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Occurrence and Distribution of *Colletotrichum* spp. on Mango (*Mangifera indica* L.) in Puerto Rico and Florida, USA

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Abstract: An extensive survey of anthracnose in mango caused by *Colletotrichum* spp. was conducted in seven locations from western, central and eastern, Florida, USA and five locations from western and southern, Puerto Rico. Morphological, serological and molecular characteristics of 183 *Colletotrichum* spp. isolates was determined. Ninety three percent of the isolates from Puerto Rico and Florida, USA were identified as *C. gloeosporioides*, while only 5% (eight isolates) as *C. acutatum*. Another 2% (3 isolates) from the same host were negative for both species. Pathogenicity tests conducted on detached mango leaves showed that both species were pathogenic. Necrotic lesions produced orange to salmon conidial masses on acervuli 7 days after inoculation. Colonies of isolates producing aerial mycelia were white, gray and/or dark gray, often with conidial masses on acidified potato dextrose agar. Conidia of isolates were hyaline, one-celled, ovoid to oblong, straight or slightly curved and ranged from 12 to 20×3.5 to 6 μm for *C. gloeosporioides* and 8 to 13×2 to 5 μm for *C. acutatum*. ELISA and PCR assays were used complementary to morphological results and confirmed *C. acutatum* causing anthracnose in mango's flower, peduncles and immature fruit in Homestead, Florida. *Colletotrichum acutatum* was neither found during this survey and has never been reported on mango in Puerto Rico. *Colletotrichum* spp. identification is essential in the development of control strategies of anthracnose disease because of differences within species to benomyl and azoxystrobin. Other fungi isolated from necrotic lesions in mango were identified as *Phomopsis* sp., *Cladosporium* sp., *Curvularia* sp., *Fusarium pallidoroseum*, *Pestalotiopsis* sp., *Alternaria infectoria* and *Nigrospora* sp. Pathogenicity tests also indicated that *A. infectoria*, *F. pallidoroseum* and *Pestalotiopsis* sp. were pathogenic to detached mango leaves under laboratory conditions.

Key words: Anthracnose, *C. gloeosporioides*, *C. acutatum*, mango, tropical fruits, survey

INTRODUCTION

In Puerto Rico, the most important disease of mango (*Mangifera indica* L.) is anthracnose, caused by *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. (teleomorph *Glomerella cingulata* (Stoneman) Spauld. and Schrenk. This pathogen reduces fruit quantity and quality, especially in hot humid tropical environments where disease incidence can reach 100% (Arauz, 2000). Typically, the pathogen mummifies immature fruits and produces sunken necrotic lesions on different organs including fruits, leaves, shoots and flowers. Infection often reduces tree vigor and productivity and causes severe post harvest fruit losses. Other species, such as *C. acutatum* Simm., also have been reported causing anthracnose of mango in Australia and Taiwan (Fitzell and Peak, 1984; Freeman *et al*., 1998; Jeffries *et al*., 1990; Ploetz, 1994; Weng and Chuang, 1995). Furthermore, *C. acutatum* has been included in quarantine list on Sweden and Latvia due to its virulence and severe yield losses in various crops (Vinnere, 2004).

Mango is the most important commercial fruit in Puerto Rico with a market value of $12.5 million and 2,347 acres committed to its production during 2004 to 2005 (Puerto Rico's Department of Agriculture, 2006; FAO, 2006). Most fruit is exported to Europe and North America. In contrast, Florida's mango production has declined from 1,700 to 1,300 acres, with a total crop value

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in 1997 of $1.45 million. Recent figures on production and crop value are no longer available because commercial shipments are very limited (Florida Agricultural Statistics Service, 2006). Despite the relative economic importance of mango in Puerto Rico, few anthracnose studies have been conducted.

