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Distribution and Fungicide Sensitivity of *Colletotrichum* Species Complexes from Rubber Tree in Hainan, China

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Abstract

Colletotrichum gloeosporioides and *C. acutatum* species complexes are causal agents of Colletotrichum leaf disease (CLD) of rubber trees worldwide. To determine the geographic distribution of *Colletotrichum* species complexes associated with CLD of rubber trees in Hainan, China, and their sensitivity to fungicides used in the region, a total of 275 *Colletotrichum* isolates were collected from 52 rubber tree plantations in 11 counties. These isolates were identified based jointly on morphological characteristics and PCR-based methodology. Of these isolates, 78 and 22% belonged to the *C. gloeosporioides* species complex (CGSC) and the *C. acutatum* complex (CASC), respectively. The incidence of CGSC isolates was greater than the CASC in all counties sampled. The incidence of CASC isolates appeared to be lower in the western and central south of Hainan than in other regions. There was no association in their presence at a given plantation between the two species complexes. The in vitro sensitivity of these two species complexes to carbendazim, chlorothalonil, and four demethylation inhibitor (DMI) fungicides (difenoconazole, propiconazole, myclobutanil, and prochloraz) was determined. Carbendazim was effective against CGSC but not against CASC with mean ED_{50} values of 0.176 and 2.182 μg/ml, respectively. CASC isolates were more sensitive to difenoconazole, propiconazole, and myclobutanil (mean ED_{50} values of 0.177, 0.129, and 1.424 μg/ml, respectively) than CGSC isolates (mean ED_{50} values of 0.710, 0.348, and 3.496 μg/ml, respectively). Mean ED_{50} values of CGSC against chlorothalonil and prochloraz were 173.341 and 0.035 μg/ml, respectively; corresponding values for CASC were 151.441 and 0.040 μg/ml. These results suggest that prochloraz, propiconazole, and difenoconazole are effective against both species complexes.

Rubber tree (*Hevea brasiliensis*), originally from the Amazon, is a perennial crop for producing natural rubber latex. Historically, they have been cropped within the equatorial zone between 10°N and 10°S in areas with all-year-round rainfall, especially in Southeast Asia, including Thailand, Indonesia, Vietnam, and Malaysia (Li and Fox 2012). Since the early 1950s, China has been investing heavily on rubber trees and successfully cultivates rubber trees in regions with cooler temperatures and distinct dry seasons, primarily in Hainan, Yunnan, and Guangdong (Pu et al. 2007).

Colletotrichum leaf disease (CLD) has been observed in most rubber growing countries. The disease often occurs when trees are producing new foliage in early spring. CLD symptoms on immature leaves of rubber trees begin at the tip of the leaf and spreads toward the base of the leaf, resulting in necrotic tissues. When semimature or mature leaves are infected, and the spots became raised and prominent as the leaf ages, leaves are covered with numerous spots having a brown margin surrounded by a yellow halo (Liyanage 1985). On susceptible genotypes, disease symptoms are mainly leaf necrosis and deformation; CLD may result in early defoliation (Guyot et al. 2001).

Previous research showed that *C. gloeosporioides* and *C. acutatum* are causal agents of CLD on rubber trees based on results from morphological, molecular, and fungicide sensitivity studies (Brown and Soepena 1994; Saha et al. 2002). By using colony color, colony growth, and conidial morphology, Jayasinghe et al. (1997) reported that *C. acutatum* is mainly responsible for CLD on rubber trees in Sri Lanka. *C. gloeosporioides* was considered to be the only CLD pathogen in China; *C. acutatum* was, however, first recorded as a causal agent of CLD in rubber trees in Yunnan Province in 2008 (Zhang et al. 2008) and in Hainan in 2010 (Li et al. 2010).

Colletotrichum species were traditionally identified mainly based on microscopic and morphological characteristics of spores and in vitro colonies, and host range (Sutton 1992). Several PCR assays for detecting *C. gloeosporioides* and *C. acutatum* have been developed. For example, PCR using primers amplifying a region of the internal transcribed spacer (ITS) of ribosomal DNA has been developed for *C. gloeosporioides* (Mills et al. 1992) and *C. acutatum* (Sreenivasaprasad et al. 1996). Species-specific primers based on the β-tubulin gene have also been developed (Talhinhas et al. 2005). Restriction fragment length polymorphisms (RFLP) of a 1-kb intron of the glutamine synthetase (GS) gene can also be used to differentiate *C. gloeosporioides* and *C. acutatum* (Liu et al. 2012).

Recent studies based on the multilocus phylogeny approach showed that both *C. gloeosporioides* and *C. acutatum* are species complexes. There are 22 species plus one subspecies within the *C. gloeosporioides* species complex (CGSC) (Weir et al. 2012) and 31 species within the *C. acutatum* species complex (CASC) (Damm et al. 2012). Although PCR primers for the ribosomal DNA-ITS region and the β-tubulin gene cannot be considered species specific because of the similarity in sequences of the ITS region and β-tubulin gene among *Colletotrichum* spp., these primers can identify isolates at the species complex level (Afanador-Kafuri et al. 2014; Álvarez et al. 2014).

CLD has been primarily managed by fungicides. In China, chlorothalonil (fungicide resistance action committee, FRAC code M5) and carbendazim (FRAC code 1) have been used to control CLD since 1990s. More recently, demethylation inhibitor fungicides (DMI) (FRAC code 3) such as difenoconazole, propiconazole, myclobutanil, and prochloraz have been registered to control CLD. However, *C. acutatum* is reported to be more sensitive to captan and triadimenol than *C. gloeosporioides*, and the opposite is true for benomyl, carbendazim, and thiophanate methyl (Greer et al. 2011; Jayasinghe and Fernando 1998). For effective management of CLD, it is important to understand the geographical

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distribution of the pathogens and their sensitivities to fungicides commonly used in the region of interest.

The objectives of the present study were to (i) determine the geographic distribution of *Colletotrichum* species complexes associated with CLD of rubber trees in different counties in Hainan; (ii) determine whether there is differential prevalence of the two species complexes (CGSC and CASC) in different regions in Hainan; and (iii) determine the sensitivity of *Colletotrichum* species complexes to chlorothalonil, carbendazim, and DMI fungicides, and the correlation in their sensitivity to these fungicides.

Materials and Methods

Sampling and isolating fungal strains. During 2014 and 2015, rubber tree leaves with CLD symptoms were collected from 52 plantations located in 11 counties in Hainan (Fig. 1). For each county, samples were taken from two to 12 plantations.

