Genetic Diversity, Fungicide Sensitivity, and Host Resistance to *Ceratocystis fimbriata* Infecting Sweetpotato in North Carolina

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Abstract

Black rot of sweetpotato, caused by *Ceratocystis fimbriata*, has recently reemerged as a significant threat to sweetpotato production in North Carolina and other states across the United States. This disease has historically been controlled largely through cultural management strategies and, in some cases, fungicide application. The sudden and destructive reemergence of this disease in 2015 created the need for rapidly evaluating disease control strategies. Genetic diversity of current *C. fimbriata* isolates infecting sweetpotato in North Carolina was assessed using ITS, TEF, and MAT-2 sequences. All 50 tested isolates were confirmed to be of a single mating type, MAT-2, based on PCR amplification. Alignment of ITS, TEF, and MAT-2 sequences revealed all isolates were identical at each locus. Fourteen common sweetpotato cultivars and advanced breeding lines were screened for black rot resistance using two isolates.

None of the cultivars were completely resistant to the disease and most were equally susceptible. ‘Stokes Purple’ and ‘Covington’ were the least susceptible, but significantly ($P < 0.05$) differed only from ‘Bellevue’, the most susceptible cultivar. Sensitivity of 50 *C. fimbriata* isolates to difenoconazole, fludioxonil, thiabendazole, dicloran, azoxystrobin, pyraclostrobin, fenamidone, and fluazinam was evaluated in vitro. Difenoconazole, thiabendazole, and fluazinam were most effective in reducing mycelia growth. Postharvest fungicide application on black rot-infected roots provided similar results. Low efficacy of dicloran, as well as a range of EC₅₀ values among isolates, suggests potential resistance to this commonly applied fungicide. Results obtained in this study provide current and useful information so that improved recommendations can be made to reduce losses in sweetpotato to black rot.

Over 106 million metric tons of sweetpotatoes were produced globally in 2013, making it the seventh most important food crop in the world (FAOSTAT 2014). The development and popularity of value-added products such as sweetpotato fries and chips, as well as increased health awareness, has led to an increase in sweetpotato consumption in the United States (Johnson et al. 2015). North Carolina is the leading producer of sweetpotatoes in the United States and is responsible for about 50% of the nation’s production, which is valued at over \$260 million in the state (NCDA&CS 2015).

Sweetpotatoes are subject to numerous diseases and disorders throughout the production season and time in storage (Clark et al. 2013; Scruggs and Quesada-Ocampo 2016a, 2016b). Black rot of sweetpotato, caused by *Ceratocystis fimbriata*, resulted in significant losses in the United States in the early 1900s (Harter and Weimer 1929) and in China to date (Hu et al. 1998). *Ceratocystis fimbriata* is capable of infecting sweetpotato plants either in the plant beds or after transplanting, resulting in black, sunken cankers on sprouts or the developing roots, leading to stunting, wilting, chlorosis, and eventually plant death. The black rot pathogen can also infect sweetpotato storage roots. Symptoms from postharvest infection include a black, firm lesion developing on the root surface (Fig. 1A), and the pathogen often produces a sweet, fruity odor on infected tissue (Clark et al. 2013). Lesion development is usually slow and typically restricted by the vascular ring (Fig. 1B). Often in the center of the lesion, dark mycelia and perithecia of *C. fimbriata* are seen protruding from the root surface. The perithecia of *C. fimbriata* produced on infected roots exude a droplet of sticky ascospores that aid in dissemination (Fig. 1C). These propagules easily contaminate harvesting, storage, and packing equipment,

potentially leading to severe postharvest losses. In the field, black rot is transmitted mainly through infected seed material or by planting sweetpotatoes in fields with known histories of *C. fimbriata* infestation (Clark et al. 2013).

