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A N I N T E R N A T I O N A L J O U R N A L

Molecular Analysis in the Differentiation of *Colletotrichum gloeosporioides* Isolates from the Cashew and Mango Trees

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ABSTRACT

The aim of the present work was to analyze the molecular methods in the differentiation of Colletotrichum gloeosporioides *isolates obtained from the cashew and mango trees. The different molecular taxonomic methods used proved to be efficient regarding intraspecific characterization. Similarly, molecular methods also proved to be efficient in differentiation of the* C. gloeosporioides *isolates in relation to host specificity. In the analysis of the ITS sequence of the ribosomal DNA, all the isolates amplified with the CgInt and ITS4 primers, confirming that they pertained to* C. gloeosporioides*. The results from this study suggested that methods based on the pathogenicity, isozyme analysis and RAPD were effective in differentiating C. gloeosporioides isolates from the cashew and mango trees.*

Key words: *Anacardium occidentale*, anthracnose, ITS-rDNA, *Mangifera indica*

INTRODUCTION

Anthracnose in the cashew (*Anacardium occidentale*) and mango (*Mangifera indica*) trees is caused most often by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., and is considered a disease of economic loss in the Northeast Brazil (Menezes and Hanlin, 1996a; Menezes and Hanlin, 1996b; Serra and Silva, 2004). *C. acutatum* J.H. Simmonds may also be the anthracnose agent in the mango trees (Freeman et al., 1998; Arauz, 2000). These species attack the leaves, branches, blossoms, stems and fruits, requiring periodic spraying with the fungicides in orchards and efficient post-harvest treatment (Ribeiro, 2005). The species cause varying degrees of harm depending on the susceptibility of the host plant and environmental conditions (Menezes, 2005; Ribeiro, 2005).

In Brazil, anthracnose in the mango trees is present in all the productive regions and is one of the greatest phytosanitary problems, especially in fruit exportation. In the Northeast, the main mango cultivation areas are located in the states of Bahia, Pernambuco and Ceará. In the cashew tree cultivation, the disease is widely disseminated in

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all the cropping areas, and is quite severe in the years with high rainfall, especially during the "cashew rains" at flowering, with a more intensive attack on the blossoms and greater harm to the production (Menezes, 2005).

Considering that the identification of *Colletotrichum* species or biotypes is nearly always difficult due to enormous variation in the morphology accepted for the different species of the genus, modern techniques have been used to integrate the morpho-physiological, biochemical and molecular methods in taxonomic studies regarding this plant pathogen (Freeman, 2000).

The shape and dimension of the conidia were basic morphological criteria used by Simmonds (1965) and later by Cox and Irwin (1988) for the separation within *C. gloeosporioides* species. Traditional morpho-physiological methods for differentiating *C. gloeosporioides* isolates include conidia morphology, appressoria formation, presence or absence of setae, presence or absence of teleomorph, color of colonies, mycelial growth rate and sensitivity to fungicides (Freeman, 2000; Serra and Silva, 2004).

Specific pathogenicity to a particular host, or host group, and cross-infection have also been used to characterize the *Colletotrichum* species as additional criteria to cultural and morphological characteristics (Arauz, 2000; Afanador-Kafuri et al., 2003). Freeman (2000) reported that a single species of *Colletotrichum* can infect different hosts. Different *Colletotrichum* species or biotypes can affect a single host, such as, for example, anthracnose in avocado and mango trees caused by *C. gloeosporioides* and *C. acutatum*, and in strawberries caused by *C. gloeosporioides*, *C. fragariae* Brooks and *C. acutatum*. According to Freeman and Shabi (1996), *C. gloeosporioides* isolates from an immense range of tropical, subtropical and temperate fruit trees have demonstrated considerable potential for cross infection.

