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MORPHOLOGICAL, MOLECULAR AND PATHOGENIC CHARACTERIZATION OF *Colletotrichum gloeosporioides* ISOLATED FROM MANGO[1]

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ABSTRACT - Mango (*Mangifera indica* L.) is a tropical fruit of great importance in the world. However, fruit postharvest losses are caused by anthracnose, a disease caused mainly by phytopathogenic fungi from the *Colletotrichum* genus. Thus, this work aimed to study morphology, genetic similarity, and pathogenicity of *Colletotrichum* spp. isolates from mango in Rio Grande do Norte state, Brazil. Morphological evaluation of 28 isolates, based on colony color, size of conidia and appressoria, and growth rate, showed that the isolates belong to the *C. gloeosporioides* species. It was later confirmed with species-specific oligonucleotides. Also, combined evaluation with RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeats) showed a genetic similarity of 0.23 between Isolates 12 and 21, and 0.71 between Isolates 2 and 4, an indication of high variability among isolates, with dissimilarity over 75%. Moreover, the dendrogram generated by the UPGMA method indicated that the isolates were arranged in eight groups, which did not correlate with the collection site. Based on pathogenicity, although most isolates showed low virulence for Tommy Atkins cultivar, some of them proved to be highly aggressive (Isolates 9, 15, 20, 21, 24, 26, and 27). Also, pathogenicity did not correlate with clusters. In conclusion, the results revealed high genetic and pathogenic variability in *C. gloeosporioides* isolated from mango cultivars in one of the main exporting regions in Brazil.

Keywords: Anthracnose. Genetical diversity. Pathogenicity. Phytopathogens.

CARACTERIZAÇÃO MORFOLÓGICA, MOLECULAR E PATOGÊNICA DE *Colletotrichum gloeosporioides* ISOLADO DE MANGA

RESUMO - A manga (*Mangifera indica* L.) é uma fruta tropical de grande importância no mundo. No entanto, as perdas pós-colheita dos frutos são causadas pela antracnose, doença causada principalmente por fungos fitopatogênicos do gênero *Colletotrichum*. Assim, este trabalho teve como objetivo estudar a morfologia, similaridade genética e patogenicidade de isolados de *Colletotrichum* spp. de manga no estado do Rio Grande do Norte, Brasil. A avaliação morfológica de 28 isolados, com base na cor da colônia, tamanho dos conídios e apressórios e taxa de crescimento, mostrou que os isolados pertencem à espécie C. *gloeosporioides*. Posteriormente, foi confirmado com oligonucleotídeos específicos para a espécie. Além disso, a avaliação combinada com RAPD (Random Amplified Polymorphic DNA) e ISSR (Inter Simple Sequence Repeats) mostrou uma similaridade genética de 0,23 entre os Isolados 12 e 21, e 0,71 entre os Isolados 2 e 4, um indicativo de alta variabilidade entre os isolados, com dissimilaridade acima de 75%. Além disso, o dendrograma gerado pelo método UPGMA indicou que os isolados foram dispostos em oito grupos, os quais não se correlacionaram com o local de coleta. Com base na patogenicidade, embora a maioria dos isolados tenha apresentado baixa virulência para a cultivar Tommy Atkins, alguns deles se mostraram altamente agressivos (Isolados 9, 15, 20, 21, 24, 26 e 27). Além disso, a patogenicidade não se correlacionou com os aglomerados. Concluindo, os resultados revelaram alta variabilidade genética e patogênica em C. *gloeosporioides* isolado de cultivares de manga em uma das principais regiões exportadoras do Brasil.

Palavras-chave: Antracnose. Diversidade genética. Patogenicidade. Fitopatógenos.

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INTRODUCTION

Mango (*Mangifera indica* L.) is one of the most important tropical fruit in the world (SANTO et al., 2018; CHENG et al., 2019), being relevant for the world economy. India is the main producing country, followed by China with almost four times lower yield (FAOSTAT, 2017). In 2019, Brazil exported approximately 222 thousand tons of mangoes, moving more than US$ 220 million (AGROESTAT / MAPA, 2019). The cultivation areas of mangoes in Brazil have increased by 21.8% a year, rising from 13.2 to 31.2 thousand hectares between 2014 and 2019 (ANUÁRIO BRASILEIRO DE HORTI E FRUTI, 2019). Brazilian production is concentrated in the Northeast region, and the state of Rio Grande do Norte ranks third as the largest producer of the country (IBGE, 2018).

Postharvest is a critical link in the mango production chain because in this phase pathogens can attack fruits, making their commercialization unfeasible (DELGADO et al., 2011). Fungi are the main phytopathogens that cause postharvest diseases in fruits (CARMONA-HERNANDEZ et al., 2019), and *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (1884) stands out for causing rots in mango (PERUMAL et al., 2016). In addition, a disease caused mainly by *Colletotrichum* species, anthracnose has gained attention due to damage in fruits and high cost of control, which has raised production costs and limited fruit export (WEIR; JOHNSTON; DAMM, 2012).

Species within the *Colletotrichum* genus are divided, according to their characteristics, into complexes. The *C. acutatum* complex comprises more than 29 species, *C. gloeosporioides* more than 22, and more than 18 have been reported in the *C. boninense* complex (DAMM et al., 2012; WEIR; JOHNSTON; DAMM, 2012). The species is generally identified by cultural and morphological characteristics such as shape and size of conidia and appressoria, presence or absence of setae, colony color, and growth rate, among others (ADASKAVEG; HARTIN, 1997). However, these morphological traits can overlap among different species and vary under different environmental conditions (KILAMBO et al., 2013). Thus, it becomes necessary to combine other techniques for accurate identification of different species within this genus (LIU et al., 2016).

Molecular tools, such as PCR (Polymerase Chain Reaction) using species-specific primers, combined with morphological analyses, have been used for accurate identification of *Colletotrichum* species (MANAMGODA et al., 2013; SCHENA et al., 2014; FAEDDA et al., 2011; RAMDEEN; RAMPERSAD, 2013; CANNON et al., 2012; VELHO et al., 2015; DOYLE et al., 2013; NOIREUNG et al., 2012). Other molecular tools widely used are markers such as RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeats). Molecular assays for genetic diversity using primers for these markers are used to elucidate genetic variation within and among species (MILGROOM, 1996). In this context, genetic variation refers to the accumulation of mutations for overcoming the host's genetic resistance (MCDONALD; LINDE, 2002). Thus, finding this resistance level is of great importance for identifying individuals with high virulence capacity. Also, studying host-pathogen interaction may aid in elucidating the disease characteristics, in addition to assessing possible variations in virulence among pathogens.

