Pathogens Causing Anthracnose and Fruit Rots of Coffee Associated with the Coffee Berry Borer and the Entomopathogenic Fungus *Beauveria bassiana* in Puerto Rico

Luz M. Serrato-Díaz,^{1,2,†} Yobana A. Mariño,¹ and Paul Bayman¹

¹ Department of Biology, University of Puerto Rico-Río Piedras, P.O. Box 23360, San Juan, PR 00931-3360, U.S.A.

² U.S. Department of Agriculture-Agriculture Research Service-Tropical Agriculture Research Station (TARS), 2200 P.A. Campos Ave., Ste. 201, Mayagüez, PR 00680-5470, U.S.A.

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ABSTRACT

Fruit rots reduce coffee production worldwide. Eight *Colletotrichum* species have been reported to cause coffee fruit rots; the most important is *C. kahawae*, the cause of coffee berry disease (CBD) in Africa. It is unknown whether these fruit rot pathogens can be dispersed by the coffee berry borer (CBB, *Hypothenemus hampei*) or whether *Beauveria bassiana* (a natural enemy of CBB) might reduce coffee fruit rots. We identified pathogens causing coffee fruits rots in Puerto Rico and evaluated whether *B. bassiana* reduced fruit rot and whether CBB could disperse pathogens. A total of 2,333 coffee fruit with CBB damage were collected; of these, 1,197 had visible growth of *B. bassiana*. *C. fructicola*, *C. siamense*, *C. theobromicola*, and *C. tropicale* were isolated and identified from the fruit using morphological traits and phylogeny of three nuclear genes. All four species caused internal and external rot after inoculation of healthy green coffee fruit. Coffee fruit treated with

B. bassiana had significantly less fruit rot than untreated fruit, suggesting *B. bassiana* can protect against fruit rot. To test whether *B. bassiana* had a protective effect, *B. bassiana* and *Colletotrichum* were coinoculated on coffee fruit. Fruit inoculated with both *B. bassiana* and *Colletotrichum* had significantly less rot than fruit inoculated with *Colletotrichum* alone. To test if CBBs dispersed the pathogens, CBBs were exposed to *Colletotrichum* conidia and placed on green fruit, which resulted in fruit rot. This study identifies new pathogens causing coffee fruit rot, shows that *C. kahawae* is not the only *Colletotrichum* that attacks green fruits, suggests a role for *B. bassiana* in disease management and demonstrates CBB can disperse the pathogens.

Keywords: anthracnose, biological control, coffee berry disease, *Colletotrichum*, disease control and pest management, mycology

Coffee berry disease (CBD) is among the most important diseases of coffee (*Coffea arabica* L.) (Talhinas et al. 2017). On green coffee fruit, lesions of CBD are dark brown, necrotic and sunken; the fruits eventually become mummified (Fig. 1) (Gaitán et al. 2015). The literature states that *Colletotrichum kahawae* subsp. *kahawae* Waller and Bridge is the sole pathogen and that the disease is limited to areas of coffee cultivation at high altitudes in Africa (Batista et al. 2017; Weir et al. 2012). The disease can cause yield losses up to 80% in Kenya, Angola, Cameroon, and Malawi (Mignucci et al. 1985; Nguyen et al. 2010; Omondi et al. 2001; Sreenivasaprasad et al. 1993; Vermeulen 1970; Waller et al. 1993). CBD management relies on fungicides and resistant cultivars (Birikunzira 2000; Etana 2018; Van der Vossen et al. 1976).

C. kahawae subsp. *kahawae* belongs to the *C. gloeosporioides* complex (Weir et al. 2012). This complex also includes the subspecies *C. kahawae* subsp. *cigarro*, not pathogenic to coffee

fruit, and 20 other species of *Colletotrichum*. Several other species in the *C. gloeosporioides* complex have been reported to cause anthracnose diseases on coffee in other countries: *C. capsici*, *C. fructicola*, *C. gloeosporioides*, and *C. siamense* as well as *C. acutatum* and *C. boninense* (Table 1) (Hocking 1966; Mignucci et al. 1985; Nguyen et al. 2010; Omondi et al. 2001; Prihastuti et al. 2009; Sreenivasaprasad et al. 1993; Vermeulen 1970; Waller et al. 1993). In Puerto Rico, only *C. gloeosporioides* has been reported to cause anthracnose on coffee, infecting various organs including fruit (Mignucci et al. 1985). No reports on control have been published in Latin America, except on resistance of hybrids of *Coffea arabica* and *Coffea canephora* Pierre ex A. Froehner to *Colletotrichum* and coffee leaf rust in Colombia (Maldonado et al. 2017). Also, fungicides and fertilization were reported to reduce anthracnose on coffee fruit in Puerto Rico (Mignucci et al. 1985).

In this report we use the term CBD to refer solely to the disease caused by *C. kahawae* in Africa. Symptoms caused by CBD are typical of an anthracnose, and other *Colletotrichum* spp. pathogens cause anthracnose-like symptoms on coffee fruit elsewhere in the world (though very few studies mention anthracnose on green fruit). Lesions caused by *Colletotrichum* are typically dark, sunken, and produce acervuli (Agrios 1997).

The coffee berry borer (CBB) *Hypothenemus hampei* (Ferrari) is the most devastating pest of coffee worldwide (Damon 2000; Infante et al. 2014; Vega et al. 2009, 2015). Damage is caused when the adult female bores into the fruit to deposit eggs; its larvae then feed on the endosperm (Damon 2000; Vega et al. 2006, 2009). High levels of CBB infestation (up to 50 to 90%) have been reported in Colombia, Jamaica, Malaysia, Mexico, Tanzania, and Uganda (Vega 2004). In Puerto Rico, CBB infestation ranged from 0 to 95% depending on year, altitude, and temperature (Mariño et al. 2017a). Integrated pest management strategies

[†]Corresponding author: L. M. Serrato-Díaz; luz.serrato@usda.gov

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including insecticides, biological control using natural enemies, and cultural control have been reported to reduce CBB populations (Aristizábal et al. 2017; Jaramillo et al. 2006; Mariño et al. 2017a).