Molecular methods have been used successfully in differentiation between the species and genotypes of *Colletotrichum* from a high number of hosts. The analysis of the nucleotide sequence of the internal transcribed spacing (ITS) of the ribosomal DNA (rDNA) from genes of □-tubulin 2 (*tub2*), histone 4 (*his4*), glutamine synthase (GS), glyceraldehyde-3-phosphate dehydrogenase (GPDH), mytocondrial DNA (mtDNA), RAPD, RFLP and AFLP markers and isozyme analysis have demonstrated the genetic complexity of *Colletotrichum* isolates obtained from diverse tropical and temperate fruit trees (Freeman, 2000; Talhinhas et al., 2005).

Reports on the diseases caused by *C. gloeosporioides* in diverse fruit trees have often demonstrated that the fungus exhibits wide phenotypic, genotypic and pathogenic variability (Menezes and Hanlin, 1996a; Afanador-Kafuri et al., 2003; Talhinhas et al., 2005) and consequently, the occurrence of pathogen populations with differentiated behavior can determine variations in the visual aspect of the disease, thereby affecting the adoption of control strategies (Freeman et al., 1998).

In recent years, a number of methods have been proposed to differentiate the species or isolates from a single fungal species, thereby contributing to precise diagnosis through the integration of methods and permitting the detection of forms on a sub-specific or even breed level. Thus, the aim of the present study was to analyze the molecular methods in the differentiation of *C. gloeosporioides* isolates obtained from the cashew and mango trees.

MATERIAL AND METHODS

Fungal cultures and growth conditions

The isolates were obtained from the cashew and mango trees from the Northeastern Brazilian states of Maranhão, Pernambuco and Paraíba in mid-2003 and codified according to the place of origin, cashew-MA (CMA), cashew-PE (CPE), cashew-PB (CPB), mango-MA (MMA), mango-PE (MPE), and mango-PB (MPB). The fungus was isolated from the leaves with symptoms of anthracnose. Following the routine cleaning and disinfection of the material, fragments from the transition region between the lesion and the healthy tissues (Menezes and Assis, 2004) were transferred to Petri dishes containing a potato-dextrose-agar (PDA) and then incubated at 25±2^{o}C in an alternating photoperiod (12h of light / 12h of darkness), adopting the method described by Menezes and Assis (2004), and the colonies were kept in tubes containing PDA.

ISOZYME CHARACTERIZATION OF *C. GLOEOSPORIOIDES* ISOLATES

Fungus culture and protein extracts

The *Colletotrichum* isolates were cultivated in 125ml of the Potato-Dextrose (PD) medium at 25±2°C for six days under a 12-h photoperiod, and gently agitated twice per day.
The mycelia was collected through filtration after two successive rinses with sterile distilled water. Next, 400mg of mycelia were ground in a chilled mortar with the addition of 150mg of sucrose, 150mg of polyvinylpyrrolidone and 1.0mL of the Tris-glycine buffer 0.125 M, pH 8.2. After a 12-h resting period, the samples were centrifuged at 14,.000 rpm for six minutes and the protein extracts were kept at 4°C (Alfenas et al., 1991).

Preparation of gel plates and electrophoresis
Polyacrylamide gels (5%) in a tris-glycine buffer at 0.125 M, pH 8.2, were prepared following the methodology described by Alfenas et al. (1991). After polymerization, the gel was placed in a horizontal tray containing the tris-glycine buffer at 0.125 M, pH 8.2. Ten microliters of the protein extract from each isolate were applied individually to the gel. Bromophenol blue was used as the marker. Electrophoresis was performed at 4°C under a constant current of 10mA until the marker line reached 6.5cm of the gel.

Detection of isoesterase and total proteins
To reveal the esterase bands, the gel was immersed in a solution containing 100mL of a phosphate buffer 0.1 M, pH 6.5, 50mg of 1% α-naphtyl acetate and 50mg of fast blue RR for one hour and kept in the dark at 37°C. To reveal the total proteins, the gel was immersed in a Coomassie blue stain for 12-h, following the technique described by Alfenas et al. (1991).
For the evaluation of the electrophoretic profiles of the isolates, the following parameters were considered: number, color intensity and relative mobility (Rf) of the bands in the polyacrylamide gel, with the latter determined through the formula: Rf = (d/D) x 100, where d = distance traveled by the molecule, and D = distance traveled by the stain marker. The genetic similarity between the isolates in the systems studied was determined using the Jaccard coefficient; matrix and genetic clustering analyses were performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using the NTSYS-pc program.

