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Research Article

GENETIC AND PATHOGENIC DIVERSITY OF COLLETOTRICHUM GLOEOSPORIOIDES, THE CAUSAL AGENT OF CASHEW ANTHRACNOSE

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ABSTRACT

Anthracnose, caused by the fungus *Colletotrichum gloeosporioides*, is one of the most important diseases in cashew (*Anacardium occidentale*) cultivation. In this study, the genetic and pathogenic diversity of this microorganism isolated from cashew crops from Pernambuco State, Brazil, were evaluated by RAPD and ribosomal DNA-RFLP analysis. Based on the RAPD analysis, considerable genetic diversity was exhibited by the evaluated isolates, and the rDNA RFLP analysis by MspI restriction demonstrated polymorphisms among the isolates. Although both techniques were efficient and reproducible, RAPD indicated higher genetic variability among the isolates when compared with the rDNA RFLP analysis. The isolates were clustered in two groups using UPGMA analysis of the RAPD and RFLP data, with Group I subdivided into five subgroups and Group II into four subgroups. A pathogenicity test performed using detached cashew leaves showed that the isolates Cg02 and Cg03 were the most aggressive. Through RAPD and rDNA RFLP analyses, this study demonstrated a correlation between the genetic groups and geographical origin of the isolates; however, no correlation was found between these groups and pathogenicity.

Key Words: *Colletotrichum* Diversity, *Anacardium Occidentale*, Anthracnose, RAPD, Rdna RFLP

INTRODUCTION

Cashew (Anacardium occidentale L., Anacardiaceae) nuts are among the most important cash crops of Northeastern Brazil. Approximately 700 000 ha are occupied with this crop, employing morethan 280,000 people and providing an annual revenue of 230 million dollars (Freire et al., 2002,Sindcaju, 2008). Anthracnose is the main disease of cashew in Brazil and is caused by Colletotrichum gloeosporioides (Penz.) Sacc. (teleomorph Glomerella cingulata), a common tropical fruit plant pathogen in avocado, banana, citrus, mango, papaya, passion fruit and guava cultivation (Afanador-Kafuri et al., 2003). Moreover, *C. gloeosporioides* is a highly genetically variable pathogen and is considered as a group species (Sutton, 1992). For this reason, molecular tools based on DNA analyses are being used in the biotyping of variants of this fungus. Many molecular markers can be used to evaluate levels of genetic diversity, phylogenetic relationships and race identification (Lopez and Lucas, 2010; Karataş and Ağaoğlu, 2010), however only one study described the genetic variability of this pathogen in cashew (Lopez and Lucas, 2010).Random Amplified Polymorphic DNA (RAPD) is a technique based on PCR that facilitates the analysis of genetic diversity and structure in natural populations and the establishment of phenotypic relationships among different species and isolates of the same species (Martin and Rygiewicz, 2005). This technique is rapid and easy to perform because it does not require knowledge of the marker sequences and can produce abundant polymorphic fragments (Karataş and Ağaoğlu, 2010).

Another important DNA fragment used as a marker is the Internal Transcribed Spacer (ITS) of the 5.8S

rRNA gene, which involves an adjusted model showing a small specific polymorphism and a high general specific variability (Martin and Rygiewicz, 2005). The amplification of this region and its restriction is an important tool to describe the genetic variability of plant pathogenic fungi.

This study aimed to evaluate the genetic variability of C. gloeosporioides isolates obtained from cashew trees in different regions of Pernambuco State, Brazil, using RAPD and rDNA-RFLP analysis and to determine the possible correlation between the region and pathogenicity level of the isolates.

MATERIALS AND METHODS

Fungal isolates

Eighteen isolates of *C. gloeosporioides* obtained from different adult cashew tree varieties situated in different locations in the state of Pernambuco, Brazil, were evaluated for genetic and pathogenic variability (Table 1). Sixteen of the specimens were isolated from symptomatic leaves, one from a branch and one from a cashew inflorescence. After surface disinfection, each plant part was then cut at the boundary of the lesions, and 10 fragments (4–6 mm) were placed onto potato dextrose agar (PDA) plates (Difco) containing 50 µg/mL chloramphenicol. After 3–7 days incubation at 28°C, the mycelium that emerged from the plant fragments were subcultured and transferred to PDA slants for later identification. The isolates were maintained in agar slants.