Morphological characterization combined with molecular tools and pathogenicity tests is crucial for disease management and control. Thus, this study aimed to assess the morphology, genetic similarity, and pathogenicity of *Colletotrichum* spp. isolated from mango in Rio Grande do Norte state, Brazil.

MATERIAL AND METHODS

Sample collection

Mango leaves and fruits with anthracnose symptoms were collected from five growing areas in Rio Grande do Norte state, Brazil, for the isolation of *Colletotrichum* sp. fungus (Table 1). The map of the collection region is shown in Figure 1.

Table 1. *Colletotrichum* spp. isolates obtained from mango in Rio Grande do Norte, Brazil.

Farm	Plant	Isolate	Plant tissue	Cultivar	City
Farm 1	Plant 1	1	Fruit	Palmer	Ipanguaçu
	Plant 2	2	Fruit	Palmer	Ipanguaçu
	Plant 3	3	Fruit	Palmer	Ipanguaçu
	Plant 4	4	Fruit	Palmer	Ipanguaçu
		5	Leaf	Palmer	Ipanguaçu
	Plant 5	6	Fruit	Palmer	Ipanguaçu

Table 1. Continuation.

Farm	Plant	Isolate	Plant tissue	Cultivar	City
Farm 2	Plant 6	7	Fruit	Tommy Atkins	Ipanguaçu
	Plant 7	8	Fruit	Tommy Atkins	Ipanguaçu
Farm 3	Plant 8	9	Leaf	Palmer	Ipanguaçu
	Plant 9	10	Fruit	Palmer	Ipanguaçu
		11	Leaf	Palmer	Ipanguaçu
	Plant 10	12	Fruit	Palmer	Ipanguaçu
	Plant 11	13	Fruit	Palmer	Ipanguaçu
	Plant 12	14	Fruit	Palmer	Ipanguaçu
	Plant 13	15	Fruit	Palmer	Ipanguaçu
		16	Leaf	Palmer	Ipanguaçu
	Plant 14	17	Fruit	Palmer	Ipanguaçu
	Plant 15	18	Fruit	Palmer	Ipanguaçu
		19	Leaf	Palmer	Ipanguaçu
	Plant 16	20	Leaf	Palmer	Ipanguaçu
Farm 4	Plant 17	21	Leaf	Tommy Atkins	Pendências
	Plant 18	22	Leaf	Tommy Atkins	Pendências
	Plant 19	23	Leaf	Tommy Atkins	Pendências
	Plant 20	24	Fruit	Tommy Atkins	Pendências
		25	Leaf	Tommy Atkins	Pendências
	Plant 21	26	Fruit	Tommy Atkins	Pendências
	Plant 22	27	Leaf	Tommy Atkins	Pendências
Farm 5	Plant 23	28	Fruit	Palmer	Assú

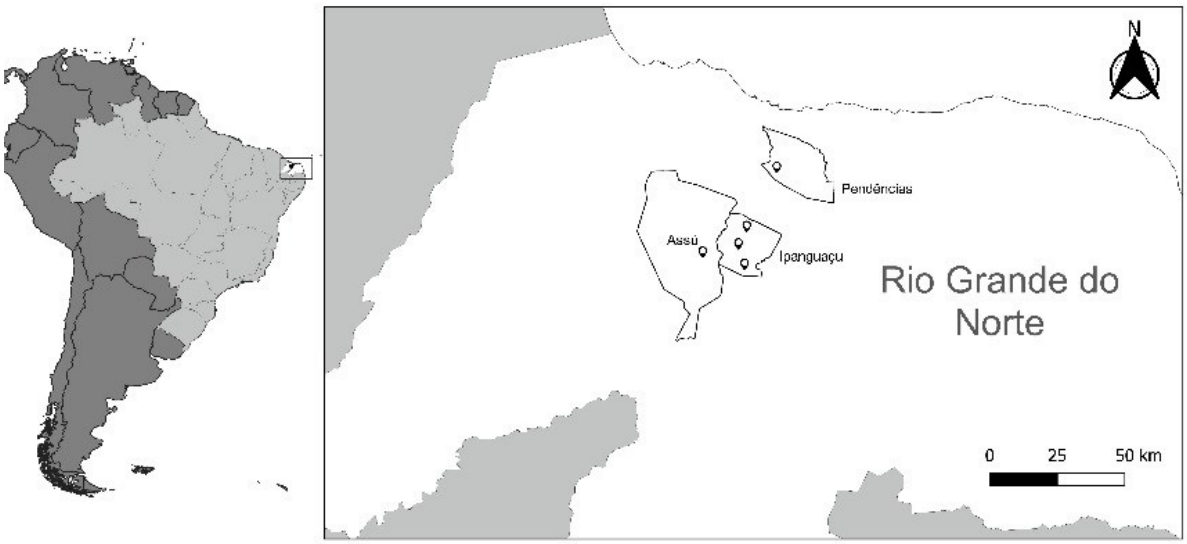

Figure 1. Map of the isolates' collection regions.

Fungus isolation

After collection, the fruits and leaves were washed in sterile distilled water and dried on sterile paper towels. To isolate fungus, tissue fragments (7 mm approximately) were removed from the disease edge (the region between healthy and diseased tissue), then disinfected in 70% alcohol for 30 seconds, followed by 1% sodium hypochlorite solution for 60 seconds, and finally in sterile distilled water. Subsequently, the fragments were placed in Potato Dextrose Agar (PDA) medium with addition of 0.05 g L^{-1} streptomycin and incubated at 28±2 ºC for seven days. After cultivation, fungal structures were transferred from the growing colonies to new PDA plates. From the pure cultures obtained by the monohyphal purification technique, disks containing fungal structures were stored at 5 ºC in flasks containing sterile distilled water (CASTELLANI, 1939).