MOLECULAR CHARACTERIZATION OF *COLLETOTRICHUM GLOEOSPORIOIDES* ISOLATES

Extraction and quantification of fungal DNA
Colletotrichum isolates were cultivated in 150ml of potato-dextrose (PD) at 25±2°C under a 12-h photoperiod, with mild agitations twice per day for six days. The DNA from the isolates was extracted from 250 mg of mycelia following the procedure described by Faleiro et al. (2004). Mycelia were collected through the filtration and ground in liquid nitrogen. Next, 700µl a lyse buffer was added (50mM Tris-HCl, pH 8.0; 50mM EDTA; NaCl 5M; 3% sodium dodecyl sulfate (SDS); 1% β-mercaptoethanol), followed by 30 minutes of incubation at 70°C, with agitations every 10 minutes. Then the samples were centrifuged at 14,000 rpm for 10 minutes and a cloroform-isoamyl alcohol solution (25:1) was added. The samples were agitated through gentle inversion and once again centrifuged. The supernatants were transferred to new tubes and the deproteinization process was repeated.
For the precipitation of DNA, NaCl 5M and chilled isopropanol were added to the supernatants. The tubes were kept at -20°C for two hours and afterward centrifuging was repeated. The precipitates were rinsed twice with 70% ethanol and dried at room temperature. Then the total nucleic acids were re-suspended in 150µl of water containing RNAse in a concentration of 40 µg/ml and placed into a water bath at 37°C for one hour for complete re-suspension and digestion of the RNA. The DNA was visually quantified in a 0.8% agar gel stained in an ethidium bromide solution at 0.05 mg/l from a comparison with a DNA standard (Low DNA Mass Ladder, Invitrogen).

RAPD analysis
Amplification reactions followed the methodology proposed by Williams et al. (1990), with the following concentrations: 3ng/µl genomic DNA, buffer 10X, 2mM $MgCl_2$, 200µM of dNTPs, 0.25µM of primer, 1U Taq DNA polymerase (Invitrogen™) and ultra pure water to complete a volume of 15µl. Amplifications were performed under the following conditions: one cycle at 94°C for 2 minutes; 40 cycles at 94°C for 1 minute,

40.4°C for 1 minute and 72°C for 2 minutes; and one cycle at 72°C for 7 minutes. A total of 30 previously selected primers were used: OPAA02, OPA04, OPA10, OPA11, OPA12, OPA15, OPA18, OPB10, OPB12, OPB17, OPC08, OPC11, OPC15, OPC20, OPD01, OPD07, OPD15, OPD18, OPT17, OPE02, OPE03, OPE04, OPV08, OPV18, OPV19, OPW06, OPX01, OPX07, PM06 and RC07. The amplification products were separated through electrophoresis in a 1.5% agar gel at 100V and stained in an ethidium bromide solution at 0.05 mg/ml. The band patterns generated were annotated as presence (1) or absence (0) and later converted to a binary matrix. Only the bands that presented adequate definition and were present in both the repetitions performed for each primer and presented lengths in the 400 to 2000bp range were considered. Genetic similarity was calculated using the Jaccard coefficient. Matrix and genetic clustering analyses were performed using the UPGMA method and the NTSYS-pc software program.

Analysis of the ribosomal DNA ITS region

The six *Colletotrichum* isolates obtained from the mango and cashew trees were subjected to PCR reaction with specific primers. For *C. gloeosporioides*, the CgInt primer (5'-GACCCTCCCGGCCTCCCGCC-3') was used, and for *C. acutatum*, the CaInt2 primer (5'-GGGGAAGCCTCTCGCGG-3') was used (Xiao et al., 2004). Both the primers were used together with the conserved ITS4 universal primer (5'-TCCTCCGCTTATTGATATTGC-3').

