Sooty blotch and flyspeck on apple: Expansion of the fungal complex, post-harvest removal, and heterogeneity of apple canopy wetness and its impact on the outcome of a disease-warning system

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I dedicate this dissertation to my husband Allen Carlson without whom I could not have accomplished this life-long goal. Thank you for your selfless encouragement, support and patience for three children and myself in realizing our potential.

ABSTRACT

Sooty blotch and flyspeck (SBFS) fungi blemish the cuticle of apple and pear fruit resulting in produce that is unacceptable to consumers. Previous studies reported that four species of fungi comprise the SBFS complex. The first portion of this is study surveyed the SBFS complex from nine orchards in four Midwestern states (USA.). Fungal morphology and the internal transcriber spacer (ITS) and large subunit (LSU) regions of rDNA of 422 fungal isolates were compared. Thirty putative species found among the Midwest samples were shown able to cause SBFS lesions on apple fruit in inoculation field trials. Two fungal species were identified as *Peltaster fructicola* and *Zygophiala jamaicensis,* which had been associated with SBFS in North Carolina. The LSU analyses inferred that all 30 SBFS fungi from Midwestern orchards were Dothideomycetes; one putative species was within the Pleosporales, 27 were within Dothideales, and two putative species could not be placed at the ordinal level. The LSU sequences of 17 Dothideales species clustered with LSU sequences of known species of *Mycosphaerella*.

Post-harvest dips in commercial disinfestants were used to remove SB and FS signs. Apples were dipped for 7 or 15 min in three dilutions of buffered sodium hypochlorite (Agclor 310 plus Decco 312 Buffer), three dilutions of hydrogen peroxide and peroxyacetic acid mixtures (Tsunami 100), or soap (Kleen 440), then brushed and rinsed for 30 s on a commercial grading line. Disease severity was assessed as percent diseased area using a quantitative rating system, and by counting the number of colonies of three mycelial types of SB and FS. Percent diseased area on apples was converted to USDA apple grade ratings and retail values. Both assessment methods provided similar results, but the percent-diseased-area method was less labor intensive. A 7-min dip in 800 ppm chlorine resulted in a mean

increase from 25% and 55% to 100% "Extra Fancy" grade for 'Jonathan' and 'Golden Delicious' apples, respectively, and increased market value by 31 and 14%, respectively. The 7-min, 200-ppm chlorine dip resulted in an increase from 28% and 45% to 92.5% and 96.5% "Extra Fancy" after treatment for 'Jonathan' and 'Golden Delicious', respectively. Blemishes were removed more effectively from 'Jonathan' and 'MacIntosh' apples than from 'Golden Delicious'. Mycelial types of the sooty blotch and flyspeck fungi were removed differentially by the disinfestant dip treatments.

Leaf wetness duration (LWD) was measured within apple tree canopies in four Iowa orchards. Variability of LWD and the timing of dew onset and dry-off were characterized for twelve positions in the canopy of trees in one Iowa orchard over three growing seasons and four Iowa orchards during one growing season. The upper and eastern portion of the canopy had the longest mean daily LWD and was the first to form dew and the last to dry. The lower, western portion of the canopy usually averaged about 2 hours of LWD per day less than the top of the canopy, and was the last zone where dew formed and the first to dry off. On about 25% of the nights when dew events occurred in the top of the canopy, no dew formed in the lower, west canopy. Differences in spatial variability of LWD were more acute during days on which dew was the sole source of wetness than during days on which rainfall occurred. Daily LWD in the upper, eastern portion of the canopy was slightly less than reference measurements made at 0.7-m height over turfgrass located near the apple trees. When LWD measurements from each sector of the canopy were input to a LWD-based warning system for the SBFS complex, timing of fungicide-spray thresholds varied by as much as 30 days among canopy positions.

CHAPTER 1. GENERAL INTRODUCTION

The sooty blotch and flyspeck (SBFS) fungal complex poses a major threat to apple production in humid climates worldwide. These fungi colonize the apple cuticle, creating blemishes that prevent apples from being marketable as fresh fruit and causing significant economic loss to growers. A calendar-based program of three to eight protectant fungicide sprays per season is applied to orchards in the eastern and midwestern U.S. in order to control SBFS. However, control failures, loss of U.S. registration of several effective fungicides, and development of fungicide resistance emphasize the need to develop more effective management strategies against the SBFS complex.

Thesis organization

This thesis is organized into five chapters. The first chapter provides an overview of the current understanding of the SBFS biology and control measures and presents information on the microclimate of apple canopies, measurement of wetness duration, and use of weather data in disease-warning systems. The second chapter is a manuscript submitted to *Mycologia* in October 2004, describing 30 species in the SBFS complex in the Midwest using genetic and morphological evidence. The third chapter, published in *Plant Disease* (Batzer et al. 2002), assessed the effectiveness of concentration and dip time in sodium hypochlorite solutions, and in mixtures of hydrogen peroxide and peroxyacetic acid, on removal of certain mycelial types caused by SBFS fungi from apples. The fourth chapter to be submitted to *Plant Disease* characterizes spatial variation in wetness duration in apple canopies of four Iowa orchards, and simulates a SBFS disease-warning system using wetness

duration input data from twelve locations in the canopy and a nearby location over turfgass. A portion of this data set has been published (Sentelhas et al. 2005). The fifth chapter presents the major conclusions of the thesis.

Literature Review

This section provides an overview of a) the biology and control of the SBFS complex and b) estimation of surface wetness duration and its use in disease-warning systems.

Introduction

Fungi of the sooty blotch and flyspeck (SBFS) complex colonize the cuticle of apple fruit (*Malus* X *domestica* Borkh.) in humid production areas worldwide. Although these fungi do not affect the growth or development of the fruit, they blemish the cuticle with dark smudges (sooty blotch) or groups of tiny black spots (flyspeck). Apples with SBFS signs are generally unacceptable to consumers, and the market value of the crop can be reduced by more than 90% (Williamson and Sutton 2000, Batzer et al. 2002). Apple growers control SBFS by applying fungicides every 10 to 14 days, from soon after bloom until shortly before harvest (Williamson and Sutton 2000). Without frequent protectant fungicide sprays, most of the apples grown in the East and Midwest U.S. would be affected. Even with an intensive fungicide spray program, sporadic control failures occur.

Historical background of SBFS taxonomy

The history of efforts to understand the taxonomy SBFS fungi is mired in confusion. A single causal agent, *Dothidea pomigena* Schw. (Schweintz 1832) was initially described as causing SBFS. From 1835 to 1920, more than 70 studies throughout Europe, Canada, and

the U.S. were published on SBFS taxonomy and control measures (Colby 1920). Colby commented on the "abundant misinformation" and attempted to "clear up the chaotic condition of misinformation" when he reported that sooty blotch was caused by *Gloeodes pomigena* (Schw.) Colby and flyspeck was caused by *Leptothyrium pomi* (Mont. & Fr.) Sacc. Colby also described the appearance of three thallus types of *G. pomigena* on apple fruit as fern-like, honeycomb, and reticulate, then described several shapes and sizes of pycnidia and conidia.

Groves (1933) also commented on "the superficial observations and unfounded deductions" of other studies. After an exhaustive search, he described distinct mycelial types of sooty blotch as ramose, punctate, fuliginous and rimate, which were adopted by subsequent researchers. Ramose thalli were described as a reticulate network of mycelia free of ridges or irregular mycelia with sparsely produced "plectenchymal bodies" becoming visible late in the season. Punctate colonies were characterized by abundant "plectenchymal bodies" with thick mycelial mats and distinct margins that generally lacked arborescent character. Fuliginous colonies were distinguished as having a smoky to dark appearance with no visible fruiting bodies. Groves presented evidence of cuticle penetration by fungi he described as rimate, which had projections of dark mycelia that extended between waxy plates of the cuticle. Photographs of the apple surface depicted rimate colonies with ridges of mycelium. No subsequent evidence of cuticle disruption by any SBFS fungus has been reported, even after electron microscopy studies (Belding 1996). The flyspeck pathogen (*Schizothyrium pomi* (Mont. & Fr.) v. Arx) uses wax as its sole carbon source without penetration into the cuticle (Nasu et al. 1987). "Thyrothecia" appear in discrete groups or colonies in late July or August and accompany a loose network of hyphae (Brown and Sutton

1993). Subsequent researchers (Hickey 1960, Sutton and Sutton 1994) observed a wide range of morphological variation among sooty blotch signs on apple fruit.

It was first assumed that the variable appearance of sooty blotch was related to differences of environment. Adding to the confusion, individual isolates of *G. pomigena* produced different morphological characteristics on different culture media (Baines and Gardner 1932, Groves 1933, Hickey 1960). Response to temperature, pH, nitrogen sources (nitrate, ammonium, amino acids), carbon sources (sugars, starches, alcohols) and toxin concentrations (malachite green, tartic acid, and sodium carbonate) differed among isolates (Baines and Gardner 1932, Groves 1933, Hickey 1960). Sutton and Sutton (1994) observed that some mycelial types were less sensitive to fungicides than others and that some mycelial types were more common in certain geographic locations.

Sooty blotch was documented as a fungal complex when Johnson and Sutton (1994) and Johnson et al. (1996, 1997) recognized three sooty blotch species: two from ramose (*Geastrumia polystigmatis* Batista & Ml.L. Farr and *Peltaster fructicola* Johnson), and one from fuliginous (*Leptodontium elatius* (G. Magenot) De Hoog) mycelial types. However, confusion still occurred in relating the causal organism isolated on media its appearance on apple. For example, the mycelial type on apple caused by *Peltaster fructicola* was initially reported to be ramose (Johnson et al. 1996), but was subsequently described as punctate (Williamson et al. 2004). Confusion also occurred regarding whether colonies that formed honeycombed ridges were rimate colonies, which disrupt areas of the cuticle (Groves 1933, Johnson et al. 1997, Sutton and Sutton 1994). Groves (1933) associated rimate colonies to fungi that produced abundant, distinctive cigar-shaped conidia, assumed to be *Gloeodes pomigena*. On the other hand, Sutton and Sutton (1994) and Johnson et al. (1997) reported

that conidia were not produced from fungi associated with rimate-like colonies. Considerable morphological variation has also been observed for flyspeck (*Schizothyrium pomi* (Mont. & Fr.) v. Arx; anamorph *Zygophiala jamaicensis* Mason) on apple fruit and in culture (Baker 1977, Lerner 1999, Nasu et al.1987).

Part of the uncertainty about the identity of the fungi that produced certain mycelial types may be related to the fact that colonies frequently overlap one another on apples resulting in mixed cultures. For example, a photograph depicting the morphology of *Peltaster fructicola* on apple shows two punctate and one ramose colony (Johnson et al. 1996). With the preponderance of conflicting reports and corrections regarding the taxonomic classification of the SBFS complex (Hickey 1960, Johnson et al. 1996, 1997, Williamson et al. 2004), it is clear why very little is known about the ecology of these fungi. Reports of identifying SBFS fungi on alternative hosts should be assessed with the understanding that the same studies erroneously identified fungi isolated from SBFS colonies obtained from apple fruit (Baines and Gardner 1932, Hickey 1960, Johnson et al. 1996). Progress in understanding the taxonomy and ecology of the SBFS complex has been slowed by difficulty in isolating, maintaining, identifying, and delineating these fungi in culture and on apple peels.

Molecular characters for the identification of fungi

Molecular identification has been used to identify plant pathogenic fungi that were otherwise difficult to classify. For example, colony morphology and ITS sequences were used to characterize endophytic fungi isolated from the vascular tissues of soybeans in the North Central United States (Harrington et al. 2000). In another work, unknown

basidiomycetes associated with bark beetles were identified to putative species using the mitochondrial small subunit and the spacer regions of the ribosomal DNA (rDNA) (Hsiau and Harrington 2003).

Ribosomal DNA, composed of conserved and variable regions, has been used to infer phylogenetic relationships of fungi at different taxonomic levels (Crous *et.* 2001, Lumbsch and Lindemuth 2001). The internal transcriber spacers (ITS) are often variable within and among species (Paulin-Mahady et al. 2002). On the other hand, the more conserved subunit 28S (LSU) of rDNA is useful in differentiating fungi at the genus and family level and has been shown to be supportive of differences inferred by analysis of the ITS region (Crous et al. 2001, Paulin-Mahady et al. 2002).

Molecular markers should not be used as the only lines of evidence to delimit species. A useful definition of species, proposed by Harrington and Rizzo (1999), is "the smallest aggregation of populations with a common lineage that share unique, diagnosable phenotypic characters." Phenotypic characters are associated with ecological adaptations that distinguish the niche of a species. In the case of SBFS fungi these characters include mycelial types on apple, appearance and growth rate of colonies on media and spore morphology.

Conventional control of SBFS on apple

In the Midwest U.S., control of SBFS with fungicide sprays is usually achieved with three to eight calendar-based sprays during the summer growing season at a direct cost of approximately $15- $25 per acre per spray (Gleason et al. 1994a). These summer fungicide sprays are often cost-effective for apple growers (Gold and Sutton 1989). For example, in a 1991-1994 field trial in northeast U.S., plots treated with fungicides had 11.6% greater yield

and 50% greater gross returns than untreated plots (Rosenberger et al. 1996). Summer fungicide applications resulted in a $17.50 return for each dollar spent. Yet even weekly fungicide applications are frequently inadequate to completely control SBFS, resulting in losses of 5 to 10% of marketable apples (Main and Gurtz, 1988). Without protectant fungicide sprays, almost 100% of the apples produced in the Southeast would be affected by SBFS (Sutton and Sutton 1994).

Conventional, calendar-based protectant fungicide sprays are not a sustainable management practice (Babadoost et al. 2004). Since the 1996 passage of the Food Quality Protection Act (FQPA), U.S. growers face possible loss of broad-spectrum fungicides that are effective against SBFS. Residues of fungicides, such as captan and mancozeb, present a health hazard to consumers because they are applied later in the season when SBFS fungi are more active (Belding 1996). Moreover, SBFS fungi have developed resistance to many base-spectrum fungicides. For example, *P. fructicola* has shown resistance to benomyl and *L. elatius* may be resistant to captan (Johnson et al. 1994, Sutton and Sutton 1994). Furthermore, growers experience sporadic control failures related to poor fungicide coverage, inadequate pruning, and environmental conditions that are highly favorable for disease development (Cooley et al. 1997).

Post-harvest dips and brushing

Effective post-harvest removal of SBFS on apples could allow growers to compensate for inadequate control in the orchard and to potentially reduce the number of fungicide sprays. Sodium hypochlorite dips can reduce populations of *Escherichia coli* strain O157:H7, *Botrytis cinerea, Mucor piriformis*, and *Penicillium expansum* on fruits and

vegetables (Beuchat et al. 1998, Honnay 1988, Jones and Sutton 1996, Spotts and Peters 1980, Winsiewsky 2000). Dips in "Javelle water" (chlorinated water) for removing sooty blotch were introduced by Colby (1920). Hendrix (1991) demonstrated that a 5- to 7-min dip in 500 ppm chlorine, followed by brushing and a fresh water rinse, reduced incidence of SB from 100% to 0% and FS from 100% to 27%. No phytotoxic reactions occurred, and weight loss of chlorine-treated apples during storage was not different than untreated apples (Hendrix 1991). In laboratory trials, the most effective method to eliminate *Botrytis cinerea* Pers. Ex Fr., *Mucor piriformis* Fischer, and *Penicillium expansum* Lk. Ex Thom. from whole apples was a 15-min dip in a solution of hydrogen peroxide and peroxyacetic acid (Winsiewsky 2000). Since these materials have demonstrated value as disinfestants in apple cider processing, their potential value in eradication of SB and FS signs on fresh-market apples may also merit investigation.

Disease-warning systems

Disease-warning systems are decision aids that help growers to efficiently time disease management practices, such as fungicide sprays (Gleason et al. 1997). They can be valuable tools for growers to improve farm profits by reducing the number of fungicide applications. Additional benefits of applying fungicides on an as-needed basis include reduced health hazards associated with human exposure to pesticides, non-target organisms and the environment, and delayed development of pesticide resistance by pathogens.

Disease-warning systems are developed from observational and/or inferential studies (Madden and Ellis 1988). Correlating observed weather conditions with disease development in the field identifies circumstances that optimize disease development. Many

successful disease-warning systems are empirically based (Gleason 2001). However, cause-and-effect relationships cannot be inferred solely from observations because confounding variables may be responsible for the observed results (Ramsey and Schafer 2002). Controlled inferential experiments, such as effects of specific environmental conditions on a host-pathogen interaction, provide a biological basis for decision rules used in disease-warning systems. For example, the influence of wetness duration on infection phases (such as sporulation, dispersal, spore germination, and incubation) under controlled conditions in a growth chamber is commonly determined in order to predict disease outbreaks (Rotem 1988, Waggoner 1974).

Leaf wetness duration

The growth and spread of many fungal pathogens are influenced by the duration of free moisture on any plant surface, defined as leaf wetness duration (LWD)(Thompson 1981). For this reason, LWD is a key input to many disease-warning systems. For example, apple scab infection periods are predicted using temperature and LWD combinations necessary for activity of *Venturia inequalis* (Cooke) G. Wint., (Mills and La Plante, 1951). The TOM-CAST warning system also uses temperature and LWD combinations to predict the onset of three fungal diseases on tomato (Gillespie et al. 1993, Pitblado et al. 1992).

In the case of SBFS on apple, the extended incubation period between inoculation and sign development complicates strategies for reducing fungicide sprays. In North Carolina, for example, SBFS inoculum is present on developing fruit in mid-May, and then SBFS signs develop from late June to mid-July (Brown and Sutton 1993). In the Midwest, inoculum is present on the apple fruit by mid-June, about 28 days after petal fall, yet signs do not develop

until mid-September, shortly before harvest (Barrett et al. 2003). In addition to LWD, factors that may affect the success of a disease-warning for SBFS include orchard slope, density of *Rubus* sp. and other reservoir hosts and their distance from the orchard border, foliage density in the canopy, and fungicide sensitivity (Cooley 1992, Cooley et al. 1997, Tarnowski et al. 2003).

Two kinds of models have been developed to forecast the appearance of SBFS. Both models utilize LWD. The New York flyspeck model (Leahy et al. 1998, Rosenberger 1996) is based on degradation of fungicide on apple fruit over time. The model assumes that the last spray for apple scab containing mancozeb (i.e. the first-cover spray) will provide residual activity for either 21 days or 3.5 inches of accumulated rainfall, whichever occurs first. Captan or ziram, on the other hand, will provide residual activity for either 14 days or 2.0 inches of accumulated rainfall, whichever occurs first. After these thresholds occur, subsequent application of fungicide containing benzimidazole is made after the accumulation of 150 hours of wetness (no minimum LWD threshold).

The most widely used SBFS warning system is based on the ecology of the dominant SBFS fungi in North Carolina. Brown and Sutton (1995) observed that first signs of SBFS usually occurred after 270 hours of accumulated leaf wetness, counting only periods of ≥ 4 hour duration starting from the first rain that occurred 10 days after petal fall. Using the 4-hour minimum threshold for counting wetness duration resulted in less variation among the eight growing seasons and three sites in the data set than using all wetness periods regardless of their duration. Brown and Sutton (1995) suggested that this reduction in variation occurred because laboratory studies showed that conidial germination and/or mycelial growth of *P. fructicola* and *Z. jamaicensis* required 4 to 5 hours of wetting (Johnson and Sutton

2000, Ocamb-Basu and Sutton 1988). They concluded that the eradicant properties (after infection, pre-symptom activity) of benzimidazole fungicides applied at 200 to 225 LWD hours could be used to obtain apples free of SBFS signs. Modified versions of this model have been tested in Massachusetts, Kentucky, and the Midwest, often saving two or more benzimidazole sprays (Babadoost et al. 2004, Ellis et al. 1998, Hall et al. 1997, Hartman 1995, 1996, and Smigell and Hartman 1997, 1998).

Factors affecting wetness in the apple canopy

Wetness is caused by rain, irrigation, and dew (Weiss et al. 1989). Rain has a significant effect on disease epidemics where rain splash results in detachment of spores from the host and allows for spore liberation into the air via raindrops and aerosols (Fitt et al. 1989). However, during the growing season in humid regions, dew is often the largest contributor to LWD and is the environmental factor that is considered to have the greatest impact on pathogen activity (Huber and Gillespie 1992). Dew forms when the surface temperature drops below the dew point of the surrounding air (Rosenberg 1974). Atmospheric factors and surface properties govern the rate of surface cooling. Energy transfer from the surface to the atmosphere is affected by radiation, latent heat, sensible heat, heat conduction, and heat storage (Rosenberg 1974). Dew events of longest durations occur on windless, clear nights (Garratt and Segal 1988).

Position within a canopy can shape the relative contributions of the ground and the atmosphere to dew formation and duration. Canopy models based on surface energy balance have been developed for several plant-soil-atmosphere combinations (Braud et al. 1995). Dew formation is influenced by factors like leaf area, plant architecture, arrangement of

plants in the field, and crop height that ultimately control the vertical profiles of temperature, vapor pressure, incoming short and long wave radiation, and wind speed (Huber and Gillespie 1992, Monteith and Unsworth 1990). The top of the canopy has the greatest LWD in humid climates (Jacobs et al. 1995, Wittich 1995), whereas the middle of the canopy tends to be the wettest in semi-arid climates (Weiss et al.1989). Decreased wind speed in the lower canopy may also delay drying of lower leaves (Huber and Itier 1990, Jacobs et al. 1995). Penrose and Nicol (1996) measured canopy wetness in semi-arid Australian apple orchards and found that the center of the canopy had the longest LWD. Calibrated, painted, electronic sensors were used to conduct a comprehensive study of LWD variability in several locations within five different crops (Sentelhas et al. 2005). The top of apple and corn canopies had longer LWD, but the bottom of young coffee plants had longer LWD, whereas no LWD differences were observed among canopy locations within short muskmelon vines and well-ventilated grape hedgerows.

Measurement of wetness duration in the apple canopy

The limit of sensor accuracy is an important consideration for the practical application of a disease-warning system. There are two kinds of leaf wetness monitoring sensors, the string type and the electrical-resistance grid. The deWit leaf wetness recorder, used by Brown and Sutton (1995) to develop the first SBFS warning system, senses wetness using a hemp string that constricts in response to wetting. This mechanical sensor is subject to sporadic malfunctions (Brown and Sutton 1995) and is not compatible with electronic digital technology, so data transfer to computer is laborious. Electronic sensors have been used to measure wetness more accurately and precisely. These sensors consist of a fine grid

network of two interlaced circuits etched on a printed circuit board. Wetness is measured using resistance; the path length of an electrical current is reduced when droplets of water bridge the gaps between the adjacent circuits, resulting in a drop in electrical resistance. Durations of wet periods are thus estimated as periods of time when resistance is below a predetermined threshold level. Resistance thresholds are determined by calibration (Potratz et al. 1994). Electronic sensors differ in shape; some have been designed to resemble leaves, whereas others are cylindrical or flat.

Sensor shape and surface texture have an effect on performance. Gillespie and Duan (1987) found that droplets of equal size evaporated faster on cylindrical sensors than on flat plate sensors in the laboratory. On the other hand, cylindrical sensors exchange radiation with both the sky and the ground, while flat plate sensors radiate to the sky only. When unpainted sensors of three differing designs were compared in apple and apricot canopies, cylindrical and flat plate sensors had similar responses to wetness inside the canopy, but flat plate sensors underestimated LWD outside the canopy (Henshall and Beresford 1997). Another approach is painting the grid with light gray latex to simulate the response of leaves to temperature and wetness. The paint absorbs water, allowing droplets smaller than the gap between the fingers of the grid to create a sensor response (Gillespie and Kidd 1978). When painted and unpainted Campbell Scientific (Campbell Scientific Inc., Logan, UT) sensors were compared side-by-side, LWD was greater for painted than unpainted sensors (Magarey 1999, Lau et al. 2000, Sentelhas et al. 2004).

It is difficult to measure or estimate LWD within a crop canopy because LWD is the product of numerous atmospheric and microclimatic factors (Magarey 1999, Thompson 1981). Sensor orientation placement has been shown to affect measurement of LWD.

Placement of flat-plate sensors at a northward-facing position, in the northern hemisphere, minimizes the interception of solar radiation, and thereby prevents premature dry of wetness after sunrise (Magarey 1999). Monitoring responses of painted flat-plate sensors during dew onset and dry-off in tomato indicated that east-facing sensors responded to dew dry-off about 20 minutes sooner than west-facing sensors (Lau et al. 2000). Prevailing winds may also influence drying of the sensor (Fisher 1992); wetness events that occur during periods of still air are more variable than when wind is present (Butt and McGlinn 1989). Tilting the plate sensor at a 45° angle to the horizontal prevents pooling of water that would result in overestimation of leaf wetness (Gillespie and Kidd 1978).

Since accuracy and precision are essential factors in determining model outcome, calibration of electronic sensors should be done by visually adjusting the wet/dry transition threshold in the field. Side-by-side comparisons of sensors in a homogeneous location, such as over turfgrass, should be made to assess sensor variation (Potratz et al. 1994). Placement of multiple sensors at the same location reduces problems with faulty sensors and lost data.

When developing the 225-hour LWD threshold for the SBFS disease-warning system in North Carolina, Brown and Sutton (1995) placed a single deWit leaf wetness recorder on the north side of a tree, approximately 1.5m above the ground, in each orchard. Likewise, a single sensor was placed in a similar location in one tree in each orchard when validating the 180-hour LWD threshold for the SBFS warning system in Kentucky. Hartman (1995, 1996) and Smigell and Hartman (1997, 1998) used Envirocaster (Neogen Corp. Lansing, Michigan) and Metos (Pest Management Supply, Inc. Hadley, Mass.) painted electronic leaf wetness sensors to determine the number of accumulated hours in wet periods $\geq$ 4 hours that occurred before the onset of first SBFS symptoms. Spray threshold differences between these studies

may have been caused by numerous factors including: sensor type, sensor variation, sensor location, orchard topography, geographic location, apple canopy microclimate, apple tree architecture, response of different SBFS species, and proximity, density of reservoir hosts. We therefore need to understand the relationships of both sensors type and canopy microclimate. Knowledge of the spatial variability of wetness in a canopy and the identification of the region of highest disease risk would enhance the development and implementation of a disease-warning system.

Thesis objectives

The objectives of my thesis were threefold. The first goal was to identify members of the SBFS complex in the Midwest U.S. based on genetic and morphological traits. My second objective was to evaluate the efficacy of techniques for post-harvest removal of SBFS blemishes. My third objective was to characterize heterogeneity of leaf wetness duration (LWD) within apple tree canopies and evaluate the impact of canopy location on the outcome of a disease-warning system for SBFS.

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CHAPTER 2. EXPANSION OF THE SOOTY BLOTCH AND FLYSPECK COMPLEX ON APPLES BASED ON ANALYSIS OF RIBOSOMAL DNA GENE SEQUENCES AND MORPHOLOGY

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ABSTRACT

Sooty blotch and flyspeck (SBFS) is a late-season disease of apple and pear fruit that cosmetically damages the cuticle, resulting in produce that is unacceptable to consumers. Previous studies reported that four species of fungi comprise the SBFS complex. We examined fungal morphology and the internal transcriber spacer (ITS) and large subunit (LSU) regions of rDNA of 422 fungal isolates within the SBFS complex from nine orchards in four Midwestern states (USA) and compared them to previously identified species. We used LSU sequences to phylogenetically place the isolates at the order or genus level and then used ITS sequences to identify lineages that could be species. We used mycelial and conidial morphology on apple and in culture to delimit putative species. Thirty putative species found among the Midwest samples were shown able to cause SBFS lesions on apple fruit in inoculation field trials. Among them, *Peltaster fructicola* and *Zygophiala jamaicensis* have been associated previously with SBFS in North Carolina. The LSU analyses inferred that all 30 SBFS fungi from Midwestern orchards were Dothideomycetes; one putative

species was within the Pleosporales, 27 were within Dothideales, and two putative species could not be placed at the ordinal level. The LSU sequences of 17 Dothideales species clustered with LSU sequences of known species of *Mycosphaerella*.

Key Words: *Cercostigmina, Colletogloeum, Dissoconium,* Dothideomycetes, *Gloeodes pomigena, Passalora,* Plant pathology, *Pseudocercospora, Pseudocercosporella, Ramularia,* SBFS, *Xenostigmina*

INTRODUCTION

Fungi of the sooty blotch and flyspeck (SBFS) complex colonize the cuticle of apple fruit (*Malus* X *domestica* Borkh.) in humid production areas worldwide. Although these fungi do not affect the growth or development of the fruit, they blemish fruit with dark smudges (sooty blotch) or groups of tiny black spots (compact speck, discrete speck, and flyspeck). Apples with signs of SBFS are generally unacceptable to consumers, and the market value of the crop can be reduced by more than 90% (Williamson and Sutton 2000, Batzer et al 2002a). Apple growers control SBFS by applying fungicides every 10 to 14 days, from soon after bloom until shortly before harvest (Hartman 1995). Without frequent protectant fungicide sprays most of the apples grown in the eastern and midwestern U.S. would be affected. Even with an intensive fungicide spray program, sporadic control failures occur.

Sooty blotch and flyspeck were initially described as having a single causal agent, *Dothidea pomigena* Schwein. (Schweintz 1832). Colby (1920) determined that sooty blotch and flyspeck were caused by two fungi, *Gloeodes pomigena* (Schwein.) Colby, and *Schizothyrium pomi* (Mont. & Fr.) Arx , respectively, and also described the appearance of three mycelial types of sooty blotch on apple fruit. Groves (1933) described distinct mycelial

types (signs) of sooty blotch as ramose, punctate, fuliginous and rimate. Subsequent researchers (Hickey 1960, Sutton and Sutton 1994) observed a wide range of variation of sooty blotch signs on apple fruit. Johnson and Sutton (1994) and Johnson et al (1997) recognized three sooty blotch species from ramose (*Geastrumia polystigmatis* Batista & M.L. Farr), punctate (*Peltaster fructicola* E.M. Johnson et al), and fuliginous [*Leptodontium elatius* (G. Mangenot) de Hoog] mycelial types. Considerable morphological variation has also been observed for flyspeck (*Schizothyrium pomi,* anamorph *Zygophiala jamaicensis* E. Mason) on apple fruit and in culture (Baker 1977, Nasu et al 1986, Lerner 1999).

Progress in understanding the taxonomy and ecology of the SBFS complex has been slowed by difficulty in isolating, maintaining, and identifying these fungi in culture, as well as scarcity of fruiting structures on apple peels (Hickey 1960). Molecular identification has been used to elucidate the etiology of plant pathogenic fungi that were otherwise difficult to identify. For example, colony morphology and ITS sequences were used to characterize pathogenic and non-pathogenic fungi isolated from the vascular tissues of soybeans in the North Central United States (Harrington et al 2000). Also, unknown basidiomycetes associated with bark beetles were identified to putative species using the mitochondrial small subunit and the spacer regions of the rDNA (Hsiau and Harrington 2003). Using a similar strategy, we examined the morphology and partial rDNA sequences of fungi within the SBFS complex from nine orchards in the Midwest in order to test the hypothesis that many species cause the same signs on apple. We used the LSU of nuclear rDNA to phylogenetically place the SBFS isolates at the order and genus level, used ITS sequences to identify lineages that

could be species, and phenotypically compared isolates from these lineages to delineate putative species (Harrington and Rizzo 1999).

MATERIALS AND METHODS

Sources of isolates

Fungi were isolated from SBFS colonies on apples harvested during the autumn of 2000 from nine orchards located in four states: near the cities of Indianola, Pella and Iowa Falls in Iowa; Rockford, Simpson and Chester in Illinois; Mooresville and New Franklin in Missouri; and New Munster, Wisconsin. Approximately 10 to 15 individual colonies on apples were arbitrarily selected from each of 10 to 12 apples from each orchard for a total of 1200 colonies. Apples were rinsed in flowing tap water for 30 min and allowed to dry in a transfer hood. Sub-samples from four quadrants within each colony were transferred aseptically to water agar acidified with a post-autoclave addition of 40 drops of 50% lactic acid per liter (AWA) and incubated at 21 to 24 C under ambient light. As mycelial growth became visible, after 1 to 3 wks, isolates were transferred to potato dextrose agar (PDA) (Difco, Detroit Michigan). A total of 422 isolates were purified and stored in glycerol at –80 C. Segments of apple peels containing the same colony from which isolates were made were also preserved by pressing the thallus and supporting peel between paper towels until they were dried. Representative cultures were deposited at the Centraalbureau voor Schimmelcultures (CBS) Utrecht, The Netherlands and specimens on apple peels were deposited at the Ada Haden Herbarium, Iowa State University Ames, Iowa (Table II).

Cultures of known members of the SBFS complex (*Leptodontium elatius, Peltaster fructicola,* two putative species of *Stomiopeltis, Geastrumia polystigmatis* and *Schizothyrium*

pomi) were kindly provided by Dr. Turner B. Sutton of North Carolina State University (NCSU) and maintained on PDA.

Polymerase chain reaction and sequencing

The ITS region (ITS1, 5.8S rDNA gene, ITS2) from one to three isolates of each distinct colony morphology type on PDA from each sampled orchard was sequenced for a total of 228 isolates. Isolates with identical ITS sequences were grouped; a portion of the LSU 28S gene was then sequenced for one to three representative isolates within each group, totaling 117 LSU sequences.

Template DNA for polymerase chain reaction (PCR) was directly obtained by scraping mycelia with a pipet tip from 4- to 6-wk-old cultures grown on PDA (Harrington and Wingfield 1995). Primer pairs used for amplification and sequencing of the ITS region were ITS-1F/ITS4 (White et al 1990), and primer pairs used for amplification and sequencing of LSU were LROR/LR5 and LROR/LR3, respectively (Vilgalys and Hester 1990). Amplification reactions consisted of 4 mM $MgCl_2$, 5% DMSO, 1X Sigma buffer, 200 μM dNTPSs, 0.5 μM of the forward and reverse primers, and 3 units of Taq polymerase (Sigma Chemical Co., St. Louis, MO). Cycling conditions (MJ Research, Inc. thermocycler, PTC-100) for amplifications were an initial denaturation at 94 C for 95 s followed by 35 cycles of denaturation at 94 C for 35 s, annealing at 49 C for LSU and at 52 C for ITS for 60 s, and extension at 72 C for 2 min. The PCR product was purified using a QIAquick DNA Purification Kit (Qiagen, Valencia, CA) and quantified on a Hoefer DyNA Quant 200 Fluorometer (Amersham Pharmacia Biotech, San Francisco, CA). Automated sequencing was performed at the Iowa State University DNA Sequencing and Synthesis Facility.

The ITS and LSU database

To identify possible relatives of the isolated SBFS fungi, BLAST searches (version 2.2.6, National Center for Biotechnical Information, Bethesda, Maryland) were conducted using representative ITS and LSU sequences. Partial sequences of taxa with high homology to SBFS fungal sequences were downloaded for phylogenetic comparisons. Additional ITS and LSU sequences of representative ascomycete taxa were also downloaded and used in analyses (Table I).

Sequence alignment and phylogenetic analysis

Sequences were imported into BioEdit (Hall 1999), and the 5'- and the 3'-ends were trimmed to aid alignment. Lengths of the ITS sequences analyzed ranged from 458 to 534 base pairs, and those of the partial LSU sequences ranged from 582 to 609 base pairs. Preliminary alignments of the ITS and LSU matrices were generated using CLUSTAL-X (Thompson et al 1997) with gap opening and gap extension parameters of 50:5, and these alignments were manually optimized. For LSU analysis, ambiguously aligned regions associated with six indels were eliminated, resulting in a matrix of 435 characters, including gaps. Taxa with redundant LSU sequences were eliminated from the data block, reducing the number of taxa in the analysis from 117 to 77. *Peziza ampelina* Pass. was used as the outgroup in the LSU phylogenetic analysis. The numerous, large insertion/deletions in the ITS data set prevented unambiguous alignment of all the sequences. Therefore, ITS sequences of subsets of taxa with similar sequences were analyzed separately.

Maximum parsimony analysis was performed using PAUP* version 4.0b10 for 32-bit Microsoft Windows (Swofford 2002). Heuristic searches were conducted with random sequence addition and tree bisection-reconnection (TBR) branch swapping algorithms,

collapsing zero-length branches, and saving all minimal length trees. Maxtrees was set at 10 000. Alignable gaps were treated as a 'fifth base.' All characters were given equal weight. To assess the robustness of clades and internal branches, a strict consensus of the most parsimonious trees was generated and a bootstrap analysis of 1000 replications was performed for ITS datasets and 200 replications for the LSU data set. Alignments and strict consensus trees were deposited in Treebase. To test alternative taxa clustering relationships based on the minimum evolution principle (Nei and Kumar 2000), a nearest neighbor joining (NJ) tree was also generated from the LSU data set using PAUP (Swofford 2002), and a bootstrap analysis of 1000 replicates was performed. Classification of taxa was based on the work of Barr (1987).

Putative species designation

Isolates were grouped into putative species based on ITS parsimony analysis, conidial characters and colony morphology on apples and on artificial media. Genus designations were determined by anamorph morphology. Putative species within each genus were assigned letter designations based on their appearance on apple, including flyspeck (FS), compact speck (CS), discrete speck (DS), ramose (RS), punctate (P), ridged- honeycomb (RH), and fuliginous (FG) (FIGS. 1 – 9) (Colby 1920, Groves 1933, Batzer et al 2002b). Letter designations of putative species were followed by numbers based on unique ITS sequences and phenotypic differences in culture.

