MORPHOPHYSIOLOGICAL AND MOLECULAR ANALYSIS IN THE DIFFERENTIATION OF *Colletotrichum gloeosporioides* ISOLATES FROM CASHEW AND MANGO TREES

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ABSTRACT

MORPHOPHYSIOLOGICAL AND MOLECULAR ANALYSIS IN THE DIFFERENTIATION OF *Colletotrichum gloeosporioides* ISOLATES FROM CASHEW AND MANGO TREES

Anthracnose has caused significant harm to tropical orchard production, requiring more in–depth studies that contribute to efficient control of this disease. The aim of the present work was to analyze morphophysiological and molecular methods in the differentiation of *Colletotrichum gloeosporioides* isolates obtained from cashew and mango trees. The different taxonomic methods used proved to be efficient regarding intraspecific characterization. The isolates exhibited variation in conidia morphology, appressoria format, formation of setae, mycelial growth, sporulation and vegetative compatibility, enabling characterization on an intraspecific level, but they did not constitute efficient separation criteria regarding host specificity. Pathogenicity was the parameter that best separated the *C. gloeosporioides* isolates according to hosts. 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 pertain to *C. gloeosporioides*. The results from this study suggest that methods based on pathogenicity, isozyme analysis and RAPD are effective in differentiating *C. gloeosporioides* isolates

from cashew and mango trees.

Index terms: *Anacardium occidentale*, anthracnose, ITS–rDNA, *Mangifera indica*, VCGs.

RESUMO

ANÁLISE MORFOFISIOLÓGICA E MOLECULAR NA DIFERENCIAÇÃO DE ISOLADOS DE *Colletotrichum gloeosporioides* OBTIDOS DE CAJUEIRO E MANGUEIRA

A antracnose tem causado significantes danos à produção, necessitando de estudos mais profundos que contribuam para um controle mais eficiente da doença. O presente trabalho objetivou analisar métodos morfofisiológicos e moleculares para diferenciação de isolados de *Colletotrichum gloeosporioides* obtidos de cajueiro e mangueira. Os diferentes métodos taxonômicos usados mostraram ser eficientes na caracterização intra–específica. Os isolados exibiram variação na morfologia dos conídios, forma dos apressórios, produção de setas, crescimento micelial, esporulação e compatibilidade vegetativa, permitindo a caracterização em nível intra–específico, mas não constituem critérios eficientes para separação em relação a especificidade hospedeira. A patogenicidade foi o parâmetro que melhor definiu os isolados de *C. gloeosporioides* de acordo com os hospedeiros. Do mesmo modo, os métodos moleculares também mostraram sua eficiência na diferenciação dos isolados de *C. gloeosporioides* em relação a especificidade hospedeira. Na análise da região ITS do DNA ribossômico, todos os isolados amplificaram com os primers CgInt e ITS4, confirmando que eles pertenciam a espécie *C. gloeosporioides*. Os resultados do presente estudo sugerem que os métodos baseados na patogenicidade, análises de isoenzimas e RAPD são efetivos para a diferenciação dos isolados de *C. gloeosporioides* do cajueiro e mangueira.

Termos para indexação: *Anacardium occidentale*, antracnose, ITS–rDNA, *Mangifera indica*., VCGs.

1. INTRODUCTION

Anthracnose in 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 importance in Northeast Brazil (Menezes & Hanlin, 1996a; Serra & Silva, 2004). *Colletotrichum acutatum* J.H. Simmonds may also be the anthracnose agent in mango trees (Arauz, 2000; Freeman *et al*., 1998). These species attack leaves, branches, blossoms, stems and fruits, requiring periodic spraying with fungicides in orchards and efficient post–harvest treatment (Ribeiro, 2005). The

species cause varying degrees of harm depending on susceptibility of the host plant and environmental conditions (Ribeiro, 2005; Menezes, 2005).

In Brazil, anthracnose in mango trees is present in all 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 cashew tree cultivation, the disease is widely disseminated in all croping areas, and is quite severe in years with high rainfall, especially during the "cashew rains" at flowering, with a more intensive attack on the blossoms and greater harm to production (Menezes, 2005).

Considering that the identification of *Colletotrichum* species or biotypes is nearly always difficult due to enormous variation in morphology accepted for the different species of the genus, modern techniques have been used to integrate morphophysiological, 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 & Irwin (1988) for separation within *C. gloeosporioides* species. Traditional morphophysiological methods for differentiating *C. gloeosporioides* isolates include conidia morphology, apressoria formation, presence or absence of setae, presence or absence of teleomorph, color of colonies, mycelial growth rate and sensitivity to fungicides (Serra & Silva, 2004; Freeman, 2000).