Adoption of integrated control practices in the mid-twentieth century led to drastic reductions in the occurrence of black rot in the United States (Clark et al. 2013). Planting of disease-free material and crop rotation are key for control of black rot disease on sweetpotatoes. To reduce severe postharvest losses during storage and transport, roots should be promptly and properly cured to allow for wound healing and toughening of the skin (Edmunds et al. 2008). Additionally, infected roots should be removed before they enter the packing line and all washing and packing equipment should be decontaminated on a daily basis to reduce secondary infections during packing and transport. Implementation of these strategies has resulted in control of black rot in the United States for many years. However, in 2015, the incidence of black rot increased significantly in North Carolina and was observed postharvest in numerous other locations throughout the United States, including Virginia, Georgia, Mississippi, Alabama, Louisiana, Arkansas, Missouri, and California, as well as in Ontario, Canada. The reemergence of black rot in the United States has prompted research to improve control options and determine the cause of the recent epidemic. Therefore, the purpose of the current study was threefold. First, susceptibility and/or resistance of 14 common sweetpotato cultivars and advanced breeding lines in the United States to black rot were examined. Second, a set of *C. fimbriata* isolates were collected from North Carolina and screened in vitro for fungicide sensitivity in an attempt to (i) determine efficacy of currently used fungicides against *C. fimbriata* and (ii) evaluate additional chemistries that could have a potential to be labeled for sweetpotato use in the future. Promising fungicides were then evaluated for black rot control in artificially inoculated sweetpotato roots. Third, multilocus sequences and mating-type data were gathered for 50 isolates to better understand the genetic diversity of *C. fimbriata* isolates infecting sweetpotatoes in North Carolina. Results of this study will aid in developing immediate disease control strategies for U.S. sweetpotato growers currently experiencing losses to black rot. The information presented here also provides foundational information for any future study involving genetic analyses and

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary table is published online.

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monitoring of *C. fimbriata* populations, fungicide resistance, and breeding resistant cultivars.

Materials and Methods

Sampling and isolation of *C. fimbriata*. Sweetpotatoes with typical black rot symptoms (Clark et al. 2013) were sampled from commercial packinghouses in North Carolina. Eighty-three isolates were collected from 10 commercial sweetpotato farms in five counties in eastern North Carolina. Diseased sweetpotatoes were selected regardless of cultivar. Roots were placed in either a plastic crate or bag, transported back to the lab, and stored at 4°C until pathogen isolation.

To isolate *C. fimbriata*, infected roots were rinsed in tap water, surface sterilized by spraying with 80% ethanol until drench, and transferred to a biosafety cabinet to air dry. Following drying, a small triangular section was cut out from the edge of the lesion and placed onto a 100-mm petri dish containing potato dextrose agar (PDA) amended with 20 mg/liter of ampicillin and 20 mg/liter of rifampicin. Plates were incubated at 23°C under continuous light. *Ceratocystis fimbriata* isolates grown on these plates were initially identified based upon cultural and morphological characteristics as described by Engelbrecht and Harrington (2005). Any fungal growth suspected to be *C. fimbriata* based on visual symptoms were then transferred to PDA in a 60-mm petri dish and allowed to grow at 23°C under continuous light. Isolates were then single-spored by adding 100 µl of sterile water onto the surface of an actively growing culture and then spreading the 100-µl droplet onto a PDA plate. After 24 h, a single, germinated spore was selected using a light microscope and transferred to another PDA plate. Isolates were stored as mycelia plugs in a 1.5-ml tube with sterile water and a hemp seed at 4°C for long-term preservation.

Genotyping of *C. fimbriata* isolates. A set of 50 *C. fimbriata* isolates, out of the total 83 isolated, were systematically selected based on diverse geographic location. For DNA extraction of the 50 selected isolates (Supplemental Table S1), a 5-mm mycelia plug from an actively growing culture was transferred to a 250-ml flask containing

50 ml of potato dextrose broth (PDB) and incubated for 7 days under light and gentle shaking on an Advanced Digital Shaker (VWR International, Radnor, PA). Tissue was collected using vacuum filtration and lyophilized in 1.5-ml tubes for 48 h. Tissue was then disrupted by adding three glass beads to each tube and homogenized for 30 s using an Omni Bead Ruptor 24 (Omni International, Kennesaw, GA). DNA extractions were performed using a standard phenol-chloroform extraction protocol (Iowa State University PTF 2003).

Total DNA was used to amplify the internal transcribed spacer (ITS) region with primers ITS1 and ITS4 (White et al. 1990). All PCR reactions were performed in a T100 Thermocycler (Bio-Rad, Hercules, CA) with the following parameters: 94°C for 3 min; 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and 72°C for 5 min. Samples were then held at 4°C. The reaction mix contained 10 µl GoTaq Green Master Mix, 7 µl sterile water, 1 µl of each the forward and reverse primer, and 1 µl of genomic DNA. The translation elongation factor (TEF) region was also amplified for all isolates using primers ef1 and ef2 (Geiser et al. 2004). These PCR reactions were performed with the following parameters: 94°C for 3 min; 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min; and 72°C for 5 min. Samples were then held at 4°C.