Perhaps one reason for the lack of anthracnose studies relates to difficulties posed by the complex taxonomy of the group. Further, characterization of *Colletotrichum* spp. based on morphology and pathogenicity is often unreliable. Traditional taxonomic criteria, such as growth, mycological characteristics (i.e., conidial morphology and size, seta and appresoria formation) and the development of the sexual stage are inconsistent. In general, environmental conditions influence the stability of many morphological traits used to differentiate species. Lately, the application of molecular techniques, like the amplification of the Internal Transcribed Spacer (ITS) region of ribosomal DNA using Polymerase Chain Reaction (PCR) technology and serological techniques such as Enzyme Linked Immunosorbent Assay (ELISA), have been used to complement traditional taxonomic methods to differentiate between *Colletotrichum* species (Afanador-Kafuri *et al*., 2003; Brown *et al*., 1996; Mills *et al*., 1992; White *et al*., 1990). More recently, diverse world wide collections of *Colletotrichum* isolates have been examined for mating-type gene sequences, mitochondrial DNA RFLP's, sequence variation of a 900 bp intron of the glutamine synthetase gene and a 200 bp intron of the glyceraldehyde-3-phosphate dehydrogenase gene to clarify phylogenetic resolutions specially among wide species complexes such as *C. gloeosporioides*, *C. acutatum* and *C. graminicola* (Du *et al*., 2005; Guerber *et al*., 2003).

Proper identification of *Colletotrichum* species is essential in the development of control strategies. For example, *Colletotrichum* species such as *C. acutatum* and *C. gloeosporioides* occurring in tropical fruits differ in sensitivity to fungicides such as benomyl and azoxystrobin, implying different management practices (Adaskaveg and Foster, 2000; Lugo-Noel, 2001).

With that key disease management constraint in mind, we conducted an extensive survey in mango farms during 1999-2001 to determine the population composition of *Colletotrichum* spp. occurring in Puerto Rico and Florida. The study included characterization of *Colletotrichum* isolates using traditional methods as well as molecular and serological techniques such PCR and ELISA. This study also provides a base to further delineate genetically distinct subgroups of species complexes among this broad genus, emphasizing those that affect tropical fruits.

MATERIALS AND METHODS

Disease survey and collection of plant material: Samples showing typical anthracnose symptoms were collected from mango orchards in Puerto Rico and Florida, during October 1999 to July 2001 (Fig. 1). Fruit samples imported from Mexico, Brazil and Nicaragua that were sold in Florida's markets were also collected to examine the possibility of the introduction of new species via fruit importation. Studies were conducted in the following locations; (I) in Puerto Rico: Juana Diaz, Ponce and Santa Isabel in the southern region; and Mayagüez and Rincon at the western region and (ii) in Florida: Bradenton, Sarasota and Pine Island in the western region; Homestead in the central region; and Palm Beach, Canal Point and Boynton Beach at the eastern region (Fig. 1).

Fungal isolation and identification: Fungi were isolated from symptomatic mango tissues after surface sterilization with ethanol (70%) and sodium hypochlorite (0.5%). Then tissues were rinsed with sterile de-ionized distilled water and transferred to potato dextrose agar acidified with 25% lactic acid (APDA). Plates were incubated at 26°C and single spore cultures were used for morphological, molecular and serological characterization of *Colletotrichum* isolates. Conidial measurements of each isolate were determined from 50 observations at 400X magnification. Morphological observations were made of colony types and from reproductive structures mounted in semipermanent slides.

Other *Colletotrichum* isolates obtained from different tropical crops such as coffee (*Coffea arabica* L.), anonna (*Annona squamosa* L.), guava (*Psidium guajava* L.), papaya (*Carica papaya* L.), soursop (*Annona muricata* L.) and starfruit (*Averrhoa carambola* L.) were included in the study for comparison. Cultures were maintained on APDA at room temperature and stored cryogenically at -80°C at the University of Florida, Gulf Coast Research and Education Center, Bradenton, FL.

Various taxonomic keys were used to identify all fungal isolates obtained from mango infected tissues (Barnett and Hunter, 1998; Dyko and Mordue, 1979; Sutton, 1980; Mordue, 1971). Isolates of *Alternaria* sp. and *Fusarium* sp. were sent to CABI Biosciences, UK for identification.