DNA extraction

C. gloeosporioides conidial suspensions (10^6 conidia/ml) were used to inoculate 100 ml Czapeck-dox liquid medium with 1% glucose (w/v) and incubated for 96 h (30°C, 250 rpm). The mycelia were harvested by filtration, washed with sterile-distilled water and stored at -20°C. The DNA was extracted according to Raeder and Broda (1985). The mycelia were ground with silica powder and liquid nitrogen and then suspended in 800 µl extraction buffer (200 mM Tris-HCI [pH 8.0], 250 mM NaCl, 25 mM EDTA and 1% SDS). After homogenisation, the mixtures were incubated for 15 min at 65°C. The DNA was then extracted (once each) with equal volumes of saturated phenol, a phenol: chloroform (1:1) mixture and a chloroform: isoamyl alcohol (24:1) mixture and precipitated with 0.3 M NaCl and 2 volumes ethanol at -20°C for 30 min. The samples were centrifuged at 10,000 x g for 15 min, and the DNA pellets were washed with 70% ethanol, air-dried, suspended in TE buffer (pH 8.0) and stored at 4°C until use.

RAPD analysis

Previously, 50 primers from the OPA, OPW and OPX kits supplied by Operon Technologies were tested using one of the DNA isolates (Cg 11) to select suitable primers for the RAPD analysis. The primers used were OPA-2, OPA-11, OPW-07, OPW-20 and OPX-07. PCR reactions were performed with these primers in a total volume of 25 µl containing 1x Taq buffer (20 mM Tris-HCl (pH 8.4) and 50 mM KCl), 25 ng genomic DNA, 0.4 mM primer, 0.25 mM dNTPs and 2.5 U Taq DNA polymerase, as described by Williams et al. (1990). The thermal cycling consisted of an initial denaturation for 4 min at 94°C, followed by 40 cycles of 1 min at 94°C, 90 s at 39°C and 2 min at 72°C, with 5 min at 72°C for the final extension. The amplification products were separated on 1.4% (w/v) agarose gels at 3 V/cm in 0.5x TBE buffer. After electrophoresis, the gels were stained with ethidium bromide (0.5 mg/ml) and photographed under UV light. Negative controls contained water instead of DNA.

rDNA-RFLP analysis

Amplification reactions were prepared in a final volume of 25 µl containing 1x Taq buffer, 50 ng template DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 12 pmols of each primer, ITS1 (5' GCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3'), and 1.25 U Taq DNA polymerase, as described by White et al (1990). The thermal cycling consisted of initial denaturation for 7 min at 94°C, followed by 30 cycles of 1 min at 94°C, 2 min at 45°C and 3 min at 72°C, with 7 min at 72°C for the final extension. The amplified fragment included the 5.8S rDNA gene and internal transcribed spacers. Aliquots of 4 µl of the amplicons were subjected to enzymatic digestion with the restriction enzymes DraI, HaeIII and MspI (Invitrogen), according to the manufacturer's

instructions. The restriction fragments were separated on 3% (w/v) agarose gels with 0.5x TBE buffer. After electrophoresis, the gels were stained with ethidium bromide (0.5 mg/ml), and the DNA fragments were visualised under UV light. The fragment sizes were estimated by a comparison with a 100 bp marker (Invitrogen).

Pathogenicity level of the isolates

Leaves of cashew variety EMBRAPA CP 09 (susceptible to anthracnose) were used for the C. gloeosporioides pathogenicity assay. Young and healthy leaves from adult trees were collected at the Experimental Station of Itapirema-IPA (Instituto Agronômico de Pernambuco). The leaves were disinfected with 2% NaOCl for 3 min, washed three times with sterile-distilled water and dried with sterilised absorbent paper. The leaves were then placed in containers, and their petioles were wrapped with cotton saturated with sterile distilled water. Discs with the mycelia of C. gloeosporioides (4 mm in diameter), originating from the seven days of growth on PDA, were placed on the abaxial surface of the leaves. The experimental design was randomised as four blocks with 19 treatments; each block contained one leaf and three repetitions of the inocula. Non-inoculated discs of PDA were used as the controls. The resulting lesions were evaluated 72 h after the inoculation, and the pathogens were re-isolated according the method described above.