Koch's Postulates

A modified version of Koch's postulates was performed to verify that the isolated fungi were capable of causing SBFS signs on apple. One to five isolates from each putative species, totaling 52 isolates, were grown on PDA for one month, excess agar was cut away

and colonies were blended with 50 mL sterile deionized water (SDW) for 60 s in a Waring^{TM} blender. Suspensions of mycelial fragments and conidia were filtered through four layers of sterile cheesecloth and then refrigerated until use, which was within 2 h of preparation. Immature apples (3–4 cm diam) on eight trees (cvs. MacIntosh and Golden Delicious) located at the Iowa State University Horticultural Research Station near Gilbert, Iowa, were surface-sterilized with 70% ethanol, allowed to dry 1 min, then swabbed with a suspension of one fungal isolate per apple. Each isolate suspension was applied to five apples on each of three trees. Ten control apples on each tree were surface sterilized and swabbed with SDW. Apples were covered with Fuji^{TM} bags (Kobayshi Bag Mfg. Co., Ltd. Iisa, Nagano, Japan) for approximately 10 wk until harvest and then examined for signs of SBFS. Orchard experiments were conducted from early July through mid-September of 2001 and 2002. Fungi were re-isolated onto AWA, and DNA was extracted from mycelia using Prepman Ultra Sample Preparation Reagent (Applied Biosystems) for PCR. Colony morphology and ITS sequences were compared to each original isolate.

Morphology of SBFS isolates on apple and *in vitro*

Signs of SBFS preserved on apple peels were described, including mycelial growth patterns and fruiting body size and density. One to five representative isolates from each putative species were grown on 1.5% malt extract agar (MEA), PDA, and carnation leaf agar (CLA) (Fischer et al 1982). Colony descriptions were made after 1 month growth on PDA and MEA at 21-24 C under intermittent ambient light. Washed and autoclaved pieces of cellulose membrane (Flexel, Inc. Covington, IN) were placed on CLA plates, mycelial plugs were transferred to the edge of the cellulose pieces, and hyphae were allowed to grow over the cellulose. When fungal structures were evident, the cellulose pieces were transferred from

the agar, mounted onto glass slides, and examined at 400x and 1000x magnification. Twenty measurements of each type of structure were taken if sufficient material was available.

Diameter growth of colonies was determined on MEA. Nine mycelial plugs (6 mm diam) from 3-wk-old colonies on MEA were placed upside down onto three plates (3 plugs per plate). After plates had been incubated upside down for 4 wk at 25 C in the dark, two perpendicular measurements of colony diameters were made, and the diameter of the plug was subtracted to determine the extent of diameter growth. Average diameters and ranges for each isolate were recorded.

RESULTS

Thirty putative species were delineated from among the SBFS isolates from apples grown in the Midwest based on ITS sequences and morphological characters. Over a period of two growing seasons, one or more representative isolates from each of the thirty putative species were verified to cause signs on inoculated fruit using a modified Koch's postulates.

Mycelial types on apple fruit.

Not all putative species could be distinguished based on mycelial type on apple fruit, but each putative species was associated with a single mycelial type. Mycelial types described by Colby (1920) and Groves (1932) were employed when appropriate, but additional mycelial types were encountered (Figs. 1-9). In some species, condiomata or spermagonia were occasionally observed to produce conidia or spermatia, respectively, on inoculated apple fruit. The term sclerotium-like bodies was employed to indicate dark hyphal structures that may have been sterile conidiomata, spermagonia, or pseudothecial-like structures. Ascospores were never observed in pseudothecial-like structures on apples, either in fall or spring following incubation in wire cages on the ground during winter, although

ascospores of *Schizothyrium pomi* and *Stomiopeltis versicola* have been reported to occur on apple fruit (Baker et al 1977, Turner B. Sutton pers. comm.). Mycelial types were classified as blotch and speck based on the presence or absence, respectively, of dark mycelial mats.

Three speck types were noted on apple fruit. Flyspeck (FS) had dark, shiny, round to oval, flattened sclerotium-like bodies ranging from 109–600 μm diam at a density of 0.5–2 structures/mm^2 (Fig. 1). Compact speck (CS) also had shiny, black, flattened sclerotium-like bodies, but these were round to irregular (35–418 μm diam) and densely arranged (5–22/mm^2) (Fig. 2). In contrast, discrete speck (DS) appeared as groupings (5–20 sclerotium-like bodies/mm^2) of tiny spheres (35–170 μm diam) with numerous setae, visible only with magnification (Fig. 3).

Sooty blotch mycelial mats with sclerotium-like bodies varied in color, density, and margin type. Ramose (RS) colonies had sclerotium-like bodies (45–278 μm diam) that appeared larger, shinier, and more convex at the center of the colony than at the margin (Figs. 4–5) and were arranged at a density of 3–10/mm^2. Sclerotium-like bodies of ramose mycelial types comprised interlocking cells. In contrast, sclerotium-like bodies of punctate (P) mycelial types (Figs. 6–7) were dull brown, comprising several overlapping hyphal strands, and appeared similar throughout the mycelial mat. Sclerotium-like bodies of punctate types ranged from 18–216 μm diam and were arranged at a density of 12–33/mm^2.

Color of sooty blotch mycelial mats with no sclerotium-like bodies ranged from pale olive to black. Ridged-honeycomb (RH) mycelial types were characterized by clumps and ridges of mycelia (Fig. 8). These mycelial clumps occasionally resembled sclerotium-like bodies, but were not well-organized when observed through a compound microscope. All

fuliginous (FG) mycelial types exhibited uniform mats of mycelia, but edges of the colonies varied from abrupt to feathered (Fig. 9).

Phylogenetic placement based on LSU sequences

Maximum parsimony (MP) analysis of the LSU sequences resulted in 766 equally most parsimonious trees. A MP tree with a topology similar to that of the neighbor-joining (NJ) tree (Fig. 11) was selected to determine phylogenetic placement (Fig. 10). Results of parsimony and distance analyses indicated that all Midwest species were Dothideomycetes sensu Barr (1987). Both MP and NJ trees placed sterile mycelium sp. RS3 within the Pleosporales (Figs. 10–11). *Peltaster* sp. CS1 and *Ramularia* sp. CS2 did not clearly fall within the Pleosporales, Dothideales, or Chaetothyriales. The NJ tree grouped *Peltaster* sp. CS1 and *Ramularia* sp. CS2 together, but the MP tree did not group these fungi with bootstrap support (Figs. 10–11). This grouping may have been based on long-branch attraction (Nei and Kumar 2000). The remainder of the Midwestern putative species, as well as the four species from North Carolina (*Schizothyrium pomi, Peltaster fructicola,* and two species of *Stomiopeltis*), were grouped with the Dothideales, which had bootstrap support of 81%. The LSU analyses clustered the most of the SBFS anamorph genera (*Peltaster, Colletogloeum, Pseudocercosporella, Dissoconium, Passalora, and Xenostigmina*) with ten *Mycosphaerella* species, and this branch had a bootstrap support of 96% (Figs. 10–11). The teleomorph *Schizothyrium pomi* and the three putative species of the anamorph genus *Zygophiala*, the teleomorph *Piedraia hortae* Fonseca & Leão, and the three putative species of the anamorph genus *Pseudocercospora* also grouped with these *Mycosphaerella* species. Within the Dothideales, *Peltaster fructicola* and *Peltaster* spp. P2.1 and P2.2 formed a strongly supported clade, with bootstrap support of 100% (Figs. 10–11).

Putative species designation based on ITS sequences and morphology

Zygophiala. Four putative species of *Zygophiala* were delineated based on ITS analysis (Fig. 12) and morphology (Table II). *Zygophiala* spp. were isolated from each of the nine orchards surveyed. The 23 Midwestern isolates of *Zygophiala* sp. FS1 had LSU and rDNA sequences similar to those of *Z. jamaicensis* (teleomorph *Schizothyrium pomi*) (Figs. 10–12) from North Carolina.

Isolates of *Zygophiala* sp. FS1 varied in colony color on PDA (Table II), but colony morphology was not associated with the minor differences in ITS sequences (Fig. 12). Colonies of all seven isolates of *Zygophiala* sp. FS2 isolates were green and white and produced a deep red soluble pigment on PDA (Table II). Cultures of *Zygophiala* spp. FS3.1 and FS3.2 were flat and zonate, whereas those of *Zygophiala* spp. FS1 and FS2 were thick, lumpy and flocculate (Table II). Conidia of *Zygophiala* spp. FS3.1 and FS3.2 were smaller than those of *Zygophiala* spp. FS1 and FS2 (Table II). Growth rate of *Zygophiala* sp. FS3.1 was faster than *Zygophiala* sp. FS3.2 (Table II).

On apple, sclerotium-like bodies of *Zygophiala* sp. FS1 were round, 290 m (155–409) in diameter and at a density of 1.5/mm^2 (Fig. 1). Sclerotium-like bodies of *Zygophiala* sp. F2 were also round, but smaller (mean diameter of 195 µm (109–364) and at a density of 2.0/mm^2. *Zygophiala* spp. F3.1 and FS3.2 sclerotium-like bodies were ovoid, larger (380 m (300–450) x 500 µm (425–600)) and were more sparsely arranged at a density of 0.5/mm^2.

Pseudocercospora. Three SBFS species were identified as belonging to *Pseudocercospora* on the basis of their morphology, but ITS and LSU sequences differed from those deposited for any *Pseudocercospora* species. Analyses of the LSU clustered *Pseudocercospora* sp. FG1.1, FG1.2 and FS4, and this clade was near *Piedrai hortea*

Fonseca & Leão (Figs. 10–11). An anamorph *Piedraia hortea* has not been reported. The ITS sequences of SBFS species FG1.1 and FS4 were similar (98% base-pair homology, 465/475 identity), and their closest match in a BLAST search was with the sequence from *Trimmatostroma abietis* Butin & Pehl (219 of 231 bases for *Pseudocercospora* sp. FS4 and 218 of 231 bases for *Pseudocercospora* sp. FG1.1). The ITS region of the single available isolate of *Pseudocercospora* sp. FG1.2 did not amplify after two attempts.

Scolecospores differed among these three putative *Pseudocercospora* species (Table II), as did their appearance on apples and colony morphology on PDA and MEA. *Pseudocercospora* sp. FG1.1 and *Pseudocercospora* sp. FG1.2 had fuliginous mycelial types with no fruiting bodies on apple whereas *Pseudocercospora* sp. FS4 had no mycelial mat and dark shields similar in size to flyspeck signs caused by *Zygophiala* spp. The soluble orange and brown pigments formed on PDA by *Pseudocercospora* spp. FS4 and FG1.2, respectively, were not observed for *Pseudocercospora* sp. FG1.1 (Table II).

Pseudocercosporella. Three species with ridged-honeycomb mycelial types (Fig. 8) on apple had *Pseudocercosporella* anamorphs. These species were found in eight of nine orchards surveyed. Isolates produced two-celled primary conidia with numerous secondary scolecospores in a slimy mass on PDA and MEA. Primary conidia varied in size and shape among the three putative species (Table II) and were formed on short conidiophores along rows of parallel hyphae, and the primary conidia did not form secondary conidia until they had separated from the conidiophore. The three species of *Pseudocercosporella* differed in colony morphology on MEA and PDA (Table II).

The 29 ITS sequences of *Pseudocercosporella* sp. RH1 grouped together with 87% bootstrap support (Fig. 13); sequence differences, of one to five base pairs, were not

associated with the highly variable mycelial characters observed among the isolates. The four ITS sequences of *Pseudocercosporella* sp. RH2.1 grouped separately from other species of *Pseudocercosporella* with bootstrap support of 94% (Fig. 13).

Colletogloeum. Twenty-six isolates from six of the nine orchards were grouped into three putative species that were morphologically similar to *Colletogloeum* (Table II). These three *Colletogloeum* spp. on apple fruit formed a dense, fuliginous mycelial mat with no sclerotium-like bodies (FG) (Fig. 9). All three putative species had thick-walled, ovoid to allantoid blastospores that were highly vacuolate, subhyaline, and truncate at the base. The NJ tree grouped the LSU sequences of the three *Colletogloeum* spp. with *Mycosphaerella marksii* Carnegie & Keane with bootstrap support of 84% (Fig. 11), but the branch was not supported by parsimony analysis (Fig. 10). The MP tree of the ITS sequence analysis delimited *Colletogloeum* sp. FG2.3 with bootstrap support of 100% and separated *Colletogloeum* sp. FG2.1 and FG2.2 with only moderate bootstrap support of 61 (Fig. 14). Mycelial color of *Colletogloeum* spp. FG2.1 and FG2.2 differed from that of *Colletogloeum* sp. FG2.3, and the growth rate of *Colletogloeum* sp. FG2.1 on MEA was less than that of *Colletogloeum* spp. FG2.2 and FG2.3 (Table II).

Ramularia. Two putative species of SBFS were morphologically placed in *Ramularia.* Single-celled conidia of *Ramularia* sp. P5 were irregular in shape, hyaline and were produced singly on brown condiophores that had dendritic branches with a single central basal cell. One of the two *Ramularia* sp. P5 isolates produced a yellow pigment on PDA, but ITS sequences of the two were identical. The closest matches to *Ramularia* sp. P5 in pair-wise comparison of ITS sequences using BLAST were sequences from *Ramularia collo-cygni* B. Sutton & J.M. Waller (97% base-pair homology, 418/429 identity) and

Ramularia sp. KC1 (98% base-pair homology, 421/427 identity). On apple fruit, *Ramularia* sp. P5 was designated as punctate mycelial type.

Eight isolates of *Ramularia* sp. CS2 produced smooth pink colonies on PDA with black reverse. The compact speck on apple had sclerotium-like bodies 183 μm (81—418) at a density of 6.8 per mm^2 (Fig. 2) but had no visible mycelial mat. Conidiophores were distinctive; the tip of the condiophore had a dark ring near its apex and produced catenate conidia attached with a narrow, curved bridge (Table II).

Dissoconium. Five putative species of SBFS were identified as members of the anamorph genus *Dissoconium*. LSU sequences of these species grouped with the teleomorph *Mycosphaerella lateralis* Crous & M.J. Wingf. (Figs. 10–11). ITS sequences of *Dissoconium* spp. DS1.1 and DS1.2 grouped with *D. aciculare* de Hoog et al with bootstrap support of 85% (Fig. 15). Although we were unable to amplify the ITS region for *Dissoconium* sp. DS2, the LSU sequences grouped *Dissoconium* sp. DS2 with *Dissoconium* spp. DS1.1, DS1.2 FG4, and FG5 with bootstrap support of 91 and 97% (Figs. 10–11). Thus the LSU analyses implies these species are within the same genus, whereas the ITS sequences and morphology indicate that they are distinct species.

Dissoconium spp. DS1.1, DS1.2, DS2 and FG4 were morphologically suggestive of *Dissoconium* anamorphs in having two forcibly discharged conidial types (Crous et al 1997). Macroconidia were hyaline, smooth, two-celled, fusiform, and with the septum at or slightly above the middle, whereas microconidia were single-celled and hyaline. Macroconidial and microconidial size and shape differed among *Dissoconium* spp. DS1.1, DS1.2, DS2 and FG4 (Table II). In contrast, *Dissoconium* sp. FG5 had a single conidial type. Conidia were one-

or two-celled, ovoid, were produced singly in whorls along a needle-like, dark brown conidiophore, and were not forcibly discharged.

On apple fruit, *Dissoconium* spp. DS1.1, DS1.2 and DS2 appeared as the discrete speck mycelial type with spherical to globose sclerotium-like bodies (Fig. 3). On occasion, sclerotium-like bodies developed into spermagonia that discharged spermatia (5 x 3 μm) through an ostiole. In contrast, *Dissoconium* spp. FG4 and FG5 appeared as the fuliginous mycelial type on apple (Fig. 9), having dense olivaceous mycelial mats with no sclerotium-like bodies.

Xenostigmina. Two species of SBFS, *Xenostigmina* sp. P3 and P4, had a similar punctate appearance on apple, with dark olivaceous, sparse, thick, ropy mycelia with feathery margins, abundant sclerotium-like bodies (Fig. 6). Despite numerous attempts to isolate from this relatively common mycelial type, only two isolates of *Xenostigmina* sp. P3 and one isolate of *Xenostigmina* sp. P4 were recovered from two orchards in Illinois. Rarely, these isolates produced slow-growing, carbonaceous colonies with dark brown, smooth, thick-walled conidia that varied in size and shape (Table II). The ITS sequences of *Xenostigmina* spp. P3 and P4 were similar (98% base-pair homology, 474/481 identity). The closest matches from BLAST searches were to sequences of *Mycosphaerella latebrosa* (243/259 identity for *Xenostigmina* sp. P3 and 223/233 identity for *Xenostigmina* sp. P4). The NJ tree grouped *Xenostigmina* spp. P3 and P4 with two *Stomiopeltis* species from North Carolina, three species with sterile mycelia (RS1, RS2.1 and RS2.2), and *Passalora* sp. FG3, with high bootstrap support of 91% (Fig. 11). The MP analysis had the branch appeared in the strict consensus tree, but there was no bootstrap support.

Sterile mycelia. Fourteen isolates from three orchards produced sterile mycelia. LSU analysis (Figs. 10—11) grouped three isolates of Sterile mycelia sp. RS3 with *Shiraia bambusicola* Henn. in the Pleosporales. LSU sequences grouped Sterile mycelia spp. RS1 and RS2 with *Stomiopeltis* sp. from North Carolina (Figs. 10—11), however, parsimony analysis of the ITS sequences separated isolates RS1 and RS2 (Fig. 16). Although species having sterile mycelia produced ramose mycelial types on fruit, with dark mycelial mats and shiny, black, convex sclerotium-like bodies (Figs. 4-5), isolates with similar ITS sequences had distinctive colony characters on PDA (Table II).

Passalora. Five isolates from an Illinois orchard could be placed in *Passalora.* Conidiophores were cylindrical, straight or curved and septate with tapered apices producing cylindrical, dark conidia (Table II). *Passalora* sp. FG3 produced a fuliginous mycelial type on apple fruit (Fig. 9). Both MP and NJ trees of LSU sequences grouped *Passalora* sp. FG3 with Sterile mycelia spp. RS1 and RS2 and *Stomiopeltis* spp. with bootstrap support of 80 and 90%, respectively (Figs. 10–11). The ITS region of *Passalora* sp. FG3 was not amplified after two attempts.

Peltaster. Anamorph morphology of four putative species was similar to *Peltaster fructicola*, having indistinct conidiophores, with single-celled ovoid conidia successively produced through a hyphal pore. Isolates with similar ITS sequences (Fig. 17) also were grouped according to colony morphology and growth rate (Table II). Isolates of *P. fructicola* (=*Peltaster* sp. P1) were among the most commonly found, although none were found in Iowa orchards. Colonies were velvety green on PDA. One isolate (MSTE4a) grew more slowly than other *P. fructicola* isolates (2.9 and 7.5 mm, respectively). *Peltaster* spp. P2.1 and P2.2 formed zonate black and green colonies on PDA and grew more slowly than *P.*

fructicola. The ITS sequences of *Peltaster* spp. P2.1 and P2.2 were 18 base pairs longer than *P. fructicola* and other sequences of SBFS isolates in the Dothideales.

Peltaster sp. CS1 did not group with other *Peltaster* anamorphs and could not be placed at the ordinal level. Although the yeast-like conidia were produced in a similar manner as other species of *Peltaster,* this putative species was readily distinguishable by mycelia in culture and appearance on the apple. Colonies did not produce visible mycelium on PDA, but they generated shiny, pink masses of ovoid conidia on short hyphae that arose from germinated conidia and by conidial budding (Table II). On apples, the compact speck mycelial type of *Peltaster* sp. CS1 also differed from the punctate mycelial type of *Peltaster* anamorphs (Figs. 2,7).

DISCUSSION

There is much more diversity within the SBFS complex than had been documented previously. We found 30 putative species of the SBFS complex in the Midwest. Two fungal species identified previously in North Carolina, *Peltaster fructicola* and *Zygophiala jamaicensis,* (Williamson et al. 2004, Ocambu-Basu and Sutton 1998) were isolated from several midwestern orchards, but the other 28 putative species were not recognized previously as being involved in the SBFS disease complex. *Leptodontium elatius* and *Geastrumia polystigmina,* which were commonly found in North Carolina (Williamson and Sutton 2000), were not recovered from midwestern orchards. Although spores were not seen in Sterile mycelia spp. RS1 and RS2, these two midwestern putative species may be related to *Stomiopeltis* species found in North Carolina.

All SBFS fungi were Dothideomycetes *sensu* Barr (1987) based on LSU analyses. However, unambiguous placement of the SBFS fungi into the Dothideales could not be made, since the LSU sequences of *Capnodium citri* (Capnodiales) and *Myriangium duriaei* (Myrangiales) also clustered in the Dothideales. Extensive morphological homoplasy has led to confusion about the classification of the Dothideomycetes (Reynolds 1991), and orders within the Dothideomycetes have not been shown to be monophyletic in other studies (Erikkson 2003, Lumbsch and Lindemuth 2001). Eriksson and Winka (1998) performed phylogenetic analyses using 18S rDNA sequences of Dothideomycetes and accepted the orders Dothideales, Patellariales, Pleosporales, Myriangiales and Capnodiales, and they elevated the Chaetothyriales to class status, but they did not classify 51 families, including Dothideaceae. Lumbsh and Lindemuth (2001) further investigated LSU and SSU sequences of isolates in the Pleosporales, Myriangiales, Melanommatales and Capnodiales, but they did not analyze the Dothideales.

The topology of the MP trees of partial sequences of the LSU was validated with the single NJ distance tree. However, when all 228 ITS sequences were aligned, parsimony analysis resulted in several thousands of trees with topologies conflicting with those of from the LSU. Therefore sub-trees of closely related species, based on LSU analyses, were constructed to delineate species. In turn, the increased taxon sampling for the ITS sub-trees allowed us to discover evolutionary changes among these species that were not apparent when using a smaller sampling of taxa used for the LSU trees. These newly discovered putative species were supported by unique culture characteristics and morphology. Although, detailed descriptions were beyond the scope of this study, efforts are being made to name these putative species. As additional species are discovered (studies in China and

Europe are underway), studies based on DNA and cultural characteristics and morphology will be required. Sequences for the elongation factor 1-α and the β-tubulin gene regions, for example, have been used to compare a large number of *Mycosphaerella* species from Eucalyptus (Crous et al 2004) and may be a valuable part of future studies.

Peltaster fructicola P1, P2.1 and P2.2 had holoblastic conidia like those of *Aureobasidium* spp. (Dothideales, Dothioraceae) (de Hoog and McGinnis 1987). Other SBFS fungi appeared related to *Mycosphaerella* based on LSU and ITS sequences analysis (Stewart et al 1999, Crous et al 2001a). Crous et al (2001b) linked 23 anamorph genera to *Mycosphaerella*. We found seven anamorph genera in the SBFS complex that appeared to be related to *Mycosphaerella* (Braun 1998, Crous et al 2001a). Morphological features of anamorphs *in vivo* used by Stewart (1999) and Crous and Wingfield (1997) for the identification of leaf blotch complex of *Eucalyptus* spp. in Australia and South Africa were useful for identification of the many anamorphs of the SBFS complex. However, colony diameter on MEA at 4 wk and color, texture and pigment production on PDA were also useful features for delimiting phenotypes of putative species.

Some of the newly identified putative species of SBFS fungi have characters similar to those ascribed by early researchers to *Gloeodes pomigena*. Descriptions and line drawings of *G. pomigena* (Colby 1920) show conidia on apple fruit similar to those of *Colletogloeum* spp. FG2.1, FG2.2 and FG2.3. Another description of *G. pomigena* made by Baines and Gardner (1932) depicted colonies grown on PDA as similar to those of *Peltaster* sp. CS1, which had distinctive pink, yeast-like production on PDA. In contrast, Groves (1933) described *G. pomigena* as having abundant masses of constricted, septate, cigar-shaped, budding spores of variable size, similar to what we observed for *Pseudocercosporella* spp.

RH1 and RH2.1. Groves also noted that *G. pomigena* was most frequently associated with the ridged-honeycomb mycelial type on apple, as we found for putative species of *Pseudocercosporella*. Hickey (1960) described a wide range of *G. pomigena* spore types and colony morphologies in culture and on fruit. Three members of the SBFS complex in North Carolina were delineated by Johnson et al (1994, 1996, 1997), but they did not report finding *G. pomigena*.

This study showed that several genetically distinct fungi in the SBFS complex may give rise to the same mycelial type on apple fruit. Therefore, mycelial type alone was not found to be a reliable character to identify a SBFS fungus to species. Mycelial type on apple was consistent within a putative species, however, and should still be considered a useful tool when observing SBFS in the field. Because results from our survey suggest that there is more than one type of speck (fruiting bodies with no mycelial mat), we described the additional mycelial types "compact speck" and "discrete speck" that differ from flyspeck. Although we occasionally recovered more than one putative species from a single sign on apple fruit, it was subsequently found that such colonies consisted of more than one mycelial type. This occurred four times in our study; in each case mixed colonies were observed when the preserved apple peel was reexamined. We have frequently observed several mycelial types growing on top of one another.

Although sclerotium-like bodies were observed on many of the colonies, none of these structures were observed to produce asci, even when infected apples were allowed to overwinter outdoors. Mature ascospores have been observed in North Carolina for *Stomiopeltis* spp. and *Schizothyrium pomi* (T. B. Sutton pers. comm.). It is possible that the longer growing season in the southern U.S. is more suitable for ascocarp maturation on the

fruit. Fungi in the SBFS complex are likely to have many suitable hosts among the wild plants surrounding orchards (Hickey 1960, Johnson and Sutton 1994, Williamson and Sutton 2000), and infestation of apple fruit may not be a primary means of survival for these fungi (Baines and Gardner 1932, Williamson and Sutton 2000).

It is reasonable to believe that there are undiscovered members of the SBFS complex in the Midwest, because only nine orchards were sampled in a single year. Annual surveys of mycelial types from apples grown throughout the Midwest have indicated that prevalence and incidence of mycelial types in the SBFS complex vary according to location and year (Batzer unpubl.). Because these fungi probably reside on plant species surrounding apple orchards (Groves 1933, Hickey 1960, Johnson et al 1997), the complex may vary from orchard to orchard depending on the local flora.

Environment may play a substantial role in the morphology of the fungi on apple fruit. In a preliminary experiment, for example, isolates from *Dissoconium* spp. DS1.1, DS1.2 and DS2, and *Pseudocercosporella* spp. RH1, RH2.1 and RH2.2 were inoculated on immature apple fruit and incubated in crispers in growth chambers at 25 C. After 4 wk, these isolates produced a light-colored, smooth-textured, mycelial mat covering the entire fruit surface, rather than the discrete speck or ridged honeycomb mycelial types found on orchard-grown apples. Because of this morphological variability on inoculated apple fruit, all Koch's postulates evaluations were conducted on intact apple fruit grown in an orchard. These assays resulted in mycelial types similar to those observed on the original apple cuticle.

Strategies for management of SBFS may need to be reassessed according to the traits of the members of the SBFS complex predominating in particular orchards and geographic regions. Members of the SBFS complex may differ in ecology and thus require different

management practices. Various SBFS mycelial types were removed from apples with different efficiency by post-harvest dip treatments (Batzer et al 2002a). In a comparison of six SBFS putative species cultured on water agar amended with the fungicides thiophanate-methyl and ziram, fungicide sensitivity varied widely among putative species (Tarnowski et al 2003). Furthermore, growth rates differed among putative species in response to nutrient composition of media (Van deVoort et al 2003). Optimal temperatures for growth on water agar of six putative species of SBFS also varied significantly (Hernandez et al 2004). Understanding the ecology of local and regional SBFS complexes may lead to strategies that reduce the economic and environmental impact of disease management.

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Table I. Sequences of representative ascomycetes used in phylogenetic analysis of the sooty blotch and flyspeck complex

Order[a]	Teleomorph	Anamorph	Genbank assession numbers	
			LSU rDNA	ITS rDNA
Pezizales	*Peziza ampelina*		AF491629	
Hypocreales	*Bionectria compactiuscula*		AF210690	
Diaporthales	*Cryphonectria parastitica*		AF277132	
Chaetothyriales	*Ceramothyrium carniolicum*		AY004339	
		Rhinocladiella atrovirens	AF050289	
		Ramichloridium anceps	AF050284	
		Curvularia eragrostidis	AF163983	
		*Leptodontium elatius**[b]	AY598931	AY598893
Pleosporales	*Cochliobolus heliconiae*		AF163978	
	Kirschsteiniothelia aethiops		AY016361	
	Melanomma radicans		U43479	
	Shiraia bambusicola		AB105798	
		Spilocaea oleaginea	AF338398	
	Venturia hanliniana	*Cladophialophora brevicatenata*	AF050290	
Myriangiales	*Elsinoe fawcettii*	*Sphaceloma fawcettii*		U28058
	Myriangium duriaei		AY016365	
	Piedraia hortae		AY016366	
Capnodiales	*Capnodium citri*		AY004337	
	Raciborskiomyces longisetosum		AY016367	
Dothideales	*Lojkania enalia*		AY016363	
		Aureobasidium pullulans	AF050239	
	Dothidea ribesia			
		Dissoconium aciculare		AF173308
		Ramularia sp. KC1		AF222848
		Ramularia collo-cygni		AF173310
		Trimmatostroma abietis		AY128699
	Mycosphaerella bixae			AF362056
	Mycosphaerella confusa	*Pseudocercospora rubi*		AF362058
	Mycosphaerella ellipsoidaea	*Uwebraunia ellipsoidaea*	AF309582	AF309593
	Mycosphaerella latebrosa			AF362067

Order[a]	Teleomorph	Anamorph	Genbank assession numbers	
			LSU rDNA	ITS rDNA
	Mycosphaerella lateralis	*Dissoconium dekkeri*	AF309583	AF309625
	Mycosphaerella marksii		AF309578	AF309588
	Mycosphaerella molleriana	*Colletogloeopsis molleriana*	AF309584	AF173313
	Mycosphaerella nubilosa			AF309618
	Mycosphaerella parkii	*Stenella parkii*	AF309579	AF173311
	Mycosphaerella pini			AF211197
	Mycosphaerella suttoniae	*Phaeophleospora epicoccoides*	AF309587	
	Stomiopeltis sp. S1-2.G2			AY160162
	Stomiopeltis sp. S1-3.G1			AY160163
	Stomiopeltis sp. S1-4.G4			AY160164
	Stomiopeltis sp. S1-5.G4			AY160165
	Stomiopeltis sp. S1-8.G1			AY160166
	Stomiopeltis sp. S1-9.G2			AY160167
	Stomiopeltis sp. S1-10.G3			AY160168
Dothideales	*Stomiopeltis* sp. S1-11.G3			AY160169
	Stomiopeltis sp. S1-12.G5			AY160170
	Stomiopeltis sp. S1-13.G3			AY160171
	Stomiopeltis sp. S1-14.G3			AY160172
	Stomiopeltis sp. S1-15.G1			AY160173.
	Stomiopeltis sp. S1-16.G1			AY160174
	Stomiopeltis sp. S1-18.G2			AY160174
	Stomiopeltis sp. S1-19.G1			AY160177
	Stomiopeltis sp. S1-21.G1			AY160177
	Stomiopeltis sp. S1-23.G1			AY160178
	Stomiopeltis sp. S1-24.G1			AY160179
	Stomiopeltis sp.*		AY598919	AY598880
	Stomiopeltis sp.*		AY598920	AY598881
	Schizothyrium pomi	*Zygophiala jamaicensis**	AY598906	AY598848
	Schizothyrium pomi	*Zygophiala jamaicensis**	AY598907	AY598849
	Schizothyrium pomi	*Zygophiala jamaicensis**	AY598908	AY598850
Mitosporic fungi		*Peltaster fructicola**	AY598927	AY598886

[a] Alexopoulos *et al.* 1996

[b] * isolates sequenced by authors

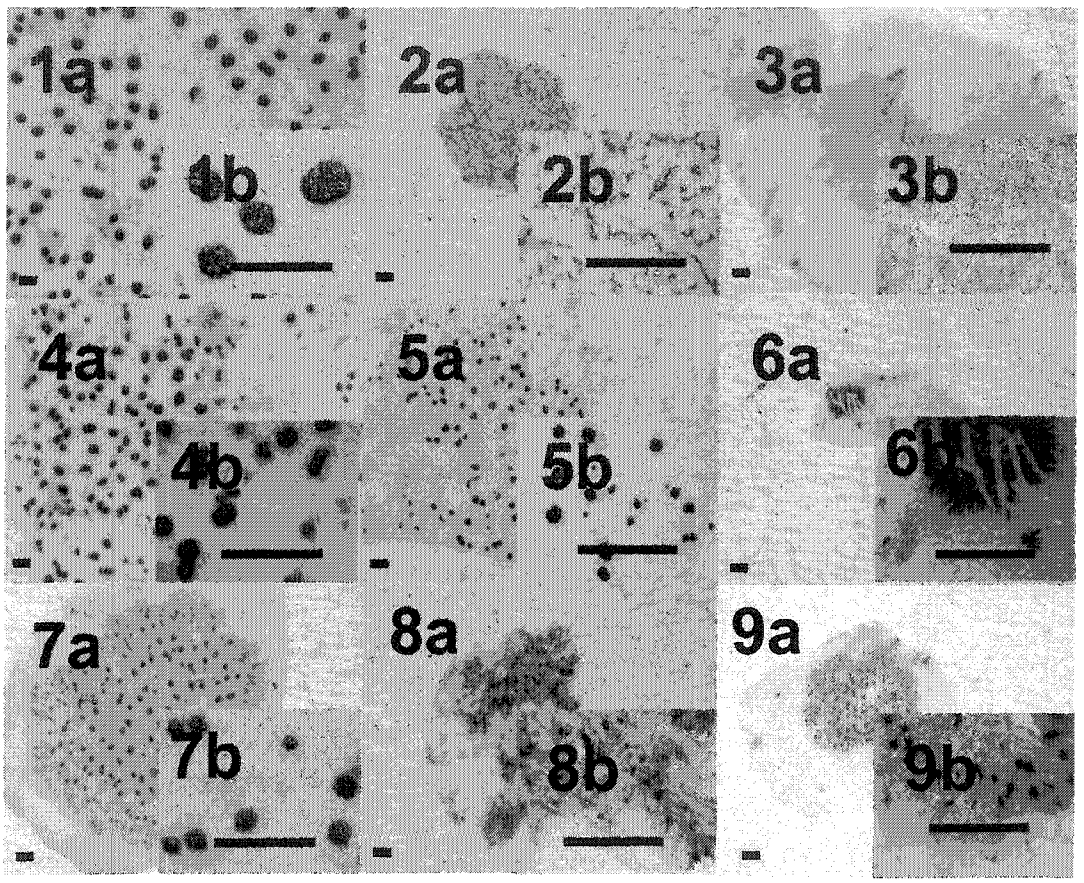
Table II. Putative species, number of isolates, number of orchards detected and distinguishing morphological characters on apple and in culture, growth on MEA at 25 C and accession numbers of LSU and ITS rDNA of isolates of 30 species of sooty blotch and flyspeck fungi from nine orchards in the Midwest.