Three 5 × 5 mm pieces of tissue were taken from the margin of CLD symptom to isolate the fungus. The tissues were surface sterilized in 70% ethanol for 30 s and 1% NaClO for 1 min, then rinsed in sterile distilled water for 30 s and finally dried on sterilized tissue paper. The surface-sterilized tissues were plated onto potato dextrose agar (PDA) and incubated at room temperature (28 to 30°C) under continuous darkness until fungal colonies had grown. Growing edges of any fungal hyphae from the tissues were then transferred aseptically to new PDA and incubated 5 days at 28°C with a 12:12 h dark/light photoperiod. For single-spore isolation, conidia were scraped off the plate using a sterile toothpick, and suspended in 1 ml sterile distilled water. Fifty microliters of the conidial suspension was spread onto a 1.5% (w/v) water agar plate and incubated at 28°C overnight. Individual germinated conidia were transferred separately onto PDA plates incubated at 28 with a 12/12 h dark/light photoperiod. From each plantation, 2 to 10 putative isolates were recovered. In total, 275 single-spore isolates were obtained (Supplementary Table S1).

Morphological and cultural characterization of isolates. An agar block (0.5 mm in diameter) was taken from the edge of a 5-day-old actively growing culture, placed onto the center of a 9-cm-diameter Petri dish containing PDA, and incubated at 28 ± 1°C with a 12:12 h dark/light photoperiod. Colony characteristics were determined visually after 5 to 7 days of incubation. Growth rate was calculated as average daily growth (mm day^{-1}) over the 5 to 7 day period. The size and shape of 20 randomly selected conidia were assessed under a compound microscope (400×). Differences between conidial dimensions and growth rates were compared using two-tailed *t* tests with $\alpha = 0.05$.

Identification of *Colletotrichum* species complexes by PCR. Mycelia were scraped with a sterile 10-µl pipette tip from the surface of colonies that had grown on PDA for 7 days at 28°C, frozen in liquid nitrogen, and ground to a fine powder. Total genomic DNA was extracted using the E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek, Germany) according to the manufacturer's instructions.

The primers for amplification of the *tub2* gene for detecting *Colletotrichum* species included the conserved primer TB5 (GGTAACC AGATTGGTGCTGCCTT) for *Colletotrichum* spp. (Talhinhas et al. 2002), and primers specific for *C. acutatum* (TBCA, 5′-CGGAGGC CTGGTTGGGTGAG-3′), and *C. gloeosporioides* (TBCG, 5′-CG GAAGCCTGGGTAGGAGCG-3′) (Talhinhas et al. 2005). Similarly, the ITS region was also amplified using the primer specific for *C. acutatum* (*Ca*Int2) (Sreenivasaprasad et al. 1996) and the primer specific for *C. gloeosporioides* (*Cg*Int) (Mills et al. 1992), in combination with the universal primer ITS4.

PCR amplifications were performed in a 25-µl reaction volume and contained 1 µl of DNA template, 12.5 µl of TaKaRa Premix Taq (Ex Taq version, TaKaRa, Tokyo, Japan), 1 µl of 5 µM of each primer. PCR reactions were performed with a C1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA, U.S.A.) using the following conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles each consisting of 30 s at 94°C, 30 s at 59°C for TBCA and TBCG, 62°C for *Cg*Int, and 60°C for *Ca*Int2, plus extension for 60 s at 72°C, with a final extension step at 72°C for 4 min.

The PCR amplification products were visualized by electrophoresis in 1.5% agarose gels stained with GoodView Nucleic Acid Stain

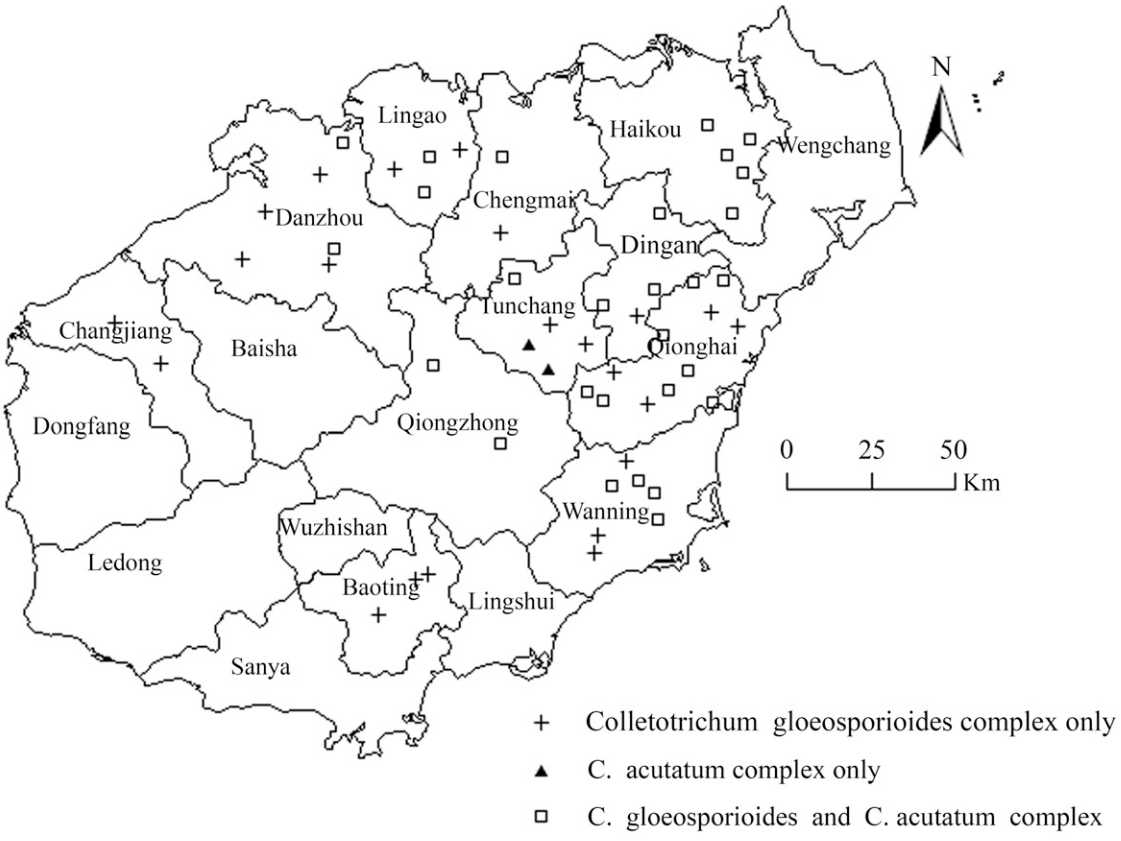

Fig. 1. Location of 52 rubber tree plantations in 11 Hainan counties sampled during 2014 and 2015 for CLD occurrence.

