Molecular and Phenotypic Characterization of *Colletotrichum* Species Associated with Anthracnose Disease of Papaya in Trinidad

Sephra N. Rampersad, The University of the West Indies, Department of Life Sciences, St. Augustine. Trinidad and Tobago

ABSTRACT

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Anthracnose disease is a major limiting factor to papaya production worldwide. Accurate identification of the pathogens responsible for this disease is important to developing disease management strategies. One hundred and three (103) isolates of *Colletotrichum* were collected from infected papaya fruits cvs. Red lady and Tainung No. 2 - F1 hybrid in Trinidad. Of all isolates, 79% were *C. gloeosporioides* and 21% were *C. truncatum*. Spore morphology, cultural characteristics, differential reaction to benomyl in addition to ITS1 and β -tubulin gene sequence comparisons unequivocally identified and separated the two species of *Colletotrichum*. Certain characteristics enabled discrimination between the two species and may be used for provisional identification of these species isolated from papaya. Isolates of *C. gloeosporioides* grew at a significantly faster rate than those of *C. truncatum*. *C. gloeosporioides* isolates were sensitive to benomyl at 1.0

$\mu\text{g/ml}$, but *C. truncatum* isolates were resistant. Pathogenicity tests revealed that *Colletotrichum* species and papaya cultivar had no significant effect on lesion diameter. In cross-infection studies, isolates of *C. gloeosporioides* from mango and *C. truncatum* from sweet pepper were able to infect and cause symptoms in wounded mature papaya fruit under controlled conditions. There was no evidence of anthracnose infection in seeds of infected fruits based on the results of growing-on tests. Phylogenetic analyses were based on comparisons of ITS1 and β -tubulin gene sequences. Both neighbor-joining and maximum parsimony methods resolved all *Colletotrichum* isolates from papaya into species-specific clusters with high bootstrap support. Additionally, a pair of species-specific primers were developed (Ct-TUB-F/R) which allowed reliable detection of *C. truncatum* isolates from papaya.

Papaya (*Carica papaya* L.) accounted for the largest share of tropical fruit production in developing countries in 2010. The leading producers are Asia and the Pacific (56%), South and Central America and the Caribbean (32%), and Africa (11%) (24). To date, imports are dominated by Europe, the United States, and Canada (74). In Trinidad, papaya is an important fruit crop, and the main cultivars grown are 'Red lady' and 'Tainung No. 2 - F1 hybrid'. Papaya production is mainly concentrated in South Trinidad (87% of production) and is the third largest orchard-type of cultivation after orange and pineapple (11). The major markets are the domestic fresh fruit market and the agro-processing industry, including export markets in other regional countries and the UK. Competition from Belize, Jamaica, and Mexico, as well as development of postharvest diseases during trans-shipment, have been identified as possible reasons for the decline in papaya exports over a 10-year period.

Colletotrichum species cause anthracnose disease in a number of hosts, including legumes, tree crops, fruits, and vegetables, and are among the most economically important plant pathogens worldwide. In some cases, several *Colletotrichum* species or biotypes can form an infection complex in a single host (27). Anthracnose of papaya is commonly caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (teleomorph *Glomerella cingulata* (Stoneman) Spauld & Schrenk) and is a major postharvest disease throughout papaya production areas in the tropics and subtropics (46). Symptoms of anthracnose infection of papaya fruit are typi-

cally seen as distinct circular sunken lesions on mature to ripe fruit. However, the fungus can also cause stem lesions, leaf spot, defoliation, and seedling damping-off (79,80).

There are several methods aimed at reducing surface rot of papaya fruit. These approaches include, singly or in combination, the use of protectant fungicides as preharvest applications and postharvest disinfectant dips for harvested fruit, prevention of mechanical and physiological injury to the fruit, modifying the storage atmosphere of stored fruit, heat treatments (14,41), chemical waxes (35,64), and irradiation (15,46). Treatments that stimulate the production of antifungal diene compounds in the fruit skin may also delay symptom development (15). However, in most cases, such methods of controlling postharvest rot have inherent problems that affect uniform ripening of the harvested fruit (41). Other studies describe a transgenic approach to delaying fruit senescence through transformation with a ripening-related acc synthase gene (ACS1/ACS2) (38,42,48) and with acc oxidase genes (CP-ACO1/CP-ACO2) from papaya fruit (7,61).

Conventional methods of detection and characterization of anthracnose fungi are based on morphology of colonies, conidia and appressoria, optimal temperature for mycelial growth, growth rate, presence or absence of setae, existence of a *Glomerella* teleomorph, affiliation with a specific host species, and benomyl sensitivity (8,27,52,67). However, the stability of morphological attributes is subject to environmental influences which can result in considerable variation at the intra- and inter-species level (27). Further, identification using spore morphology is time-consuming, tedious, and subjective as spore morphology can be confused with those of other fungal pathogens (27). Differentiation among *Colletotrichum* species based on host range may also not be reliable, since certain species, such as *C. gloeosporioides*, *C. acutatum*, and *C. truncatum* (*C. truncatum* syn. *C. capsici* [17]), infect a wide range of host plants (8,67).

Molecular methods are used in conjunction with traditional methods of diagnosis. Polymerase chain reaction (PCR)-based methods include but are not limited to internal transcribed spacer (ITS) sequence analysis of the rDNA region using universal primers and species-specific primers that target conserved regions of

Corresponding author: Sephra N. Rampersad,
E-mail: sephra.rampersad@sta.uwi.edu

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the ITS1-5.8S-ITS2 region (5,44,65). Restriction fragment length polymorphism (RFLP) analysis of rDNA, analysis of A+T-rich mitochondrial DNA (mtDNA), comparison of arbitrarily primed (AP)-PCR or random amplified polymorphic DNA (RAPD)-PCR-generated DNA fingerprints (25,27–29,68) have also been used to differentiate populations of *Colletotrichum* species. Hyde et al. (36) and Cai et al. (8) both described the usefulness of a polyphasic approach to characterizing these species and suggested the use of multiple loci sequence comparisons of different genes (e.g., partial β -tubulin, actin, calmodulin, glutamine synthase, and glyceraldehyde-3-phosphate-dehydrogenase) in resolving the taxonomic status of closely related *Colletotrichum* species.

There are two distinct types of symptoms associated with anthracnose disease of papaya in Trinidad. One type is observed as sunken circular lesions, sometimes with a pinkish gelatinous effect, and the other type is seen as large dark brown to black circular lesions. Both symptoms can appear on either papaya cultivar, usually on mature yellow fruit, but also on fruit at the “three-stripe” maturity stage. In Trinidad, anthracnose disease incidence is between 90 and 100% for most fields, and yield loss is estimated at 40 to 60% for retailers who cater to the fresh fruit market. It was hypothesized that more than one *Colletotrichum* species may be responsible for these symptoms. The objectives of this work were to identify and characterize *Colletotrichum* species responsible for anthracnose disease in papaya in Trinidad based on a polyphasic approach (8).

Materials and Methods

Isolate collection and maintenance. Between December 2009 and February 2010, symptomatic fruits were collected from the main production areas in Trinidad (Caura Valley, Princes Town, Tableland, Macoya, Valencia, Aranguez, Sangre Grande, Penal) and from retailers throughout Trinidad. Fruits were surface sterilized by rinsing in 70% ethanol for 2 min followed by another rinse in sterile distilled water for 2 min. A 4-mm³ block taken from the edge of the lesion was plated on water agar (BactoAgar, Difco) supplemented with 50 mg/liter each of streptomycin, tetracycline, and chloramphenicol (Sigma-Aldrich Co. Ltd.). Cultures were incubated at 25 $\pm$ 1°C for 5 days. Emerging colonies were then transferred to potato dextrose agar (PDA) (Oxoid Ltd.). Monoconidial cultures were maintained on PDA slants at 4°C for short-term storage and were subcultured every 4 weeks (39). Conidia were kept in glycerol at –70°C for long-term storage. Morphological characterization was carried out on the first pure culture obtained for each isolate.

Morphological and cultural characterization of isolates. A 4-mm³ agar block taken from the advancing edge of a 5-day-old actively growing culture was transferred to PDA. Morphological features of colonies and conidia for all 103 isolates were recorded after 5 days incubation at 25 $\pm$ 1°C on PDA with a 12:12 alternating dark and light photoperiod (67). The colony diameter (orthogonal measurements) was recorded daily, and the rate of growth was calculated based on linear regression. Colony characteristics were also recorded. The size and shape of 20 conidia from each culture were recorded under a stereomicroscope ($\times$ 100 obj.) The temperature requirements for optimal growth of these isolates were also evaluated at seven temperature ranges: 15 to 17°C, 18 to 20°C, 21 to 23°C, 24 to 26°C, 27 to 29°C, 30 to 32°C, and 33 to 35°C. Morphological characterization and determination of optimal temperature for growth in vitro were based on triplicate cultures, and experiments were conducted twice.