Putative species	No. of isolates	Orchards detected	Distinguishing morphological characters				LSU rDNA		ITS rDNA	
			On Apple	Mycelium on PDA	Conidia on CLA (μm)	Extent of diameter growth on MEA (mm)[a]	Strain	GenBank	Strain	Genbank
Zygophiala jamaicensis (FS1)	24	9	Flyspeck	Pink, orange, white or green, sectored, thick	20--25 x 5--7	4.5--20.5	CUA1a	AY598895	AHA2a, GTA1a	AY598851, AY598852
Zygophiala sp. FS2	10	2	Small flyspeck	Green and white, thick; red soluble pigment	13.5--21 x 3.5--9	3.0--10.0	FVA2c	AY598896	FVA2a	AY598854
Zygophiala sp. FS3.1	6	2	Large flyspeck	Thin, gray	10--17 x 7--9	13.5--22.5	MSTA8a	AY598897	MSTA8a, GTA4b	AY598853, AY598855
Zygophiala sp. FS3.2	4	1	Large flyspeck	Thin, gray	11.5--18 x 6	2.5--4.5			MWA1a	AY598856
Pseudocercospora *sp. FS4*	2	1	Flyspeck	Olive, rounded; orange soluble pigment	100--350 x 4	3.0--5.5	MWA4b	AY598900	MWA4a	AY598857
Pseudocercospora sp. FG1.1	3	2	Fuliginous	Green, rounded; brown soluble pigment	20--77 x 2--3	2.0--3.0	MWF7a	AY598898	MWF7a	AY598858
Pseudocercospora sp. FG1.2	1	1	Fuliginous	Light brown, thick, lumpy	27--107 x 1.5	ND[b]	MSTF5a	AY598899		
Pseudocercosporella sp. RH1	29	8	Ridged - honeycomb	Pale to dark green, deeply folded	1° conidia 17--63 x 1--5.5; apices tapered	2.0--10.5	MWD1a	AY598901	CUD3b, MWD7a, AHD4a, MWD1a, GTD2a, UMD4	AY598859, AY598860, AY598861, AY598862, AY598863, AY598864
Pseudocercosporella sp. RH2.1	4	3	Ridged - honeycomb	Colonies tough, gelatinous, dirty white	1° conidia 20--67 x 2--4; apices blunt	14.5--19.0	UMD1a	AY598902	UMD6a, UMD1a, AHE9a	AY598865, AY598866, AY598867
Pseudocercosporella sp. RH2.2	4	3	Ridged - honeycomb	Colonies tough, gelatinous, dirty white	1° conidia 12.5--22 x 8--10; hourglass-shaped	3.0--12.5	GTC1a UID2	AY598904, AY598903	GTC1a, UID2	AY598868, AY598869

Putative species	No. of isolates	Orchards detected	Distinguishing morphological characters			Extent of diameter growth on MEA (mm)[a]	LSU rDNA		ITS rDNA	
			On Apple	Mycelium on PDA	Conidia on CLA (μm)		Strain	GenBank	Strain	Genbank
Colletogloeum sp. FG2.1	3	2	Fuliginous	Light brown, mounded, felty, thick	6--19 x 2.5--4.5	5.5--7.5	AHF4a	AY598908	AHF4a	AY598870
Colletogloeum sp. FG2.2	18	6	Fuliginous	Beige, flat, thin edges	5--19 x 2--5	8.0--14.5	MWF1a	AY598907	MWF1a	AY598871
Colletogloeum sp. FG2.3	2	2	Fuliginous	Pale green and white, felty	7--11 x 1--2	8.0--10.0	CUF2d	AY598909	CUF2d	AY598872
Ramularia sp. P5	2	2	Punctate	Green, pink, white, sectored, folded	5.2--14.5 x 1.5--7; Ovularia-type	8.0--9.5	UME2	AY598910	UME2	AY598873
Ramularia sp. CS2	8	3	Compact speck	Pink, dense, smooth; surface cracks revealing black interior; red soluble pigment	13--41 x 1.5--2.2; Catenate, attached with narrow, curved bridge	12.9--14.0	GTE4b	AY598911		
Dissoconium sp. DS1.1	12	4	Discrete speck	Pale pink/white; black sclerotia embedded in media	Macroconidia 11--27 x 4.5--10; microconidia obovate 6--17 x 4.5--10	6.0--13.0	MSTB4b	AY598912	CUB2c	AY598874
Dissoconium sp. DS1.2	2	1	Discrete speck	White; black sclerotia embedded in media; yellow soluble pigment	Macroconidia 16--19 x 5--9; microconidia obovate 9--16 x 4--8	7.5--14.0	UMB4b	AY598913	UMB4a	AY598875
Dissoconium sp. DS2	3	1	Discrete speck	Gray-green, white patches	Macroconidia 27--36 x 7--11; microconidia spherical, beaked 5--10 x 5--10	15.5--26.5	MWB6	AY598914		
Dissoconium sp. FG4	5	1	Fuliginous	Olive, folded, dense	Macroconidia 13--23 x 4--5.5; microconidia ovoid 3--4 x 2--3	10.2--19.0	MSTF2	AY598915	MSTF3a	AY598876
Dissoconium sp. FG5	4	2	Fuliginous	Dark olive, highly mounded	12--17 x 3--4; not forcibly discharged	2.5--4.5	UIF3	AY598916	UIF3	AY598877

Putative species	No. of isolates	Orchards detected	Distinguishing morphological characters			Extent of diameter growth on MEA (mm)[a]	LSU rDNA		ITS rDNA	
			On Apple	Mycelium on PDA	Conidia on CLA (μm)		Strain	GenBank	Strain	Genbank
Xenostigmina sp. P3	2	1	Punctate	Carbonaceous	35--70 x 14--27; Clavate dictyospores	1.0--1.5	AHE7a	AY598917	AHE7a	AY598878
Xenostigmina sp. P4	1	1	Punctate	Carbonaceous, highly mounded	80--128 x 8--12.5	0.5--1.0	UIE3	AY598918	UIE3	AY598879
Sterile mycelia sp. RS1	4	3	Ramose	Olive tan, lobed, mounded; brown sclerotia	None	0.5--2.0	PEC6a	AY598921	PEC6a	AY598882
Sterile mycelia sp. RS2	7	3	Ramose	Blue-green, felty, highly ridged, arborescent submerged margins	None	0.25--2.5	AHC3a, UMC2	AY598922, AY598923	AHC1a, UMC2	AY598882, AY598883
Sterile mycelia sp. RS3	3	1	Ramose	Gray, flat	None	NA	MWC6c			
Passalora sp. FG3	5	1	Fuliginous	Blackish brown, convoluted	Dark, catenate, with flattened ends	2.0--3.0	GTF3a	AY598926		
Peltaster fructicola (sp. P1)	17	4	Punctate	Young colonies with yellow yeast-like growth, turning green velvety with age	1.5--10 x 1.5--4.0	6.5--9.5	MSTE1a	AY598928	MSTE1a	AY598887
Peltaster sp. P2.1	4	2	Punctate	Black and green, zonate, convoluted; orange exudates on older colonies	4--7.5 x 2--4	3.0--5.0	GTE9a	AY598929	GTE6d	AY598888
Peltaster sp. P2.2	4	2	Punctate	Black colonies; pink exudate on older colonies	2.5--6.5 x 1.5--3	1.2--2.0	CUB2a	AY598930	CUE2b, UIE11b	AY598889, AY598890
Peltaster sp. CS1	8	3	Compact speck	Pink yeast-like growth, turning black with age; no mycelium	5--10 x 2.5--5	0.5--2.5	PEE1 UIE17b	AY598905 AY598906	PEE2 MSTEb	AY598891 AY598892

[a]Diameter of colonies, minus plug diameter, measured after 4 wk on MEA at 25 C. [b]ND=not determined



Figures 1–9. Mycelial types of fungi in the sooty blotch and flyspeck complex on apple fruit in overview (a) and close up view (b). 1. Flyspeck (FS), caused by *Zygophiala jamaicensis*. 2. Compact speck (CS), caused by *Ramularia* sp. CS2. 3. Discrete speck (DS), caused by *Dissoconium* sp. DS1. 4. Ramose (RS), caused by Sterile mycelia sp. RS2. 5. Ramose, caused by Sterile mycelia sp. RS3. 6. Punctate (P), caused by *Xenostigmina* sp. P4. 7. Punctate (P), caused by *Peltaster fructicola*. 8. Ridged honeycomb (RH), caused by *Pseudocercosporella* sp. RH1. 9. Fuliginous (FG) caused by *Dissoconium* sp. FG4. Scale bar = 500 μm in all photos.

Figure 10. One of 766 equally most parsimonious trees of partial sequences of the 28S large subunit (LSU) region of rDNA from sooty blotch and flyspeck species and other ascomycetes. The tree is rooted to *Peziza ampelina*. Branches in bold are supported by strict consensus of the most parsimonious trees. Total tree length = 907; retention index = 0.7593; consistency index = 0.4377; rescaled consistency index = 0.7593; 169 parsimony informative characters. Bootstrap values >80% are indicated above branches. Taxa in bold were obtained from apples infested with sooty blotch and flyspeck complex in the Midwest. Taxa designated by * were previously identified members of the SBFS complex obtained from T.B. Sutton, North Carolina State University. Numbers within parentheses indicate the number of isolates with identical LSU sequences.

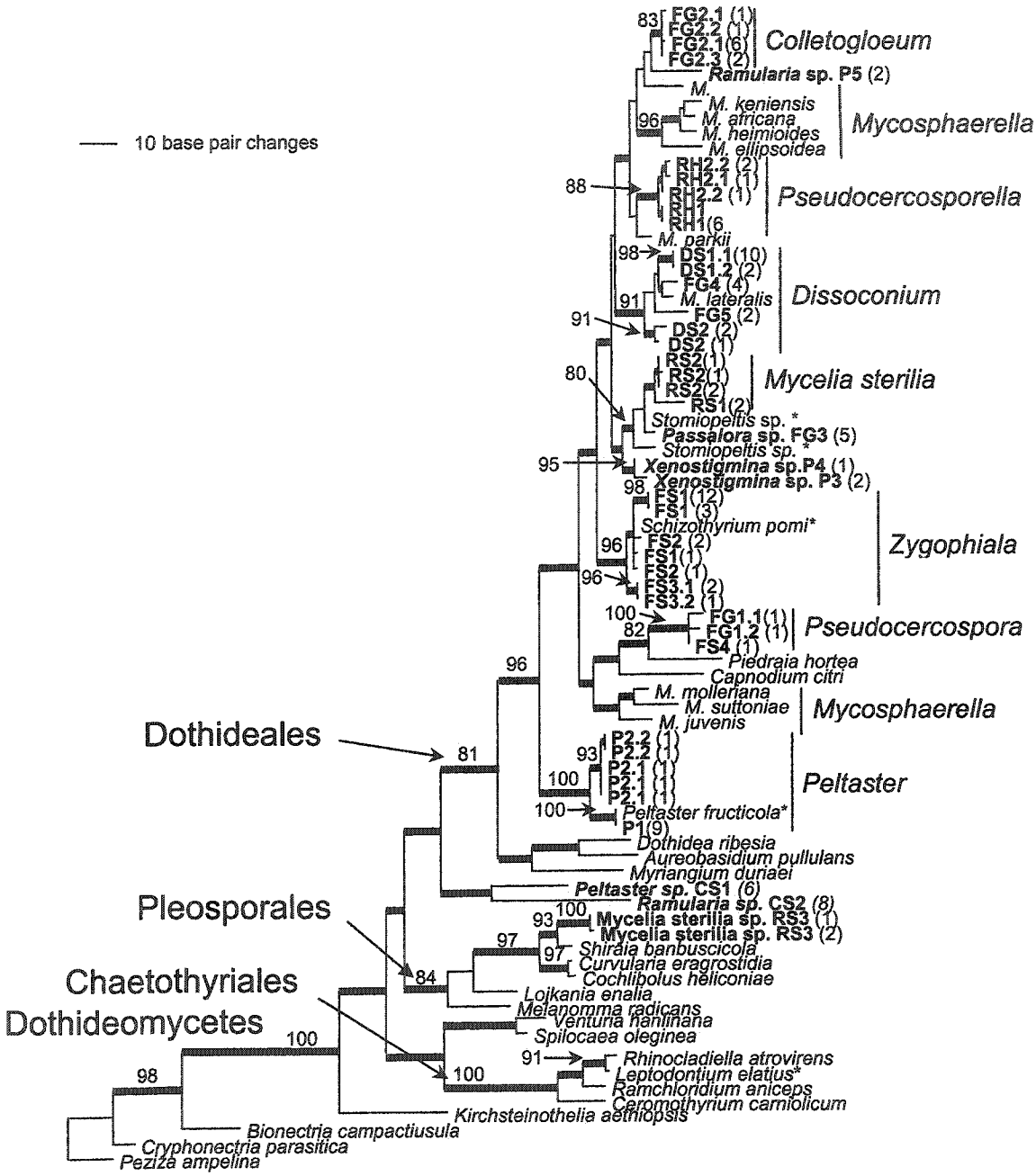

10 base pair changes
Colletogloeum
Mycosphaerella
Pseudocercosporella
Dissoconium
Mycelia sterilia
Zygophiala
Pseudocercospora
Mycosphaerella
Peltaster
Dothideales
Pleosporales
Chaetothyriales
Dothideomycetes
FG2.1 (1)
FG2.2 (1)
FG2.1(6)
FG2.3 (2)
Ramularia sp. P5 (2)
M.
M. keniensis
M. africana
M. heimioides
M. ellipsoidea
RH2.2 (2)
RH2.1 (1)
RH2.2 (1)
RH1
RH1(6
M. parkii
DS1.1(10)
DS1.2 (2)
FG4 (4)
M. lateralis
FG5 (2)
DS2 (2)
DS2 (1)
RS2(1)
RS2(1)
RS2(2)
RS1(2)
Stomiopeltis sp. *
Passalora sp. FG3 (5)
Stomiopeltis sp.
Xenostigmina sp.P4 (1)
Xenostigmina sp. P3 (2)
FS1(12)
FS1 (3)
Schizothyrium pomi*
FS2 (2)
FS1(1)
FS2 (1)
FS3.1 (2)
FS3.2 (1)
FG1.1(1)
FG1.2 (1)
FS4 (1)
Piedraia hortea
Capnodium citri
M. molleriana
M. suttoniae
M. juvenis
P2.2 (1)
P2.2 (1)
P2.1 (1)
P2.1 (1)
P2.1 (1)
Peltaster fructicola*
P1(9)
Dothidea ribesia
Aureobasidium pullulans
Myriangium duriaei
Peltaster sp. CS1 (6)
Ramularia sp. CS2 (8)
Mycelia sterilia sp. RS3 (1)
Mycelia sterilia sp. RS3 (2)
Shiraia banbuscicola
Curvularia eragrostidia
Cochliobolus heliconiae
Lojkania enalia
Melanomma radicans
Venturia hanliniana
Spilocaea oleginea
Rhinocladiella atrovirens
Leptodontium elatius*
Ramichloridium aniceps
Ceramothyrium carniolicum
Kirchsteinothelia aethiopsis
Bionectria campactiusula
Cryphonectria parasitica
Peziza ampelina

Figure 10

Figure 11. Neighbor joining tree using partial sequences from the large subunit rDNA from sooty blotch and flyspeck species and other ascomycetes. *Peziza ampelina* was used as the outgroup taxon. Bootstrap values >80% are indicated above branches. Taxa in bold were obtained from infested apples from the Midwest. Taxa designated by * were previously identified members of the SBFS complex obtained from T. B. Sutton, North Carolina State University. Numbers within parentheses indicate the number of isolates with identical LSU sequences.

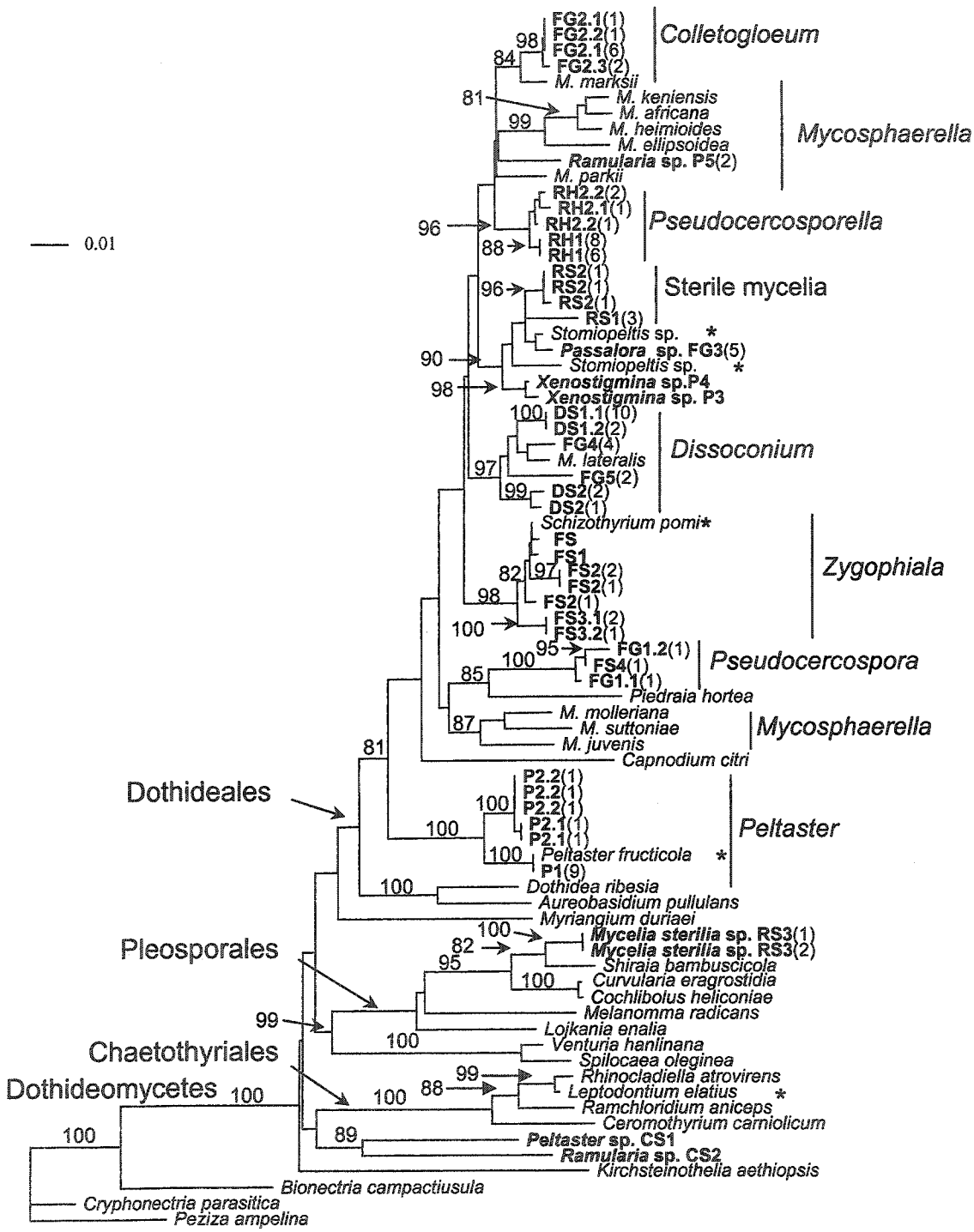

0.01
FG2.1(1)
FG2.2(1)
FG2.1(6)
FG2.3(2)
M. marksii
Colletogloeum
M. keniensis
M. africana
M. heimioides
M. ellipsoidea
Ramularia sp. P5(2)
M. parkii
Mycosphaerella
RH2.2(2)
RH2.1(1)
RH2.2(1)
RH1(8)
RH1(6)
Pseudocercosporella
RS2(1)
RS2(1)
RS2(1)
RS1(3)
Sterile mycelia
Stomiopeltis sp. *
Passalora sp. FG3(5)
Stomiopeltis sp. *
Xenostigmina sp.P4
Xenostigmina sp. P3
DS1.1(10)
DS1.2(2)
FG4(4)
M. lateralis
FG5(2)
DS2(2)
DS2(1)
Dissoconium
Schizothyrium pomi*
FS
FS1
FS2(2)
FS2(1)
FS2(1)
FS3.1(2)
FS3.2(1)
Zygophiala
FG1.2(1)
FS4(1)
FG1.1(1)
Pseudocercospora
Piedraia hortea
M. molleriana
M. suttoniae
M. juvenis
Mycosphaerella
Capnodium citri
Dothideales
P2.2(1)
P2.2(1)
P2.2(1)
P2.1(1)
P2.1(1)
Peltaster fructicola *
P1(9)
Peltaster
Dothidea ribesia
Aureobasidium pullulans
Myriangium duriaei
Pleosporales
Mycelia sterilia sp. RS3(1)
Mycelia sterilia sp. RS3(2)
Shiraia bambuscicola
Curvularia eragrostidia
Cochlibolus heliconiae
Melanomma radicans
Lojkania enalia
Venturia hanlinana
Spilocaea oleginea
Chaetothyriales
Rhinocladiella atrovirens
Leptodontium elatius *
Ramichloridium aniceps
Ceromothyrium carniolicum
Dothideomycetes
Peltaster sp. CS1
Ramularia sp. CS2
Kirchsteinothelia aethiopsis
Bionectria campactiusula
Cryphonectria parasitica
Peziza ampelina

Figure 11

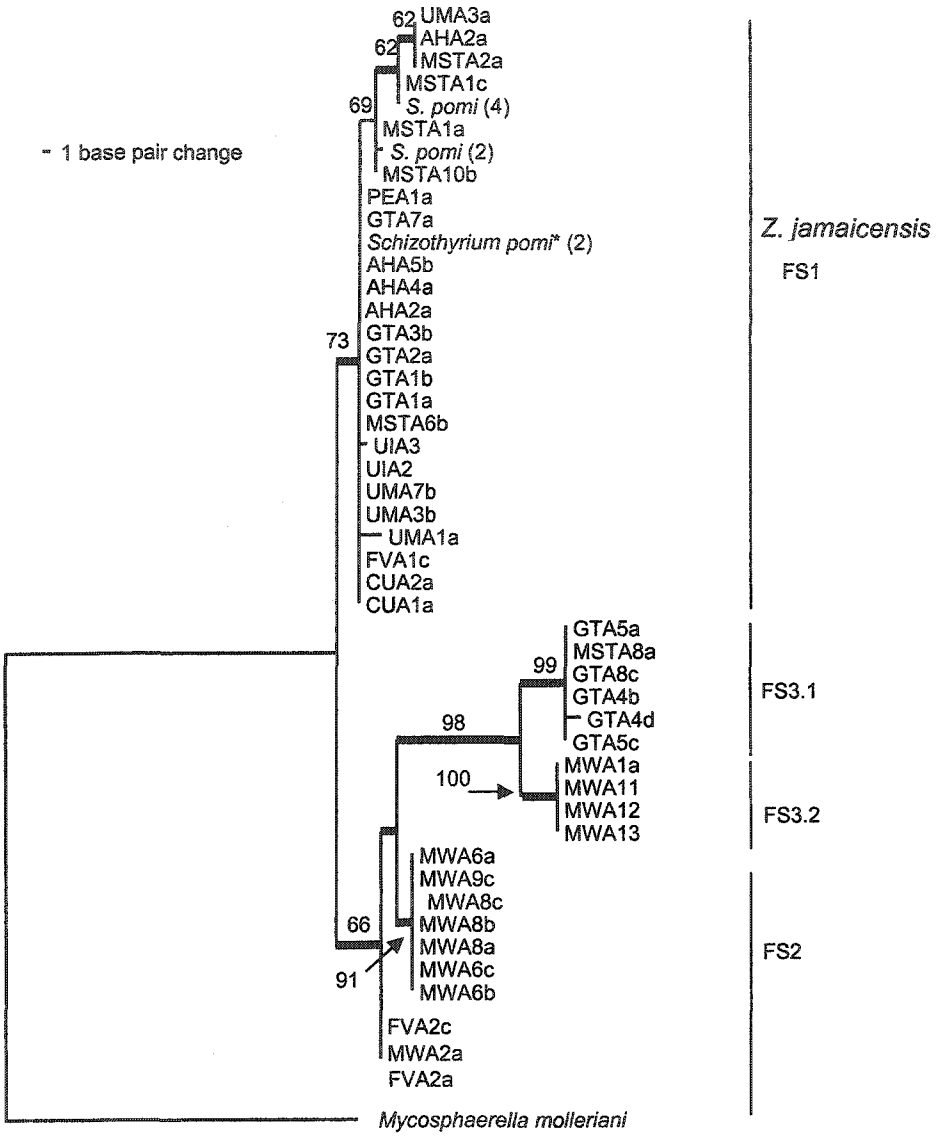


Figure 12. One of 22 most parsimonious trees of sequences of the ITS region of rDNA from *Schizothyrium pomi* and *Zygophiala* species isolated from apples with flyspeck (FS) in the Midwest. Putative species were inferred by parsimony analysis based on 478 characters, including gaps, of the ITS-1, 5.8S, and ITS-2 regions of the rDNA operon. Gaps were treated as a fifth state. The tree is rooted to *Mycosphaerella molleriana.* Bootstrap values greater than 50% are denoted above branches. Branches in bold are supported by strict consensus of all trees. Total tree length = 142; retention index = 0.9577; consistency index = 0.8951.

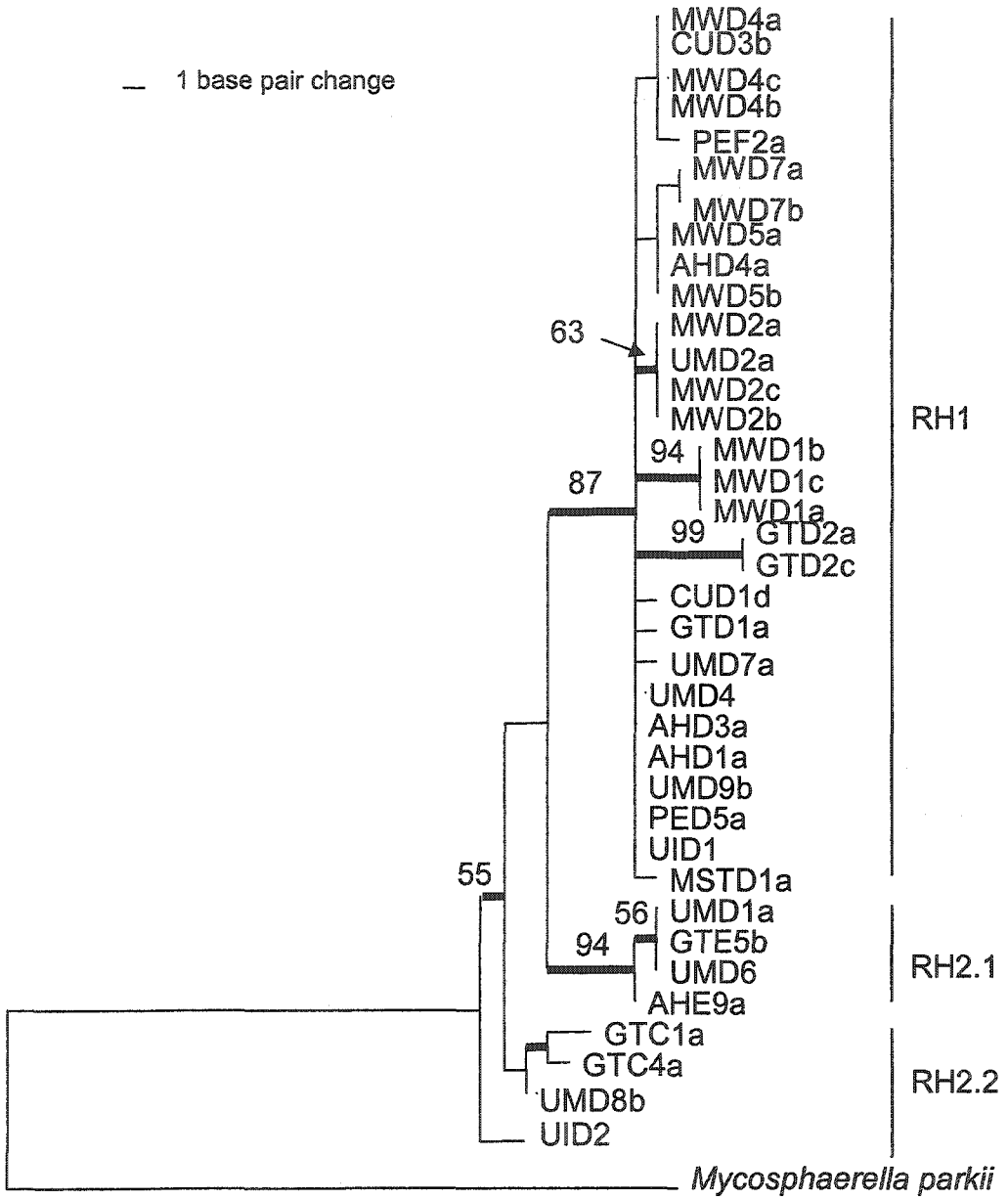


Figure 13. One of seven most parsimonious trees of sequences of the ITS region of rDNA from *Mycosphaerella parkii* and three *Pseudocercosporella* species isolated from apples with ridged-honeycomb (RH) sooty blotch in the Midwest. Putative species were inferred by parsimony analysis based on 476 characters, including gaps, of the ITS-1, 5.8, and ITS-2 regions of the rDNA operon. Gaps were treated as a fifth state. The tree is rooted to *Mycosphaerella parkii.* Bootstrap values greater than 50% are denoted above branches. Branches in bold are supported by strict consensus of all trees. Total tree length = 89; retention index = 0.9048; consistency index = 0.9101.

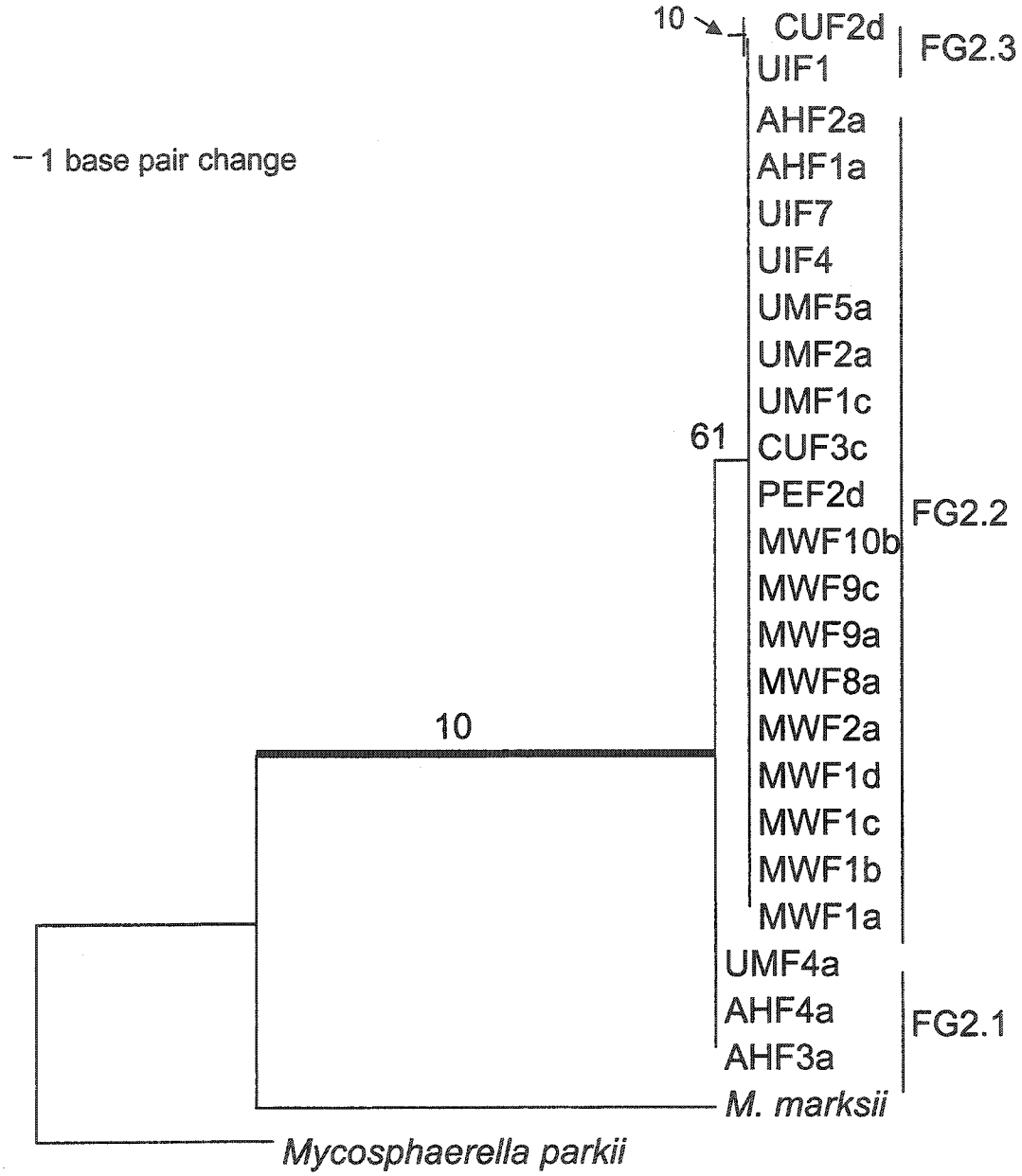


Figure 14. One of four most parsimonious trees of sequences of the ITS region of rDNA from *Mycosphaerella parkii, M. marksii* and three *Colletogloeum* species isolated from apples with fuliginous (FG) sooty blotch in the Midwest. Putative species were inferred by parsimony analysis based on 469 characters, including gaps, of the ITS-1, 5.8 S, and ITS-2 regions of the rDNA operon. Gaps were treated as a fifth state. The tree is rooted to *Mycosphaerella parkii*. Bootstrap values greater than 50% are denoted above branches. Branches in bold are supported by strict consensus of all trees. Total tree length = 849; retention index = 0.9259; consistency index = 0.9762.

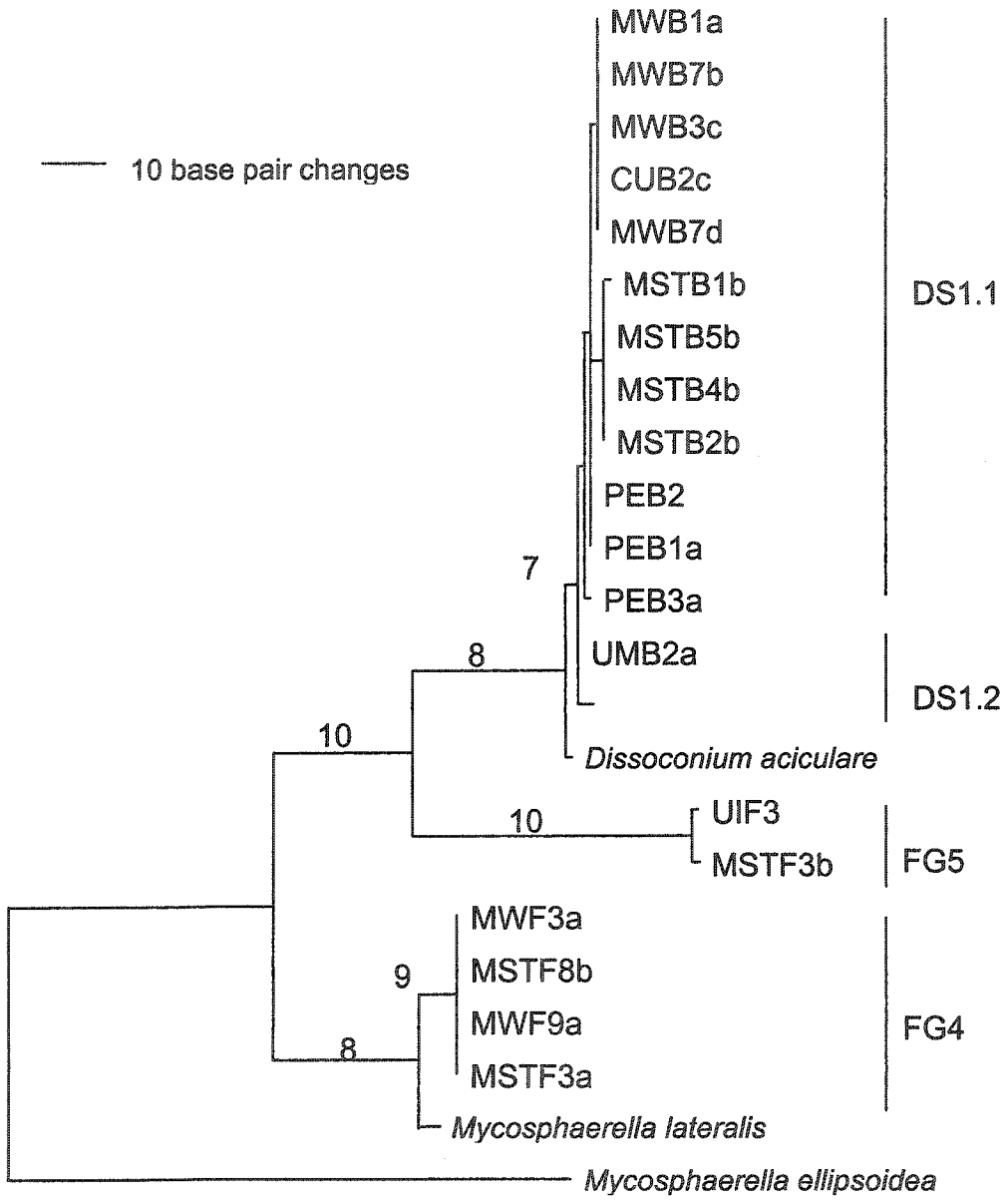


Figure 15. The single most parsimonious tree of sequences of the ITS region of rDNA from *Mycosphaerella lateralis, M. ellipsoidea, Dissoconium aciculare* and four *Dissoconium* species isolated from apples with discrete speck (DS) and fuliginous (FG) sooty blotch in the Midwest. Putative species were inferred by parsimony analysis based on 504 characters, including gaps, of the ITS1, 5.8 S, and ITS2 regions of the rDNA operon. Gaps were treated as a fifth state. The tree is rooted to *Mycosphaerella ellipsoidea.* Bootstrap values above 50% are denoted above branches. Total tree length = 265; retention index = 0.9522; consistency index = 0.9245.

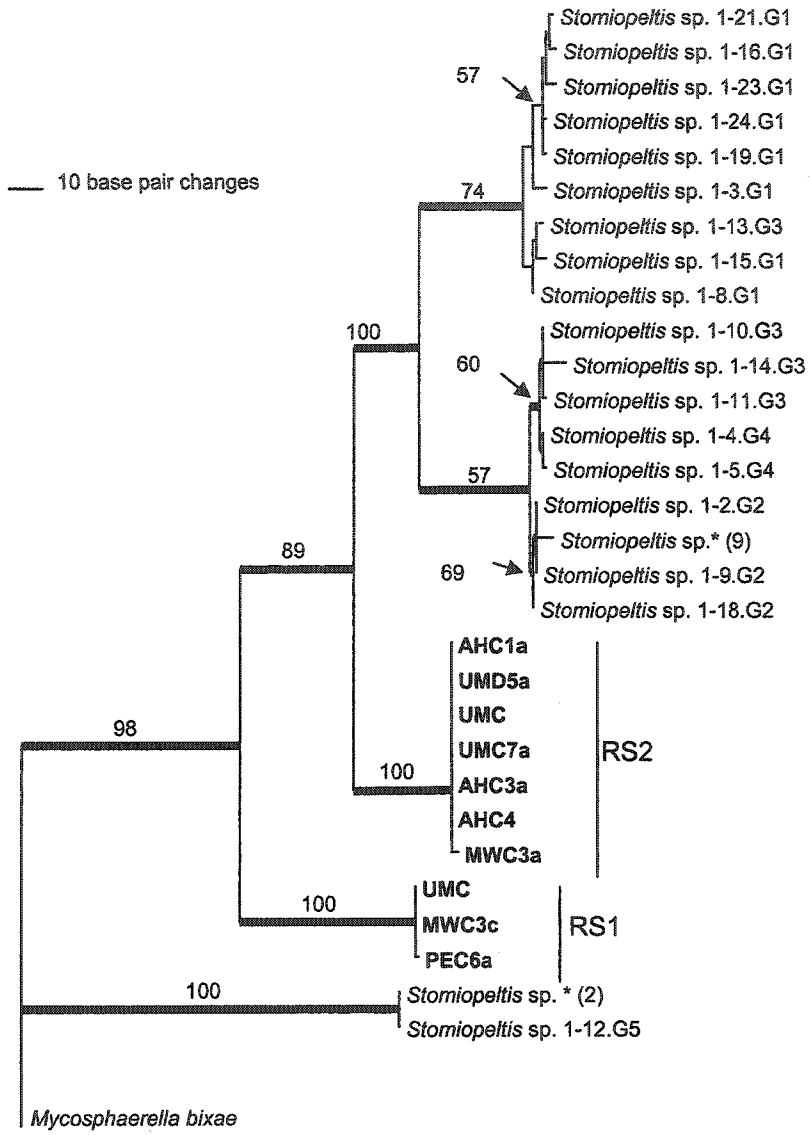


Figure 16. One of 108 most parsimonious tree of sequences of the ITS region of rDNA from *Mycosphaerella bixae,* 20 *Stomiopeltis* species (isolates sequenced by authors are denoted with an *), and two species with sterile mycelia isolated from ramose (RS) sooty blotch in the Midwest (denoted in bold). Putative species were inferred by parsimony analysis based on 522 characters, including gaps, of the ITS1, 5.8 S, and ITS2 regions of the rDNA operon. Gaps were treated as a fifth state. The tree is rooted to *Mycosphaerella bixae.* Bootstrap values above 50% are denoted above branches. Branches in bold are supported by strict consensus of all trees. Total tree length = 280; retention index = 0.9277; consistency index = 0.8321.

