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RESEARCH PAPERS

Effect of temperature and nutrient concentration on the growth of six species of sooty blotch and flyspeck fungi

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Summary. We assessed the effects of temperature and nutrient concentration on the growth of commonly occurring members of the sooty blotch and flyspeck (SBFS) complex in the Midwest United States. Radial growth *in vitro* of two isolates of each of six SBFS species (*Dissoconium aciculare*, *Colletogloeum* sp. FG2, *Peltaster* sp. P2, *Sybren* sp. CS1, *Pseudocercosporella* sp. RH1, and *Peltaster fructicola*) was measured at 10, 15, 20, 25, 30 and 35°C for 7 weeks. Optimal growth for all six 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. Differences in growth rate among species were evident at 10, 15, and 35°C. In a separate trial, the same isolates were incubated at 25°C in darkness on cellulose membrane (12–14 kDa) placed on Noble agar that had been amended to obtain concentrations of 0, 0.01, 0.05 or 0.5% apple juice. After 3 weeks, colonies were digitally photographed and colony opacity was assessed. The presence and concentration of apple juice strongly impacted colony morphology as evidenced by changes in colony tone, and some species were more sensitive to changes in apple juice concentration than others. These findings are the first published evidence of differences among newly described SBFS species in response to temperature and nutrient concentration.

Key words: apple skin, mycelial type, luminosity, *Monosporascus cannonballus*.

Introduction

The sooty blotch and flyspeck (SBFS) complex is a group of epiphytic fungi that colonizes the epicuticular wax layer of the fruit of apple (*Malus* × *domestica* Borkh.) and pear (*Pyrus communis* L.) as well as the leaves, stems, and fruit of many other plants. In regions with moist summer weather, blemishing of apples by SBFS results in considerable economic loss to growers and necessitates frequent applications of protectant fungicides (Batzer *et al.*, 2002; Rosenberger *et al.*, 1996; Williamson and Sutton, 2000). In the eastern United States, fungicides are typically sprayed on apples three to 10 times per growing season to protect against losses of up to 90% of market value caused by SBFS (Williamson and Sutton, 2000; Batzer *et al.*, 2002).

Until recently, the identity of the causal organisms of SBFS was poorly defined. From 1920 to 1994 the flyspeck mycelial type, designated as colonies that develop clusters of shiny black, round to ovoid, sclerotium-like bodies and lack a visible mycelial mat, was ascribed to a single species, *Schizothyrium pomi* (Mont. & Fr.) Arx (Baines and Gardner, 1932; Williamson and Sutton, 2000). Sooty blotch mycelial

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types that were designated as colonies that possessed a visible mycelial mat were ascribed to a different single species, *Gloeodes pomigena* (Schwein.) Colby (Groves, 1933). This paradigm changed when Johnson *et al*. (1994, 1996, 1997, 2000) distinguished four sooty blotch species, *Peltaster fructicola* Eric M. Johnson, T.B. Sutton & Hodges, *Stomiopeltis versicolor* (Desm.) Arx, *Geastrumia polystigmatis* Bat. & M.L. Farr, and *Leptodontidium elatius* (F. Mangenot) de Hoog. Wrona (2004b) suggested two additional species, *Phialophora sessilis* de Hoog and *Tripospermum myrti* (Lind) S. Hughes; however Koch's postulates have not yet been fulfilled with these species since the colony morphology on inoculated fruit has not been compared to the morphology of the original colonies from which the isolate was obtained. With the aid of molecular tools, the SBFS complex was recently expanded to more than 60 putative species (Batzer *et al.,* 2005; Díaz, 2007). Pathogenicity of these species was verified by inoculating isolates on immature apples in an orchard, and subsequently comparing ITS sequences and colony morphology on ripened fruit with those of the original colonies (Batzer *et al.,* 2005; Díaz, 2007; Hemnani *et al.,* 2007).

