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*pel*B gene in isolates of *Colletotrichum gloeosporioides* from several hosts

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ABSTRACT. *Colletotrichum gloeosporioides* is an important pathogen for a great number of economically important crops. During the necrotrophic phase of infection by *Colletotrichum* spp, the degradative enzymes of plant cell walls, such as pectate lyase, clearly increase. A gene *pel*B that expresses a pectate lyase was identified in isolates of *C. gloeosporioides* in avocado pathogens. Various molecular studies have identified a kind of specialization of *C. gloeosporioides* isolates with specific hosts; however, there have been no studies of this gene in isolates from hosts other than avocado. The same is true for other species of *Colletotrichum*. We examined genetic variability in order to design primers that would amplify *pel*B gene fragments and compared the products of this amplification in *C. gloeosporioides* isolates from different hosts. Genetic variability was assessed using ISSR primers; the resultant data were grouped based on the UPGMA clustering method. Primers for the *pel*B gene were designed from selected GenBank sequences using the Primer 3 program at an annealing temperature of 60°C

and product amplification of nearly 600 bp. The ISSR primers were efficient in demonstrating the genetic variability of the *Colletotrichum* isolates and in distinguishing *C. gloeosporioides*, *C. acutatum* and *C. sublineolum* species. The gene *pel*B was found in *C. gloeosporioides*, *C. acutatum* and *C. sublineolum*. Amplified restriction fragments using *Msp*I did not reveal differences in *pel*B gene structure in isolates from the three different host species that we investigated.

Key words: *Colletotrichum*; CgInt; ISSR; *pel*B gene; Pectate lyase

INTRODUCTION

Fungi of the genus *Colletotrichum* and its teleomorph *Glomerella* are among the main pathogens studied and are responsible for huge losses in many tropical, subtropical and temperate crops. Cereal, vegetable, ornamental, and fruit crops can be seriously affected by fungus, leading to significant reductions in production. In the case of fruit crops, even though the fungus infects different aerial parts of the plant, the most significant economic losses stem from the attack on the fruits. In addition, infection can occur and remain quiescent until post-harvest, when the typical symptoms develop, extending losses to merchants and consumers (Bailey and Jeger, 1992; Freeman et al., 1998, 2000). Due to the high genetic plasticity and the great dependence on environmental factors, pathogenic agents are subject to constant physio- and morphological changes in pathogenic behavior (Michereff, 2000). With the existence of species-specific primers based on nucleotide sequences of internal transcribed spacer region 1 (ITS1) of rDNA, polymerase chain reaction (PCR) has become a powerful tool to aid in the identification of *Colletotrichum* species (Brown et al., 1996). Other techniques such as the analysis of the inter-simple sequence repeats (ISSR) region, oligonucleotides of microsatellites have been used to demonstrate the intraspecific genetic variability of fungi of agronomic interest (Vainio et al., 1998; Yu et al., 2006; Chadha and Gopalakrishna, 2007; Bayraktar et al., 2008).

Knowledge of the molecular basis of the action of plant pathogens including the structure and evolution of genes related to pathogenicity is of fundamental importance to disease control programs (Perfect et al., 1999).

The enzymes that degrade the cell wall and other physical barriers, such as cutin and pectin of plants, may be essential for pathogenicity (Lebeda et al., 2001). In the necrotrophic phase of infection by *Colletotrichum* species, enzymes that degrade the plant cell wall such as endopolygalacturonase and pectate lyase show clear increase in expression (Perfect et al., 1999). In the *Glomerella cingulata* teleomorph of *C. gloeosporioides*, efforts have been made to characterize these pectate lyases. Such a gene called *pel*B coding for a pectate lyase was identified in isolates of *C. gloeosporioides* and mutants with deletions of this gene showed reduced pathogenicity to avocado (*Persea amarican* Mill.) (Yacoby et al., 2001). So far, studies on the *pel*B gene, which encodes a pectate lyase, were performed on isolates of *C. gloeosporioides* from avocado but were not extended to other isolates of the same species from other hosts, thus requiring knowledge of its distribution and variations in sequence. The objectives of this study were to assess genetic variation and the presence of the pathogenic *pel*B gene and to compare the amplification products of this gene in isolates from different hosts of *C. gloeosporioides.*