***In vitro* pathogenicity tests:** Pathogenicity tests were conducted *in vitro* on detached mango leaves (var. Keitt) under controlled laboratory conditions at 25°C for 110 isolates of *Colletotrichum* species. Leaves were surface sterilized with 70% ethyl alcohol, 0.5% sodium hypochlorite for 1 min each, rinsed with sterile de-ionized distilled water and placed in plastic humidity chambers

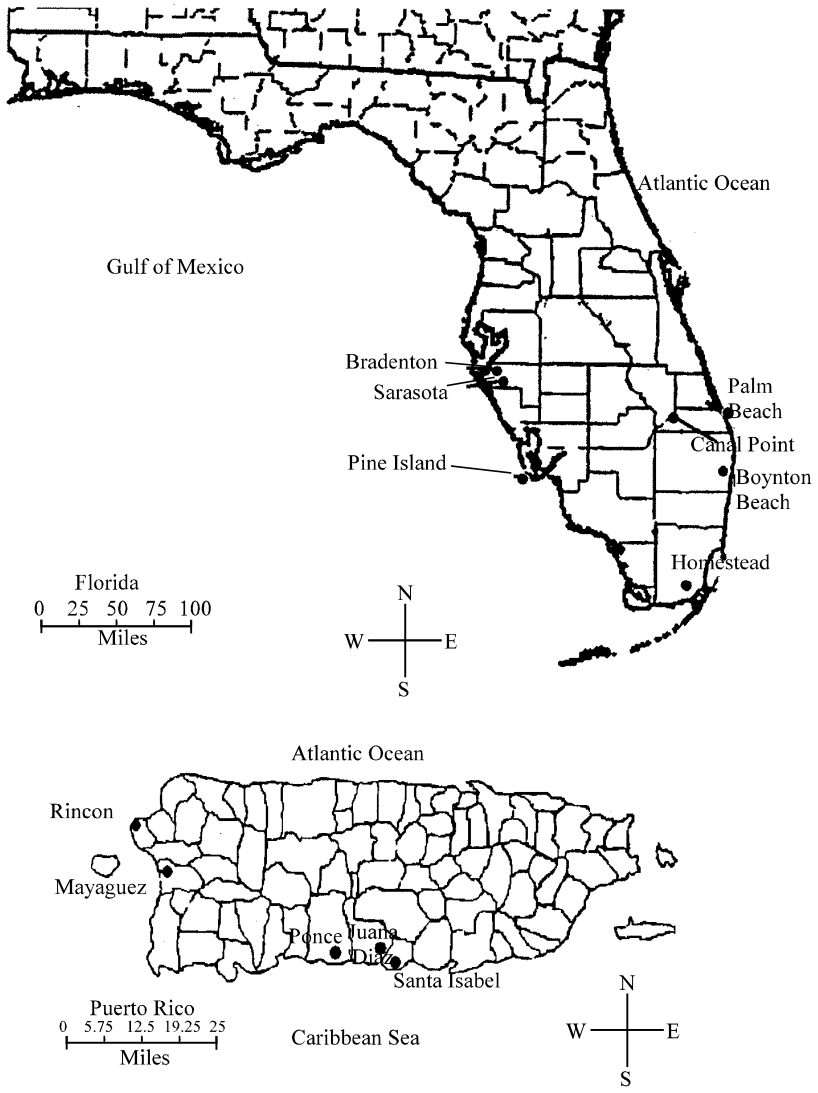

Fig. 1: Location of mango farms and collection sites sampled for anthracnose in Florida and Puerto Rico

(i.e., 91×41×15 cm). Inoculations were made by placing mycelial plugs (4 mm diam.) of *Colletotrichum* spp. from 7-day-old fungal cultures on the upper surface of the leaves after an incision made with a sterile dissection needle. Controls were inoculated with APDA disks after an incision. Humidity chambers were incubated at 25°C at 90-100% HR in randomized complete blocks. The experiment was replicated three times.