Early research assessed the influence of environmental factors on *in vitro* growth and development of *Gloeodes pomigena* (Baines and Gardner, 1932; Groves, 1933; Hickey, 1960); however, morphological descriptions of the isolates used in these trials suggest that several different fungal species were inadvertently studied, compromising the reliability of the findings. More recently, it was shown that optimal radial growth of *P. fructicola* occurred between 12 and 24°C, compared to 16 to 28°C for *L. elatius* (Johnson and Sutton, 2000). In single-species experiments, the anamorph of *S. pomi* produced conidia at 16 to 20°C, whereas the optimal temperature for ascospore germination was 16 to 28°C (Ocamb-Basu and Sutton, 1988). Maturation of *S. pomi* fruiting bodies (thyrothecia) on *Rubus* canes in Massachusetts was found to be influenced by accumulation of heat units (Cooley *et al.,* 2007). These findings suggest that environmental conditions could differentially affect the risk of infestation of apples by SBFS species.

Wrona (2004a) linked the timing of appearance of SBFS blemishes to increased concentration of sugars on the fruit surface. In her studies, the first SBFS blemishes observed on apple fruit were associated with surface sugar concentrations greater than 0.15%. Amino acids were also detected on the fruit surface, and it was shown that conidial germination of *P. fructicola* was triggered by 0.1% sugar (fructose and glucose) concentrations whereas hyphal growth required amino acids (Wrona and Gleason, 2005).

Examining environmental influences on the growth of newly discovered SBFS fungi can both increase understanding of pathogen ecology and provide physiological evidence to help delineate species (Harrington and Rizzo, 1999). Our objectives were to 1) determine the influence of temperature on mycelial growth of six commonly occurring SBFS species, and 2) determine the effect of apple juice concentration on colony morphology.

Materials and methods

Fungal isolates

Putative species used in this study were obtained from a survey of nine orchards in four midwestern US states in 2000 (Batzer *et al.,* 2005). Species were selected on the basis of being relatively prevalent (occurring in at least five of the nine orchards) and being prolific spore producers (Batzer *et al.,* 2005) (Table 1). Reference strains are maintained in the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, and at Iowa State University (Table 1). With the exception of *P. fructicola* and *Dissoconium aciculare*, species designations are putative and descriptions are under way. Two isolates each of *Colletogloeum* sp. FG2, *P. fructicola*, *Peltaster* sp. P2, *Sybren* sp. CS1, *Pseudocercosporella* sp. RH1, and *Dissoconium aciculare* were recovered from -80°C glycerol storage, transferred to 2% malt extract agar (MEA, Difco® Becton Dickson and Co., Sparks, MD, USA) and incubated at 25°C in darkness for 2 weeks.

Temperature experiment

Inoculum preparation and transfer

After 2-week-old cultures were scraped with a flame-sterilized rubber policeman, mycelial fragments and conidia were suspended in tubes containing 5 mL of sterile deionized water (SDW) and vortexed for 15 seconds. One mL of the suspension was transferred onto each of four MEA plates and spread with a sterile bent glass rod to obtain a uniform distribution of mycelium; plates were incubated at 25°C in darkness for 3 weeks.

Table 1. Species, strain, Centraalbureau voor Schimmelcultures (CBS) accession number and geographic origin of the sooty blotch and flyspeck (SBFS) fungal isolates used in the temperature and nutrition experiments.