MATERIAL AND METHODS

Colletotrichum isolates

Twenty isolates of *Colletotrichum* from different hosts attacked by anthracnose were used. They were provided by the Culture Collection - URM (University of Recife - Mycology) of the Mycology Department, Federal University of Pernambuco and the Pathology Sector of the Luis de Queiroz School of Agriculture (ESALQ-USP) (Table 1). Isolates of *C. acutatum* and *C. sublineolum* were used for comparative analysis of genetic variability and structure of the *pel*B gene.

Table 1. *Colletotrichum* isolates according to host origin.

Fungus	Accession number	Geographic origin	Substratum or host
C. gloeosporioides	URM4626	Brejão/PE	Stalk of onion
	URM4627	Belém de S. Francisco/PE	Leaf of onion
	URM4628	Brejão/PE	Inflorescence of onion
	URM4629	Petrolina/PE	Leaf of onion
	URM4894	Brejão/PE	Leaf of cashew
	URM4896	Garanhuns/PE	Leaf of cashew
	URM4900	São João/PE	Leaf of cashew
	URM4905	Igarassu/PE	Leaf of cashew
	URM4908	Igarassu/PE	Inflorescence of cashew
	URM4852	Garanhuns/PE	Leaf of mango
	URM4854	Itambé/PE	Leaf of mango
	URM4856	Igarassu/PE	Leaf of pink mango
	URM4857	Igarassu/PE	Leaf of mango
	URM4858	Brejão/PE	Leaf of pink mango
	URM4859	Recife/PE	Leaf of pink mango
	Ci	São Paulo/SP	Leaf of cyclamen
	PI13	São Paulo/SP	Fruit of chili
	PI15	São Paulo/SP	Fruit of chili
C acutatum	Acu	São Paulo/SP	Fruit of peach
C. sublineolum	Sub	São Paulo/SP	Leaf of sorghum

PE = Pernambuco; SP = São Paulo.

Obtaining the mycelium and DNA extraction

Conidia of each isolate were suspended in 3 mL 0.1% Tween 20 (v/v) (10^6 conidia/mL) and transferred to Erlenmeyer flasks containing 100 mL liquid minimal medium. Vials were shaken at 27 rpm for 120 h at 28°C for fungal growth. Subsequently, the mycelium was collected by vacuum filtration and washed with autoclaved distilled water. The wet weight was determined and the sample stored at -20°C until use for DNA extraction. DNA was extracted following the technique described by Kuramae-Izioka (1997). The mycelial mass was ground with liquid nitrogen and transferred to microtubes, to which 700 µL extraction buffer (1 M Tris-HCl, pH 8.0, 250 mM NaCl, 0.5 mM EDTA, pH 8.0, 10% sodium dodecyl sulfate) was added. After homogenization, the microtubes were incubated at 65°C for 30 min and gently shaken by inversion every 10 min, after which 500 µL 5 M potassium acetate was added, followed by homogenization and centrifugation at 14,500 *g* for 10 min. The supernatant was extracted with 1 volume of chloroform-isoamyl alcohol (24:1), and subsequently, centrifugation was carried out at 14,500 *g* for 10 min. One volume of isopropanol was added to the recovered aqueous phase, and the mixture cooled to 4°C for DNA precipitation. Samples were again subjected to centrifugation at 14,500 *g* for 10 min. The precipitate was washed with 70%

ethanol, centrifuged for 10 min, dried at room temperature, resuspended in TE buffer, pH 8.0 (1 M Tris-HCl, 0.5 M EDTA) and stored in a refrigerator at -20°C.

The DNA concentration was estimated by electrophoresis on a 0.8% agarose gel at 3 V/cm distance between the electrodes with 1X Tris-borate-EDTA (TBE) running buffer in comparison with the lambda phage DNA molecular weight marker (Invitrogen Life Technologies). After electrophoresis, the gel was stained in ethidium bromide solution (1X TBE/0.5 µg/mL EtBr; Sambrook et al., 1989) for 30 min, observed with an ultraviolet transilluminator and photographed using a Sony digital camera (effective 7.2 megapixels).