Benomyl sensitivity. Molten PDA was amended with benomyl (active ingredient [a.i.] 50% Benlate) to achieve four different final concentrations of a.i.: 0.0 μ g/ml, 0.1 μ g/ml, 1.0 μ g/ml, and 10.0 μ g/ml. Benomyl was prepared in DMSO (dimethyl sulfoxide; Sigma-Aldrich) as a 0.2 g/ml stock solution. Various volumes of this stock were then added to molten PDA to obtain the required concentrations of benomyl and to keep the final concentration of DMSO below 0.2%. The amended agar was swirled vigorously to ensure that the contents were thoroughly mixed. A 4-mm³ agar

block was removed from the advancing edge of an actively growing colony and placed in the center of the amended PDA plate so that mycelium was in contact with the surface of the medium. Plates were incubated at 25°C for 5 days, and the radial diameter of each colony was measured (mm). Six isolates from papaya and two isolates from mango and sweet pepper were tested in duplicate, and the experiment was conducted twice. Mean values of both experiments were presented as percent growth of the isolates compared with the nonamended control.

Pathogenicity tests. Pathogenicity tests were conducted to determine the ability of the isolates to evoke symptoms on detached fruits of two cultivars of papaya (cvs. Red lady and Tainung No. 2 - F1 hybrid) under controlled conditions. Conidia were harvested by flushing the surface of an active 5-day-old culture grown on PDA with 10 ml of sterile distilled water. The surface of the plate was scraped with a sterile scalpel blade, and the resulting suspension was filtered through two layers of mira cloth. The concentration of conidia was adjusted to a final working concentration of 2.0×10^4 conidia per ml. Fruits that were at the first color break stage of ripening (i.e., the one-stripe stage: one yellow stripe just visible at the blossom end) were first surface-sterilized then inoculated. Ten microliters of conidial suspension were dispensed at each wound and at nonwounded sites (6 sites per fruit along the longitudinal axis) on the fruits using the wound/nonwound-microdroplet method of Than et al. (75). Inoculated fruits (for each isolate, four fruits of each cultivar were inoculated) were placed in large plastic containers that were partially sealed to maintain relatively high humidity. Negative controls were fruits treated with sterile distilled water. Fruits were monitored for the onset of symptoms for 7 days. Additionally, two isolates of *C. gloeosporioides* from mango (*Mangifera indica* L. ‘Long mango’) and two isolates of *C. truncatum* from sweet pepper (*Capsicum annum* L. ‘Aristotle’) were used in cross-pathogenicity tests of both cultivars of papaya. The pathogenicity test was conducted twice.

Detection of *C. gloeosporioides* in papaya seeds. Growing-on tests were conducted to determine whether seeds from symptomatic fruit were infected with *C. gloeosporioides* and *C. truncatum* and were able to transmit these pathogens to seedlings. Seeds were removed from infected papaya fruit (20 fruits of Red lady and 20 fruits of Tainung No. 2 - F1 hybrid), rinsed in running water, and then imbibed in sterile distilled water for 24 h. Seeds then were sown in sterile potting mix in seedling trays. Total nucleic acids were extracted from the leaves of 50 3-week-old seedlings of each cultivar. Leaf and root sections were plated in triplicate as previously described. The experiment was conducted twice.

PCR amplification and sequencing. Molecular identification of the isolates was based on ITS rDNA and partial β -tubulin gene sequence comparisons. Total genomic DNA was isolated using the E.Z.N.A. SP fungal DNA extraction kit (Omega Bio-tek Inc.). PCR amplification was carried out using the universal primer pair ITS4/5 (ITS region of the nuclear ITS1-5.8S-ITS2 rDNA) (83), *C. gloeosporioides*-specific primers CgInt/ITS4 and *C. acutatum*-specific primers CaInt2/ITS4 (65), *Colletotrichum*-specific primers Col1/2 (43) and Bt2a/b primers (partial β -tubulin gene) (31). For a single reaction of 25 μ l, PCR components (Invitrogen by Life Technologies) included 1 $\times$ PCR buffer; 1.5 mM MgCl₂, 0.2 mM dNTP, 1% Tween 20, 0.4 mg/ml BSA (Sigma-Aldrich Co.), 2.5 U *Taq* DNA Polymerase, and 50 pmol of each primer (Integrated DNA Technologies). Standard PCR amplification conditions were applied using a Techne Thermocycler (Techne Co.): initial denaturation of 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, with a final extension of 5 min at 72°C. PCR products were visualized on 1.4% agarose gels stained with 10 mg/ml ethidium bromide. PCR products were sequenced in both directions (Amplicon Express). Based on the β -tubulin sequence data for *C. truncatum* isolates from papaya, a pair of *C. truncatum*-specific primers were developed (Ct-TUB-F/R) using the PrimerSelect Module of Lasergene version 7.0 software (DNASTAR, Inc.). The degree of specificity of these primers to other potential target sequences available in the public databases

was determined using Primer Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

Phylogenetic analyses. Initial comparisons of cognate sequences available in the GenBank and EMBL public databases were made using the gapped BLAST algorithm. Multiple sequence alignments were performed with the MEGALIGN module of Lasergene using the CLUSTALW algorithm. Adjustments for delimiting the start and stop positions for the alignments were done manually using the EDITSEQ module. Phylograms based on ITS1 (45) and partial β -tubulin gene (75) sequence comparisons were constructed using the neighbor-joining (NJ) (58) and maximum parsimony (MP) methods in MEGA 4 – Molecular Evolutionary Genetic Analysis Software version 4 for Windows (69,70). NJ analysis was conducted using the maximum composite likelihood (MCL) model for estimating evolutionary distances (d_{ij}) between DNA frequencies. The MP tree was obtained using the close-neighbor-interchange algorithm. Model parameters were empirically determined from the sequences. Bootstrap (BS) analysis with 1,000 repetitions was performed to test the significance of the nodes and the robustness of the trees (23). A bootstrap threshold of

50% was retained for all branches. In addition to the sequences generated in this study, interspecific multiple alignments included other ITS1 and β -tubulin sequences obtained from GenBank. *C. crassipes* was used as the outgroup (13).

Statistical analyses. Analyses of variance (ANOVA) were conducted to determine the significance of differences in conidia dimensions, growth rates, and lesion diameters postinoculation with *C. gloeosporioides* and *C. truncatum* isolates. Percentages were arcsine-transformed prior to analyses. Means were separated using Fisher's least significant difference (LSD) test with a significance threshold of $P \leq 0.05$. Replications did not serve as sources of variation, and the mean values and standard error of the mean for all experiments were reported. All statistical analyses were computed using MINITAB 15.0 software package for Windows (MINITAB Inc.).

Results

Identification of isolates. One hundred and three (103) isolates of *Colletotrichum* were collected from infected papaya fruits Red lady and Tainung No. 2 - F1 hybrid. Symptoms of field-infected fruit are shown in Figure 1A and B. Identification of these isolates was based on morphological attributes and sequence comparisons of the ITS1 region of rDNA and β -tubulin gene. Seventy-nine percent of all isolates were *C. gloeosporioides* and 21% were *C. truncatum*. Of the Red lady fruits tested, 85% of isolates collected were identified as *C. gloeosporioides* and 15% as *C. truncatum*. Of the Tainung No. 2 - F1 hybrid fruits tested, 81% of the isolates collected were identified as *C. gloeosporioides* and 19% as *C. truncatum*. The study also identified four isolates of *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk 1903, the teleomorph stage of *C. gloeosporioides* based on sequence data.

Fig. 1. Symptoms of anthracnose disease in papaya in Trinidad. **A**, Fruit with sunken lesions with salmon to pink-colored spore masses toward the center indicative of *Colletotrichum gloeosporioides* infection. **B**, Fruit with dark brown to black lesions associated with *C. truncatum* infection.

Fig. 2. Morphotypes of isolates of *Colletotrichum gloeosporioides* and *C. truncatum* from papaya. Bar represents 25 μ m. Colony characteristics: Plates in column A, reverse view; B, aerial view; and C, typical conidia shape.