(Beijing SBS Genetech, Beijing, China) in 1.0× Tris-acetate acid EDTA (TAE) buffer and photographed under UV light. Images were captured using the gel reader software Gel Doc 2000 (Bio-Rad Laboratories, Inc.).

Pathogenicity tests. A number of isolates from different species complexes were selected for pathogenicity tests on detached leaves of rubber tree (Reyan 7-33-97, one of the main clones in China) under controlled conditions; 20 isolates from each species complex were tested. Leaves at the light green stage without disease symptoms were wounded by piercing with a sterilized needle. Four wounds on either side of the midrib of each leaf were made. Four PDA agar plugs (5 mm diameter) containing actively growing mycelium from the same isolate were placed onto the wounded sites on each leaf. Sterile agar plugs were used as a negative control. Treated leaves were placed on moist tissue paper, maintained in a moist chamber at 28°C, and monitored daily for lesion development. Lesion diameter at each inoculation site was measured 7 days after inoculation. The experiment follows a completely randomized design with three replicates per isolate, each replicate with three leaves. The experiment was performed twice.

Co-occurrence between the two species complexes. A generalized linear model with a Poisson error distribution was used to assess whether the two species complexes occurred together at a given plantation. Poisson sampling means that a fixed amount of effort was used to sample the diseased leaves across plantations, which were then categorized into the presence/absence of the two species complexes at each plantation. The number of plantations for the presence/absence of the two species complexes was summarized as a 2 × 2 contingency table. Then Fisher's exact test was used to assess whether the presences of the two species complexes at a given plantation were independent.

Fungicide sensitivity assays. Thirty-five isolates from CGSC and 20 from CASC, selected based on geographical origin and species complex, were tested for their sensitivities against carbendazim, chlorothalonil, and four DMI fungicides (difenoconazole, propiconazole, myclobutanil, and prochloraz) (Table 1). Each chemical was prepared in sterile distilled water and in acetone, respectively, adjusted to a concentration of 1,000 mg/ml as the stock solution. PDA was cooled to 50°C and then amended with different amounts of fungicide using the stock solution to obtain final concentrations of fungicide (Table 2). Fungicide-amended medium was poured into 9-cm-diameter Petri dishes (20 ml per dish).

Mycelial plugs (5 mm in diameter) were removed from the margin of actively growing colonies of 5-day-old cultures on PDA and placed onto the center of plates with fungicide-amended PDA. Plates were incubated at 28°C in dark to reduce any risk of fungicide photolysis. Each isolate-fungicide concentration combination had three replicate plates. When colonies in the control treatment (with application of acetone instead of DMI fungicides or water instead of carbendazim or chlorothalonil) covered 50 to 75% of the plate area (incubated 5 and 7 days for CGSC and CASC, respectively), colony diameters across two perpendicular directions were measured for all plates. The experiment was repeated once, i.e., each isolate was tested twice.

The 50% effective dose (ED_{50}) was used to measure fungicide sensitivity (Wong and Midland 2007; Wong and Wilcox 2002). The log ED_{50} was calculated from the interception of the regression line with an RG value of 50% and ED_{50} values were calculated by taking the antilog of the resulting log ED_{50} value. Fungal relative growth (RG) at a given fungicide concentration was calculated as percent (%) of fungal growth of the control plates. Regression analysis was performed to describe the relationship between RG and the log fungicide concentration and only the regressions with $P < 0.05$ were used in determination of ED_{50}. Mean log ED_{50} values for CGSC and CASC for the same fungicide were compared using two-tailed t tests with $\alpha = 0.05$. ANOVA was performed on the

Table 1. Chemical names, manufacturer, FRAC code of fungicides according to fungicide resistance action committee (FRAC) website (http://www.frac.info), and fungicide concentrations for sensitivity assays[y]

FRAC code	Chemical name[z]	Manufacturer	Concentration (μg of a.i. per ml)
1	Carbendazim 50% WP	Shanghai Shenglian Agricultural Chemical Co. Ltd., China	0, 0.0156, 0.0625, 0.25, 1, 4 for CGSC 0, 0.0625, 0.25, 1, 4, 16 for CASC
M5	Chlorothalonil 75% WP	Hainan Zhengye Zhongnong High-tech Co. Ltd., China	0, 56.25, 112.5, 225, 450, 900
3	Prochloraz 97%	Jiangsu Huifeng Agricultural Chemical Co. Ltd., China	0, 0.0039, 0.0156, 0.0625, 0.25, 1
3	Difenoconazole 95%	Shandong Weifang Rainbow Chemical Co. Ltd., China	0, 0.03125, 0.125, 0.5, 2, 8
3	Propiconazole 95%	Hainan Zhengye Zhongnong High-tech Co. Ltd., China	0, 0.03125, 0.125, 0.5, 2, 8
3	Myclobutanil 96%	Shandong Weifang Rainbow Chemical Co. Ltd., China	0, 0.0625, 0.25, 1, 4, 16

[y] CGSC = *Colletotrichum gloeosporioides* species complex, CASC = *C. acutatum* species complex.
[z] WP = wettable powder.

Table 2. Percentage of isolates recovered from foliage of rubber trees grouped according to *Colletotrichum gloeosporioides* complex and *C. acutatum* complex for each of the sampled counties in Hainan, China

			C. gloeosporioides complex		*C. acutatum* complex	
County	Number of plantations sampled	Number of isolates	Percentage of plantations	Percentage of isolates	Percentage of plantations	Percentage of isolates
Wanning	7	37	100.00	64.86	57.14	35.14
Qionghai	12	57	100.00	66.67	66.67	33.33
Haikou	5	30	100.00	60.00	100.00	40.00
Dingan	4	21	100.00	66.67	75.00	33.33
Tunchang	5	24	60.00	62.50	40.00	37.50
Qiongzhong	2	14	100.00	57.14	100.00	42.86
Baoting	3	13	100.00	100.00	0.00	0.00
Danzhou	6	31	100.00	87.10	33.33	12.90
Chengmai	2	15	100.00	86.67	50.00	13.33
Lingao	4	23	100.00	78.26	50.00	21.74
Changjiang	2	10	100.00	100.00	0.00	0.00

log ED_{50} values to compare the sensitivity of each fungal complex to the fungicides tested where the fungicide and isolate were treated as factors. To determine levels of cross-sensitivity between pairs of the fungicides tested, coefficient of correlation in the log ED_{50} value between any two fungicides was calculated for each species complex.