PCR products were cleaned up using ExoSAP-IT (Affymetrix, Santa Clara, CA) and sequenced at the Genomic Sciences Laboratory, North Carolina State University, on a LifeTech 3730xl DNA Analyzer (Life Technologies, Carlsbad, CA) using the forward primer that was used to amplify the DNA. Electropherogram files from the LifeTech 3730xl DNA Analyzer were viewed for quality of reaction in FinchTV (Geospiza, Inc., Seattle, WA) and sequence reactions were repeated when necessary. Sequences were compared against the GenBank database using BLAST (Altschul et al. 1990). ITS and TEF sequences for each of the 50 isolates were deposited into GenBank.

Mating-type determination. Total DNA was used to amplify a portion of the high mobility group (HMG) box of the *MAT-2* mating-type gene using primers CFM2-1 and CFM2-2 (Witthuhn et al. 2000) in each of the 50 selected *C. fimbriata* isolates. Reactions were

Fig. 1. Typical symptoms of black rot on sweetpotato caused by *Ceratocystis fimbriata* are shown. Infection of storage roots results in **A**, dry, dark, circular lesions that **B**, do not extend below the vascular tissue inside the root. **C**, Black perithecia protrude from the center of the lesion and give rise to sticky droplets of conidia and ascospores. **D**, Typical cultural morphology of an actively growing 14-day-old *C. fimbriata* isolate is shown.

performed as described above with the following parameters: 94°C for 1 min and 35 s; 35 cycles of 94°C for 35 s, 58°C for 1 min, and 72°C for 1 min; and 72°C for 15 min. Samples were then held at 4°C. PCR products were run on a 1% agarose gel for 45 min and visualized with UV light. Amplified samples were considered mating-type 2 (MAT-2) whereas failure to amplify indicated mating-type 1 (MAT-1) (Witthuhn et al. 2000). PCR products of the MAT-2 samples were cleaned up and sequenced as described above for phylogenetic analysis. MAT-2 sequences for the 50 isolates were deposited into GenBank.

Phylogenetic analysis. Sequences were aligned using ClustalWS (Larkin et al. 2007) in the Jalview 2.9.0 (Waterhouse et al. 2009) software package. Alignments were visualized using Jalview 2.9.0 and the sequence ends were manually trimmed to remove low-quality bases. The ITS, TEF, and MAT-2 datasets were concatenated to produce a total sequence of 1018 bp for each isolate. Haplotypes were identified using the haplotype block workflow in the Moby SNAPP Workbench (Price and Carbone 2005; Monacell and Carbone 2014) with a minimum of a one base-pair difference to determine haplotypes. Gaps were considered a fifth state. No additional analysis was conducted due to lack of variation among the sequenced loci.

Evaluating cultivar susceptibility to black rot. Fourteen sweetpotato cultivars and advanced breeding lines were evaluated for resistance to black rot in the storage roots. Sweetpotatoes used for this study were grown at the Horticultural Crops Research Station in Clinton, NC, in 2014 using standard production practices. Following harvest, roots were properly cured at 29°C and stored long-term at approximately 15°C. Roots were stored for five months before being used for the current experiment. Prior to inoculation, roots were rinsed with water and surfaced sterilized by soaking in 5% bleach for five minutes. Roots were rinsed once more and allowed to air dry in a biosafety cabinet.

Two *C. fimbriata* isolates, AS186 and AS236, were used for cultivar evaluation. These two isolates were selected due to differences in isolation year and location. Eighteen roots of each sweetpotato cultivar were used for each experiment. Among them, six roots were used for control, six roots for inoculation with isolate AS186, and six roots for inoculation with isolate AS236. Roots of each cultivar were first wounded by inserting a sterile 5-mm toothpick and then inoculated by pipetting 20 µl of a 10⁶ spores per ml *C. fimbriata* spore suspension into the wound. After inoculation, roots were placed in pairs into clear, 28 cm × 20 cm × 14 cm plastic containers, each with three sterile paper towels dampened with 15 ml of sterile water to maintain high humidity as previously described (Scruggs and Quesada-Ocampo 2016 a and b). Control roots were wounded and inoculated with 20 µl of sterile water. Roots were stored in the dark at approximately 23°C. Results were recorded by measuring lesion diameter at 4, 7, 11, 14, 18, and 21 days post inoculation (dpi). The experiment, with a completely randomized design (CRD) and six replications, was repeated two additional times. Area under disease progress curve (AUDPC) values were calculated based on lesion-diameter measurements (mm) for each root over a 21-day period using the trapezoidal method (Madden et al. 2007). AUDPC values were log transformed

to meet statistical assumptions and were subject to an analysis of variance (ANOVA). A post-hoc Bonferroni correction was applied to separate treatments.