Morphological examination of colony and conidia characteristics. *C. gloeosporioides* isolates were grouped into four distinct morphological types (morphotypes 1 to 4), and *C. truncatum* had only one morphotype (morphotype 5) based on colony attributes (Fig. 2). Thirty percent of all isolates belonged to morphotype 1, which had olive-colored colonies with a dark gray conidial mass in the center. Isolates belonging to morphotype 2 were the least common (6%) and had cream-to-salmon-colored colonies with a salmon-colored conidial mass in the center. Isolates belonging to morphotype 3 (30%) had cream-to-pink-colored colonies with a light gray-colored conidial mass in the center. Diurnal zonation was apparent for morphotypes 1 to 3. Sixteen percent of the isolates belonged to morphotype 4. These isolates had colonies that were white and flocculose with no apparent conidial mass in the center. Colonies of morphotype 5 (18%) were tan-colored with no apparent conidial mass in the center and were exclusively *C. truncatum* type isolates (Table 1). Some of the isolates did not sporulate readily in culture despite varying incubation conditions and media type. Nevertheless, conidia morphology was distinct for each *Colletotrichum* species. Conidia of *C. gloeosporioides* isolates were cylindrical and rounded at both ends, sometimes more pointed at one end, whereas conidia of *C. truncatum* isolates were falcate.

Growth rate and optimum temperature for growth. The optimum temperature for growth of *C. gloeosporioides* and *C. truncatum* isolates was 25 to 27°C. No growth was observed for any isolates incubated above 31°C. Isolates of *C. gloeosporioides* grew at significantly faster rates ($P = 0.001$) than those of *C. truncatum* (Table 1).

Benomyl sensitivity. All isolates grew on PDA amended with 0.1 µg/ml benomyl. However, isolates showed a differential reaction to benomyl at 1.0 µg/ml. Isolates of *C. gloeosporioides* from

mango and from papaya were equally sensitive to benomyl at concentrations ≥ 1.0 µg/ml, but *C. truncatum* isolates from papaya and sweet pepper were resistant even at 10.0 µg/ml (Table 2). Isolates with mean diameters representing $>60\%$ of growth compared to the nonamended control were considered resistant to benomyl.

Pathogenicity tests. It was found that site-specific inoculation of nonwounded detached fruit did not result in lesion formation for either cultivar despite using a longer incubation period (7 to 10 days resulted in severely decayed unusable fruits) and a higher concentration of inoculum (10^7 conidia per ml rather than 10^4 conidia per ml). Also, Red lady ripened at a much faster rate than Tainung No. 2 - F1 hybrid. At each inoculation site, however, whitish-to-gray mycelial growth was observed. A 4-mm³ block of fruit tissue was taken from each inoculation site, including the negative control site that was treated with sterile distilled water, and plated as previously described. No colonies grew for the negative controls. Colonies that originated from fruit tissue cultures were confirmed to be *C. gloeosporioides* and *C. truncatum*.

There were no significant differences in mean lesion diameter (mm) that developed on wound-inoculated Red lady and Tainung No. 2 - F1 hybrid cultivars. ANOVA results revealed that isolate and papaya cultivar had no effect on lesion diameter ($P = 0.928$ and $P = 0.448$, respectively). *C. gloeosporioides* isolates from mango and *C. truncatum* from sweet pepper caused lesions to develop in inoculated fruit of both cultivars, but these were not significantly different in size compared to those caused by *C. gloeosporioides* and *C. truncatum* isolates from papaya ($P = 0.073$; Table 3). Koch's postulates were fulfilled with the reisolation of the pathogens from symptomatic fruit.

Growing-on tests. In a growing-on test, 3-week-old seedlings were grown from seeds taken from infected fruit of Red lady and

Table 1. Morphological and cultural characteristics of *Colletotrichum* species isolated from papaya

<i>Colletotrichum</i> species	Colony appearance	Morpho-type	% Isolates ^x	Conidia length (L) Mean $\pm$ SE	Conidia width (W) Mean $\pm$ SE	L:W ratio	Conidia shape	Growth rate ^y Mean $\pm$ SE
<i>C. gloeosporioides</i>	Olive-colored colony with dark gray-colored conidial mass in center	1	30	13.6 $\pm$ 0.6 b ^z	3.4 $\pm$ 0.1 a	4.04 b	Cylindrical with rounded ends	11.8 $\pm$ 0.2 b Fast growing
<i>C. gloeosporioides</i>	Cream to light salmon-colored colony with no visible conidial mass in center	2	6	12.5 $\pm$ 0.2 b	2.9 $\pm$ 0.1 ab	4.33 b	Cylindrical with rounded ends	11.6 $\pm$ 0.1 b Fast growing
<i>C. gloeosporioides</i>	Cream to pink-colored colony with grayish-colored conidial mass in center	3	30	12.5 $\pm$ 0.2 b	2.9 $\pm$ 0.1 ab	4.34 b	Cylindrical with rounded ends	10.1 $\pm$ 0.5 c Moderate growing
<i>C. gloeosporioides</i>	White flocculose colony with no visible conidial mass in center	4	16	11.7 $\pm$ 0.3 c	2.8 $\pm$ 0.1 ab	4.27 b	Cylindrical with rounded ends	12.3 $\pm$ 0.3 a Fast growing
<i>C. truncatum</i>	Tan-colored colony with no visible conidial mass	5	18	19.5 $\pm$ 0.3 a	2.3 $\pm$ 0.1 b	8.75 a	Falcate	7.2 $\pm$ 0.2 d Slow growing

^x Percentages rounded to nearest whole number.

^y Conidia length and width measured in µm. Growth rate measured as lesion diameter in mm per day.

^z Mean values in columns followed by the same letter are not significantly different ($P \leq 0.05$) according to an LSD test.

Table 2. Percent growth in amended agar assays to compare benomyl sensitivity of *Colletotrichum truncatum* and *C. gloeosporioides* isolates from papaya

Host	Isolate	<i>Colletotrichum</i> species	Benomyl/a.i. µg/ml			Reaction ^z
			0.1	1.0	10.0	
<i>Carica papaya</i>	PAW-41	<i>C. gloeosporioides</i>	100.0	0.0	0.0	S
<i>C. papaya</i>	PAW-65	<i>C. gloeosporioides</i>	94.3	0.0	0.0	S
<i>C. papaya</i>	PAW-62	<i>C. gloeosporioides</i>	66.0	0.0	0.0	S
<i>C. papaya</i>	PAW-7	<i>C. truncatum</i>	88.9	84.5	86.7	R
<i>C. papaya</i>	PAW-14	<i>C. truncatum</i>	100.0	100.0	100.0	R
<i>C. papaya</i>	PAW-20	<i>C. truncatum</i>	88.5	71.5	70.0	R
<i>Mangifera indica</i>	L1-Man	<i>C. gloeosporioides</i>	88.0	0.0	0.0	S
<i>M. indica</i>	L2-Man	<i>C. gloeosporioides</i>	100.0	0.0	0.0	S
<i>Capsicum annuum</i>	PEP-CC1	<i>C. truncatum</i>	87.2	98.6	94.1	R
<i>C. annuum</i>	PEP-CC2	<i>C. truncatum</i>	91.7	98.0	97.3	R

^z Reaction to benomyl: S = sensitive ($<60\%$ growth compared to control), R = resistant ($>60\%$ growth compared to control).

Tainung No. 2 - F1 hybrid cultivars to determine whether seeds and seedlings can serve as reservoirs of the pathogens. Neither *C. gloeosporioides* nor *C. truncatum* was found in these seedlings, and tissue sections taken from leaves of these seedlings showed no evidence of infection by either *Colletotrichum* species.

PCR and sequencing. PCR amplification with universal primers ITS4/5 and genus-specific primers Col1/2 directed the synthesis of single amplicons that were ~600 bp and ~425 bp in length, respectively. Representative ITS1-5.8S-ITS2 sequences for isolates of *C. gloeosporioides* and *C. truncatum* were submitted to GenBank (HQ287578 and HQ287579, respectively). Species-specific PCR amplification with CgInt/ITS4 resulted in a single amplicon that was ~450 bp for only *C. gloeosporioides* isolates. DNA from *G. cingulata* did not amplify with CgInt/ITS4 primers. No PCR products of expected band size (~425 bp) were obtained with *C. acutatum*-specific primers for any of the isolates. Amplification using primer pair Bt2a/b generated a single amplicon that was ~500 bp in length for all isolates. Representative β -tubulin sequences for isolates of *C. gloeosporioides* and *C. truncatum* were submitted to GenBank (HQ287580 and HQ287581, respectively). Primers (Ct-TUB-F: 5'-AGAACATCTCTGGCGAGCAT-3' and Ct-TUB-R: 5'-GCACGGGTACGTACTTGT-3') were designed for the detection of *C. truncatum* isolates from papaya. These primers generated a single amplicon that was 174 bp in length for only *C. truncatum* isolates (Fig. 3). Negative controls consisted of *C. gloeosporioides* isolates from papaya (PAW-41, PAW-46, PAW-53) in Trinidad, *F. solani* from pumpkin (*F. solani* pu) and sweet pepper (*F. solani* swp) in Trinidad, and *C. gloeosporioides* isolate from papaya (Cg-Mexico) in Mexico. The positive control was a *C. truncatum* isolate from papaya in Mexico (Ct-Mexico).