Morphological analysis

For the fungus morphological analysis, disks (5 mm in diameter) containing fungal structures from the isolated cultures were placed in Petri dishes containing PDA medium and incubated at 25±2 °C and 12-hour photoperiod for seven days, recording their coloring during the incubation. Also, diameter and growth rate (mm day^{-1}) of mycelia of the isolated colonies were daily evaluated. Moreover, for the conidium and appressorium evaluation, the microculture method was used. A piece of agar containing fungal structures was deposited onto slides covered by coverslips, which were then placed onto Petri dishes lined with filter paper moistened with sterile distilled water (MENEZES; ASSIS, 2004). The plates were incubated at 25±2 ºC in BOD for five days. After incubation, the slides were removed from the Petri dishes and evaluated under an optical microscope. For each isolate, length and width of 100 appressoria and conidia randomly selected were determined using the ImageJ software, version 1.52a. Analyses were performed in triplicate, and means were grouped by the Scott-Knott test at 5% probability level using the Sisvar software version 5.7 (Build 91).

DNA extraction

Mycelial mass from the fungal isolates was obtained from PDA plates grown at 28±2 ºC for seven days. The mycelium was macerated in liquid nitrogen using a mortar, and the genomic material was extracted by the Ferreira and Grattapaglia's (1998) method with modifications, using 700 µL extraction buffer solution (1.4 M NaCl; 100 mM Tris -HCl pH 8.0; 20 mM EDTA; 0.5% SDS; 0.2% β-Mercaptoethanol, and Milli-Q water) and incubation at 65 ºC for 60 min. Then, chloroform solution was added: isoamyl alcohol (24:1, v:v) with subsequent precipitation with 400 mL isopropyl alcohol. The precipitates were centrifuged at 15,000 g for 10 min. Subsequently, the precipitates were washed in cold 70, 80, and 90% ethanol, sequentially, and dried at room temperature. The samples were then suspended in sterile water with addition of RNAse, then subjected to a water bath at 37 °C for 30 min, and subsequently stored at -20 °C. The DNA obtained was quantified by electrophoresis in 1% agarose gel, stained with ethidium bromide (0.5 µg mL^{-1}) in TBE buffer, at 120 V for 30 min. Subsequently, the gel was visualized under UV light using the gel documentation system (ProteinSimple, AlphaImager HP system).

Molecular identification

Molecular identification was performed using specific primers for the *Colletotrichum* genus, Cc1F1 (5'-ACC TAA CTG TTG CTT CGG CG-3') and Cc2R1 (5'-AAA TTT GGG GGT TTT ACG GC-3') as described by Cullen et al. (2002), and for *C. gloeosporioides* species, CgInt (5'-GGC CTC CCG CCT CCG GGC GG-3 ") and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3 ') as described by Mills et al. (1992). For PCR, 20 µL final volume was used, containing 25 ng DNA, 1x PCR buffer solution (50 mM KCl, 200 mM Tris-HCl, pH 8.4; Synopsis inc), 2.5 mM each dNTP (Synopsis inc.), 1.5 mM $MgCl_2$, 2 mM Primer, and 1 U Taq polymerase (Synopsis inc.). The DNA amplifications were performed in Amplitherm DNA thermocycler, programmed to operate with an initial denaturation at 95 ºC for 3 min, followed by 35 cycles at 94 ºC for 40 s, 60 ºC for 1 min, and 72 ºC for 1 min, and a final extension at 72 ºC for 10 min. The PCR products were separated on 1.2% agarose gel, stained with ethidium bromide (0.5 µg mL^{-1}) in TBE buffer, under 120 V for 1.5-2 h, and then visualized under UV light using the gel documentation system (ProteinSimple, AlphaImager HP system). The size of the amplified fragments was compared with a 100 bp molecular weight standard (Plus DNA Ladder, Thermo Fisher Scientific).

Genetic diversity

For genetic diversity evaluation, nine ISSR and 12 RAPD primers were used to assess the polymorphism of 28 *C. gloeosporioides* isolates (Table 2). Amplification reactions for the RAPD primers were performed using 12 µL final volume, containing 10 ng DNA, 5 µM primer, 2 mM each dNTP, 1x Buffer solution (50 mM KCl, 200 mM Tris-HCl, pH 8.4), 1.5 mM $MgCl_2$, 5 µg BSA, and 1 U Taq DNA polymerase. The thermocycler was programmed to operate with an initial denaturation at 96 °C for 1 min, followed by 40 cycles of denaturation at 94 °C for 1 min,

annealing at 40 °C for 1 min, and extension at 72 °C for 2 min, and finally an extension at 72 °C for 5 min. In turn, amplification reactions for the ISSR primers were performed using 12 µL final volume containing 10 ng DNA, 0.2 µM primer, 2 mM each dNTP, 1x Buffer solution (50 mM KCl, 200 mM Tris-HCl, pH 8.4), 1.5 mM $MgCl_2$, 5 µg BSA, and 1 U Taq DNA polymerase. Reactions consisted of an initial denaturation at 94 ºC for 4 min, followed by 35 cycles at 94 °C for 40 s, annealing temperature of each primer for 40 s and 72 ºC for 1 min, and a final extension at 72 °C for 5 min.

Table 2. DNA sequence and annealing temperature of the ISSR and RAPD primers used.

Primer number	Primer code	Primer sequence (5' → 3')	Annealing temperature
ISSR primer			
1	DiGA5'CR	CRGAGAGAGAGAGAGAGA	50
2	TriCAC'RC	CACCACCACCACCACRC	55
3	TriGTG	GTGGTGGTGGTGGTG	55
4	TriGTG3'yC	GTGGTGGTGGTGGTGyC	52
5	TriGTG5'Cy	CyGTGGTGGTGGTGGTG	55
6	TriTGT5'Cy	CyTGTTGTTGTTGTTGT	50
7	TriTCA3'RC	TCATCATCATCATCARC	50
8	TriTCC3'RC	TCCTCCTCCTCCTCCRC	50
9	TriCGA3'RC	CGACGACGACGACGARC	45
RAPD primer			
1	OPD 02	GGACCCAACC	40
2	OPD 03	GTCGCCGTCA	40
3	OPD 07	TTGGCACGGG	40
4	OPD 08	GTGTGCCCCA	40
5	OPD 13	GGGGTGACGA	40
6	OPD 18	GAGAGCCAAC	40
7	OPD 20	ACCCGGTCAC	40
8	OPM 01	GTTGGTGGCT	40
9	OPM 02	ACAACGCCTC	40
10	OPM 12	GGGACGTTGG	40
11	OPM 15	GACCTACCAC	40
12	OPM 20	AGGTCTTGGG	40

The PCR products were separated in 1.2% agarose gel, stained with ethidium bromide (0.5 µg mL^{-1}) in 1x TBE buffer, under 120 V for 1.5-2 h, and then visualized under UV light using the gel documentation system (ProteinSimple, AlphaImager HP system). The size of the amplified fragments was compared with a 100 bp molecular weight standard (Plus DNA Ladder, Thermo Fisher Scientific).