In vitro fungicide sensitivity assays. Fungicides used in the in vitro experiments were commercial grade, similar to comparable studies by Bolton et al. (2012) and McManus et al. (1999). Active ingredients (a.i.) tested were difenoconazole (Inspire; Syngenta, Basel, Switzerland), fludioxonil (Maxim 4FS; Syngenta), thiabendazole (Mertect 340-F; Syngenta), dicloran (Botran 75-W; Gowan Co., Yuma, Arizona), azoxystrobin (Dynasty; Syngenta), pyraclostrobin (Cabrio EG; BASF, Ludwigshafen, Germany), fenamidone (Reason; Bayer CropScience, France), and fluazinam (Omega 500F; Syngenta). In a preliminary test, 15 *C. fimbriata* isolates were evaluated for in vitro sensitivity to thiabendazole at a.i. concentrations of 0, 0.1, 1.0, 5.0, 10, 50, and 100 ppm. Due to high levels of growth inhibition at 5.0 ppm, concentrations of 50 and 100 ppm were excluded from further study.

Stock solutions of each fungicide were incorporated into potato dextrose agar (PDA) medium to provide active ingredient concentrations of 0, 0.1, 1.0, 5.0, and 10 ppm. Mycelial disks (5-mm) of *C. fimbriata* were removed from the edge of a 14-day-old culture and transferred to the center of a 60-mm petri dish containing fungicide-amended PDA media. A set of 50 current *C. fimbriata* isolates from North Carolina was selected for this study. The experiment was conducted in a randomized complete block design with experiment replication as the blocking factor. Two biological replicates were used per treatment and the experiment was conducted twice, for four total replicates. Percent inhibition of radial growth as compared with the untreated control was calculated for each fungicide and concentration after 12 days of incubation at 23°C under continuous fluorescent light. The percent inhibition values of the two replicated experiments were used for statistical analyses. Data from control plates were removed from the data set prior to statistical analyses because no inhibition was observed. Data residuals were calculated, checked for normality using the Shapiro-Wilk normality test, Q-Q plots, and boxplots, and for equality of variances with Levene's test using R (R Core Team 2013). Once residuals were found to follow statistical assumptions, the data were subjected to Analysis of Variance (ANOVA) using the aov function in R (R Core Team 2013). Effects were considered significant at 0.05 and means separated using Tukey's test.

The concentration that reduced mycelia growth by 50% (EC₅₀) was determined using the EC₅₀ Plus v1.0 online software (Vargas 2000). Extrapolations were not made for EC₅₀ values below 0.1 ppm or above 10.0 ppm.

Postharvest in vivo fungicide sensitivity assays. 'Covington' sweetpotatoes were wounded by scraping a single side of each root with a handheld cheese grater, to produce a scrape-type wound that extended just below the root periderm. After wounding, 70 wounded sweetpotatoes were placed into a 121-liter bin containing 45 liters of water. Roots were inoculated by placing 12 black rot infected roots into the bin of water, gently agitating to ensure uniformity throughout the bin, and letting them soak for 60 min. Black rot-infected roots were selected based upon lesion size and sporulation area from commercial packing facilities. Following inoculation, roots were taken out of the water bin and allowed to air dry. Roots were then laid out on the ground with the wounded side up in seven groups of 40 roots each. Each group was randomly assigned a given treatment (Table 1) that was sprayed over the roots to simulate a fungicide spray along a commercial packing line. Tested rates were selected based upon current labeled recommendations. Roots were sprayed using a CO₂-powered backpack sprayer with a spray pressure 45 psi calibrated to deliver approximately 8.75 ml of fungicide spray per root. After treatment application, roots were placed in groups of 10 into clear, plastic containers (40 by 50 by 17.9 cm) and incubated at 15°C and 95% relative humidity for 21 days. The experiment with a complete randomized design (CRD) was with four replications per treatment was conducted twice. Roots were visually rated for disease severity 21 days after inoculation. Data were fit to a generalized linear mixed model using the lme4 package in R and pairwise comparisons between treatments with Tukey corrections were identified

Table 1. List of fungicides, active ingredients, tested rates, and average percent disease severity recorded 21 days after inoculation during the postharvest evaluation of fungicides for black rot control

Treatment	Active ingredient	Tested rate	% Disease severity ± SE ^z
Untreated Control	-	-	50.6 ± 5.4 a
Botran	Dicloran	1.2 g/liter	45.6 ± 6.1 a
Omega	Fluazinam	4.1 ml/liter	12.5 ± 2.5 b
Inspire	Difenoconazole	3.4 ml/liter	5.0 ± 0.9 c
Scholar	Fludioxonil	2.5 ml/liter	4.4 ± 1.8 c
Mertect	Thiabendazole	8.4 ml/liter	2.5 ± 1.3 cd
Stadium	Azoxystrobin + difenoconazole + fludioxonil	6.1 ml/liter	0.6 ± 0.6 d

^z % Disease severity is reported as mean ± standard error. Means followed by the same letter in a column do not significantly differ ($P = 0.05$) based on Tukey Test.

using the multcomp package in R at $P < 0.05$ significance level (R Core Team 2013).