The most commonly used biological control agent against CBB is the fungus *Beauveria bassiana* (Costa et al. 2002; De La Rosa et al. 2000; Haraprasad et al. 2001; Wraight et al. 2018). *B. bassiana* also inhibits in vitro growth of several phytopathogenic fungi including *Colletotrichum* (Ownley et al. 2010; Sahab 2012; Sanivada and Challa 2014). Other studies have demonstrated a protective effect of *B. bassiana* in vivo against *Fusarium oxysporum* f. sp. *lycopersici* race 3 and *Rhizoctonia solani* (Culebro-Ricaldi et al. 2017; Ownley et al. 2004; Ownley et al. 2008).

We hypothesize that more than one species of *Colletotrichum* causes anthracnose diseases of green coffee fruit, that they may be responsible for causing different degrees of internal versus external rots of coffee fruit, and that infection with *B. bassiana* can protect coffee fruit from infection by the causal pathogens. Furthermore, CBB may be a dispersal agent of these pathogens. Also, we hypothesized that some of the pathogens of coffee fruit in Puerto Rico cause symptoms indistinguishable to those attributed to CBD in Africa. Thus, this study has four objectives: (i) to identify the causal agents responsible for symptoms of anthracnose on coffee fruit in Puerto Rico; (ii) to explore the interactions among anthracnose, CBB, and *B. bassiana*; (iii) to differentiate between pathogens that may cause external or internal symptoms in coffee fruit; and (iv) to interpret the results in light of the debate on identity and distribution of CBD.

Fig. 1. Coffee fruit rot on fruit of *Coffea arabica* ‘Limani’. Brown and red sunken lesions show early stages of the disease; black, mummified fruit (upper center) show advanced stages. Green fruit as well as mature fruit are infected. Several fruits show holes caused by the coffee berry borer, which bores the hole in the fruit to deposit its eggs.

MATERIALS AND METHODS

Collection of plant material and isolation of fungi. Surveys were conducted in 2016 and 2017 from August to December (when coffee fruit are developing and maturing in Puerto Rico). A total of 2,333 full-sized green coffee fruits (*Coffea arabica* ‘Limani’ and ‘Catuai’) with entry holes of CBB were collected from commercial and experimental orchards in Puerto Rico. Two of the orchards were in Adjuntas (18°10’28.73’’N, 66°44’35.83’’W, 510 m above sea level (m.a.s.l.) and 18°10’12.42’’N, 66°47’43.21’’W, 636 m.a.s.l.), one in Ciales (18°18’12.24’’N, 66°33’7.55’’W, 373 m.a.s.l.), and one in Utuado (18°15’51.52’’N, 66°47’14.61’’W, 580 m.a.s.l.). Selected fruit had no external symptoms of fruit rot. Of the 2,333 fruit with CBB damage, 1,197 had white fungal growth typical of sporulating *B. bassiana* colonies collected from dead CBBs in the entrance hole or on the fruit adjacent to the hole where the CBB entered. These colonies were cultured and examined by light microscopy to confirm the morphology was congruent with that of *B. bassiana*. Coffee fruit were cut in half and disease severity was evaluated by visually estimating the percentage of the cut surface that was rotted, in comparison with the scale in Figure 2.

Pieces of coffee fruit with internal lesions were surface-sterilized in 70% ethanol, followed by 1% sodium hypochlorite, rinsed in sterile distilled water (1 min each) and placed in Petri plates on potato dextrose agar (PDA) amended with streptomycin at 0.125 g/liter and ampicillin at 0.25 g/liter. Cultures were incubated at 23°C with an approximately 12 h photoperiod for 8 days. Pure cultures were obtained by hyphal tip transfer. Isolates that sporulated were identified to genus, using standard morphological characters: colony color and texture, growth rate, size and shape of conidia, formation of structures (pycnidia, acervuli, perithecia, sporodochia, among others) and size of asci and ascospores if present (Barnett and Hunter 1998).

Morphological characterization of *Colletotrichum*. Fungal colonies suspected of being *Colletotrichum* spp. were transferred to PDA and incubated for 8 days at 23°C with an approximately 12 h photoperiod. Isolates were grouped in morphospecies based on colony color and texture, shape and size of conidia and appressoria, formation of perithecia, and size of asci and ascospores, if produced. A total of 50 measurements were taken per isolate for each morphological trait that was assessed microscopically at 100× or 400× (depending on the trait). The traits were used to identify the *Colletotrichum* species using taxonomic keys (Weir et al. 2012).

DNA extraction, PCR, and sequencing. Fourteen representative *Colletotrichum* isolates (including representatives of all morphospecies) were selected and grown in potato dextrose broth (PDB) under the conditions described above, DNA was extracted from approximately 0.1 to 0.25 g of mycelium using a Dneasy Plant Mini Kit (Qiagen, Valencia, CA). Three gene regions commonly used to identify *Colletotrichum* species were amplified by PCR using an Amplitaq Gold (Applied Biosystems, Waltham, MA) DNA polymerase according to Weir et al. (2012). The gene regions amplified were: the internal transcribed spacer (ITS)

TABLE 1. Species of *Colletotrichum* causing anthracnose of coffee fruit and coffee berry disease worldwide

<i>Colletotrichum</i> species	Country	Reference
<i>C. acutatum</i>	Vietnam, Papua New Guinea	Nguyen et al. 2010; Kenny et al. 2012
<i>C. boninense</i>	Vietnam	Nguyen et al. 2010
<i>C. capsici</i>	Vietnam	Nguyen et al. 2010
<i>C. coffeanum</i>	East Africa, Kenya	Hocking 1966; Vermeulen 1970
<i>C. fructicola</i>	Thailand	Prihastuti et al. 2009
<i>C. gloeosporioides</i>	Puerto Rico, Vietnam, Papua New Guinea	Mignucci et al. 1985; Nguyen et al. 2010; Kenny et al. 2012
<i>C. kahawae</i>	Kenya, Angola, Cameroon, and Malawi	Waller et al. 1993; Omondi et al. 2001
<i>C. siamense</i>	Thailand	Prihastuti et al. 2009

ITS1-5.8S-ITS2 region of the nuclear ribosomal DNA, part of the chitin synthase (CHS) gene, and part of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (Weir et al. 2012). The ITS region was amplified using primers ITS1/ITS4 (White et al. 1990). Partial sequences of the CHS and GAPDH genes were amplified using primers CHS-79F/CHS-345R (Carbone and Kohn 1999) and GDF/GDR (Templeton et al. 1992), respectively. Amplicons ranged from 300 to 550 bp. PCR products were purified using a QIAQuick PCR Kit (Qiagen) and sequenced at the Sequencing and Genotyping Facility at the University of Puerto Rico (UPR, San Juan, PR). Sequences were edited using Sequencher (version 5.0, Gene Codes Corporation, MN) and deposited in NCBI GenBank (Table 2).