Specific pathogenicity to a particular host, or host group, and cross–infection have also been used to characterize *Colletotrichum* species as additional criteria to cultural and morphological characteristics (Arauz, 2000; Afanador *et al*., 2003). Freeman (2000) reported that a single species of *Colletotrichum* can infect different hosts. The author goes on to describe that different *Colletotrichum* species or biotypes can affect a single host, citing the examples of 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 & 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 species and genotypes of *Colletotrichum* from a high number of hosts. Analysis of the nucleotide sequence of the internal transcribed spacing (ITS) of the ribosomal DNA (rDNA) from genes of β–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 diseases caused by *C. gloeosporioides* in diverse fruit trees have often demonstrated that the fungus exhibits wide phenotypic, genotypic and pathogenic variability (Menezes & Hanlin, 1996a; Talhinhas *et al*., 2005; Afanador *et al*., 2003) 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 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 different taxonomic methods in the differentiation of *C. gloeosporioides* isolates obtained from cashew and mango trees.

2. Material and Methods

2.1. Fungal cultures and growth conditions

The isolates were obtained from cashew and mango trees from the states of Northeastern Brazil: Maranhão (MA), Pernambuco (PE) and Paraíba (PB), and codified according to 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 leaves with symptoms of anthracnose, adopting the method described by Menezes & Assis (2004), and the colonies were kept in tubes containing PDA.

2.2. Morphological characterization of *Colletotrichum gloeosporioides* isolates

For the morphological studies, all *C. gloeosporioides* isolates were evaluated regarding shape and dimensions of the conidia, the formation of appressoria and the presence or absence of setae, employing a microculture technique (Menezes & Assis, 2004). Incubation was performed at 25±2ºC three to seven days under a photoperiod of 12 hours. Fifty conidia from each isolate were measured. Conidia dimensions were

determined through a micrometric ocular at 400x, annotating average length and width, as well as amplitude of variation. The predominant shape of the conidia was also observed, thereby obtaining an evaluation of morphological variability.

2.3. Physiological and pathogenic characterization of *Colletotrichum*

Mycelial growth and sporulation

For the study of physiological characteristics, mycelial disks (5.0mm in diameter) were removed from the young colony of each isolate and transferred to the center of a Petri (9.0mm in diameter) dish containing PDA, and incubated at 25±2°C under a photoperiod of 12 hours. Growth was evaluated by measuring the diameter of the colony in opposite directions at 24–hour intervals using a millimeter ruler. Readings began 48 hours after installation of the experiment until the colonization of the entire surface of the culture medium, which corresponded to seven days of incubation. Growth rate was calculated in according to Lilly & Barnett (1951).

For the determination of sporulation after mycelial growth evaluation, it was prepared a suspension of conidia from all the isolates. The concentration was quantified in a Neubauer chamber.

The experimental design was completely randomized, with four replications for each treatment. Data were subjected to analysis of variance and averages were compared through the Scott–Knott test at 5% probability level.

Cross–pathogenicity of the isolates

For pathogenic behavior evaluation, the isolates were inoculated in young leaves removed from cashew and mango trees following the cross schematic, that is, those obtained from mango were inoculated in cashew and those from cashew were inoculated in mango. The inocula consisted of mycelial disks (5mm in diameter) removed from five day old pathogen colonies grown in PDA. Inoculation was performed using the injury method and depositing the inocula at three equidistant points on the leaf surface. Next, the leaves were conditioned on plastic trays lined with filter paper and kept in a humidity chamber at 25±2°C under a 12 hours photoperiod. For the controls, the same procedures were repeated, but only disks from the PDA culture medium were deposited. The severity of disease symptoms was assessed seven days after inoculation through a reading of the diameter of the necrotic tissue and observation of lesion characteristics.

The experimental design was completely randomized, with four replications (represented by trays), with each plot made up of five leaves / tray. The data obtained were submitted to analysis of variance and averages were compared through the Scott–Knott test at 5% probability level.

2.4. Isozyme characterization of *Colletotrichum gloeosporioides* isolates

Fungal culture and protein extracts

The *Colletotrichum* isolates were cultivated in 125ml of the Potato–Dextrose (PD) medium for six days at 25±2ºC under a 12 hours photoperiod, and gently agitated twice for 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 polyvinyl pyrrolidone and 1mL of the Tris–glycine buffer 0.125 M, pH 8.2. After a 12–hour resting period, samples were centrifuged at 14.000 rpm for 6 minutes and the protein extracts were kept at 4ºC (Alfenas *et al*., 1991).

Preparation of gel plates and electrophoresis

Gel plates were prepared with 5%–polyacrylamide in a tris–glycine buffer at 0.125 M, pH 8.2, following the methodology described by Alfenas *et al*. (17). After polymerization, the gel was placed in a horizontal tray containing the tris–glycine buffer at 0.125 M, pH 8.2. Ten mL of the protein extract from each isolate were applied individually in a small cavity of the gel. Bromophenol blue was used as the marker. Electrophoresis was performed at 4ºC 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 37ºC. To reveal the total proteins, the gel was immersed in a Coomassie blue stain for 12 hours, 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 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.