Through the analysis of the band pattern produced by both RAPD and ISSR primers for the 28 isolates, a binary matrix was built, attributing the number one to the presence and zero to the absence of polymorphic bands, considering only clear and reproducible bands. Polymorphism Information

Content (PIC) was generated for each selected marker. Thereby, a dendrogram was constructed based on the Jaccard's similarity coefficient using the unweighted pair group method with arithmetic mean (UPGMA) in NTSYS software (ROHLF, 1993).

Pathogenicity test

Virulence of isolates was assessed in Tommy Atkins mango fruits purchased from growing areas in Rio Grande do Norte, Brazil. After washing in running water, the fruits were disinfected by immersion in 1.5% sodium hypochlorite (NaCl) solution for 2 min, then washed again in sterile distilled water and dried at room temperature (30 ± 1°C). Afterward, three wounds (7 mm in diameter) were made on fruit epidermis using a sterile metal rod, and PDA disks (5 mm in diameter) from the edge of the colony were cut out aseptically from the 7-day-growth PDA plates and transferred to each wound. The control group consisted of fruits inoculated with PDA disks without fungal growth. After inoculation, fruits were placed in plastic trays (previously disinfected with 70% alcohol) containing cotton wool moistened with sterile distilled water, which was then covered with plastic wrap to maintain moisture in fruits. Subsequently, the trays with fruits were kept at room temperature (30±1 °C) under natural photoperiod.

The experimental design was completely randomized with six replicates per isolate, and one wound as the experimental unit. Virulence evaluation was performed seven days after fruit inoculation, by measuring the average diameter of lesions (cm). The isolates were considered pathogenic when they injured an area in fruits greater than the initial 7 mm diameter wound. Virulence of the *C. gloeosporioides* isolates were analyzed by ANOVA, and means were grouped by the LSD test at 5% probability level. Statistical analyses were performed in Sisvar software version 5.7 (Build 91).

RESULTS AND DISCUSSION

Morphological analysis

Differences in colony coloring resulted in six distinct groups for the 28 *C. gloeosporioides* isolates (Figure 2). Isolates from Group 1 (Isolate 1) produced slightly grayish colonies with no visible conidial mass. From Group 2 (Isolates 5, 7, 14, 15, 16, 17, 22, 27 and 28), isolates showed slightly grayish mycelium with little visible orange conidial mass. Group 3 isolates (Isolates 6, 8, 10, 11, 20 and 23) showed a gray mycelial mass with dark conidial mass in the center and lateral dark regions. Group 4 (Isolates 2, 9, 12, 13, 21, 18 and 25) showed a slightly salmon color with orange conidial mass, and dark in the center. Group 5 (Isolates 19 and 24) showed salmon color with dark conidial mass and dark concentric rings from center to border of the colony. Group 6 (Isolates 4 and 26) showed concentric color variations ranging from salmon to light gray and dark gray with visible dark conidial mass. Furthermore, colony growth rate was significantly different among isolates (Table 3), ranging from 11.13 (Isolate 26) to 17.73 mm day^{-1} (Isolate 14).

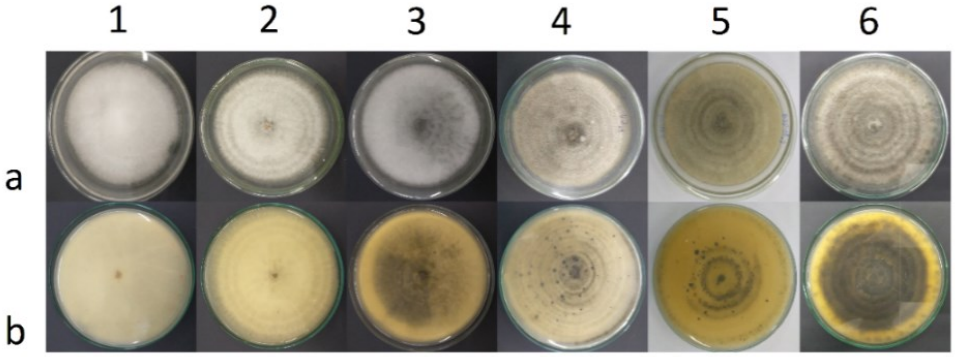

Figure 2. Six morphotypes found for *Colletotrichum gloeosporioides* isolated from mango. a) upper side of the colony; b) lower side of the colony.

Color diversity observed among the fungal colonies may be due to phenotypic expressions being influenced by environmental conditions. Also, the release of secondary metabolites, which have been associated with *Colletotrichum* isolates, may have contributed to morphocultural variations (BAILEY; JERGER 1992; BONETT et al., 2010). The mycelial growth rate was significantly different even for isolates belonging to the same morphocultural coloring group, which indicates that there is no relationship between color and mycelial growth rate. In addition, a significant difference in growth was observed between *C. gloeosporioides* isolated from different tissues of the same plant, such as fruit and leaf tissues, respectively, as between isolates 10 and 11, 15 and 16, 18 and 19, and 24 and 25. This result

suggests a possible growth variation, even at the genetic level, in isolates obtained from different host tissues.

Three morphotypes of conidia and appressoria according to shape were identified for the evaluated isolates (Figure 3): straight with rounded ends (1), straight with constriction (2), and straight and tapered at one end (3) for conidium; and irregular and lobulate (1), irregular and slightly lobulate (2), and rounded and smooth (3) for appressorium. Also, 25 isolates had in general straight with rounded ends conidia, whereas three isolates showed straight and tapered at one end conidia. Regarding appressorium, 19 isolates had rounded and smooth structures, while nine isolates showed irregular and slightly lobulate ones. In addition, septate conidia were observed in 8 of the 28 isolates. Relative to size, conidia ranged from 2.10 to 4.55 µm in width and 7.75 to 14.28 µm in length, while appressoria ranged from 4.89 to 7.05 µm in width and 7.13 to 9.90 µm in length (Table 3).