Phylogenetic inference. Phylogenetic analysis included 71 ingroup taxa (Supplementary Table S1), with *C. boninense* ex-types MAFF306094 and MAFF305972 as outgroups according to Weir et al. (2012). Both *Colletotrichum* isolates from the field and those recovered from pathogenicity experiments were included in the analysis. Sequences were aligned using SATé (Kato and Standley 2013) and MAFFT with RAxML for the tree estimator with 10 iterations. The three genes were concatenated using Sequence Matrix to give 1,232 characters including alignment gaps. Maximum likelihood trees were constructed to infer the phylogeny

of *Colletotrichum* spp. using RAxML with GTRCAT as the default model, 25 gamma categories and the automatic bootstrap MRE implemented in CIPRES Science Gateway portal (Miller et al. 2010; Stamatakis 2006a, b).

Location of field experiments and plant material. All field experiments described below were conducted at the UPR Agricultural Experiment Station in Adjuntas, Puerto Rico, a center of coffee research and production. The experiments were conducted in a plot planted with cultivar Limaní. The plot is located at 18°10'12.24''N, 66°47'43.21''W, with an altitude of 434 m.a.s.l. and mean temperature of 22.5°C. Coffee plants were grown under partial shade of *Gliricidia sepium* (mother-of-cocoa, Fabaceae: Faboideae). Lab experiments described below were conducted at the UPR-Río Piedras location. Both lab and field experiments were repeated (2017 and 2018). For all experiments, we selected full-sized, green coffee fruit approximately 80 days old (Gallardo and González 2015).

Inoculations with *Colletotrichum* spp. only. Pathogenicity tests were used to determine whether the *Colletotrichum* isolates described above (Table 2) were pathogens or saprotrophs, and to determine if damage caused by CBB could be an entry point for *Colletotrichum* spp. to infect coffee fruit. Since there is often considerable variation in aggressiveness within species (Balardin

Fig. 2. Examples of differences in severity of internal rot. The severity of symptoms was rated by estimating the percent area of the cut with internal fruit rot. The two halves of each fruit are shown.

TABLE 2. Isolate and accession numbers for the sequences of the ITS1-5.8S-ITS2, chitin synthase and glyceraldehyde 3-phosphate dehydrogenase genes from various *Colletotrichum* isolates collected from internal rots in coffee fruit in Puerto Rico

Isolate	Species	Isolate source	GenBank accession numbers		
			ITS	CHS	GADPH
C16P	<i>Colletotrichum fruticola</i>	Field	MK790665	MK796193	MK796222
C38PP	<i>Colletotrichum fruticola</i>	Recovered—pathogenicity test	MK790664	MK796192	MK796221
C2P	<i>Colletotrichum siamense</i>	Field	MK790642	MK796170	MK796199
C3P	<i>Colletotrichum siamense</i>	Field	MK790643	MK796171	MK796200
C13PA	<i>Colletotrichum siamense</i>	Field	MK790647	MK796175	MK796204
C13PC	<i>Colletotrichum siamense</i>	Field	MK790648	MK796176	MK796205
C14P	<i>Colletotrichum siamense</i>	Field	MK790649	MK796177	MK796206
C17P	<i>Colletotrichum siamense</i>	Field	MK790670	MK796198	MK796227
C19P	<i>Colletotrichum siamense</i>	Field	MK790650	MK796178	MK796207
C111P	<i>Colletotrichum siamense</i>	Field	MK790652	MK796180	MK796209
C54PP	<i>Colletotrichum siamense</i>	Recovered—pathogenicity test	MK790655	MK796183	MK796212
C62PP	<i>Colletotrichum siamense</i>	Recovered—pathogenicity test	MK790656	MK796184	MK796213
C63PP	<i>Colletotrichum siamense</i>	Recovered—pathogenicity test	MK790657	MK796185	MK796214
C88PP	<i>Colletotrichum siamense</i>	Recovered—pathogenicity test	MK790658	MK796186	MK796215
C89PP	<i>Colletotrichum siamense</i>	Recovered—pathogenicity test	MK790659	MK796187	MK796216
C124PP	<i>Colletotrichum siamense</i>	Recovered—pathogenicity test	MK790660	MK796188	MK796217
C136PP	<i>Colletotrichum siamense</i>	Recovered—pathogenicity test	MK790661	MK796189	MK796218
C162PP	<i>Colletotrichum siamense</i>	Recovered—pathogenicity test	MK790668	MK796196	MK796225
C9P	<i>Colletotrichum theobromicola</i>	Field	MK790645	MK796173	MK796202
C12PA	<i>Colletotrichum theobromicola</i>	Field	MK790646	MK796174	MK796203
C120P	<i>Colletotrichum theobromicola</i>	Field	MK790653	MK796181	MK796210
C122P	<i>Colletotrichum theobromicola</i>	Field	MK790654	MK796182	MK796211
C51PP	<i>Colletotrichum theobromicola</i>	Recovered—pathogenicity test	MK790669	MK796197	MK796226
C53PP	<i>Colletotrichum theobromicola</i>	Recovered—pathogenicity test	MK790667	MK796195	MK796224
C67PP	<i>Colletotrichum theobromicola</i>	Recovered—pathogenicity test	MK790662	MK796190	MK796219
C68PP	<i>Colletotrichum theobromicola</i>	Recovered—pathogenicity test	MK790663	MK796191	MK796220
C20P	<i>Colletotrichum tropicale</i>	Field	MK790651	MK796179	MK796208
C165PP	<i>Colletotrichum tropicale</i>	Recovered—pathogenicity test	MK790666	MK796194	MK796223

et al. 1997; Denoyes-Rothan et al. 2003; Hong et al. 2008; Ratanacherdchai et al. 2007), 16 *Colletotrichum* isolates representing four species were selected.