2.5. Vegetative compatibility of *Colletotrichum gloeosporioides* isolates

Generation and characterization of *nit* mutants

For the study of vegetative compatibility groups (VCGs), auxotrophic *nit* mutants were used that were incapable of utilizing nitrate as the only source of nitrogen. Mutants were generated using the methodology described by Correll *et al*. (1993). The isolates were cultivated in a complete medium (CM) for six days, with nitrate as the only nitrogen source. Ten mycelial disks (5.0mm in diameter) from each tested isolate were transferred to a Petri dish (one disk per dish) containing potato–dextrose–chlorate (PDC). The dishes were kept in the continual darkness at 25 ±2º C until the appearance of sectors of rapid colony growth that had initially been restricted. Sub–cultures were transferred from all sectors to the Minimum Medium (MM) and the colonies with shallow growth (few or no aerial mycelia) in this medium were considered *nit* mutant.

For the phenotype characterization of the *nit* mutants obtained from each isolate, the visual aspects of the colonies were observed in a basal medium (MB) supplemented with four different sources of nitrogen: MM (MB with nitrate); MN (MB with nitrite); MH (MB with hypoxanthine; and MA (MB with ammonia). The *nit* mutants were classified into four phenotype classes: a) *nit*M – mutants incapable of utilizing nitrate and hypoxanthine; b) *nit*1 – mutants capable of only utilizing nitrate; c) *nit*2 – mutants capable of only utilizing ammonia; and d) *nit*3 – mutants capable of utilizing both ammonia and hypoxanthine (Brooker *et al*., 1991).

Complementation tests

Complementation tests between the *nit*M mutant obtained from the MPB isolate and the *nit*1, *nit*2 and *nit*3 mutants obtained from the isolates (with the exception of the CPB isolate, which only generated *nit*2 mutants) were conducted in Petri dishes containing the MM medium. The mutants were paired in all possible combinations so as to identify possible compatibility groups and thereby use a representative isolate

for each detected group for subsequent pairings. A tester mutant was chosen from each isolate for the remaining pairings. After this selection, pairing was performed between the tester mutants from each *Colletotrichum* isolate. One mycelial disk (5mm in diameter) from each tester isolate (*nit*1/*nit*2/*nit*3) was placed 1 cm equidistant from the others in diverse combinations in the same medium cited above. The dishes were kept in continual darkness for 10 days at 25±2º C. Complementation was visualized through accentuated growth in the contact zones between compatible mutants.

2.6. 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 hours 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 filtration and ground in liquid nitrogen. Next, 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 70ºC, with agitations every 10 minutes. Next, samples were centrifuged at 14,000 rpm for 10 minutes and a cloroform–isoamyl alcohol solution (25:1) was added. 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 –20ºC for two hours and afterward centrifuging was repeated. The precipitates were rinsed twice with 70% ethanol and dried at room temperature. Next, the total nucleic acids were re–suspended in 150ml of water containing RNAse in a concentration of 40mg/ml and placed into a water bath at 37ºC for 1 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.*

(21), with the following concentrations: 3ng/µl genomic DNA, buffer 10X, $MgCl_2$ (2mM), dNTPs (2.5mM), primer (10ρmol), Taq DNA polymerase (5unidades/µl) and ultra pure water to complete a volume of 15µl. Amplifications were performed under the following conditions: one cycle at 94ºC for 2 minutes; 40 cycles at 94ºC for 1 minute, 40.4ºC for 1 minute and 72ºC for 2 minutes; and one cycle at 72ºC 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 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 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 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, 200mM of dNTPs, 1.5mM $MgCl_2$, 1.25 U Taq DNA polymerase (Invitrogen^{tm}) and ultra pure water to complete the final volume. Amplification was carried out under the following conditions: one cycle at 95ºC for 5 minutes; 40 cycles at 95ºC for 30 seconds, 65ºC for 30 seconds and 72ºC for 90 seconds; and one final cycle at 72ºC 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.

3. Results and Discussion

3.1. Morphological characterization of *Colletotrichum gloeosporioides* isolates

The conidia from the different *C. gloeosporioides* isolates from mango and cashew were hyaline, unicellular, single–nuclear, cylindrical or straight with an obtuse apex, demonstrating variation in length and width (12–16.4µm x 3.4–4.5µm), as displayed in Table 1. This variation was observed for all the isolates and is within the limits indicated for *C. gloeosporioides* according to Freeman (2000). In studying *C. gloeosporioides* isolates from different tropical fruit trees, Serra & Silva (2004) observed similar results regarding morphological characteristics such as conidia dimensions; the authors verified a variation ranging from 14.8 to 18.4µm in conidia length. Menezes & Hanlin (1996a) also observed considerable variability in the conidia morphology of *C. gloeosporioides* isolates obtained from avocado in different regions of Northeast Brazil.

It is important to point out that cashew isolates formed a sexual phase corresponding to *Glomerella*. The conidia were produced in acervuli in succession at the extremity of phialidic conidiophores or the apex of setae (Figure 1). Although it has no taxonomic value (Menezes & Hanlin, 1996b), the presence of setae and the production of appressoria were observed in all isolates, with the exception of the

Table 1. – Morphologic characteristics and size of conidia of *Colletotrichum gloeosporioides* isolates from cashew and mango trees.