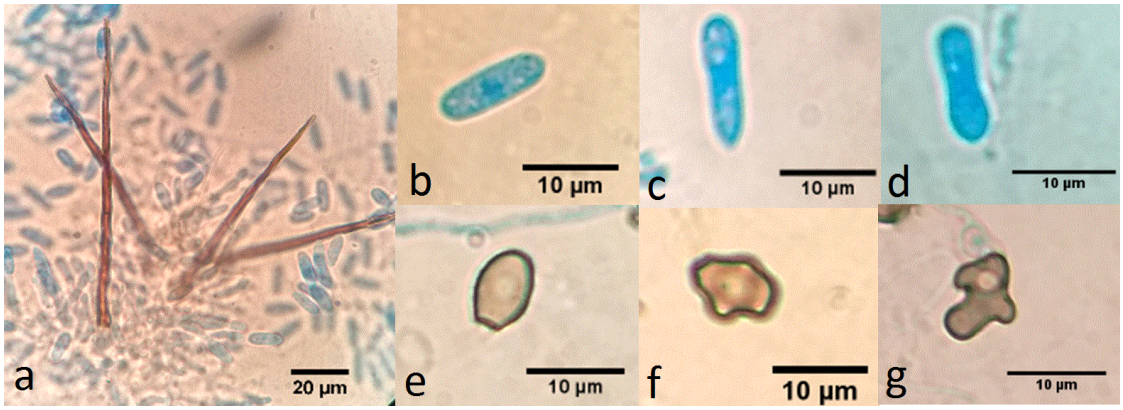

Figure 3. Structures found in the studied isolates. Presence of setae (a). Conidium shape: straight with rounded ends (b), straight and tapered at one end (c), and straight with constriction (d). Appressorium shape: rounded and smooth (e), irregular and slightly lobulate (f), and irregular and lobulate (g).

Table 3. Growth rates, characterization of conidia and appressoria, and presence of setae in *C. gloeosporioides* isolates.

Isolate	Presence of setae	Growth rate (mm day^{-1})	Conidium			Appressorium		
			Width (µm)	Length (µm)	Most found shape	Width (µm)	Length (µm)	Most found shape
1	-	15.47±0.32 e	3.85±0.32 c	11.23±0.86 f	1	6.21±0.78 e	8.62±1.17 e	3
2	+	13.93±0.19 d	3.54±0.27 b	11.34±1.06 f	1	6.34±0.70 f	9.10±1.08 f	2
3	-	14.53±0.24 d	4.55±0.37 f	7.75±0.87 a	1	6.15±1.43 e	8.19±1.29 d	3
4	-	13.20±0.22 c	4.17±0.28 e	11.26±0.97 f	1	6.32±0.70 f	8.21±0.95 d	3
5	-	13.93±0.27 d	3.95±0.29 e	12.17±0.69 h	3	5.13±0.44 a	7.39±0.88 a	3
6	-	13.73±0.26 d	2.82±0.43 a	9.46±1.40 c	1	6.03±0.75 d	8.08±1.19 c	3
7	-	12.40±0.30 b	2.93±0.37 b	10.91±0.59 e	1	5.27±0.67 b	7.41±1.07 a	3
8	-	11.20±0.15 b	2.46±0.45 a	8.32±1.12 b	3	5.88±0.77 d	8.31±0.81 d	2
9	-	13.40±0.16 c	3.54±0.28 c	11.20±0.51 e	1	6.10±0.69 d	7.91±1.30 c	2
10	-	15.60±0.22 e	2.10±0.39 a	10.98±1.27 e	1	5.78±0.79 c	7.73±1.11 b	2
11	-	13.73±0.16 d	3.52±0.32 b	11.86±0.84 g	1	4.89±0.80 a	7.48±1.29 a	2
12	-	14.20±0.26 d	3.65±0.34 c	11.63±1.36 f	1	6.54±0.87 f	8.43±0.97 d	2
13	-	14.33±0.14 d	2.89±0.59 b	9.89±1.23 c	1	5.17±0.75 a	7.13±1.20 a	2
14	+	17.73±0.38 g	3.50±0.23 b	10.52±0.57 d	1	5.56±0.68 b	7.41±0.83 a	3
15	-	14.07±0.23 d	4.03±0.19 e	11.73±0.55 g	1	5.32±0.80 b	7.72±1.17 b	3
16	-	17.40±0.30 g	4.19±0.34 e	12.20±0.62 h	3	6.24±0.64 e	9.30±1.32 g	3
17	+	16.07±0.19 e	4.09±0.37 e	11.67±0.76 g	1	6.09±0.66 d	8.24±0.95 d	3
18	+	14.07±0.22 d	2.94±0.52 b	11.15±1.29 e	1	5.86±0.50 c	8.13±0.68 d	2
19	+	16.27±0.13 f	4.27±0.40 f	12.79±0.84 i	1	6.87±0.59 g	9.60±0.88 h	3

Most found shape. Conidium: straight with rounded ends (1), straight with constriction (2), straight and tapered at one end (3). Appressorium: irregular and lobulate (1), irregular and slightly lobulate (2), and rounded and smooth (3). Mean ± standard error. Means with different letters in the same column are significantly different by the Scott-Knott test ($p \leq 0.05$).

Table 3. Continuantion.