Results

Identification of *Colletotrichum* species complexes by morphological characterization and PCR. Based on colony characteristics, conidial morphology, growth rates, and PCR results, the 275 isolates were divided into two groups: CGSC (198) and CASC (77). Colonies of CGSC varied from white to dark gray with dense pale gray aerial mycelium; colonies of CASC were white with sparse aerial mycelium and orange conidial masses near the inoculum point (Fig. 2A). CGSC produced oblong to elliptical conidia while conidia of CASC were elliptical to fusiform (Fig. 2B). There were significant differences ($P < 0.01$) in colony growth rates between the two species complexes: 14.26 ± 0.97 mm $day^{-1}$and 8.43 ± 1.23 mm day^{-1} for CGSC and CASC, respectively. CGSC isolates had a growth rate of 11.4 to 15.7 mm day^{-1} while CASC grew 5.37 to 10.3 mm day^{-1}.

Primers *Cg*Int (specific) and ITS4 amplified a 450-bp DNA fragment for 198 isolates, which were identified as CGSC by morphological characters. For all 77 isolates of the CASC, primers *Ca*Int2 (specific) and ITS4 amplified a 450-bp DNA fragment. These results were confirmed by using primers TBCG and TBCA combined with TB5, which amplified a fragment of about 330 bp for both complexes (Fig. 3).

Pathogenicity tests. All selected CGSC and CASC isolates were pathogenic irrespective of the symptom types from which they were originally isolated. Typical disease lesions were water-soaked, darker, and circular (Fig. 2C).

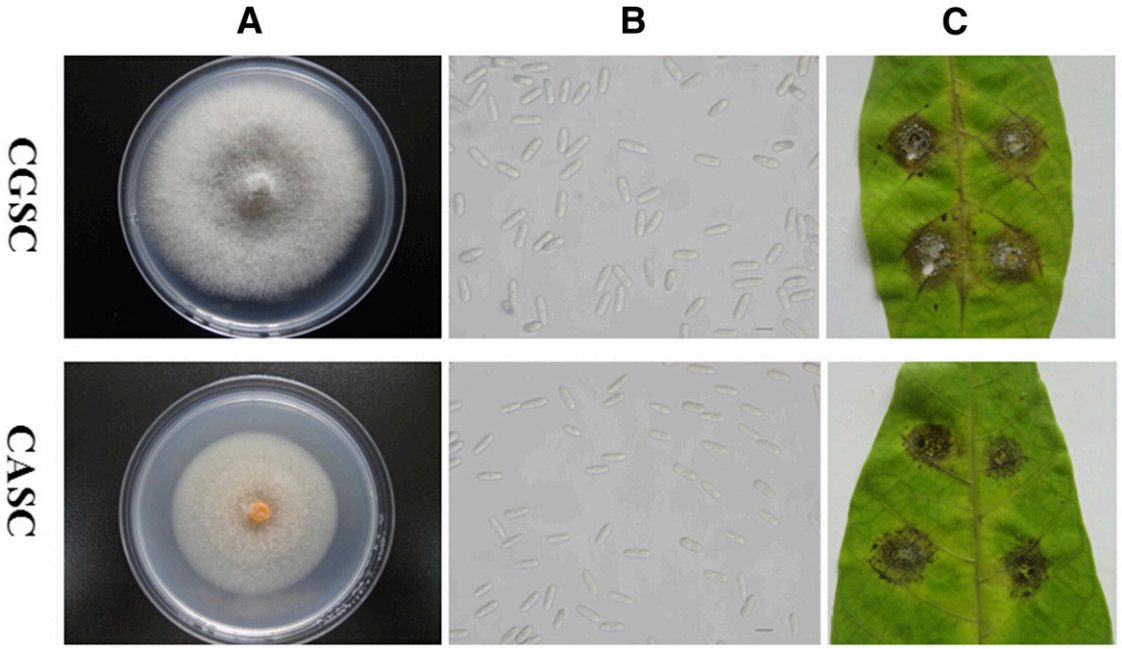

Fig. 2. A, Colony characters of *Colletotrichum gloeosporioides* complex (CGSC) and *C. acutatum* complex (CASC) isolates on PDA 7 days after inoculation. **B,** Shapes of conidia of CGSC and CASC. Scale bar = 10 μm. **C,** typical symptoms of detached rubber tree leaves inoculated with mycelial agar plugs of isolates of CGSC and CASC.

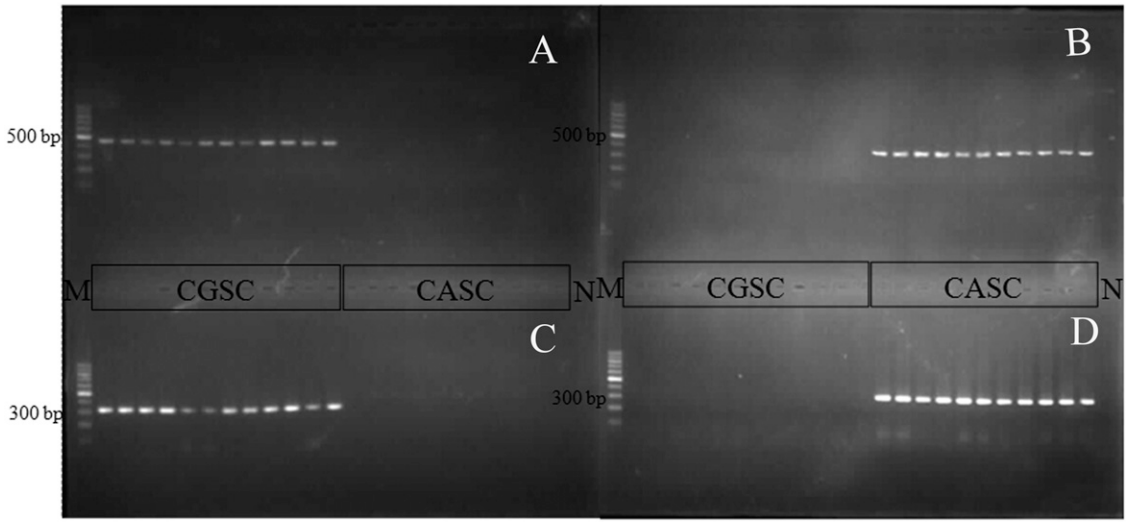

Fig. 3. Agarose gel electrophoresis of PCR products obtained with DNA of the *Colletotrichum* spp. isolates using rRNA gene-ITS primers and β-tubulin 2 primers specific for *C. gloeosporioides* complex (CGSC) and *C. acutatum* complex (CASC). **A,** CGSC-specific products generated with primers CgInt and ITS4; **B,** CASC-specific products generated with primers CaInt2 and ITS4; **C,** CGSC-specific products generated with TB5 and TBCG; **D,** CASC-specific products generated with primers TB5 and TBCA. M: 100-bp ladder and N: negative control.

