Aggressiveness of *Colletotrichum sublineola* Strains from *Sorghum bicolor* and *S. halepense* to Sweet Sorghum Variety Sugar Drip, and Their Impact on Yield

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Abstract

Sweet sorghum (*Sorghum bicolor*) has been grown in the southeastern United States for more than 150 years on a relatively limited scale, primarily for forage and for the production of table syrup. However, interest in the crop has increased recently due to its potential as a feedstock for biofuels. *Colletotrichum sublineola* is the causal agent of anthracnose on cultivated sorghum and on the wild sorghum relative Johnsongrass (*S. halepense*). Anthracnose is an important disease of grain sorghum worldwide, but little is known about its impact on sweet sorghum in the U.S. The aggressiveness of four *C. sublineola* isolates collected from sweet and grain sorghum and from Johnsongrass at various locations across Kentucky was measured as disease incidence and severity on the susceptible heirloom sweet sorghum inbred

Sugar Drip in inoculated field trials. The isolate from sweet sorghum was the most aggressive, while the two Johnsongrass isolates caused only minimal disease symptoms. Disease incidences of up to 99%, and severities of up to 16.7% of leaf area affected, had no negative effect on the yield of biomass, grain, juice, or Brix. Removal of sorghum seed heads increased Brix in the stalks and leaves, but did not affect susceptibility to anthracnose. The same group of fungal isolates was evaluated for aggressiveness in greenhouse assays on juvenile plants, and in the laboratory on seedlings and detached leaf sheaths. These protocols will be useful for prescreening sorghum germplasm for new sources of resistance or for characterizing the aggressiveness of new fungal isolates.

Sorghum [*Sorghum bicolor* (L.) Moench] is an annual C4 grass characterized by its high photosynthetic efficiency and its relative resistance to environmental stresses, including drought, salinity, marginal soils, and high temperatures (Hallam et al. 2001; Sasaki and Antonio 2009). In the United States, sorghum is the second most important source of grain for animal feed (Maunder 2008). Sorghum ranks fifth in importance among cereal crops worldwide, and it is especially significant as a major source of food for some of the poorest regions of the world (Doggett 1995; Kresovich et al. 2005; Maunder 2008; Mekbib 2006).

In addition to grain sorghum varieties and hybrids that produce a large, starchy seed head on a short stalk, there are also sweet sorghum types. These produce more biomass than the grain varieties, with many reaching heights of three meters or more, and they accumulate large amounts of soluble sugar in the stalks. Sweet sorghum varieties are not as commonly grown as the grain varieties, and are used traditionally for forage and for the production of sweeteners (Rooney et al. 2007). Recently, interest in sweet sorghum has increased in the U.S. and worldwide because of its potential for the production of ethanol biofuel from cellulosic biomass or directly from sorghum sugar (Bitzer 1997; Cutz et al. 2013; Koçar and Civaş 2013; Pederson 2010; Sarath et al. 2008; Sipos et al. 2009; Wheals et al. 1999; Yu et al. 2014; Zegada-Lizarazu and Monti 2012). Sweet sorghum is well adapted for cultivation in the southeastern U.S., where the crop

has been grown on a small scale for more than 150 years for the production of table syrup. Kentucky (KY) and its neighboring southeastern states are responsible for 90% of the total U.S. output of sweet sorghum syrup (Bitzer 1997; Vermerris et al. 2008).

Anthracnose is an important disease of grain sorghum worldwide, and has been reported to cause reductions of as much as 50% in grain yield (Harris et al. 1964; Thomas et al. 1996). *Colletotrichum sublineola* (Henn.) causes anthracnose on *S. bicolor*, and can infect all parts of the plant including the stalk, peduncle, panicle, grain, and leaf (Ferreira and Warren 1982). Foliar anthracnose is the most common phase of the disease in grain sorghum. Foliar anthracnose occurs everywhere in the world where grain sorghum is produced, and is especially severe under conditions of high temperature and humidity, as are typical in the southeastern U.S. (Ali and Warren 1987). Infection of the peduncle can result in premature death of the head. Infection of the stalk produces a disease called red stalk rot, which can result in lodging. The occurrence of anthracnose on grain sorghum has been associated with stresses during grain filling including drought, soil saturation, cold weather, and nutritional deficiencies (Dodd 1980a).

During World War II, epidemics of foliar anthracnose severely limited the productivity of sweet sorghum that was grown in the Gulf Coast region to provide a substitute for cane sugar (LeBeau et al. 1951). Anthracnose is still common on sweet sorghum in the southeastern U.S. (Bitzer 1997; Hagan 2015), but little is known about the impact of this disease on yield, or its potential for damage if sweet sorghum is grown more widely for biofuel as has been suggested in the future.

The main strategy for the management of foliar anthracnose in grain sorghum is the use of resistant cultivars (Chala et al. 2010a; Mughogho 1982). However, resistance fails frequently, and this is thought to be related to the large number of pathogenic races in the population of *C. sublineola* (Casela et al. 1998, 2000). New sources of resistance must constantly be developed and deployed in order to manage the pathogen effectively. Another strategy recommended for the management of anthracnose on sorghum is to use crop rotation with other plant genera. However, *C. sublineola* also causes foliar lesions on the ubiquitous weed Johnsongrass (Cardwell 1989), which could serve as a refuge for *C. sublineola* during crop rotation schemes. Furthermore, Johnsongrass could be a source of novel pathogenic races, since this

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weed is genetically diverse (Karimi and Saeidi 2016). One prior study suggested that Johnsongrass isolates did not significantly contribute to the populations of *C. sublineola* on nearby grain sorghum in Texas (Gale 2002; Rosewich 1996). However, the aggressiveness of Johnsongrass strains to *S. bicolor* has never been tested directly in field experiments.