In the field, each isolate was inoculated on 12 coffee fruit (*Coffea arabica* 'Limani') per branch, on three different branches per plant, and on five plants, for a total of 180 fruit inoculated per isolate. Each fruit was marked, and a hole made using a sterilized wire dissecting needle (0.5 to 0.8 mm diameter) to mimic CBB damage. Subsequently, coffee fruit were inoculated using a Pasteur pipette with approximately 10 µl of a suspension of 4×10^6 conidia/ml from 8-day-old pure cultures of *Colletotrichum* spp. grown on PDA as previously described. Untreated controls were inoculated with sterilized distilled water only. Inoculated coffee branches were covered with plastic bags for 15 days to maintain humidity and to prevent access to CBB. Fruit rot was evaluated at 15 days after inoculation (DAI) (a period sufficient for fruit to become diseased but not sufficient for other fungi to invade). Percent internal and external fruit rot was estimated visually: external fruit rot was evaluated before fruit were cut in half to evaluate internal fruit rot as described above (Fig. 2). In both cases severity of fruit rot was estimated based on the percentage scale (0 to 100%), where 0 represents healthy fruit and 100% indicates the entire fruit was rotted.

Diseased tissue was plated on PDA to complete Koch's postulates (Agrios 1997). Fourteen isolates were recovered from pathogenicity tests, identified by grouping into morphotypes and sequencing of the three genes as described above to determine that the isolates recovered were the same as those used for the inoculations.

Dual inoculations with *Colletotrichum* spp. and *B. bassiana*. To test the hypothesis that infection by *B. bassiana* could protect coffee fruit from rot caused by *Colletotrichum*, pathogenicity tests were conducted in the field and laboratory. *C. siamense* and *C. tropicale* were chosen for inoculation because *C. siamense* was the most common species isolated from the coffee fruit, while *C. tropicale* caused the most severe external rot; in both field and laboratory experiments a mixture of 2×10^6 conidia of each species was applied using a Pasteur pipette with 10 µl of a suspension following the procedure described above.

Each test included five treatments and 300 coffee fruit (*Coffea arabica* 'Limani') (60 fruit per treatment). Three treatments were negative controls (fruit with holes to mimic CBB damage inoculated with *B. bassiana* [T1], fruit with holes alone [T2], and intact fruit [T3]), one treatment was a positive control (fruit with holes and inoculated with *Colletotrichum* [T4]), and one treatment was the experimental treatment (fruit with holes inoculated with *B. bassiana* and *Colletotrichum* [T5]) (Table 3). For the field experiments, five typical coffee plants were selected, and five branches on each plant were randomly assigned to treatments T1 to T5; any CBB perforated or diseased fruit were removed from branches to avoid cross contamination. Twelve apparently healthy, full-sized green fruit per branch were chosen and marked, and a hole was made with a sterilized wire dissecting needle (0.5 mm diameter) to mimic CBB damage.

Inoculation of fruit with *B. bassiana* was performed 2 days before the inoculation with *Colletotrichum* spp. *B. bassiana* isolate N5 was used. It was originally isolated from coffee fruit in Adjuntas, PR and was chosen because it is highly aggressive to CBB (*unpublished*

observations); isolate N5 is stored in culture collections at UPR-RP and at the U.S. Department of Agriculture-Agricultural Research Service Mycology and Nematology Genetic Diversity and Biology Laboratory, Beltsville, MD. Preparation and inoculation of *B. bassiana* was the same as described above for *Colletotrichum*.

For laboratory tests, three coffee branches containing at least 12 apparently healthy, full-sized green fruit were detached from the coffee bushes and placed in Erlenmeyer flasks with distilled water (to prevent wilting) and inoculated as previously described. At 15 DAI, fruit were cut in half and the percent severity of fruit rot was visually estimated for each fruit and diseased tissue was plated on PDA to complete Koch's postulates.

CBB as a dispersal agent of *Colletotrichum*. The hypothesis that CBB could disperse *Colletotrichum* to coffee fruit was tested in the laboratory and in the field. For laboratory experiments groups of adult female CBBs were exposed to a suspension of conidia of *C. siamense* and *C. tropicale* (mixed as described above). For field experiments, the four species of *Colletotrichum* were tested separately.

CBBs were grown on the artificial diet CENIBROCA as previously described (Mariño et al. 2017b). Adult females were surface-disinfected by submersion in 1% sodium hypochlorite with 0.02% Tween 80, followed by two rinses with sterilized water for 1 min each. For both lab and field experiments the insects were surface-inoculated with spores of *Colletotrichum* spp. A filter paper in a Petri dish was impregnated with 500 µl of a conidial suspension of one species of *Colletotrichum* at a time (field experiments) or a 50:50 mix (laboratory experiments) of *C. siamense* and *C. tropicale* (4×10^6 conidia/ml in both cases). The CBBs were placed in the Petri dishes for 1 h.