Isolates*	Length (mm) mean amplitude)	Width (mm) mean (amplitude)	(%) Cylindrical conidia	Setae **	Apressoria **
CMA	13.6 (11.5 - 16.5)	4.5 (3.3 - 4.9)	100	+	...
CPE	12.0 (11.5 - 16.5)	4.5 (3.3 - 4.9)	100	+	...
CPB	15.8 (13.2 - 18.1)	4.0(3.3 - 6.6)	100	-	...
MMA	13.2 (9.9 - 18.1)	3.7 (3.3 - 4.9)	90	+	...
MPE	16.4 (11.5 - 23.1)	3.5 (3.3 - 4.9)	80	+	..
M PB	15.9 (13.2 - 19.8)	3.4 (3.3 - 4.9)	100	+	..
C.V	6.55	8.94			

*CMA (cashew tree-MA), CPE (cashew tree-PE), CPB (cashew tree-PB), MMA (mango tree-MA), MPE (mango tree-PE), MPB (mango tree-PB).
** Presence(+), absence (-) of setae; apressoria production abundant (...) and median (..).

CPB isolate (cashew–PB), which presented no setae. The appressoria produced varied in shape and quantity, with a dark brown color and an oval to irregular shape. Regarding appressoria production, the isolates were grouped as to abundant production (...) and median production (..), as displayed in Table 1.

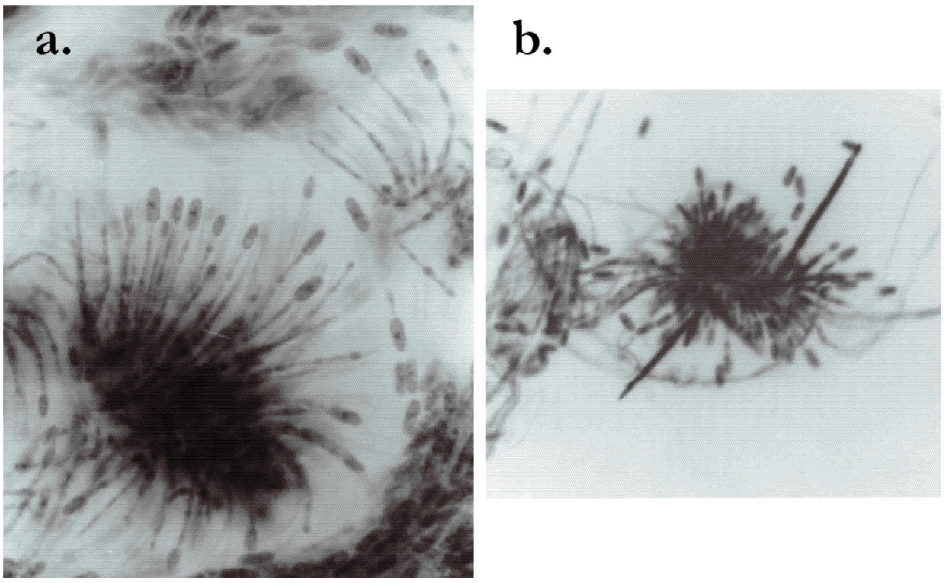

Figure 1. — Conidial production of *Colletotrichum gloeosporioides* isolates at the extremity of phialidic conidiophores and at the apex of setae: a. MMA (mango – MA); b. CPE (cashew – PE).

3.2. Physiological and pathogenic characterization of *Colletotrichum gloeosporioides*

Mycelial growth and sporulation

The mycelial growth of the different *C. gloeosporioides* isolates obtained from cashew and mango trees after seven days of incubation are displayed in Table 2, revealing significant differences ($P \leq 0.05$) between averages and thereby permitting classification into three groups: MG–1 (MPE, MMA and CPE), MG–2 (CPB) and MG–3 (MPB and CMA). According to the standard established by Freeman (2000), all isolates demonstrated compatible growth with that observed for the species *C. gloeosporioides*. These results are consistent with other studies that used mycelial growth to differentiate *Colletotrichum* spp. isolates from other hosts (Bernstein et al., 1995).

Table 2. – Mycelial growth after seven days of incubation, growth rate and sporulation of *Colletotrichum gloeosporioides* isolates from cashew and mango trees.

Isolates*	Micelial growth (cm)		Growth rate (mm/day)**		Sporulation (x 10^5conidia/ml)	
		MG[1]		RG		SG
MPE	8.50 a	1	14.04 a	1	1.25 c	3
MMA	8.47 a	1	14.82 a	1	7 a	1
CPE	8.42 a	1	14.58 a	1	0 d	4
CPB	7.60 b	2	14.46 a	1	1.22 c	3
MPB	6.95 c	3	11.58 b	2	1.32 c	3
CMA	6.27 c	3	13.68 a	1	2.65 b	2

*CMA (cashew tree-MA), CPE (cashew tree-PE), CPB (cashew tree-PB), MMA (mango tree-MA), MPE (mango tree-PE), MPB (mango tree-PB).
**Txc = (C96-C48)/T (16). Mean of four repetitions. Means followed by the same small letter in the same column do not significantly differ (P?0,05), according to Scott-Knott test.
[1]MG - micelial growth group; RG - growth rate group; SG - sporulation group.

However, in characterizing *C. gloeosporioides* and *C. acutatum* isolates from almond and strawberry, respectively, Freeman *et al*. (1998) observed no significant difference in the mycelial growth of the isolates.