The primary goal of the work described here was to compare the aggressiveness of KY *C. sublineola* isolates from *S. bicolor* and *S. halepense* on the sweet sorghum variety Sugar Drip, and to evaluate the impact of anthracnose on yield of biomass and juice, and on sugar concentrations. Sugar Drip is an heirloom variety that is still grown in KY, even though it is known to be susceptible to anthracnose. Sorghum breeders have developed some anthracnose-resistant varieties of sweet sorghum for the southeastern U.S., but the pace and importance of this work has recently increased as the potential of sorghum for biofuels production has become more widely recognized (Broadhead et al. 1970; Cuevas et al. 2015; Day et al. 1995; Vermerris et al. 2008). Field screening for anthracnose resistance is resource-intensive, and results are complicated by the presence of other abiotic and biotic stresses (Erpelding and Prom 2004; Sharma et al. 2012). Rapid, high throughput greenhouse or laboratory assays would allow prescreening of a larger number of sorghum germplasm sources against more pathogen strains, speeding the process of development of resistant varieties. Thus, a second goal of the work described here was to compare the results of field experiments with the results of greenhouse and laboratory assays.

Materials and Methods

Fungal isolates. Some of the *Colletotrichum* isolates used in this study were collected during the summer of 2012 from sweet sorghum and Johnsongrass on the University of Kentucky (UK) Agronomy farm in Fayette County, and during a visit to several KY sweet sorghum growers in Clay and Russell counties. Foliar anthracnose lesions were relatively rare on sweet sorghum, but common on Johnsongrass in these locations. Panicle rot, and red stalk rot and lodging due to stalk disease, were not observed. One isolate from the sweet sorghum variety Sourless (SS1.001, from Fayette County) and two from Johnsongrass (JG9.001 and JG18.001, from Russell and Fayette counties, respectively) were used in all of the experiments (Table 1). Grain sorghum is produced only in western KY. One *C. sublineola* isolate from symptomatic grain sorghum leaves collected in Hopkins County in 2013 (GS1.001) was also used for all experiments (Table 1). Our laboratory standard *C. sublineola* strain (CgS11) was collected from grain sorghum in Indiana in the early 1980s (Jamil and Nicholson 1987). This strain was not used for field assays, due to the need for containment (Table 1). An isolate of *C. graminicola* collected from maize at the UK Agronomy farm in 2012 (M30.001) was included as a negative (nonpathogen) control in all experiments (Table 1).

The isolates were recovered from small pieces of plant tissue cut from the edges of foliar lesions, soaked in 70% ethanol for 30 s, transferred to 10% sodium hypochlorite (Clorox) for 2 min, and then washed three times with sterile water. Disinfested pieces were blotted on sterile paper towels and then placed on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) containing ampicillin (0.1 mg/ml). The isolates were genetically purified by single-sporing, then were preserved on silica gel at -80°C (Tuite 1969). All fungal strains were routinely cultured on PDA at 23°C under continuous fluorescent light.

Strains were identified based on their morphology, including the characteristic falcate spore shape and presence of acervuli with setae, and on BLASTN matches of the ribosomal DNA internal transcribed spacer (ITS) sequences (>99% identity) to the *C. sublineola* species type (accession NR_111191) or to the *C. graminicola* species type (NR_111190) (Table 1). Fungal genomic DNA was isolated by the method of Thon et al. (2000) and the primer pair ITS4 and ITS5 was used to amplify the ITS (White et al. 1990). PCR reactions were done in a final volume of 25 μl containing: 25 ng of genomic DNA, 10 $\times$ PCR buffer, 50 mM MgCl_2 , 200 μM dNTPs, one U of *Taq* DNA polymerase, and 5 μM of each primer. The PCR cycling conditions included: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing for 30 s at 51°C ; extension at 72°C for 1 min; with a final extension at 72°C for 10 min. Forward and reverse primers were used to sequence the amplicons with the BigDye Terminator cycle-sequencing chemistry (Applied Biosystems, Foster City, CA) on an ABI 3730 DNA Capillary Sequencer following the manufacturer's protocol, except in a reaction volume of 10 μl .

Sorghum. The heirloom sweet sorghum inbred Sugar Drip was used for all experiments. Seeds were obtained from Dr. Todd Pfeiffer at the University of Kentucky, and increased in 2012 at the UK Agronomy Farm in Lexington, KY. Greenhouse conditions for the production of sorghum seedlings and plants included 14 h of light, irrigation every other day to saturation by using an automated overhead watering system, and application of a solution of 150 ppm of Peters 20-10-20 fertilizer (Scotts-Sierra Horticultural Product Co., Marysville, OH) once a month, beginning one week after seedling emergence.

Preparation of fungal inoculum. Falcate spores were harvested from 2-week-old PDA cultures by adding 10 ml of sterile water to each plate and scraping the surface gently. The spores were collected by filtering through sterile cheesecloth, washed three times with sterile water to remove the mucilage, and quantified by using a hemocytometer. After adjusting the concentration (see details below), spore suspensions were used directly for laboratory assays to inoculate sorghum seedlings or leaf sheaths. Fungal inoculum for field and greenhouse experiments was produced by using a modified protocol of Prom et al. (2009). Briefly, 40 g of autoclaved Sugar Drip sorghum grains were placed aseptically in a Petri dish. A volume of a quantified spore suspension was added to achieve a final concentration of 5×10^6 spores per gram of sorghum grain. An equivalent volume of sterile water was added instead of the spore suspension to some of the dishes, in order to obtain mock-inoculated grains that were used for water control treatments. The treated grains were incubated for 14 days at 23°C in the dark before they were used as inoculum.

Field experiments. Field experiments were conducted during 2014 and 2015 at the UK South Farm in Lexington, KY. Two different experimental plots, both with Maury silt loam soils, were used. A single application of fertilizer (19-19-9 NPK) was made to the fields at a rate of 56 kg/ha in 2014, and 89 kg/ha in 2015, prior to planting. The rate in 2015 was greater because the plot used that year had previously been in pasture. Weeds were controlled mechanically without the use of herbicides, and drip irrigation was applied as necessary throughout the growing season. Records of rainfall, temperature, and relative humidity were obtained from the KY Mesonet weather station, which is located less than 1 km from the two experimental sites (http://www.kymesonet.org/historical_data.php).