Species	Strain	CBS accession	Geographic origin (city or county, state)
Dissoconium aciculare	PEB4a	CBS 118958	Pella, IA
D. aciculare	MSTB3a	CBS 118967	New Munster, WI
Colletogloeum sp. FG2	MWF1a	CBS 118943	Indianola, IA
Colletogloeum sp. FG2	AHF4a	CBS 118944	Mooresville, MO
Pseudocercosporella sp. RH1	MWD1a	CBS 119462	Indianola, IA
Pseudocercosporella sp. RH1	AHD1a	CBS 118960	Mooresville, MO
Sybren sp. CS1	PEE1	CBS 118951	Pella, IA
Sybren sp. CS1	UIE17b	CBS 118952	Simpson, IL
Peltaster sp. P2	CUE2b	CBS 118953	Rockford, IL
Peltaster sp. P2	GTE5a	CBS 119463	Chester, IL
Peltaster fructicola	GTE1a	n.a.	Chester, IL
P. fructicola	Pf002	n.a.	Moore County, NC

n.a., not available

Individual 12-well polystyrene plates (Costar®, Corning Incorporated, Corning, NY, USA) were designated as the experimental unit. After each 22-mm-diameter well was filled with 2 mL of 2% water agar (WA) the plates were sealed in plastic wrap to prevent drying. Placement of each isolate in individual wells was randomly assigned for each plate. Six-mm-diameter plugs of mycelium, obtained from 3-week-old cultures on MEA, were transferred mycelium-side down to the center of each well. Six replicate plates (36 in total) were incubated at 10, 15, 20, 25, 30, or 35°C in darkness. An electronic sensor (Watchdog Model 450, Spectrum Technologies Inc., Plainfield, IL, USA) was placed in each incubator to monitor temperature. The experiment was conducted twice.

Measurement of mycelial growth

Every 7 to 11 days for 8 weeks, two measurements of colony diameter, along perpendicular axes, were made using a ruler and stereo microscope. Mean diameter growth for each isolate was calculated by averaging colony diameter measurements and subtracting the plug diameter. The area under the mycelial growth curve (AUMGC) was then calculated using colony diameter and the difference in time (days) between successive dates on which measurements were made (Campbell and Madden, 1990). AUMGC estimates were calculated by $\sum_{i=1}^{n-1}[(y_{i+1}+y_i)/2][t_{i+1}-t_i]$, where n= total number of observations, y_i = first colony diameter measurement expressed in mm at the the ith observation, y_i+1 = second colony diameter measurement expressed in mm at the ith observation, and $t_{i+1}-t_i$ = time (days after inoculation) at the ith observation.

Data analysis

Differences among treatments were assessed by analysis of variance (ANOVA) using PROC MIXED in SAS (Version 9.1, SAS Institute, Cary, NC, USA). The response variable was AUMGC; fixed effects were trial, temperature, species, isolates nested within species, and the resultant interactions. The random effects were the replications nested to species, trial × species, and trial × species × temperature; isolate × replication was also nested to species, trial × species, and species × temperature.

Temperature optima were determined using a one-way analysis of variance with isolate and replication as error. The optimal temperature of mycelial growth for all species ranged from 20 to 25°C. Growth at 20°C did not differ from that at 25°C (P=0.68) (Batzer, unpublished data). The F-test resulted in a significant (P<0.001) interaction of species with temperature, however; suggesting that the species responded differently at one or more of the other

temperatures used in this study. We therefore investigated temperature sensitivity for each species. Growth at sub-optimal temperatures, expressed as a proportion of optimal growth, was compared using Fisher's least significant difference (LSD) test. The baseline for optimal growth was established as the pooled mean of AUMGC values at 20 and 25°C for each species and growth data for 10, 15, 30 and 35°C were expressed as a proportion of optimal growth.

Nutrition experiment

The appearance of SBFS species on agar media can differ markedly from that on the apple fruit; however, when these fungi are cultured on firm surfaces such as parafilm, plastic, or cellulose, their appearance mimics that on apple (Ocamb-Basu and Sutton, 1988; Johnson and Sutton, 1997; Belding *et al.,* 2000). We therefore used cellulose membranes on top of agar media to obtain a fungal morphology similar to that on apple skin.