For the laboratory tests, coffee branches with fruit as described for the previous experiment were placed in Erlenmeyer flasks with distilled water. There were three replicate branches for each treatment. The treatments were CBBs previously inoculated with conidia of *Colletotrichum* spp. (T1), CBBs not inoculated with conidia of *Colletotrichum* spp. (T2), CBBs exposed to sterilized distilled water only and intact fruit, a negative control (T3), and fruit manually perforated to mimic CBB damage and subsequently inoculated with *Colletotrichum* spp., a positive control (T4). In the field, five coffee plants were selected, and three replicate branches with at least 20 apparently healthy, full-sized green fruit were used per *Colletotrichum* species. Experiments included five treatments: CBBs not exposed to conidia of *Colletotrichum* spp., a negative control (T1), CBBs exposed to conidia of *C. fructicola* (T2), CBBs exposed to conidia of *C. siamense* (T3), CBBs exposed to conidia of *C. tropicale* (T4), and CBBs exposed to conidia of *C. theobromicola* (T5). In both the laboratory and the field experiments, any CBB perforated or diseased fruit were removed from branches to avoid cross contamination. Forty CBBs exposed to the conidial suspension were placed on each branch and the branches covered with plastic bags to maintain humidity and prevent the CBBs from escaping. Percent fruit rot was evaluated at 15 DAI; diseased fruit tissue and CBBs were plated on PDA to confirm that the pathogens were the same as the fungi that were inoculated.

Statistical analysis. One-way analysis of variance (ANOVA) was performed to determine significance of treatment effects, and

TABLE 3. Treatments to test the protective effect of preinoculation of green coffee fruit with *Beauveria bassiana* against coffee fruit rot caused by *Colletotrichum* spp.

Treatment	Bored	<i>B. bassiana</i>	<i>Colletotrichum</i> spp.
T1: Bored fruit ^a + <i>Beauveria bassiana</i>	X	X	
T2: Bored fruit (negative control)	X		
T3: Intact fruit (negative control)			
T4: Bored fruit + <i>Colletotrichum</i> spp.	X		X
T5: Bored fruit + <i>Colletotrichum</i> spp. + <i>B. bassiana</i> (experimental)	X	X	X

^a Bored fruit: each fruit was penetrated with a 0.5 mm wire needle to simulate coffee berry borer damage.

Tukey's honest significant differences (HSD) post hoc test ($\alpha = 0.05$) was used to explore the differences between the mean values of the treatments (severity of fruit rot among *Colletotrichum* species or among inoculation treatments). All analyses were calculated using R (R Core Team 2017). ANOVAs tests were performed with the aov() function and pairwise post hoc tests with TukeyHSD() function with a confidence interval of 95%. Severity data on fruit rot were transformed using the Bliss angular transformation (Bliss 1937) to fulfill requirements of normality and homogeneity of variance. Back transformed mean fruit rot severity values are presented.

RESULTS

Fungal diversity and rot in coffee fruit with and without *B. bassiana*. A total of 323 fungi in 11 genera were isolated from the rotted internal tissues of the sampled coffee fruit. The most common fungi were *B. bassiana* (175 isolates) followed by *Fusarium* spp. (72 isolates) and *Colletotrichum* spp. (49 isolates). Other genera were isolated less frequently (<5 isolates each): *Pestalotiopsis*, *Phomopsis*, *Aspergillus*, *Guignardia*, *Cladosporium*, and *Penicillium* (Supplementary Table S2).

Coffee fruit with sporulating colonies of *B. bassiana* had significantly less fruit rot than coffee fruit without *B. bassiana* ($F = 126.06$, $P < 0.0001$), suggesting *B. bassiana* could be a potential biological control agent against anthracnose (Fig. 3). The result formed the basis for subsequent experiments.

Morphological identification of *Colletotrichum* spp. Four species belonging to the *C. gloeosporioides* complex were isolated from rotted coffee fruit tissues and identified based on morphology (Weir et al. 2012).

C. fruticola. On PDA, colonies were gray with aerial mycelia. Conidia did not form acervuli in culture. Conidia ($n = 50$) were $13.9 \times 4.5 \mu\text{m}$, one-celled, smooth-walled, hyaline, cylindrical to oblong, rounded at both ends. Appressoria were 6.5 to 8.5 μm in diameter. Perithecia were subglobose to ovoid, semi-immersed in PDA. Asci ($n = 20$) were $55 \times 10 \mu\text{m}$, clavate, unitunicate, thin-walled and contained 8 ascospores. Ascospores ($n = 50$) were $10.9 \times 4.3 \mu\text{m}$, one-celled, hyaline, slightly curved and rounded at both ends (Fig. 4A).

C. siamense. On PDA, colonies were grayish-white with aerial mycelium and conidial masses (acervuli visible in culture). Conidia ($n = 50$) were $15.2 \times 4.3 \mu\text{m}$, one-celled, smooth-walled, hyaline, cylindrical to oblong, and rounded at both ends. Appressoria were 4.5 to 5.0 μm in diameter, and perithecia were absent (Fig. 4B).

C. theobromicola. On PDA, colonies were grayish turning dark gray with age, with aerial mycelium. Conidia did not form acervuli in culture. Conidia ($n = 50$) were $15 \times 4.5 \mu\text{m}$, subcylindrical to clavate. Appressoria were 7 to 10 μm in diameter and perithecia were absent (Fig. 4C).

C. tropicale. On PDA, colonies were grayish-white with aerial mycelium. Conidia did not form acervuli in culture. Conidia ($n = 50$) were $19.8 \times 5.3 \mu\text{m}$, cylindrical, hyaline, straight, and rounded at both ends. Appressoria were 8 to 10 μm in diameter and perithecia were absent (Fig. 4D).

C. kahawae was not isolated.

Phylogenetic inference. The best maximum likelihood tree clustered the *Colletotrichum* isolates in four clades corresponding to the species described above (Fig. 5). Of the 28 isolates of *Colletotrichum* sequenced, eight clustered with *C. siamense* (bootstrap support [BS] = 61), four with *C. theobromicola* (BS = 99), one with *C. fruticola* (BS = 83), and one with *C. tropicale* (BS = 99).

Inoculations with *Colletotrichum* spp. alone. All four species of *Colletotrichum* tested (*C. fruticola*, *C. siamense*, *C. theobromicola*, and *C. tropicale*) were pathogenic on green coffee fruit, causing fruit rot. All four species caused both internal

and external fruit rots at 15 DAI. With external rots, the epicarp turned from green to dark brown, in some cases with mycelial growth visible on the fruit surface. With the internal rots, the endosperm turned brown and water-soaked lesions were observed in the mesocarp (Fig. 6B). Fruit from untreated controls (inoculated with sterilized distilled water only) showed no symptoms of fruit rot.