Table 1. *Colletotrichum* isolates used in this study

Strain	Host	Species	State	County	Year Collected	ITS sequence accession #	Reference
CgS11	Grain sorghum	<i>C. sublineola</i>	IN	Tippecanoe	1982	KY006070	Jamil and Nicholson 1987
SS1.001	Sweet sorghum	<i>C. sublineola</i>	KY	Fayette	2012	KY006074	This study
GS1.001	Grain sorghum	<i>C. sublineola</i>	KY	Hopkins	2013	KY006071	This study
JG9.001	Johnsongrass	<i>C. sublineola</i>	KY	Russell	2012	KY006073	This study
JG18.001	Johnsongrass	<i>C. sublineola</i>	KY	Fayette	2012	KY006072	This study
M30.001	Maize	<i>C. graminicola</i>	KY	Fayette	2012	KY006075	This study

Sorghum seeds were sown on 4 May 2014, and 12 May 2015, in 72-cell flats (each cell was 1.27 cm L × 3.81 cm W and 5.72 cm deep) containing Pro-Mix BX (Premiere Horticulture, Ltd, Riviere du Loup, PQ, Canada) in the greenhouse. After 4 to 6 weeks, the seedlings were transplanted into the field with a between-row spacing of 1.4 m, and a within-row spacing of 0.7 m. Transplants were used in order to achieve equal spacing of plants and a uniform emergence time, thus controlling for plant age and microclimate, e.g., local humidity levels or light intensities, which can be affected by uneven stands. These factors are known to influence anthracnose disease severity (Dodd 1980b; Duncan 1984; Hammerschmidt and Nicholson 1977; Negeri et al. 2013; Schall et al. 1980). Transplants of early maturing varieties sometimes flower prematurely (Bitzer 1994), and this occurred during both years of the study. The main stalk of each plant was cut back to the crown at approximately 5 weeks after transplanting, prior to panicle emergence, which promoted the development of multiple tillers per plant. The tillers matured and flowered at the normal time for sorghum in the area, and were used for the inoculation experiments.

The experimental treatments included five *Colletotrichum* isolates (SS1.001; GS1.001; JG9.001; JG18.001; M30.001) and a water control. Prior studies have suggested that removing the seed head from sorghum plants (de-heading) increases the concentration of sugar in the stalks (Erickson et al. 2012), and other studies have reported a correlation between increased concentrations of stalk sugar and decreased susceptibility of maize to anthracnose stalk rot (Mortimore and Ward 1964). Head-intact and de-headed subtreatments were included for each treatment in a 2 by 6 factorial design. De-heading was accomplished by removing all the developing panicles from the plant tillers as they appeared, from boot stage until harvest (a period of approximately 30 days). The treatments were arranged in a randomized complete block design with four blocks. Each treatment replicate included two rows of five plants each. Each of the replicates was separated from the others by a row of maize to prevent the dissemination of inoculum between treatments. Maize seeds (inbred Mo17) were planted in these border rows 2 or 3 weeks prior to transplanting the sorghum.

Tillers ($n = 2$ per plant) were inoculated at the V5 or V6 growth stage by using a modified protocol as outlined by Prom et al. (2009). Twenty sorghum grains colonized with one of the five *Colletotrichum* isolates were placed into the whorl of each tiller. Mock-inoculated grains were used for the water control treatment. The optimal growth stage for inoculation was determined in preliminary experiments demonstrating that maximal levels of disease resulted when plants were inoculated at this stage (Xavier et al., unpublished data). A total of 20 tillers were inoculated per replicate.

Anthracnose disease symptoms were evaluated on a weekly basis beginning at 14 days post inoculation for a period of 4 weeks. A standardized rating scale was used, with values ranging from 0 to 5, based on one that has been commonly used for assessment of anthracnose in grain sorghum (Cardwell et al. 1987; Casela et al. 1998; Valério et al. 2005). A “0” indicates no symptoms or minor red flecks; a “1” is defined as small red dots on the leaves; a “2” indicates small-to-large red spots on the leaves; a “3” is lesions with necrotic centers but without acervuli; a “4” specifies necrotic lesions with acervuli in the centers of the lesions; and a “5” indicates the presence of coalescing necrotic lesions and abundant production of acervuli. This scale divides samples into two classes: resistant, including values of 0 to 3; and susceptible, including values of 4 and 5. Disease incidence was calculated as the number of infected (susceptible, ratings of 4 or 5) plants at each time point.

Disease severity was evaluated as the percentage of leaf tissue affected at a single time point 6 weeks after inoculation. Leaves were collected from the 6th and 7th internodes from five randomly chosen inoculated tillers per treatment replicate, brought back to the laboratory, and scanned using an Epson GT-1500 scanner. The percentage of discolored (necrotic, chlorotic, or red) tissue versus healthy green tissue was evaluated for each leaf by using the software Quant (Vale et al. 2003).

Inoculated tillers were harvested by cutting them at the crown at physiological maturity, 45 days after flowering. Five inoculated tillers were randomly selected from each treatment replicate for yield measurements. In 2014, seed heads were removed from the tillers, but leaves were retained, while in 2015, both leaves and seed heads were removed prior to analysis. The tillers were weighed, and then pressed at 12.4 MPa of pressure in a grooved, two-roller mill (manufacturer unknown) powered by a 1.5-HP electric motor. The more than 100-year-old mill was originally horse-driven, and later converted to a PTO drive. The juice was collected, weighed, and the Brix was measured immediately with a handheld refractometer (ATAGO, Tokyo, Japan). Mature seed heads were collected from the stems and pooled for each replication of the head-intact treatments, and the grain was threshed and weighed.

Greenhouse experiments. The ability of *Colletotrichum* isolates to cause disease on Sugar Drip was evaluated in the UK Department of Plant Pathology greenhouse in Lexington, KY. Four sorghum seeds were sown per 20.32 cm pot, in a 3:2 mixture of Pro-Mix BX (Premiere Horticulture, Ltd, Riviere du Loup, PQ, Canada) and sterile topsoil. Experimental treatments included five *Colletotrichum* isolates (SS1.001; GS1.001; JG9.001; JG18.001; M30.001) and a water control. Pots were arranged on the greenhouse benches in a randomized complete block design with four blocks. Each treatment replicate included three pots containing a total of 12 plants. Plants were grown to the V5 or V6 stage, and then inoculated by placing 15 colonized sorghum grains into the whorl of each plant. Mock-inoculated grains were used for the water control treatment. Disease incidence was evaluated weekly for a period of 4 weeks by using the same method already described for the field experiments. Disease severity was not measured. The experiment was repeated twice.