Regarding the mycelial growth rate as evaluated in mm/day, the isolates were classified into two groups (Table 2); the first group was formed by the MMA, MPE, MPB, CMA, CPE and CPB isolates, and the second group was formed by a single MPB representative with a lower growth rate ($P \leq 0.05$) in relation to the others. All isolates exhibited a growth rate >10mm/day, which is considered typical for the species *C. gloeosporioides* according to Adaskaveg & Foster (2000). Studies carried out by Serra & Silva (2004) with *C. gloeosporioides* isolates from different tropical fruit trees allowed the classification of isolates into three physiological groups based on the mycelial growth rate. Mycelial growth and the mycelial growth rate do not present a decisive taxonomic value in the differentiation of *Colletotrichum* species, but may play an important role in intraspecific variability.

Regarding sporulation evaluated after seven days of incubation, cluster analysis based on the Scott–Knott test made possible the classification of isolates into four groups (Table 2). SG–1 (MMA) presented the greatest amount of conidia (7x 10^5conidia/ml), significantly differing from the remaining isolates, SG–2 (CMA),

SG–3 (MPB, MPE and CPB) and SG–4 (CPE), which stood out for not producing conidia after seven days of incubation in the PDA medium. This isolate, together with CMA and CPB, formed the teleomorphic sexual phase (*Glomerella*) in the culture medium. Sporulation is a little–utilized criterion for differentiating *C. gloeosporioides* isolates, as this characteristic can be variable in relation to the aggressiveness of the isolates. In studying *C. gloeosporioides* isolates obtained from mango trees in Mexico regarding the characteristics of germination, growth, sporulation and pathogenicity, Gutierrez *et al.* (2001) verified that the more aggressive isolates were also those that presented greater sporulation. However, Assis (2001) verified no significant correlation between sporulation and aggressiveness of *C. gloeosporioides* isolates obtained from mango trees.

Cross pathogenicity of the isolates

All isolates were pathogenic in the leaves detached from mango and cashew trees, inducing symptoms of anthracnose in the form of lightly indented dark spots. The evaluation of isolate pathogenicity as measured through the diameter of the lesions revealed differences ($P \leq 0.05$) between isolates. Moreover, through the Scott–Knott test, it was possible to separate isolates into three groups according to aggressiveness: Group 1– greater aggressiveness, formed by the MMA, MPE and MPB isolates inoculated in mango; Group 2– intermediate aggressiveness, made up of CPE isolates inoculated in mango, and CPB and MMA isolates inoculated in cashew; Grup 3– lower aggressiveness, formed by the remaining isolates (Table 3). Through aggressiveness, it was possible to separate the *C. gloeosporioides* isolates obtained from cashew and mango; isolates from mango presented greater aggressiveness when inoculated in their congenial host. A number of authors have observed similar results, demonstrating that *C. gloeosporioides* and *C. acutatum* isolates appear to be more aggressive in their specific hosts than in other hosts (Freeman & Shabi, 1996; Swart, 1999). In general, the isolates from cashew presented lower aggressiveness when inoculated in mango as well as in cashew, with the exception of the CPE and CPB isolates, which presented intermediate aggressiveness when inoculated in mango and cashew, respectively.

Cross and specific pathogenicity are parameters used in diverse studies for the characterization of *Colletotrichum* isolates. In evaluating the cross infection of 290 *C. gloeosporioides* isolates from avocado and mango, Swart (1999) demonstrated the occurrence of variation in the aggressiveness of the isolates, and that avocado isolates

Table 3. - Pathogenicity of *Colletotrichum gloeosporioides* isolates from cashew and mango trees inoculated in the crossed system, and formation of aggressiveness groups.

Isolates*/Inoculated plant	Pathogenicity ** Diameter of the lesions (cm)	Aggressiveness group (AG)
MMA - mango tree	1.62 a	1
MPE - mango tree	1.45 a	1
MPB - mango tree	1.45 a	1
CPB - cashew tree	0.92 b	2
CPE - mango tree	0.90 b	2
MMA - cashew tree	0.80 b	2
CMA - cashew tree	0.62 c	3
CPE - cashew tree	0.60 c	3
MPE - cashew tree	0.60 c	3
MPB - cashew tree	0.60 c	3
CMA - mango tree	0.60 c	3
CPB - mango tree	0.60 c	3
C.V. =	17.43	

*CMA (cashew tree-MA), CPE (cashew tree-PE), CPB (cashew tree-PB), MMA (mango tree-MA), MPE (mango tree-PE), MPB (mango tree-PB).
**Mean of four repetitions. Means followed by the same letter in the same column, do not significantly differ (P?0,05), according to Scott-Knott test.

inoculated in avocado induce greater lesions than mango isolates in avocado.

Lesion size was consistent with what has been described for avocado and mango (Freeman & Shabi, 1996; Swart, 1999), with avocado isolates being more aggressive than mango isolates. In the present study, the isolates from mango exhibited high aggressiveness than those from cashew. These differences may be attributed to the adaptation of the pathogen to a less susceptible host (Swart, 1999) or the fact that the isolates from cashew were forming the sexual phase.