With the external rots, significant differences in aggressiveness were observed among species ($F = 6.98$; $P < 0.0001$). The most aggressive species was *C. tropicale*, followed by *C. theobromicola*, *C. fruticola*, and *C. siamense*, respectively. With the internal rots, differences among species were also significant ($F = 11.0$; $P = 0.012$); *C. theobromicola* and *C. fruticola* were the most aggressive species (Fig. 6A).

Phylogenetic analysis of species reisolated from the pathogenicity tests confirmed that they belonged to the same four *Colletotrichum* species that had been used for inoculation, fulfilling Koch's postulates (Figs. 5 and 6B).

Dual inoculations with *Colletotrichum* spp. and *B. bassiana*. In both laboratory and field experiments, coffee fruit inoculated with *B. bassiana* before inoculation with *Colletotrichum* (treatment T5) had significantly lower severity (percent rot per fruit) than fruit inoculated with *Colletotrichum* alone (positive controls, T4) (Fig. 7A and C; lab experiments: $F = 16.30$, $P < 0.0001$; field experiments: $F = 15.74$, $P < 0.0001$). However, under field conditions the negative controls (T1 and T2) were not different from the positive controls (T4): there was growth of *Fusarium* spp., indicating that *Fusarium* also plays a role in coffee fruit rot and thus may complicate observations on the interaction between *Colletotrichum* spp. and *B. bassiana* (Fig. 7D). *Colletotrichum* was reisolated from fruit inoculated with *Colletotrichum* alone but not from fruit inoculated with both *Colletotrichum* and *B. bassiana* (except for 2 isolates recovered from a sample in a plastic bag that opened in the field, permitting entry of inoculum from outside). In the field, *Fusarium* was the most frequent fungal genus isolated from control fruit and fruit inoculated with *Colletotrichum* and *B. bassiana* (Fig. 7B and D).

CBB as a dispersal agent of *Colletotrichum*. In lab experiments, fruit damaged by CBBs that were previously exposed to conidia of *C. siamense* and *C. tropicale* (mixed 1:1, T1) showed significantly higher fruit rot than fruit damaged by CBBs not exposed to conidia (CBB alone, T2) and negative controls of fruit with no damage (T3) ($F = 201.8$; $P < 0.0001$) (Fig. 8A). Both fruit damaged by CBBs exposed to *Colletotrichum* (T1) and positive

Fig. 3. Mean percent severity of rot in field-collected coffee fruit visibly infected with *Beauveria bassiana* (Bb) versus noninfected fruit. All fruit had been penetrated by the coffee berry borer. Bars with different letters are statistically different according to Tukey's honest significant differences post hoc test ($\alpha = 0.05$). Bars show +1 standard error.

controls (T4, fruit manually damaged and inoculated with *Colletotrichum*) showed fruit rot symptoms, and *Colletotrichum* was recovered from the diseased fruit. *Colletotrichum* was not recovered from fruit damaged by CBBs but not exposed to conidia (T2, Fig. 8B).

In field experiments, the CBBs were exposed to conidia of each *Colletotrichum* species separately. Similar to the pathogenicity tests described above (see Inoculations with *Colletotrichum* spp. only), fruit damaged by CBBs exposed to *C. tropicale* (T4) and *C. theobromicola* (T5) showed significantly more external fruit rot ($F = 9.60$; $P < 0.0001$) than fruit damaged by CBBs exposed to *C. fructicola* (T2) and *C. siamense* (T3) (and control fruit perforated by CBBs not exposed to any fungus, negative control [T1]) (Fig. 9A). The results rating the severity of internal rots support those from the pathogenicity tests: *C. theobromicola* was the most aggressive and *C. tropicale* the least aggressive. Differences between *C. theobromicola* and the other three species were significant ($F = 11.1$; $P < 0.0001$) (Fig. 9B). *Colletotrichum* was recovered from fruit damaged by CBBs with *Colletotrichum* but not from fruit damaged by CBBs but not exposed to conidia (Fig. 9C).

DISCUSSION

The morphological and phylogenetic studies and the pathogenicity tests all confirmed that four *Colletotrichum* species, *C. fructicola*, *C. siamense*, *C. theobromicola*, and *C. tropicale*, cause rot of green coffee fruit in Puerto Rico. Koch's postulates were fulfilled. To the best of our knowledge, this is the first report of

C. theobromicola and *C. tropicale* causing anthracnose on coffee fruit, although *C. theobromicola* and *C. siamense* have been reported causing anthracnose on coffee leaves in Mexico (Cristóbal-Martínez et al. 2017). *C. fructicola* and *C. siamense* were reported causing anthracnose in Thailand (Prihastuti et al. 2009) (Table 1). Our results reaffirm that report.

Pathogenicity tests with *C. fructicola*, *C. siamense*, *C. theobromicola*, and *C. tropicale* showed that all four cause both internal and external rots of green fruit. More severe external fruit rot was caused by *C. tropicale*, *C. theobromicola*, and some isolates of *C. siamense*, while more severe internal fruit rot was caused by *C. theobromicola* and *C. fructicola*. Worldwide, previous reports of CBD have described the disease as an external fruit rot with slightly sunken, brown blight lesions on green and ripening fruit (Gaitán et al. 2015; Hocking 1966; Kenny et al. 2012; Nguyen et al. 2010; Omondi et al. 2001; Vermeulen 1970; Waller et al. 1993). Internal fruit rot, as we describe here, is not mentioned in the cited literature. It is likely that internal rot is more common when CBB damage (or other damage) provides entry to internal tissues, as was found in field-collected fruit with CBB damage, which was also mimicked in the experiments described here.

Colletotrichum spp. can penetrate plant tissues through stomata, wounds or directly through the cuticle via the production of a penetration peg from the appressorium (Kumar et al. 2001; Moraes et al. 2013; Panday et al. 2012; Smith et al. 1999). All *Colletotrichum* spp. identified in the study produced appressoria in culture, a key structure for fungal penetration of the host. Appressoria may have been responsible for the external fruit rot

Fig. 4. *Colletotrichum* spp. identified from internal rot of coffee fruit in Puerto Rico. Colony, conidia, and appressoria of **A**, *C. fructicola*. Asci and ascospores were observed only for *C. fructicola*, **B**, *C. siamense*, **C**, *C. theobromicola*, and **D**, *C. tropicale*.