Laboratory experiments. *Seedling assay.* Sugar Drip sorghum seeds were sown in 72-cell flats (each cell was 1.27 cm L × 3.81 cm W and 5.72 cm deep) containing Pro-Mix BX (Premiere Horticulture, Ltd, Riviere du Loup, PQ, Canada) in the greenhouse. After 14 days, seedlings were brought to the laboratory and inoculated by pipetting 10 μ l of a spore suspension (10^7 spores per ml), or of sterile water as a control, into the whorl. Treatments included six *Colletotrichum* isolates (CgS11; SS1.001; GS1.001; JG9.001; JG18.001; M30.001) and the water control. The treated seedlings were arranged in a completely randomized design that included 11 replicates of each treatment. The inoculated seedlings were maintained at 23°C under continuous light in closed clear plastic containers lined with moistened germination paper to maintain high levels of humidity (~100%) for 10 days. Disease evaluation was performed by using a dissecting stereoscope. Disease incidence was recorded as the number of replicates within each treatment that developed acervuli and pathogen sporulation. The experiment was repeated twice.

Detached leaf sheath assay. The detached sheath assay was based on the protocol described by Kankanala et al. (2007), as modified by Torres et al. (2014). Sorghum seedlings grown in the greenhouse were cut at the soil line at the V5 stage and brought back to the laboratory. Leaf sheaths of the youngest fully expanded leaves were removed and cut into 7-cm-long segments. A pipette was used to fill the detached segments with 150 μ l of fungal spore suspension (5×10^5 spores per ml), or the same volume of sterile water as a control. The filled sheaths were supported, with the midrib at the bottom, inside a Petri dish containing moistened Whatman paper (Whatman No. 1, Whatman, Hillsboro, OR) to maintain high humidity, and incubated at 23°C under continuous light for up to 108 h. After incubation, the leaf blades were removed from each sheath segment, leaving only the midrib section. This was inverted on a wet microscope slide, and a single-edged razor blade was used to remove excess tissue from the abaxial surface until only a thin layer of mostly epidermal cells remained that were then observed under the light microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY).

To optimize the leaf sheath method for determination of disease incidence, colonization of Sugar Drip leaf sheaths by *C. sublineola* strain CgS11 was characterized at 0, 12, 24, 36, 48, 60, 72, and 108 h post inoculation (hpi). Fifty infection sites (consisting of

appressoria and associated structures, if any) on each of 10 leaf sheaths were observed, and the developmental stage of the infection for each was recorded. The experiment was repeated three times.

Once the method had been optimized, the aggressiveness of six *Colletotrichum* strains (SS1.001; GS1.001; JG9.001; JG18.001; CgSI1; M30.001) was compared on Sugar Drip leaf sheaths. The Petri dishes containing the treated sheaths were arranged in a completely randomized design with 20 replicates of each treatment. A total of 50 fungal infection sites on each of the 20 replicate leaf sheaths for each treatment were observed at 60 hpi (a total of 1,000 infection sites per strain), and the number in which more than one host cell had been invaded was recorded. The experiment was repeated twice.

Table 2. Mean daily temperature and relative humidity, and total rainfall data for the 30 days before and after inoculation of Sugar Drip sweet sorghum with *Colletotrichum* spp. in 2014 and 2015

Parameter	Year	30 days before inoculation	30 days after inoculation
T (°C) ^x	2014	23.5	21.9
	2015	23.9	21.1
Humidity (%) ^y	2014	71.0	76.7
	2015	77.5	69.2
Precipitation (mm) ^z	2014	81.3	168.7
	2015	246.6	52.8

^x Mean daily temperature for the 30 day period.

^y Mean daily relative humidity for the 30 day period.

^z Total rainfall amount for the 30 day period.

Statistical analysis. Data for the field experiment were analyzed as a 2 by 6 factorial arranged in a randomized complete block design by a two-way analysis of variance (ANOVA), performed with PROC GLM (versions 9.3 or 9.4, SAS Institute, Inc., Cary, NC). We tested the effect of removing the seed head of the plants, and we evaluated the relationships between plant yield and Brix and the aggressiveness of the fungal strains expressed as area under the disease progress curve (AUDPC). Disease incidence values from the field and greenhouse studies were used to calculate the AUDPC by the trapezoidal integration method (Campbell and Madden 1990). Multiple mean comparisons among the different treatments were performed by using *t* tests with the least squares estimates of marginal means (LSMEANS statement). *P*-values of less than 0.01 were considered significant. For the analyses of grain yield, disease incidence and severity at single time points, and seedling and detached leaf laboratory assays, treatment means were compared by using the Tukey's multiple comparison test procedure of the SAS software package. In these cases, *P*-values of less than 0.05 were considered significant.

Results

Field experiments. Temperatures were similar in both years of the study, but the amount of rainfall and the relative humidity during the 30 days before versus after inoculation varied. Greater rainfall occurred in 2015 than 2014 prior to inoculation, and the mean relative humidity was greater. However, after inoculation the opposite was true, with 2015 being much drier than 2014 (Table 2). Prior to inoculation in 2015, many of the plants became damaged and showed signs of nutritional stress, possibly due to the saturated soil, and to the violent rainstorms or the prolonged presence of excess water in

Fig. 1. A, Examples of plant damage, apparently due to excess rain, observed prior to inoculation with *Colletotrichum* spp. in 2015. B-E, Representative anthracnose disease symptoms resulting from inoculation of sweet sorghum variety Sugar Drip with four *Colletotrichum sublineola* isolates, and with a *C. graminicola* nonpathogen control and a mock-inoculated water control, in the B, field experiment, V5-V6 tillers inoculated with colonized sorghum grains applied to the whorls; C, greenhouse experiment, V5-V6 plants inoculated with colonized sorghum grains applied to the whorls; D, whole seedling inoculation, 14-day-old seedlings inoculated by application of spore suspensions to the whorl (scale bar = 0.6 cm); E, detached leaf sheath assay, detached leaf sheaths from V5 plants inoculated by filling them with spore suspensions (scale bar = 50 μ m).

the whorl (Fig. 1A). These plants recovered and appeared to develop normally later in the season.

In both years, small, elliptical, reddish spots were visible within 2 days post inoculation (dpi) on the midrib and on the leaf blades of inoculated plants. These increased in number and size, eventually coalescing to cover larger portions of the leaves (Fig. 1B). Signs (acervuli containing conidia and setae) were produced in the necrotic centers of the lesions within 5 dpi. The pathogen spread from the inoculated tillers, resulting in the development of symptoms and signs on the leaves of neighboring, uninoculated tillers by the end of the experiment in the most heavily affected treatments. The water and the nonpathogen negative control treatments also developed

reddening, particularly along the midvein (Fig. 1B), but these reddened areas did not expand or spread beyond the inoculated leaves, nor progress to necrosis, and signs of the pathogen were not observed in these control treatments. This indicated that there was no natural infection, and no interplot pathogen movement.