Results

Genetic diversity and mating-type determination. All 50 tested isolates were verified as mating-type 2 based on PCR amplification. PCR products of the *MAT-2* gene from the 50 isolates determined to be mating-type 2 were sequenced to evaluate genetic diversity at this locus. Alignment of the 50 ITS, TEF, and *MAT-2* sequences resulted in trimmed datasets of 455 bp, 412 bp and 151 bp, respectively. Datasets were concatenated to produce a 1018-bp sequence for each isolate. The concatenated dataset of 50 isolates was collapsed into a single haplotype in the Mobyle SNAP Workbench indicating no variation in the set of isolates at the sequenced loci, thus, no additional phylogenetic analysis was conducted.

In vivo inoculation assays to evaluate cultivars and breeding lines. Postharvest evaluation of 14 common sweetpotato cultivars and advanced breeding lines for susceptibility to black rot using artificial inoculation of *C. fimbriata* revealed no complete resistance among the tested cultivars and breeding lines. All sweetpotato cultivars produced firm, black, dry lesions around the point of inoculation (Fig. 2) and lesions did not extend below the vascular tissue. Characteristic perithecia of *C. fimbriata* were observed on the surface of infected tissue. Although all tested cultivars were susceptible, analysis of AUDPC values calculated over a three-week period revealed some cultivars were less susceptible than others (Fig. 3). Stokes Purple, a purple-fleshed sweetpotato, proved to be the least susceptible of all tested cultivars (Fig. 2A). Covington was second to Stokes Purple and was the least susceptible orange-flesh cultivar (Fig. 2B). While Stokes Purple and Covington were not significantly different than most cultivars at the 95% confidence level, they were significantly different ($P = 0.01$) than the orange-fleshed cultivar Bellevue, which was the most susceptible cultivar in the current study (Fig. 2F). All other tested cultivars were not significantly different from any of the cultivars tested (Fig. 3).

In vitro fungicide sensitivity assay. Mean EC_{50} values calculated for mycelial growth of 50 *C. fimbriata* isolates on fungicide amended media were lowest for difenoconazole (Table 2) and fell below the lowest tested active ingredient concentration. Difenoconazole significantly inhibited mycelial growth more than any other active ingredient at concentrations of 0.1 and 1.0 ppm (Table 2). At higher concentrations of 5.0 and 10.0 ppm of difenoconazole, inhibition was not significantly different than thiabendazole at similar concentrations. Frequency distributions of active ingredients that resulted in EC_{50} values outside of our tested concentrations were not generated to avoid extrapolation of the data.

Thiabendazole resulted in the second-lowest EC_{50} value, and inhibition at 5.0 and 10.0 ppm was highest for this active ingredient. The 50 tested isolates resulted in EC_{50} values ranging from <0.1 to 1.46 ppm, with most isolates falling between 0.1 and 0.4 ppm (Fig. 4A). Mycelial inhibition of fluazinam at 5.0 and 10.0 ppm was significantly higher than that recorded for fludioxonil, dicloran, azoxystrobin, pyraclostrobin, and fenamidone. EC_{50} values for fluazinam ranged from 0.27 to 2.08 ppm with the majority of isolates having an EC_{50} between 0.4 and 1.4 ppm (Fig. 4B). Fludioxonil (Fig. 4C) and dicloran (Fig. 4D) also had mean EC_{50} values within the tested range and inhibition for both ingredients was significantly higher than that of azoxystrobin, pyraclostrobin, and fenamidone at concentrations of 1.0, 5.0, and 10.0 ppm (Table 2). Fludioxonil EC_{50} values ranged from 0.64 to 9.25 ppm with most isolates having an EC_{50} between 0.6 and 3.0 ppm and dicloran EC_{50} values ranged from 2.42 to >10.0 ppm with the majority falling between 3.0 and 8.0 ppm. Eight isolates had EC_{50} values above the highest tested concentration for dicloran.