Isolate	Presence of setae	Growth rate (mm day^{-1})	Conidium			Appressorium		
			Width (µm)	Length (µm)	Most found shape	Width (µm)	Length (µm)	Most found shape
20	+	11.33±0.14 a	4.29±0.58 f	14.28±2.62 j	1	5.90±0.74 d	8.62±1.06 e	2
21	+	15.53±0.25 e	4.32±0.33 f	12.28±0.67 h	1	7.05±0.76 g	9.90±1.01 h	3
22	+	15.13±0.23 e	3.98±0.21 e	11.83±1.48 g	1	5.74±0.46 c	7.92±0.78 c	3
23	-	14.80±0.29 e	3.88±0.26 d	11.31±0.75 f	1	5.67±0.45 c	7.91±0.81 c	3
24	-	12.87±0.14 c	3.65±0.38 c	12.29±0.74 h	1	5.85±0.62 c	8.91±0.95 f	3
25	-	15.40±0.38 e	3.92±0.25 d	13.04±0.98 i	1	5.44±0.50 b	8.16±0.86 d	3
26	-	11.13±0.27 a	3.88±0.23 d	11.68±0.69 g	1	5.21±0.81 b	7.82±1.25 c	3
27	-	16.00±0.36 f	4.26±0.54 f	10.85±1.18 d	1	5.12±0.90 a	7.34±1.92 a	3
28	-	16.47±0.08 f	4.02±0.29 d	12.45±0.85 h	1	6.30±0.79 e	9.59±1.84 g	3

Most found shape. Conidium: straight with rounded ends (1), straight with constriction (2), straight and tapered at one end (3). Appressorium: irregular and lobulate (1), irregular and slightly lobulate (2), and rounded and smooth (3). Mean ± standard error. Means with different letters in the same column are significantly different by the Scott-Knott test ($p \leq 0.05$).

Most isolates had a predominance of straight conidia with rounded ends, a characteristic shape in *C. gloeosporioides* (SUTTON, 1992). Also, according to Sutton (1992), conidia in this species are 12 to 17 µm in length and 3.5 to 6 µm in width. However, conidia in the present study were smaller in size (7.75 µm in length and 2.10 µm in width). Other studies (TOZZE JÚNIOR et al., 2015; ANDRADE, 2007) also reported different results from those of Sutton (1992). Thus, as stated by Damm et al. (2012), variability in the expression of these traits turns the identification of this species based on its morphology ambiguous.

Although morphological and molecular characterization of *Colletotrichum* species have been studied worldwide, such studies are still scarce for Rio Grande do Norte (RN) state, a great mango exporter in Brazil. The combination of morphological and molecular traits in the present study confirmed *C. gloeosporioides* as one of the etiological agent of anthracnose in mango in RN. Also, we identified, by molecular markers, that 100% of the isolates obtained here (28) belong to this species complex. Specific primers for *C. gloeosporioides* also successfully detected this species in tropical fruits, such as tamarind, passion fruit, and mango (AFANADOR-KAFURI et al., 2003). These results confirm the reliability of these primers for accurate detection, especially when morphological identification is difficult (WHITELAW-WECKERT et al., 2007). In addition, PCR-based tools are of great interest since they allow a fast and accurate diagnosis of this species.

Molecular analysis

Molecular identification

DNA of the 28 isolates from mango leaves and fruits were amplified, respectively, by the Cc1F1/Cc2R1 and CgInt/ITS4 primers, which are specific for *C. gloeosporioides* genus and species (Figure 4). Both primers generated a 450 bp DNA fragment, as expected.

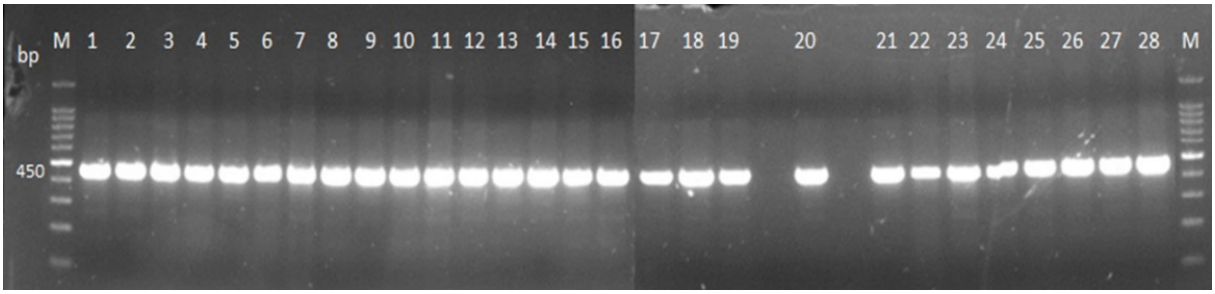

Figure 4. PCR amplification product by the CgInt/ITS4 primer pair for the detection of *Colletotrichum gloeosporioides*.

Genetic diversity

In ISSR and RAPD primer profiles, an apparent reliability and reproducibility of PCR amplifications were observed. Amplifications using the 12 RAPD primers (Figure 5) produced 167 loci, of which 128 (76.6%) were polymorphic (Table 4), being highly distinct bands. The number of amplified

bands per primer ranged from 7 (OPD08) to 22 (OPM01). Although none of the 12 primers obtained 100% polymorphism, the OPM01 primer showed the highest polymorphism rate (90%). Also, the highest PIC value was observed for OPD07 (0.440), while the lowest one was observed for OPM02 (0.265). The average PIC for the RAPD primer was 0.364.

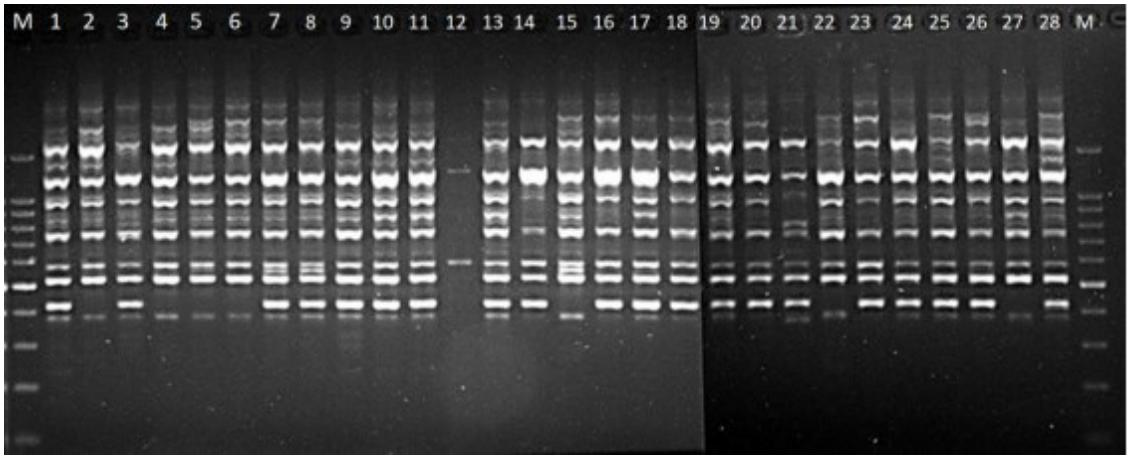

Figure 5. PCR amplification products obtained with RAPD marker for 28 *C. gloeosporioides* isolates after electrophoresis on agarose gel. 'M' represents the 100 bp molecular weight marker. Channels 1 through 28 represent each of the 28 isolates.