Media preparation

Noble agar (1.5%) (Difco) was prepared in four 500-mL flasks and autoclaved for 20 minutes. Filter-sterilized apple juice (Gerber$_{TM}$) was added to cooled media in order to obtain concentrations of 0, 0.01, 0.05, and 0.5%, which were selected to represent the range of sugar concentrations detected on the cuticle of ripened apple fruit (Wrona, 2004a). Full-strength Gerber$_{TM}$ apple juice contains 0.6% fructose, 0.25% glucose, and 3% sucrose (USDA, 2008). Three-ml aliquots of amended agar were dispensed into each well (3.0 cm × 3.5 cm) of eight-well rectangular culture plates (Nalge Nunc International, Rochester, NY, USA). A reduced Latin square experimental design was employed to neutralize position effects and minimize cross-contamination. A row of paired, adjacent wells, containing the same apple juice concentration, was located at each possible position within each set of four replicate plates. Rinsed, sterile 2.8 × 3.3-cm pieces of dialysis membrane (12–14 kDa Spectra/Por RC, Spectrum Labs, Rancho Domingues, CA, USA) were placed on top of 1-day-old media in the wells. In preliminary trials, the pH was 5.27, 5.28, 5.31, and 4.83 for 0, 0.01, 0.05, and 0.5% apple juice medium, respectively. Plates were stored for 4 days in a sealed crisper at 25°C to verify sterility. The experimental unit consisted of a single eight-well plate, with eight plates per isolate for a total of 96 plates in each experiment. The experiment was repeated once.

Inoculum preparation

To obtain conidia, 2-week-old cultures were scraped using a flame-sterilized rubber policeman; mycelial fragments and conidia were suspended in tubes containing 5 mL of SDW and vortexed for 15 seconds. One mL of each suspension was transferred onto each of two potato dextrose agar (PDA) plates, spread with a sterile bent glass rod, and incubated at 25°C. After 4 days, conidia from these plates were transferred to 1 mL of SDW using a flame-sterilized wire loop, vortexed for 30 seconds, and centrifuged for 2 minutes. Following centrifugation, the supernatant was removed and conidia were re-suspended in 1 mL SDW; the process was then repeated in order to thoroughly remove nutrients remaining from agar culture. With the aid of a hemacytometer the suspension was diluted to approximately five conidia 10 μL^{-1}. A 10-μL drop of the suspension was dispensed onto each well of the eight-well culture plates containing the range of apple juice concentrations.

Measurement of fungal growth

After 3 weeks of incubation at 25°C in darkness, dialysis membranes were transferred to WA and digitally photographed under a dissecting microscope, using a 3-second exposure. White balance was adjusted to a standard value at the beginning of each photographing session. Digital photographs were converted to black and white using PhotoShop 6.0 (Adobe, Minneapolis, MN, USA). For each image, margins of individual colonies were traced using the PhotoShop 6.0 slice tool to delineate them from the surrounding agar. The histogram feature was then used to assess brightness (a scale from 0 to 255; dark to light) for each colony. 'Colony tone' values (a proportional value that is lower for lighter colonies) were obtained by subtracting the brightness value for a given apple juice concentration in a plate from 255 (highest brightness value), then dividing by 255 and multiplying by 100.

Data analysis

To evaluate the relative sensitivity of isolates and species to changes in apple juice concentration, the 'colony tone' of the lower apple juice concentration was subtracted from the next higher apple juice concentration of each isolate grown on the same eight-well plate. Differences in species, isolate and replication were compared with ANOVA using PROC GLM in SAS (Version 9.1, SAS Institute)

(Table 3). Since isolates within species did not differ (P=0.1072), comparisons were made among species. Mean change in 'colony tone' between paired apple juice concentrations (0 vs. 0.01%, 0.01 vs. 0.05% and 0.05 vs. 0.5%) for each species was then compared using Fisher's protected LSD test at $P \geqq 0.05$.