Distribution of *Colletotrichum* species complexes in different counties. CGSC was obtained in all 11 counties sampled, whereas CASC was not obtained in Baoting and Changjiang (Table 2). CGSC isolates were found in all sample plantations except two in Tunchang; in contrast, CASC was obtained at every plantation only in Haikou and Qiongzhong. The percentage of isolates from CASC in western Hainan (Danzhou, Chengmai, Lingao, and Changjiang) and central south of Hainan (Baoting) was <22%, while it was >33% in other counties (Table 2).

Co-occurrence between the two species complexes. There was a significant difference in the number of isolates between the two species complexes across all plantations sampled (P = 0.0001). There was no association between the two species complexes in their presence at a given plantation (P = 0.497, Fisher's exact test).

Sensitivity to fungicides. CGSC isolates were more sensitive to carbendazim than CASC with the mean ED_{50} values of 0.176 and 2.182 μg/ml, respectively (P = 0.0001) (Fig. 4A; Table 3). However, CASC isolates were more sensitive to difenoconazole, propiconazole, and myclobutanil (mean ED_{50} values of 0.177, 0.129, and 1.424 μg/ml, respectively) than those for CGSC (mean ED_{50} values of 0.710, 0.348, and 3.496 μg/ml, respectively) (P = 0.0001) (Fig. 4C, D, and E; Table 3). The mean ED_{50} values for CGSC to chlorothalonil and prochloraz were 173.341 and 0.035 μg/ml, respectively, whereas for CASC they were 151.441 and 0.040 μg/ml, respectively (Table 3). There were no significant differences in the ED_{50} values against chlorothalonil and prochloraz between the two species complexes (P = 0.171 and P = 0.387 for chlorothalonil and prochloraz, respectively) (Fig. 4B and F).

There were significant differences in the sensitivities to different fungicides within each species complex (P = 0.0001). Among the fungicides tested, prochloraz was the most effective against the two species complexes with mean ED_{50} values of 0.035 μg/ml (ranging from 0.004 to 0.076 μg/ml) and 0.040 μg/ml (ranging from 0.008 to 0.061 μg/ml) for CGSC and CASC, respectively (Table 3). Difenoconazole and propiconazole were also effective against the two species complexes with the mean ED_{50} values of 0.710 and 0.348 μg/ml for CGSC and 0.177 and 0.129 μg/ml for CASC. ED_{50} values against myclobutanil were higher than against other DMI fungicides for both species complexes.

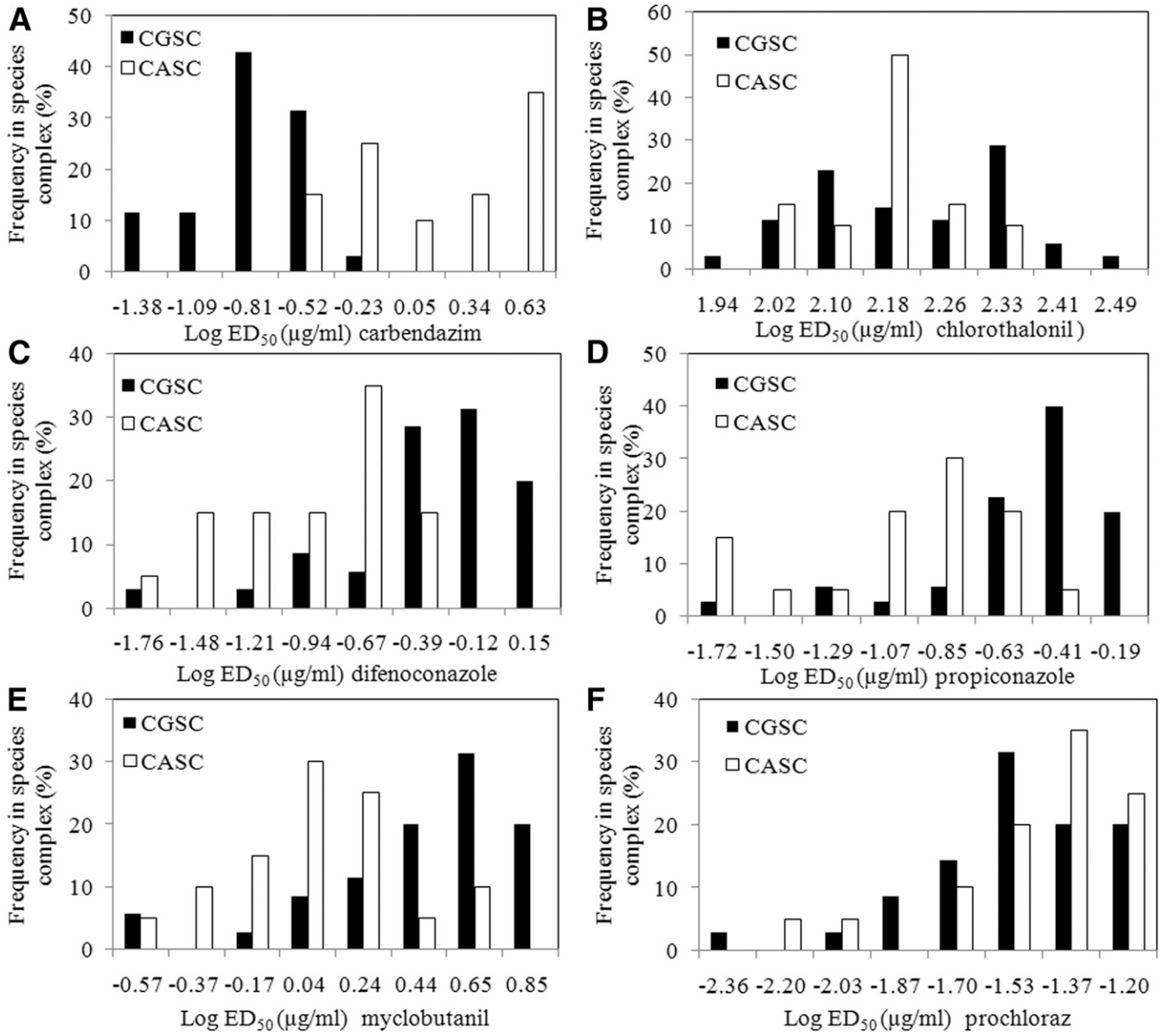

Fig. 4. Frequency distributions of sensitivities of *Colletotrichum gloeosporioides* complex (CGSC) (n = 35) and *C. acutatum* complex (CASC) (n = 20) sampled from foliage of rubber trees to carbendazim (**A**), chlorothalonil (**B**), and four demethylation inhibitor (DMI) fungicides difenoconazole (**C**), propiconazole (**D**), myclobutanil (**E**), and prochloraz (**F**) in Hainan, China. Log 50% effective dose (ED_{50}) values are used to reflect the log-normal distribution of the data and indicated values represent the midpoint value of each sensitivity category.

