.8	19.9	20.3	19.4	ND	25.1	24.3	25.4	24.5
	LWD ^e	26	96	78	129	42	ND	133	ND	120	127
7	Start date	24 Aug	28 Aug	28 Aug	27 Aug	28 Aug	26 Aug	16 Aug	11 Aug	13 Aug	7 Aug
	End date	7 Sep	11 Sep	11 Sep	10 Sep	11 Sep	9 Sep	30 Aug	25 Aug	27 Aug	21 Aug
	No. of apples	8	10	16	15	12	19	15	22	23	27
	Mean temperature	ND	ND	ND	17.4	18.1	ND	25.4	23.3	25.3	24.5
	LWD	ND	ND	ND	186	ND	ND	93	ND	4	16

^a Exposure period treatments were 2-week-long periods during which apples were uncovered to allow for inoculum deposition and infection by SBFS fungi. The initial exposure period for all apples started at the onset of petal fall and continued for 12 to 21 days; the seven exposure periods then proceeded consecutively until approximately 2 weeks before harvest.

^b All orchard sites had cv. Golden Delicious, except cv. Liberty at Apple Ridge.

^c Start dates for each 2-week-long exposure period throughout growing season.

^d Mean daily temperature for each exposure period was monitored using an electronic sensor (Spectrum Technologies) placed within the apple tree canopy.

^e Leaf wetness duration (LWD) for each exposure period was monitored using an electronic sensor (Spectrum Technologies) placed within the apple tree canopy. The numbers shown represent the total hours of LWD during the exposure period.

^f ND = Not determined.

the beginning of each 2-week-long exposure period, five arbitrarily selected apples per tree on each of the five trees per plot were uncovered, and then recovered at the start of the next exposure period. Treatments included the seven consecutive exposure periods as well as two control treatments: full-season coverage by fruit bags (from the start of the first bagging period until harvest) and full-season exposure (never bagged). Exposure periods were denoted using distinct colors of flagging tape that was tied to the branch below each apple. At harvest, fruit bags were removed and apples were counted, sorted by exposure period, placed in perforated plastic bags, and stored at 2°C for 6 weeks until colonies were counted and sampled.

Monitoring of leaf wetness duration. Leaf wetness duration (LWD), defined as the cumulative number of hours that free water is present on surfaces, was monitored in each orchard during 2009 and 2010 (Table 1). This variable was identified in previous studies as a key driver of SBFS infection and colony development (Gleason et al. 2011; Williamson and Sutton 2000). Wetness sensors (WatchDog; Spectrum Technologies, Inc., Plainfield, IL) were deployed at 1.5-m height under the tree canopy, facing north at a 45-degree angle to horizontal, in study plots from fruit set until harvest.

Assessment of impact of fruit bags. Although deployment of fruit bags was deemed essential to pinpoint timing of infection periods in the orchards, the presence of fruit bags was expected to alter microenvironmental conditions at the fruit surface. Therefore, the impact of the use of fruit bags on each SBFS taxon was estimated in two ways. First, the total number of colonies for all orchard-years in the full-season-exposure control treatment was compared with the cumulative number of colonies arising from infections that were initiated during the seven consecutive 2-week-long exposure periods (Fig. 3). For the full-season-exposure control treatment, we estimated the total number of colonies of the mycelial type that characterized each SBFS genus (Batzer et al. 2012). Second, impact of fruit bags on temperature and relative humidity was assessed at HS during a 10-week period in Jun-Aug 2012. In this trial, one of a pair of adjacent apples in each of three trees was covered with a fruit bag and the other fruit was not covered; air temperature and relative humidity inside each fruit bag and adjacent to each non-covered fruit were monitored hourly using WatchDog sensors and data loggers (Spectrum Technologies).

SBFS colony characterization. After 6 weeks of postharvest storage at 2°C, SBFS colonies were assessed. On apples from bagging treatments, all SBFS colonies on each apple were excised with their subtending peels, pressed, and labeled. All pressed colonies from bagged apples were identified to species using PCR-RFLP analysis.