Fig. 5. Maximum likelihood tree of different *Colletotrichum* species based on the concatenated sequences of internal transcribed spacer, chitin synthase, and glyceraldehyde 3-phosphate dehydrogenase genes. Bootstrap support values are shown at the nodes. Isolates from coffee fruit collected in the field in Puerto Rico and isolates recovered from pathogenicity tests are indicated by colored star symbols. The tree was rooted to the outgroups *C. boninense* ex-type MAFF306094 and MAFF305972.

Fig. 6. Internal and external fruit rot of coffee caused by four species of *Colletotrichum* isolated from diseased fruit in Puerto Rico. **A**, Mean percent internal and external fruit rot caused by each isolate of *C. siamense*, *C. theobromicola*, *C. tropicale*, and *C. fructicola* is indicated. Bars show ± 1 standard error. **B**, Coffee fruit rot/anthracnose symptoms on fruit inoculated with each species of *Colletotrichum* (top), external and internal fruit rots (center), and isolates recovered after pathogenicity tests (bottom).

observed, but we did not study this. A penetration peg from the appressorium of *C. kahawae* can generate 2.6 Mpa, about twice the osmotic pressure of green coffee fruit, and this mechanical pressure is enough to break the cuticle (Chen et al. 2004).

This study demonstrates that coffee fruit rot is a disease complex caused by several *Colletotrichum* species, some of which cause more external rot and others more internal rot. This suggests the possibility of differences in ecological niches among the species in the complex, perhaps related to differences in their modes of infection. Inoculation experiments on intact fruits are needed to better understand interactions and differences among these pathogens.

In experiments in both the lab and field, inoculation with *B. bassiana* protected coffee fruit from rot caused by *Colletotrichum*.

Previous studies have reported that *B. bassiana* inhibits various phytopathogenic fungi (Culebro-Ricaldi et al. 2017; Ownley et al. 2000, 2004, 2010; Sanivada and Challa 2014). *B. bassiana* produces chitinases and secondary metabolites (i.e. beauvericin, beauverolides, bassianolides, oosporein, cyclosporin A, and oxalic acid) with antifungal, antibacterial, and insecticidal activities (Ownley et al. 2010). Protection by *B. bassiana* against *Colletotrichum* may be due to a combination of secondary metabolites and physical exclusion by colonization of plant tissue. *B. bassiana* has been reported as an endophyte of coffee (Vega et al. 2010) and is common on coffee farms in Puerto Rico (Gallardo et al. 2010).

There are many examples of insects that act as dispersal agents of plant pathogens, including viruses, bacteria, and fungi

Fig. 7. Protective effect of preinoculation with *Beauveria bassiana* against coffee fruit rot/anthracnose caused by *Colletotrichum* spp. in lab and field experiments. **A**, Mean percent severity for fruit rot in dual inoculations (*Colletotrichum* and *Beauveria bassiana*) in lab experiments (T5) compared with inoculations with *Colletotrichum* alone and controls (T1 to T4). **B**, Surface-sterilized coffee fruit plated on potato dextrose agar (PDA). For fruit in T4, *Colletotrichum* was recovered, but with fruit in T5, *Colletotrichum* was not recovered. **C**, Mean percent severity for fruit rot in dual inoculations (*Colletotrichum* and *B. bassiana*) in field experiments. **D**, Coffee fruit plated on PDA. For fruit in T4, *Colletotrichum* was recovered, but for fruit in T5 *Colletotrichum* was not recovered. In treatments T1, T2, and T5, *Fusarium* spp. were recovered for field experiments and in T1 and T2 for lab experiments. Bars with different letters are statistically different according to Tukey's honest significant differences post hoc test ($\alpha = 0.05$). Error bars show +1 standard error.

(Eigenbrode et al. 2018; Perilla-Henao and Casteel 2016). For example, bark beetles of the genus *Scolytus* (mainly *S. scolytus* and *S. multistriatus*, in the same family as CBB) are vectors of the fungus *Ophiostoma ulmi*, causal agent of Dutch elm disease (Faccoli and Santini 2016). In our experiments, when CBBs were exposed to conidia of *Colletotrichum* spp. and subsequently given access to coffee fruit, the fruit became diseased (Fig. 9), indicating that CBB can be a dispersal agent of coffee fruit pathogens. Although *Colletotrichum* was isolated from internal tissues after infestation with *Colletotrichum*-exposed CBBs, external rots were significantly more common, suggesting that *Colletotrichum* conidia are left on the fruit surfaces as well as carried into the mesocarp. Several studies have shown that CBBs can carry fungal spores: masses of conidia of *Penicillium brocae* and several unknown fungi have been found on the head and ventral parts of the abdomen, especially at the base of setae and asperites (Carrión and Bonet 2004; Peterson et al. 2003). The internal fruit rot caused by *Colletotrichum* in coffee may be facilitated by perforations left by CBB; conidia may be transported through the perforation into the fruit by wind or water, or be carried by the CBB itself. The CBB bores a single hole, approximately 0.78 mm in diameter in each coffee fruit using its mandibles (Vega et al. 2019). Normally, there is a single hole per fruit, but this hole reaches the endosperm and provides entry to internal tissues (Damon 2000).

C. kahawae is regarded in the literature as the sole pathogen causing CBD: “Coffee berry disease is confined to date to the African continent and is the major economic threat to the production of arabica coffee normally growing at high altitudes...” (Gaitán et al. 2015). It can cause losses of up to 80%, due to “...its unparalleled adaptation of infecting green coffee berries” (Silva et al. 2012). *C. kahawae* is “...a specialist pathogen with the ability to infect green coffee berries, an ecological niche previously unoccupied by other fungi” (Vieira et al. 2019). It is characterized by dark or pale, sunken lesions or anthracnose on fruit, which can progress to sporulation, fruit drop or mummification (Gaitán et al. 2015; Waller et al. 1993).