The amplification reaction was performed following the procedure described by Afanador-Kafuri et al. (2003) for a final volume of 25µl, containing 10 to 70ng of genomic DNA, 0.25µM each primer, 200µM of dNTPs, 1.5mM $MgCl_2$, 1.25 U Taq DNA polymerase (Invitrogen™) and ultra pure water to complete the final volume. Amplification was carried out under the following conditions: one cycle at 95°C for 5 minutes; 40 cycles at 95°C for 30 seconds, 65°C for 30 seconds and 72°C for 90 seconds; and one final cycle at 72°C for 5 minutes. The amplification products were separated in 1.5% agar gel in the Tris-acetate-EDTA buffer, through horizontal electrophoresis at 80V for 2 hours. The gel was stained in an ethidium bromide solution at 0.5mg/l and the products were observed under ultraviolet light.

RESULTS AND DISCUSSION

Protein and isoesterase characterization of *Colletotrichum gloeosporioides* isolates through electrophoresis in polyacrylamid gel

Between the two systems analyzed in the present study (Table 1), esterase presented a variation from one to three bands, with a predominance of three, in all the isolates, except MPB (mango-PB), which presented only one band (Est 6) with Rf 104.61 of weak intensity. In general, the intensity of the bands varied, demonstrating that the isolates from the cashew had higher isoenzymatic activity (Est3, Est5 and Est6) in relation to the isolates from the mango (Table 1). This activity was characterized by the uniformity in the coloration intensity of the bands. All the isolates, whether from the cashew or mango, presented just one band in common (Est6), and therefore, the same relative mobility of the molecules in the gel (Rf =104.61), suggesting that this band was a characteristic of the species *C. gloeosporioides*.

The protein pattern of the *C. gloeosporioides* isolates also demonstrated variations in the phenotypes exhibited in the polyacrylamid gel. The number of bands ranged from two (isolates from cashew) to three (isolates from mango), with a predominance of medium to weak intensity (Table 1). Regarding the relative mobility (Rf), a difference was clearly observed between the uniformity of the protein pattern presented by the isolates from the cashew: Pr1 (Rf =58.46), Pr3 (Rf =69.23) and Pr4 (Rf =76.92), and that of the isolates from mango: Pr2 (Rf =61.53) and Pr4 (Rf =76.92). As seen in the isoesterase system, there was the presence of a single common band in the protein system as well (Pr4), with Rf =76.92 for all the isolates analyzed, indicating similarity in the behavior within each system, maintaining a relationship between the isolates revealed by the presence of common bands.

The dendrogram constructed through the NTSYS-pc software using the Jaccard coefficient separated the isolates in two groups according to the isoesterase and protein profiles; one group was formed by the isolates from the MPB, MPE and MMA mangos and the second group was made up of the isolates from the CMA, CPE and CPB cashews, which presented 100% similarity (Fig. 1), demonstrating strict uniformity between the isolates from the cashew in the enzyme systems studied. In *C. graminicola*, Horvath and Vargas Jr.

(2004) separated the isolates obtained from four different hosts into two groups by means of isoenzymatic analysis, with isolates from sorghum (*Sorghum* spp.) and corn (*Zea mays* L.) presenting differences in genetic distance in comparison to the isolates obtained from *Poa annua* L. and *Agrostis palustris* Huds., which are species of grass.

Table 1 - Total number, intensity and relative mobility of the esterases bands and total proteins presented by *Colletotrichum gloeosporioides* isolates.

Isolates*	**No. of bands / Esterase**	**Intensity of the bands**			**Relative mobility (Rf) [1]**					
		strong	medium	weak	Est1	Est2	Est3	Est4	Est5	Est6
CMA	3	1	2	0			70.76		90.76	104.61
CPE	3	1	2	0			70.76		90.76	104.61
CPB	3	1	2	0			70.76		90.76	104.61
MMA	3	1	1	1	46.15	64.61				104.61
MPE	3	0	3	0	46.15			78.46		104.61
MPB	1	0	0	1						104.61
	No. of bands / total protein	**Intensity of the bands**			**Relative mobility (Rf) [1]**					
		strong	medium	weak	Pr1	Pr2	Pr3	Pr4		
CMA	3	0	1	2	58.46		69.23	76.92		
CPE	3	1	1	1	58.46		69.23	76.92		
CPB	3	1	1	1	58.46		69.23	76.92		
MMA	2		0	2		61.53		76.92		
MPE	2		1	1		61.53		76.92		
MPB	2		1	1		61.53		76.92		