On fruit from the full-season-exposure control treatment, SBFS colonies were too numerous to identify efficiently using DNA-based methods; therefore, they were identified to genus by using mycelial type, based on previous PCR-RFLP studies conducted in the same orchards during 2006 to 2008 (Batzer et al. 2012) and previous studies (summarized in Gleason et al. 2011) showing that each mycelial type was associated with a distinct SBFS genus. We counted the total number of colonies of each mycelial type on each apple (Batzer et al. 2012; Gleason et al. 2011) and then used the number of colonies of each mycelial type to estimate the number of colonies associated with each genus. No SBFS colonies were observed on apples that had been covered by fruit bags for the entire 14-week treatment period.

Genomic DNA extraction. Fungal DNA was extracted directly from colonies on apple peels (Duttweiler et al. 2008) using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA). After 8 µl of PrepMan reagent was pipetted onto the colony, mycelium was scraped and suspended in the buffer; this suspension was then transferred by pipet to 25 µl PrepMan buffer. DNA was extracted directly from these samples following the manufacturer's instructions. The tubes were incubated in a thermocycler for 30 min at 56°C followed by 10 min at 100°C (Duttweiler et al. 2008). Tubes containing DNA template were stored at -20°C until PCR amplification.

Polymerase chain reaction. Amplification of the partial ribosomal DNA (rDNA) was performed with primer pair ITS-1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns 1993) and Myc1-R (5'-ACTCGTCGAAGGAGCTACG) (Duttweiler et al. 2008). The 25-µl reaction mixture contained 0.25 pM of primers, 200 µM of dNTPs, one unit of *Taq* DNA polymerase (Promega Corporation, Madison, WI), 2.5 mM of MgCl₂, and 1.0 µl of fungal DNA template. The reactions were performed in a thermocycler (PTC-100, MJ Research Inc., Waltham, MA) using the following cycling parameters: denaturation at 95°C for 95 s; 35 cycles of denaturation at 95°C for 60 s; annealing at 58°C for 60 s; extension at 72°C for 5 min; and cooling at 4°C. Five-microliter aliquots from each reaction were separated on 1% agarose gel (BioRad, Hercules, CA) at 100V in 10× Tris-borate EDTA (TBE) buffer. After electrophoresis, the gel was stained with ethidium bromide, de-stained with distilled water, and checked for the expected size of PCR product.

Restriction fragment length polymorphism (RFLP) analysis. Three units of restriction enzyme *Hae*III (Invitrogen, Carlsbad, CA) were used to digest 15-µl aliquots of PCR products with 5 µl of the reaction buffer in a final volume of 20 µl (Díaz Arias et al. 2010). The reactions were incubated at 37°C for 30 min. Electrophoresis was performed in 2% agarose gel in 10× TBE for 2 h. A 1-kb Plus DNA ladder (Invitrogen Corp.) was used to determine the size of RFLP bands. Gels were stained with ethidium bromide for 10 min and then rinsed with distilled water. Gels were photographed using UV transillumination. Banding patterns were compared with those of previously identified SBFS species or genera (Duttweiler et al. 2008).

DNA sequencing. To verify RFLP-based identifications, a subsample consisting of two SBFS colonies per banding pattern was sequenced to confirm that the RFLP banding patterns matched the expected SBFS species or genus (Duttweiler et al. 2008). For samples whose banding patterns did not match with a previously identified SBFS taxon, PCR products were sequenced using primers ITS1-F and Myc1-R. PCR products were purified (QIAquick DNA Purification Kit, QIAGEN, Valencia, CA) and automated sequencing was performed with a DNA Analyzer (Model 3730xl; Applied Biosystems) at the Iowa State University DNA Sequencing and Synthesis Facility (Ames, IA). Edited DNA sequences were provisionally identified using nBLAST searches (National Center for Biotechnology Information, NCBI, Bethesda, MD) and aligned with sequences of previously identified fungi using BioEdit (Hall 1999).

Quantification of SBFS colonies. Mean number of colonies per apple (CPA) and mean number of colonies per cm² of apple surface area (CPSA) were calculated for each exposure period. In estimating CPSA, surface area of apples was calculated based on changes in apple diameter (cv. Golden Delicious) that had been measured over the course of the 2010 growing season at the HS orchard (Katuuramu 2012). Mean estimated diameter of apples during each 2-week-long exposure period (EP1-EP7) was 25.2, 35.5, 44.5, 52.4, 59.3, 65.4, and 70.8 mm, respectively (Katuuramu 2012). Apple fruit surface area at each exposure period was calculated from estimated apple diameter using the standard spherical fruit equation (Clayton et al. 1995). We used the cumulative mean number of colonies per apple to determine the time when 50% of infections occurred for each species by graphing the cumulative CPA resulting from each consecutive 2-week-long exposure period versus the middle date of each exposure period, then using the graph to determine the date on which 50% of the cumulative total CPA occurred.