Amplification with primers CgInt/ITS4

For specific confirmation of *Colletotrichum* isolates, we used the species-specific primer CgInt (5'-GGCCTCCCGCCTCCGGGCGG-3') in combination with the universal primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), according to Freeman et al. (2000). The amplification reactions were performed in a final volume of 25 µL containing: buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 mM primer, 0.04 U Taq DNA polymerase (Invitrogen Life Technologies) and 25 ng DNA. The thermal cycler Techne TC-512 was used with the following schedule: initial denaturation at 95°C for 5 min, 40 cycles at 95°C for 30 s, 50°C for 30 s and 72°C for 1 min and 30 s. Amplified products were separated by electrophoresis on a 1% agarose gel at 3 V/cm in 0.5X TBE running buffer, pH 8.0, using a 100-bp molecular weight marker (Invitrogen Life Technologies). After electrophoretic migration, the gel was stained in ethidium bromide solution for 30 min, visualized with an ultraviolet transilluminator and photographed with a Sony digital camera (effective 7.2 megapixels).

ISSR by PCR

DNA samples were subjected to PCR with primers $(GTG)_5$, $(GACA)_4$ and M13. The amplification reactions were performed in a final volume of 25 µL under the following conditions: buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 0.75 mM $MgCl_2$, 0.25 mM dNTP, 0.25 mM primer, 0.1 U Taq DNA polymerase (Operon Technologies) and 25 ng DNA, as described by de Barros Lopes et al. (1996). The amplification cycles consisted of an initial denaturation step at 95°C for 5 min followed by 40 cycles of 20 s at 95°C, 45 s at 55°C and 1 min at 72°C, followed by a final extension of 3 min at 72°C. Amplified products were separated by electrophoresis on a 1.4% agarose gel at 3 V/cm in 1X TBE running buffer, pH 8.0, using a 100-bp molecular weight marker (Invitrogen Life Technologies). Subsequently, the gel was stained in ethidium bromide solution for 30 min, visualized with an ultraviolet transilluminator and photographed with a Sony digital camera (effective 7.2 megapixels). The data obtained from the amplification with the microsatellite markers were analyzed with the Numerical Taxonomy System of Multivariate Programs - NTSYS-PC (Bussab et al., 1990; Rohlf, 2002), using the Jaccard coefficient. The dendrogram was generated by the clustering method UPGMA (unweighted pair group method with arithmetic average).

Design of specific primers for the *pel*B gene and optimization of PCR

Primers for PCR were designed from sequences of *C. gloeosporioides* selected from GenBank (National Center for Biotechnology Information, http://www.ncbi.

nlm.nih.gov) using the Primer 3 program (Rozen and Skaletsky, 2000). The primers were named P1 (5'-ACGAAAACCACCAACCACA-3') with Tm of 53.1°C and P6 (5'-TGGAGGCGTGAGTGATGTC-3') with Tm of 53.7°C, and the amplification products had an expected size of approximately 600 bp. The PCR conditions were optimized in thermal cycler with a temperature gradient. The optimization reaction showed that 60°C was the optimal annealing temperature for the pair of primers designed. The amplification reactions were performed in a final volume of 25 µL under the following conditions: 1X buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.3 mM dNTP, 0.2 mM of each primer, 0.04 U Taq DNA polymerase (Invitrogen Life Technologies) and 25 ng DNA. PCR involved an initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The amplification products of the *pel*B gene were separated by electrophoresis on a 1% agarose gel at 3 V/cm in 0.5X TBE running buffer, pH 8.0, using a 100-bp molecular weight marker (Invitrogen Life Technologies). The gel was stained in ethidium bromide solution for 30 min, visualized with an ultraviolet transilluminator and photographed with a Sony digital camera (effective 7.2 megapixels).