Data analysis

Based on the RAPD and rDNA-RFLP data, dendrograms were constructed based on the Jaccard coefficient (Sj) using band positions (for each pattern, the Sj divides the number of corresponding bands by the total number of bands in both patterns) and UPGMA (unweighted pair group method with averages) cluster analysis. A consensus tree was generated using Winboot software (Yap and Nelson 1996), with the bootstrap replicate number test at 1000.

The medium diameters of the lesions were transformed by y=senx for data normalisation. These data were then analysed according to the variance (ANOVA) and the medians were compared with the Tukey test ($P > 0.05$) using the 7.2 beta version of the ASSISTAT program (Silva, 2004).

RESULTS AND DISCUSSION

Fungal genetic variability

Among the fifty RAPD primers, five of them (OPA-2, OPA-11, OPW-07, OPW-20 and OPX-07) showed variability for the C. gloeosporioides isolates (Figure 1A-E). Using these data, a combinatory cluster analysis was performed, showing that the isolates were separated into two main groups, with similarities of 15% (Figure 1F). Group I includes the majority of the isolates from the Meridional “Agreste” region of Pernambuco State (located in Brejão, São João and Garanhuns), and Group II includes the isolates from the Zona da Mata (Itambé, Goiana and Igarassu) of Pernambuco State. Moreover, Group I was subdivided into three subgroups, with similarities between 50 and 60% (Groups IA, IB and IC), a group with 40% similarity (ID) and another group (IE) formed only by Cg14. Within Group II, two subgroups were observed (IIB and IIC) with 60% similarity, and the other two subgroups (IIA and IID) formed by the isolates Cg08 and Cg17 showed less similarity with the others. Several authors have used the RAPD technique to demonstrate a large genetic variability of C. gloeosporioides isolates originating from Stylosanthes (Munaut et al., 1998; Kelemu et al., 1999; Munaut, Hamaide and Maraite, 2002), strawberries, citrus (MacKenzie et al., 2007; Kuramae-Izioka et al., 1997), banana, apple (Zakaria et al, 2009) and mango (Gupta et al., 2010). Of the eighteen C. gloeosporioides isolates used in the present work, six demonstrated a sexual teleomorphic state that is characterised as Glomerella cingulata (data not shown). The presence of sexual reproduction (Freeman and Shabi, 1996) in populations of C. gloeosporioides may have contributed to their increase in genetic variability.

The amplification of the ITS1-5.8S-ITS2 of the rDNA region using the universal primers ITS1 and ITS4 produced a fragment of approximately 590 bp for all of the C. gloeosporioides isolates, confirming previously reported results (Martinez-Culebras et al., 2000; Abang et al., 2002; Saha et al., 2002, Vila Nova et al., 2011). However, we detected a second fragment of 620-630 bp for the Cg15 isolate in all of

the repetitions (Figure 2A). A secondary fragment was also detected in Fusarium solani isolates that was associated nonspecifically with a genomic region different from the ITS of the rDNA region (Brasileiro, 2004). However, this F. solani fragment was irregular in number and size, varying from 400 to 700 bp; this result was different from the fragment observed in the present study, which was consistently observed in all of the replicates. The occurrence of multiple copies of these rDNA clusters per genome allows intra-individual polymorphisms. The presence of a secondary band for isolate Cg15, non-pathogenic (Table 2) and from an inflorescence, seems to distinguish it from the others.

Table 1: Isolates of Colletotrichum gloeosporioides from cashew trees.

Isolates	URM[a]	Geographical orign[b]	Cashew varities
Cg10	4903	Igarassu 07° 34' 37"S 34° 90'64"W	Common cashew
Cg14	4907		Precoce cashew
Cg17	4910	Itambé 07° 24' 37" S 35° 06' 46' W	Embrapa C.P. 09
Cg11	4904	Goiana 07° 33' 38"S 35° 00' 09"W	Embrapa C.P. 1001
Cg12	4905		Embrapa C.P. 09
Cg15	4908		Embrapa C.P. 22
Cg16	4909		Embrapa C.P. 76
Cg03	4896	Garanhuns 08° 53' 25"S 36° 29' 34"W	Common cashew
Cg04	4897		
Cg06	4899		
Cg13	4906		
Cg05	4898	São João 08° 52' 32"S 36° 22' 00"W	Common cashew
Cg07	4900		
Cg18	4911		
Cg01	4894	Brejão 09° 01' 49" S 36° 34' 07" W	Common cashew
Cg02	4895		
Cg08	4901		
Cg09	4902		