Postharvest fungicide application. Disease severity of untreated roots was 50.6% (Table 1). Azoxystrobin + difenoconazole + fludioxonil (Stadium) provided the lowest level of disease severity at 0.6% and was significantly different from all other treatments except thiabendazole (Mertect), which had the second lowest level of disease at 2.5%. Fludioxonil (Scholar) and difenoconazole (Inspire) offered the third- and fourth-best reduction in disease severity at 4.4% and 5.0%, respectively. These fungicides did not significantly differ from each other, as

well as from thiabendazole, but did provide significantly better disease protection than fluazinam (Omega) at 12.5% disease and dicloran (Botran) at 45.6% disease. Inoculated roots treated with fluazinam had significantly lower disease levels than those treated with dicloran, which also did not significantly differ from the untreated control (Table 1).

Discussion

The current study identified no genetic diversity among 50 *C. fimbriata* isolates collected from diseased sweetpotato roots in North

Fig. 2. Observed symptoms 21 days postinoculation (dpi) with *Ceratocystis fimbriata* on the select cultivars A, Stokes Purple, B, Covington, C, NC04-531, D, Bayou Belle, E, Beauregard, and F, Bellevue.

Carolina based on ITS, TEF, and *MAT*-2 sequencing. All 50 isolates were confirmed to be mating-type 2 (*MAT*-2) based on PCR amplification. This finding is consistent with previous reports by Webster and Butler (1967), where most field isolates were found to possess the *MAT*-2 gene. Lack of genetic diversity among pathogen isolates may suggest the potential recent introduction of a new pathogen strain as demonstrated in previous studies (Engelbrecht et al. 2004). This hypothesis is consistent with field observations of recent dramatic increases in black rot incidence on sweetpotato in North Carolina and failure of historically effective disease management strategies.

Numerous population-genetic studies have been conducted across the *C. fimbriata* species complex infecting woody hosts (Baker et al. 2003; Thorpe et al. 2005; Harrington et al. 2011), but limited research has been done on isolates infecting sweetpotato. Use of ITS and *MAT*-2 sequences is common for phylogenetic analysis in *C. fimbriata* due to normally high variation (Baker et al. 2003), which is why these loci were selected in the current study. *Ceratocystis fimbriata* is also shown to have strong host specificity. For instance, Baker et al. (2003) identified a sweetpotato-associated lineage but all sweetpotato isolates within this lineage had identical ITS sequences, even though isolates were obtained from diverse geographical locations. Furthermore, using a panel of 16 simple sequence repeats (SSRs),

Steimel et al. (2004) found low levels of polymorphisms on isolates from sweetpotato but increased polymorphisms in isolates from other hosts. Lack of genetic diversity among *C. fimbriata* strains from sweetpotatoes could have resulted from clonal movement of the pathogen across the globe on infected sweetpotato roots (Engelbrecht and Harrington 2005). Our finding of no genetic variation within the ITS and *MAT*-2 loci of our *C. fimbriata* isolates is consistent with these previously mentioned reports for sweetpotato. While it seems these loci are sufficient for determining genetic differences due to host specialization and/or geographic location, there is not enough resolution to differentiate between isolates within the sweetpotato lineage (Baker et al. 2003). Future work should examine *C. fimbriata* populations from sweetpotato using molecular tools with higher powers of resolution. Similar to the recent reemergence of black rot in North Carolina on sweetpotatoes, *C. fimbriata* is also responsible for a recent epidemic of 'Ōhi'a wilt in Hawaii (Keith et al. 2015). This raises additional questions that should be answered in future host-pathogenicity assays and population-genetic analyses that include pre- and post-epidemic isolates from diverse hosts and geographic regions.

This study also demonstrated the ability of *C. fimbriata* to cause black rot on stored sweetpotato roots of 14 common cultivars and advanced breeding lines in the United States. Due to reduction in black

Fig. 3. Relative susceptibility of 14 common sweetpotato cultivars and advanced breeding cultivars to black rot disease based on area under the disease progress curve (AUDPC) values calculated over a three-week period. Cultivars marked with similar letters do not significantly differ at $\alpha = 0.05$ under a Bonferroni correction.