Phylogenetic analyses. ITS1 and β -tubulin gene sequences of all papaya isolates were compared to cognate sequences of epitype strains of *C. truncatum* (62) and *C. gloeosporioides* (9). Phylograms were generated to confirm interspecific separation of *C. truncatum* and *C. gloeosporioides* isolates from papaya from various countries and to determine whether any intra-specific differences among only *C. truncatum* and *C. gloeosporioides* isolates from papaya can be revealed using ITS and β -tubulin sequences. *C. truncatum* isolates from papaya shared 99 and 98% similarity with sequences of an epitype strain (AY376526 and EF683602) for ITS and β -tubulin gene sequences, respectively. *C. gloeosporioides* isolates from papaya shared 100 and 99% similarity with sequences of an epitype strain for ITS and β -tubulin gene sequences, respectively (FJ972609 and FJ907445). NJ and MP phylograms were constructed and compared for ITS1 and β -tubulin gene sequences (Only the NJ phylograms are shown in Figures 4 and 5). Phylogenetic relatedness of the ITS1 region of 49 taxa of *Colletotrichum* was inferred using neighbor-joining and maximum parsimony methods. The ITS analysis included sequences of several papaya isolates based on their availability from GenBank: eight *C. truncatum* isolates from Mexico, one *C. gloeosporioides* isolate from Malaysia, three *C. gloeosporioides* isolates from South Af-

rica, and two *C. gloeosporioides* isolates from Mexico (Table 4). The NJ-ITS1 tree positioned the *Colletotrichum* isolates into species-specific clades. *C. gloeosporioides* isolates from papaya were placed in a well-resolved cluster with other *C. gloeosporioides* isolates regardless of geographic origin. *C. gloeosporioides* isolates from papaya shared between 98.5 to 100.0% sequence similarity. *C. truncatum* isolates from papaya were positioned with other *C. truncatum* isolates in a species-specific cluster with high bootstrap support. Sequences of *C. truncatum* isolates from papaya shared between 99.6 and 100.0% similarity. The MP-ITS1 tree had similar topological characteristics to the NJ-ITS1 tree except that species-specific clades were supported by higher bootstrap values. Tree statistics were calculated where the consistency index (CI) was 0.6868 and the retention index (RI) was 0.9168. There were 188 positions in the final dataset, of which 177 were parsimony informative.

The NJ- β -tubulin tree resolved 36 *Colletotrichum* taxa into species-specific clades. *C. gloeosporioides* and *C. truncatum* isolates from papaya shared 96.6 to 99.4% and 95.8 to 99.6% sequence similarity, respectively. Tree topology was similar for NJ and MP trees with respect to placement of taxa. For the most part, the member isolates of these subgroups were consistent for both MP-ITS1 and MP- β -tubulin trees. The CI was 0.6079 and the RI was 0.8726. There were 318 positions in the final dataset, of which 317 were parsimony informative. For both ITS and β -tubulin trees, Trinidad isolates were not separated according to field location, original host cultivar, or morphotype.

Discussion

A polyphasic approach was undertaken with the aim of identifying and characterizing the *Colletotrichum* species responsible for

Fig. 3. Polymerase chain reaction (PCR) amplification of *Colletotrichum truncatum* isolates from papaya using species-specific primers. **A**, PCR amplification, using Ct-TUB-F/R, resulted in a single PCR product (~174 bp) for only *C. truncatum* isolates from Trinidad and one from Mexico, and **B**, PCR amplification, using Ct-TUB-F/R, for negative controls consisting of *C. gloeosporioides* isolates from papaya (PAW-41, PAW-46, PAW-53) in Trinidad, *Fusarium solani* from pumpkin (*F. solani* pu) and sweet pepper (*F. solani* swp) in Trinidad, and one *C. gloeosporioides* isolate from papaya from Mexico (Cg-Mexico).

Table 3. Pathogenicity of *Colletotrichum truncatum* and *C. gloeosporioides* isolates on detached papaya fruit 'Red lady' and 'Tainung No. 2 - F1 hybrid'

Host - cultivar	Isolate	<i>Colletotrichum</i> spp.	Lesion diameter in mm $\pm$ SE ^z	
			Red lady	Tainung No. 2 - F1 hybrid
Papaya - Tainung No. 2 - F1 hybrid	PAW-53	<i>C. gloeosporioides</i>	10.2 $\pm$ 0.2 c	15.5 $\pm$ 0.1 a
Papaya - Tainung No. 2 - F1 hybrid	PAW-2	<i>C. gloeosporioides</i>	10.1 $\pm$ 0.1 c	15.2 $\pm$ 0.6 a
Papaya - Red lady	PAW-60	<i>C. gloeosporioides</i>	14.2 $\pm$ 0.4 a	14.2 $\pm$ 0.2 a
Papaya - Red lady	PAW-63	<i>C. gloeosporioides</i>	13.0 $\pm$ 0.5 ab	14.1 $\pm$ 0.2 a
Papaya - Tainung No. 2 - F1 hybrid	PAW-48	<i>C. truncatum</i>	15.0 $\pm$ 0.4 a	14.0 $\pm$ 0.2 a
Papaya - Tainung No. 2 - F1 hybrid	PAW-21	<i>C. truncatum</i>	14.3 $\pm$ 0.6 a	13.2 $\pm$ 0.4 ab
Papaya - Tainung No. 2 - F1 hybrid	PAW-49	<i>C. truncatum</i>	15.1 $\pm$ 0.4 a	13.0 $\pm$ 0.6 ab
Mango - Long	L1-Man	<i>C. gloeosporioides</i>	13.3 $\pm$ 0.5 ab	14.1 $\pm$ 0.3 a
Mango - Long	L2-Man	<i>C. gloeosporioides</i>	15.0 $\pm$ 0.5 a	11.9 $\pm$ 0.3 b
Sweet pepper - Aristotle	PEP-CC1	<i>C. truncatum</i>	12.0 $\pm$ 0.0 ab	9.5 $\pm$ 0.0 c
Sweet pepper - Aristotle	PEP-CC2	<i>C. truncatum</i>	13.0 $\pm$ 0.0 ab	13.4 $\pm$ 0.0 ab

^z Values in columns followed by the same letter are not significantly different ($P \leq 0.05$) according to an LSD test. Mean lesion diameters used in the comparison were those calculated for two replicates, four fruits per cultivar for each isolate.

causing anthracnose disease in papaya in Trinidad. Spore morphology, cultural characteristics, differential reaction to benomyl, and ITS1 and β -tubulin gene sequence comparisons identified and separated two species of *Colletotrichum*, *C. gloeosporioides* and *C. truncatum*, responsible for anthracnose disease in papaya in Trinidad.

Isolates of *C. gloeosporioides* and *C. truncatum* were pathogenic to mature papaya fruit in Trinidad. Both species were also found to infect papaya in Mexico (71), Florida (72), Japan (84), and Thailand (60). *C. truncatum* is considered to be of quarantine importance for papaya importation into Chile and is associated with

Fig. 4. Phylogram generated from ITS data using the neighbor-joining method. The optimal tree is shown. Percentage of replicate trees in which associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branch nodes with a bootstrap threshold greater than 50%. All positions containing gaps and missing data were eliminated from the dataset.

anthracnose in peppers (*Capsicum* spp.) (3,75), but there are more than three hundred host-pathogen combinations reported from different geographical regions worldwide for this *Colletotrichum* species (22). Phoulivong et al. (54) reported that fungal pathogens belonging to two *Colletotrichum* species complexes (*C. gloeo-*

sporioides species primarily and *C. acutatum* to a lesser extent) were responsible for anthracnose disease in a number of tropical fruits in Laos and Thailand. Preliminary designations for the *C. acutatum* species complex included *C. simmondsii*, *C. fiorinae*, *C. lupinii*, and possibly *C. trichellum*. Members of the *C. gloeo-*

Fig. 5. Phylogram generated from partial β -tubulin sequences using the neighbor-joining method. The optimal tree is shown. Percentage of replicate trees in which associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branch nodes with a bootstrap threshold greater than 50%. All positions containing gaps and missing data were eliminated from the dataset.

sporioides species complex were *C. asianum*, *C. fructicola*, *C. hori*, *C. hymenocallidis*, *C. kahawae*, *C. siamense*, *C. gloeosporioides*, and several other species (e.g., *C. musae*) that were yet to be epitypified.