[a] Number of accession in the mycological collection – URM (Recife-PE, Brazil)
[b] Geographical coordinates of the regions from which the cashew trees were sampled

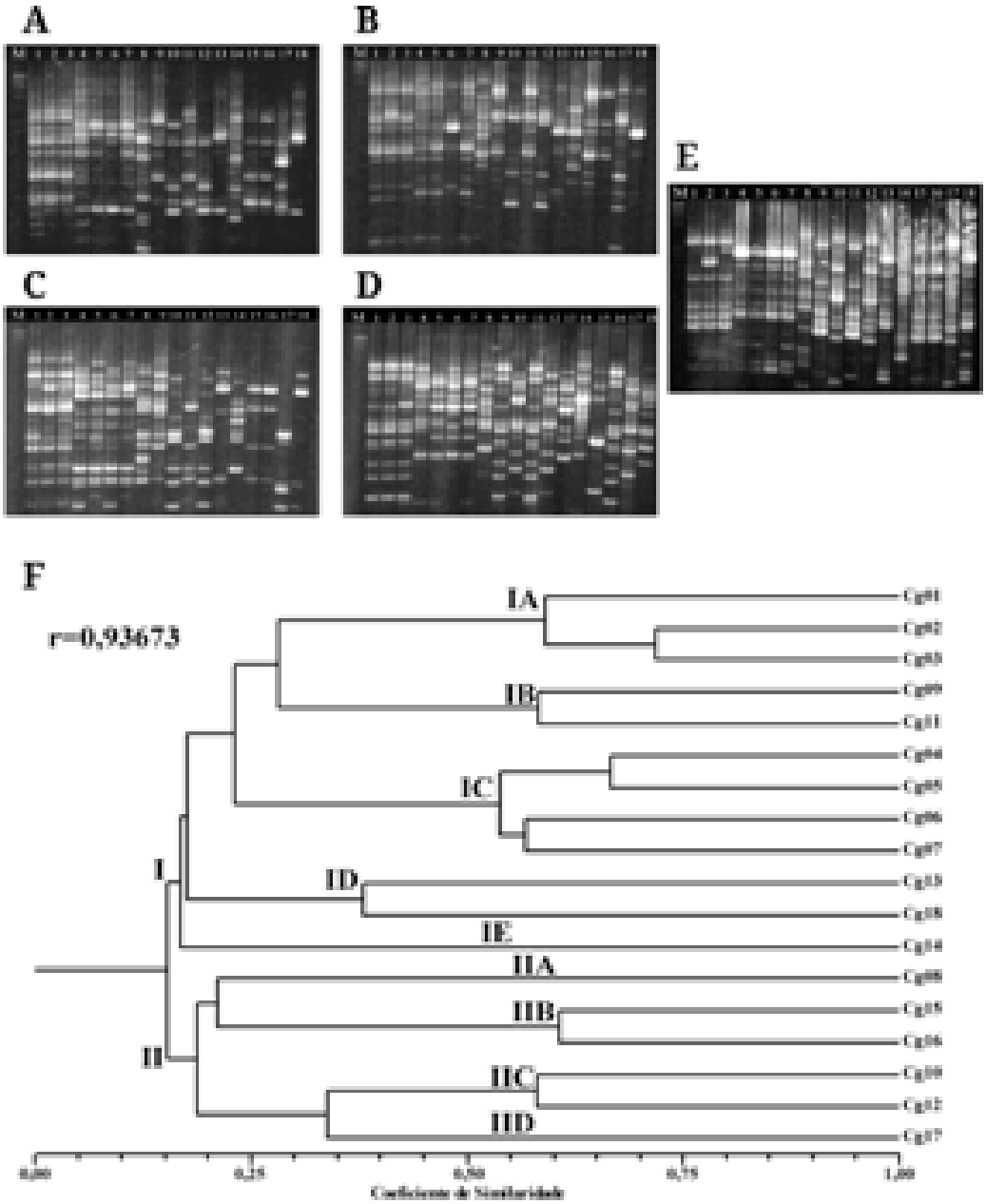
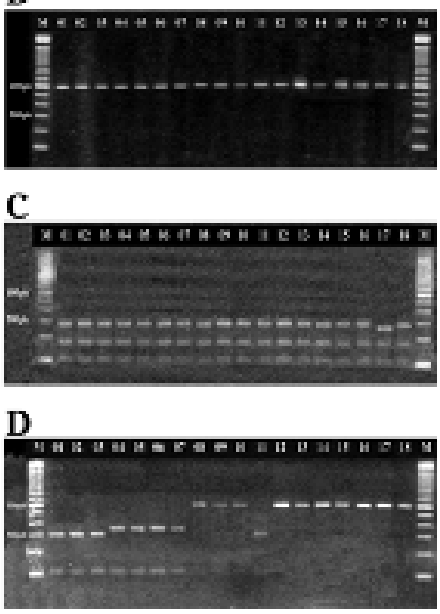