Table 2. Percent inhibition of mycelial growth achieved across 50 *Ceratocystis fimbriata* isolates at four concentrations of eight fungicides tested and the respective EC_{50} value calculated for each fungicide

Active ingredient	Percent (%) mycelial growth inhibition ^a				EC_{50} (ppm)
	0.1 ppm	1.0 ppm	5.0 ppm	10.0 ppm	
Difenoconazole	76.2 ± 1.9 a	89.1 ± 1.2 a	97.6 ± 0.5 a	99.4 ± 0.3 a	<0.1
Thiabendazole	34.9 ± 3.5 b	39.5 ± 2.5 c	99.3 ± 0.2 a	99.9 ± 0.1 a	0.52
Fluazinam	21.2 ± 1.0 c	45.4 ± 1.1 b	79.3 ± 1.0 b	85.4 ± 0.7 b	0.93
Fludioxonil	9.5 ± 1.1 d	43.3 ± 2.0 bc	72.0 ± 2.4 c	76.4 ± 2.2 c	2.18
Dicloran	5.2 ± 0.7 d	33.1 ± 0.8 d	46.9 ± 0.9 d	53.7 ± 0.8 d	7.99
Azoxystrobin	5.6 ± 0.7 d	10.1 ± 1.0 ef	16.2 ± 1.2 e	24.5 ± 1.6 e	>10
Pyraclostrobin	8.4 ± 0.8 d	12.7 ± 0.7 e	18.0 ± 0.9 e	24.4 ± 1.2 e	>10
Fenamidone	6.0 ± 0.5 d	6.0 ± 0.6 f	8.7 ± 0.7 f	13.6 ± 1.0 f	>10

^a % inhibition is reported as mean ± standard error. Means followed by the same letter in a column do not significantly differ at $P = 0.05$ level based on Tukey Test.

rot incidence in the United States in the mid 1900s, no recent studies have evaluated cultivar susceptibility on commonly grown cultivars in the United States. Nielsen and Yen (1966) evaluated black rot disease resistance of more than 600 sweetpotato cultivars from around the world but found none to have complete resistance, though cultivars did differ quantitatively in susceptibility. This is in agreement with results found in the current study. Harter et al. (1926) also found all tested cultivars to

be susceptible to black rot in their early experiments and similar observations were made by Cheo (1953) and Martin (1954). More recently, a study by Lewthwaite et al. (2011) evaluated commonly grown cultivars in New Zealand and found all tested cultivars to be susceptible. Beau-regard, although susceptible, demonstrated the lowest levels of disease severity. To our knowledge, there has been no report of a sweetpotato cultivar with complete resistance to black rot.

Fig. 4. Frequency distributions of EC_{50} values from 50 *Ceratocystis fimbriata* isolates collected across five counties in North Carolina for **A**, thiabendazole, **B**, fluzinam, **C**, fludioxonil, and **D**, dicloran. Distributions are shown for fungicides where a majority of calculated EC_{50} values fell within the tested concentration range of 0.1 to 10.0 ppm. EC_{50} values were calculated using the EC_{50} Plus v1.0 online software.

The underlying mechanisms of quantitative resistance to black rot in sweetpotato have been studied extensively, largely based on enzyme activity within infected tissues (Weber et al. 1967). Peroxidase and polyphenoloxidase show increased activity in diseased tissue and are suggested to play a role in induced resistance (Stahmann et al. 1966). The activity of these enzymes is stimulated by an increase in ethylene production by *C. fimbriata* infected sweetpotato tissues (Hyodo and Uritani 1984; Stahmann et al. 1966). It has also been suggested that levels of resistance depend upon the host's ability to transport peroxidases and polyphenoloxidases to infection sites in a timely manner (Weber et al. 1967). Considering the high similarity in susceptibility among the commonly grown cultivars in the United States, much work is needed to identify sources of resistance to black rot and incorporate them into a commercially acceptable cultivar before host resistance can be relied upon as a primary mechanism of disease control.

Another objective of the present study was to evaluate the in vitro fungicide sensitivity of recent North Carolina *C. fimbriata* isolates to fungicides currently used and to some that may have potential use in the future. The heavy reliance in the past on dicloran for general disease control in sweetpotato and thiabendazole for control of black rot followed by this sudden increase in disease occurrence suggests the possibility of fungicide-resistant isolates. Unfortunately, since no similar in vitro experiments previously have been conducted in this system, we have no baseline sensitivities for historic comparison; however, early work identified thiabendazole as being effective for control of black rot in sweetpotato (Martin 1971; Martin 1972). Evaluation of isolate sensitivity to thiabendazole in the current study revealed that thiabendazole is very effective in reducing mycelia growth of *C. fimbriata* in vitro and no isolates displayed strong resistance to this chemistry. Thiabendazole is a high-risk fungicide for resistance development and resistance has been observed in other fungi in the Ascomycota (Dekker 1976), but to our knowledge no resistance has been reported in the genus *Ceratocystis*.