Table 4. Data obtained from PCR amplification by RAPD and ISSR primers for total loci (TL), monomorphic loci (ML), polymorphic loci (PL), percentage of polymorphism (%P), and Polymorphic Information Content (PIC).

Primer	TL	ML	PL	%P	PIC
RAPD primers					
OPD 02	10	1	9	90	0.387
OPD 03	8	2	6	75	0.356
OPD 07	9	1	8	88.9	0.440
OPD 08	7	2	5	71.4	0.328
OPD 13	12	7	5	41.7	0.363
OPD 18	17	3	14	82.4	0.290
OPD 20	20	2	18	90	0.328
OPM 01	22	2	20	90.9	0.406
OPM 02	17	3	14	82.4	0.265
OPM 12	15	8	7	46.7	0.414
OPM 15	12	3	9	75	0.378
OPM 20	18	5	13	72.2	0.408
Total	167	39	128	76.6	-
Mean	13.9	3.25	10.7	-	0.364
ISSR primers					
DiGA5'CR	8	0	8	100	0.356
TriCAC'RC	7	1	6	85.7	0.215
TriGTG	10	0	10	100	0.353
TriGTG3'yC	8	2	6	75	0.460
TriGTG5'Cy	7	0	7	100	0.439
TriTGT5'Cy	5	0	5	100	0.364
TriTCA3'RC	6	0	6	100	0.401
TriTCC3'RC	6	0	6	100	0.446
triCGA3'RC	6	2	4	66.7	0.385
Total	63	5	58	92.1	-
Mean	7	0.5	6.4	-	0.380

Amplifications obtained with the nine ISSR primers produced 63 loci, of which 58 (92.1%) were polymorphic (Table 4). The number of amplified bands per ISSR primer ranged from five (TriTGT5'Cy) to ten (TriGTG). From the nine primers, six showed 100% polymorphism (DiGA5'CR, TriGTG, TriGTG5'Cy, TriTGT5'Cy, TriTCA3'RC, and TriTCC3'RC). The highest PIC value was obtained with ISSR10 (0.460), while the lowest one was obtained with ISSR08 (0.215), 0.380 on average per primer. Data obtained with both primers were combined to generate the genetic similarity.

Polymorphic variation was 66.7 to 100% for ISSR and 41.7 to 90.9% for RAPD among the isolates, with high polymorphism rate for ISSR primers. Other studies have identified the high capacity of ISSR primers to assess genetic diversity (ROCHA et al., 2012; ABADIO et al., 2012; SANTANA et al., 2011; SILVA et al., 2011; SHOUHANI et al., 2014). However, some authors revealed a higher percentage of polymorphism from RAPD primers, compared to ISSR, attributing this performance to greater capacity in accessing microsatellite regions that are more informational in the genome (TANYA et al., 2011). In the present study, PIC values were 0.380 on average for ISSR (ranging from 0.215 to 0.460) and 0.364 for RAPD (ranging from 0.290 to 0.440). Differently, Prittesh et al. (2018) found higher PIC values for the ISSR and RAPD primers (0.767 and 0.705, respectively) when evaluating the genetic diversity among *C. falcatum* isolates.

Based on the cut-off line used for the genetic separation of the isolates, the dendrogram (Figure 6) formed eight groups with 0.51 similarity: Group 1 (Isolates 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13 , 14, 15, 16, 19, 27 and 28), Group 2 (Isolates 22 and 26), Group 3 (Isolates 20, 24 and 25), Group 4 (Isolate 18), Group 5 (Isolate 23), Group 6 (Isolate 17), Group 7 (Isolate 21), and Group 8 (Isolate 12). The similarity coefficient ranged from 0.23 (Isolates 12 and 21) to 0.71 (Isolates 2 and 4). Thus, although low PIC values were obtained in our study, low similarity coefficients obtained with both primers revealed their capacity in accessing the genetic diversity among *C. gloeosporioides*.

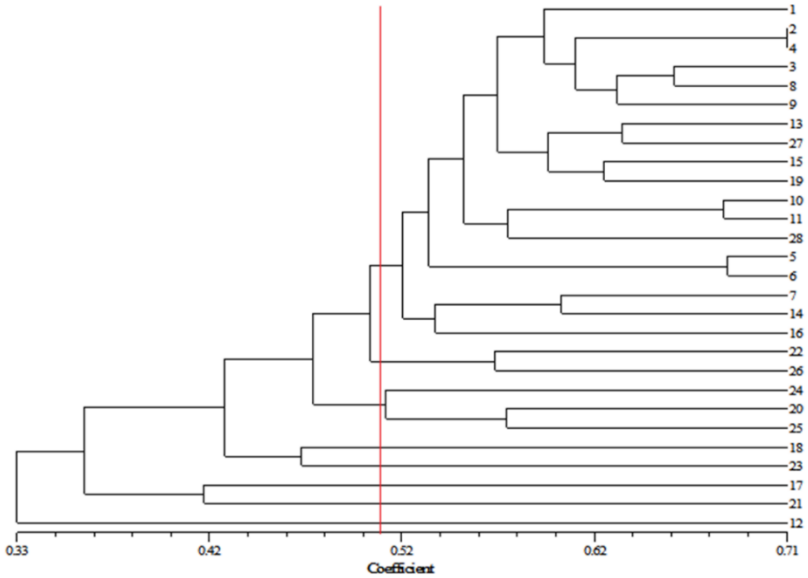

Figure 6. Dendrogram generated by the UPGMA grouping method from the combination of ISSR and RAPD primers. Red line means the cut generated for the evaluation of the obtained groups.

The genetic diversity was found among isolates collected from the same plant, thus identifying the high genetic variation in *C. gloeosporioides* affecting mango in Rio Grande do Norte, Brazil. In citrus, on the other hand, the molecular characterization of *Colletotrichum* did not group isolates collected from different tissues in the same plant (SILVEIRA et al., 2016).