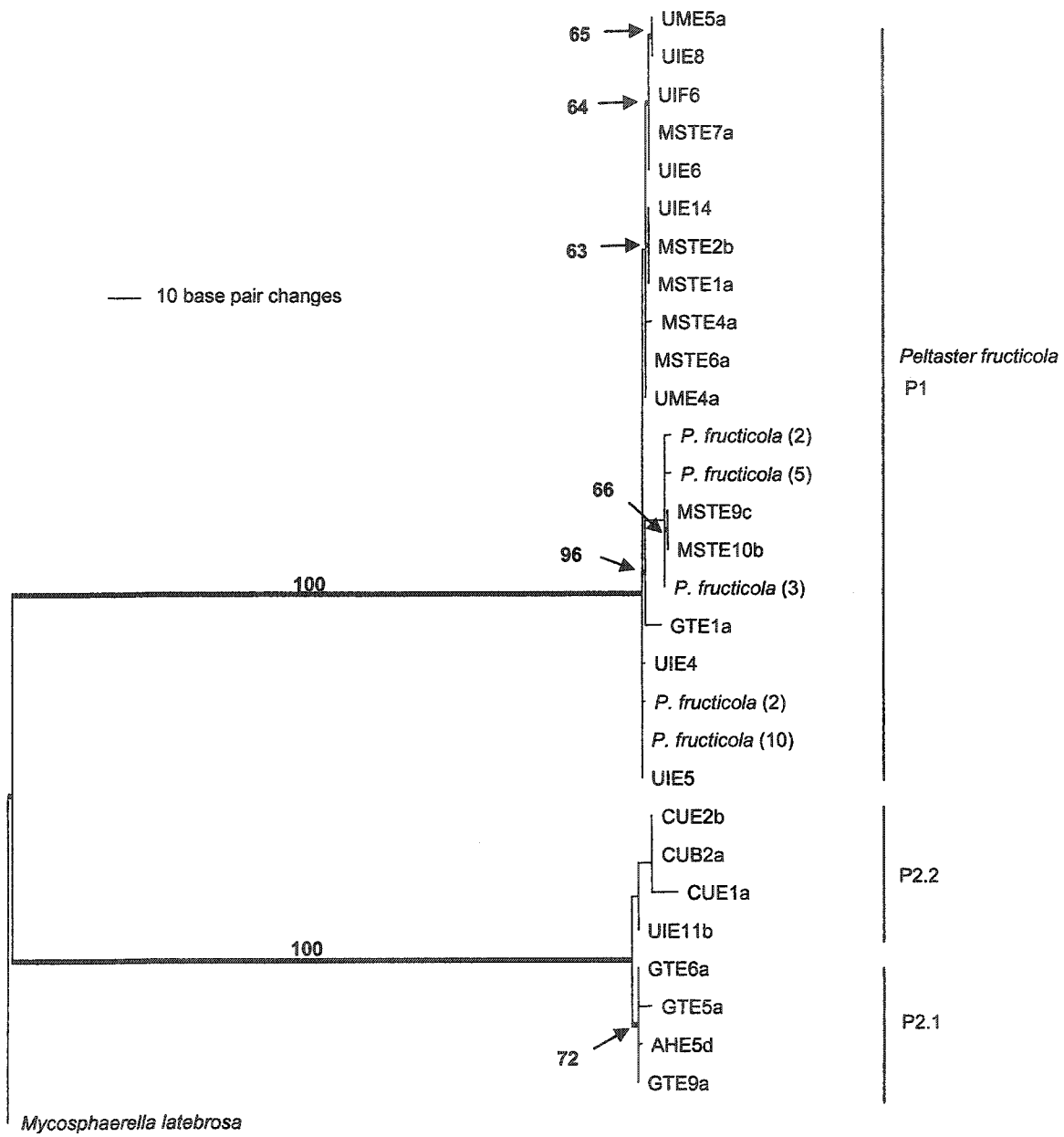


Figure 17. One of 83 most parsimonious trees of sequences of the ITS region of rDNA from *Mycosphaerella latebrosa* and three *Peltaster* species isolated from apples with punctate (P) sooty blotch in the Midwest. Putative species were inferred by parsimony analysis based on 537 characters, including gaps, of the ITS1, 5.8 S, and ITS2 regions of the rDNA operon. Gaps were treated as a fifth state. The tree is rooted to *Mycosphaerella latebrosa*. Bootstrap values above 50% are denoted above branches. Branches in bold are supported by strict consensus of all trees. Total tree length = 308; retention index = 0.9876; consistency index = 0.9643.

CHAPTER 3. EVALUATION OF POST-HARVEST REMOVAL OF SOOTY BLOTCH AND FLYSPECK ON APPLES USING SODIUM HYPOCHLORITE, HYDROGEN PEROXIDE WITH PEROXYACETIC ACID, AND SOAP[1]

A paper published in the journal *Plant Disease*[2]

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ABSTRACT

Post-harvest dips in commercial disinfestants were used to remove signs of the flyspeck pathogen (FS), *Schizothyrium pomi*, and the sooty blotch (SB) complex (*Peltaster fructicola, Leptodontium elatius*, and *Geastrumia polystigmatis*). Apples were dipped for 7 or 15 min in buffered sodium hypochlorite (Agclor 310 plus Decco 312 Buffer) at 200, 400, 500, 600 or 800 ppm chlorine, a mixture of hydrogen peroxide and peroxyacetic acid

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(Tsunami 100) at 60 ppm/80 ppm, 120 ppm/160 ppm, or 360 ppm/480 ppm, respectively, or soap (Kleen 440), then brushed and rinsed for 30 s on a commercial grading line. Disease severity was assessed as percent diseased area using a quantitative rating system, and by counting the number of colonies of three mycelial types of SB and FS. Percent diseased area on apples was converted to USDA apple grade ratings and retail values. Both assessment methods provided similar results, but the percent-diseased-area method was less labor intensive. A 7-min dip in 800 ppm chlorine resulted in a mean increase from 25% and 55% to 100% "Extra Fancy" grade for 'Jonathan' and 'Golden Delicious' apples, respectively, and increased market value by 31 and 14%, respectively. The 7-min, 200-ppm chlorine dip resulted in an increase from 28% and 45% to 92.5% and 96.5% "Extra Fancy" after treatment for 'Jonathan' and 'Golden Delicious', respectively. Blemishes were removed more effectively from 'Jonathan' and 'MacIntosh' apples than from 'Golden Delicious'. Mycelial types of the sooty blotch and flyspeck fungi were removed differentially by the disinfestant dip treatments.

Key words: disease assessment, epidemiology

INTRODUCTION

Flyspeck (FS), caused by *Schizothyrium pomi* (Mont. & Fr.) Arx. (anamorph: *Zygophiala jamaicensis* E. Mason), and the sooty blotch complex (SB), caused by at least three different fungal species [*Peltaster fructicola* Johnson, Sutton & Hodges, *Leptodontium elatius* (G. Mangenot) de Hoog, and *Geastrumia polystigmatis* Batista & M.L. Farr (Williamson and Sutton 2000)], are major diseases of commercial apples (*Malus* x *domestica* Borkh.) in eastern North America and worldwide. The SB and FS fungi grow on the

epicuticular wax layer of apples (Johnson et al. 1997, Nasu and Kunoh 1987, Ocamb-Basu and Sutton 1988). No cultivar resistance has been reported. Several different SB and FS fungi often co-exist in the same orchards and on the same fruit (Johnson and Sutton 2000, Williamson and Sutton 2000). Sooty blotch appears as a dark smudge-like blemish on the apple surface, whereas FS resembles fecal spots left by insects (Brown and Sutton 1993). The fungi of the SB complex have been categorized into various mycelial types including punctate, ramose, rimate and fuliginous (Campbell and Madden 1990, Colby 1920, Groves 1933, Johnson et al. 1996, Johnson et al. 1997). The presence of either SB or FS on the apple surface lowers quality and market value of the fruit (Jones and Sutton 1996).

Control of SB and FS with fungicide sprays is often cost effective for apple growers (Rosenberger et al. 1996). However, in regions of high rainfall and relative humidity, such as the southeastern U.S., even weekly fungicide applications are frequently inadequate to completely control these diseases, resulting in 5 to 10% reduction in marketable apples (Main and Gurtz 1988). Without protectant fungicide applications, almost 100% of the apples produced in the Southeast would be affected by SB and FS (Sutton and Sutton 1994).

Growers in the Upper Midwest usually can control SB and FS with frequent fungicide applications, but growers experience sporadic control failures related to poor fungicide coverage, inadequate pruning, and environmental conditions that are highly favorable for disease development (Cooley et al. 1997). Moreover, late-season fungicide sprays contribute to pesticide residues on harvested apples (Belding et al. 1998). Since the 1996 passage of the Food Quality Protection Act (FQPA), U.S. growers face possible loss of broad-spectrum fungicides that are effective against both SB and FS. Furthermore, differential efficacy of fungicides against certain fungi within the SB complex has been shown. For example,

benomyl is less effective against *P. fructicola* than *L. elatius* (Johnson 1994), whereas captan is less effective against *L. elatius* than *P. fructicola* (Belding et al. 1998, Brown and Sutton 1993). Effective post-harvest removal of SB and FS on apples could allow growers to compensate for inadequate control of these diseases in the orchard and to potentially reduce the number of fungicide sprays.

Sodium hypochlorite dips can reduce populations of *Escherichia coli* strain O157:H7, *Botrytis cinerea, Mucor piriformis*, and *Penicillium expansum* on fruits and vegetables (Beuchat et al. 1998, Jones and Sutton 1996, Spotts and Peters 1980, Williamson and Sutton 2000), and reduce severity of SB and FS (Colby 1920, Hendrix 1991, Winsiewsky et al. 2000). In 1920, Colby reported the effectiveness of "Javelle water" (chlorinated water) in removing SB (Colby 1920). Hendrix demonstrated that a 5- to 7-min dip in 500 ppm chlorine, followed by brushing and a fresh water rinse, reduced incidence of SB from 100% to 0% and FS from 100% to 27% (Hendrix 1991). In laboratory trials, the most effective method to eliminate *E. coli* strain O157:H7 from whole apples was a 15-min dip in a solution of hydrogen peroxide and peroxyacetic acid (Honnay 1988, Sapers and Simons 1998, Winsiewsky et al. 2000). Since these materials have demonstrated value as disinfestants in apple cider processing, their potential value in eradication of SB and FS signs on fresh-market apples also merits investigation.

In order to adequately quantify the benefits of various dips, reliable and cost-efficient assessment methods are needed. In this study, two different methods were used to assess the effectiveness of concentration and dip time in sodium hypochlorite solutions, and in mixtures of hydrogen peroxide and peroxyacetic acid, on removal of SB and FS from 'Jonathan' and 'Golden Delicious' apples. The influence of disinfestant dips on the removal of various SB

and FS mycelial types was also evaluated. A preliminary report has been published (Batzer et al. 2001). A preliminary experiment using sodium hypochlorite and soap to remove SB and FS from 'MacIntosh' and 'Golden Delicious' apples is also presented.

MATERIALS AND METHODS

Experiment 1

'MacIntosh' apples were harvested from the Iowa State University Horticulture Station near Gilbert, IA on 10 Sept 1998 and 'Golden Delicious' apples were harvested from a commercial orchard near Indianola, IA, on 18 Sept 1998. 'MacIntosh' apples were very lightly blemished (< 3% of the apple surface). 'Golden Delicious' apples were severely blemished on only one side of the apple due to a fungicide application failure. Fruit were arbitrarily sorted into seven crates of 50 apples and stored at 1°C for 2 weeks until treatment.

SB and FS were rated separately on each apple. 'MacIntosh' apples were rated according to total number of SB and FS colonies on the apple. On 'Golden Delicious' apples, percent coverage on the hemisphere with severe SB and FS infections was rated as follows: 0 = none; 1 = 0.1-4%; 2 = 10-19%; 4 = 20-30%; 5 = 30-50%; and 6= >50%. Apples were rated before and after treatment. Rater 1 evaluated replications 1 and 3; rater 2 evaluated replications 2 and 4.

Dip treatments of 5 to 7 min in a 568-liter polypropylene tank were used to remove SB and FS signs. Buffered sodium hypochlorite solutions [Decco Agclor 310 (Elf Atochem Corp., Monrovia, CA) plus an equal volume of Decco 312 Buffer (Elf Atochem Corp., Monrovia, CA) to achieve a pH of 6.5] with chlorine concentrations of 200, 400, 600 or 800

ppm were prepared by incrementally adding the necessary amount of sodium hypochlorite and buffer to the dump tank beginning with non-chlorinated well water, and increasing chlorine and buffer concentrations by 200 ppm (0.852 liter sodium hypochlorite and buffer) after each treatment until the 800 ppm concentration was achieved. After the tank was drained and rinsed, 3.8 liter of Kleen 441 (Elf Atochem Corp., Monrovia, CA) was added to 568 liter non-chlorinated well water (label rate). For each disinfestant and concentration combination, including water-dip controls, 50 'Golden Delicious' and 50 'MacIntosh' apples per replication were dipped for 5 to 7 min while turning them continuously to ensure uniform exposure.

Apples were transferred from the plastic tubs to the conveyor belt of a commercial grading line using a dip net. No-dip controls were placed directly on the grading line. Apples were passed over a series of dry brushes for 10 s, rinsed with tap water for 10 s under spray nozzles, then passed over additional brushes and a series of foam pads for 10 s. If necessary, apples were pushed along the grading line with a rubber squeegee to ensure uniform time on the brushes. Immediately following treatments, apples were bagged and stored at 1° C for 4 to 5 wk until post-treatment evaluation.

Replications were conducted on four different days. A total of 2,800 apples were included in the experiment (2 cultivars x [4 concentrations of chlorine, soap, water control, and no-dip control] x 4 replications x 50 apples per treatment). Effects of disinfestant, concentration combination, and rater were evaluated using the differences of mean values of "before" and "after" severity ratings for 'Golden Delicious' and colony numbers of SB and FS for 'MacIntosh' (N=4). Disinfestant/concentration dips were compared using a t-test after

an analysis of variance was performed for each cultivar. All statistical analyses were performed using SAS software (version 6.2, SAS Institute, Cary, NC).

Experiment 2

Apples (cv. 'Jonathan' and 'Golden Delicious') were harvested from a commercial orchard near Indianola, IA on 9 and 10 Sept 1999. Trees had not received fungicide applications since approximately 2 weeks after petal fall. Moderately severe levels of SB and FS were observed on all harvested apples. Apples with light SB and FS signs (< 10 colonies or < 2% of the apple surface covered by SB and FS) or otherwise defective fruit were not used in the study. Fruit ranging in severity from 3 to 65% of the surface affected by SB and FS were stored in wooden crates at 1°C for 1 to 4 weeks until they were evaluated for disease severity.

One person performed all disease assessments since, in Experiment 1, significant ($P<$ 0.05) differences between individual raters were observed. Using an indelible marker, apples were numbered at the calyx and a line bisecting the fruit was drawn from the stem end to the calyx. Disease severity data were recorded for each side of the apple before and after treatment. "Before" and "after" ratings were paired for the same apple by noting its individual number. Two methods of disease assessment were compared. The percent diseased area method involved estimating the percentage of diseased area on the front and back halves of each apple (determined by placement of the line drawn on the fruit) with reference to standard area diagrams depicting diseased areas ranging from 3% to 48%. Standard area diagrams were constructed by projecting photographs of 10 'Golden Delicious' and 10 'Jonathan' apples, displaying a wide range of disease severity, onto acetate sheets. After tracing the outline of each apple, a marker was used to blacken the areas displaying SB

or FS signs. The blackened areas on each acetate sheet were quantified using a Delta T Leaf Area Meter (Decagon Devices, Inc., Pullman, WA). Next, the outline of each apple on the acetate sheet was copied onto white paper and cut out. The area of the apple was determined using the same instrument. Percent diseased area was determined as the total diseased area (sum of blackened areas on the acetate sheet) divided by the total area of the paper cutout. Paper images of diseased apples, representing a wide range of disease severity, were pasted onto a poster board and each diagram was labeled with its respective percent diseased area. These standard area diagrams were then used as a reference to estimate percent diseased area of each apple assessed.

The second method of disease assessment involved counting the number of colonies of three distinct mycelial types: flyspeck (FS), having dark fruiting structures (thyrothecia) in discrete groups with no visible mycelial mat; fuliginous (FG), having distinct mycelial mats with no fruiting structures; and punctate/ramose (PR), having dark pycnothyria-like fruiting structures and visible mycelial mats (Williamson and Sutton 2000). Mycelial types of SB and FS on apples were identified under a circular fluorescent light at 5X magnification (Model 8MC-100, Dazor, St. Louis, MO). After disease assessment, 10 apples per treatment were arbitrarily selected, placed in perforated plastic bags, and refrigerated at 1° C for 1 to 7 days to preserve freshness until apples were subjected to the SB and FS removal process.

Solutions were prepared in 31-liter plastic tubs. Buffered sodium hypochlorite solutions were made from Decco Agclor 310 and an equal volume of Decco 312 Buffer to achieve chlorine concentrations of 200, 500, or 800 ppm. Tsunami 100 (a solution of 11% hydrogen peroxide and 15% peroxyacetic acid (Ecolabs, St. Paul, MN)) was prepared at concentration ratios of 60:80, 120:160, or 360:480 ppm hydrogen peroxide: peroxyacetic

acid (HPPA), respectively. Solutions were prepared 30 min prior to use and tubs were covered when not in use. Apples were placed in tubs for 7 or 15 min and turned continuously to ensure uniform exposure. Water controls were placed in non-chlorinated well water in the same manner as disinfestant treatments. Apples were mechanically brushed and rinsed as described for Experiment 1. No-dip controls were placed directly on the grading line.

One replication was conducted each day, for eight separate days. There were 2400 apples included in the experiment (2 cultivars x [(((2 disinfestants x 3 concentrations) + water control) x 2 dip times) + no-dip control] x 8 replications x 10 apples per replication). After effects of cultivar, dip time, and disinfestant were evaluated using percent reduction of "before" and "after" ratings for each apple, the mean percent reduction data for each 10-apple experimental unit were transformed by the natural log prior to analysis. All effects were tested using variability between experimental units. A two-factor ANOVA using cultivar and treatment was performed with complete factorial treatment arrangement. The 15 treatments in this analysis represent unique combinations of disinfestant, concentration, and dip time. The cultivar x disinfestant x concentration interaction was evaluated; if significant, disinfestant x concentration combination effects were evaluated separately for each cultivar using a one-way ANOVA. The effects of disinfestant, concentration, and dip time on reduction of colony number and percent diseased area were compared using Tukey's studentized range test. Statistical analyses were performed using SAS software (version 6.2, SAS Institute), except for the analysis of disease assessment methods, which was done using SPSS Sigma Plot 2000.

All effects were tested using variability between experimental units, which were groups of ten apples. Differences between mycelial types and interactions of mycelial type with disinfestant/concentration were compared using a split plot analysis of variance, in which disinfestant, concentration and cultivar were the main plot effects and mycelial type was the sub-plot effect. If these interactions were significant, the differences between mycelial types were calculated separately for each combination of cultivar, disinfestant and concentration. Interactions of cultivar x mycelial type and cultivar x compound x mycelial type were tested.

To test the hypothesis that the assessment methods (estimates of percent diseased area and counts of the number of SB and FS colonies) were in good agreement, assessment methods were compared by i) regressing mean percent diseased area (y) against colony number (x) in post-treatment observations, and ii) regressing reduction in percent diseased area (y) against reduction in colony number (x) for each disinfestant concentration. Counting colony number was believed to be less subjective than estimating percent diseased area, so colony count data were used as the independent variable in regression equations (Nutter et al. 1993, Nutter and Schultz. 1995). Agreement between the percent diseased area estimates and colony number was evaluated for both apple cultivars using the slopes and y-intercepts of the regression lines relating percent reduction in colony counts (x) and percent reduction in percent diseased area (y) (Nutter et al 1993, Nutter and Schultz. 1995, O'Brien and van Bruggen 1992). Precision was evaluated using the coefficient of determination (r^2), which indicates how much variation in one method (percent diseased area) is explained by another method (colony number), and the standard error of the estimate (SEy), which is a measure of variation about a predicted y (Nutter et al. 1991).

The economic impact of post-harvest removal using disinfestants was estimated after converting the percent diseased area data to USDA grade scores. USDA grade standards (USDA 1995), based on the percentage of apple fruit surface covered by FS and SB signs, were as follows: "Extra Fancy," <5%; "Fancy," 5 to 10%; "Utility," 11 to 33% and "Non-graded," >33%.

Retail values (dollars per bushel) of fresh-market apples ("Extra Fancy" and "Fancy," collectively marketed locally as "No. 1") and cider apples ("Utility" and lower grades) were obtained from four commercial growers in Iowa who marketed their products directly to the public at farmers' market or roadside stands. We assumed that one bushel contained 160 medium size apples and weighed 18.16 kg. We then compared the estimated retail value of the apples used for each disinfestant and concentration combination before and after the 7-min dip. Retail value estimates were determined in two ways. For sorted apples, dollar values were determined by the proportion of fresh-market and cider apples in the population. For unsorted apples, the presence of any cider-quality apples resulted in the entire population being considered cider quality.

RESULTS

Experiment 1

A high degree of variability was observed in disease severity ratings and colony counts within and between runs of the experiment, especially for pre-treatment values. Pre-treatment and post-treatment colony counts for 'MacIntosh' ranged from 0 to 34 colonies and 0 to 4 colonies, respectively. Disease severity ratings for 'Golden Delicious' ranged from 0 to 6 before treatment and 0 to 4 after treatment. Soap was most effective for the removal of

SB signs for 'MacIntosh' and 'Golden Delicious' apples, on which percent reduction was 90% and 62%, respectively (Table 1). No-dip and water controls were as effective as chlorine for removing SB from 'MacIntosh'. Pre-treatment ratings of FS on 'MacIntosh' were not significantly different from post-treatment ratings, except for the water control (Table 1), but mean percent reduction of FS colonies ranged from 78 to 89% (Table 1). Chlorine at 800 ppm was the most effective treatment for removing FS from 'Golden Delicious.'

Experiment 2

Both methods of disease assessment (reduction in percent diseased area and reduction in colony number) provided similar results. Overall differences between disinfestant and concentration combinations were significant ($P<0.0001$). Reduction of SB and FS severity was significantly ($P<0.0001$) greater on 'Jonathan' than 'Golden Delicious' apples (data not shown). There were significant ($P=0.039$`) differences between dip times when cultivar-disinfestant/concentration combinations were analyzed together. Overall, increasing the soak time from 7 to 15 min resulted in the removal of an additional 11% of SB and FS (data not shown). However, extending the dip time to 15 min did not significantly increase percent reduction of SB and FS for any dip except for 'Jonathan' in 500 ppm chlorine (Table 2).

The 500 and 800 ppm concentrations of chlorine were more effective than 200 ppm chlorine and all HPPA concentrations in reducing SB and FS, except for 7 min of chlorine at 500 ppm (Table 2). In most cases, reduction of diseased area and number of colonies by HPPA, water dips, and the "no dip" control was not significantly different, except for 'Jonathan' at the highest concentration of HPPA (Table 2). There was a significant cultivar-disinfestant/concentration ($P=0.0002$); for example, 25% more colonies were removed on

'Jonathan' than on 'Golden Delicious' in the 15-min dip at the highest rate of HPPA (Table 2).

Agreement between disease assessment methods

Individual colonies were, on average, 53% larger on 'Jonathan' than 'Golden Delicious' (Fig. 1A). When both assessment methods were standardized by converting the data to percent reduction (Fig. 1B), slopes of the regression lines for both cultivars were not significantly different than 1.0, indicating good agreement between the assessment methods (Nutter and Schultz. 1995). There was a significant (P<0.001) linear relationship between reduction of colony number (x) and reduction of percent diseased area (y) for both 'Golden Delicious' and 'Jonathan' apples after treatment (Fig 1B). The y-intercepts for 'Golden Delicious' and 'Jonathan' were significantly (P=0.0000387 and P=0.035, respectively) less than zero. Estimates of reduction in percent diseased area for 'Golden Delicious' and 'Jonathan' were 14.2 and 13.5% higher, respectively, than reduction in colony number (Fig. 1B).

Colony number explained 98.8% and 97.4% of the variation in percent diseased area on 'Golden Delicious' and 'Jonathan' apples, respectively (Fig. 1A). Moreover, colony number predicted percent diseased area (SEEy) in 'Golden Delicious' and 'Jonathan' within 0.2% and 0.3%, respectively (Fig. 2A), and percent reduction in colony number explained 98.3% and 93.3% of the variation in percent reduction in diseased area (Fig. 1B). Percent reduction in colony number predicted estimates of percent diseased area (SEEy) within 2.9% and 4.7% for 'Golden Delicious' and 'Jonathan', respectively (Fig. 1B).

Effect of mycelial type on removal of SB and FS

Removal of FS, and the SB mycelial types PR and FG, was differentially affected by disinfestant and concentration combination ($P<0.0001$) (Fig. 2) and cultivar ($P<0.0001$). There was also an interaction of mycelial type and cultivar for FS and FG ($P=0.0006$ and $P=0.0002$, respectively). On 'Golden Delicious', for example, HPPA, water, and "no-dip" removed FS and PR more effectively than FG ($P<0.05$). At high concentrations of chlorine, however, significant differences between PR and FG were not observed on 'Golden Delicious' apples, and less FS than PR or FG was removed with chlorine. On 'Jonathan' apples, significantly ($P<0.0007$) less FS was removed than PR and FG at all rates of chlorine. The highest rate of HPPA removed significantly ($P<0.006$) more PR and FG than FS from 'Jonathan' apples. The water dip removed significantly more PR than FS or FG ($P<0.0033$ and $P<0.0153$, respectively).

Effect of post-harvest removal of SB and FS on USDA grade and market value

The 7-min 800-ppm chlorine dip resulted in 100% "Extra Fancy" apples for both cultivars (Fig. 3). HPPA dips resulted in 68 to 86% "Extra Fancy," water dips resulted in 60 and 72% "Extra Fancy," and the no-dip control resulted in 47 and 56% "Extra Fancy" on 'Jonathan' and 'Golden Delicious', respectively.

Retail prices obtained in March 2001 from four commercial apple growers in Iowa indicated large differences between fresh-market and cider apple prices. Cider apples sold for $2.00 per bushel at all orchards while the mean price of fresh-market apples was $32 per bushel when sold at roadside stands and farmers' markets. Retail values ranged from $22 to

$48 per bushel depending upon the local market, cultivar, and fruit size. The growers interviewed in this study did not market apples as processing fruit.

The greatest increase in retail value was obtained with chlorine dips (Table 3). If a vendor sorted the apples manually into fresh-market and cider grades, the 7-min dip in 800 ppm chlorine increased the value of 'Golden Delicious' and 'Jonathan' apples by $4.50 and $9.76 per bushel, respectively. The HPPA dips also increased retail value substantially, since "Fancy" and "Extra Fancy" apples were considered to have the same retail value. If apples were left unsorted and orchards with mixed grades were marketed as cider apples at $2.00 per bushel, then the 7-min dip in 800 ppm chlorine for 'Jonathan' and 'Golden Delicious' apples and the 200 and 500 ppm chlorine dips for 'Golden Delicious' increased retail value by $30.00 per bushel. The other disinfestant/concentration dips substantially increased the retail value of the apples, but did not eliminate the need to sort the apples in order to gain economic benefits.

DISCUSSION

The results of both experiments indicated that chlorine reduced FS and SB signs on 'Golden Delicious' apples by 50 to 85%. While the design of Experiment 1 prevented close examination of the effects of disinfestant/concentration, cultivar, and mycelial type on the removal process, mean percent reduction of SB and FS was similar to results of Experiment 2. Experiment 2 was designed to reduce the data variability obtained in Experiment 1 by using a single rater, developing a standard area diagram, labeling the apples individually, pairing before- and after- treatment ratings for the same apple, and increasing the number of runs from four to eight.

Results from Experiment 2 showed that a 7-min dip in 800 ppm chlorine, followed by 30 s of brushing and rinsing, reduced SB and FS sufficiently to yield 100% "Extra Fancy" grade apples, regardless of initial disease level. The 200-ppm (label rate) and 500-ppm chlorine treatments were also highly effective, resulting in 92.5% and 96.5% "Extra Fancy" post-treatment 'Jonathan' and 'Golden Delicious' fruit, respectively. However, these lower chlorine concentrations resulted in a small percentage of 'Jonathan' apples (4%) that remained in the "Utility" grade. Water and HPPA treatments were not effective for the removal of SB and FS as evidenced by the relatively large number of "Utility" apples after treatment. Increasing dip time to 15 min increased the effectiveness of removal of SB and FS by 11%, but one or two "Utility" grade apples per batch were present even after 15 min in 200 ppm chlorine. Our study indicates that increasing the dip time from 7 to 15 min would not be a cost-effective strategy to increase USDA grade scores of apples. Furthermore, increasing the dip time to 15 min may be impractical because it would slow the rate of apple processing unacceptably.

Disease assessment methods in our study differed from those of Hendrix (Hendrix 1991). We evaluated disease severity, whereas Hendrix evaluated disease incidence, which obscures differences among levels of disease severity. Hendrix reported that a 5- to 7-min dip in 500 ppm chlorine removed all SB and 73% of FS from apples, whereas we obtained a reduction of 81% of SB and 75% of FS colonies for 'Golden Delicious' and a reduction of 85% of SB and 70% of FS colonies for 'Jonathan' apples after a 7-min dip in 500 ppm chlorine. A possible explanation for these differences is that the fungal species and mycelial types in Georgia may differ from those present in our Iowa study. The amount of brushing the apples received after treatment also may have differed between the two studies. Hendrix

(Hendrix 1991) reported that apple cultivar ('Red Delicious', 'Golden Delicious', 'Granny Smith', 'Rome Beauty', 'Gala', 'Jonathan', 'Stayman', and 'Empire') did not affect removal of SB and FS, whereas our results clearly indicated that more SB and FS were removed from 'Jonathan' and 'MacIntosh' than 'Golden Delicious'. The chemical composition of epicuticular wax varies among apple cultivars (Belding et al. 1998), and characteristics of the wax may have influenced the removal of SB and FS by disinfestant compounds and the action of the brushes.

Converting percent diseased area assessment data to USDA grade values provided a means to evaluate treatment effects in economic terms, although the benefits to sale value by post-harvest dip treatments will vary according to season and location. In Iowa, locally grown apples are marketed as "No.1," which includes "Extra Fancy" and "Fancy" grades. The USDA shipping point values are based on "Extra Fancy," "Peelers" and "Cider" grades. Manually sorting out these "Utility" grade apples would substantially add to the cost of treatment (Gold and Sutton 1989). The cost of sorting the apples must be considered when determining the feasibility of post-harvest treatment, and may be more affordable for high-value cultivars or in markets with less exacting cosmetic standards.

The percent diseased area assessment method provided an accurate evaluation of disease severity on three-dimensional surfaces. Counting colonies required an average of 90 s per apple. In contrast, assessing percent diseased area provided an accurate estimation of disease severity percent and required an average of only 15 s per apple, regardless of disease severity. Although the reduction in percent diseased area consistently overestimated reduction in colony number by about 14% for both cultivars, this bias was constant and did not affect our conclusions regarding comparisons of disinfestant compound, concentration,

dip time, or cultivar. Raters tend to overestimate disease severity when large numbers of small lesions are present (Campbell and Madden 1990). The occasional differences in disinfestant dip effects between the two assessment methods may have resulted from partial removal of colonies during treatment, more frequent removal of small colonies than large colonies, or the fact that colony number counts ignored colony size. Percent diseased area took into account the size of the colonies and provided a measure of disease severity, whereas colony number provided a measure of the frequency of mycelial types (FS, PR, FG). The diseased area assessment method developed in this study has potential for application to other three-dimensional sampling units beyond the apple/SBFS pathosystem.

The present study is the first to document differential removal of FS and SB mycelial types and interactive effects between treatment and cultivar on effectiveness of post-harvest treatments. Therefore, mycelial type and cultivar should be considered when evaluating effectiveness of a post-harvest treatment on SB and FS removal. Differences in the composition of epicuticular wax of 'Jonathan' and 'Golden Delicious' apples (Belding et al. 1998) may have been a factor in differential removal of mycelial type, as well as an explanation of the 53% greater colony size on 'Jonathan' compared to 'Golden Delicious'. Although the effectiveness of disinfestant treatments may vary with cultivar, mycelial type, and environmental conditions during the growing season, post-harvest removal of SBFS is an effective tool in increasing the retail value of fresh-market apples.

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Table 1. Differences in SBFS disease severity on apples, and percent disease reduction, resulting from post-harvest dips in sodium hypochlorite (Cl), soap, water, or a no-dip control followed by 30 s of scrubbing on a commercial grading line in Experiment 1.

Treatment	Dip time (min)	Pathogen Type	P-value of difference pre- vs. post-treatment [w]		% Reduction in disease	
			MacIntosh[x]	Golden Delicious[y]	MacIntosh[x]	Golden Delicious[y]
No dip	7	SB	0.014	0.014	63 ab [z]	27 b
Soap	7		0.004	0.086	90 a	62 a
Water	7		0.051	0.009	59 b	27 b
200 ppm Cl	7		0.003	0.006	73 ab	36 ab
400 ppm Cl	7		0.011	0.006	81 ab	39 ab
600 ppm Cl	7		0.029	0.005	82 ab	51 a
800 ppm Cl	7		0.024	0.0134	86 ab	54 a
No dip	7	FS	0.167	0.112	80 a	33 c
Soap	7		0.101	0.003	87 a	46 bc
Water	7		0.042	0.007	86 a	51 b
200 ppm Cl	7		0.121	0.015	82 a	50 b
400 ppm Cl	7		0.082	0.028	89 a	48 b
600 ppm Cl	7		0.081	0.050	85 a	53 ab
800 ppm Cl	7		0.086	0.003	78 a	65 a

[w] Means of 50 apples were analyzed separately for each combination of cultivar, disinfestant/concentration, and pathogen type (N=4).

[x] Percent reduction based on number of colonies of SB and FS before and after treatment.

[y] Percent reduction based on a disease severity of SB and FS on a 0 to 6 scale observed before and after treatment.

[z] Numbers within each column followed by the same letter are not significantly different ($P>0.05$). Comparisons were made within cultivar and pathogen type.

Table 2. Reduction in sooty blotch and flyspeck on Golden Delicious and Jonathan apples as a result of post-harvest dips in sodium hypochlorite (Cl), a combination of hydrogen peroxide and peroxyacetic acid (HPPA), water, or no-dip control followed by 30 s scrubbing on a commercial grading line in Experiment 2

Treatment	Concetration (ppm)	Dip time (min)	% Reduction in disease[y] Golden Delicious		Jonathan	
			Number of colonies	Percent diseased area	Number of colonies	Percent diseased area
No dip			29 a [z]	19 a	47 a	31 a
Water		7	36 a	23 a	64 abc	52 ab
		15	42 ab	26 ab	59 abc	49 ab
HPPA (hydrogen peroxide/ peroxyacetic acid)	80 /60	7	40 a	35 ab	56 abc	56 abc
	80 /60	15	46 abc	33 ab	57 abc	57 abc
	160 /120	7	47 abc	40 ab	71 abcd	62 abcd
	160 /120	15	47 abc	37 ab	69 abcd	60 abcd
	480 /360	7	43 ab	33 ab	72 abcd	65 bcd
	480 /360	15	53 abc	46 b	78 bcd	70 bcd
Cl (sodium hypochlrorite)	200	7	63 bc	56 cd	81 cd	75 bc
	200	15	66 cd	62 de	77 bcde	72 bcd
	500	7	78 de	72 ef	84 de	77 de
	500	15	83 e	76 fg	91 ef	88 f
	800	7	86 ef	80 gh	92 f	87 ef
	800	15	91 f	85 h	94 f	89 f

[y] Values based on the percent reduction of 1) number of colonies of sooty blotch and flyspeck2) percent of apple surface area with sooty blotch and flyspeck observed before and after treatment.

[z] Numbers followed with same letter within each column are not significantly different at $P>0.05$ according to Tukey's studentized range for log reduction. N=80.

Table 3. Estimated increase in retail value of apples resulting from removal of SB and FS signs by a 7-min post-harvest dip in sodium hypochlorite (Cl), a combination of hydrogen peroxide and peroxyacetic acid (HPPA), water, or no-dip control followed by 30 s of scrubbing and a water rinse on a commercial grading line.

		Increased retail value ($/kg[x])			
	Concentration	Golden Delicious		Jonathan	
Compound	(ppm)	Sorted[y]	Unsorted[z]	Sorted[y]	Unsorted[z]
Cl (sodium hypochlorite)	200	0.21	0	0.47	0
	500	0.21	1.65	0.45	0
	800	0.25	1.65	0.54	1.65
HPPA (hydrogen peroxide/ peroxyacetic acid)	80/60	0.07	0	0.33	0
	160/120	0.23	0	0.41	0
	480/360	0.19	0	0.41	0
Water		0.14	0	0.35	0
No dip		0.12	0	0.29	0

[x]Dollar values of apples were based on the proportion of USDA grades that could be marketed as fresh-market or cider apples, assuming $1.76/kg and $0.11/kg for No.1 fresh-market and cider apples and 18.16kg/bu for medium-size apples.