Statistical analysis. CPA and CPSA were compared among SBFS species. The 10 orchard-years were treated as replicates because preliminary statistical analysis showed significant interactions of orchard and year ($P = 0.0014$ and $P = 0.0007$, respectively). To test the hypothesis that the timing of apple infection differed among SBFS species, we used a broad sense inference comparing the fixed effects of species and exposure period to the consistency of these effects across orchard-year (narrow-sense) (PROC MIXED type 3)

(SAS Inc., Durham, NC). Least square means of exposure period for each species were compared using $P \leq 0.05$ as the threshold and orchard-year as the error term.

Results

Species identification. A total of 1,061 apples were evaluated from treatments that had been covered by fruit bags for a portion of the season during one of the 10 orchard-years, and DNA was extracted directly from 1,513 SBFS colonies and subjected to PCR-RFLP analysis. Amplicons were obtained from 1,462 colonies (96.6%) using the Capnodiales-specific primer set, and 1,417 (93.6%) of all colonies produced RFLP patterns matching those of previously identified SBFS taxa (Duttweiler et al. 2008). Forty-five SBFS colonies (3.0%) produced RFLP patterns that did not match those of previously identified SBFS taxa and were further examined using direct sequencing. BLASTn searches on GenBank indicated that these sequences came from two putative species that were not previously associated with SBFS: a *Mycosphaerella* sp. was recovered in three orchard-years and a *Penidiella* sp. was obtained once. These new ITS sequences were submitted to GenBank (Table 2). A total of 15 SBFS species were detected using PCR-RFLP and sequence analysis.

For the no-bagged control apples that had been exposed throughout the season, subsampled SBFS colonies of each mycelial type were identified to genus. The number of colonies per apple for each genus was estimated from the mycelial type counts on apple, and the corresponding genus name was based on previous PCR-RFLP (Duttweiler et al. 2008) results as follows: flyspeck (*Schizothyrium*), ridged honeycomb (*Microcyclosporella*), ramose (*Stomiopeltis*-like), discrete speck (*Dissoconium*), fuliginous (*Colletogloeopsis*-like), and punctate (*Peltaster*). No SBFS colonies were observed on apples that had been covered since 10 to 21 days after petal fall (bagged control).

Species prevalence. The eight most prevalent species (*Schizothyrium pomi*, *Microcyclosporella mali*, *Stomiopeltis* sp. RS1, *Stomiopeltis* sp. RS2, *Dissoconium aciculare*, *Colletogloeopsis*-like sp. FG2, *Peltaster* sp. P2, and *Peltaster fructicola*) were detected in either nine or 10 orchard-years and either six or seven exposure periods (Table 2). Less prevalent species, including *Diatractium*-like sp., *Uwebraunia commune*, *Ramularia* sp. P5, *Pseudoveronaea* sp., and *Mycosphaerella* sp., were detected in six or fewer orchard-years. Two species (*Phaeothecoidiella* sp. and *Penidiella* sp.) were detected in a single orchard-year (Table 2).

Phenological patterns of infection. Differences in total CPA and CPSA were detected among the seven exposure periods ($P = 0.0514$ and $P < 0.0001$, respectively) and the 15 SBFS species ($P = 0.0014$ and $P = 0.0030$, respectively) using ANOVA. In contrast, no significant differences occurred among the 10 orchard-years ($P = 0.0966$ and $P = 0.0738$, respectively). Infection period patterns were highly consistent ($P = 1.0$ for CPA and $P = 0.9990$ for CPSA) for the interaction of orchard-year $\times$ exposure period $\times$ species. Infection period patterns for each species (exposure period $\times$ species), using orchard-year for the error term, were consistent for CPSA ($P = 0.0006$) but not for CPA ($P = 0.0599$).