RAPD and ISSR detected variation in polymorphism and were able to discriminate individuals, which indicates the effectiveness of both primers for genetic diversity analysis. Despite that, the isolates were not grouped according to their location, as shown in the dendrogram. On the other hand, when assessing the genetic diversity among *C. falcatum* in pepper by ISSR, Ratanacherdchai et al. (2010) also reported that diversity among individuals was correlated with geographic

distribution. Therefore, results in the present study suggest the occurrence of cross-infection by *Colletotrichum* from different locations, and the genetic exchange among regions, which thus did not allow grouping isolates from different origins. Also, the dispersion may lead to contamination by more pathogenic isolates over RN region, which can increase production costs to control it and lead to production losses.

Pathogenicity evaluation

Virulence was significantly different among the *C. gloeosporioides* isolates ($P \leq 0.05$) (Figure 7). Only three isolates (Isolates 5, 8 and 23) did not lesion the fruits (0.7 cm compared to initial wound) (Table 5). In contrast, the other isolates caused typical anthracnose lesions. The largest injuries were caused by Isolates 24 (2.7 cm), 21 (2.6 cm), and 20 (2.3 cm).

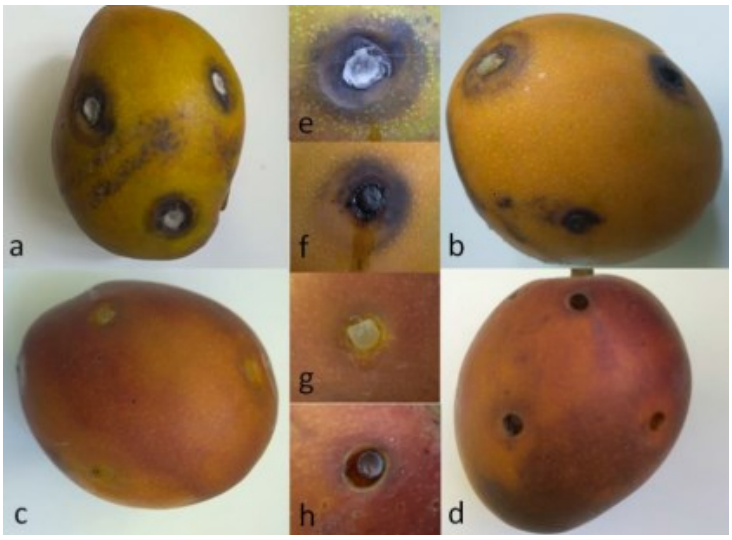

Figure 7. Pathogenicity of *C. gloeosporioides* in Tommy Atkins mangoes seven days after fruit inoculation. Lesions caused by the most virulent isolates: Isolates 24 (a) and 21 (b), and detail of their respective lesions (e and f). And two non-pathogenic isolates: Isolates 5 (c) and 8 (d), and their inoculation sites (g and h).

Table 5. Average diameter of lesion in mangoes caused by *C. gloeosporioides* isolates.

Isolate	Lesion (cm)	Isolate	Lesion (cm)
Control	0.7 a	15	2.1 ef
1	0.82 ab	16	0.95 b
2	0.88 ab	17	0.85 ab
3	0.78 ab	18	0.77 ab
4	0.75 ab	19	0.72 ab
5	0.7 a	20	2.3 f
6	1.2 c	21	2.6 g
7	0.73 ab	22	0.73 ab
8	0.7 a	23	0.7 a
9	2.1 e	24	2.7 g
10	0.82 ab	25	0.8 ab
11	0.82 ab	26	1.65 d
12	0.75 ab	27	1.77 d
13	0.75 ab	28	0.82 ab
14	0.95 b		

Different letters show significant differences ($P \leq 0.05$) according to the t test (LSD).

Regarding *Colletotrichum* virulence, 67% of isolates from Tommy Atkins cultivar were virulent, causing lesions over 1 cm in diameter in fruits, while only 21% from Palmer showed virulence.

Lima et al. (2015) observed that Tommy Atkins cultivar was more resistant against *C. gloeosporioides* pathogenicity than Keith and Palmer. It was also observed in the present study, in which most of the *Colletothricum* isolates were not virulent for Tommy Atkins cultivar, which can be due to characteristics of the fruits and isolates. According to Lima et al. (2015), this low virulence may be associated with fruits from this cultivar being more resistant to postharvest deterioration, which makes them less perishable and partially resistant to anthracnose, and, therefore, more attractive for production and export.

Regarding locations, the most pathogenic isolates were from Farm 3 (Isolates 9, 15, and 20) located in Ipanguaçu, RN, and from Farm 4 (Isolates 21, 24, 26, and 27) located in Pendências, RN. The isolates from the other farms were less virulent. Lima et al. (2015) also found highly virulent isolates for mango in Pendências, the same location as Farm 3, causing lesions of 6 cm in diameter 10 days after fruit inoculation. The results indicate that isolates from these areas may have a highly virulent genetic load, which sustains the need for strict management practices to control this fungus.

Ten isolates were obtained from fruits and leaves from five different plants. It was noted that isolates from fruits (15 and 24 from plants 13 and 20, respectively) were more virulent than isolates from leaves (16 and 25 from plants 13 and 20, respectively). The other isolates (4, 5, 10, 11, 18, and 19) showed low virulence, which was not significantly different among them.

Although genetic diversity among *Colletotrichum* isolates could be assessed using ISSR and RAPD markers, the genetic similarity was not correlated with pathogenicity levels, thus some groups included both pathogenic and non-pathogenic individuals. It may be related to the hypothesis that pathogenic isolates may have originated from pre-existing non-pathogenic populations or they had lost their pathogenicity (GORDON; MARTYN, 1997). Also, as pathogenic traits can be controlled by several genes, another justification is that the ISSR and RAPD markers may not have accessed these regions in the genome. Therefore, these markers could not distinguish the pathogenic isolates from the non-pathogenic ones.

CONCLUSION

Our findings reveal a great species diversity of the *C. gloeosporioides* complex in mango from growing areas in Rio Grande do Norte, Brazil. Therefore, extensive research on the characterization and pathogenicity of *Colletotrichum* species allows knowing their virulence potential in the producing regions.

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