[y] Cost of sorting is not included in the analysis.

[z] All apples were used for cider if any portion of the population was lower than USDA grade "Fancy."

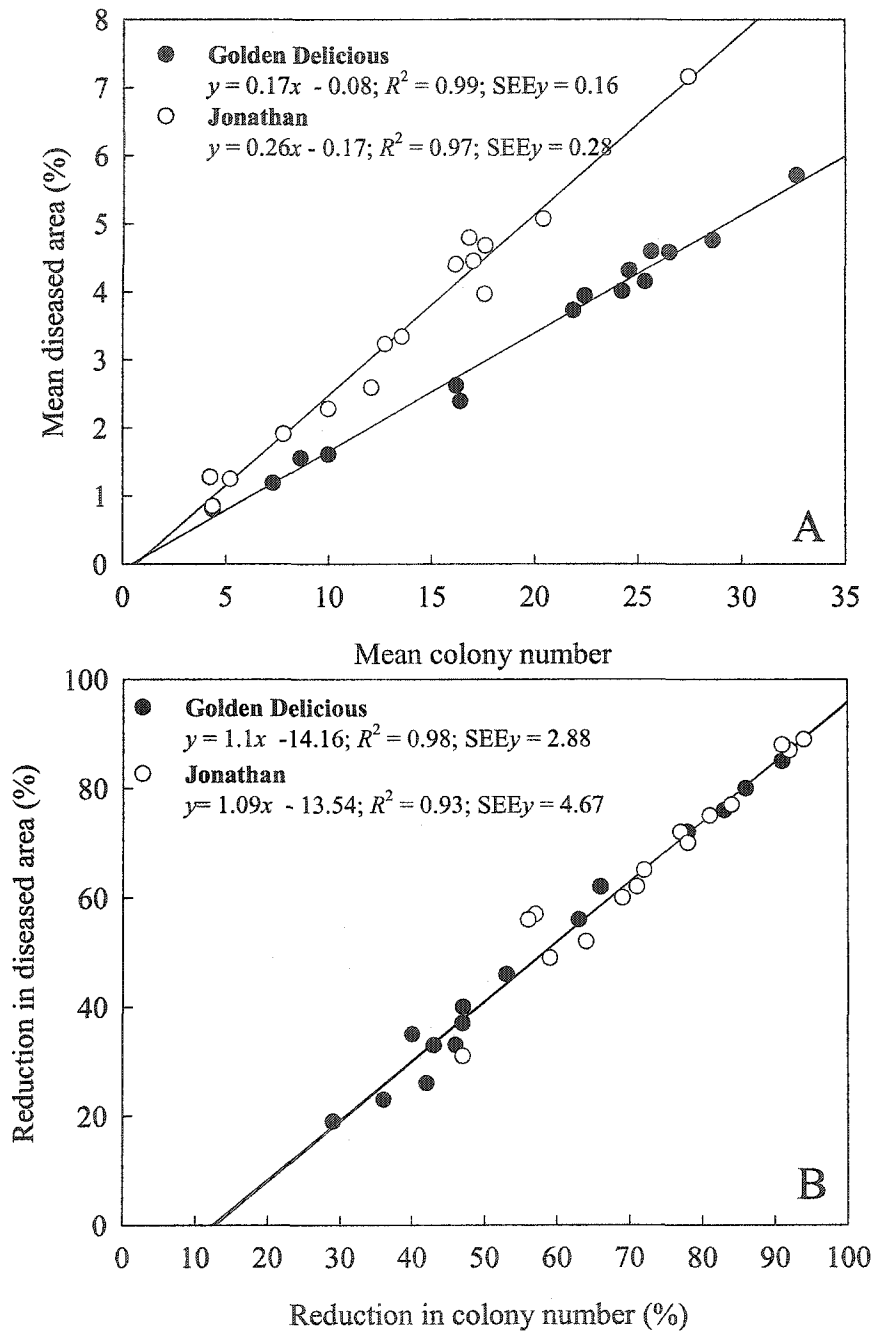

Figure 1. Regression analysis of A) mean colony number of SB and FS (x) vs. mean percent diseased area of SB and FS (y) on apples after treatment and B) mean reduction in colony number (x) vs. mean reduction in percent diseased area of SB and FS (y) after dipping 'Golden Delicious' and 'Jonathan' apples for 7 or 15 min in buffered sodium hypochlorite, a combination of hydrogen peroxide and peroxyacetic acid, or water, followed by 30 s brushing on a commercial grading line. Treatments were combined (N=1,200).

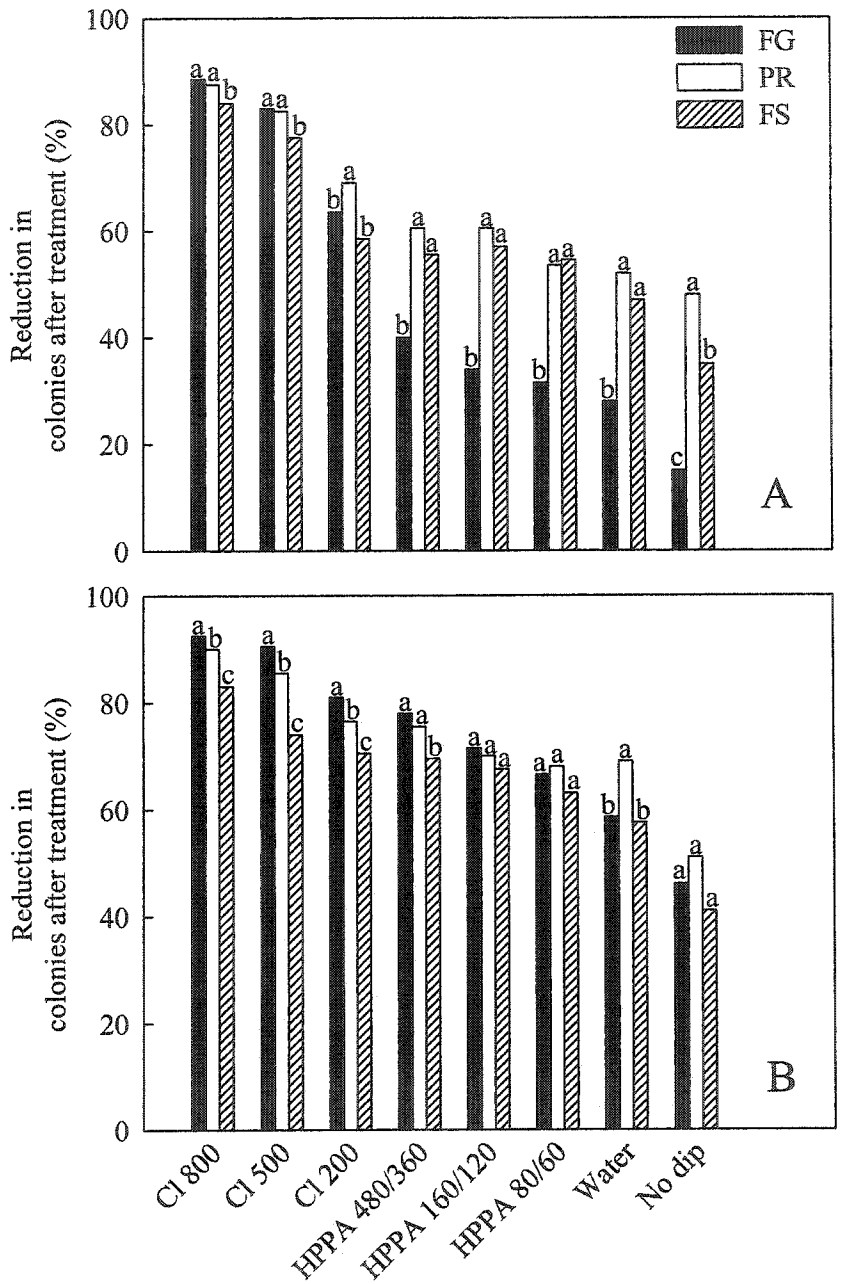

Figure 2. Percent reduction in number of three mycelial types of the FS and SB complex on 'Golden Delicious' (A) and 'Jonathan' (B) apples after a dip in two disinfestants, water or "no-dip" followed by 30 s brushing on a commercial grading line. Disinfestant treatments included three concentrations of buffered sodium hypochlorite (800-, 500-, or 200-ppm Cl), three concentrations of a combination of hydrogen peroxide and peroxyacetic acid (HPPA) (480-ppm hydrogen peroxide (HP) and 360-ppm peroxyacetic acid (PA), 160-ppm HP and 120-ppm PA, or 80-ppm HP and 60-ppm PA). Each compound was analyzed separately and bars with the same letter within each compound grouping are not significantly different at $P>0.05$ according to Student's t-test. Mycelial types were distinguished as: FS (flyspeck), having fruiting structures (thyrothecia) with no visible mycelial mat; FG (fuliginous), having distinct mycelial mats and without fruiting structures; and PR (punctate/ramose), having pycnothyria-like fruiting structures and dark mycelial mats. Dip times (7 and 15 min) were combined. N=160.

Figure 3. USDA grade of 'Jonathan' (A, C, E) and 'Golden Delicious' (B, D, F) apples before and after a 7-min dip in 200, 500, or 800 ppm buffered sodium hypochlorite or a mixture of hydrogen peroxide/peroxyacetic acid (HPPA) at 80 ppm hydrogen peroxide (HP) and 60 ppm peroxyacetic acid (PA), 160 ppm HP and 120 ppm PA, or 480 ppm HP and 360 ppm PA, then brushed and rinsed for 30 s on a commercial grading line. N=80. USDA grade standards were defined as follows: "Extra Fancy" fruit have <5% of the surface covered by SB and FS; "Fancy" fruit have 5 to 10% of the surface covered by SB and FS; "Utility" fruit have 11 to 33% of the surface covered by SB and FS.

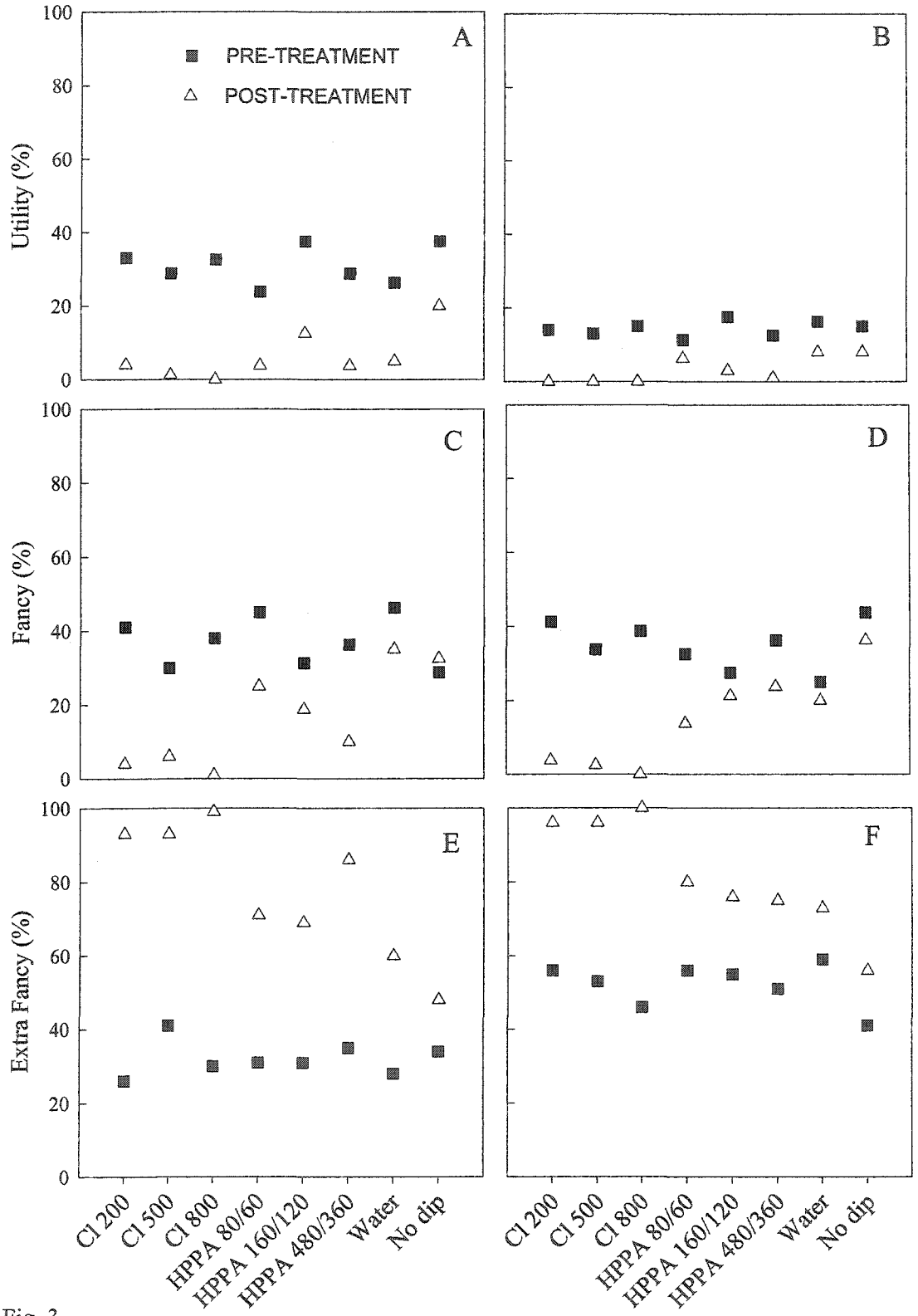
A
B
C
D
E
F
PRE-TREATMENT
POST-TREATMENT
Utility (%)
Fancy (%)
Extra Fancy (%)
0
20
40
60
80
100
Cl 200
Cl 500
Cl 800
HPPA 80/60
HPPA 160/120
HPPA 480/360
Water
No dip

Fig. 3

CHAPTER 4. INFLUENCE OF SPATIAL VARIABILITY OF LEAF WETNESS DURATION IN APPLE TREE CANOPIES ON THE PERFORMANCE OF A WARNING SYSTEM FOR SOOTY BLOTCH AND FLYSPECK

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ABSTRACT

Leaf wetness duration (LWD) was measured within apple tree canopies in four Iowa orchards. Variability of LWD and the timing of dew onset and dry-off were characterized for twelve positions in the canopy of trees in one Iowa orchard over three growing seasons and four Iowa orchards during one growing season. The upper and eastern portion of the canopy had the longest mean daily LWD and was the first to form dew and the last to dry. The lower, western portion of the canopy usually averaged about 2 hours of LWD per day less than the top of the canopy, and was the last zone where dew formed and the first to dry off. On about 25% of the nights when dew events occurred in the top of the canopy, no dew formed in the lower, west canopy. Differences in spatial variability of LWD were more acute during days on which dew was the sole source of wetness than during days on which

rainfall occurred. Spatial variability of LWD was sufficiently large that a sensor located in a give sector of a tree would not indicate rain and dew events accurately for the entire tree. Daily LWD in the upper, eastern portion of the canopy was slightly less than reference measurements made at 0.7-m height over turfgrass located near the apple trees. When LWD measurements from each sector of the canopy were input to a LWD-based warning system for the sooty blotch and flyspeck complex, timing of fungicide-spray thresholds varied by as much as 30 days among canopy positions.

Keywords: Disease forecasting, microclimate

INTRODUCTION

The sooty blotch and flyspeck (SBFS) fungal complex poses a major threat to apple production in humid climates worldwide. These fungi colonize the apple cuticle, creating blemishes that prevent apples from being marketable as fresh fruit. Apples with SBFS are usually sold for cider, reducing the crop value by up to 90% (Williamson and Sutton 2000, Batzer et al. 2002b). Growers control SBFS by applying fungicides every 10 to 14 days, from soon after bloom until shortly before harvest (Williamson and Sutton 2000) at a cost of approximately $15–$25 per acre per spray.

A conventional, calendar-based protectant fungicide spray program for SBFS management may not be sustainable (Babadoost et al. 2004). Since the 1996 passage of the Food Quality Protection Act, U.S. growers face restriction and possible loss of broad-spectrum fungicides that are effective against SBFS. Residues of fungicides, such as captan and mancozeb, present a health hazard to consumers because they are applied later in the season when SBFS fungi are more active (Belding 1996). Moreover, SBFS fungi have developed resistance to many broad-spectrum fungicides (Johnson 1994, Sutton and Sutton

1994). Growers experience sporadic control failures related to poor fungicide coverage, inadequate pruning, and environmental conditions that are highly favorable for SBFS development (Cooley et al. 1997).

Disease-warning systems can be valuable tools for growers to reduce input costs by minimizing the number of fungicide applications while continuing to safeguard the crop from disease losses. Additional benefits of applying fungicides on an as-needed basis may include reduced hazards associated with pesticides to humans, protection of non-target organisms and the environment, and delayed development of fungicide resistance by pathogens.

The extended incubation period between inoculation of apples by SBFS fungi and development of visible signs complicates strategies for reducing fungicide sprays. In North Carolina, for example, SBFS inoculum is present on developing fruit in mid-May, whereas SBFS signs develop from late June to mid-July (Brown and Sutton 1993). In Iowa, inoculum is present on apples by mid-June but signs do not develop until mid-September, shortly before harvest (Barrett et al. 2003). The success of a SBFS disease-warning system is dependent upon thorough spray coverage and adequate understanding of the environmental conditions that favor the growth and spread of the fungal complex (Williamson and Sutton 2000). Favorable conditions for disease development are mediated by leaf wetness duration (LWD) within the canopy, proximity of apples trees to *Rubus* sp. and other sources of inoculum, foliage density in the canopy, sensitivity of the SBFS fungi to fungicides, and the rate of fungicide degradation (Brown and Sutton 1995, Cooley 1996, Cooley et al. 1997, Tarnowski et al. 2003).

The most widely used SBFS disease-warning system is based on knowledge of the ecology of the dominant SBFS fungi in North Carolina. Brown and Sutton (1995) observed

that the first signs of SBFS usually occurred after 270 hours of accumulated LWD, counting only periods of $\geq$ 4 hour duration starting from the first rain that occurred 10 days after petal fall. Using the 4-hour minimum threshold for counting wetness duration resulted in less variation among the eight growing seasons and three sites in the data set than using all wetness periods regardless of their duration. Brown and Sutton (1995) suggested that this reduction in variation occurred because laboratory studies showed that conidial germination and/or mycelial growth of *P. fructicola* and *Z. jamaicensis* required 4 to 5 hours of wetting (Johnson and Sutton 2000, Ocamb-Basu and Sutton 1988). They concluded that the benzimidazole fungicides applied at 200 to 225 LWD hours could eradicate existing SBFS fungi on the fruit to obtain apples free of SBFS blemishes. Modified versions of this model have been tested in Massachusetts, Kentucky, and the Midwest, often saving two or more fungicide sprays per season (Babadoost et al. 2004, Ellis et al. 1998, Hall et al. 1997, Hartman 1995, 1996, and Smigell and Hartman 1997, 1998).

During the growing season in humid regions, dew is often the largest contributor to LWD and can exert a profound impact on pathogen activity (Huber and Gillespie 1992). Dew forms when the surface temperature drops below the dew point of the surrounding air (Rosenberg 1974). Atmospheric factors and surface properties govern the rate of surface cooling. Energy transfer from the surface to the atmosphere is affected by radiation, latent heat, sensible heat, heat conduction, and heat storage (Rosenberg 1974). Dew events of longest durations occur on windless, clear nights (Garratt and Segal 1988, Madeira et al. 2002).

Canopy position mediates the relative contributions of the ground and the atmosphere to dew formations and duration (Magarey 1999). Dew formation is influenced by factors

such as leaf area, plant architecture, arrangement of plants in the field, and crop height that ultimately control the vertical profiles of temperature, vapor pressure, incoming short and long wave radiation, and wind speed (Huber and Gillespie 1992, Monteith and Unsworth 1990). The top of the plant canopy experiences the greatest LWD in humid climates, whereas the middle of the canopy tends to be the wettest in semi-arid climates (Jacobs et al. 1995, Weiss et al. 1989, Wittich 1995). For example, Penrose and Nicol (1996) found that LWD in semi-arid Australian apple orchards was greatest in the center of the canopy. Decreased wind speed in the lower canopy may also delay drying of lower leaves (Huber and Itier 1990, Jacobs et al. 1995). Sentelhas et al. (2005) characterized LWD variability within five different crop canopies. The top of apple and corn canopies had longer LWD than the lower portions of the canopy. In contrast the lower canopy of young coffee plants in Brazil had longer LWD than top positions. On the other hand, no substantial LWD differences were observed among within muskmelon canopies or well-ventilated grape hedgerows.

A thorough understanding of the crop canopy, including identification of canopy microclimates that are most conducive to disease outbreak, is essential for implementing a disease-warning system that relies on weather data as inputs. The objectives of this study were to 1) characterize spatial LWD within apple canopies of Iowa orchards and 2) simulate the use of a SBFS disease-warning system for different regions of the canopy. Portions of these data have been published previously (Batzer et al. 2002, Batzer et al. 2003, Sentelhas et al. 2005).

METHODS

Study sites

Leaf wetness duration data were collected from the canopy of apple trees during three growing seasons at four central Iowa orchards located within 100 km of each other. Mature (11-16 ft tall) semi-dwarf trees (cv. Golden Delicious) were well pruned and under conventional management for insects and diseases. Study sites were in Gilbert, (42.1087, -93.6500, trees planted in a north-south row orientation), Cambridge (41.8972, -93.5204, tree row planted east-west row orientation), Jefferson (41.9933, -94.4292, trees rows planted east-west) and Fort Dodge (42.5075, -94.2531 W, trees rows planted north-south), Gilbert and Cambridge were upland, level, unobstructed. The Jefferson site was among gentle hills surrounded by woods and the Fort Dodge site was in sloping terrain in a river valley. In Gilbert, sensors were placed in three apple trees from 5 July to 30 August 2000 (57 days), in four apple trees, from 9 May to 18 September 2001 (130 days) and in a single tree from 5 May to 30 September 2003 (145 days). Single trees in Fort Dodge, Jefferson and Cambridge were monitored from 5 May to 30 September 2003 (145 days). Additional sensors were erected 0.7 m above mowed turfgrass, facing north, on an unobstructed site located 3 to 100 m away from apple trees. Two sensors were deployed above turfgrass at Fort Dodge, Jefferson, and Cambridge and four sensors at Gilbert.

Leaf wetness sensors

Flat, printed-circuit sensors (Model 237, Campbell Scientific, Logan, UT) were used to measure LWD. Sensors consisted of a 1-mm-thick circuit board with a grid comprised of interlacing gold-plated copper circuits. Sensors were painted with off-white latex paint of proprietary composition (R. Olson, Savannah, GA, personal comm.) to increase sensitivity to

small droplets of moisture and heat-treated (60-70°C for 12 h) to remove or deactivate hygroscopic components of the paint. Sensors were mounted on the end of a section of PVC pipe at an inclination of 45° to horizontal. Prior to placement within apple canopies, sensors were compared to each other for 5 days at 0.7-m height over turfgrass at three resistance thresholds (500, 1000 and 1500 kΩ). Condensation on the sensors was detected by measuring the electrical resistance of the grid using a datalogger (Model CR10, Campbell Scientific, Logan UT). Wetness (resistance) was measured at 5-minute intervals and data were recorded as percent time wet per hour. Precipitation events were recorded by tipping-bucket rain gauge located at an unobstructed site adjacent to each orchard.

Sensor locations in the canopy

Hourly averages of LWD were obtained at 12 canopy positions within each apple tree (Fig. 1). Sensors were placed facing north, at an inclination of 45° to horizontal, at four east-west horizontal positions about 0.5 m apart, within the canopy at approximately 3.5 m (upper canopy), 2.5 m (middle canopy), and 1.2 m (lower canopy), depending on the height and width of each tree. Sensors placed in upper and outer sections of apple trees were mostly exposed to the sky, while the leaf canopy surrounded sensors in lower regions of the apple trees. The middle, outer sensors had differing exposures to the sky depending on orchard row-orientation.

Data analysis

The 'temporal' data set consisting of data obtained from Gilbert, Iowa during the 2000, 2001 and 2003 growing seasons, was analyzed separately from the 'regional' data set, which consisted of 2003 data obtained at Gilbert, Cambridge, Jefferson, and Fort Dodge orchards. Daily data sets began at 11 am and ended at 10 am to avoid dividing dew periods

and were further partitioned into dew days (days with no measured rain) and rain days (days with measured rainfall $\geq$ 0.25mm).

The hypothesis that all canopy positions had equivalent daily LWD was evaluated using an F-test, and least squares means were then compared using Fisher's least significant differences for multiple comparisons. Analyses were done using the SAS Mixed procedure to incorporate correlations corresponding to tree canopy spatial patterns. Responses on different days were assumed to be independent. Daily LWD data obtained from sensors over turfgrass were correlated with data obtained from regions A and C in the canopies of the four trees in Gilbert during 2001 and the canopies of the 'regional' data set.

Relative dew onset and dry-off within the apple canopy was assessed for four trees in Gilbert during the 2001 growing season. Sixty-nine nights during which dew events were not associated with rainfall were assessed. Since the time of dew onset ranged from early evening to early morning, relative times of dew onset and dry-off were compared within the canopy for each dew event. This was done by designating the start of a dew event as the time the first sensor in any canopy location recorded wetness for >30 min per hour. Onset times for the remaining canopy locations were noted relative to this first occurrence of dew. Relative dew dry-off for locations that had been wet were similarly determined; the end of a dew event occurred when the first sensor in any canopy location became dry for >30 min per hour and remained dry for at least 3 hours. Means of time of dew onset relative to initial dew onset and times of dew dry-off relative to initial dry-off were calculated for each canopy location. However, comparisons of means using statistical tests were not performed because scarcity of dew events in the lower western canopy (region C in Fig.1) obscured relative differences.

Simulation of a SBFS-warning system

We used the Sutton/Hartman SBFS disease-warning system to determine the timing of the second-cover fungicide spray (Brown and Sutton 1995, Hartman 1995, 1996, and Smigell and Hartman 1997, 1998). A second-cover fungicide spray is recommended when cumulative LWD following application of the first-cover spray reaches 175 hours. Wetness hours were accumulated based on the following decision rules: 1) when hourly LWD was detected <30 min/hr, the hour was scored as dry (0) and when wetness was detected ≥ 30 min/hr, the hour was scored as wet (1); 2) wetness periods were counted only when ≥4 h; and 3) wetness periods ended when ≥2 consecutive dry h were recorded (Babadoost et al. 2004).

A simulation of operation the SBFS-warning system was conducted for the 2001 and 2003 data using the actual first-cover spray dates: 29 May 2001 (Gilbert), 24 May 2003 (Gilbert, Cambridge, Fort Dodge) and 20 May 2003 (Jefferson).

RESULTS

Comparisons of adjacent LWD sensors over turfgrass

Comparison of resistance thresholds of LWD sensors destined for placement in apple canopies indicated that the 1000-kΩ threshold had the least hourly LWD variability and was adequately sensitive to wetness when placed over turfgrass for 5 days. No significant differences in hourly LWD were found within the four groups of 12 sensors (p-values of 0.9984, 0.9999, 0.9999, 0.9840) using the 1000-kΩ threshold. Therefore, sets of sensors placed in each tree were equivalent in sensitivity to wetness and differences among sensor readings could be attributed to spatial heterogeneity.

Spatial variation of LWD within apple tree canopies

Since the F-test rejected the hypothesis that means of LWD were equal, multiple comparisons were conducted for both data sets (Fig. 2 a,b). Therefore, no two sensors on the tree were assumed to have exactly the same environment. Similar spatial patterns of LWD were observed for the 'temporal' and 'regional' data sets (Fig. 2 a,b). Canopy wetness microclimates were delineated into three regions (Fig. 1). Wetness duration was greatest in region A and least in region C (Figs. 1 and 2 a,b).

Temporal data set. Differences among canopy positions were the greatest during non-rain days(Fig. 2a). In position C, for example, daily LWD was 4.5 hours shorter (65% less) than in position A during non-rain days. In contrast, LWD in these positions differed by about only 2.5 hours (33%) during rain days. Rain days comprised an average of 28% of all days in the 'temporal' data set (27 of 59 days during 2000, 29 of 130 days during 2001, and 37 of 145 days during 2003). Non-rain days were the largest component (72%) of the growing season (32 of 59 days during 2000, 101 of 130 days during 2001, and 108 of 145 days during 2003). As a result, patterns of spatial variation in LWD observed for non-rain days were similar for all days (non-rain days + rain days).

Significant differences ($p< 0.0001$) in daily LWD were detected for 'height' (upper, middle, lower canopy levels) and 'direction' (east vs. west positions) (Table 1). Differences in LWD from east to west were not as pronounced in the upper canopy as the lower canopy (Fig. 2a). Likewise, differences among heights were less acute on the outer eastern (2 h) than the outer western (4.2 h) canopy positions. Mean daily LWD tended to be relatively similar for horizontal positions closer to the tree trunk and more dissimilar for positions near the

periphery of the canopy (in/out). Significant interactions of 'height,' 'direction,' and 'in/out' factors were observed for non-rain days, but not for days with rain (Table 1).

Regional data set. Significant differences ($p < 0.0001$) were observed among the four orchards in 2003 (Table 2). There were no apparent interactions among orchards and canopy positions, indicating that trees at all study sites had similar patterns of variation in LWD with canopy position. On non-rain days, LWD differed among 'height,' 'direction,' and 'in/out,' and interactions between 'height' with 'in/out' and 'level' with 'direction' were observed. On rain days, however, LWD differed only for 'height' during rain days, and no interactions of 'height' 'direction,' and 'in/out' were detected.

In the 'regional' data set greater differences among canopy positions were observed for non-rain days than rain days, similar to the 'temporal' data set, (Fig. 2b) region C (Fig. 1) was the driest part of the canopy, regardless of orchard site, topography or row orientation, and Region A was consistently the wettest.

Timing of dew onset and dry-off

Dew onset was usually detected on sensors between 10 pm and midnight, but occasionally as late as 5 am. On nights when dew was detected by all sensors in a canopy, dew onset occurred gradually over a period of about three hours, beginning at the top of the canopy. Region A was first to get wet, usually within an hour of dew onset (Fig. 3a), and was last to dry (Fig. 3b).

Dew dry-off usually occurred between 7 and 9 am and usually began in region C. The entire canopy typically became dry within two hours. Of the 69 dew events in the Gilbert orchard during the 2001 growing season, dew was not detected on sensors in region C on 16 nights (23% of dew events). In contrast, sensors did not detect dew an average of only

two nights (3% of dew events) in region A and 4 to 5 times (7% of dew events) in region B. Therefore, differences in dew onset and dry-off among canopy locations were even more extreme than indicated in Fig. 3, since dew was frequently not detected in region C, thus were not included in the data.

Estimation of canopy LWD from turf sensors

When sensors over turfgrass recorded dew, there was an 86 to 90% chance that region A of the nearby apple tree canopy was experiencing dew (Table 3). Daily mean LWD from sensors placed over turfgrass only slightly overestimated LWD in the top of the apple canopy, but greatly overestimated hourly LWD for region C, except for the Jefferson site (Figs. 4 and 5). In Jefferson sensors over turfgrass registered lower LWD and thus estimated region C more accurately. This may be explained by the fact that both turfgrass sensors were placed only 3 m away from trees in a partially obstructed area within an opening in the orchard row where two trees had been removed. In other orchards, however, the turfgrass sensors slightly overestimated LWD in region A.

Simulation of SBFS-warning system using LWD from different canopy positions

Simulation studies of the SBFS-warning system using LWD data obtained from sensors over turfgrass resulted in conservative second cover spray recommendations that still saved at least one spray (Figs. 4 and 5). For example, a calendar based spray program would typically apply a cover spray every 10 days from first-cover, but the SBFS-warning system using LWD data from nearby sensors over turfgrass made a spray recommendation 22 to 29 days after first-cover, saving one or two sprays.

Both mean daily LWD from the first- to second-cover fungicide spray (Figs. 4a and 5a) and the number of days from first to second cover spray recommended by the simulation

study (Figs. 4b and 5b) showed a wide range of relationships among canopy location and duration of the period between first-cover and second-cover. Three types of relationships were discerned between daily LWD and the SBFS disease-warning system simulation outcomes. The most common response (Type I) (Table 4) occurred in four of the eight simulation trials, where there was a simple linear association between mean daily LWD and days from first- to second-cover fungicide spray (Fig. 6a, e, g, h). Canopy locations with longer daily LWD had shorter inter spray periods than those with shorter daily LWD. For Type I outcomes warning system thresholds occurred was 15 to 30 days earlier in canopy region A than in region C (Table 4). The warning system decision rule requiring a minimum period of 4 hour of wetness to initiate the tallying of hours toward 175 did not appear to exert a substantial influence on duration of the interspray period for canopy regions A or C. Although region C of Jefferson had seven dew events excluded from the tallied hours, there was still a considerable delay of the second cover spray recommendation for region C, consistent with other study sites classified as Type I (Table 4).

Simulation outcomes classified as Type II had only 3- to 5- day differences between region A and C in duration of the interspray period (Table 4 and Fig. 4b). The fact that differences in daily LWD between region A and C differed by only 0.3 and 0.4 h for Gilbert trees 2 and 4 and explained the similar interspray differences of 1 and 3 days, respectively (Table 4). In the case of Gilbert Tree 3, however, the difference of 1.7 h between region A and Region C may have partially minimized by the warning system decision rules which excluded three more dew events from region A than region C. When interspray periods were plotted against mean daily LWD (Fig. 6b, c, d), each additional hour of mean daily LWD resulted in a reduction of five days.

A single study site, Cambridge, was classified as outcome Type III. When days from first to second cover were plotted against mean daily LWD there was no apparent relationship (Fig. 6f). The lack of fit for the linear equation suggests that LWD was not the only factor in determining the warning system outcome. Anomalous differences in warning system threshold timing occurred among canopy positions. For example, mean daily LWD was about 2 hours longer in region A than region C, but the simulation study recommended that region C receive the second cover spray ten days earlier than region A (Table 4). Also, the spray recommendation for the middle, inner, west position was 20 days later than the middle, inner, east position, although mean daily LWD differed by only 0.1 h for the Cambridge study site (Fig. 5). Region C required two more dew events than region A to reach 175 hours (Table 4), but decision rules did not eliminate any wetness events from being tallied towards the warning system. Several wetness hours were discarded when the average LWD hours for the entire canopy were used.

DISCUSSION

Our data documented clearly that apple tree canopies in central Iowa were characterized by substantial spatial heterogeneity in LWD, and that this heterogeneity was more pronounced when the wetness was caused by dew than by rainfall. In a mature orchard, only the top of the canopy has unobstructed exposure to the sky and is generally the first to exhibit wetness, both during dew and rain events. The daily mean difference in LWD between canopy regions A and C was about 2 h, which agreed with results of Wittich (1995). East-west differences in LWD were consistent for all study sites on non-rain days and were apparently related to the westerly winds that are predominant in Iowa during the growing

season. Dew duration was longer in the upper than the lower canopy, presumably because overhanging leaves created a barrier that reduced heat loss and radiant heat from the ground delayed the cooling of surfaces in the lower canopy, and therefore delayed dew formation (Sentelhas et al. 2005). Shorter dew events in the lower canopy were the result of slower dew onset and more rapid dry-off than the upper canopy. Compared to dew, rainfall ≥ 0.25 mm tended to create much more uniform LWD throughout the canopy, reducing but not removing differences among canopy heights. These spatial patterns were consistent for same-cultivar trees of similar size and pruning amount, despite differing row orientation (north-south and east-west), topography (river valley and upland prairie within central Iowa) and growing season.

The Sutton/Hartman SBFS-warning system (Brown and Sutton 1995, Hartman 1995, 1996, Smigell and Hartman 1997, 1998) was developed from wetness sensors placed in Region C (Fig. 1), the most variable location within the tree, according to this study. It is possible, however, that the microclimate of apple canopies in Kentucky and North Carolina differ from those in Iowa. Furthermore, wetness sensors were used in the North Carolina and Kentucky studies may have differed in LWD sensitivity to those used in our study, which could have influenced warning system outcomes. For example, the deWit hemp string wetness sensors used by Brown and Sutton (1995) are less sensitive to dew than the painted printed-circuit sensors used in this study. The relative importance of rain and dew in the disease cycle of SBFS fungi remains unclear.

The Sutton/Hartman SBFS-warning system is likely to be highly robust since it has been validated successfully over numerous growing seasons (Babadoost et al. 2004, Brown and Sutton 1995, Ellis et al. 1998, Hall et al. 1997, Hartman 1995, 1996, and Smigell and

Hartman 1997, 1998). However, reliable outcomes of the SBFS-warning system may be difficult to obtain for individual growers because the influence of canopy heterogeneity in LWD accumulation. Although the geometry of LWD pattern was consistent, simulation studies of the disease-warning system using data from different canopy locations led to unpredictable outcomes. In five of eight trees in the simulation study, sensor location differences of only 0.5 m within the canopy changed the second-cover spray day recommendation by ≥ 7 days. Such discrepancies could hinder successful outcome of this disease-warning system and result in excess sprays or inadequate disease control, especially if only a single sensor is placed in the middle or lower portion of the canopy. This study suggests that estimates for LWD hours for the wettest part of the canopy could be consistently obtained from sensors placed either at the top of the canopy or above nearby turfgrass, and may be usable for implementing the SBFS-warning system or other waning system that rely on LWD input data.