Although the lesions produced by both species in field-infected fruit were distinct, in vitro tests did not allow this difference to be recorded, as the rapid rate of fruit decay terminated the pathogenicity test before any difference in lesion appearance could be noted. In field-infected fruits, sunken lesions with salmon-to-pink-colored spore masses toward the center were indicative of *C. gloeosporioides* infection and represented the more common of the two lesion types. *C. truncatum* infection usually began as small sunken lesions that turned dark brown to black with age. However, identification of these species based on differences in lesion appearance in field-infected fruits should not be encouraged, as other fungal pathogens of papaya can evoke symptoms similar to anthracnose. *Fusarium solani* also causes sunken lesions that may or may not have a dark brown to black appearance (46,74,82); *Alternaria alternata* can cause blackish lesions to appear on papaya fruit (2); and *Phoma caricae-papayae* causes a watery, sunken lesion up to 4 mm in diameter that turns black with age (74,82).

Using spore morphology alone can also lead to misdiagnosis, as immature macroconidia and microconidia of *F. solani* can be confused with conidia of *C. truncatum* and *C. gloeosporioides*. Morphological attributes are affected by environmental conditions and may vary considerably among isolates of a single species (27,52). Based on the results of this study, morphological traits of colonies and lesion appearance should not be the only parameters by which *Colletotrichum* isolates from papaya are identified. *C. gloeosporioides* and *C. truncatum* had distinctly different conidia size and shape. Isolates of *C. gloeosporioides* had similar conidia size and shape even though the colonies were different morphologically. Intraspecific discrimination among *C. gloeosporioides* and *C. truncatum* isolates could not be carried out based on conidia dimensions alone. Than et al. (75) explained that there may be morphological overlap with respect to conidia dimensions at both the intraspecific and interspecific levels.

In this study, the temperature range for optimum mycelial growth of both *C. truncatum* and *C. gloeosporioides* isolates was 25 to 27°C. Tasiwal and Benagi (73) and Kumara and Rawal (37) reported an optimum range of 28 to 30°C for most *C. gloeosporioides* isolates from papaya in India. However, the majority of

Table 4. Reference sequences used in phylogenetic analyses of *Colletotrichum truncatum* and *C. gloeosporioides* isolates from papaya^z

<i>Colletotrichum</i> species	Sequence	GenBank accession no.	Strain	Host	Country	Reference
<i>C. truncatum</i>	ITS	AY376526	STE-U 5304	<i>Arachis hypogaea</i>	Tanzania	61
		HM562709	Ccg15	<i>Carica papaya</i>	Mexico	69
		HM015005	CAPFV	<i>C. papaya</i>	Mexico	69
		HM562707	Ccg13	<i>C. papaya</i>	Mexico	69
		HM562706	Ccg12	<i>C. papaya</i>	Mexico	69
		EU056740	Cgb3	<i>C. papaya</i>	Mexico	69
	β-tubulin	EU056739	Cgb2	<i>C. papaya</i>	Mexico	69
		DQ454056	HKUCC 10852 'R5'	<i>Capsicum annum</i>	Thailand	69
		EF683602**	HKUCC10828/CBS 120709**	<i>C. frutescens</i>	India	61
		DQ454057	HKUCC 10880 'R12'	<i>C. annum</i>	Thailand	76
<i>C. gloeosporioides</i>	ITS	AY376532	STE-U 4295	Unknown	Unknown	39
		AY371022	IMI 356878	<i>Citrus</i> sp.	Italy	9
		FJ972609**	CBS 953.97**	<i>Citrus</i> sp.	Italy	9
		HM562711	Cgg2	<i>C. papaya</i>	Mexico	69
		HM562710	Cgg1	<i>C. papaya</i>	Mexico	69
		AY188932	CGP1	<i>C. papaya</i>	South Africa	Unpublished
		AY188933	CGPMS1	<i>C. papaya</i>	South Africa	Unpublished
		AY188934	CGPMS3	<i>C. papaya</i>	South Africa	Unpublished
		GU066699	140CP/L	<i>C. papaya</i>	Malaysia	Unpublished
	β-tubulin	AY376583	STE-U4450	Unknown	Unknown	40
		AY376585	STE-U 4455	Unknown	Unknown	40
		AY376584	STE-U4450	Unknown	Unknown	40
		DQ454043	M1	<i>Mangifera indica</i>	Thailand	Unpublished
		FJ907445**	CBS 953.97**	<i>Citrus</i> sp.	Italy	9
<i>C. orbiculare</i>	ITS	AY841133	MX-2-153-Mexico	Unknown	Mexico	Unpublished
		AB269941	MAFF 306685	Unknown	Japan	Unpublished
		EF570872	XG-1	Unknown	China	Unpublished
	β-tubulin	AY376589	STE-U5296	Unknown	Unknown	40
<i>C. dematium</i>	ITS	GU227819	CBS 125.25**	<i>Epidendrum campestre</i>	France	17
		GU227820	CBS125340**	Apiaceae	Czech Rep.	17
	β-tubulin	GU228118	CBS125346	<i>Xanthium</i> sp.	Unknown	17
		GU228117	CBS123729	<i>Genista</i> sp.	Unknown	17
<i>C. acutatum</i>	ITS	EF143974	BRIP28519**	<i>C. papaya</i>	Australia	75
		AJ301981	BBA 71370	<i>Cyclamen</i> sp.	Unknown	67
		AJ301982	BBA 71371	<i>Cyclamen</i> sp.	Unknown	67
	β-tubulin	FJ907444	CBS29467	<i>C. papaya</i>	Australia	75
		EF143970	BRIP28519**	<i>C. papaya</i>	Australia	75
		AJ748635	PD89-582	<i>Cyclamen</i> sp.	The Netherlands	67
<i>C. fragariae</i>	ITS	AJ536221	IMI 345046	<i>Fragariae</i> sp.	USA	43
		AJ536222	IMI 348167	<i>Fragariae</i> sp.	USA	43
	β-tubulin	EU635506	CBS142.31	<i>Fragariae</i> sp.	Unknown	18
<i>C. crassipes</i>	ITS	AJ536230	IMI 309371	<i>Oncidium</i> sp.	The Netherlands	43
	β-tubulin	AY376578	STE-U 4445	Unknown	Unknown	40

^z Abbreviations: HKUCC – Hong Kong University Culture Collections; STE-U – The culture collection of the Department of Plant Pathology at the University of Stellenbosch, South Africa; CBS – Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; IMI – International Mycological Institute (CABI), UK. ** Isolates designated as epitypes in references indicated.

C. truncatum isolates had a significantly slower growth rate in vitro than isolates of *C. gloeosporioides*. This parameter may be used in combination with other characters to distinguish between the two species. Substantial work conducted by Shenoy et al. (62) on morpho-molecular characterization of *C. truncatum* did not report on comparative growth rate of this species. *C. truncatum* from chili peppers had a slower growth rate than isolates of *C. gloeosporioides* (75). Sutton (67) concluded that *C. gloeosporioides* can be differentiated from *C. acutatum* by its significantly faster growth rate. *C. asianum* can also be distinguished from *C. fruticola* and *C. siamense* from coffee by its significantly slower growth rate (55). This is the first instance where growth rate on PDA may be used as a marker by which isolates of *C. gloeosporioides* can be separated from isolates of *C. truncatum* from papaya.

There was a differential reaction to benomyl at a concentration of 1.0 µg/ml by both species. All *C. gloeosporioides* isolates tested were sensitive to 1.0 µg/ml benomyl. Benomyl sensitivity at 1.0 µg/ml by *C. gloeosporioides* isolates from citrus was reported by Peres et al. (51). *C. truncatum* isolates from papaya were resistant. Benomyl resistance has also been reported for *C. truncatum* isolates from chili (59). *C. acutatum* can be differentiated from *C. gloeosporioides* species based on benomyl sensitivity at 1.0 µg/ml (27,52). Based on the results of this study, papaya isolates of *C. gloeosporioides* and *C. truncatum* may also be distinguished by their relative sensitivity to benomyl at 1.0 µg/ml.

In pathogenicity tests involving nonwounded detached papaya fruit, no lesions developed even though both *Colletotrichum* species were recovered from the inoculation sites. Stanghellini and Aragaki (66) studied infection in attached nonwounded immature fruit. They concluded that *C. gloeosporioides* was a wound-pathogen that primarily infects mitotically inactive fruit tissues in the field. Dickman and Alvarez (19) sought to prove the existence of latent infection in papaya in the field and attempted two inoculation methods. The first method involved spray-inoculation of the entire immature fruit column followed by an observation period of 22 to 26 weeks; the second method was based on site-specific inoculation of immature attached fruit. Criticisms of the first method include the inability to determine whether symptoms that developed were attributable to artificial inoculation or to natural field infection that was quiescent on the immature fruit. With respect to the second method, it was necessary to culture a plug of fruit tissue taken from the inoculation site in order to recover the fungus and to determine its presence and ability to survive in vitro because there were no visible symptoms that could be recorded. This is similar to what occurred in this study.