3.3. Protein and isoesterase characterization of *Colletotrichum gloeosporioides*

Isolates through electrophoresis in polyacrylamid gel

Between the two systems analyzed in the present study (Table 4), 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

Table 4. - Total number, intensity and relative mobility of the esterases bands and total proteins presented by *Colletotrichum gloeosporioides* isolates.

Isolates*	No. of bands	Intensity of the bands			Relative mobility (Rf) [1]					
		strong	medium	weak						
Esterase										
					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
Total protein										
					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 (17).

Rf 104.61 of weak intensity. In general, the intensity of the bands varied, demonstrating that the isolates from cashew have greater isoenzymatic activity (Est3, Est5 and Est6) in relation to the isolates from mango (Table 4); this activity was characterized by uniformity in the coloration intensity of the bands. All the isolates, whether from 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 is 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 4). Regarding relative mobility (Rf), a difference was clearly observed between the uniformity of the protein pattern presented by the isolates from 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 behavior within each system, maintaining a relationship between 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 isolates from the MPB, MPE and MMA mangos and the second group was made up of isolates from the CMA, CPE and CPB cashews, which presented 100% similarity (Figure 2), demonstrating strict uniformity between isolates from cashew in the enzyme systems studied. In *C. graminicola*, Horvath & Vargas Jr. (2004) separated 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 isolates obtained from *Poa annua* L. and *Agrostis palustris* Huds., which are species of grass.

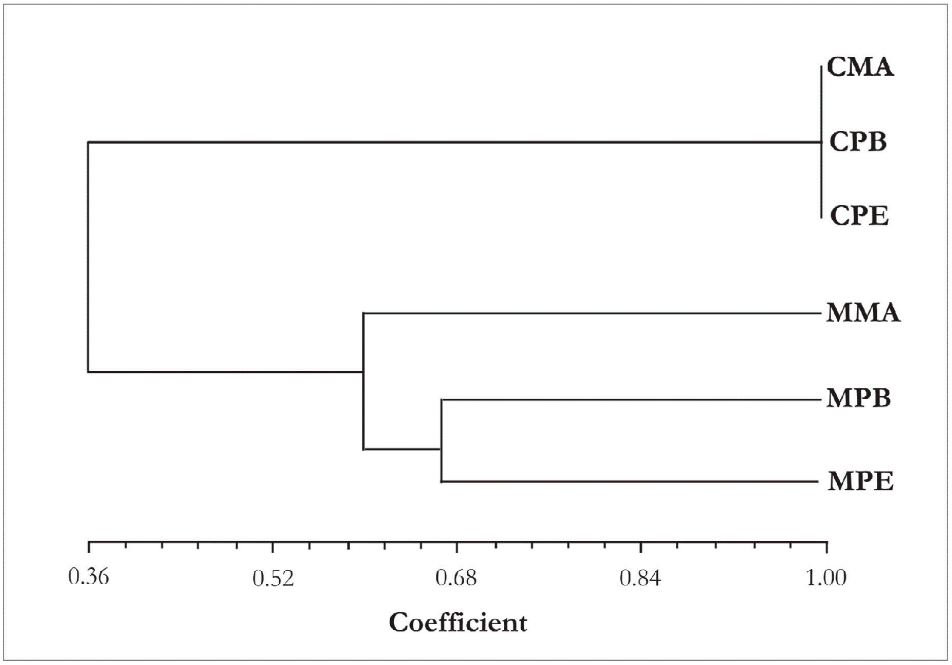

Figure 2. — Dendrogram of *Colletotrichum gloeosporioides* isolates from cashew and mango trees, based on the isozyme analyses, using the method UPGMA and the coefficient of Jaccard through the program NTSYS–pc.

Some studies have been developed with the aim of comparing protein and isoenzymatic patterns to differentiate 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 bands formed in polyacrylamid gel, allowing the visualization of similarity groups between *C. gloeosporioides* isolates obtained from rubber trees. Kaufmann & Weidmann (1996) verified striking polymorphism between 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 oppose this, as the isolates that presented the sexual phase (CMA, CPE and CPB) exhibited no variability in the isoenzymatic and protein phenotypes.

3.4. Vegetative compatibility of *Colletotrichum gloeosporioides* isolates

All the *C. gloeosporioides* isolates produced sectors in the PDC (1.5% $KClO_3$). The appearance of sectors of rapid growth that are resistant to chlorate was observed

starting on the eighth day of incubation. Differences were verified between the frequencies of sector formation per isolate. The isolates from mango exhibited a greater formation of sectors in relation to those from cashew.

Regarding the characterization of the *nit* mutants, it was not possible to obtain four phenotype classes related to the nitrate assimilation metabolism for all isolates. The *nit*1, 2 and 3 phenotypes were obtained for all isolates, with the exception of the CPB (cashew–PB) isolate, which only presented *nit*1 mutants. The *nit*M mutant was only formed by the MPB (mango–PB) isolate. Várzea *et al.* (2002) obtained similar results to those verified in the present study; the authors did not observe the formation of four phenotype classes among 31 *Colletotrichum* spp. isolates obtained from coffee plants.