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Table 1. The 'Temporal' data set was subjected to an analysis of variance of leaf wetness duration (LWD, hours) for twelve canopy locations within four trees sampled in a Gilbert apple orchard during 2000, 2001 and 2003 growing seasons. Fixed location factors included 'Height' (upper, middle and lower), 'In/out' (inner versus outer edge of canopy) and 'Direction' (east or west of canopy center). Data were sub-divided into Rain Days and Non-rain days.

Source	df	All Days *p*-value	Rain Days *p*-value	Non-rain Days *p*-value
Main effects				
Height	2	<0.0001	<0.0001	<0.0001
In/out	1	0.2750	0.7722	0.178
Direction	1	<0.0001	0.0178	<0.0001
Interactions of fixed factors				
Height*inout	2	0.0002	0.5228	<0.0001
Height*direction	2	0.0005	0.2477	<0.0001
In/out*direction	1	0.0002	0.2193	<0.0001
Height*in/out*direction	2	0.0045	0.4644	0.0012

Table 2. The 'Regional' data set was subjected to an analysis of variance of average leaf wetness duration (LWD, hours) for twelve canopy locations within single trees in four central Iowa apple orchards during 2003. 'Orchard' (study site) was treated as a random effect. Fixed canopy location factors included 'Height' (upper, middle and lower), 'In/out' (inner versus outer edge of canopy) and 'Direction' (east or west of canopy center). Data were sub-divided into Rain Days and Non-rain days.

Source	df	All Days *p*-values	Rainy Days *p*-values	Non-rain Days *p*-values
Main effects				
Orchard	3	<0.0001	<0.0001	<0.0001
Height	2	<0.0001	<0.0001	<0.0001
Direction	1	<0.0001	0.0730	<0.0001
In/out	1	0.0122	0.3930	0.0040
Interactions of fixed factors				
Height *in/out	3	0.0489	0.5869	0.0169
Height *direction	2	0.0011	0.2827	0.0007
In/out*direction	1	0.3638	0.5686	0.3569
Height *in/out*direction	2	0.2718	0.4415	0.3380
Interactions with orchard				
Orchard*height	6	0.7128	0.7208	0.7738
Orchard*in/out	3	0.1887	0.8433	0.0635
Orchard*direction	3	0.1602	0.5203	0.0293
Orchard* height *in/out	3	0.6972	0.9196	0.3839
Orchard* height *direction	6	0.4399	0.5031	0.2239
Orchard*in/out*direction	3	0.5878	0.7150	0.6577
Orchard*height*in/out*direction	3	0.8846	0.9254	0.5089

Table 3. Accuracy (r^2) of using sensors over turf near orchard to predict daily LWD for the wettest and driest part of the apple canopy.

Year	Orchard	Region A[1]	Region C[2]
2000	Gilbert	0.87	0.68
2001	Gilbert	0.90	0.77
2003	Gilbert	0.89	0.57
2003	Fort Dodge	0.86	0.55
2003	Cambridge	0.86	0.72

[1] Wettest portion of canopy was located in the upper, outer, eastern location in Region A

[2] Driest portion of canopy was located in the lower, outer, western location in Region C

Table 4. Effect of canopy position on spray recommendation made by SBFS-warning system. Leaf wetness duration (LWD) from day of first cover spray through fifty days.

YEAR	2001			2001			2001			2001			2003			2003			2003			2003		
STUDY SITE	Gilbert Tree 1			Gilbert Tree 2			Gilbert Tree 3			Gilbert Tree 4			Jefferson			Cambridge			Fort Dodge			Gilbert Tree 1		
WARNING SYSTEM OUTCOME TYPE	I[1]			II[1]			II			II			I			III[1]			I			I		
CANOPY REGION	A[2]	C[3]	X[4]	A	C	X	A	C	X	A	C	X	A	C	X	A	C	X	A	C	X	A	C	X
LWD 1st cover + 50 days																								
Mean hours	7.1	4.0	5.4	5.7	5.4	5.7	6.4	4.7	6.0	6.3	5.9	6.1	7.6	5.0	5.8	6.8	3.9	6	7.4	4.6	5.4	7.4	3.1	5.1
Region A – Region C	3.1			0.3			1.7			0.4			2.6			2.9			2.8			4.3		
Days to 2nd cover spray																								
	26	49	33	30	31	34	29	33	30	30	33	31	25	40	26	46	31	46	23	48	30	37	67	43
Region C – Region A	23			1			4			3			15			-15			25			30		
Decision rule effect																								
Zero h counted as +1	0	0	3	0	0	0	3	1	2	0	1	1	2	1	1	0	0	2	3	1	3	0	1	4
1 h dew event not counted	0	0	0	0	0	2	2	1	1	2	2	0	0	1	5	0	0	1	1	2	2	2	2	11
2 h dew event not counted	0	0	2	0	0	2	2	1	1	0	0	2	0	2	3	0	0	5	1	2	2	0	0	3
3 h dew event not counted	0	0	6	0	0	1	3	2	2	2	4	2	0	4	1	0	0	2	3	2	1	2	4	4
Number of dew events >4 h to reach 175 h	19	23	23	22	24	24	21	25	22	20	19	22	17	21	19	22	24	17	21	25	19	20	19	19

1 The relationships between daily LWD and disease-warning system outcomes could be grouped into three types. Type I response had earlier spray days at locations with longer daily LWD. Type II had similar spray days regardless of canopy position. Type III had no consistent relationship between canopy location and second cover spray day.

2 Single wettest location in region A; upper outer east

3 Single driest location in region C; lower outer west

4 All 12 positions in canopy regions A, B, C were averaged before applying disease-warning system

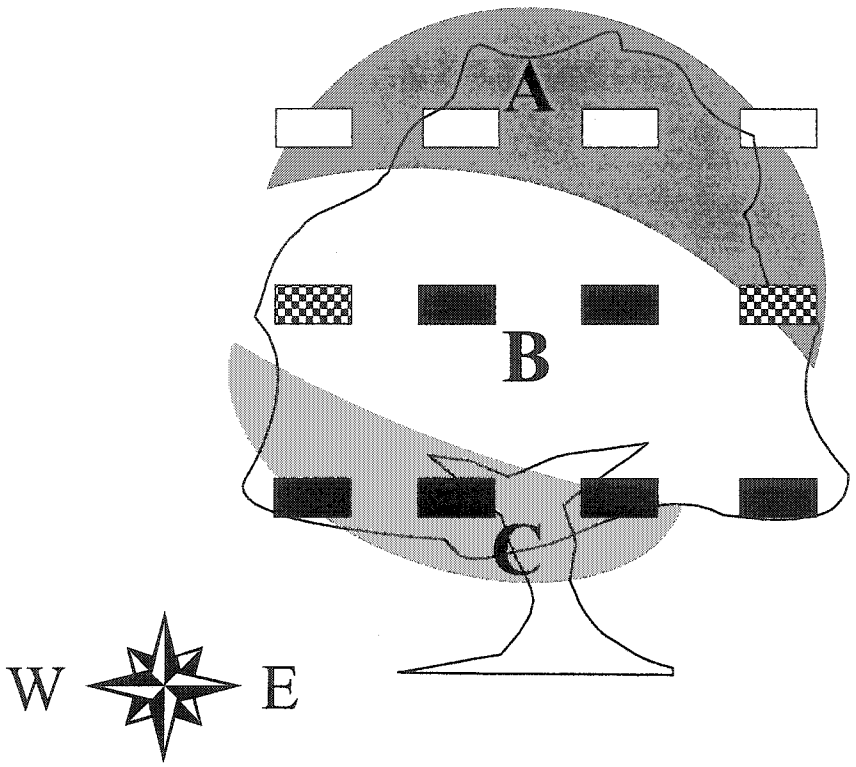

Figure 1. Sideways view of apple tree facing northward with sensors placed within canopy at three levels and four directions (west to east). Electronic sensors were mounted with a northern inclination of 45°. Sensors are denoted by rectangles. White rectangles denote sensors that were exposed > 50% to the sky. Sensors in shade ≥ 50% are represented in black. Checkered rectangles signify sensors exposed mostly to sky when in orchards with north-south rows and mostly in shade when in orchards with east-west rows. Apple canopies were subdivided into three regions indicated by shaded areas A, B, and C.

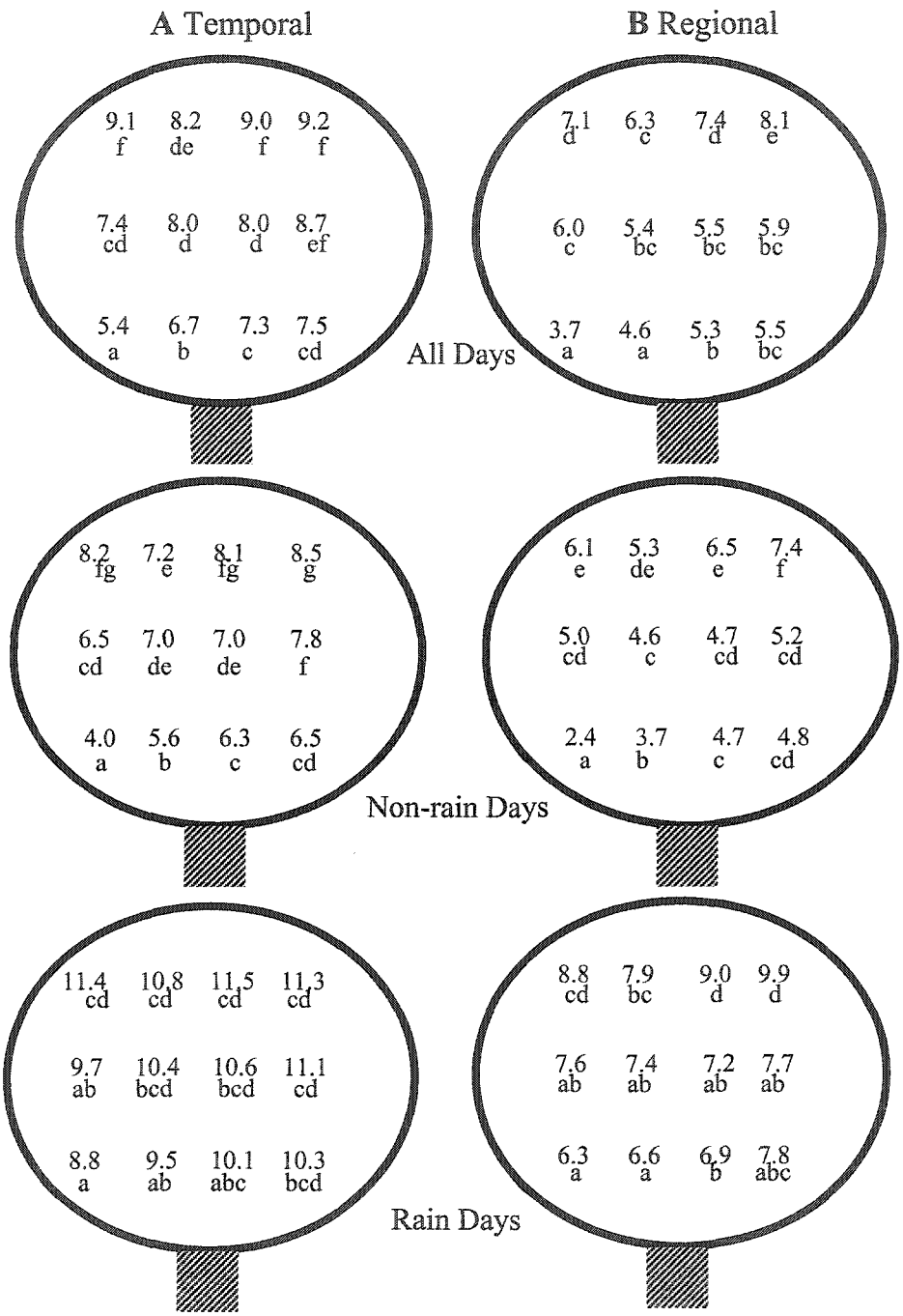

Figure 2. Mean daily wetness durations (hours) were determined for twelve canopy positions for data sets, A ('Temporal' three years one orchard) and B ('Regional' one year four orchards). Data sets were sub-divided into All Days (11am to 10 am), Non-rain Days, and Rain Days. Differing letters denote significant differences ($p<0.05$) of daily wetness duration for canopy positions within each data sub-set.

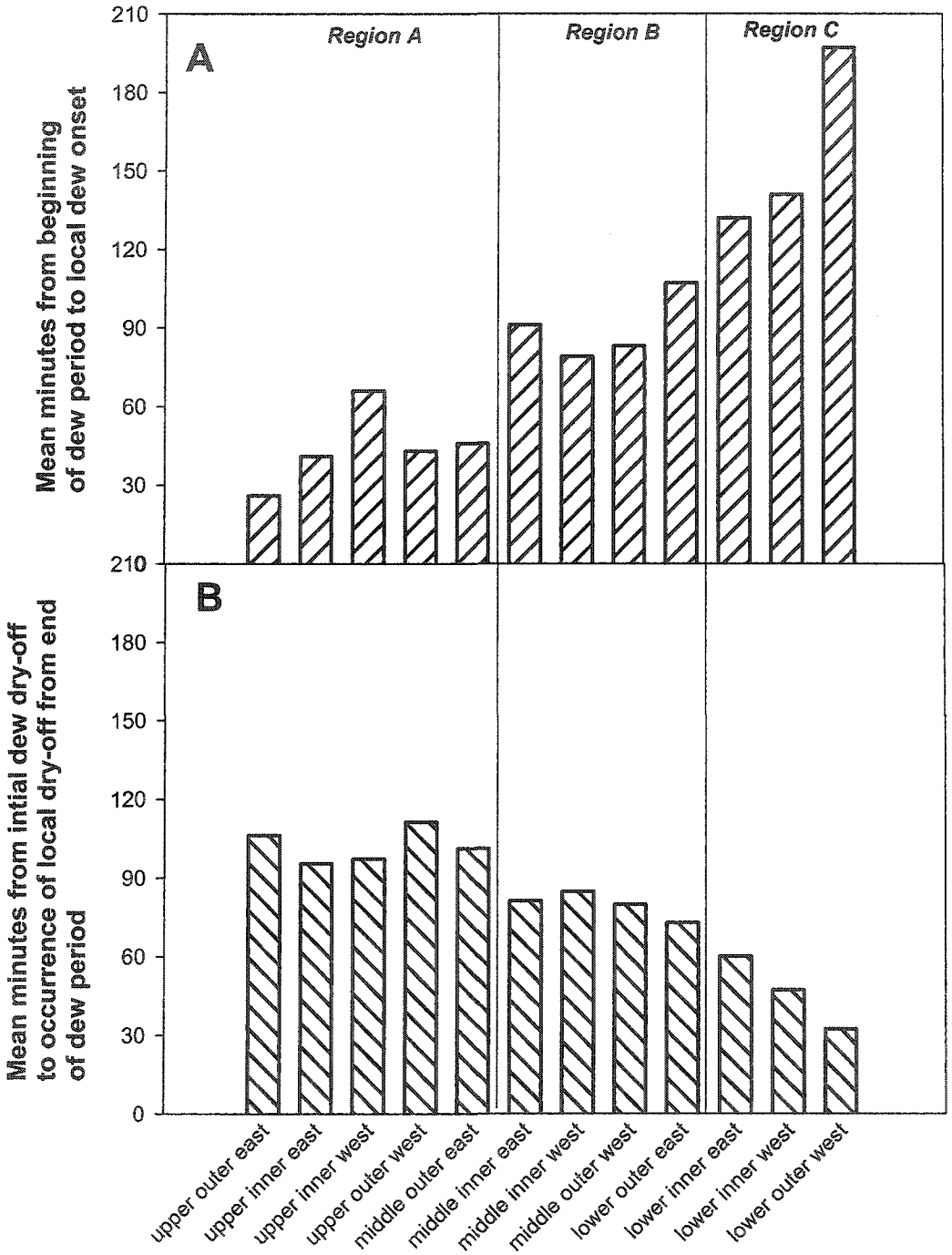

Figure 3. Mean relative onset and dry off times of dew events for twelve canopy positions for four trees in Gilbert during 2001. Time of dew onset (A) was determined when any canopy location registered as wet for greater than 30 min. Dew dry off (B) was determined to begin when any canopy location that had been wet registered as dry for more than 30 mir for more than two consecutive hours. Events associated with rain were eliminated from the data set. Canopy regions A, B and C (Fig 1) are delineated.

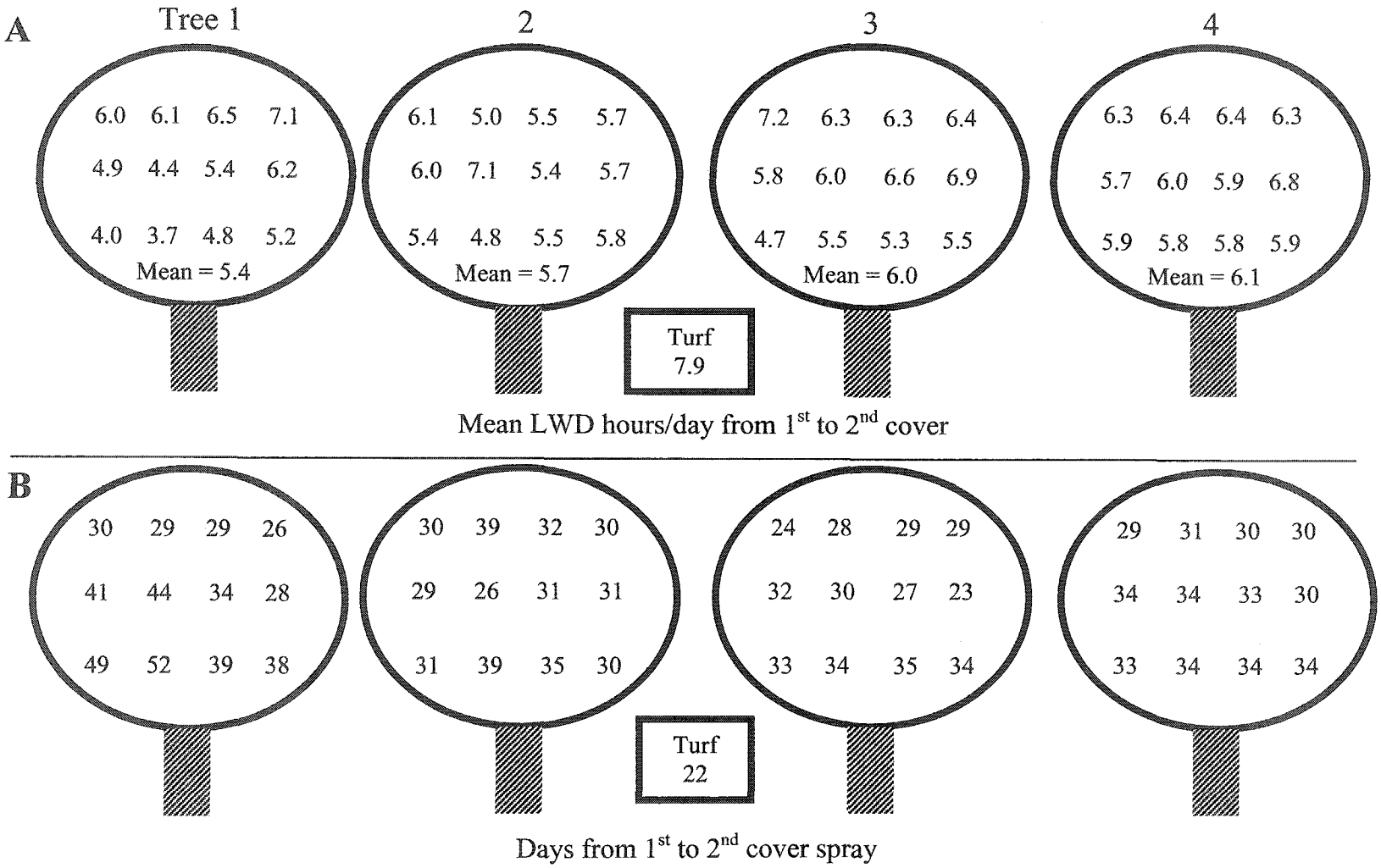

Figure 4. A. Mean daily wetness durations (hours) for canopy positions in four trees and over turf (mean of 4 sensors) 100 m north of Gilbert study site, 2001, beginning at time of first cover for 50 days. **B.** Days from 1st to 2nd cover spray for each canopy position and over turf when LWD hours were used in simulation of a SBFS disease-warning system.

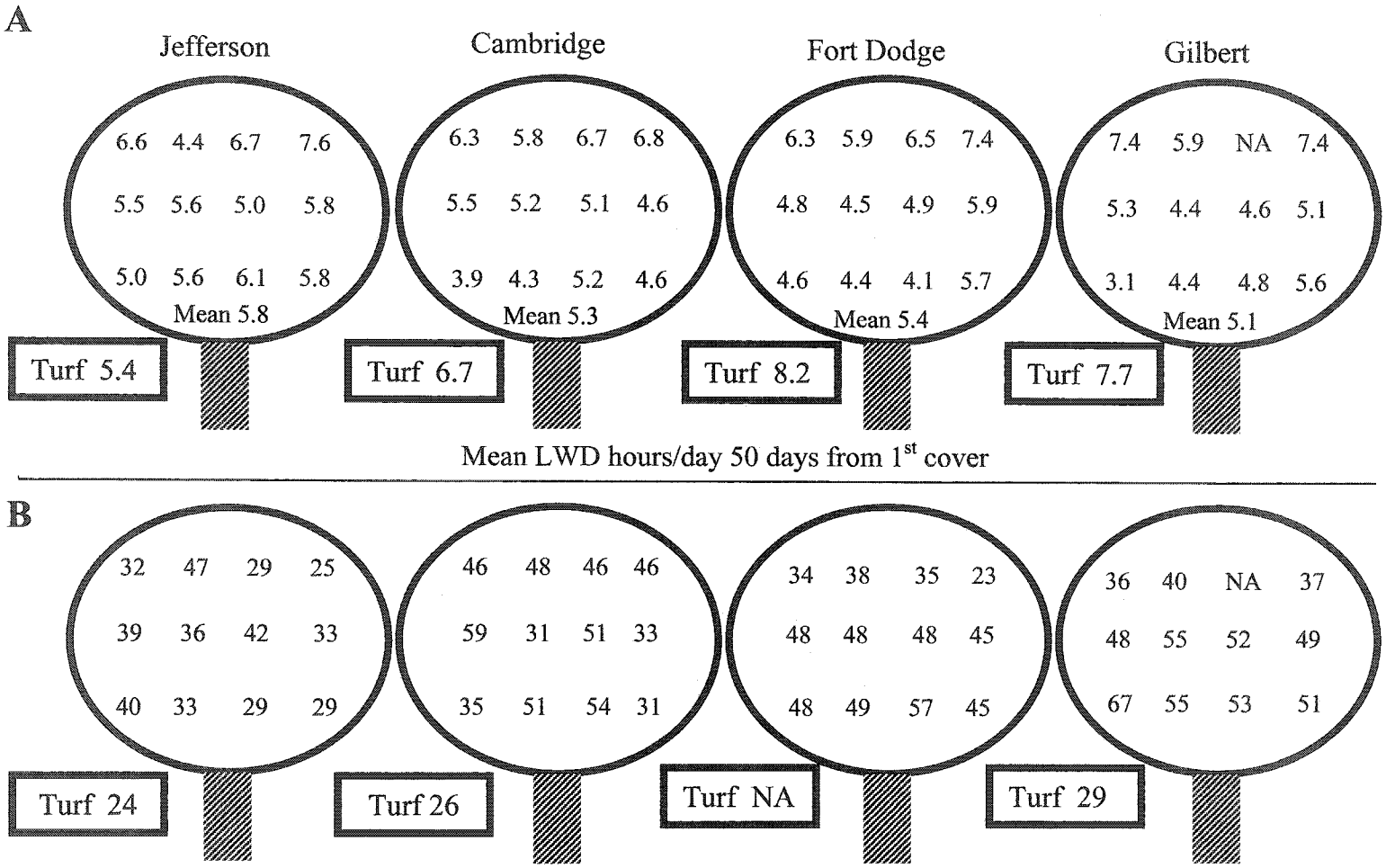

Figure 5. A. Mean daily wetness durations (hours) for canopy positions in trees at four Iowa orchards and over turf (mean of 2 sensors) near each study site, 2003, beginning at time of first cover for 50 days. **B.** Days from 1st to 2nd cover spray for each canopy position and over turf when LWD hours were used in simulation of a SBFS disease warning system.

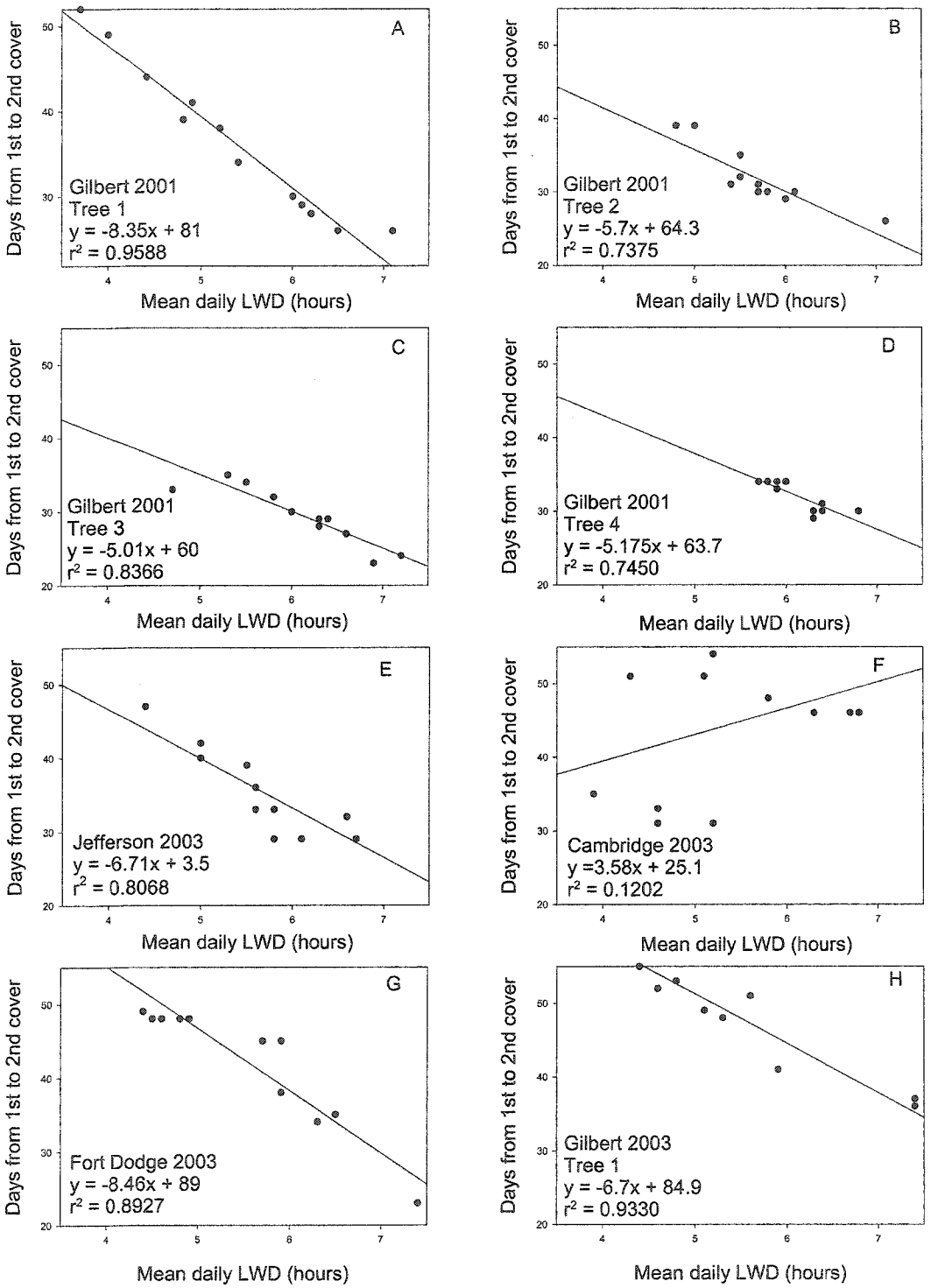

Figure 6. Mean daily wetness hours for the first 50 days after first cover spray were plotted against the number of days from first to second cover spray as determined from simulation studies of a SBFS-disease warning system for twelve positions for four trees in Gilbert 2001 (A, B, C, D) and trees in Jefferson 2003 (E), Cambridge 2003 (F), Fort Dodge 2003 (G) and Gilbert Tree 1 2003 (H). Simple linear regression analysis is presented.

CHAPTER 5. GENERAL CONCLUSIONS

Fungi in the sooty blotch and flyspeck (SBFS) complex are epiphytes that cause significant economic loss to growers. Control failures, loss of U.S. registration of several effective fungicides, and development of fungicide resistance emphasize the need to develop more effective management strategies against the SBFS complex. The rationale for the research presented in this dissertation was to address problems in understanding the ecology of the pathogens, including identification of the causal organisms and characterization of the apple canopy environment, and to develop alternative control measures.

The research conducted has provided new insights about the pathogen complex. Thirty components of the SBFS complex were revealed using ribosomal DNA sequences and morphological evidence. All SBFS fungi were grouped with the Dothideomycetes *sensu* Barr (1987) based on LSU analyses. This study showed that several genetically distinct fungi in the SBFS complex might give rise to the same mycelial type on apple fruit. Members of the SBFS complex may differ in ecology and thus require different management practices. Several of the newly recognized SBFS fungi exhibited a diversity of fungicide sensitivity, temperature optima, and media response (Tarnowski et al. 2003, Hernandez et al. 2004, Van deVoort et al. 2003). Since these fungi probably reside on plant species surrounding apple orchards, the complex may vary depending on the local flora. Further work is needed to understand the ecology of local and regional SBFS complexes. This may lead to strategies that reduce the economic and environmental impact of disease management.

Environment may play a substantial role in colonization of apple fruit by these saprophytes. Therefore, a thorough understanding of the apple canopy environment may

provide information in the implementation of disease-warning systems. This study compiled a thorough assessment of spatial heterogeneity of LWD within the canopy of apple trees in four Iowa orchards. The uppermost part of the canopy was generally the first area to exhibit wetness, during both dew and rain events. Dew events in the lower canopy were shorter than in the upper canopy as a result of slower dew onset and more rapid dry-off. Compared to dew, rainfall tended to create more uniform wetness duration throughout the canopy, reducing, but not eliminating, LWD differences among canopy heights. This spatial pattern was consistent for same-cultivar trees of similar size and pruning, despite differing row orientation, topography, and growing season.

Although the geometry of LWD pattern was consistent, simulation studies of a SBFS-warning system, using data from different canopy locations, sometimes led to unanticipated outcomes. When LWD measurements from each sector of the canopy were input to the warning system, timing of occurrence of the fungicide-spray threshold varied by as much as 30 days among canopy positions. This variability in spray recommendation could result in excess fungicide sprays or inadequate disease control when using the warning-system driven by a single sensor in the canopy. This study also provided preliminary evidence that reasonably accurate estimates for LWD hours for the wettest part of the canopy could be consistently obtained from sensors placed at either the top of the canopy or above nearby turfgrass estimates and may be usable for disease control guidance.

This study also furthered the development of post-harvest removal of SBFS signs. Dipping apples for 7 min in 800 ppm chlorine followed by 15 sec of brushing and a 30-sec rinse and brush increased the percentage of apples rated as "Extra Fancy" from 25% and 55% to 100% for 'Jonathan' and 'Golden Delicious' apples, respectively, and increased

market value by 31 and 14%, respectively. Blemishes were removed more effectively from 'Jonathan' and 'MacIntosh' apples than from 'Golden Delicious.' This study was the first to document differential removal of distinct mycelial types of SBFS as interactive effects between treatment and cultivar. Therefore, SBFS mycelial type and apple cultivar should be considered when evaluating effectiveness of a post-harvest treatment.

The work presented in this dissertation has enhanced the understanding of several aspects of the management of SBFS on apple. New information about the diversity of the fungal complex, the microclimate of the apple canopy and the refinement of a post-harvest control measure will be useful to researchers and growers interested in developing better management strategies for SBFS.

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Tarnowski, T.B., Batzer, J.C., Gleason, M.L., Helland, S., and Dixon, P. 2003. Sensitivity of newly identified clades in the sooty blotch and flyspeck complex on apple to thiophanate-methyl and ziram. Online. Plant Health Progress doi:10.1094/PHP-2003-12XX-01-RS.

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Many individuals made this life-long goal and research possible. I would like to sincerely thank those in the Gleason and Harrington labs, and those on my POS committee, Tom Harrington, Lois Tiffany, Ken Koehler, and Elwynn Taylor.

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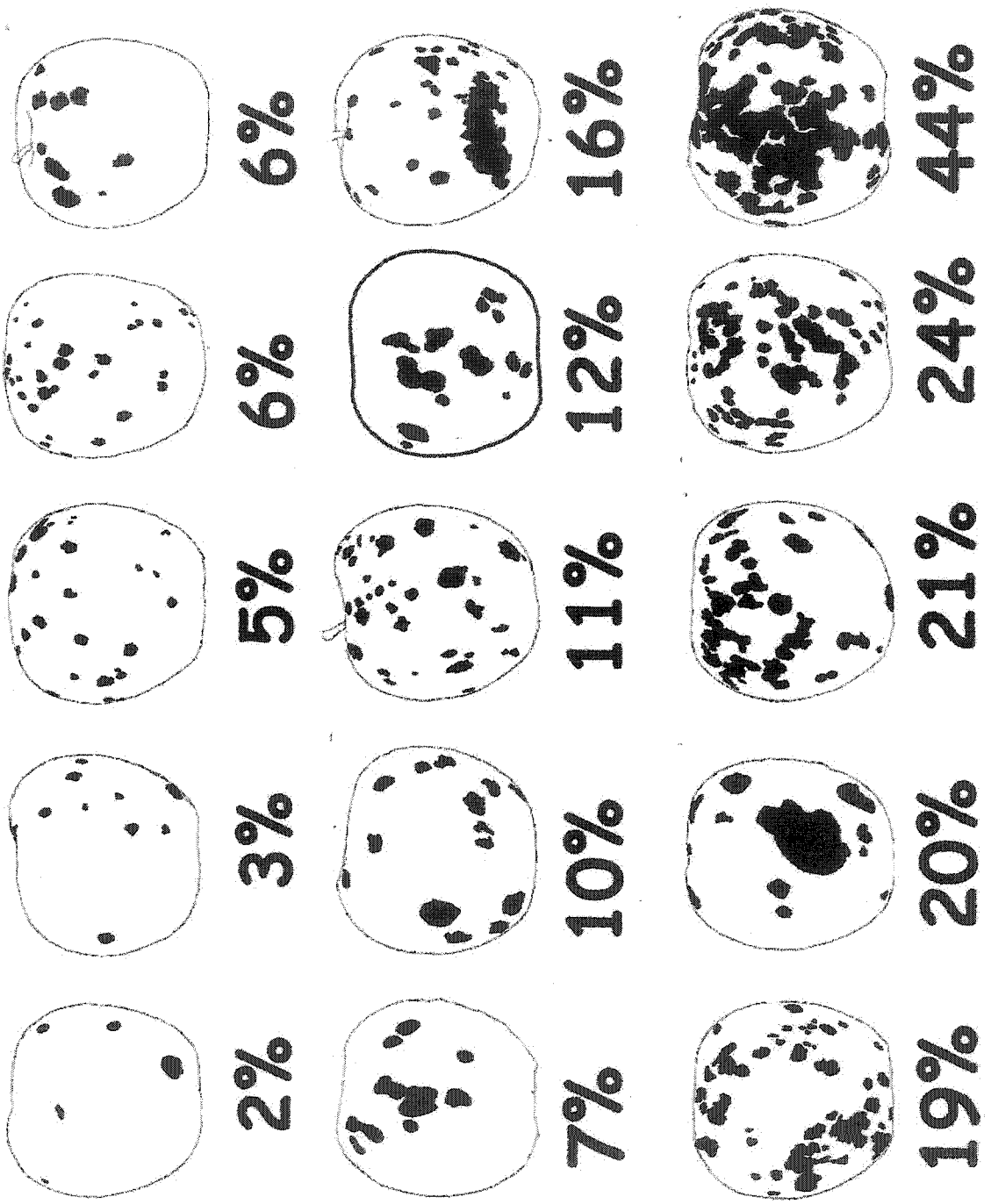
A special thanks to Lois Tiffany, who shared my enthusiasm for every new fungal structure (and there were many) that I wanted her to see—even when they weren't the discovery of the century that I sometimes hoped they were. You never made me feel foolish, but seemed to truly enjoy my interest in fungi.

Also thank you to Tom Harrington, who modeled the ideal of the scientific process. Your guidance and intellect has greatly contributed to the quality of my work and how I hope to approach future research.

There were many people integral to the success of my project. Ed Braun selflessly spent many hours teaching me to prepare and photograph fungi. Sandra Hernández and Miralba Agudelo patiently and carefully cultured the fungi. Chen Wei also worked tirelessly when amplifying and sequencing hundreds of isolates. Joe Steimel unconditionally advised me on molecular techniques.

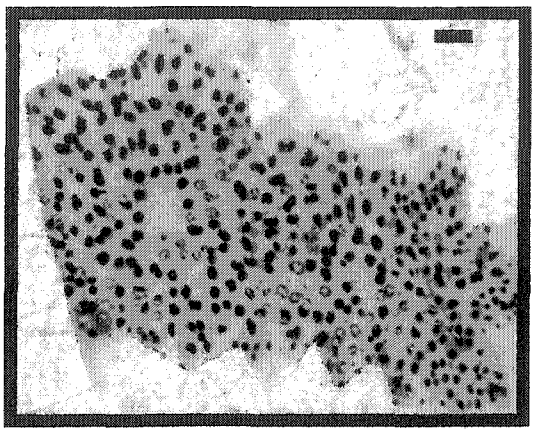
APPENDIX 1



Standard area diagram for percentage of apple surface with blemishes.