Brix values were 17.5% greater for the de-headed treatments versus the head-intact treatments in 2014, and 7.9% greater in 2015 ($P < 0.0001$) (Table 3, Supplementary Table S1). The year-by-treatment interaction was not significant ($P = 0.1783$). Removal of the seed heads did not significantly affect the yield of fresh biomass ($P = 0.4618$) or juice ($P = 0.7136$) in either year. Overall yield of both biomass and juice were reduced by more than 70% in 2015 in comparison with the year before ($P < 0.0001$), while Brix increased by 21% ($P < 0.0001$).

De-heading did not have a significant effect on final disease incidence or severity levels ($P > 0.05$) or on AUDPC ($P > 0.01$), for any treatment in either year (Table 4, Supplementary Tables S2, S3, and S4). In 2014, inoculation with any of the four *C. sublineola* strains resulted in greater incidences of disease than the water or nonpathogen negative controls (P -values ≤ 0.0003) (Table 4). Disease incidence reached as high as 99% of the tillers inoculated with the sweet sorghum strain SS1.001 in 2014. The two isolates from *S. bicolor* (SS1.001 and GS1.001) caused significantly more disease than the two isolates from Johnsongrass (JG9.001 and JG18.001) ($P < 0.0001$) (Table 4). Overall disease levels in 2015 were lower than in the previous year. The maximal incidence of 81% was achieved by SS1.001. The two *S. bicolor* isolates resulted in significantly greater disease incidences than the water and nonpathogen controls

Table 3. Effect of de-heading Sugar Drip sweet sorghum on Brix, weight, and juice content^x

Year	Treatment	Brix, degrees ^{y,z}	Fresh biomass, kg ^z	Juice content, kg ^z
2014	Intact head	12.0 a	4.7 a	2.4 a
	De-head	14.1 b	5.0 a	2.5 a
P-value		<0.0001	0.2788	0.0690
2015	Intact head	15.2 c	1.2 b	0.7 b
	De-head	16.4 d	1.1 b	0.6 b
P-value		<0.0001	0.5700	0.4254

^x Averages within each column followed by the same letter are not significantly different according to least squares means (significant at $P < 0.01$).

^y One (1) degree Brix corresponds to 1 kg of solute in 100 kg of solution.

^z Average of five inoculated tillers per treatment replicate.

Table 4. Effect of de-heading inoculated Sugar Drip sweet sorghum on final anthracnose disease incidence and severity levels, and on the area under the disease progress curve (AUDPC)^x

Treatments	2014			2015		
	Incidence ^y	AUDPC ^z	Severity	Incidence ^y	AUDPC ^z	Severity
GS1.001 intact head	15.0 b	48.5 b	8.2 bc	9.2 b	25.0 b	7.9 abcd
GS1.001 de-head	14.8 b	51.8 b	9.5 b	7.9 b	20.0 b	11.4 abc
SS1.001 intact head	18.3 ab	66.8 a	12.4 ab	16.1 a	59.3 a	16.8 a
SS1.001 de-head	19.8 a	72.8 a	16.7 a	15.5 a	59.5 a	12.8 ab
JG18.001 intact head	6.0 d	33.0 c	2.8 d	1.1 c	5.8 cd	3.1 bcd
JG18.001 de-head	7.0 cd	27.8 cd	3.9 cd	1.4 c	7.4 c	2.6 cd
JG9.001 intact head	10.5 c	19.0 d	3.2 cd	2.4 c	1.6 cd	4.7 bcd
JG9.001 de-head	8.3 cd	20.5 d	2.4 d	2.1 c	5.7 cd	5.8 bcd
M30.001 intact head	0.0 e	0.0 e	0.8 d	0.0 c	0.0 d	2.0 cd
M30.001 de-head	0.0 e	0.0 e	2.2 d	0.0 c	0.0 d	2.0 cd
Water intact head	0.0 e	0.0 e	1.1 d	0.0 c	0.0 d	2.9 cd
Water de-head	0.0 e	0.0 e	1.6 d	0.0 c	0.0 d	1.2 d
ANOVA treatment P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

^x Averages within each column followed by the same letter are not significantly different according to least squares means (significant at $P < 0.01$) for the AUDPC data, and according to the Tukey's multiple means comparison test for the incidence and severity data (significant at $P < 0.05$).

^y Incidence = average number of infected tillers out of 20 at the last evaluation time point.

^z Incidence data for all time points was used to calculate the AUDPC.

Table 5. Effect of inoculation of Sugar Drip sweet sorghum with different *Colletotrichum* spp. on weight of biomass and grain, and on juice content

Treatments	2014			2015		
	Biomass ^w	Juice ^w	Grain ^x	Biomass ^w	Juice ^w	Grain ^x
GS1.001	4.9 a ^y	2.3 a ^y	146.4 ba ^z	1.1 a ^y	0.6 a ^y	58.1 a ^z
JG18.001	4.8 a	2.5 a	113.3 ba	1.2 a	0.7 a	55.9 a
JG9.001	4.6 a	2.5 a	174.1 a	1.1 a	0.6 a	83.5 a
M30.001	4.7 a	2.6 a	148.1 ba	1.0 a	0.6 a	109.1 a
SS1.001	4.9 a	2.5 a	79.2 b	1.1 a	0.6 a	52.7 a
Water	5.4 a	2.4 a	134.8 ba	1.2 a	0.7 a	62.0 a
ANOVA Treatment P-value	0.2779	0.6698	0.0426	0.1738	0.4708	0.1388

^w Average weight in kg from five inoculated tillers.

^x Average grams of seed from five seed heads.

^y Averages in this column followed by the same letter are not significantly different according to Least Squares Means (significant at $P < 0.01$).

^z Averages in this column followed by the same letter are not significantly different according to Tukey's multiple comparison test procedure (significant at $P < 0.05$).