For most SBFS species, CPSA per exposure period was highest in the first two exposure periods and then decreased over the course of the remaining exposure periods (Fig. 1), although the rate of decrease differed among species. *Schizothyrium pomi* infections were highest during the first 8 weeks after fruit set, then declined gradually until harvest (Fig. 1A). *Microcyclosporella mali* had the largest number of CPSA for the first exposure period; it then decreased by 48% during the second exposure period and remained low throughout subsequent exposure periods (Fig. 1B). Infection patterns for *Stomiopeltis* sp. RS1 and *Stomiopeltis* sp. RS2 were similar to that of *M. mali* in that most infections occurred during the first 2 weeks after fruit set, then declined significantly ($P < 0.05$) until the end of the 14-week-long exposure period (Fig. 1C and D). The CPSA of *Dissoconium aciculare*, *Colletogloeopsis*-like sp. FG2, and *Peltaster* sp. P2 initiated during the first 4 weeks after fruit set was significantly ($P < 0.05$) higher than for subsequent exposure periods (Fig. 1E, F, and G). For *Peltaster fructicola* (Fig. 1H), however, there were no significant differences in CPSA among exposure periods; infection peaks were also not detected for less common species.

Time to 50% of colony infections. By 20 days after fruit set, *Colletogloeopsis*-like sp. FG2 had reached 50% of the total number of infections that ultimately resulted in colonies (Fig. 2). In contrast, *P. fructicola* required more than twice as long—43 days—to reach the 50% infection level; these two species differed significantly ($P < 0.05$) from each other. Other prevalent species varied in time to 50% infection from 23 to 34 days, but were statistically indistinguishable from each other.

Impact of fruit bags. No SBFS colonies were visible on control apples that had been covered continuously by fruit bags since 12 to 21 days after petal fall. In contrast, apples that were exposed throughout the fruit development period (non-bagged control) displayed many more SBFS colonies than the cumulative number of colonies formed on apples that had experienced 2 weeks of exposure and

Table 2. Prevalence of SBFS species in 10 orchard-years (6 orchards in 2009 and 4 orchards in 2010) on apples exposed during seven 2-week-long periods

Species identified ^a	Representative rDNA ITS sequence	Number of orchard-years detected ^b	Number of orchard-years detected per exposure period ^c						
			1	2	3	4	5	6	7
<i>Schizothyrium pomi</i>	AY598851	10	9	10	9	8	6	6	7
<i>Microcyclosporella</i> spp. RH	FJ425196	10	7	7	7	6	8	8	5
<i>Stomiopeltis</i> sp. RS1	AY598882	10	5	7	5	4	5	5	3
<i>Stomiopeltis</i> sp. RS2	AY598883	10	8	5	4	6	6	6	3
<i>Dissoconium aciculare</i>	AY598874	10	7	7	5	0	3	3	2
<i>Colletogloeopsis</i> -like sp. FG2	FJ425193	9	8	5	2	2	5	3	2
<i>Peltaster</i> sp. P2	AY598888	9	6	6	4	6	5	5	1
<i>Peltaster fructicola</i>	AY598887	9	4	2	0	2	2	3	3
<i>Diatractium</i> -like sp.	JQ347531	6	4	1	1	2	5	1	1
<i>Uwebraunia commune</i>	AY598876	5	4	0	1	0	1	3	0
<i>Ramularia</i> sp. P5	AY598873	4	0	1	1	1	2	0	0
<i>Pseudoveronaea</i> sp.	AY598877	3	1	0	0	1	0	0	0
<i>Mycosphaerella</i> sp. ^d	KF922739	3	1	2	0	1	0	0	0
<i>Phaeothecoidiella</i> spp.	AY598878, AY598879	1	1	1	0	0	0	0	0
<i>Penidiella</i> sp. ^d	KF922740	1	1	0	0	0	0	0	0

^a Species were delineated using a PCR-RFLP analysis of rDNA (13) and a subset was verified with sequencing.

^b Prevalence (out of 10 orchard-years).

^c Apples were covered with fruit bags within 12 to 21 days after petal fall, then for a single 2-week-long exposure period, and were subsequently rebagged until harvest. There were seven exposure periods, designated by the column headings.

^d Not previously reported as a member of the SBFS complex.

Fig. 1. Infection period patterns for colonies of the eight most prevalent SBFS species on apples during 10 orchard-years in central Iowa in 2009 and 2010. Apples were covered by fruit bags and subsamples of apples were exposed in a series of seven 2-week-long periods beginning 12 to 20 days after petal fall. Mean colonies per cm² is defined as number of colonies observed at harvest, divided by the estimated surface area of the apples during the time when the apples were exposed. Bars with the same letters are not significantly different from each other ($P < 0.05$). **A**, *Schizothyrium pomi*; **B**, *Microcyclosporella mali*; **C**, *Stomiopeltis* sp. S1; **D**, *Stomiopeltis* sp. RS2; **E**, *Dissoconium aciculare*; **F**, *Colletogloeopsis*-like sp. FG2; **G**, *Peltaster* sp. P2; **H**, *Peltaster fructicola*.