The ED_{50} values against chlorothalonil were the highest with the values for most isolates greater than 100 μg/ml (Table 3).

Cross-sensitivity to fungicides. Correlation coefficient (r) in ED_{50} values between carbendazim, chlorothalonil, and DMI fungicides were all below 0.3 for the two *Colletotrichum* species complexes ($P > 0.05$) (Table 4). However, strong correlations were obtained between difenoconazole and propiconazole for the two *Colletotrichum* species complexes ($r = 0.70$, $P < 0.01$). The correlation was also strong between myclobutanil and difenoconazole and between myclobutanil and propiconazole for CGSC ($P < 0.05$) but not for CASC (Table 4).

Discussion

All isolates from rubber tree leaves with CLD symptoms can be divided into two groups based on morphological traits and PCR results, corresponding to the two *Colletotrichum* species complexes. Of the two complexes, CGSC was predominant in Hainan, accounting for 72% of the isolates recovered. Results also indicate that morphological characters can be used to differentiate CGSC from CASC, agreeing with previous reports (Afanador-Kafuri et al. 2014; Álvarez et al. 2014; Jayasinghe and Fernando 1998; Pardo-De la Hoz et al. 2016; Than et al. 2008).

The relative importance of the two species complexes varies with respect to region. CASC (34 out of 52 isolates) was identified as the main cause of CLD in Sri Lanka (Jayasinghe et al. 1997), and CGSC was predominant in Hainan as shown in this study. CGSC was considered the causal agent of CLD of rubber tree in Hainan when the disease was first reported in 1956. CASC was first reported in Hainan in 2010 (Li et al. 2010) and appears to be less common in the western and central southern Hainan than in other areas. This differential distribution may be explained by several factors. CASC is more important in temperate regions whereas CGSC is more common in the tropics (Tarnowski 2009). Temperature in western and central southern Hainan is relatively high, compared with northern and central Hainan. In addition, there is less rainfall in the western region as rainfall is an important factor affecting CLD epidemics (Guyot et al. 2005). Host genotypes likely play an important role in the distribution of major *C. cereale* clades in North American turfgrass (Beirn et al. 2014). Both CGSC and CASC have a broad host range, including mango, coffee, citrus, and strawberry (Damm et al. 2012; Freeman et al. 2000; Jeffries et al. 1990; Weir et al. 2012), which are all grown to different extents in different counties of Hainan. Finally, significant differences in the sensitivity to several fungicides have been reported previously (Greer et al. 2011; Jayasinghe and Fernando 1998), as well as in the present study. There was no significant association in the presence/absence between the two species complexes at any given plantation, mostly likely because CGSC was present at all plantations except for two plantations in Tunchang.

Species of *Colletotrichum* may exhibit large differences in their sensitivity to fungicides. For example, most wild-type *C. gloeosporioides* isolates are highly sensitive to benzimidazoles fungicides while *C. acutatum* is inherently resistant to them (Greer et al. 2011; Jayasinghe and Fernando 1998). The present study confirmed that CGSC isolates were more sensitive to carbendazim than CASC. CASC isolates were more sensitive to difenoconazole, propiconazole, and myclobutanil than CGSC. The significant differences in the sensitivity of the two species complexes against carbendazim, difenoconazole, propiconazole, and myclobutanil indicated the importance of understanding their relative prevalence in a given region/plantation. The difference in sensitivity to the two fungicides may also partly explain why CASC, although first reported on rubber tree only in 2010 in Hainan, already accounted for 28% of all isolates sampled in Hainan. Carbendazim is widely used to control CLD in Hainan, which may have led to the increase of CASC at the expense of CGSC.

Differences in efficacy among DMI fungicides have been reported for *C. cereal*, which was more sensitive to tebucanazole and propiconazole than to myclobutianil (Wong and Midland 2007). Our results indicate that *Colletotrichum* spp. isolated from rubber tree in Hainan were most sensitive to prochloraz, less sensitive to propiconazole and difenoconazole, and least sensitive to myclobutanil. Therefore, prochloraz, propiconazole, and difenoconazole would be expected to provide better control than myclobutanil.

Correlation coefficients in the fungal sensitivity between carbendazim, chlorothalonil, and DMI fungicides were below 0.3 for both *Colletotrichum* species complexes. These may be related to the different mode of actions of these fungicides. Although cross-resistance is common for fungicides that share a similar mode of action, it is not the case for DMI's on *C. cereale* (Wong and Midland 2007) or with *Colletotrichum* spp. from peach (Chen et al. 2016). Also in the present study, there were strong correlations in sensitivity between difenoconazole and propiconazole for both CGSC and CASC; and between myclobutanil and difenoconazole, and myclobutanil and propiconazole for CGSC only. These may be related with the DMI resistance, which might result from multiple mechanisms such as point mutations

Table 4. Cross-sensitivity between carbendazim, chlorothalonil, and demethylation inhibitor fungicides for isolates of *Colletotrichum gloeosporioides* complex and *C. acutatum* complex sampled from foliage of rubber trees in Hainan, China[x]

Chemical name	Carben[y]	Chlor	Difen	Propi	Myclo	Proch
Carben	-[z]	0.18	0.11	0.17	0.05	0.02
Chlor	0.02	-	0.19	0.04	0.20	0.14
Difen	0.22	0.12	-	0.70*	0.10	0.28
Propi	0.11	0.16	0.70*	-	0.39	0.03
Myclo	0.05	0.18	0.40*	0.73*	-	0.10
Proch	0.01	0.22	0.10	0.07	0.04	-

[x] Correlation coefficient (r) for *C. gloeosporioides* species complex ($n = 35$) is presented in the upper triangular part of the table, while for *C. acutatum* species complex ($n = 20$) is presented in the lower triangular part. r determined by comparison of log 50% effective concentration values for each fungicide from the individual species complex; an asterisk (*) indicates correlation coefficient significant at $P = 0.05$.

[y] Carben = carbendazim, Chlor = chlorothalonil, Difen = difenoconazole, Propi = propiconazole, Myclo = myclobutanil, Proch = prochloraz.

[z] Not analyzed.