($P < 0.0001$), whereas the two Johnsongrass isolates did not differ from the controls in 2015 ($P > 0.01$) (Table 4). Symptoms induced by the Johnsongrass strains did not differ from those observed on the negative controls (water and nonpathogen) in either year ($P > 0.05$) (Table 4). In contrast, both isolates from *S. bicolor* produced disease symptoms that were significantly more severe than the water and nonpathogen controls in both years ($P < 0.05$) (Table 4). Disease severity did not exceed a mean of 16.7% of the leaf area damaged on the inoculated tillers in either year (Table 4). The sweet sorghum isolate SS1.001 caused the most severe foliar symptoms in both years.

Since de-heading had no significant effect on yield of juice and fresh biomass, the intact and de-headed treatments were pooled for analysis of the effect of inoculation with different fungal strains on these parameters. Yield did not differ from the water and nonpathogen negative controls for any of the pathogen treatments, for any level of disease that was achieved during the 2-year study ($P > 0.01$) (Table 5, Supplementary Table S5). Grain yield was also unaffected by inoculation with the disease treatments ($P > 0.05$) (Table 5). Disease did not affect Brix in either intact or de-headed plants in 2015 ($P > 0.01$). In 2014, the intact-head water controls had a reduction of 17% Brix compared with the other treatments (P -values ≤ 0.0048) (Table 6, Supplementary Table S6).

Greenhouse experiments. Symptoms in the greenhouse began to appear within 2 dpi as red spots on the leaf blade and midrib of treated plants. Within 5 dpi, necrotic lesions containing *Colletotrichum*

fruiting structures were visible. Symptoms produced in the greenhouse were similar to those in the field, although they were typically much milder (Fig. 1C). The mean AUDPC during the first greenhouse trial (which occurred in the late summer) was approximately 1/10 that observed in the second trial (which took place in the winter) (Table 7). In the first trial, only the isolate SS1.001 from *S. bicolor* produced statistically greater levels of disease than the water and nonpathogen control treatments ($P < 0.0001$) (Table 7, Supplementary Table S7). In the second trial, both of the *S. bicolor* strains (SS1.001 and GS1.001) were equally aggressive ($P = 0.382$), and both produced more disease than the controls ($P < 0.0001$) (Table 7). The strains from Johnsongrass did not differ statistically from the water or nonpathogen controls in either trial ($P > 0.01$) (Table 7). The combined results of the greenhouse trials confirmed that the two isolates from *S. bicolor* were more aggressive on Sugar Drip than the two isolates from *S. halepense* (Table 7).

Laboratory experiments. *Seedling assay.* All three isolates from *S. bicolor* (CgS11, SS1.001, and GS1.001) produced dark, sporulating lesions on the seedlings within 10 dpi (Fig. 1D). In the first replication of the study, these three isolates could be statistically separated based on incidence, with CgS11 being the most aggressive of the three, and GS1.001 the least ($P < 0.0001$). In the second replication, the isolates were not statistically different from each other ($P > 0.05$) (Fig. 1C, Table 7). The isolates from Johnsongrass caused only relatively mild reddening, similar to the nonpathogen control, and they did not sporulate on the seedlings in either trial (Fig. 1C, Table 7).

Detached leaf sheath assay. At 0 hpi, only ungerminated spores were visible on the surface (Fig. 2A). The pathogen had formed appressoria by 12 hpi (Fig. 2B), and small primary hyphae could be observed within the host epidermal cells by 24 to 48 dpi (Fig. 2C and D). By 60 hpi, the hyphae in a compatible interaction had progressed to colonize adjacent cells (Fig. 2E). Colonization continued to expand into additional cells by 72 hpi (Fig. 2F), and narrower secondary hyphae had begun to develop as branches from the hyphae behind the colonization front (data not presented). By 108 hpi, tissue collapse was evident, and sporulation had occurred (Fig. 2G).

Based on observations of hundreds of individual inoculation sites, it was determined that whenever the pathogen was able to colonize more than one plant cell within 60 hpi, it almost always progressed to sporulation. On the other hand, if the pathogen had not colonized beyond one cell in that period of time, its growth was arrested and it almost always failed to complete its life cycle even up to 6 days after inoculation. Thus, an infection was successful if the pathogen progressed beyond one plant cell within 60 hpi.

This sheath assay was used to compare the ability of six *Colletotrichum* isolates to penetrate and colonize Sugar Drip. The isolates were statistically different from one another ($P < 0.0001$). The laboratory standard grain sorghum isolate CgS11 was the most aggressive

Table 6. Effect of inoculation of Sugar Drip sweet sorghum with different *Colletotrichum* spp. on Brix

Treatments	BRIX (degrees) ^y	
	2014 ^z	2015 ^z
GS1.001 de-head	14.4 a	16.3 abcd
JG18.001 de-head	13.6 ab	16.4 abc
JG9.001 de-head	14.3 a	16.8 ab
M30.001 de-head	14.5 a	16.1 abcd
SS1.001 de-head	14.6 a	17.1 a
Water de-head	13.6 ab	15.9 abcd
JG18.001 intact head	12.2 c	15.1 d
GS1.001 intact head	12.6 bc	15.1 d
JG9.001 intact head	12.0 c	15.7 bcd
M30.001 intact head	12.5 bc	15.1 d
SS1.001 intact head	12.3 c	15.1 d
Water intact head	10.5 d	15.3 cd
ANOVA treatment <i>P</i> -value	<0.0001	0.0166

^y One (1) degree Brix corresponds to 1 kg of solute in 100 kg of solution.

^z Average of five inoculated tillers per treatment replicate. Averages in each column followed by the same letter are not significantly different according to least squares means (significant at $P < 0.01$).