The *nit* mutants selected from each isolate as mutant testers were CPB1 (*nit*1), CPE5 (*nit*3), CMA2 (*nit*1), MPB8 (*nit*1), MPE1 (*nit*1) and MMA2 (*nit*1). These mutants were obtained achieved by means of complementation with the *nit*M mutant from the MPB isolate. Tester mutants were crossed between one another for the analysis of the complementation test. All isolates proved self–incompatible when paired; there was no formation of aerial mycelia among the colonies. Other authors have observed similar results when studying *Colletotrichum* spp. (Brooker *et al*., 1991; Várzea *et al*., 2002).

However, complementation between different *nit* mutants was observed through the growth of the aerial mycelia in the contact zone of the mycelia from the two mutant colonies. In the present study, an isolate was only placed into a vegetative compatibility group (VCG) when it exhibited the formation of stable heterocarion (Figure 3). Among the isolates analyzed, there was the formation of two groups: VGC–1, made up of the MMA, MPB, CPB and CPE isolates; and VGC–2, made up of the CMA and MPE isolates. Abang *et al*. (2004) characterized forty–one *C. gloeosporioides* isolates from yams in 28 vegetative compatibility groups. Várzea *et al.* (2002) characterized 31 isolates from different species of *Colletotrichum* from coffee plants in five vegetative compatibility groups; according to the authors, it was the first time the existence of genetically distinct groups and subgroups separated by barriers of incompatibility was demonstrated in *Colletotrichum* spp. isolates from coffee plants.

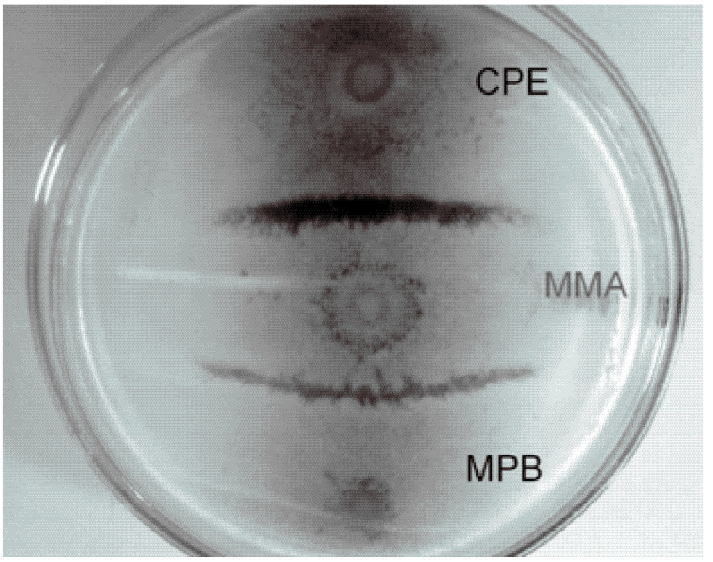

Figure 3. — Compatible reaction among of *Colletotrichum gloeosporioides* isolates belonging to the same group of vegetative compatibility, involving two auxotrofic mutants in the route of nitrate assimilation. Mycelial disks: down, isolate (*nit*1) – MPB (mango tree – PB), above, isolate (*nit*3) – CPE (cashew tree – PE) and of the center, isolate (*nit*1) – MMA (mango tree – PB).

3.5. 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 cashew and mango for a total of 293 bands, 255 of which were polymorphic (Table 5).

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 (Figure 4), isolates were separated in two groups. The first group was made up of isolates from the MA, PE and PB cashews and the PE mango; the second group was made up of 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 (Figure 5). In studying the genetic variability of *C. acutatum* in almonds, Forster & Adasgaveg

Table 5. - Total of amplified bands and of bands polymorphics and monomorphics goes primer, observed by *Colletotrichum gloeosporioides* isolates by RAPD.

Primer	Bands	Total number of Bands		% polimorfism
	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%
OPA 15	6	1	7	86%
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	

(1999) observed similar results, as did Freeman *et al.* (1998) in strawberries; the latter