Table 3. Summary of the sensitivity to carbendazim, chlorothalonil, and demethylation inhibitor fungicides for isolates of *Colletotrichum gloeosporioides* complex and *C. acutatum* complex sampled from foliage of rubber trees in Hainan, China[y]

		C. gloeosporioides complex			*C. acutatum* complex		
		ED_{50} (μg/ml)			ED_{50} (μg/ml)		
FRAC code	Chemical name	No. isolates	Range	Mean[z]	No. isolates	Range	Mean
1	Carbendazim	35	0.030 to 0.448	0.176* e	20	0.265 to 5.870	2.182* b
M5	Chlorothalonil	35	79.472 to 340.656	173.341 a	20	100.791 to 226.889	151.441 a
3	Difenoconazole	35	0.013 to 1.939	0.710* c	20	0.019 to 0.439	0.177* c
3	Propiconazole	35	0.015 to 0.824	0.348* d	20	0.019 to 0.339	0.129* c
3	Myclobutanil	35	0.236 to 8.939	3.946* b	20	0.211 to 5.085	1.424* b
3	Prochloraz	35	0.004 to 0.076	0.035 f	20	0.008 to 0.061	0.040 d

[y] Data were transformed using Log 10 transformation before statistical analysis.

[z] Means followed by * are significantly different between different species complexes based on the two-tailed t tests of transformed data with $\alpha = 0.05$. Means followed by the same letter are not significantly different between fungicides within each species complex based on the least significant difference tests of transformed data with $\alpha = 0.05$.

within the P450 demethylation inhibitor (*CYP51*) genes, increased expression of the *CYP51* gene, and expression of ABC transporters (Hulvey et al. 2012; Sang et al. 2015).

Chlorothalonil is considered to be at low risk for resistance development due to its multisite activity. However, resistance to chlorothalonil was reported in other pathogens (Fairchild et al. 2013; Sujkowski et al. 1995). Resistance to benzimidazole and DMI fungicides has also been reported for *Colletotrichum* species, including *C. gloeosporioides* and *C. acutatum* on hosts other than rubber tree (Kim et al. 2007; Peres et al. 2004; Wong and Midland 2007; Wong et al. 2008). In the present study, a wide range of sensitivity to the fungicides was found for both species complexes. The ED_{50} value against carbendazim of a CGSC isolate from a region that did not use this fungicide was 0.064 μg/ml (Luo et al. 2003). The mean ED_{50} value of all tested CGSC isolates in the present study for carbendazim was 0.176 μg/ml. The ED_{50} values of a CGSC and a CASC from rubber tree were 104.78 and 306 μg/ml against chlorothalonil and 0.021 and 0.029 μg/ml aginst prochloraz, respectively (Cai et al. 2008). In the present study, ED_{50} values were 173.341 and 151.441 μg/ml to chlorothalonil and 0.035 and 0.040 μg/ml to prochloraz in the present study, indicating possible loss of efficacy.

This study reported the distribution and fungicide sensitivity of *Colletotrichum* species complexes from rubber tree in Hainan, which can optimize selection of the most efficacious fungicides. However, each species complex has several species. Further work is needed to identify the *Colletotrichum* spp. affecting rubber tree in Hainan using the multilocus phylogeny approach and to analysis the relationship of species abundance and the influence of environmental variables.

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Literature Cited

Afanador-Kafuri, L., González, A., Gañán, L., Mejía, J. F., Cardona, N., and Álvarez, E. 2014. Characterization of the *Colletotrichum* species causing anthracnose in Andean blackberry in Colombia. Plant Dis. 98:1503-1513.

Álvarez, E., Gañán, L., Rojas, A., Mejía, J. F., Llano, G. A., and González, A. 2014. Diversity and pathogenicity of *Colletotrichum* species isolated from Soursop in Colombia. Eur. J. Plant Pathol. 139:325-338.

Beirn, L. A., Clarke, B. B., and Crouch, J. A. 2014. Influence of host and geographic locale on the distribution of *Colletotrichum cereale* lineages. PLoS One 9:e97706.

Brown, A., and Soepena, H. 1994. Pathogenicity of *Colletotrichum acutatum* and *C. gloeosporioides* on leaves of *Hevea* spp. Mycol. Res. 98:264-266.

Cai, Z. Y., Li, J. Z., Wang, J. Q., Zhang, C. X., He, M. X., and He, L. Q. 2008. Sensitivity test of *Colletotrichum gloeosporioides* and *Colletotrichum acutatum* isolated from rubber to the fungicides. Yunnan Nong Ye Da Xue Xue Bao 23:787-790.

Chen, S. N., Luo, C. X., Hu, M. J., and Schnabel, G. 2016. Sensitivity of *Colletotrichum* species, including *C. fioriniae* and *C. nymphaeae*, from peach to demethylation inhibitor fungicides. Plant Dis. 100:2434-2441.

Damm, U., Cannon, P. F., Woudenberg, J. H. C., and Crous, P. W. 2012. The *Colletotrichum acutatum* species complex. Stud. Mycol. 73:37-113.

Fairchild, K. L., Miles, T. D., and Wharton, P. S. 2013. Assessing fungicide resistance in populations of *Alternaria* in Idaho potato fields. Crop Prot. 49:31-39.

Freeman, S., Shabi, E., and Katan, T. 2000. Characterization of *Colletotrichum acutatum* causing anthracnose of anemone (*Anemone coronaria* L.). Appl. Environ. Microbiol. 66:5267-5272.

Greer, L. A., Harper, J. D. I., Savocchia, S., Samuelian, S. K., and Steel, C. C. 2011. Ripe rot of south-eastern Australian wine grapes is caused by two species of *Colletotrichum*: *C. acutatum* and *C. gloeosporioides* with differences in infection and fungicide sensitivity. Aust. J. Grape Wine Res. 17:123-128.

Guyot, J., Omanda, E. N., Ndoutoume, A., Otsaghe, A. A. M., Enjalric, F., and Assoumou, H. G. N. 2001. Effect of controlling *Colletotrichum* leaf fall of rubber tree on epidemic development and rubber production. Crop Prot. 20:581-590.

Guyot, J., Omanda, E. N., and Pinard, F. 2005. Some epidemiological investigations on *Colletotrichum* leaf disease on rubber tree. Crop Prot. 24:65-77.

Hulvey, J., Popko, J. T., Sang, H., Berg, A., and Jung, G. 2012. Overexpression of *ShCYP51B* and *ShatrD* in *Sclerotinia homoeocarpa* isolates exhibiting practical field resistance to a demethylation inhibitor fungicide. Appl. Environ. Microbiol. 78:6674-6682.

Jayasinghe, C. K., and Fernando, T. H. P. S. 1998. Growth at different temperatures and on fungicide amended media: two characteristics to distinguish *Colletotrichum* species pathogenic to rubber. Mycopathologia 143:93-95.

Jayasinghe, C. K., Fernando, T. H. P. S., and Priyanka, U. M. S. 1997. *Colletotrichum acutatum* is the main cause of *Colletotrichum* leaf disease of rubber in Sri Lanka. Mycopathologia 137:53-56.