Others established that the cuticle is a barrier to fungal entry (77), but *Colletotrichum* species can form appressoria, infection pegs, germ tubes, and subcuticular hyphae in immature field-infected papaya fruit which explain, ultra-structurally, the nature of quiescent or symptomless infection in papaya fruit (12). Dickman and Alvarez (19) also purported the effects of fungistatic and toxic compounds that exist in the latex, pulp, and skin and/or seeds of papaya fruit (tannins, alkaloids, benzylglucosinolate [BG], and benzyl isothiocyanate [BITC]) and the formation of periderm and callose deposition upon mechanical injury (66) as reasons for symptomless infections in papaya. The initial signals perceived by anthracnose fungi prior to penetration are not properly understood, and different surface signals enable other pathogenicity factors, e.g., cutinase secretion by the fungus (20). A complex interaction of factors may be involved for papaya more so than for other tropical fruits (12).

Although Dickman and Alvarez (19) attempted to explain the lack of symptoms after site-specific inoculation on nonwounded attached fruit, Stanghellini and Aragaki (64) and Hewajulige et al. (35) reasoned that anthracnose is symptomatic in senescing tissues and development of symptoms is supported by wounding of the fruit surface. Tarnowski and Ploetz (72) and Nascimento et al. (47) both reported on pathogenicity tests conducted on wounded mature papaya fruit. These responses may be unique to papaya, as there

are no similar reports for other fruit hosts (e.g., strawberry [43,49] and bell peppers [39]) except for Than et al. (76), who reported that wounding encouraged infection in chili peppers inoculated with conidial suspensions of *C. truncatum*. Than et al. (75) also indicated that one *Colletotrichum* isolate from chili pepper could not evoke symptoms in a specific chili cultivar (PBC 932) without wounding and concluded that the cuticle may be more of a barrier to infection to some isolates than others.

Alternatively, perhaps a more appropriate method to artificially inoculate nonwounded detached or attached papaya fruit is still to be developed. Brown and Ogle (6) argued that detached fruit consists of biochemically active cells, and while there is an important difference in the physiology of a fruit that is attached to the plant and one that is detached, senescence will still occur albeit at a different rate.

In this study, *C. gloeosporioides* isolates from mango and *C. truncatum* isolates from sweet pepper were able to infect and cause symptoms in wounded mature papaya fruit under controlled conditions. Freeman et al. (27) reported that *C. gloeosporioides* from mango can infect other tropical fruit hosts including papaya. These results add to the growing evidence that species of *Colletotrichum* have tremendous cross-infection potential under laboratory conditions (1). Some pathogenic *Colletotrichum* species, including *C. gloeosporioides*, are also commonly found in a mutualistic or even commensalistic association with a range of plant species not known to be hosts, but certain host-related triggers can affect the outcome of symbiosis (53,57). It should be noted, however, that the forced simulated conditions of the inoculation procedure may not accurately reflect what occurs under field conditions. Further studies on infection capability of these two *Colletotrichum* species from papaya under field conditions should be carried out using appropriate methods of inoculation.

Various species of *Colletotrichum* have been reported to cause seedborne infections which may or may not be manifested by symptoms on seedlings (4,10,32,33,56,78). The results of this study indicate that the papaya seedlings were not infected with either *Colletotrichum* species based on a growing-on test. Seedborne infections have serious epidemiological implications, as growers use seeds from fruits to prepare successive fields.

In the present study, two independent DNA regions were used to phylogenetically separate isolates of *C. gloeosporioides* and *C. truncatum* isolates from papaya; 10 isolates each of *C. gloeosporioides* and *C. truncatum* were placed in common clades with other isolates of like species. ITS sequence comparisons allowed interspecific discrimination among *Colletotrichum* isolates but were unable to resolve intraspecific differences. Similar findings were reported by Hyde et al. (36), Cai et al. (8), and Van Hemelrijck et al. (81). β -tubulin gene sequences detected a higher level of intraspecific variation among *C. gloeosporioides* and *C. truncatum* isolates from papaya using the maximum parsimony algorithm. Geographical distribution, original host cultivar, or morphotype were not indicative of phylogenetic relatedness. Cooke et al. (16) and Forster et al. (26) explained that this divergence may arise because these characters are homoplasious and would have evolved independently over evolutionary time.

C. acutatum is an anthracnose pathogen of many economically important crop hosts, and is subject to statutory quarantine requirements in Europe (29/2000 Directive EC). The taxonomy of *C. acutatum* is imprecise, as the species is morphologically complex and intraspecies classification has been hampered by either incomplete or inadequate characterization (77). *C. acutatum* was not isolated from papaya in this study, nor were there any reports of *C. acutatum* in papaya in Japan, Malaysia, more recently in Florida and in Brazil (47). Interestingly, the holotype, paratype, and epitype specimens listed for *C. acutatum* all originated from papaya in Australia (63,77). One explanation as to why this species has not been reported in papaya outside of Australia since 1966 may be that the infection in papaya is far less common than *C. gloeosporioides* and *C. truncatum*, and more extensive sampling may be required for detection. Additionally, the different cultivars of pa-

papaya grown in various countries may affect the frequency of infection occurrence. Lewis-Ivey et al. (39) and Harp et al. (34) demonstrated that *C. acutatum* more commonly infects immature bell peppers while *C. gloeosporioides* affects ripe bell peppers; hence, there can be different pathogenic outcomes even for a given host species.

Accurate identification of fungal pathogens is essential for making quarantine decisions, informing disease management schemes, and for the development of resistant cultivars. Different *Colletotrichum* species may also vary in their reactions to different fungicides, which would have serious implications to deploying chemical control strategies. It was also essential to understand the genetic diversity of the affecting species so that appropriate molecular tools can be developed for more rapid detection and definitive identification. Further, different species of *Colletotrichum* may have different conidial dispersal capacities, which will affect epidemiology of anthracnose disease as is the case with strawberry (49). With respect to quarantine control, accurate species identification is critical, especially as papaya fruit is a high-value export commodity. *C. truncatum* infection in papaya is of quarantine importance to certain countries, but other *Colletotrichum* species, such as *C. gloeosporioides*, are less important due to their wide geographic distribution and host range (21). The findings presented here will significantly enhance our understanding of this pathosystem and provide baseline information for devising management strategies which, in the absence of commercially available resistant or tolerant papaya cultivars, should include screening a range of fungicides with differently acting chemistries for use in commercial papaya production in Trinidad.