Results

Temperature study

Differences among species were more pronounced at 10 and 15°C than at 30 or 35°C (Table 2). No significant differences (P>0.05) were found among isolates within a given species (Batzer, unpublished data), so comparisons were made among species. *Dissoconium aciculare* was the least inhibited species at 10 and 15°C whereas *P. fructicola* was the most inhibited species at 10°C (Table 2). No differences among species were observed at 30°C when comparing proportion of growth to optimum. Three species grew at 35°C and three species did not (Table 2).

Nutrition study

Colony morphology changed for all isolates when they were subjected to increased concentrations of apple juice (Figure 1). Colony color values increased as mycelium darkened or colonies developed sclerotium-like bodies. Differences among species between paired apple juice concentrations were significant (P<0.001) (Batzer, unpublished data); however,

Table 2. Comparison of mycelial growth of six sooty blotch and flyspeck (SBFS) species [a] on water agar in response to temperature. Data shown are the ratio of growth measured at sub-optimal temperatures to maximum growth (maximum growth is defined as mean growth at 20 and 25°C).

Species	Temperature (°C)			
	10°	15°	30°	35°
Dissoconium aciculare	0.74 a [b]	0.78 a	0.09 a	0.00 a
Colletogloeum sp. FG2	0.35 b	0.56 b	0.31 a	0.00 a
Pseudocercosporella sp. RH1	0.35 b	0.57 bc	0.16 a	0.00 a
Sybren sp. CS1	0.33 b	0.40 d	0.26 a	0.03 b
Peltaster sp. P2	0.27 b	0.42 cd	0.26 a	0.02 b
Peltaster fructicola	0.17 c	0.41 d	0.08 a	0.01 ab
LSD	0.09	0.14	0.24	0.02

[a] Means of two isolates per species.
[b] Means within columns followed by the same letter are not significantly different ($P \leq 0.05$) according to Fisher's least significant difference. Data from two trials were combined.

Table 3. Differences in colony tone[a] of six sooty blotch and flyspeck (SBFS) species grown at 25°C on Noble agar amended with apple juice to obtain concentrations of 0, 0.01, 0.05 and 0.5% apple juice.

Species	Differences in colony tone between paired treatments (% apple juice in media)		
	0.01–0	0.05–0.01	0.50–0.05
Sybren sp. CS1	12.0 a[b]	12.6 a	20.1 a
Pseudocercosporella sp. RH1	6.2 b	7.5 ab	10.5 bc
Peltaster sp. P2	3.7 b	7.8 ab	16.5 ab
Dissoconium aciculare	3.8 b	8.5 ab	9.8 bc
Peltaster fructicola	4.9 b	7.3 ab	8.3 bc
Colletogloeum sp. FG2	6.2 b	6.9 b	6.7 c
LSD	4.8	5.4	8.4

[a] Darker colonies have higher colony tone values.
[b] Means within columns followed by the same letter are not significantly different ($P \leq 0.05$) according to Fisher's least significant difference. Data from two separate trials were combined.