*CMA (cashew tree-MA), CPE (cashew tree-PE), CPB (cashew tree-PB), MMA (mango tree-MA), MPE (mango tree-PE), MPB (mango tree-PB).

[1] Rf= (d/D) x100

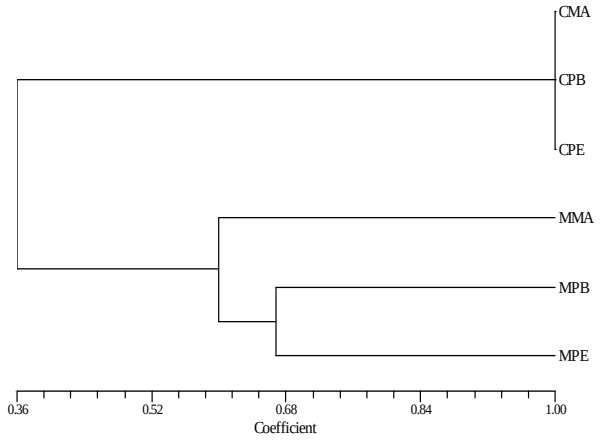

Figure 1 - Dendrogram of *Colletotrichum gloeosporioides* isolates from cashew and mango trees, based on the isozyme analyses, using the method UPGMA and the coefficient of Jaccard through the program NTSYS-pc.

Several studies have been conducted with the aim of comparing the protein and isoenzymatic patterns to differentiate the species or even differentiate an isolate within a single species of *Colletotrichum*. Furtado et al. (1999) observed variation in the number, intensity and relative mobility (Rf) of the bands formed in polyacrylamid gel, allowing the visualization of similarity groups between *C. gloeosporioides* isolates obtained from the rubber trees. Kaufmann and Weidmann (1996) verified striking polymorphism between the populations of *C. gloeosporioides* from different hosts and different locations, attributing these results to the

occurrence of sexual reproduction or some other mechanism of genetic variability. The results from the present study contradicted this, as the isolates that presented the sexual phase (CMA, CPE and CPB) exhibited no variability in the isoenzymatic and protein phenotypes.

MOLECULAR CHARACTERIZATION OF *COLLETOTRICHUM GLOEOSPORIOIDES* ISOLATES

RAPD analysis

All the primers tested generated amplification products for the genomic DNA of the *C. gloeosporioides* isolates from the cashew and mango for a total of 293 bands, 255 of which were polymorphic (Table 2).

Genetic distance analysis allowed the identification of the CPB and CPE isolates as the genetically closest, with 75% similarity; and the CMA and MPB isolates as the most distant, with 27% similarity. As seen in the dendrogram (Fig. 2), the isolates were separated in two groups. The first group was made up of the isolates from the MA, PE and PB cashews and the PE mango; the second group was made up of the isolates from the MA and PB mangos. Despite the CMA, CPE and CPB isolates presenting the sexual form and originating in different locations, they proved to be very close genetically, which was clearly observed in most of the primers used (Fig. 3). In studying the genetic variability of *C. acutatum* in the almonds, Forster and Adasgaveg (1999) observed similar results, as did Freeman et al. (1998) in strawberries; the latter authors observed considerable genetic uniformity among the isolates.

Table 2 - Total of amplified bands and of bands polymorphics and monomorphics goes primer, observed by *Colletotrichum gloeosporioides* isolates by RAPD.