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Literature Cited

- Alahakoon, P. W., Brown, A. E., and Sreenivasaprasad, S. 1994. Cross-infection potential of genetic groups of *Colletotrichum gloeosporioides* on tropical fruits. *Physiol. Mol. Plant Pathol.* 44:93-103.
- Alvarez, A. M., and Nishijima, W. T. 1987. Postharvest diseases of papaya. *Plant Dis.* 71:681-687.
- Anonymous. 2010. Biosecurity in New Zealand. <http://www.biosecurity.govt.nz/regs/exports/plants/icpr/chile/tissue-culture-papaya>
- Ben-Daniel, B., Bar-Zvi, D., and Tsrur, L. 2010. Transmission of *Colletotrichum coccodes* via tomato seeds. *Phytoparasitica* 38:167-174.
- Brown, A. E., Sreenivasaprasad, S., and Timmer, L. W. 1996. Molecular characterization of slow-growing orange and Key lime anthracnose strains of *Colletotrichum* from citrus as *C. acutatum*. *Phytopathology* 86:523-527.
- Brown, J. F., and Ogle, H. J. 1997. Plant pathogens and plant diseases. Endorsed by the Australasian Plant Pathology Society Inc., Armidale, N.S.W. Rockvale Publications for the Division of Botany, School of Rural Science and Natural Resources, University of New England, pp. 533-540.
- Burns, P., Chubunmee, S., Pinon, P., and Kumdee, O. 2007. Identification of two 1-aminocyclopropane-1-carboxylate oxidase genes (CP-ACO1 and CP-ACO2) from *Carica papaya* and characterisation of putative CP-ACO1 promoter using agrobacterium infiltration. *Acta Hort.* 740:163-168.
- Cai, L., Hyde, K. D., Taylor, P. W. J., Weir, B. S., Waller, J., Abang, M. M., Zhang, J. Z., Yang, Y. L., Phoulivong, S., Liu, Z. Y., Prihastuti, H., Shivas, R. G., McKenzie, E. H. C., and Johnston, P. R. 2009. A polyphasic approach for studying *Colletotrichum*. *Fungal Divers.* 39:183-204.
- Cannon, P. F., Buddie, A. G., and Bridge, P. D. 2008. The typification of *Colletotrichum gloeosporioides*. *Mycotaxon* 104:189-204.
- Cardwell, K. F., Hepperly, P. R., and Frederiksen, R. A. 1989. Pathotypes of *Colletotrichum graminicola* and seed transmission of sorghum anthracnose. *Plant Dis.* 73:255-257.
- Central Statistical Office (CSO). 2004. Tree crops under cultivation. Ministry of Planning and Development, The Republic of Trinidad and Tobago.
- Chau, K. F., and Alvarez, A. M. 1983. A histological study of anthracnose on *Carica papaya*. *Phytopathology* 73:1113-1116.
- Chung, W.-H., Ishii, H., Nishimura, K., Fukaya, M., Yano, K., and Kajitani, Y. 2006. Fungicide sensitivity and phylogenetic relationship of anthracnose fungi isolated from various fruit crops in Japan. *Plant Dis.* 90:506-512.
- Coates, L. M., and Johnson, G. I. 1993. Effective disease control in heat disinfected fruit. *Postharv. News Inform.* 4:35N-40N.
- Coates, L. M., and Johnson, G. I. 1997. Postharvest diseases of fruits and vegetables. Pages 533-547 in: *Plant Pathogens and Plant Diseases*. J. F. Brown and H. K. Ogle, eds. Australasian Plant Pathology Society.
- Cooke, D. E. L., Drenth, A., Duncan, J. M., Wagels, G., and Brasier, C. M. 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal Genet. Biol.* 30:17-30.
- Damm, U., Woudenberg, J. H. C., Cannon, P. F., and Crous, P. W. 2009. *Colletotrichum* species with curved conidia from herbaceous hosts. *Fungal Divers.* 39:45-87.
- Debode, J., Van Hemelrijck, W., Baeyen, S., Creemers, P., Heungens, K., and Maes, M. 2009. Quantitative detection and monitoring of *Colletotrichum acutatum* in strawberry leaves using real-time PCR. *Plant Pathol.* 58:504-514.
- Dickman, M. B., and Alvarez, A. M. 1983. Latent infection of papaya caused by *Colletotrichum gloeosporioides*. *Plant Dis.* 67:748-750.
- Dickman, M. B., Patil, S. S., and Kolattukudy, P. E. 1982. Purification, characterization, and role in infection of an extracellular cutinolytic enzyme from *Colletotrichum gloeosporioides* Penz. on *Carica papaya* L. *Physiol. Plant Pathol.* 20:333-347.
- European Plant Protection Organization (EPPO). 2010. Online. <http://www.eppo.org>
- Farr, D. F., Rossman, A. Y., Pal, M. E., and McCray, E. B. 2007. Fungal Databases, Systemic Botany and Mycology Laboratory, ARS, USDA. Online. <http://nt.ars-grin.gov/fungal-databases/>
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.
- Food and Agriculture Organization (FAO). 2010. Production statistics for papaya. FAO.
- Ford, R., Banniza, S., Photita, W., and Taylor, P. W. J. 2004. Morphological and molecular discrimination of *Colletotrichum truncatum* causing anthracnose on lentil in Canada. *Australas. Plant Pathol.* 33:559-569.
- Forster, H., Cummings, M. P., and Coffey, M. D. 2000. Phylogenetic relationships of *Phytophthora* species based on ribosomal ITS1 DNA sequence analysis with emphasis on Waterhouse groups V and VI. *Mycol. Res.* 104:1055-1061.
- Freeman, S., Katan, T., and Shabi, E. 1998. Characterization of *Colletotrichum* species responsible for anthracnose diseases of various fruits. *Plant Dis.* 82:596-605.
- Freeman, S., Minz, D., Jurkevitch, E., Maymon, M., and Shabi, E. 2000. Molecular analyses of *Colletotrichum* species from almond and other fruits. *Phytopathology* 90:608-614.
- Freeman, S., Pham, M., and Rodriguez, R. J. 1993. Molecular genotyping of *Colletotrichum* species based on arbitrarily primed PCR, A-T-rich DNA, and nuclear DNA analyses. *Exp. Mycol.* 17:309-322.
- Garrido, C., Carbú, M., Fernández-Acero, F. J., Budge, G., Vallejo, I., Collyer, A., and Cantoral, J. M. 2008. Isolation and pathogenicity of *Colletotrichum* spp. causing anthracnose of strawberry in south west Spain. *Eur. J. Plant Pathol.* 120:409-415.
- Glass, N. L., and Donaldson, G. C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* 61:1323-1330.
- Gramarnick, A., Frydman, R. B., and Barreta, D. 1994. Prevention of infection of soybean seeds by *Colletotrichum truncatum* by polyamide biosynthesis inhibitors. *Phytopathology* 84:1445-1448.
- Grover, R. K., and Bansod, R. D. 1970. Seed borne nature of *Colletotrichum capsici* in chilli seeds and its control by seed dressing fungicides. *Indian Phytopathol.* 23:664-668.
- Harp, T. L., Pernezny, K., Lewis Ivey, M. L., Miller, S. A., Kuhn, P. J., and Datnoff, L. 2008. The etiology of recent pepper anthracnose outbreaks in Florida. *Crop Prot.* 27:1380-1384.
- Hewajulige, I. G. N., Sultanbawa, Y., Wilson Wijeratnam, R. S., and Wijesundara, R. L. C. 2009. Mode of action of chitosan coating on anthracnose disease control in papaya. *Phytoparasitica* 37:437-444.
- Hyde, K. D., Cai, L., Cannon, P. F., Crouch, J. A., Crous, P. W., Damm, U., Goodwin, P. H., Chen, H., Johnston, P. R., Jones, E. B. G., Liu, Z. Y., McKenzie, E. H. C., Moriwaki, J., Noireung, P., Pennycook, S. R., Pfennig, L. H., Prihastuti, H., Sato, T., Shivas, R. G., Tan, Y. P., Taylor, P. W. J., Weir, B. S., Yang, Y. L., and Zhang, J. Z. 2009. *Colletotrichum* – names in current use. *Fungal Divers.* 39:147-182.
- Kumara, K., and Rawal, R. D. 2008. Influence of carbon, nitrogen, temperature and pH on the growth and sporulation of some Indian isolates of *Colletotrichum gloeosporioides* causing anthracnose disease of papaya (*Carica papaya* L.). *Trop. Agric. Res. Ext.* 11:7-12.
- Laurena, A. C., Magdalita, P. M., Hidalgo, M. S. P., Villegas, V. N., Mendoza, E. M. T., and Botella, J. R. 2002. Cloning and molecular characterization of ripening-related ACC synthase from papaya fruit (*Carica papaya* L.). *Acta Hort.* 575:163-169.
- Lewis-Ivey, M. L., Nava Diaz, C., and Miller, S. A. 2004. Identification and management of *Colletotrichum acutatum* on immature bell peppers. *Plant Dis.* 88:1198-1204.
- Lubbe, C. M., Denman, S., Cannon, P. F., Groenewald, J. Z., Lamprecht, S. C., and Crous, P. W. 2004. Characterization of *Colletotrichum* species associated with diseases of Proteaceae. *Mycologia* 96:1268-1279.
- Lurie, S. 1998. Postharvest heat treatments – A review. *Postharv. Biol. Technol.* 14:257-269.
- Magdalita, P. M., Laurena, A. C., Yabut-Perez, B. M., Mendoza, E. M. T.,