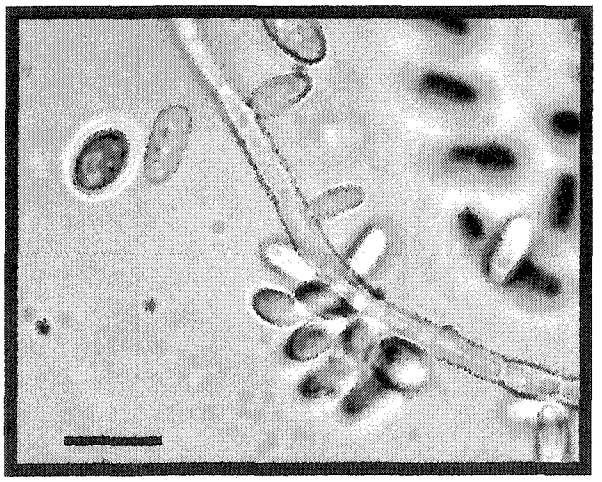
APPENDIX 2

Peltaster sp. CS1

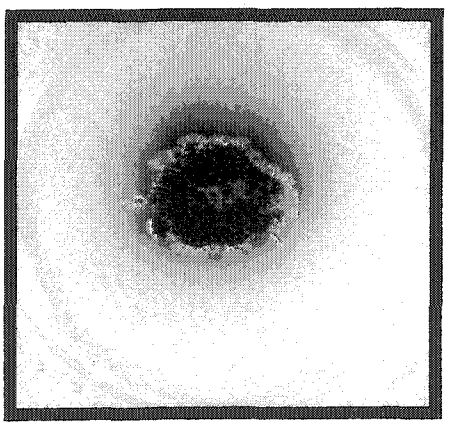


Appearance on apple. Compact speck mycelial type of *Peltaster* sp. CS1 strain mwe2.

Mycelial mat slightly discolored, but no visible mycelia at 40x magnification. Sclerotium-like bodies numerous, compactly arranged, black, shiny, slightly raised, circular to oblong frequently coalescing. A black ring remains when removed from cuticle. Density 22.3/mm^2. Diameter 35–357mm.
Bar = 0.5μm



Conidia subhyaline, single–celled, ellipsoid with tapered base 2.5–8.7 x 1.5–4 μm.
Pink slimy masses of ovoid conidia are produced on short hyaline hyphae and by conidial budding.
Bar = 5μm

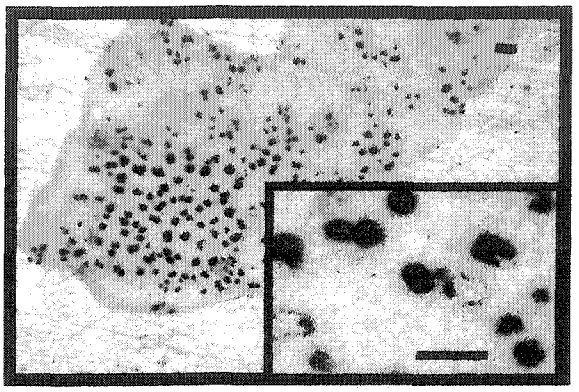


Appearance on PDA. Three-month old growth of *Peltaster* sp. CS1 strain pee2 (left).

Growth on media. *PDA.* Upper: pink yeast-like slime with no mycelia, turning black with age. Lower: black. *MEA.* Upper: black, shiny, carbonaceous, crusty, uneven edges; pink yeast-like slime exuded from centers. Lower: black. Diameter in 4 weeks on MEA: PEE1 0.5–1.0mm; UIE17 2.0 1.5–2.5mm

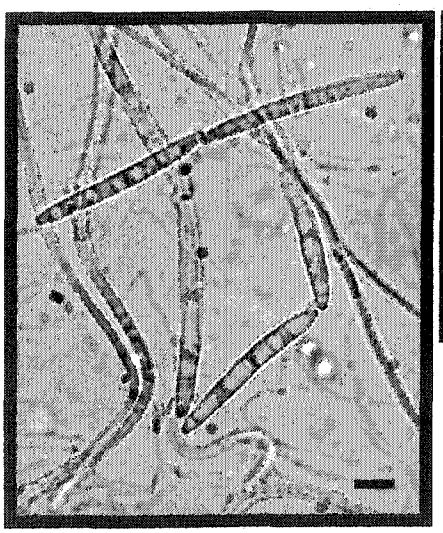
APPENDIX 3

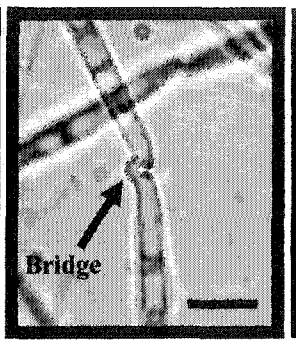
Ramularia sp. CS2 strain

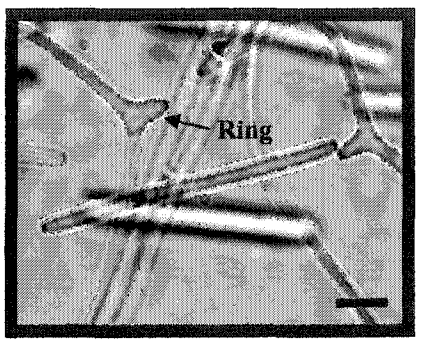


Appearance on apple. Compact speck mycelial type of *Ramularia* sp. CS2 strain gte2.

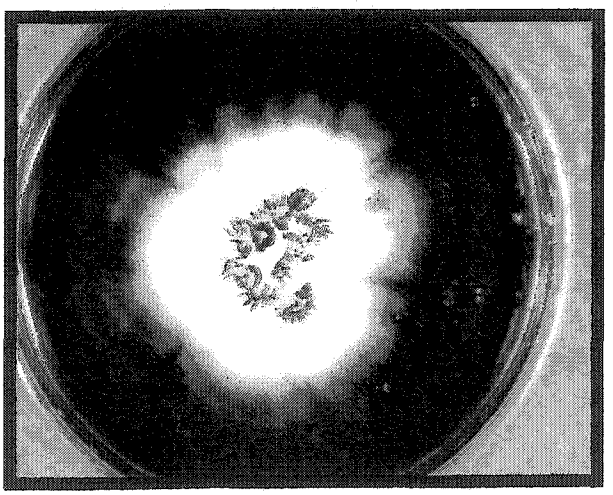
No visible mycelial mat. Sclerotium-like bodies dark brown, compactly constructed (6.8/mm^2) circular to oblong, slightly raised. Diameter: 81–418μm. Bar = 0.5mm







Conidia. Hyaline, two-celled, cylindrical, tapered truncate, both ends dark. 14.5–41 X 1.5–2.5μm. Produced in aerial chains (left), sometimes branched. Conidia attached to each other with curved bridge 0.5 X 1μm (above center). Conidiophore rounded; dark ring near tip (above right). Bar = 5μm

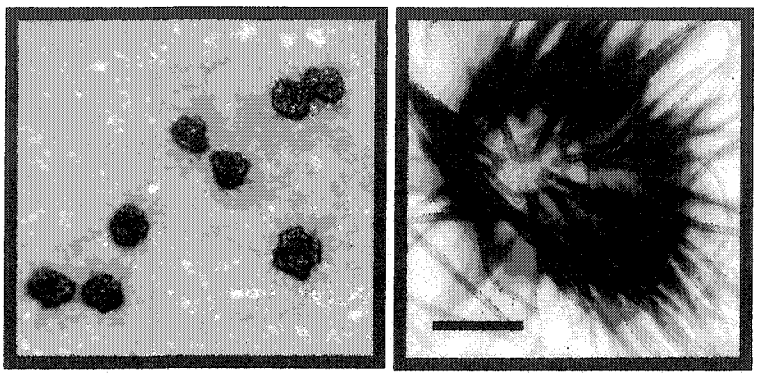


Appearance on PDA. Three-month old growth of *Ramualria* sp. P7 strain gte2 (left).

Growth on media. *PDA.* Upper: pale pink, dense, smooth. Surface cracks revealing black interior. Lower: black. Pigment: red. *MEA.* Upper: white, flat with aerial mycelia; numerous black sclerotia formed at bottom of plate. Diameter at 4 weeks on MEA 12.8–14.0mm.

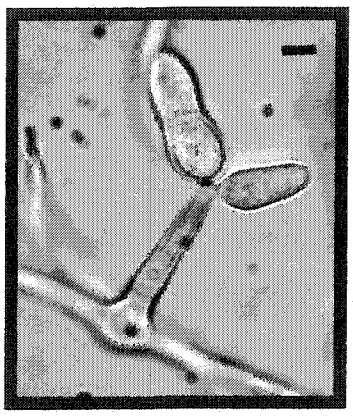
APPENDIX 4

Dissoconium sp. DS1.1 and DS1.2



Appearance on apple. Discrete speck of *Dissoconium* sp DS1.1 strain mstb9 on apple (left). Bar = 0.5mm.

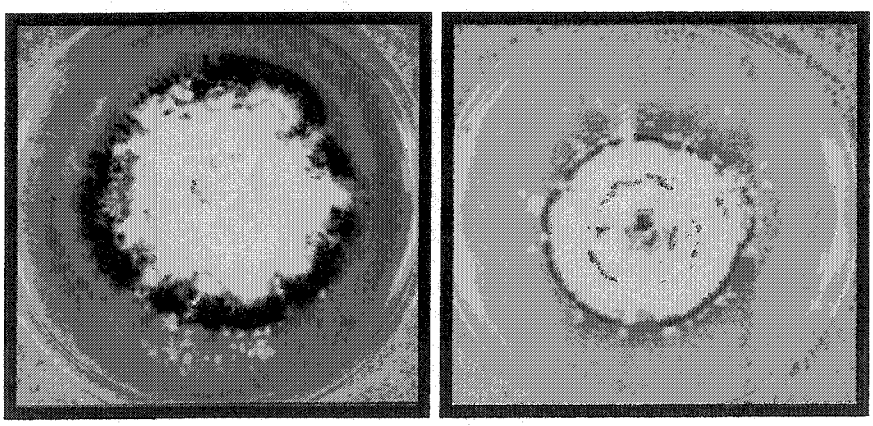
Spermagonium on carnation leaf agar. *Dissoconium* sp DS1.1 strain mstb9 on carnation leaf agar (right). Bar = 50μm.



Conidia. Macroconidia two-celled obtuse, truncate at septum. *Dissoconium* sp. DS1.1: 11–27 x 4.5–10μm; *Dissoconium* sp. DS1.2: 16–19 x 4–8μm.

Microconidia single-celled obovate usually fused to macroconidia. *Dissoconium* sp. DS1.1: 6–17 x 4.5–10μm; *Dissoconium* sp. DS1.2: 9–16 x 4–8μm.

Conidiophore elongate, lengthens with age, swollen at base, tapered apex 38–66 x 2–7μm. Light brown, scars, conidia forcibly ejected together. Bar = 5μm.

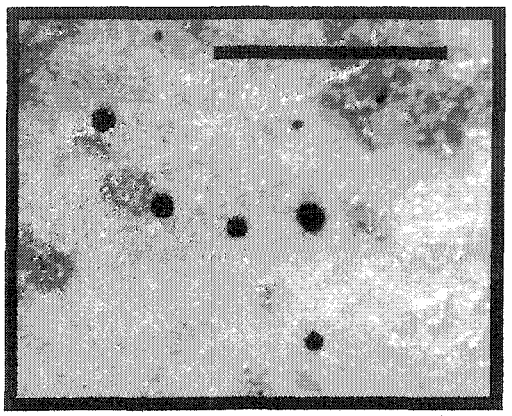


Appearance on PDA. Three-month-growth on PDA of *Dissoconium* sp. DS1.1 strain cub2a (left) and *Dissoconium* sp. DS1.2 strain umb4 (note yellow pigment) (right).

Growth on media. *PDA.* Upper: pale pink, felty, raised center; black schlerotia (<1mm) embedded within media. Lower: black. Yellow soluble pigment for *Dissoconium* sp. DS1.2 (only observed difference between these two species). *MEA.* Upper: pale gray, flat, thin, white aerial mycelia; black sclerotia (<1mm) embedded within media. Edges not well defined. Lower: black center with tan margins. Diameter at 4 weeks on MEA: *Dissoconium* sp. DS1.1 6.0–13.0mm; *Dissoconium* sp. DS1.2 7.5–14.0mm.

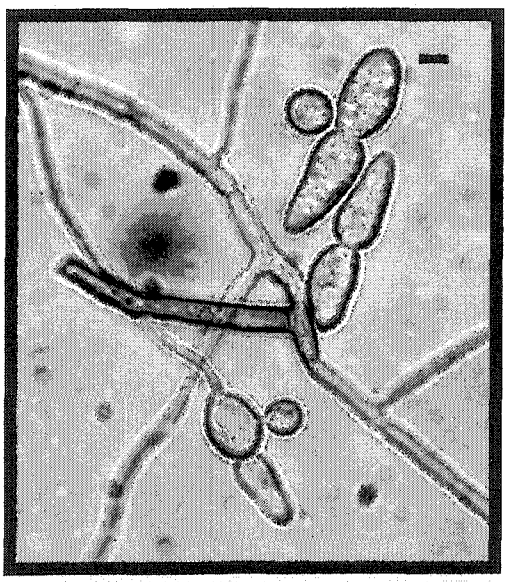
APPENDIX 5

Dissoconium sp. DS2



Appearance on apple. Discrete speck mycelial type on apple Bar = 0.5mm.

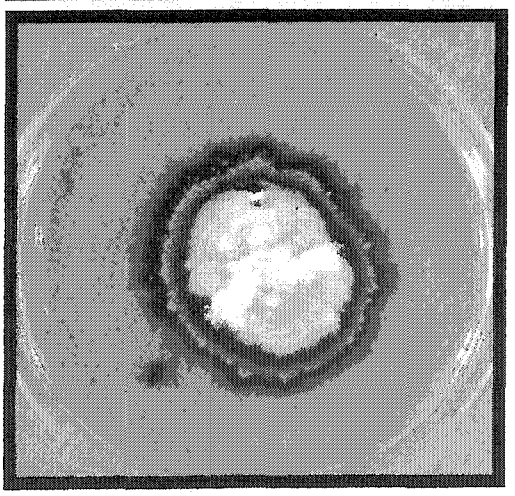
Mycelial mat absent, sclerotium-like bodies (4–5/mm^2) scattered, black, flattened, no setae observed. Diameter 35–208µm.



Conidia. Macroconidia hyaline, two-celled. Basal cell obtuse, truncate at septum; apical cell obpyriform, 27–36 x 7–11µm.

Microconidia single-cell, spherical with beak-like projection; sometimes fused to macroconidia obovate, 5–10 x 5–10µm.

Conidiophores pigmented, septate, elongate, swollen at base, tapered apex, 22–55 (lengthens with age) x 3.5–5.5µm. Light brown scars, forcibly ejected together. Bar = 5µm

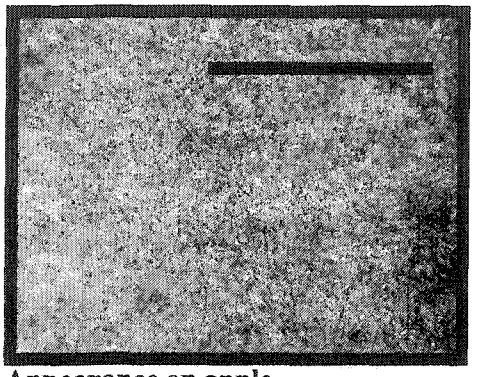


Appearance on PDA. Three-month-old growth on PDA of *Dissoconium* sp. DS2 strain mwb5a.

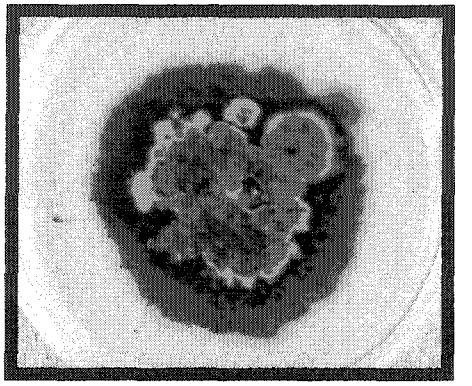
Growth on media. *PDA*. Upper: gray-green colony, some white mycelia. Lower: black. *MEA*. Upper: thin gray, flat, no aerial mycelia. Lower: black. Diameter at 4 weeks on MEA 15.5–26.5mm.

APPENDIX 6

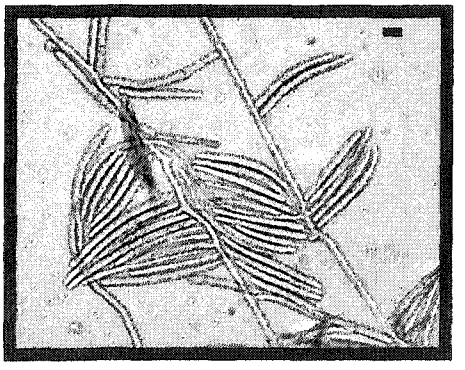
Pseudocercospora sp. FG1.1 and FG1.2

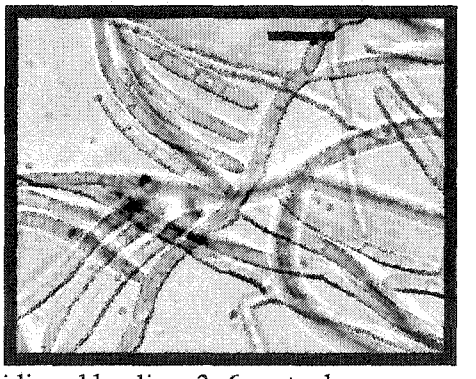


Appearance on apple. P*seudocercospora* sp. FG1.1 strain mwf7. Light fuliginous mycelial type on apple. Bar = 0.5mm.

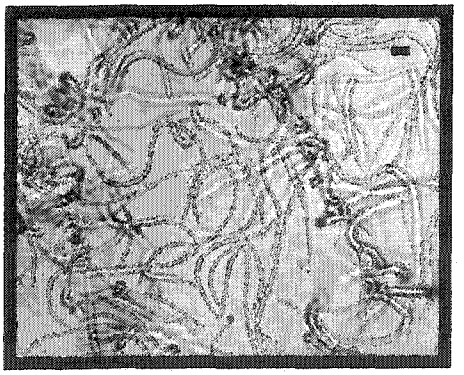


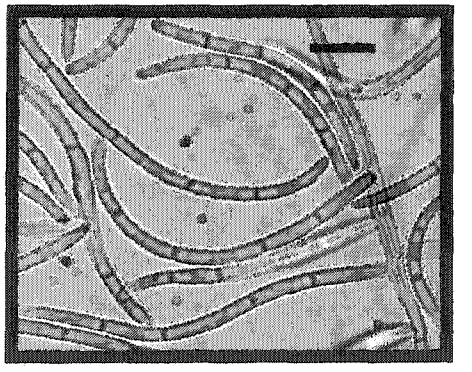
Appearance on PDA. Three-month growth of P*seudocercospora* sp. FG1.1 strain mwf7.





P*seudocercospora* sp. FG1.1 strain mwf7. Conidia subhyaline, 3–6 septa; long flexuous, broader at base with no scar and straight apex; 20–77 x 2–3 µm. Conidiophore is swollen hyphal cell, scars, pore. Bar = 5µm.



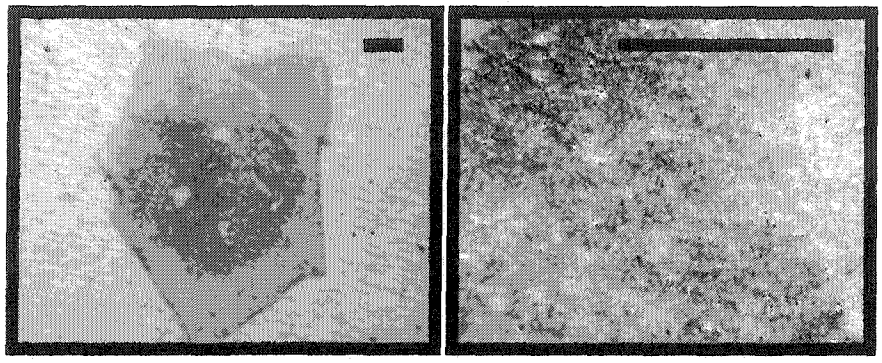


P*seudocercospora* sp. FG1.2 strain mstf5. Conidia subhyaline, 3–10 septa, long flexuous, scar base; 27–107 x 1.5µm. Conidiophore is swollen hyphal cell, scars, pore. Bar = 5µm.

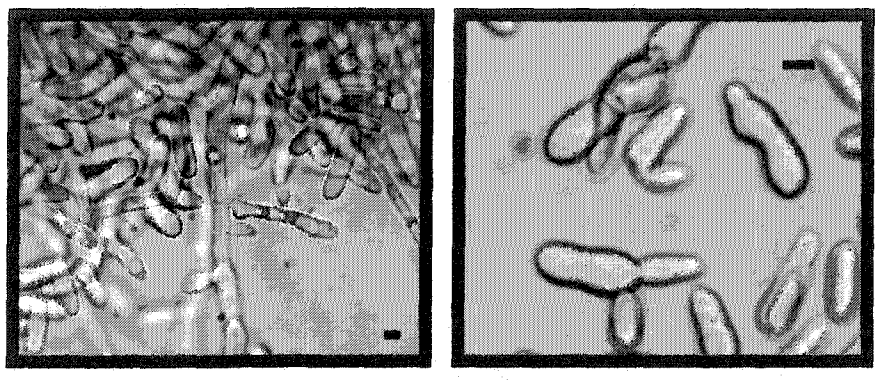
Growth on media. ***Pseudocercospora*** **sp. FG1.1** *PDA.* Upper: light brown, tufted, lumpy, thick colony. Lower: brown. Pigment: brown. *MEA.* Upper: rounded, tan, fuzzy. Lower: black. Diameter at 4 weeks on MEA 2.0–3.0 mm.
Pseudocercospora **sp. FG1.2** *PDA.* Upper: green, rounded, velvety. Lower: black. No pigment. *MEA.* Upper: flat, black, thin edges. Lower: black.

APPENDIX 7

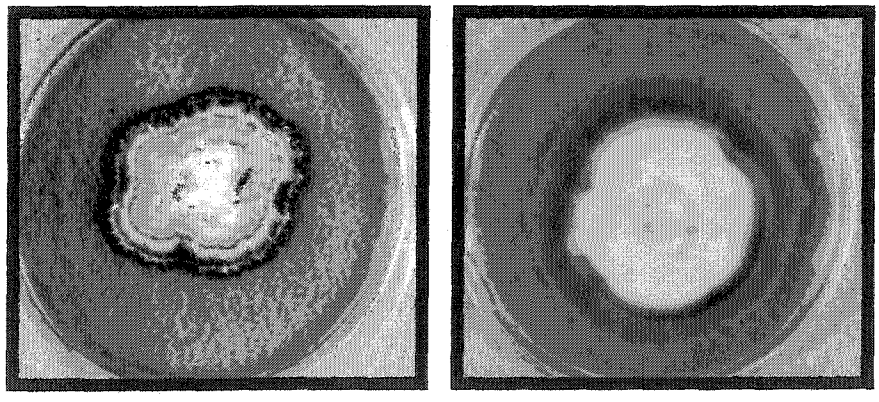
Colletogloeum spp. FG2.1, FG2.2 and FG2.3



Appearance on apple. Fuliginous mycelial type of *Colletogloeum* sp. FG2.1 strain uif7. Bar = 0.5mm



Conidia. *Colletogloeum* sp. FG2.1 strain uif7. Conidia subhyaline, 1 or 2-celled ovoid, allantoid, base truncate. No scars. Borne singly on swollen hyphal cells. Abundant secondary conidiation forming yellow slimy masses in young cultures. Bar = 5µm.



Appearance on PDA. Three-month-growth of *Colletogloeum* sp. FG2.2 strain mwf1a (left) and *Colletogloeum* sp. FG2.3 strain cuf2a (right).

Growth on media.

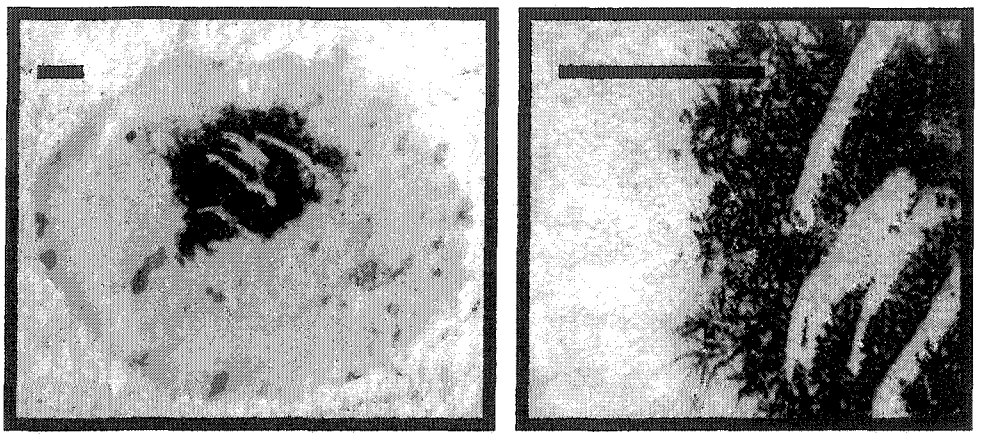
***Colletogloeum* sp. FG2.1** *PDA.* Upper: pale green and white felty, rounded with drops of translucent brown liquid. Lower: brown. *MEA.* Upper: pale green, fuzzy, rounded, thin. Lower: olive. Diameter at 4 weeks on MEA: 5.5–7.5mm.

***Colletogloeum* sp. FG2.2** *PDA.* Upper: light brown, rounded, felty. Thick colony. Lower: brown. *MEA.* Upper: light brown, fuzzy clumps of hyphae, submerged. Lower: brown. Diameter at 4 weeks on MEA: 8.0–10.0mm.

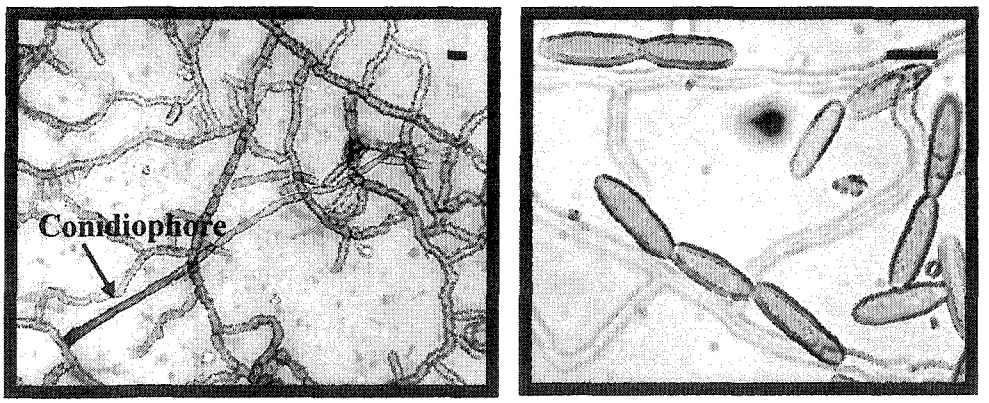
***Colletogloeum* sp. FG2.3** *PDA.* Upper: beige, flat thin edge. Lower: tan with brown center. *MEA.* Upper: tan, fuzzy, rounded, thin margins, tufts of white aerial hyphae. Lower: olive-brown. Diameter at 4 weeks on MEA: 8.0–14.5mm.

APPENDIX 8

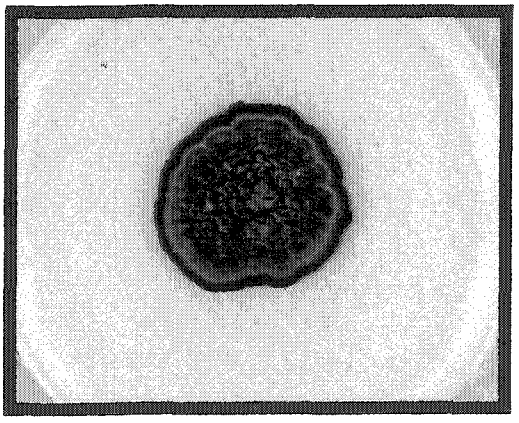
Passalora sp. FG3



Appearance on apple. Fuliginous mycelial type of *Passalora* sp. F3 strain gtf7 on apple. Bar = 0.5 mm



Conidia. *Passalora* sp. FG3 strain gtf1a. Conidia subhyaline to brown, 1 or 2-celled, cylindrical, ends tapered, flat, dark, scarred; 10–16 x 2–3.5 µm. Borne in chains on brown thick-walled, smooth, cylindrical, septate (1-8) conidiophores. Youngest conidia at apex. Bar=5µm

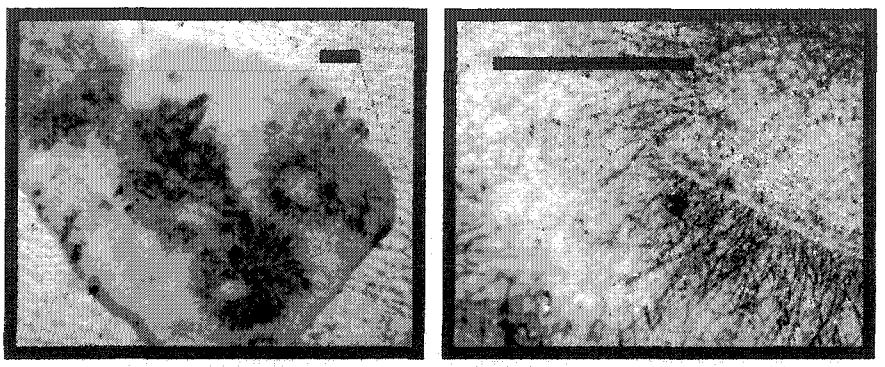


Appearance on PDA. Three-month-old growth of *Passalora* sp. FG3 strain gtf2.

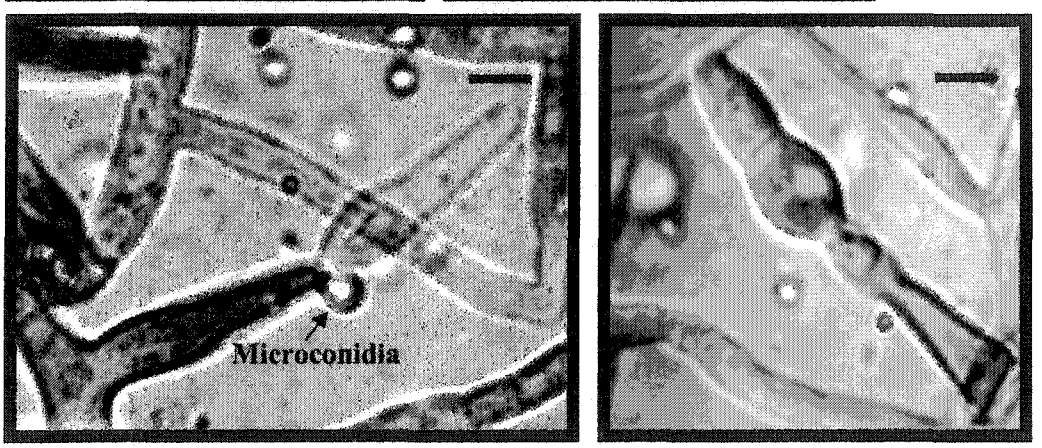
Growth on media. *PDA.* Upper: blackish brown, convoluted, submerged, reticulated edges. Dark aerial mycelium. Lower: blackish brown. *MEA.* Upper: olive, mostly above surface, dark green aerial mycelia. Diameter in 4 weeks on MEA: 2.0-3.0mm.

APPENDIX 9

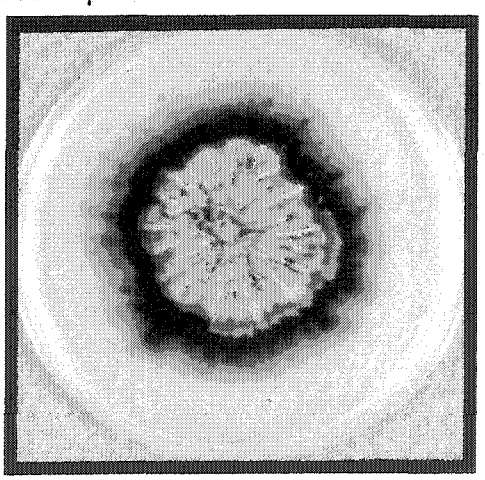
Dissoconium sp. FG4



Appearance on apple. Fuliginous mycelial type of *Dissoconium* sp. FG4 strain mstf4. Bar = 0.5mm.



Conidia. Macroconidia subhyaline. Basal cell obconic, truncate at septum; apical cell ovoid, truncate at septum;13–23 x 4–5.5µm. Microconidia single-cell, ovoid, (not common) 3–4 x 2–3 µm. Conidiophore pigmented, septate, elongate, swollen at base, tapered apex 14.5–35.0 (lengthens with age) x 3.0–4.5µm. Light brown scars, forcibly discharged. Bar = 5µm.

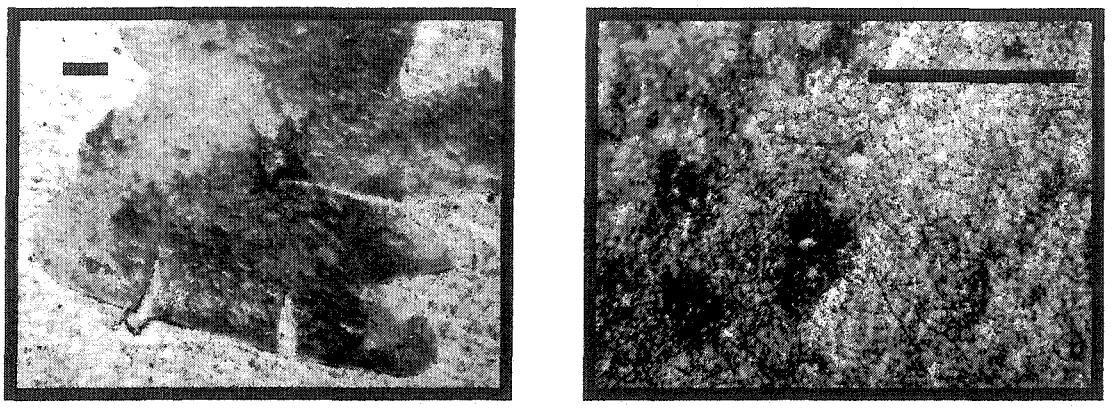


Appearance on PDA.
Three-month-growth of *Dissoconium* sp. FG4 strain mstf6.

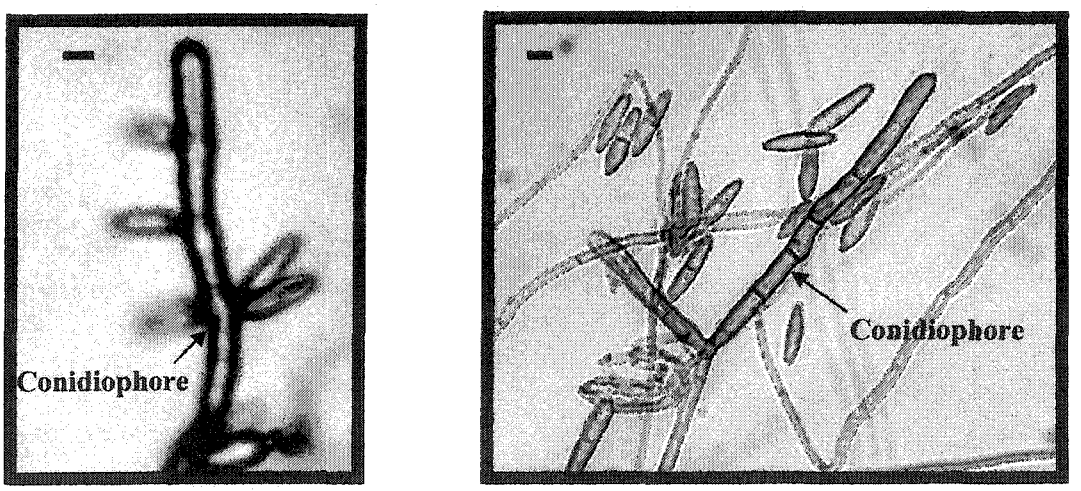
Growth on media.
PDA. Upper: olive green, dense, felty with slight convolutions. Lower: black.
MEA. Upper: olive green, thin edges, tan aerial mycelia, slightly submerged. Lower: dark center. Diameter at 4 weeks on MEA 10.2–19.0mm.

APPENDIX 10

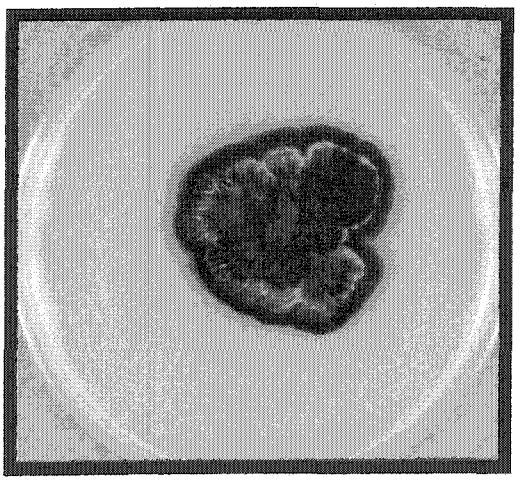
Dissoconium sp. FG5



Appearance on apple. Fuliginous mycelial type of *Dissoconium* sp. FG5 strain uif3 on apple. Bar = 0.5mm.