Primer	bands		Total n°. of bands	Total polymorphism
	Polymorphic	Monomorphic		
RC 07	5	1	6	83%
PM 06	6	3	9	67%
OPX 01	12	1	13	92%
OPX 07	9	1	10	90%
OPA A02	4	2	6	67%
OPA 04	10	2	12	83%
OPA 10	14	2	16	88%
OPA 11	10	3	13	77%
OPA 12	4	0	4	100%
OPA18	3	1	4	75%
OPB 10	10	0	10	100%
OPB 12	12	0	12	100%
OPB 17	6	1	7	86%
OPC 08	4	1	5	80%
OPC 11	11	3	14	79%
OPC 15	4	1	5	80%
OPC 20	7	1	8	88%
OPD 01	7	1	8	88%
OPD 07	12	1	13	92%
OPD 15	8	0	8	100%
OPD 18	10	2	12	83%
OPE 02	12	3	15	80%
OPE 03	9	0	9	100%
OPE 04	8	0	8	100%
OPW 06	13	2	15	87%
OPV 08	9	2	11	82%
OPV 18	11	2	13	85%
OPV 19	13	1	14	93%
OPT 17	6	0	6	100%
Total	**255**	**38**	**293**	

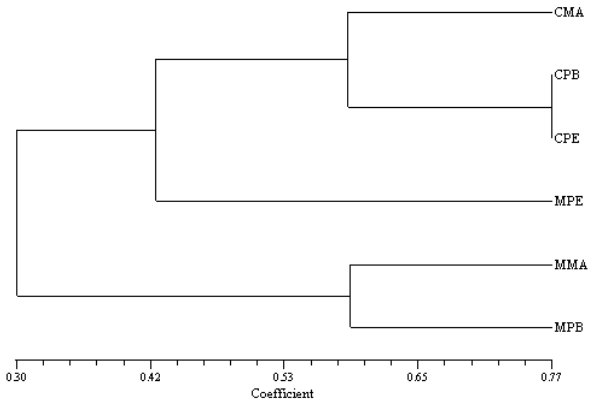

Figure 2 - Dendrogram of *Colletotrichum gloeosporioides* isolates from cashew and mango trees, based on markers RAPD, using the method UPGMA and the coefficient of Jaccard through the program NTSYS-pc.

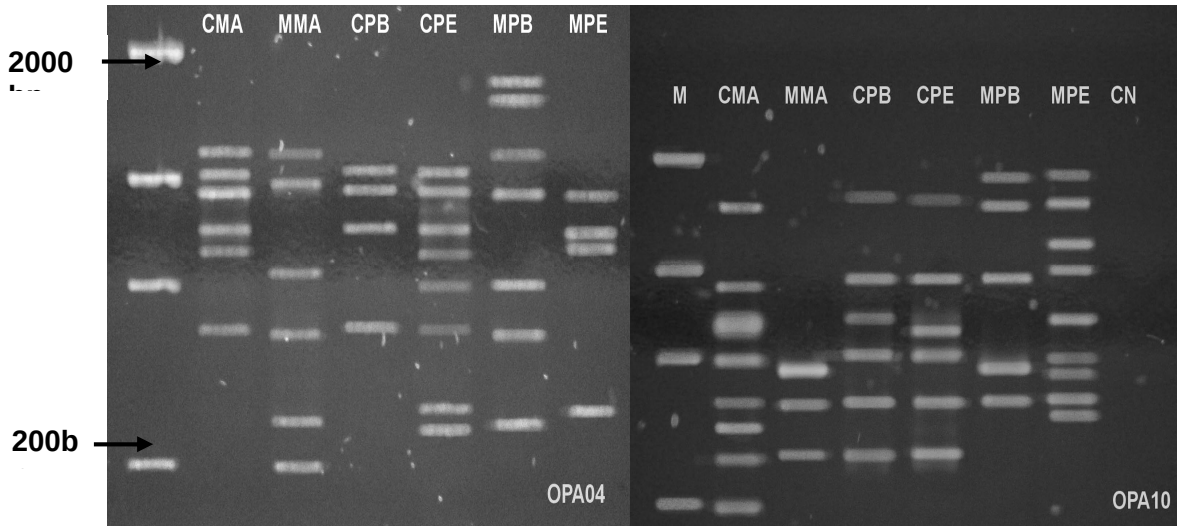

Figure 3 - Products of amplification of genomic DNA of *Colletotrichum gloeosporioides* isolates from cashew and mango trees, with the RAPD primers.