Various *Colletotrichum* species attack a wide spectrum of fruit, ripe and unripe, and the number of fruit available on coffee farms present a resource for any species that can colonize them and sporulate. Thus, it is not surprising that species of *Colletotrichum* other than *C. kahawae* subsp. *kahawae* have the capacity to infect and rot green coffee fruit causing typical symptoms of anthracnose and additional external and internal rot symptoms. It has been proposed that *C. kahawae* made a recent host jump to coffee in Africa, where coffee originated (Silva et al. 2012). Coffee was introduced to other parts of the world and planted on a large scale (Bigger 2006), and so it is reasonable to speculate that other *Colletotrichum* species may have made coffee fruit a new host tissue once the plant became available (Kenny et al. 2012; Nguyen et al. 2010; Prihastuti et al. 2009). Likewise, it is not surprising that a complex of *Colletotrichum* species cause coffee fruit rots, since this has been shown for other fruit diseases (Serrato-Díaz et al. 2017; Serrato-Díaz et al. 2020). Our results indicate niche differentiation among species of *Colletotrichum* infecting coffee fruit in Puerto Rico; some species caused more external rot and others more internal rot. These species were included in the *C. gloeosporioides* complex and were recently described as separate species based on DNA sequence data (Weir et al. 2012). Furthermore, it is likely that fungi other than *Colletotrichum* also cause rots of coffee fruit, although there are few reports in the literature: in one field experiment, *Fusarium* was frequently isolated from control fruit and fruit inoculated with *Colletotrichum* (Fig. 8).

C. kahawae was not isolated in this study and has never been reported from Puerto Rico. As noted, other *Colletotrichum* species were isolated from green fruit with anthracnose lesions and caused rot when inoculated either artificially or through CBB into green coffee fruit, fulfilling Koch’s postulates for pathogenicity. Furthermore, they caused internal as well as external rots; internal rot has not been reported previously in relation to pathogens of coffee fruit. The term “coffee berry disease” should not be used for these rots, since by convention its use is restricted to the disease caused by *C. kahawae* on coffee in Africa (Gaitán et al. 2015). However, all the

Fig. 8. Results of a field experiment testing whether the coffee berry borer (CBB) can be a dispersal agent of *Colletotrichum*. **A**, Mean percent severity of fruit rot in fruit with CBB damage and where the CBBs were previously inoculated with conidia of *Colletotrichum* (T1), fruit damaged by CBBs that were not previously exposed to conidia (T2), and fruit that were artificially damaged to mimic CBB boring and subsequently inoculated with *Colletotrichum* (T4). Bars with different letters are statistically different according to Tukey’s honest significant differences post hoc test ($\alpha = 0.05$). Error bars show +1 standard error. **B**, Coffee fruit rot symptoms 15 days after inoculation; *Colletotrichum* and *Fusarium* were recovered from inoculated fruit. *Fusarium* (red colonies) was observed in fruit damaged by CBBs but not exposed to *Colletotrichum* (CBB alone, T2).

symptoms described for CBD caused by *C. kahawae* in Africa were also observed in this study (Batista et al. 2017). These symptoms include rotting of green coffee fruit, eventually resulting (in advanced stages) in black and mummified fruit.

The literature on CBD has focused on a single pathogen on a single continent, for historical reasons and also because of its alarming virulence (Batista et al. 2017). The data presented here indicate that the relationships among coffee fruit diseases, their symptoms, and the fungal species that cause them may be much more complex than previously considered. We predict they will be found in most coffee-growing areas with high humidity. Their distribution, identification, incidence, and effect on yield should be studied, both in and out of Africa. Furthermore, phytosanitary regulations implemented to prevent the spread of *C. kahawae* out of Africa may be ineffective if they overlook pathogens causing the same symptoms that are already present in other countries such as Vietnam and Thailand (Nguyen et al. 2010; Prihastuti et al. 2009).

Greater awareness of anthracnose among plant pathologists, agricultural extension agents, and coffee farmers is needed, and

could reduce losses in coffee yields and quality. In Puerto Rico, there are no management practices for anthracnose because the growers do not yet recognize it as a disease, as stated above. Indeed, farmers and agricultural extensionists have told us that the fruit rot we describe was caused by sun damage or poor plant nutrition, rather than a pathogen. Fertilization and fungicides were reported to reduce anthracnose substantially but not completely (Mignucci et al. 1985). There may be abiotic factors that contribute to the disease, but identification and recognition of the pathogens involved will be critical for its future control.

Conclusions. We have provided evidence that the CBB could be a dispersal agent of coffee fruit pathogens. The possible dual use of *B. bassiana* against CBB and coffee fruit rot could help farmers by (i) reducing the applications of different products if one product containing *B. bassiana* helps control a pest and a disease simultaneously, and (ii) indirectly reduces the damage caused by *Colletotrichum* by controlling the CBB.

Anthracnose of coffee fruit is complex and consists of both external and internal rots, caused by different *Colletotrichum*

Fig. 9. Results of a field experiment testing whether the coffee berry borer (CBB) was a dispersal agent of *Colletotrichum* spp. to green coffee fruit. **A**, Mean percent severity of external fruit rot in coffee fruit damaged by CBBs previously exposed or not exposed to different *Colletotrichum* spp. Error bars show +1 standard error. **B**, Mean percent internal fruit rot in coffee fruit damaged by CBBs previously exposed or not exposed to different *Colletotrichum* spp. Bars with different letters are statistically different according to Tukey's honest significant differences post hoc test ($\alpha = 0.05$). **C**, Coffee fruit 15 days after inoculation; *Colletotrichum* spp. recovered after exposure of fruit to CBBs (*C. fructicola*, *C. siamense*, *C. tropicale*, and *C. theobromicola*, respectively).

pathogens. Our results suggest further that *Fusarium* spp. also play a role in some coffee fruit rots. Studies with *Fusarium* spp. are needed to better understand the disease in the field and the role of the CBB as a dispersal agent under natural conditions.

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