DNA extraction and ITS region amplification: DNA was extracted from all *Colletotrichum* isolates using the CTAB method (Graham *et al*., 1994). DNA amplification was performed using the following primers: ITS4 (TCCTCCGCTTATTGATATGC) coupled with primers for *C. gloeosporioides* (*Cg*Int) (GGCCTCCCGCCTCCGGGCGG) and *C. acutatum* (*Ca*Int2) (GGGGAAGCCTCTCGCGG) in a 50 μL reaction volume (Brown *et al*., 1996; Mills *et al*., 1992; White *et al*., 1990). PCR reaction mixture contained 1X Taq buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl), 0.2 mM of each dNTP (Promega, Madison, WI), 25 pmol of each primer, 1 U Taq polymerase (Fisher Scientific, Pittsburgh, PA or Sigma, St. Louis, MO), 1 mM spermidine, 1.5 or 3.75 mM $MgCl_2$ (for the *C. gloeosporioides* or the *C. acutatum* specific reactions, respectively) and 10-100 ng of template DNA. Amplification was performed using a MJ Research PTC100 thermocycler (Watertown, MA). PCR conditions were the following: initial DNA denaturation for 5 min at 95°C, followed by 34 cycles of 45 sec at 95°C, 75 sec at 55°C for *Cg*Int or 69°C for *Ca*Int2, 2 min (+3 sec/cycle) at

72°C. After all cycles, a final elongation was conducted for 5 min at 72°C. Following PCR, the amplification product was stored at 4°C until subjected to electrophoresis. PCR products were visualized in 1% agarose gels using ethidium bromide and UV light. DNA samples from any isolates that failed to react with either primer set were further purified by an additional isopropanol precipitation and then the PCR was repeated. Each individual analysis was replicated twice.

ELISA: ELISA was used to differentiate *C. acutatum* from other *Colletotrichum* species. This assay utilized a *Colletotrichum acutatum* Identikit (ADGEN Limited, Ayr, Scotland, UK) following the manufacturer's instructions. Samples tested were conidial suspensions made from *Colletotrichum* spp. cultures grown on acidified carnation leaf agar (ACLA). Results of the ELISA were read at 405 nm (reference point = 550 nm), on the Easy Reader EAR 400AT plate reader (SLT Labinstruments, Australia).

RESULTS

Disease survey, collection of plant material and fungal isolation: Twelve sites were sampled from different geographical regions in Puerto Rico and Florida. Sites surveyed, number of samples per plant organ and location are shown in Table 1. A total of 183 fungal isolates were collected, of these 149 isolates were obtained from typical anthracnose lesions from mango leaves, flowers, fruits and panicles. Eighty and sixty nine isolates were obtained from Puerto Rico or Florida, respectively. The majority of the isolates were obtained from fruits (46%) and leaves (50%) (Table 1). Eighty seven percent of the isolates were from mango including nine additional isolates from imported fruits from Mexico, Brazil and Nicaragua that were sold in Florida's markets. Another 5% (10 isolates) were obtained from other tropical fruits including anonna, guava, papaya, soursop, coffee and starfruit. Eight percent (14 isolates) were either contaminated or lost during manipulation.

Characterization of *Colletotrichum* spp. from mango: Different types of colonies were commonly observed on acidified potato dextrose agar: i) white, cottony mycelium with not obvious sporulation (11%); ii) white, cottony mycelium with gray concentric rings with not obvious sporulation (19%) and iii) gray and/or white mycelium with different levels of sporulation showing orange masses of conidia (70%) (Fig. 2).

Based on morphological observations, fungal colonies producing cylindrical conidia with obtuse ends or slightly ellipsoid, such as *Colletotrichum gloeosporioides*, was frequently isolated from mango leaves and fruits at both locations, whereas fungal colonies producing fusiform conidia, with one acute end, likely *C. acutatum*, were obtained from peduncles, flowers and immature fruits only in Homestead, FL (Fig. 3 A and B). Ninety five percent of the isolates examined were identified as *C. gloeosporioides*; while only 5% were identified as *C. acutatum*.