Figure 1: **Analysis of the genetic variability of isolates of Colletotrichum gloeosporioides from cashew trees using RAPD with the primers (A) OPA-2, (B) OPA-11, (C) OPW-07, (D) OPW-20 and (E) OPX-07 supplied by Operon Technologies. The numbers 1-18 are the isolates Cg01, Cg02, Cg03, Cg04, Cg05, Cg06, Cg07, Cg08, Cg09, Cg10, Cg11, Cg12, Cg13, Cg14, Cg15, Cg16, Cg17 and Cg18, respectively, and M is the 100 bp molecular marker (Invitrogen). (F) UPGMA dendrogram based on the coefficient of Jaccard, illustrating the genetic relationships among the isolates of C. gloeosporioides. The numbers in the dendrogram indicate the percentage of similarity of the groups to each node occurred in the tree.**

A

Isolates	ITS (bp)	ITS-RFLP (bp)	
		*Hae*III	*Msp*I
Cg10	590	280+170+110	590
Cg14	590	280+170+110	590
Cg17	590	270+170+110	590
Cg11	590	280+170+110	300
Cg12	590	280+170+110	590
Cg15	590+620	280+170+110	590
Cg16	590	280+170+110	590
Cg03	590	280+170+110	300+110
Cg04	590	280+170+110	350+110
Cg06	590	280+170+110	350+110
Cg13	590	280+170+110	590
Cg01	590	280+170+110	300+110
Cg02	590	280+170+110	300+110
Cg08	590	280+170+110	590
Cg09	590	280+170+110	590
Cg05	590	280+170+110	350+110
Cg07	590	280+170+110	350+110
Cg18	590	280+170+110	590



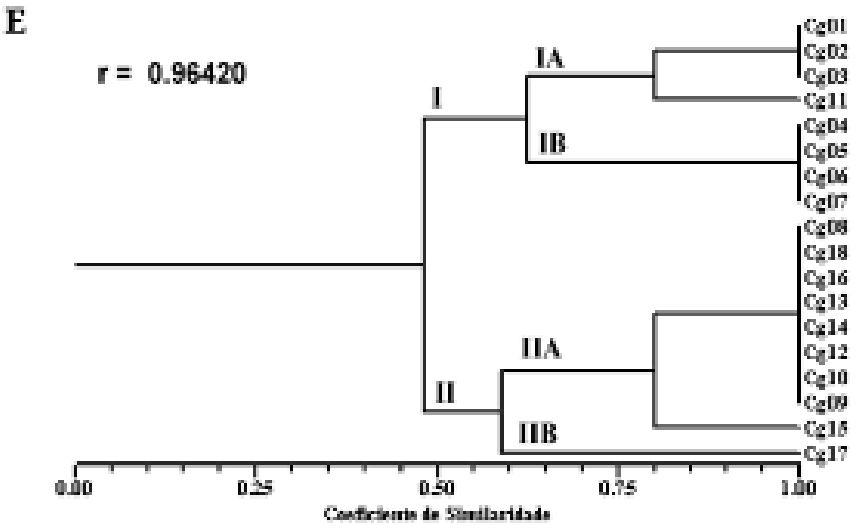


Figure 2: **Analysis of the genetic variability of isolates of Colletotrichum gloeosporioides from cashew tree using rDNA-RFLP. Number and size of ITS fragment generated by the amplification of ITS1-5.8S-ITS2 of the rDNA region (A) and the digestion products using the enzymes DraI (B), HaeIII (C) and MspI (D). The numbers 1-18 are the isolates Cg01, Cg02, Cg03, Cg04, Cg05, Cg06, Cg07, Cg08, Cg09, Cg10, Cg11, Cg12, Cg13, Cg14, Cg15, Cg16, Cg17 and Cg18, respectively, and M is the 100 bp molecular marker (Invitrogen). UPGMA dendrogram based on the coefficient of Jaccard, illustrating the genetic relationships among the isolates of C. gloeosporioides. The numbers in the dendrogram indicate the percentage of similarity of the groups to the right of the node occurred in the tree (E).**