- and Botella, J. R. 2002. Progress in the development of transgenic papaya: Transformation of solo papaya using ACC synthase antisense construct (*Carica papaya* L.). *Acta Hort.* 575:171-176.
43. Martinez Culebras, P. V., Querol, A. A., Suarez Fernandez, M. B., Garcia-Lopez, M. D., and Barrio, E. 2003. Phylogenetic relationship among *Colletotrichum* pathogens of strawberry and design of PCR primers for their identification. *J. Phytopathol.* 151:135-143.
44. Mills, R. R., Sreenivasaprasad, S., and Brown, A. E. 1992. Detection and differentiation of *Colletotrichum gloeosporioides* isolates using PCR RAPD band patterns of *Colletotrichum* isolates from banana from Australia. *FEMS Microbiol. Lett.* 98:137-144.
45. Moriwaki, J., and Tsukiboshi, T. 2002. Grouping of *Colletotrichum* species in Japan based on rDNA sequences. *J. Gen. Plant Pathol.* 68:307-320.
46. Morton, J. F. 1987. Papaya *Carica papaya* L. Pages 336-346 in: *Fruits of Warm Climates*. J. F. Morton, ed. Creative Resource Systems, Miami, FL.
47. Nascimento, R. J., Mizubuti, E. S. G., Câmara, M. P. S., Ferreira, M. F., Maymon, M., Freeman, S., and Michereff, S. J. 2010. First report of papaya fruit rot caused by *Colletotrichum magna* in Brazil. *Plant Dis.* 94:1506-1506.
48. Neupane, K. R., Mukatira, U. T., Kato, C., and Stiles, J. I. 1998. Cloning and characterization of fruit-expressed ACC synthase and ACC oxidase from papaya (*Carica papaya* L.). *Acta Hort.* 461:329-337.
49. Ntahimpera, N., Wilson, L. L., and Ellis, M. A. 1999. Comparison of rain effects on splash dispersal of three *Colletotrichum* species infecting strawberry. *Phytopathology* 89:555-563.
50. Organization for Economic Co-Operation and Development (OECD). Consensus document on compositional considerations for new varieties of papaya (*Carica papaya* L.): Key food and feed nutrients, anti nutrients, toxicants environment. Environment, health and safety publications series on the safety of novel foods and feeds no. 21. [http://www.oecd.org/dataoecd/16/28/46815336.env/jm/mono\(2010\)28](http://www.oecd.org/dataoecd/16/28/46815336.env/jm/mono(2010)28)
51. 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.
52. Peres, N. A. R., Timmer, L. W., Adaskaveg, J. E., and Correll, J. C. 2005. Lifestyles of *Colletotrichum acutatum*. *Plant Dis.* 89:784-796.
53. Photita, W., Taylor, P. W. J., Ford, R., Hyde, K. D., and Lumyong, S. 2005. Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand. *Fungal Divers.* 18:117-133.
54. Phoulivong, S., Cai, L., Chen, H., McKenzie, E., Abdelsalam, K., Chuksatirote, E., and Hyde, K. 2010. *Colletotrichum gloeosporioides* is not a common pathogen on tropical fruits. *Fungal Divers.* 44:33-43.
55. Prihastuti, H., Cai, L., Chen, H., McKenzie, E. H. C., and Hyde, K. D. 2009. Characterization of *Colletotrichum* species associated with coffee berries in northern Thailand. *Fungal Divers.* 39:89-109.
56. Ravi, S., Doraiswamy, S., Valluvaparasian, V., and Jeyalakshmi, C. 2000. Seed-borne infection of French bean by *Colletotrichum lindemuthianum*. *Acta Phytopathol. Entomol. Hungarica* 34:283-291.
57. Redman, R. S., Dunigan, D. D., and Rodriguez, R. J. 2001. Fungal symbiosis from mutualism to parasitism: Who controls the outcome, host or invader? *New Phytol.* 151:705-716.
58. Saitou, N., and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406-425.
59. Sariah, M. 1989. Detection of benomyl resistance in the anthracnose pathogen, *Colletotrichum capsici*. *J. Isl. Acad. Sci.* 2:168-171.
60. Sepiah, M. 1994. Efficacy of propiconazole against fungi causing postharvest disease on Eksotika papaya. International Conference, Chaing Mai (Thailand), 19 July 1993. Australian Center for International Agricultural Research, Canberra, A.C.T. Australia.
61. Sew, Y. S., Lam, P. F., and Abu Bakar, U. K. 2007. Isolation and cloning of two 1-aminocyclopropane-1-carboxylate oxidases from *Carica papaya* var. Eksotika I and Eksotika II. *Acta Hort.* 740:153-162.
62. Shenoy, B. D., Jeewon, R., Lam, W. H., Bhat, D. J., Than, P. P., Taylor, P. W. J., and Hyde, K. D. 2007. Morpho-molecular characterisation and epitypification of *Colletotrichum capsici* (Glomerellaceae, Sordariomycetes), the causative agent of anthracnose in chilli. *Fungal Divers.* 27:197-211.
63. Simmonds, J. H. 1966. A study of the species of *Colletotrichum* causing ripe fruit rots in Queensland. *Queensl. J. Agric. Anim. Sci.* 22:437-459.
64. Sivakumar, D., Hewardthamagae, N. K., Wilson Wijeratnam, R. S., and Wijesundera, R. L. C. 2002. Effect of ammonium carbonate and sodium bicarbonate on anthracnose of papaya. *Phytoparasitica* 30:486-492.
65. Sreenivasaprasad, S., Mills, P. R., and Brown, A. E. 1994. Nucleotide sequence of the rDNA spacer 1 enables identification of isolates of *Colletotrichum* as *C. acutatum*. *Mycol. Res.* 98:186-188.
66. Stanghellini, M. E., and Aragaki, M. 1966. Relation of periderm formation and callose deposition to anthracnose resistance in papaya fruit. *Phytopathology* 56:444-450.
67. Sutton, B. C. 1992. The genus *Glomerella* and its anamorph *Colletotrichum*. Pages 1-26 in: *Colletotrichum: Biology, Pathology and Control*. J. A. Bailey and J. J. Jeger, eds. CAB International, Wallingford, UK.
68. Talhinhas, P., Sreenivasaprasad, S., Neves-Martins, J., and Oliveira, H. 2005. Molecular and phenotypic analyses reveal association of diverse *Colletotrichum acutatum* groups and a low level of *C. gloeosporioides* with olive anthracnose. *Appl. Environ. Microbiol.* 71:2987-2998.
69. Tamura, K., Dudley, J., Nei, M., and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24:1596-1599.
70. Tamura, K., Nei, M., and Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Nat. Acad. Sci. USA* 101:11030-11035.
71. Tapia-Tussell, R., Quijano-Ramayo, A., Cortes-Velazquez, A., Lappe, P., Larque-Saavedra, A., and Perez-Brito, D. 2008. PCR-based detection and characterization of the fungal pathogens *Colletotrichum gloeosporioides* and *Colletotrichum capsici* causing anthracnose in papaya (*Carica papaya* L.) in the Yucatan Peninsula. *Mol. Biotechnol.* 40:293-298.
72. Tarnowski, T. B. L., and Ploetz, R. C. 2010. First report of *Colletotrichum capsici* causing postharvest anthracnose on papaya in South Florida. *Plant Dis.* 94:1065.
73. Tasiwal, V., and Benagi, V. I. 2009. Studies on the cultural and nutritional characteristics of *Colletotrichum gloeosporioides*, the causal organism of papaya anthracnose. *Karnataka J. Agric. Sci.* 22:787-789.
74. Teixeira da Silva, J. A., Rashid, Z., Nhut, D. T., Sivakumar, D., Gera, A., Teixeira Souza, M., Jr., and Tennant, P. F. 2007. Papaya (*Carica papaya* L.) Biology and Biotechnology. *Tree Forestry Sci. Biotechnol.* 1:47-73.
75. 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 on chilli (*Capsicum* spp.) in Thailand. *Plant Pathol.* 57:562-572.
76. Than, P. P., Prihastuti, H., Phoulivong, S., Taylor, P. W., and Hyde, K. D. 2008. Chilli anthracnose disease caused by *Colletotrichum* species. *J. Zhejiang Univ. Sci.* 9:764-778.
77. Than, P. P., Shivas, R. G., Jeewon, R., Pongsupasamit, S., Marney, T. S., Taylor, P. W. J., and Hyde, K. D. 2008. Epitypification and phylogeny of *Colletotrichum acutatum* J.H. Simmonds. *Fungal Divers.* 28:97-108.
78. Thomas, G. J., and Sweetingham, M. W. 2003. Fungicide seed treatments reduce seed transmission and severity of lupin anthracnose caused by *Colletotrichum gloeosporioides*. *Australas. Plant Pathol.* 32:39-46.
79. Trujillo, E. E., and Obrero, F. P. 1969. Anthracnose of papaya leaves caused by *Colletotrichum gloeosporioides* Plant Dis. Rep. 53:323-325.
80. Uchida, J. Y., Kadooka, C. Y., Aragaki, M., and Manshardt, R. 1996. Papaya seedling blight and damping off caused by *Colletotrichum gloeosporioides* in Hawaii. *Plant Dis.* 80:712.
81. Van Hemelrijck, W., Debode, J., Heungens, K., Maes, M., and Creemers, P. 2010. Phenotype and genetic characterization of *Colletotrichum* isolates from Belgian strawberry fields. *Plant Pathol.* 59:853-861.
82. Ventura, J. A., Costa, H., and da Silva Tatagiba, J. 2004. Papaya diseases and integrated control. Pages 201-235 in: *Diseases of fruits and vegetables-Diagnosis and management*, Vol. II. S. A. M. H. Naqvi, ed. Kluwer Academic Publishers, Dordrecht, Netherlands.
83. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: *PCR Protocols: A Guide to Methods and Applications*. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, New York.
84. Yaguchi, Y., Nakanishi, Y., Saito, T., and Nakamura, S. 1995. Anthracnose of *Carica papaya* L. caused by *Colletotrichum capsici*. *Ann. Phytopathol. Soc. Jpn.* 61:222.