Conidial size of *C. gloeosporioides* and *C. acutatum* ranged from 12-20×3.5-6 μm and 8-13×2-5 μm, respectively. Both species' bear unicellular and hyaline conidia produced in setose acervuli (Fig. 3 C). Perithecia containing asci and ascospores of *C. gloeosporioides* teleomorph, *Glomerella cingulata*, were observed in culture plates (Fig. 3D). As we had foreseen, the accurate identification of a group of isolates required further studies using PCR and ELISA to confirm their identification due to instability of traits such as colony type, conidial morphology and production in APDA. In addition, some isolates did not sporulate profusely on culture media and their characterization required molecular or serological techniques.

Table 1: Location of mango groves examined during this survey and number of *Colletotrichum* spp. isolates collected per plant organ

Mango groves location	No. of sites examined	Sites examined	No. of isolates per plant organs examined: Leaves	Fruits	Panicles	Flowers	Total isolates collected
Puerto Rico							
Southern	3	Ponce	8	0	0	0	8
		Juana Diaz	2	0	0	0	2
		Santa Isabel	16	12	0	0	26
Western	2	Rincon	4	0	0	0	4
		Mayaguez	37	1	0	0	38
Subtotal	5		67	13	0	0	80
Florida							
Western	3	Bradenton	4	2	0	0	6
		Sarasota	0	18	0	0	17
		Pine Island	0	11	0	0	11
Central	1	Homestead	3	10	4	2	20
Eastern	3	Palm Beach	0	3	0	0	3
		Canal Point	0	5	0	0	5
		Boynton Beach	0	7	0	0	7
Subtotal	7		7	56	4	2	69
Total	12		74	69	4	2	149

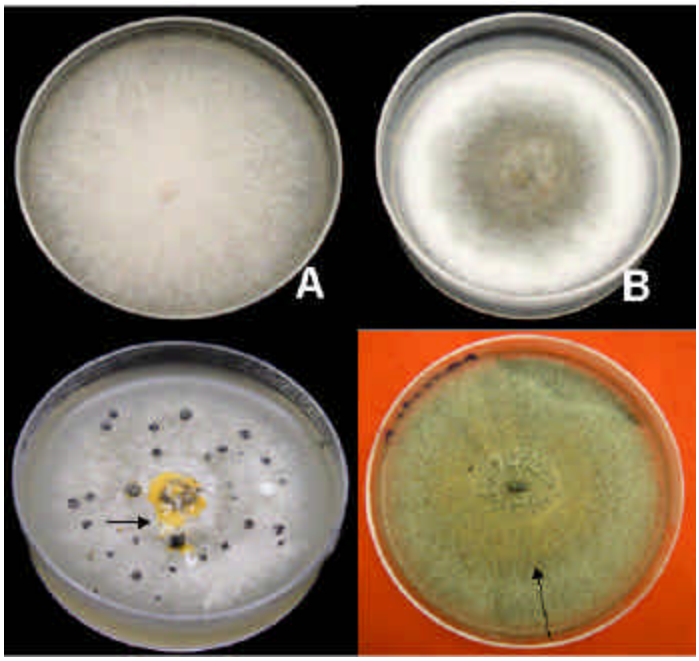

Fig. 2: *Colletotrichum* spp. colony types grown on acidified potato dextrose agar were: A) white, cottony mycelium with no obvious sporulation; B) white, cottony mycelium with gray center with no obvious sporulation and C) white or D) gray mycelium with different levels of sporulation, showing orange masses of conidia (→)