Tests on the sensitivity of current *C. fimbriata* populations to dicloran, which has been used extensively in sweetpotato production both in the seed beds and postharvest application, reveals poor efficacy for inhibiting mycelia growth in vitro. A wide range of EC₅₀ values among the 50 tested isolates was observed. This variation in sensitivity could be indicative of selection of tolerant isolates; however, to our knowledge, there no sensitivity data of historical isolates for pre-outbreak comparison. Earlier work by Martin (1971), however, demonstrated dicloran was not effective in controlling black rot in vivo, although a broad range of isolates were not tested. Sixteen percent of the tested isolates in the current study had EC₅₀ values above 10 ppm, further suggesting dicloran is not an effective control option for black rot in sweetpotato. While no resistance to dicloran has previously been identified in *Ceratocystis*, resistant isolates have been identified in other fungal genera (Bolton 1976). Future dicloran sensitivity tests with pre-outbreak isolate collections will provide further clarification on the reasoning for lack of efficacy in controlling *C. fimbriata*.

The demethylation inhibitor (DMI) fungicide difenoconazole was the most effective chemistry tested in the current experiment for reducing mycelia growth of *C. fimbriata*, with an average EC₅₀ value below that of our lowest-tested concentration. Limited work has been conducted evaluating fungicide efficacy for control of *C. fimbriata*; however, Wilson and Forse (1997) found that difenoconazole was very effective in reducing the growth rate of *C. fagacearum* in vitro. Based on our findings, difenoconazole could be considered as an alternative to thiabendazole for black rot control with a different mode of action to prevent fungicide resistance. Fludioxonil provided moderate inhibition of *C. fimbriata* in the present experiment while the quinone outside inhibitor (QoI) fungicides azoxystrobin, pyraclostrobin, and fenamidone were not effective at inhibiting mycelial growth of *C. fimbriata*.

A postharvest fungicide trial on artificially inoculated roots was also conducted to provide further evidence of fungicide efficacy for control of black rot in sweetpotato. Fungicides for this experiment were selected based on activity found in the in vitro assays. Dicloran

was included as an industry standard. Efficacy results from the in vivo experiment correspond to the levels of inhibition observed in the in vitro assays. While only single-active-ingredient fungicides were used for the in vitro assays, a premix fungicide of azoxystrobin + difenoconazole + fludioxonil (Stadium) was tested in vivo and provided the most disease control. Dicloran was the least effective among tested fungicides at controlling postharvest black rot and did not significantly differ from the untreated control. This relates to the relative lack of inhibition observed by dicloran in the in vitro assays. Thiabendazole, fludioxonil, difenoconazole, and fluazinam all resulted in significant disease reduction in the postharvest fungicide trials. To our knowledge, this is the first postharvest fungicide trial for control of black rot in sweetpotato. This data compliments our in vitro findings to provide support for future labeling of additional effective fungicides that can help reduce losses from black rot in sweetpotato.

In summary, the reemergence of black rot of sweetpotato in North Carolina stems from a seemingly genetically uniform *C. fimbriata* population of the MAT-2 mating-type. While diversity among *C. fimbriata* isolates infecting sweetpotato has been previously reported to be low, additional work should further characterize the current North Carolina population and compare it to that of spatially and temporally diverse populations. The range in fungicide sensitivity among isolates, as seen in the present study, further exemplifies the need for additional population-genetics work in this pathosystem. Based on in vitro studies, difenoconazole was the most effective fungicide for inhibiting mycelia growth. This finding is further supported by the in vivo postharvest fungicide test demonstrating Stadium (azoxystrobin + difenoconazole + fludioxonil) as the most effective in controlling black rot. Thiabendazole and fluazinam were also efficacious in vitro and in vivo. Dicloran was ineffective in controlling black rot in vivo and in reducing mycelia growth of *C. fimbriata* in vitro. The lack of efficacy of dicloran for this reemerging disease demonstrates the importance of identifying and registering additional fungicides for disease control in sweetpotato. Evaluation of 14 sweetpotato cultivars and advanced breeding lines revealed no complete resistance to black rot, with most cultivars being equally susceptible. Future work should identify sources of host resistance and integrate the trait into commercially accepted cultivars. The current study provides key information with immediate application for control of this reemerging pathogen. With this information, research-based recommendations can be provided to sweetpotato growers in the United States. The results presented here also provide a baseline on which additional research can be conducted in sweetpotato and may also offer valuable information toward control of other *C. fimbriata*-caused diseases, such as the recent rise in 'Ōhi'a wilt in Hawaii. Future work should also examine alternative methods for control of black rot in sweetpotato.

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