Table 7. Incidence of foliar anthracnose resulting from inoculation of Sugar Drip with *Colletotrichum* strains in field, greenhouse, and laboratory assays

Treatments	Field		Greenhouse		Lab/seedlings	
	AUDPC ^{y,w}		AUDPC ^{y,w}		Incidence ^y	
	2014	2015	1	2	1	2
CgS11	NA ^x	NA	NA	NA	2.7 a ^y	6.5 a ^y
GS1.001	50.1 b ^z	22.5 b ^z	1.3 b ^z	23.3 a ^z	1.0 c	6.0 a
SS1.001	69.8 a	59.4 a	3.7 a	22.0 a	1.7 b	6.5 a
JG18.001	30.4 c	6.6 c	0.0 b	0.3 b	0.0 d	0.0 b
JG9.001	19.8 d	3.6 cd	0.0 b	0.0 b	0.0 d	0.0 b
M30.001	0.0 e	0.0 d	0.0 b	0.0 b	0.0 d	0.0 b
Water	0.0 e	0.0 d	0.0 b	0.0 b	0.0 d	0.0 b
ANOVA Treatment <i>P</i> -value	<0.0001	<0.0001	0.0004	<0.0001	<0.0001	<0.0001

^y Incidence = number of infected V5-V6 tillers out of 20 in field studies (pooled for intact and de-head treatments); number of infected V5-V6 plants out of 12 in the greenhouse; number of sporulating 14-day-old seedlings out of 11 in the lab.

^w Least squares means for area under the disease progress curve (AUDPC) for the greenhouse and field assays.

^x NA = this strain was not included in these experiments.

^y Averages within this column followed by the same letter are not significantly different according to Tukey's multiple comparison test procedure (significant at $P < 0.05$).

^z Averages within this column followed by the same letter are not significantly different according to least squares means (*t* test significant at $P < 0.01$).

(Fig. 1E and 3). SS1.001 and GS1.001 did not differ from one another, but both were significantly more aggressive than the Johnsongrass isolates, which were not different from the nonpathogen control (the maize isolate M30.001) (Fig. 1E and 3). Thus the sheath assay also confirmed that the isolates from *S. bicolor* were more aggressive on Sugar Drip than the isolates from *S. halepense*.

Discussion

Foliar anthracnose, which causes significant losses of grain sorghum worldwide, is relatively common on cultivated and wild sorghum throughout the southeastern U.S. In KY, anthracnose is managed in sweet sorghum grown for syrup primarily by the use

of resistant varieties. However, sorghum cultivation in the state is currently very limited, and there is the potential that the disease could have a more significant impact, and that the durability of current sources of resistance would be reduced, if the crop was more widely grown for biofuels. Although red rot and lodging have resulted in total losses of sweet sorghum crops (Bitzer 1997), there is little information available about the damage potential of foliar anthracnose on sweet sorghum yield or Brix in the southeastern U.S. The wild sorghum relative Johnsongrass is ubiquitous in the region, but the potential impact of *C. sublineola* isolates from Johnsongrass on cultivated sweet sorghum is unknown.

Fig. 2. Representative micrographs illustrating the infection process during the interaction between *Colletotrichum sublineola* strain CgSI1 and sweet sorghum variety Sugar Drip leaf sheaths. Leaf sheaths were removed from V5 plants and filled with spore suspensions, then incubated in moist chambers for 0 to 108 hours post inoculation (hpi). **A**, an ungerminated falcate spore (arrow) on the plant surface at T = 0; **B**, appressoria (arrows) produced from germinated falcate spores at T = 12 hpi; **C**, small infection hypha (arrow) visible at T = 36 hpi; **D**, larger infection hypha (arrow), still restricted to one cell, at T = 48 hpi; **E**, infection hyphae colonizing at least two cells at T = 60 hpi; **F**, infection hyphae colonizing three or more cells at T = 72 hpi; and **G**, formation of acervuli with setae at T = 108 hpi.

Sugar Drip is an heirloom variety of unknown origin that was commonly grown in Kentucky up through the 1980s. It is well adapted to the state and produces high quality syrup (Bitzer 1997). However, it is susceptible to several sorghum diseases, including anthracnose, and for this reason it has been largely displaced by more resistant varieties, although it remains popular among specialty growers. Sugar Drip was chosen for this study in order to investigate the impact of foliar anthracnose on yield. Transplants were used to control for variation in microclimate caused by uneven plant stands, but this resulted in the plants flowering early in the field during both years of the study. The early-flowering stems did not resemble normal growth, producing relatively few photosynthetic leaves on shortened internodes. Therefore, the main stem was cut back to promote the maturation of tillers. Inoculations were done on individual tillers that were similar in age (between V5 and V6), which then developed normally throughout the season, flowering at the same time as other conventionally grown Sugar Drip plants in the area.

Incidence of foliar anthracnose on inoculated tillers of up to 99%, and severities of up to 16.7% of the leaf area affected, were achieved on Sugar Drip in the field experiments, and secondary spread of the disease to neighboring uninfected tillers in the most heavily affected plots occurred frequently. However, these disease levels had no negative impact on the yield of biomass, grain, juice, or Brix. No red rot or lodging was observed, suggesting that the pathogen did not significantly colonize the stalk from the foliage when it was inoculated at V5 to V6. Given that the levels of foliar anthracnose disease in the experimental fields were much greater than observed at the collection sites in Fayette, Clay, and Russell counties, it was concluded that, at present, the economic impact of this disease on yield in Kentucky is probably minimal.

Removal of seed heads has been proposed as a way to increase yield of sugar for syrup (Bitzer 1997). Maize anthracnose has been reported to be negatively affected by increases in stalk sugars (Dodd 1980b; Mortimore and Ward 1964), and so it was determined whether removal of seed heads would also increase resistance of sweet sorghum to anthracnose. In the current study, removal of seed heads increased Brix by as much as 17.5%, but this had no significant effect on the levels of foliar anthracnose. Two tillers were inoculated per plant. Most of the plants eventually produced up to 10 additional tillers, and these may have compensated for the tissue loss by remobilizing photosynthate to the diseased tillers (Slewisinski 2012). However, Brix levels were in line with expectations for this variety, and similar to plants that were grown traditionally in nearby fields in

the same year, and the levels did not vary relative to disease levels, suggesting that any impact of mobilization of carbon was probably minimal.

The amount of anthracnose disease (incidence and severity) varied significantly between years. This was probably due to environmental factors, especially rainfall and humidity, which are strongly associated with anthracnose disease severity in grain sorghum (Chala et al. 2010b; Frederiksen 1983; Ngugi et al. 2002). In 2014, rainfall levels were unusually high post inoculation. The increased levels of leaf moisture during and subsequent to inoculation probably favored the development of the pathogen in 2014, leading to more disease. The two Johnsongrass isolates (JG9.001 and JG18.001) did not differ from controls in 2015, but they did in 2014. This suggests that increased leaf wetness may be an especially important determinant of infectivity for less aggressive isolates. Similar conclusions have been made for anthracnose on grain sorghum (Chala et al. 2010a; Prom et al. 2015). Despite greater levels of disease, yield of biomass, grain, and juice were greater in 2014 than in 2015. The yield reductions may have been related to the fact that plants were noticeably damaged and stunted early in the season in 2015, possibly as a result of the unusually high levels of rainfall and flooding that occurred just after transplanting.