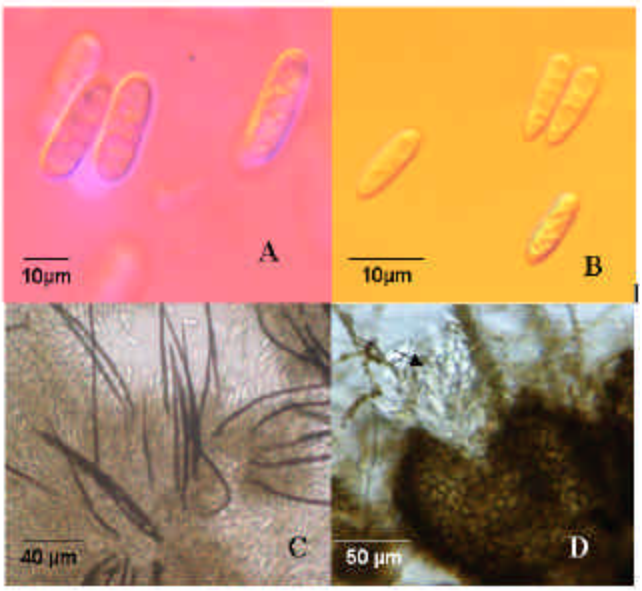

Fig. 3: Conidia of (A) *C. gloeosporioides* and (B). *C. acutatum*. (C) Acervulus of *C. gloeosporioides*, observed black setae and (D) perithecia of *C. gloeosporioides* teleomorph, *Glomerella cingulata*, containing asci and ascospores (→)

Table 2: Identification of *Colletotrichum* spp. using PCR and ELISA assays

Mango groves location	PCR assay[a/]					
	Primers *Cg*Int		Primers *Ca*Int		ELISA Assays[b/]	
	+	-	+	-	+	-
Puerto Rico						
Southern	36	0	0	36	0	36
Western	40	2	0	42	0	42
Subtotal	76	2	0	78	0	78
Florida						
Western	33	1	0	34	0	34
Central	12	8	8	12	8	12
Eastern	15	0	0	15	0	15
Subtotal	60	9	8	61	8	61
Total	136	11	8	139	8	139

[a/] Primers set used were *Cg*Int/ITS4 for *C. gloeosporioides* and *Ca*Int2/ ITS 4 for *C. acutatum*. Two different PCR reactions were conducted. Key: + = positive or - = negative reactions, [b/]A commercial ELISA test for *C. acutatum* from Adgen Limited, Scotland, UK was used. Key: + = positive and - = negative reactions for *C. acutatum*

Other fungi isolated during the studies: Various fungal species were isolated from necrotic lesions in mango and were identified as *Phomopsis* sp., *Cladosporium* sp., *Curvularia* sp., *Fusarium pallidoroseum*, *Pestalotiopsis* sp., *Alternaria infectoria* and *Nigrospora* sp. Pathogenicity tests indicated that *A. infectoria*, *F. pallidoroseum* and *Pestalotiopsis* sp. were pathogenic to detached mango leaves under laboratory conditions (data not shown).

PCR amplification of rDNA ITS region and ELISA assays: Ninety three percent of the mango isolates tested positive for *C. gloeosporioides*, while only 5% (eight isolates from Florida) were identified as *C. acutatum* using specific primers for each species (Table 2; Fig. 4 and 5). Three isolates representing 2% (one fruit isolate from Sarasota, Florida and 2 leaf isolates from Mayagüez, Puerto Rico) initially identified as *C. gloeosporioides* were not confirmed at species level using PCR and ELISA assays. Seven isolates from guava, soursop and starfruit tested positive for *C. gloeosporioides*, while isolates from annona (PR), papaya (FL) and coffee (PR) were negative for both *C. gloeosporioides* and *C. acutatum*. All *Colletotrichum* spp. isolates obtained from Florida's markets imported from Mexico, Brazil and Nicaragua were identified as *C. gloeosporioides*.

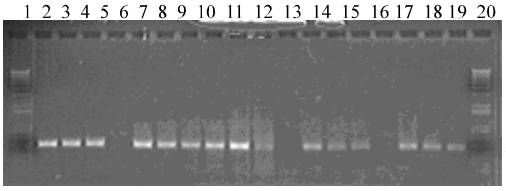

Fig. 4: *Colletotrichum* isolates from mango identified as *C. gloeosporioides* using primers *Cg*Int and ITS 4. Isolates tested were: 1820-2 (lane 2 and 13), 1820-3 (lane 3 and 14), Mxed 01 (lane 4 and 15), *C. acutatum* control (lane 5 and 16), 1815-3 (lane 6 and 17), 1821-3 (lane 7 and 18), 1820-4 (lane 8 and 19), 1820-5 (lane 9), *C. gloeosporioides* + control (lane 10 and 11), water (lane 12) and 1kb ladder (lanes 1 and 20)