The products of the PCR ITS rDNA digestion with DraI revealed that no restriction sites were present (Figure 2B). The digestion with HaeIII resulted in a characteristic pattern of three fragments in all of the isolates (Figure 2A and C). Cg17 presented a fragment of 270 bp, which was different from the other

isolates. The digestion with MspI demonstrated more distinct polymorphisms among the isolates, yielding fragments of variable molecular weights (Figure 2A and D). The in-group analysis of the rDNA ITS restriction patterns using HaeIII and MspI revealed two other groups, with 65% of similarity (Figure 2E).
The two groups formed by the cluster analysis with RAPD profiles were similar for both groups based on the restriction patterns of their rDNA ITS regions. The only exception was the isolate Cg09, which grouped with the isolates from Meridional "Agreste" by RAPD and has grouped with the isolates of the Zona da Mata by RFLP.
Using both techniques, the Cg17 isolate was distant in comparison to the other isolates, indicating a possible different taxon. An important fact is that, among the eighteen isolates used in this work, only Cg15 and Cg17 were derived from the inflorescence and stem, respectively; the others were all isolated from leaves, suggesting a possible correlation among the plant tissue and the type of pathogen. Another relevant observation is that, under the tropical conditions from which the Colletotrichum isolates were obtained, the cultivation of many hosts, such as mango, papaya, passion fruit, banana, avocado, guava, citrus and cashew, in neighbouring areas or within the same area, favours a rapid dissemination of pathogens from one host to another (Peres et al., 2002). This cross-infection may contribute to the permutation among individuals of different hosts, acting as a potential source of variability. In this way, the Cg15 and Cg17 isolates may have been derived from cross-infection among the different hosts found in the respective regions of isolation.

Pathogenicity of isolates

The eighteen isolates of C. gloeosporioides were tested according to their pathogenic severity on the cashew leaves. The isolates that caused the largest lesion diameters were Cg02 and Cg03 followed by Cg16, Cg09, Cg06 and Cg18. The other isolates were not significantly different from the control (Table 2).

[a]The diameters of the lesions were transformed by y=sen x for data normalisation. Values with the same letter are not significantly ($P > 0.05$) different according to Tukey's test. The results are the means of four replicates for each isolate.

The isolates Cg04, Cg07 and Cg15 did not cause lesions on the leaves during the experimental period, however lesions on the leaves were observed after an additional five days. Despite the fact that the majority of the isolates (83.3%) caused leaf lesions, the intact cuticle created a physical barrier that could interfere with the infection caused by the C. gloeosporioides isolates; in comparison, only seven isolates (33.3%) attained a lesion size of medium diameter that was significantly different from the control. The late appearance of the lesions by Cg04, Cg07 and Cg15 can be attributed to the intracellular infection strategy (O'Connell et al., 1985; Latunde-Data et al., 1996), as reflected in the absence of pathogenicity during the experimental period. All of the isolates could be re-isolated from the leaves and showed the same morphological characteristics that were observed upon the initial isolation.
According to the pathogenicity assay, no correlation was observed among the pathogenicity and the genetic groups distinguished by the RAPD and rDNA ITS techniques. However, the present study confirmed that providing good molecular markers will improve the understanding of the population structure of C. gloeosporioides and help to further effective measures of cashew-anthracnose control and breeding programs.

Table 2: Pathogenicity of inoculated Colletotrichum gloeosporioides isolates on the leaves of cashew variety EMBRAPA C.P. 09

Isolate	Lesion diameter[a]	
Cg02	0.01490	a
Cg03	0.01265	ab
Cg16	0.00916	bc
Cg09	0.00887	bc
Cg06	0.00734	cd
Cg18	0.00596	cde
Cg10	0.00385	def
Cg11	0.00298	def
Cg08	0.00261	ef
Cg17	0.00182	ef
Cg01	0.00138	ef
Cg14	0.00131	f
Cg05	0.00116	f
Cg12	0.00116	f
Cg13	0.00058	f
Cg04	0.00000	f
Cg07	0.00000	f
Cg15	0.00000	f
Control	0.00000	f

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