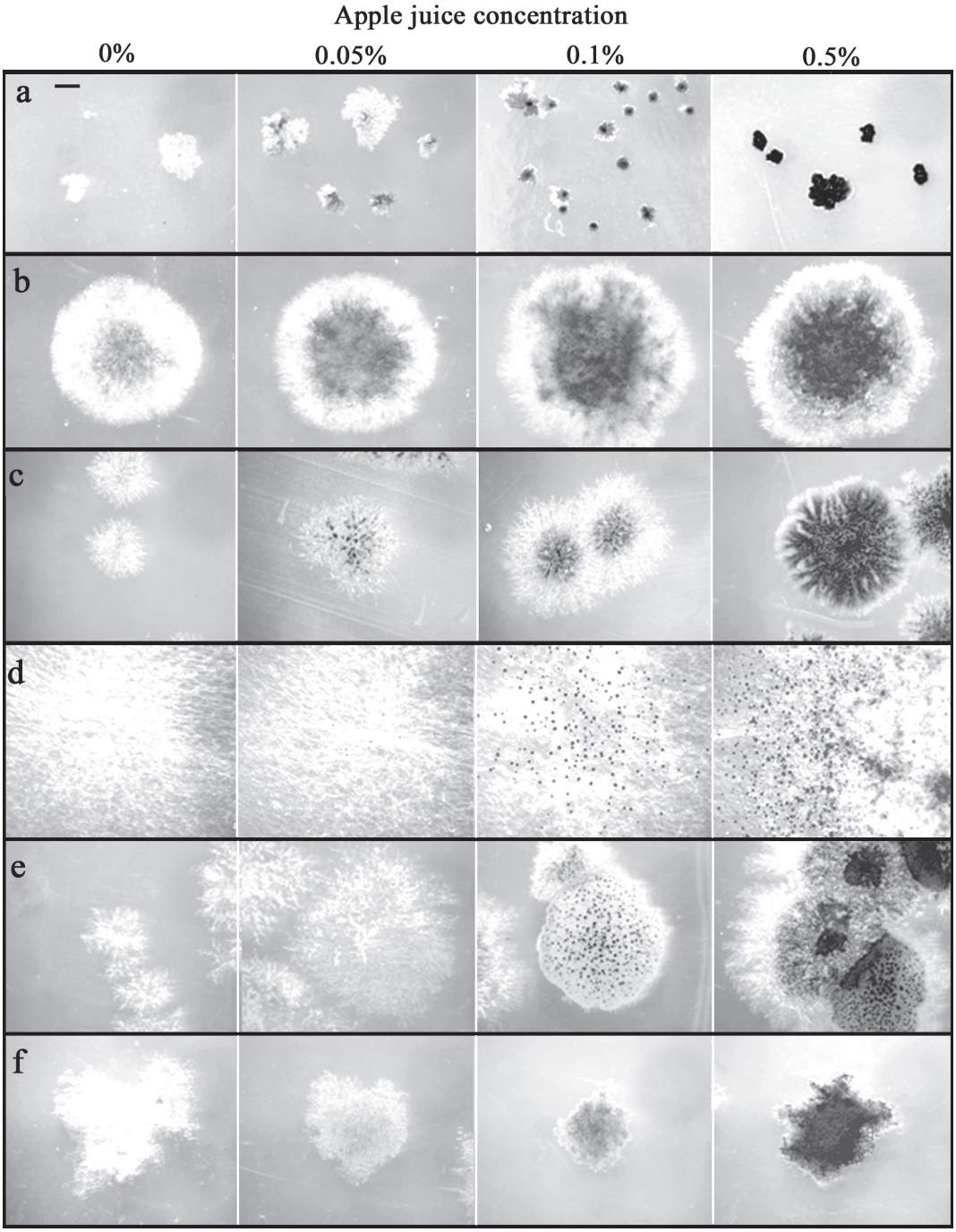

Figure 1. Growth of six species of SBFS fungi after 3 weeks at 25°C on cellulose membranes placed on Noble agar amended with apple juice (0, 0.01, 0.05 or 0.5%). a) *Sybren* sp. CS1; b) *Pseudocercosporella* sp. RH1; c) *Peltaster* sp. P2; d) *Dissoconium aciculare*; e) *Peltaster fructicola*; and f) *Colletogloeum* sp. FG2. Digital photographs were converted to black and white using PhotoShop 6.0. After that the program's drawing tool was used to delineate colony margins from the surrounding media, mean brightness was assessed for each colony using the histogram feature. Data were then converted to colony tone (dark colonies with higher values) for statistical analysis. Bar = 1 mm.

isolates within species and experimental repetitions did not differ ($P>0.05$) (Batzer, unpublished data). Therefore, further paired t-test comparisons were based on species.

Sybren sp. CS1 was the most sensitive to apple juice; colony color values changed more than other species between successive apple juice concentrations (Table 3). *Colletogloeum* sp. FG2 was the least sensitive to increases in apple juice concentration. Moderately sensitive species, *D. aciculare, P. fructicola, Peltaster* sp. P2 and *Pseudocercosporella* sp. RH1 were similar to each other in response to increased apple juice concentration.