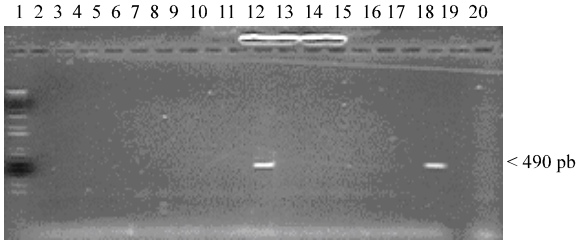

Fig. 5: *Colletotrichum* isolates from mango were identified as *C. acutatum* using primers *Ca*Int2 and ITS 4. Isolates tested were: 1824-4 (lane 2), 1866 (lane 3), 1805A (lane 4), 1805C (lane 5), Mcg 27(lane 6), Mcg 36 (lane 7), Mcg 12 (lane 8), Mcg 73 (lane 9), Mcg 25 (lane 10), Mcg 89 (lane 11), Mxed 02 (lane 12), Mxed 12 (lane 13), Mxed 13 (lane 14), Mxed 14 (lane 15), *C. acutatum* + control (lane 18), water (lane 20) and 1kb ladder (lane 1)

Pathogenicity tests: Pathogenicity tests conducted with 110 selected isolates on detached mango leaves showed that 100% were pathogenic on mango leaves. Necrotic lesions and typical orange conidial masses were often observed on leaves 5 to 7 days after inoculation (Table 3). Puerto Rico's isolates of

Table 3: Pathogenicity tests of *C. gloeosporioides* and *C. acutatum* isolates collected from mango tissues from Puerto Rico and Florida. Data represent mean lesion dia. (mm) per number of isolates tested on detached mango leaves *in vitro* after 5 to 7 days of incubation at 25°C

Isolates from	Mean lesion diam. (mm), isolate origin and No. of isolates tested			
	Leaf	Fruit	Panicle	Flower
Florida				
C. gloeosporioides	11.87 (n = 6)	14.02 (n = 32)	ND	ND
C. acutatum	ND	17.40 (n = 32)	12.16 (n = 4)	18.00 (n =2)
Puerto Rico				
C. gloeosporioides	27 (n = 59)	40 (n = 7)	ND	ND

C. gloeosporioides caused larger lesion diameter (mm) on detached mango leaves than Florida's isolates. Fruit isolates caused larger lesions than those obtained from leaves. Florida's isolates of *C. acutatum* obtained from flowers produced larger lesions than those obtained from panicles. There were no differences in lesion size among Florida's *C. gloeosporioides*.

DISCUSSION

Present results verified and corroborated the identity of *C. acutatum* causing anthracnose on mango flowers, peduncles and immature fruit from Homestead, Florida. Previous studies in Florida using cluster analysis of randomly amplified polymorphic DNA (RAPD) indicated five distinctive *Colletotrichum* groups. Davis (1999) applied boot-strap analysis and stated that RAPD groupings were not rigorous and that RAPD groupings could not be used to evaluate phylogenetic relationships among groups because membership of individual isolates within a particular group could be considered equivocal. Based on spore shape and virulence on mango inflorescence, Davis (1999) suggested that one group of isolates might be classified as *C. acutatum* and not *C. gloeosporioides*.

The findings reported herein facilitate and improve current detection systems of diseases through the application of DNA technology (PCR). An important fact is that *C. acutatum* is reportedly more resistant to fungicides such as benomyl and azoxystrobin than the more common species *C. gloeosporioides,* which is critical for disease management purposes (Adakaveg and Foster, 2000: Lugo- Noel, 2001). This might imply future significant changes in distribution and prevalence of *Colletotrichum* populations in sampled regions of Florida.

In Puerto Rico, *C. acutatum* was not isolated from mango and has not been reported, even though, fungal isolations were made strictly from fruits and leaves. Studies on the genomic and pathogenic diversity of *Colletotrichum* spp. are needed to improve the scope of our knowledge of the anthracnose causing populations in different tropical fruits in Puerto Rico.

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