Conidia. *Dissoconium* sp. FG5 strain mstf3a. Conidia subhyaline, 1 or 2-celled, narrowly ovoid, base darkly scarred; 12–17 x 3–4 μm. Borne singly in whorls along conidiophore. Conidiophore cylindrical, straight or bent, septate, elongates with age. Bar = 5μm.

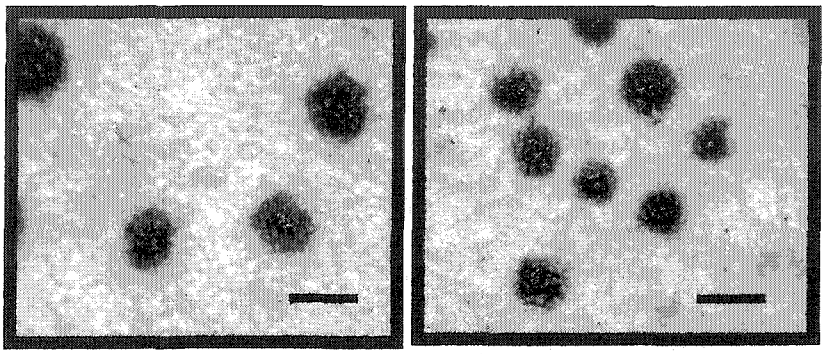


Appearance on PDA. Three-month-old growth of *Dissoconium* sp. FG5 strain uif3.

Growth on media. *PDA.* Upper: high, rounded colony; dark olive. Lower: black. *MEA.* Upper: dark olive, flat, feathery edges. High, rounded colony; dark olive.Lower: black. Diameter in 4 weeks on MEA: 2.5–4.5mm.

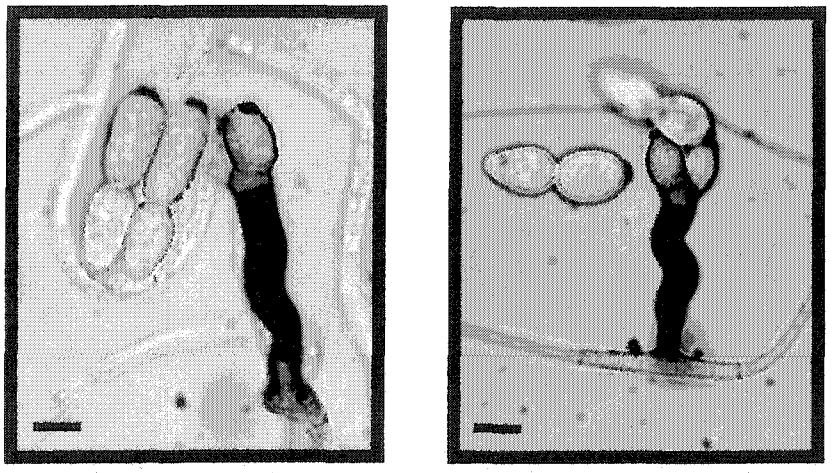
APPENDIX 11

Zygophiala jamaicensis (sp. FS1) and sp. FS2

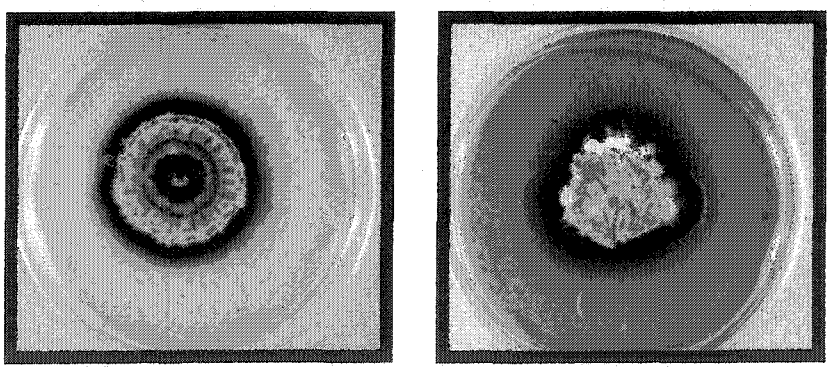


Appearance on apple. Flyspeck mycelial type of *Z. jamaicensis* (left) and *Zygophiala* sp. FS2 (right).

Sclerotium-like structures blister-like rounded. When removed from fruit, often leaves a dark ring. FS1: 1.5/mm^2; 155–409µm diam. FS2: 2/mm^2; 109–364µm diam Bar = 0.5mm.



Conidia. Sub-hyaline, basal cell obtuse; apical cell obpyriform. Borne on multi-celled conidiophore with sinuous stipe, pentagonal terminal cell, and ovate sporogenous cell.
FS1(left) 20–25 x 5-7µm.
FS2 (right) 13.5–21 x 3.5–9µm.
Bar = 5µm.

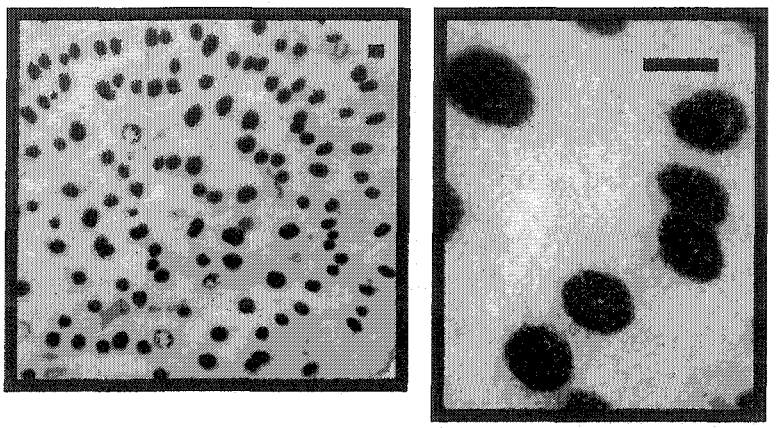


Appearance on PDA. Three-month old growth of *Z. jamaicensis* strain mwa9 (left) and *Zygophiala* sp. FS2 stain FVA2 (right).

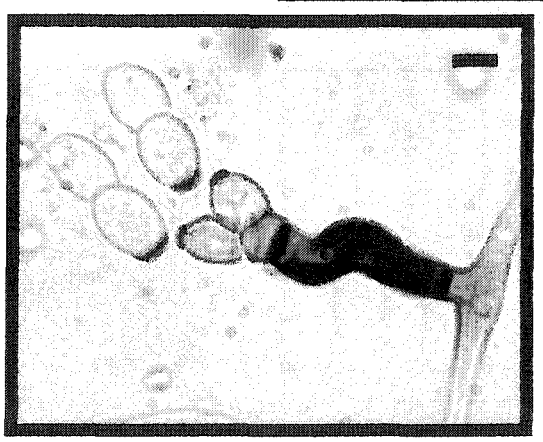
Growth on media. ***Z. jamaicensis*** (FS1) *PDA*: Upper: pink, orange, white, or green patches of dense, lumpy mycelia, thin uneven edge, sclerotia within medium. Lower: black. *MEA:* Upper: thin, beige with brown/ green center; uneven edges, submerged mycelia; sparse conidiophores (< 20 per plate). Aerial white mycelia. Lower: black/tan. Diameter at 4weeks on MEA. ITS clade: a 12.5–20.5mm ITS clade b 4.5–9.5mm.
***Zygophiala* sp. FS2** *PDA:*Upper: lumpy, flocculate, dense; white with dark green patches. Lower: black.Pigment: red. *MEA*:Upper: beige and pale green, thin transluscent; dark patches of olive black dense mycelia or sclerotia in center and submerged within media; scant white aerial mycelia. Prolific conidiophores (several hundred per plate). .Lower: tan with black center. Diameter at 4 weeks on MEA 3.0–10.0 mm.

APPENDIX 12

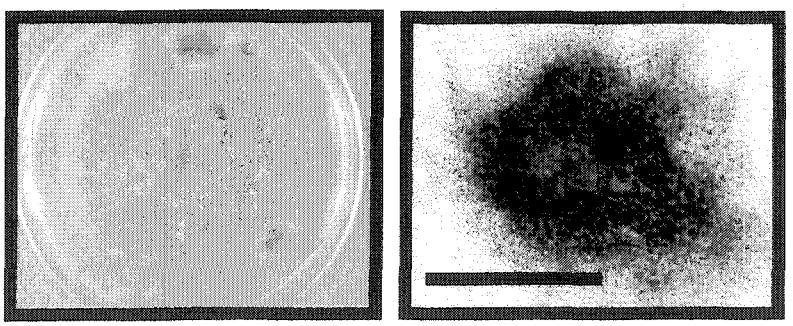
Zygophiala sp. FS3.1 and FS3.2



Appearance on apple. Flyspeck mycelial type of *Zygophiala* sp. FS3.1 strain gta4 and *Zygophiala* sp. FS3.2 strain mwa1. No visible mycelial mat. Sclerotium-like bodies dark, shiny ovoid, large 300–450 x 425–600μm and sparsely arranged at a density of 0.5/mm^2. Bar = 0.5mm.



Conidia. Two-celled; formed in couplets. basal and apical cell ovoid, truncate at septa FS3.1 10–17 x 7–9μm ; FS3.2 11.5–18 x 6μm. Borne on multi-celled condiophore with sinuous brown stipe 15–21 x 4.5–5.8μm; pentagon terminal cell 4–4.5 x 3–3.5μm; sub-hyaline scarred sporogenous cell 4–10 x 3.5–5.5μm. Bar = 5μm.

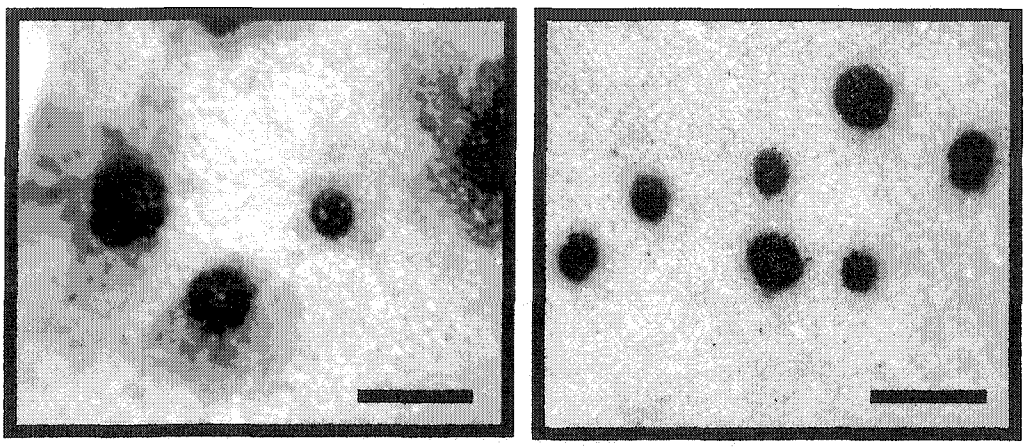


Appearance on PDA. Three-month old growth on PDA *Zygophiala* sp. FS3.1 strain gta4 (right). Sclerotium-like bodies (lower left) comprised of interlocking cells. Bar = 0.5mm.

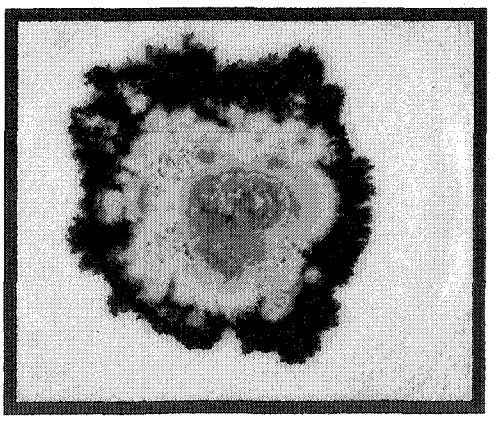
Growth on media. *Zygophiala* sp. FS3.1 and FS3.2. *PDA.* Upper: flat, zonate, dense pale green center, white margins; center region with black sclerotia on surface. Lower: brown, yellow margins. *MEA.* Upper: flat, thin, dark green center, white translucent margins; scant white aerial mycelia center region with black sclerotia on surface and embedded within medium; mycelia deeply submerged. Moderate numbers of conidiophores (less than 100 per plate). Lower: green. Diameter growth at 4 weeks on MEA. FS3.1: 13.5–22.5mm; FS3.2: 2.5–4.5mm.

APPENDIX 13

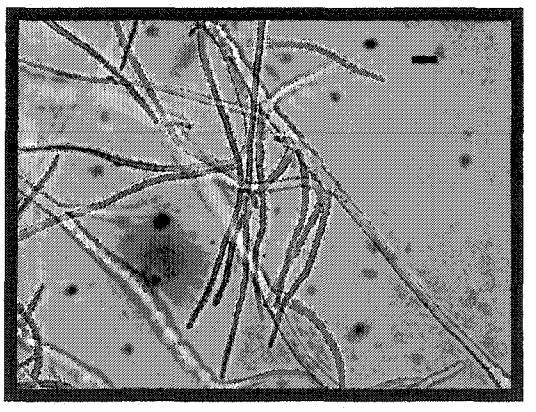
Pseudocercospora sp. FS4



Appearance on apple. Flyspeck mycelial type of *Pseudocercospora* sp. FS4 strain mwa10b (left) and strain mwa5 (right). Sclerotium-like bodies dark, shiny ovoid, large 245–464 x 209-345μm and sparsely arranged at a density of 0.44/mm^2. Bar = 0.5mm.



Appearance on PDA. Three-month old growth of *Pseudocercospora* sp. FS4 strain mwa10b.

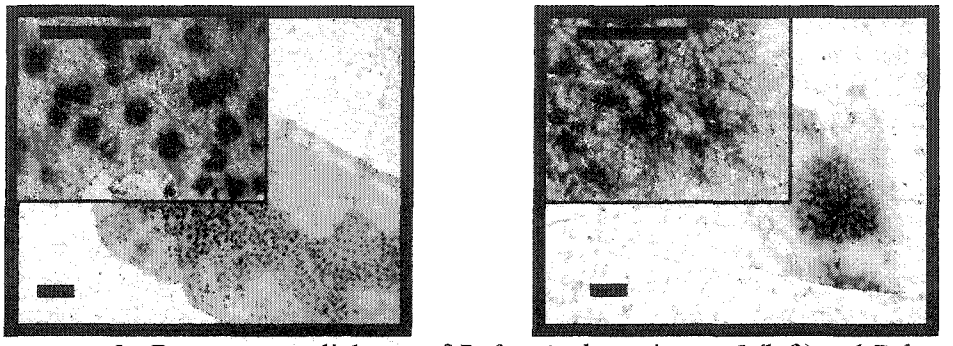


Conidia. *Pseudocercospora* sp. FS4 strain mwa5. Conidia subhyaline, 6–10 septa; long flexuous; 250-350 x 4μm (left). Bar = 10μm.

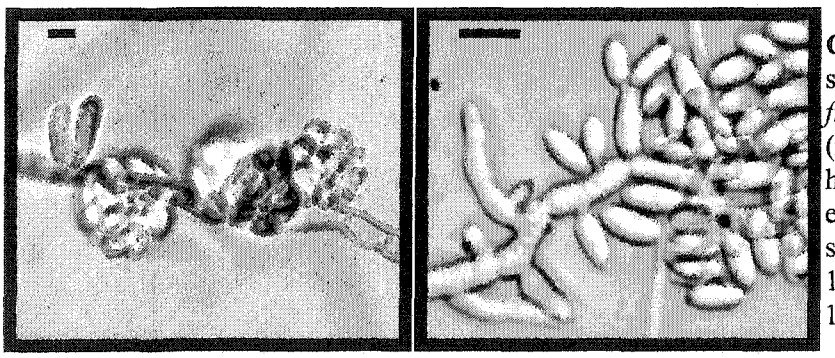
Growth on media. *Pseudocercospora* sp. FS4 strain mw10b. *PDA:* Upper: light olive, dense, tufted, highly rounded. Lower: black. Pigment: Orange. *MEA*: Upper: Dark olive, thickly tufted tan aerial mycelia, highly rounded. Lower: black. Diameter at 4 weeks on MEA: 3.0–5.5 mm.

APPENDIX 14

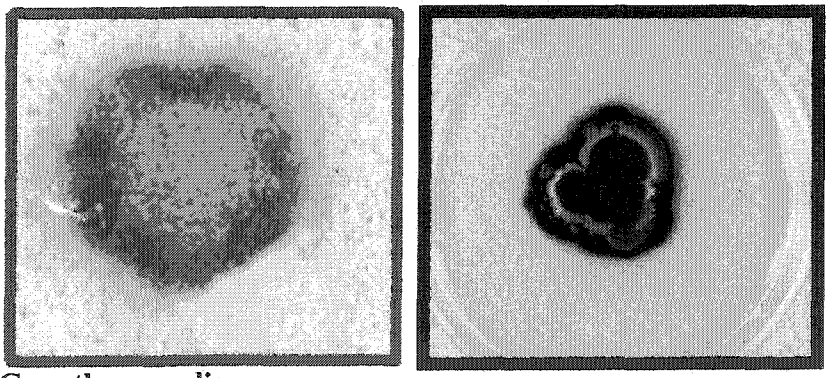
Peltaster fructicola (= sp. P1) and *Peltaster* spp. P2.1 and P2.2



Appearance on apple. Punctate mycelial type of *P. fructicola* strain ume5 (left) and *Peltaster* sp. P2.2 strain cue2 (right). Dark mycelial mat. Sclerotium-like bodies composed of overlapping hyphal strands, not well-delineated from mycelial mat. Similar in appearance througout colony. P1: 12–33/mm^2; diameter 18-216μm. P2.1 & P2.2: 14–23/mm^2; diameter 18-153μm. Bar=0.5mm.



Conidia. *Peltaster* sp. P2.2 strain cue2a (left) and *P. fructicola* strain mste2a (right). Conidia holoblastic, hyaline, 1 or 2-celled ellipsoidal. No scars. Borne singly forming clusters. P1: 1.5–10 x 1.5–4; P2: 2.5-8.7 x 1.5–3μm. Bar = 5μm.



Appearance on PDA. Three-month old growth on PDA of *P. fructicola* (left) and *Peltaster* sp. P2.2 (right).

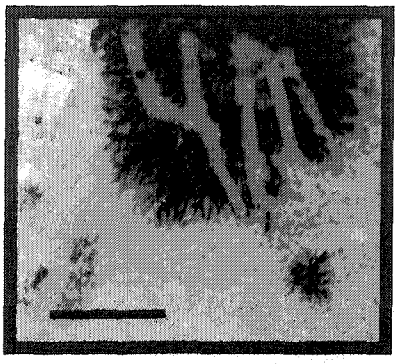
Growth on media.

Peltaster fructicola. *PDA.* Upper: light olive, velvety rounded, thick colony with defined edge. Very young colonies with shiny areas of yellow yeast-like growth. Lower: olive, black or brown. *MEA.* Upper: light olive, flat, mostly submerged, even edges, scant tuft of green aerial mycelia. Lower: black or dark olive. Diameter at 4 weeks on MEA: 6.5–9.5 mm.

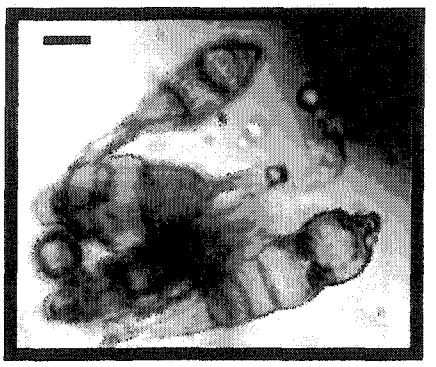
***Peltaster* sp. P2.1 and P2.2.** *PDA.* Upper: black and green zonate (P2.1) or black (P2.2), highly mounded; abrupt edge. Drops of pale orange (P2.1) or pink (P2.2) exudates comprised of conidia. Reddish brown pigment in media. Lower: black. *MEA.* Upper: black to dark olive, mostly submerged; center third of colony above media, rounded, convoluted. Lower: Black or gray. Diameter at 4 weeks on MEA: P2.1: 3–5mm. P2.2: 1.2–2mm.

APPENDIX 15

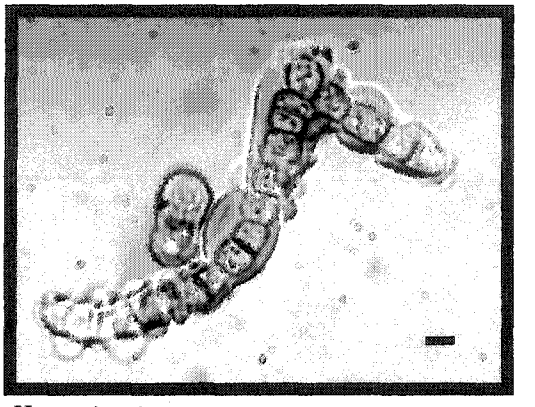
Xenostigmina sp. P3 and P4



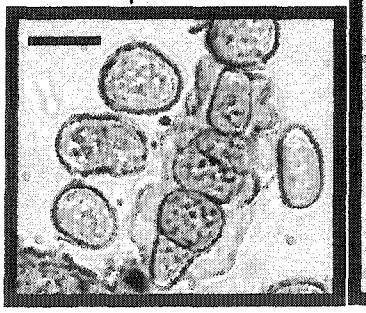
Appearance on apple. Punctate mycelial type of *Xenostigmina* sp. ahe3 (left) and *Stigmina* sp. uie3 (right). Bar = 0.5mm
Sclerotium-like structures numerous, irregular, with distinct margins, flat. P3 density 20/mm^2; diameter 7–81µm. P4 density 21/mm^2; diameter 27-155µm. Mycelial mat dark, reticulate, very small colonies.

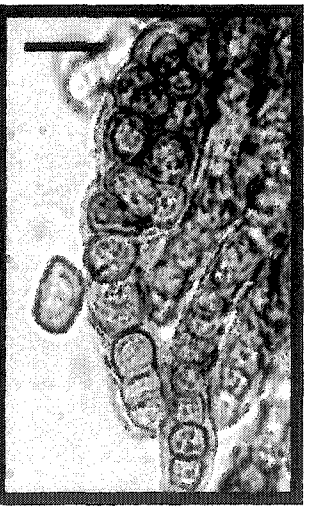


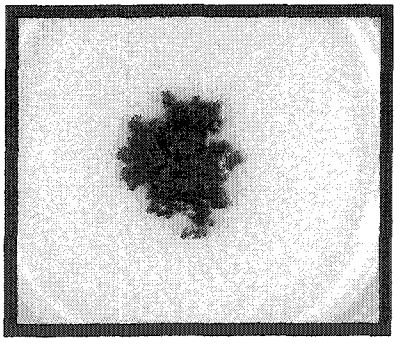
Conidia. *Xenostigmina* sp.P3 ahe3 (above). Brown, clavate with short rostrum; 2-5 septa, muriform; 35 – 70µm x 14 – 27µm, borne singley.



Conidia. *Xenostigmina* sp. P4 (above, right and below). Brown, elongate, comprised of ~12 sub-units (1-2 cells) that easily break apart. Bar = 5µm.





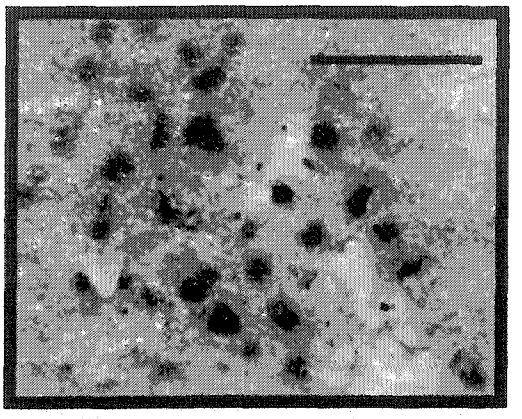


Appearance on PDA. Three-month old growth of *Xenostigmina* sp. P3 ahe3

Growth on media. ***Xenostigmina* sp. P3.** *PDA and MEA.* Upper: black, carbonaceous, crumbly, completely above surface of medium. Lower: black. Diameter in 4 weeks on MEA 1.2mm. ***Xenostigmina* sp. P4.** *PDA and MEA.* Upper: black, carbonaceous, crumbly, completely above surface of medium; highly mounded above surface (>5mm). Lower: black. Diameter in 4 weeks on MEA 0.5–1.0 mm.

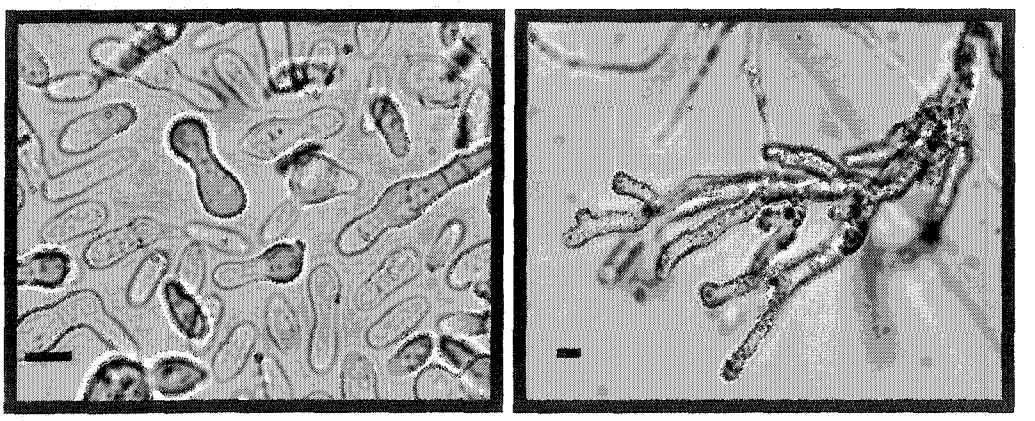
APPENDIX 16

Ramularia sp. P5

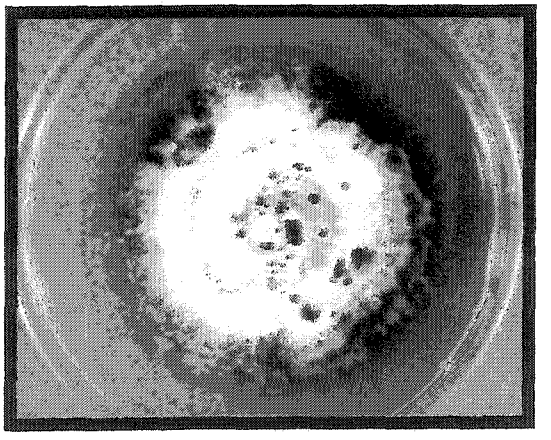


Appearance on apple.
Punctate mycelial type on apple.
Visible mycelial mat ranging from dark to light.

Sclerotium-like structures numerous, circular to oval, flat. Density 22/mm^2. Diameter 63–180um. Bar = 0.5 mm.



Conidia. Single-celled conidia of *Ramularia* sp. P5 (left) were irregular in shape (ovoid, hourglass, pyriform) hyaline (5.2–14.5 x 1.5–7 µm) and were produced singly on brown condiophores that had dendritic branches with a single central basal cell (right). Bar = 5µm.

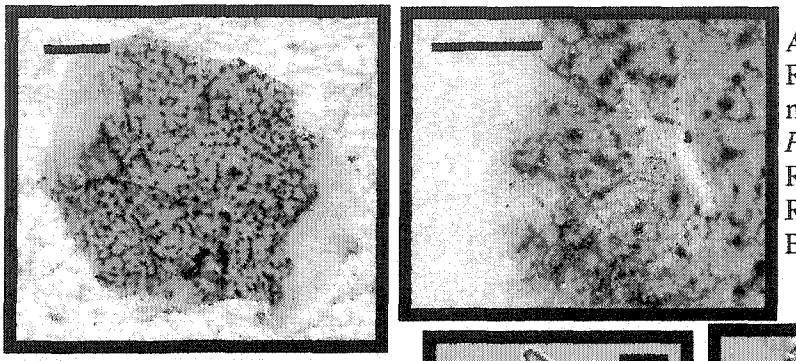


Appearance on PDA.
Three-month-old growth on PDA of *Ramularia* sp. P5 strain ume2a.
One of the two isolates produced a yellow pigment on PDA.

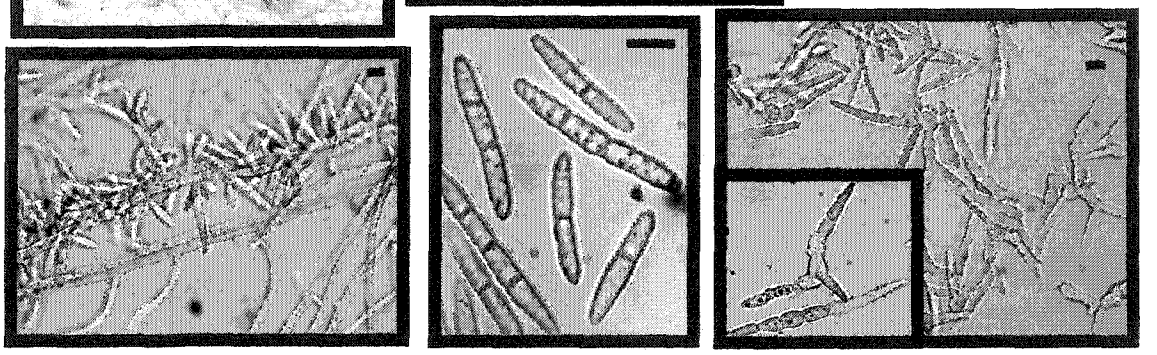
Growth on media. *PDA.* Upper: green, white and pink, sectored; aerial mycelia, slightly mounded, folded, partly submerged. Lower: green, pink. *MEA.* Upper: tan, green center, mostly submerged, uneven margins. Lower: tan. Diameter at 4 weeks 8.0–9.5mm.

APPENDIX 17

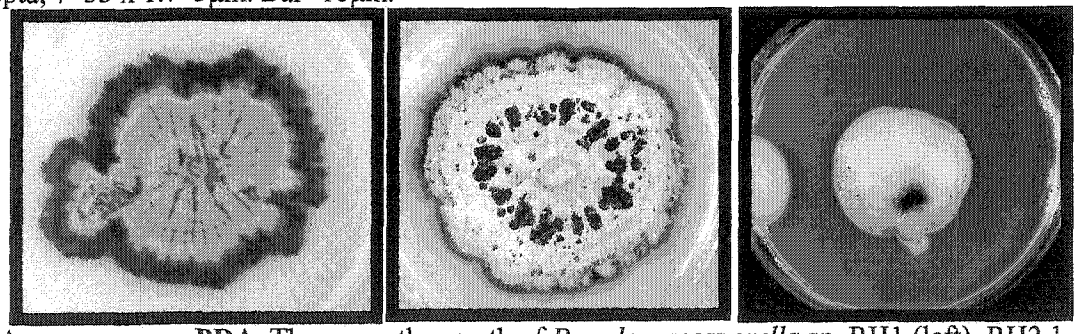
Pseudocercosporella sp. RH1, RH2.1 and RH2.2



Appearance on apple. Ridged honeycomb mycelial type of *Pseudocercosporella* sp. RH1 strain mwd1 (left) and RH2 strain uid1a (right). Bar = 0.5mm.



Conidia. Borne singly on hyphae that are often grouped together in rope-like structures (left). Detached primary and secondary conidia (center). Secondary conidia branching from detached older primary conidia (right). Primary conidia subhyaline; scolecospores 17–63 x 1–5.5µm, truncated base, tapered apex, 4–6 septa becoming constricted with age. Detached conidia develop prolific secondary conidia. Secondary conidia cigar shaped, 0–3 septa; 7–33 x 1.7–3µm. Bar=10µm.



Appearance on PDA. Three month growth of *Pseudocercosporella* sp. RH1 (left), RH2.1 (center), RH2.2 (right).

Growth on media.

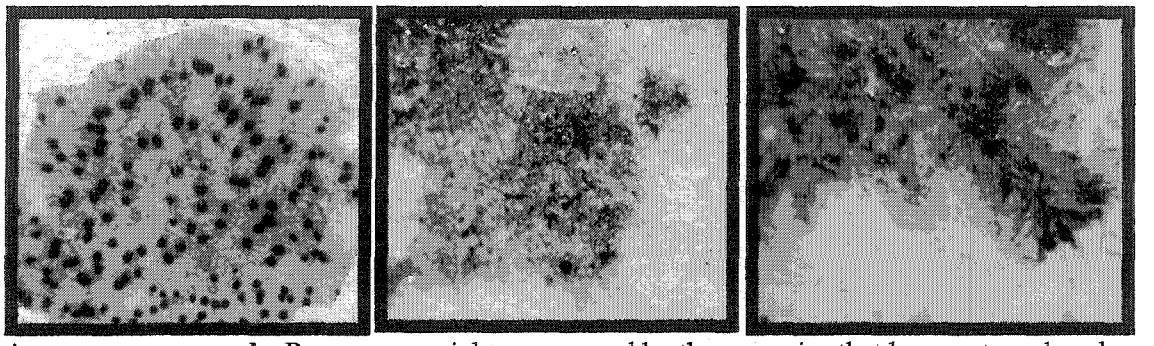
***Pseudocercosporella* sp. RH1** *PDA.* Upper: dark green, highly mounded, deep folds, scant white aerial hyphae; beige slimy masses (conidia) on surface. Lower: black, pulls up from plate. *MEA:*Upper: flat, black, dense; scant white tufts of aerial mycelia. Lower: black. Diameter at 4 weeks on MEA: 2.0–10.5 mm.

***Pseudocercosporella* sp. RH2.1** *PDA.*Similar to RH1 but light olive upper colony.

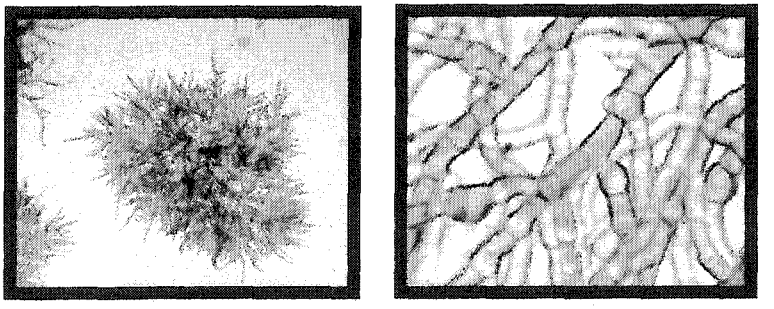
***Pseudocercosporella* sp. RH2.2** *PDA.* Upper: dirty white, rounded, shiny, firm gelatinous hard growth ultimately replaced by spore producing hyphae, dry with black drops of spore-filled exudates. Lower: brown *MEA:*Upper: green, thin, flat; large, thick tufts of green aerial mycelia, deeply submerged. Lower: brown Diameter at 4 wks on MEA: 3.0–19.0 mm.

APPENDIX 18

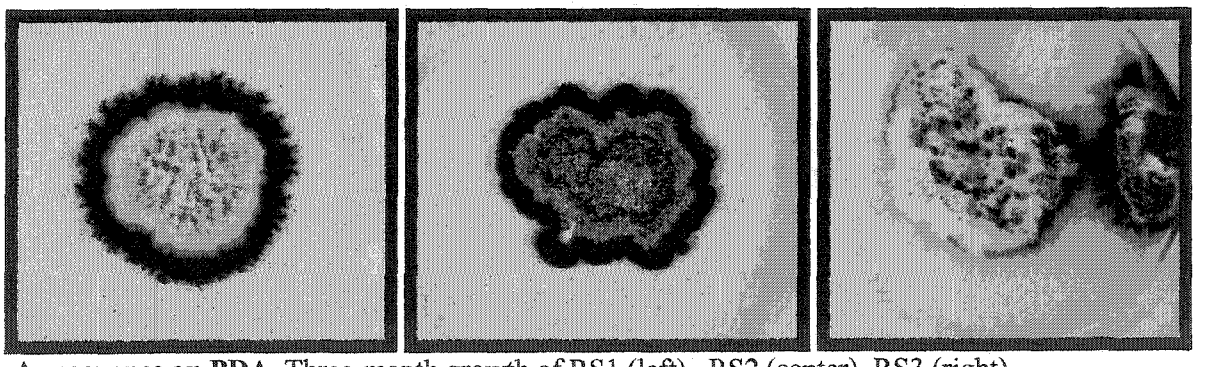
Sterile mycelia spp. RS1, RS2, RS3



Appearance on apple. Ramose myceial types caused by three species that have not produced conidia in culture **RS1** (left) Mycelial mat web-like with dark strands, edge abrupt. Sclerotium-like bodies scattered, black, shiny, raised, but often sunken in center, more developed in center of colony; 4–10/mm^2 . Diameter 93–140mm. **RS2** (center) Mycelial mat olive, loosely woven, hirsute margins. Sclerotium-like bodies partially concentric, large (0.5mm) black, shiny, raised, but edges not strictly limited. Structures more developed in center of colony. **RS3** (right) Mycelial mat dark, dense, irregular shape, thick feathered margins. Sclerotium-like bodies Scattered, black, shiny raised.



Appearance in culture. Sterile mycelia on media with arborescent margins (100x objective) (left) and thick, dark, hyphal strands (1000x objective) (right).



Appearance on PDA. Three-month growth of RS1 (left) , RS2 (center), RS3 (right).

Growth on media.

RS1 *PDA.* Upper: olive tan, lobed, mounded, folded, uneven edges. Lower: brown. *MEA.* Upper: olive, tufts of aerial mycelia, black sclerotia on upper surface, rounded. Lower: black. Diameter in 4 weeks on MEA: 0.5-2.0mm.

RS2 *PDA.* Upper: blue green, felty, highly mounded and ridged, margins submerged into medium with arborescent edges. Lower: black. *MEA.* Upper: olive, arborescent edges, aerial mycelia. Lower: black. Diameter in 4 weeks on MEA: 0.35-2.0mm.

RS3 *PDA.* Upper: gray, flat. Lower: black. *MEA.* Upper: black, arborescent edges. Lower: black.