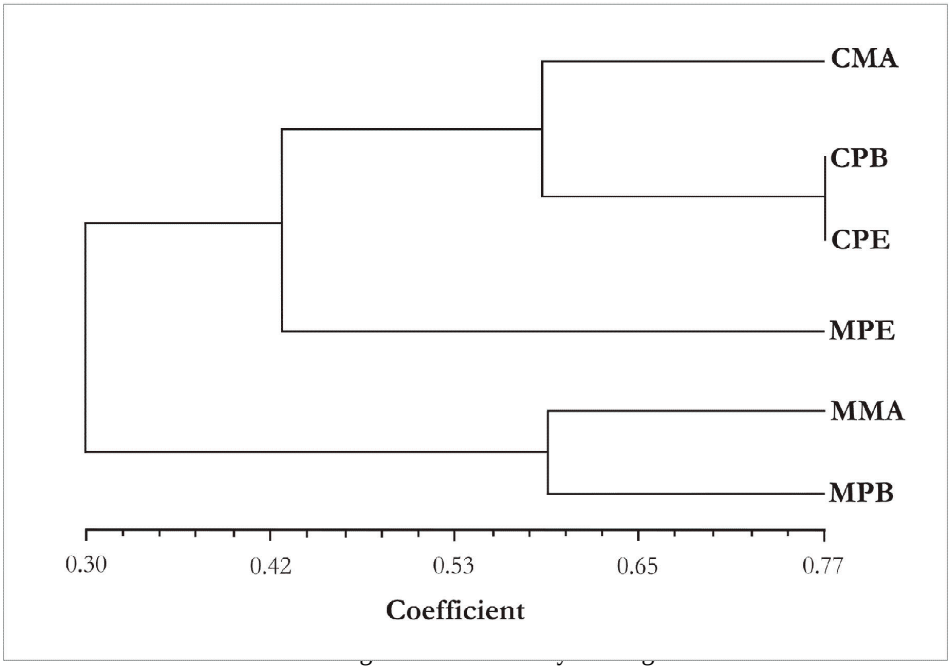

Figure 4. — Dendrogram of *Colletotrichum gloeosporioides* isolates from cashew and mango trees, based on markers RAPD, using the method UPGMA and the coefficient of Jaccard through the program NTSYS–pc.

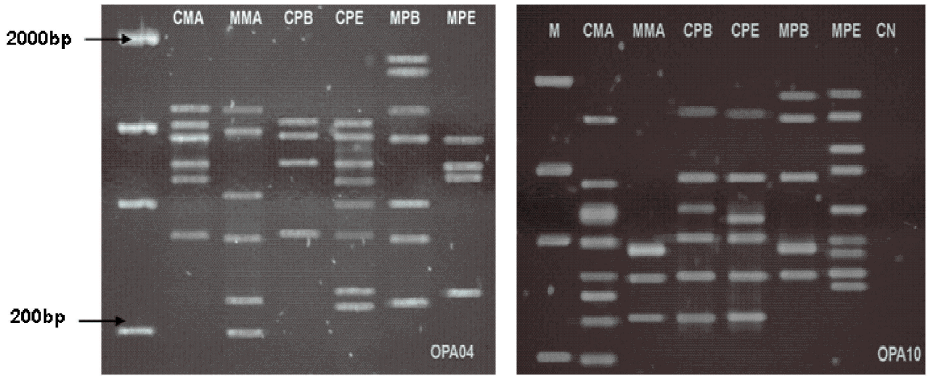

Figure 5. — Products of amplification of genomic DNA of *Colletotrichum gloeosporioides* isolates from cashew and mango trees, with the RAPD primers.

However, the isolates obtained from mango exhibited genetic diversity among one another, which may be explained by the different geographic origins. A number

of studies have demonstrated the genetic diversity of *C. gloeosporioides* isolates (Freeman *et al*., 1998; Bernstein *et al*., 1995; Swart, 1999). In characterizing *Colletotrichum* isolates from tamarind, mango and passion fruit, Afanador–Kafuri *et al*. (2003) observed striking genetic diversity among isolates from 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 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 pertain to *C. gloeosporioides*. The PCR products of the isolates are displayed in Figure 6. Afanador–Kafuri *et al.* (2003), carried out studies demonstrating that the CaInt2 and CgInt primers were efficient in differentiating *Colletotrichum* isolates obtained from 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 mango were identified as *C. gloeosporioides.*

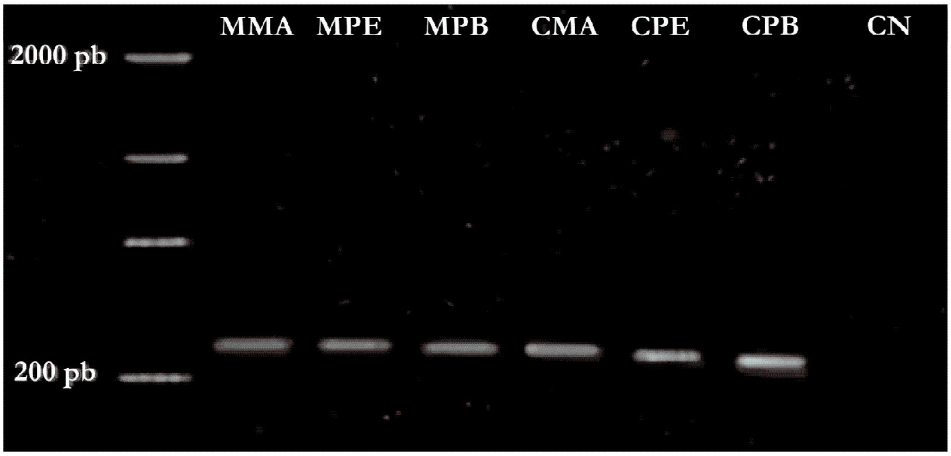

Figure 6. — Amplification of the area ITS and specific identification of *Colletotrichum gloeosporioides* isolates from cashew and mango trees, using the primer CgInt in combination with ITS4.

The different morphophysiological and molecular methods used in the differentiation of *C. gloeosporioides* isolates from 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 mango in relation to growth, sporulation and pathogenicity.

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

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