12 weeks of bagging during the 14-week treatment period. All genera of SBFS fungi that developed colonies in the non-bagged control treatment also developed colonies in the bagged treatments (Fig. 3). Paired T-tests of each SBFS genus over the 10 orchard-years indicated that the fruit bags significantly ($P < 0.05$) reduced the total number of colonies of *Stomiopeltis*-like spp., *Dissoconium* sp., and *Peltaster* spp. by 80, 93, and 97%, respectively. In contrast, fruit bags did not significantly affect the number of *Schizothyrium* sp., *Microcyclosporella* sp., and *Colletogloeopsis*-like sp. colonies. Differences

in total number of colonies between the non-covered control treatment and the summed 2-week-long exposure treatments were greater during 2010, which was a much wetter growing season than 2009 (Table 1; Ismail, unpublished data).

Relationship of leaf wetness duration to SBFS infection patterns.

Linear regression of cumulative hours of LWD with either CPA or CPSA during individual 2-week-long exposure periods over the 10 orchard-years revealed no significant relationships for any SBFS taxon (Ismail, unpublished data).

Fig. 2. Time to 50% infection for the eight most prevalent SBFS species on apples from 10 orchard-years in central Iowa in 2009 and 2010. Date to 50% infection was determined by graphing the number of colonies of each species on the middle date of each exposure period for each orchard-year, then estimating the mean days to 50% infection. Bars followed by the same letters are not significantly different ($P \leq 0.05$) based on Fishers Least Significant Difference test.

Fig. 3. Mean number of colonies associated with the six prevalent SBFS genera from 1) non-covered control apples exposed all season and 2) the total number of colonies on apples exposed during the seven 2-week-long exposure periods in 2009 and 2010. Differing letters for each genus indicate significant ($P < 0.05$) differences between control and treatment apples determined by paired T-tests based on orchard-year ($n = 10$ orchard-years).

Microenvironmental differences inside versus outside fruit bags.

Ten weeks of monitoring the microenvironment within fruit bags and ambient conditions immediately outside the bags during the 2012 growing season at HS orchard revealed that the ranges of mean daily relative humidity and maximum daily air temperature inside the bags were 74 to 77% and 24.0 to 25.0°C, respectively, compared with 73 to 77% and 24.5 to 24.7°C outside the bags.

Discussion

This study provides the first evidence of taxon-specific patterns in the timing of infection by SBFS fungi on apple fruit. Our findings build on recent evidence of taxon-specific patterns in the timing of late-season SBFS colony appearance (Batzer et al. 2012) by pinpointing the timing of an earlier event in the life cycles of these fungi. Combining knowledge of key events in the biology of SBFS taxa with recognition of which taxa are most prevalent in each region (Díaz Arias et al. 2010; Gleason et al. 2011) and how each responds to environmental conditions on the apple surface (Batzer et al. 2010; Cooley et al. 2007) is foundational in understanding fungal communities that reside on the apple surface. Our study is also the first to focus on community-wide phenological patterns of SBFS infection. Cooley and coworkers (2007) characterized the temperature dependency of thyriothecia development of *Schizothyrium pomi*, a prevalent SBFS species, on the reservoir host species *Rubus allegheniensis* in Massachusetts, but little is known about the timing of spore formation, release, and deposition for other SBFS species. Brown and Sutton (1993) pinpointed the timing of the start of the infection period for apple by the SBFS species *Gloeodes pomigena* and *S. pomi* in North Carolina as 10 to 21 days after petal fall. Our study supports this finding, since we did not detect infections on the control apples that were bagged from 12 to 21 days after petal fall until harvest. Gao et al. (2014) recently documented the development and subsequent collapse of hyphae linking clusters of sclerotium-like bodies of *Schizothyrium pomi* on apple fruit; however, the environmental biology of the events occurring between infection and colony appearance has not yet been characterized for this or any other SBFS taxa.