Control plots (mock-inoculated or inoculated with the nonpathogenic maize *Colletotrichum* isolate) were disease-free, indicating that there was little or no natural inoculum in plots. This is in spite of the fact that Johnsongrass was common in the grassy areas surrounding the fields, and that *C. sublineola* could be readily recovered from these plants. To our knowledge, this is the first study to use Johnsongrass isolates to inoculate sorghum in the field. The results suggest that crop rotation could be an effective way to manage anthracnose in sweet sorghum, even though infected Johnsongrass is common in the region. Although significantly less aggressive, both Johnsongrass isolates were nonetheless able to infect the inoculated Sugar Drip plants in the field and complete their life cycle. Thus, if inoculum loads are high and levels of resistance are low, Johnsongrass could serve as a reservoir for the pathogen during crop rotation schemes, and as a source for new races.

Field studies are limited by time and resources, and it is possible to test only relatively small numbers of local isolates. The performance of three alternative greenhouse and laboratory assays that would facilitate more rapid evaluation of larger numbers of strains, and exotic strains that require containment, was evaluated. All three of the assays confirmed that the two Johnsongrass isolates were significantly

Fig. 3. Leaf sheath assay of six strains of *Colletotrichum* spp. on Sugar Drip. Disease incidence was measured as the number of infection sites in which the pathogen was able to colonize at least two plant cells at 60 h after inoculation. This figure shows the averaged data of two trials. Columns labeled with different letters were statistically different ($P < 0.05$).

less aggressive to Sugar Drip than the two isolates from cultivated sorghum. In the greenhouse and laboratory assays, however, the two Johnsongrass isolates appeared to be nonpathogenic, while the field assays demonstrated that these isolates were capable of completing their life cycles on Sugar Drip sweet sorghum.

There were greater levels of disease in the field in both years than in the greenhouse. Similar results were observed by Ferreira and Warren (1982) when evaluating 23 grain-sorghum cultivars for resistance to *C. sublineola* in the greenhouse versus field (Ferreira and Warren 1982). Plants in the field are exposed to multiple stresses including insects, wounds that facilitate pathogen entry, abiotic stressors (wind, high temperature, drought or flooding, hail, etc.), and biotic disease (bacteria and other fungi). Stress is well-known to predispose grain sorghum to anthracnose (Dodd 1980a). Plants in the greenhouse are maintained at optimal conditions of temperature and moisture and protected from most other biotic and abiotic stressors. In the current study, disease incidence was greater in the second trial of the greenhouse experiment than in the first. The second trial took place in the midwinter months, whereas the first was done in the late summer, and thus light intensity may have played a role in this difference. Increased light intensities result in reduced anthracnose disease severity in maize, due to an increase in the ability of the plant to express resistance (Hammerschmidt and Nicholson 1977; Negeri et al. 2013; Schall et al. 1980). In the current study, supplemental lighting was provided in the greenhouse, but it did not match natural summer light intensities.

The sheath assay provides a uniquely detailed view of the host-pathogen interaction in living tissues. Snyder et al. (1991) described the induction of red vesicles, by the nonpathogen *C. graminicola* at the infection site (Snyder et al. 1991). Wharton and Julian (1996) described how leaf sheaths from resistant plants responded to infection by *C. sublineola* by accumulation of red vesicles, which eventually burst and released their contents, filling the colonized cells and often also coating the fungal hyphae. In contrast, in interactions with leaf sheaths from susceptible plants, the accumulation of pigmented vesicles was relatively slow, and the pigmentation was more orange than red (Wharton and Julian 1996). Snyder et al. (1991) demonstrated that the dark red vesicles formed in incompatible interactions contained 3-deoxyanthocyanidin phytoalexins, which have been strongly implicated in the resistance of sorghum to *C. sublineola* (Ibraheem et al. 2010; Lo et al. 1999; Nicholson et al. 1988). Observations in sweet sorghum leaf sheaths in the current study (Fig. 1D) were consistent with previous observations of grain sorghum. A rapid and intense production of vesicles containing a bright red pigment was observed when leaf sheaths from Sugar Drip were inoculated with the nonpathogen, *C. graminicola*. None of the *C. sublineola* strains produced as intense a reaction in Sugar Drip sheaths. Strains SS1.001 and GS1.001 (both virulent) induced the production of a diffuse reddish pigment throughout the colonized cells, whereas inoculation with CgS11 (virulent) or JG9.001 (avirulent) did not result in any change in color. Strain JG18.001 (avirulent) caused only a slight accumulation of red pigments surrounding the infection hyphae. Production of these more diffuse reddish compounds was not correlated with resistance, and it is possible that they are not related to the 3-deoxyanthocyanidins previously described. Biochemical assays will need to be performed to address the question of their identity and significance. The sheath assays are well-suited for this kind of detailed analysis of the host-pathogen interaction.

As sweet sorghum is increasingly being explored as a potential bioenergy feedstock crop for the southeastern U.S., there is an urgent need for more information about the threat posed by *C. sublineola* populations in the region. Field studies demonstrated that *C. sublineola* has a significant potential to cause foliar anthracnose disease on sweet sorghum in KY when conditions are favorable (i.e., with a combination of a susceptible host and favorable environmental conditions). Two Johnsongrass isolates of *C. sublineola* were much less aggressive than two isolates from *S. bicolor*, suggesting that crop rotation could be an effective disease management tool even when Johnsongrass is present. However, both Johnsongrass strains were still able to complete their life cycles on sweet sorghum plants when

inoculum loads were high, implying that Johnsongrass could serve as a reservoir for the pathogen during crop rotation schemes, and could also be a source of new races. This is expected to complicate disease management, and efforts to develop new, durable resistant varieties of sweet sorghum for the region. Greenhouse and laboratory assays will be useful for prescreening sorghum germplasm for new sources of resistance, but should not be expected to provide an accurate prediction of damage potential, since plants in the field were more severely impacted when compared with these controlled studies.

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