Jeffries, P., Dodd, J. C., Jeger, M. J., and Plumbley, R. A. 1990. The biology and control of *Colletotrichum* species on tropical fruit crops. Plant Pathol. 39:343-366.

Kim, Y. S., Min, J. Y., Kang, B. K., Bach, N. V., Choi, W. B., Park, E. W., and Kim, H. T. 2007. Analyses of the less benzimidazole-sensitivity of the isolates of *Colletotrichum* spp. causing the anthracnose in pepper and strawberry. Plant Pathol. J. 23:187-192.

Li, J. F., Liu, X. B., Cai, J. M., Lin, C. H., and Huang, G. X. 2010. Identification and the internal transcribed spacer regions analysis of pathogen causing rubber anthracnose. Chin. Agricul. Sci. Bull. 26:221-226.

Li, Z., and Fox, J. M. 2012. Mapping rubber tree growth in mainland Southeast Asia using time-series MODIS 250 m NDVI and statistical data. Appl. Geogr. 32:420-432.

Liu, B., Louws, F. J., Sutton, T. B., and Correll, J. C. 2012. A rapid qualitative molecular method for the identification of *Colletotrichum acutatum* and *C. gloeosporioides*. Eur. J. Plant Pathol. 132:593-607.

Liyanage, A. de. S. 1985. Diseases of rubber and their control. Bull. Rubber Res. Inst. Sri Lanka. 20:5-9.

Luo, D. Q., Zheng, Z. F., Fan, H. Y., and Ye, S. B. 2003. Laboratory determination of anthracnose of *Hevea brasiliesis* resistance to carbendazim fungicide in Hainan. Chin. J. Trop. Agic. 23:8-10.

Mills, P. R., Sreenivasaprasad, S., and Brown, A. E. 1992. Detection and differentiation of *Colletotrichum gloeosporioides* isolates using PCR. FEMS Microbiol. Lett. 98:137-143.

Pardo-De la Hoz, C. J., Calderón, C., Rincón, A. M., Cárdenas, M., Danies, G., López-Kleine, L., Restrepo, S., and Jiménez, P. 2016. Species from the *Colletotrichum acutatum, Colletotrichum boninense* and *Colletotrichum gloeosporioides* species complexes associated with tree tomato and mango crops in Colombia. Plant Pathol. 65:227-237.

Peres, N. A. R., Souza, N. L., Peever, T. L., and Timmer, L. W. 2004. Benomyl sensitivity of isolates of *Colletotrichum acutatum* and *C. gloeosporioides* from citrus. Plant Dis. 88:125-130.

Pu, J. J., Zhang, X., Qi, Y. X., Xie, Y. X., Zhang, H. Q., and Zhang, H. 2007. First record of *Corynespora* leaf fall disease of *Hevea* rubber tree in China. Australas. Plant Dis. Notes 2:35-36.

Saha, T., Kumar, A., Ravindran, M., Jacob, C. K., Bindu, R. O. Y., and Nazeer, M. A. 2002. Identification of *Colletotrichum acutatum* from rubber using random amplified polymorphic DNAs and ribosomal DNA polymorphisms. Mycol. Res. 106:215-221.

Sang, H., Hulvey, J., Popko, J. T., Lopes, J., Swaminathan, A., Chang, T., and Jung, G. 2015. A pleiotropic drug resistance transporter is involved in reduced sensitivity to multiple fungicide classes in *Sclerotinia homoeocarpa* (FT Bennett). Mol. Plant Pathol. 16:251-261.

Sreenivasaprasad, S., Sharada, K., Brown, A. E., and Mills, P. R. 1996. PCR-based detection of *Colletotrichum acutatum* on strawberry. Plant Pathol. 45:650-655.

Sujkowski, L. S., Fry, B. A., Power, R. J., Goodwin, S., Peever, T. L., Hamlen, R. A., and Fry, W. E. 1995. Sensitivities of Mexican isolates of *Phytophthora infestans* to chlorothalonil, cymoxanil, and metalaxyl. Plant Dis. 79: 1117-1120.

Sutton, B. C. 1992. The genus Glomerella and its anamorph Colletotrichum. Pages 1-26 in: *Colletotrichum*: Biology, Pathology and Control. J. A. Bailey and M. J. Jeger, eds. CAB International, Wallingford, U.K.

Talhinhas, P., Sreenivasaprasad, S., Neves-Martins, J., and Oliveira, H. 2002. Genetic and morphological characterisation of *Colletotrichum acutatum* causing anthracnose of lupins. Phytopathol. 92:986-996.

Talhinhas, P., Sreenivasaprasad, S., Neves-Martins, J., and Oliveira, H. 2005. Molecular and phenotypic analyses reveal association of diverse *Colletotrichum acutatum* groups and low level of *C. gloeosporioides* with olive anthracnose. Appl. Environ. Microbiol. 71:2987-2998.

Tarnowski, T. L. B. 2009. Using molecular analysis to investigate phylogenetic relationships in two tropical pathosystems: witches'-broom of cacao, caused by *Moniliophthora perniciosa*, and mango anthracnose, caused by *Colletotrichum* spp. D.Sc. Thesis, University of Florida. Available online: http://etd.fcla.edu/UF/UFE0041003/tarnowski_t.pdf

Than, P. P., Jeewon, R., Hyde, K. D., Pongsupasamit, S., Mongkolporn, O., and Taylor, P. W. J. 2008. Characterization and pathogenicity of *Colletotrichum* species associated with anthracnose disease on chilli (*Capsicum* spp.) in Thailand. Plant Pathol. 57:562-572.

Weir, B. S., Johnston, P. R., and Damm, U. 2012. The *Colletotrichum gloeosporioides* species complex. Stud. Mycol. 73:115-180.

Wong, F. P., De la Cerda, K. A., Hernandez-Martinez, R., and Midland, S. L. 2008. Detection and characterization of benzimidazole resistance in California populations of *Colletotrichum cereale*. Plant Dis. 92:239-246.

Wong, F. P., and Midland, S. L. 2007. Sensitivity distributions of California populations of *Colletotrichum cereale* to the DMI fungicides propiconazole, myclobutanil, tebuconazole, and triadimefon. Plant Dis. 91:1547-1555.

Wong, F. P., and Wilcox, W. F. 2002. Sensitivity to azoxystrobin among isolates of *Uncinula. necator*: Baseline distribution and relationship to myclobutanil sensitivity. Plant Dis. 86:394-404.

Zhang, C. X., He, M. X., Li, J. Z., Cai, Z. Y., and Wang, J. Q. 2008. Identification of the pathogen causing *Colletotrichum* leaf disease on rubber in Xishuangbanna Yunnan. Plant Prot. 34:103-106.