Taxon-specific phenological patterns have begun to emerge for the SBFS assemblage in Iowa orchards, but these patterns remain to be discovered for SBFS assemblages that are characteristic of other regions of the world. Nevertheless, the present study corroborated previous findings from Poland, Germany, and Brazil (Grabowski and Wrona 2004; Mayr et al. 2010; Spólti et al. 2011) that most SBFS infections occur during the first half of the fruit development period, although these studies did not discriminate among SBFS taxa. In the present study, the number of SBFS infections was greatest in the first half of the fruit development period, which may be due to relatively high rates of inoculum deposition during that time. However, verification of this assumption would require spore trapping accompanied by species identification of the spores.

As anticipated, the use of fruit bags for studying time-dependent patterns of infection impacted the number of infections caused by some SBFS taxa. Nevertheless, no SBFS genus was excluded from the postharvest colony counts as a result of bagging. Furthermore, monitoring of microenvironmental conditions at HS orchard during 2012 revealed that maximum, minimum, and mean daily RH and temperature differed very little inside versus outside the bags. Nevertheless, it is reasonable to assume that other microclimate alterations inside bags, such as shorter duration of wet periods and protection from rainfall and ultraviolet radiation, influenced the growth, spread, and pigmentation of SBFS fungi on apples (Batzer, unpublished data).

Another experimental bias associated with the use of fruit bags is that SBFS spores landing on apples during late summer exposure periods had less time to develop into visible colonies than spores landing during earlier exposure periods. Although it is an inevitable result of isolating multiple time periods across a growing season to infer phenological patterns, this bias was highly unlikely to have impacted the general conclusion that most SBFS infections that later became visible colonies occurred during the early weeks of the fruit development

period. Because SBFS colonies do not become visible to the naked eye until several weeks to several months after infection (Gleason et al. 2011) and apples were not examined until harvest (about 2 weeks after the end of the last exposure period) plus 3 months of 2°C storage, only the exposure periods that occurred latest in the growing season may have encountered a time bias. Furthermore, nutrient availability on the apple surface increases rapidly during the last few weeks before harvest, so SBFS growth can proceed more rapidly at that time than earlier in the season (Wrona and Grabowski 2004), which could compensate in part for reduced post-infection time in the later exposure periods. Despite the recognized limitations of fruit bags, they proved to be valuable tools for uncovering species-specific phenological patterns of apple infection.

The present study showed that *D. aciculare* infected apple fruit early in the season. Interestingly, a prior study in Iowa found that its colonies did not become visible until late in the season (Batzer et al. 2012). It therefore appears that this species infects at about the same time as other SBFS fungi but is much slower to develop visible colonies. Compared with other SBFS taxa tested in vitro, *D. aciculare* grew relatively slowly under the high (30 to 35°C daily maximum) temperatures typical of the middle of the fruit development period (July and August) in Iowa, but grew more rapidly than other taxa at 15°C, which is closer to the daily mean temperature in Iowa in September, during the final month of fruit maturation (Batzer et al. 2010).

Our experiments provide indirect evidence supporting Batzer et al.'s (2012) conclusion that the most prevalent operational taxonomic units of the SBFS assemblage in Iowa orchards exhibited monocyclic patterns of apple infection; that is, a single cycle of infection per growing season. For most of the taxa in the present study, total number of colonies per apple was only marginally higher on non-covered control apples than on the bagged apples summed over all exposure periods (Fig. 3), suggesting that secondary cycles of spore production and colony development did not occur on apple fruit. However, *Peltaster* spp. had >20-fold more colonies per apple in the non-covered (exposed all season) control than the bagged treatments, suggesting that colony numbers increased on individual fruit in the non-covered apples. *Peltaster* spp. are known to produce abundant yeast-like blastospores as secondary inoculum on apples under wet conditions (Batzer et al. 2010; Johnson et al. 1997; Wrona and Grabowski 2004), unlike the other SBFS genera in this study. Additional field research under controlled wetting conditions is needed to confirm the nature of epidemiological patterns of spread of these species on apples.

Two new potential SBFS species were detected in the study based on unique ITS sequences. It is reasonable to hypothesize that there are additional undiscovered SBFS species in Iowa orchards, since a wide diversity of reservoir hosts that surround most Iowa apple orchards may contribute to the sources of inoculum for SBFS epidemics (Gleason et al. 2011; Hemnani et al. 2008). This study also confirms previous findings (Gleason et al. 2011) identifying the eight most prevalent and abundant SBFS species that colonize apples in central Iowa.

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