Discussion

This is the first study contrasting responses of several SBFS species to environmental factors. Results of some previous studies (Groves, 1933; Hickey, 1960) are in question because identity of the SBFS species could not be confirmed. Other comparative studies focused on only two species (Belding, 2000; Johnson and Sutton, 2000; Wrona, 2004a). Our evidence supports the hypothesis that species in the SBFS complex have differing responses to nutrients and temperature. Physiological data, in addition to molecular and morphological characters, are valuable for defining fungal species (Harrington and Rizzo, 1999). The fact that isolate response to temperature and nutrient concentration was consistent within each of our putative species, but differed among species, strengthens the designation of these taxa as species.

The variability in growth at different temperatures suggests that climatic conditions could also influence the regional distribution of certain SBFS species. For example, 2000 and 2005 surveys of apple orchards in 14 eastern US states (Batzer *et al.,* 2005; Díaz, 2007) revealed that *D. aciculare*, which is tolerant to 10°C, was limited to the northern regions of the U.S. Conversely, *P. fructicola,* which grew little at 10 and 15°C, was among the most abundant species in the warmer southern states but was less common in the cooler northern states (Díaz, 2007). Therefore, differences in the distribution of SBFS species among geographic regions are likely to be influenced by climate, and consequently tactics used to control SBFS may need to be customized to the ecological requirements of the SBFS assemblage in each region.

Integrated management of SBFS requires detailed knowledge of pathogen ecology. Our study showed marked differences in tolerance to suboptimal temperatures. Johnson and Sutton (2000) also found that two species, *P. fructicola* and *L. elatius*, differed in tolerance to 32°C when grown in low humidity. They hypothesized that these two species may respond differently to periods of drought. Likewise, our data suggest that tolerance to cool or hot weather in the orchard may differentially influence the growth of six commonly occurring species in the Midwest US. Growth rates of SBFS species at suboptimal temperatures that commonly occur during the growing season in the Midwest U.S. could be factors to consider in the development of a disease-warning system. Using a disease-warning system developed in the southeastern US, for example, Duttweiler *et al.* (2008) showed that relative humidity predicted the timing of appearance of SBFS colonies in the midwestern US more accurately than leaf wetness duration, which is the standard weather input in the Southeast (Brown and Sutton, 1995). This difference between regions was attributed to substantial divergence in their prevailing climatic conditions (Duttweiler *et al.,* 2008). The data presented here, however, suggest that temperature should be investigated for its role in disease development in the field.

In a previous study, lack of growth by *P. fructicola* and *L. elatius* on purified apple waxes and on parafilm provided indirect evidence that SBFS fungi rely on nutrients that leak through the cuticle (Belding *et al.,* 2000). Our findings are the first evidence that morphology of SBFS fungi can be differentially affected by nutrients that increase in concentration on the surface of the apple cuticle as fruit ripen (Wrona, 2004a).

The relative insensitivity of *Colletogloeum* sp. FG2 to changes in apple juice concentration may account for recent observations that the number of colonies of this species in Iowa orchards did not increase appreciably as fruit ripened (Sisson *et al.,* 2008). Sisson (2008) also found that *Schizothyrium pomi* and *Stomiopeltis* sp. RS1 blemishes occurred in high numbers as apples first began to ripen. Although these species were not included in the present study, evidence from preliminary experiments suggested that they were highly sensitive to changes in apple juice concentration (Le Corronc *et al.,* 2006). Results of our *in vitro* study suggest that species-specific patterns in timing of SBFS colony appearance in apple orchards could be attributed in part to their differential response to changes in

nutrient availability during ripening. Because the assemblage of SBFS species varies substantially by geographic region (Díaz, 2007), refinement of SBFS warning systems needs to account for differential response to climate and nutrient availability among regionally predominant SBFS species.

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