However, the isolates obtained from the mango exhibited genetic diversity among one another, which might be explained by the different geographic origins. A number of studies have demonstrated the genetic diversity of *C. gloeosporioides* isolates (Bernstein et al., 1995; Freeman et al., 1998; Swart, 1999). In characterizing the *Colletotrichum* isolates from the tamarind, mango and passion fruit, Afanador-Kafuri et al. (2003) observed striking genetic diversity among the isolates from the mango and passion fruit. This genetic heterogeneity could be the result of the presence of the teleomorph phase.

Analysis of the ribosomal DNA ITS region

The DNA from the *Colletotrichum* isolates obtained from the cashew and mango was amplified with the primers specific to *C. gloeosporioides* (CgInt) and *C. acutatum* (CaInt2). All the isolates amplified with the CgInt and ITS4 primers, confirming that the isolates pertained to *C. gloeosporioides*. The PCR products of the isolates are displayed in Figure 4. Afanador-Kafuri et al. (2003), carried out studies demonstrating that the CaInt2 and CgInt primers were efficient in differentiating *Colletotrichum* isolates obtained from the tamarind and mango at the species level

in *C. acutatum* and *C. gloeosporioides*. Based on the analysis of the ITS region of the rDNA with the specific primers, the authors were able to confirm that all the isolates obtained from the mango were identified as *C. gloeosporioides.*

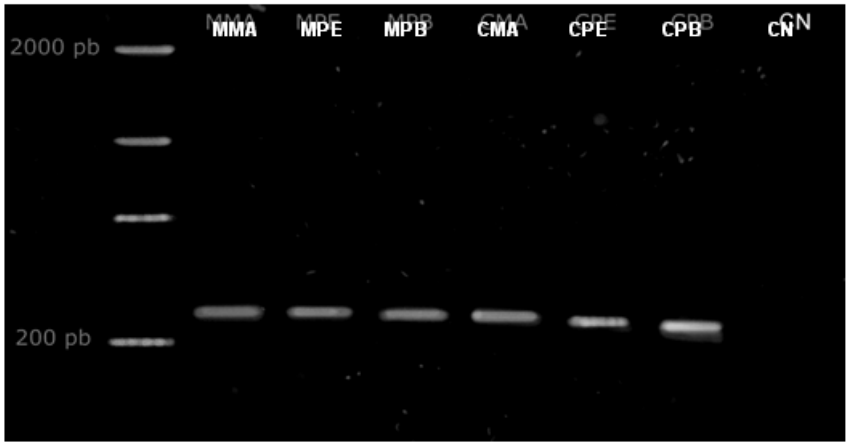

Figure 4 - Amplification of the area ITS and specific identification of *Colletotrichum gloeosporioides* isolates from cashew and mango trees, using the primer CgInt in combination with ITS4.

The different morphophysiological and molecular methods used in the differentiation of *C. gloeosporioides* isolates from the cashew and mango proved efficient with regard to intraspecific characterization, highlighting sporulation, with the formation of four groups, and mycelial growth and pathogenicity, with the formation of three physiological groups. The analysis of Pearson's correlation between the variables of mycelial growth, growth rate, sporulation and pathogenicity revealed no significance at a 5% level of probability. Similar results were found by Assis (2001), who verified no significant correlation in the *C. gloeosporioides* pathosystems from the mango in relation to growth, sporulation and pathogenicity.

Regarding host specificity of the *C. gloeosporioides* isolates obtained from the cashew and mango, pathogenicity was the method that best separated the isolates according to their host. This results corresponded with those obtained from the analyses based on the isoenzymatic systems and RAPD markers. This corroborated the results found by Swart (1999), who observed that pathogenicity and RAPD analysis were the most effective methods in separating the *C. gloeosporioides* isolates from avocado and mango according to host and geographic origin.

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