Enzymatic digestion of PCR products of the *pel*B gene

The enzymatic digestion was performed by mixing 6 µL amplification products of *pel*B with 20 µL restriction mix containing 0.1 U restriction enzyme *Msp*I (Invitrogen Life Technologies) in buffer for specific restriction. After incubation for 1 h at 37°C with the enzyme, the resulting fragments were separated by electrophoresis on a 1.5% agarose gel at 3 V/cm distance between electrodes in 0.5X TBE running buffer, pH 8.0, using a 100-bp molecular weight marker (Invitrogen Life Technologies). The restriction fragments were stained as described above.

RESULTS

Specific characterization with primers CgInt/ITS4

The amplification using primers CgInt/ITS4 (Figure 1) was positive for all isolates of *C. gloeosporioides*, generating fragments of approximately 450 bp and negative for isolates of *C. acutatum* and *C. sublineolum*. The use of species-specific primers confirmed the identity of isolates of *C. gloeosporioides* stored in the RMU fungal collection identified by classical methodology.

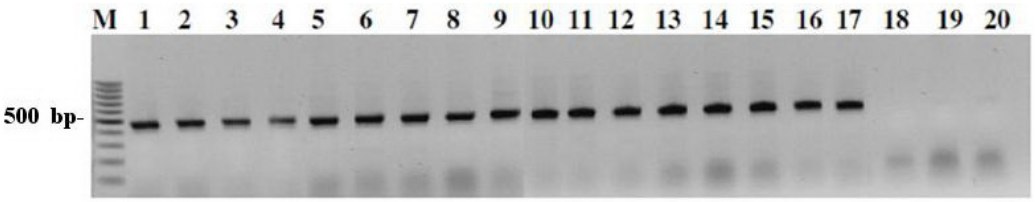

Figure 1. Profiles of amplification of specific fragments of 20 *Colletotrichum gloeosporioides* isolates with the primer CgInt/ITS4. M = Molecular weight size markers, 100 bp. *Lanes 1-20* = corresponding to strains URM4626, 4627, 4628, 4629, 4894, 4896, 4900, 4905, 4908, 4852, 4854, 4856, 4857, 4858, 4859, Ci, PI13, PI15, Acu, and Sub, respectively.

Analysis of ISSR regions

The amplification profiles of the ISSR region, using the primers $(GTG)_5$, $(GACA)_4$ and M13 are illustrated in Figure 2A, B and C, respectively. The dendrogram generated from the amplification profiles with the primer $(GTG)_5$ (Figure 3) showed four groups at the similarity level of fragment size of 85%. The first group is represented by four isolates of *C. gloeosporioides*: 4626 (onion peduncle/PE, which means Pernambuco), 4627 (onion leaf/PE), 4628 (onion inflorescence/PE), and 4629 (onion leaf/PE). The second group is formed by the species *C. gloeosporioides*: 4854 (sword mango leaf/PE), 4856 (pink mango leaf/PE), 4857 (sword mango leaf/PE), 4859 (pink mango leaf/PE), and 4858 (pink mango leaf/PE). The third group is made up of two representatives of *C. gloeosporioides*: 4894 (cashew leaf/PE) and 4896 (cashew leaf/PE), and the fourth is formed by the species *C. acutatum* PI15 (pepper/SP, which means São Paulo) and Acu (peach/SP). Representatives of the first group had 100% similarity in fragment size and 65% similarity with the isolate 4905 (cashew leaf/PE) of *C. gloeosporioides*. The isolates from the second and third groups showed 100% similarity in fragment size, while the representatives of the fourth group showed 100% similarity among themselves and 20% similarity with the isolate Sub (sorghum/SP) of *C. sublineolum*. Analyzing the dendrogram, the isolates 4905 (cashew leaf/PE), Ci (cyclamen/SP), 4908 (cashew inflorescence/PE), 4900 (cashew leaf/PE), PI13 (pepper/SP), 4852 (sword mango leaf/PE), and Sub (sorghum/SP) were not grouped with other isolates of the same species, although this marker differentiated *C. gloeosporioides*, *C. acutatum* and *C. sublineolum*.

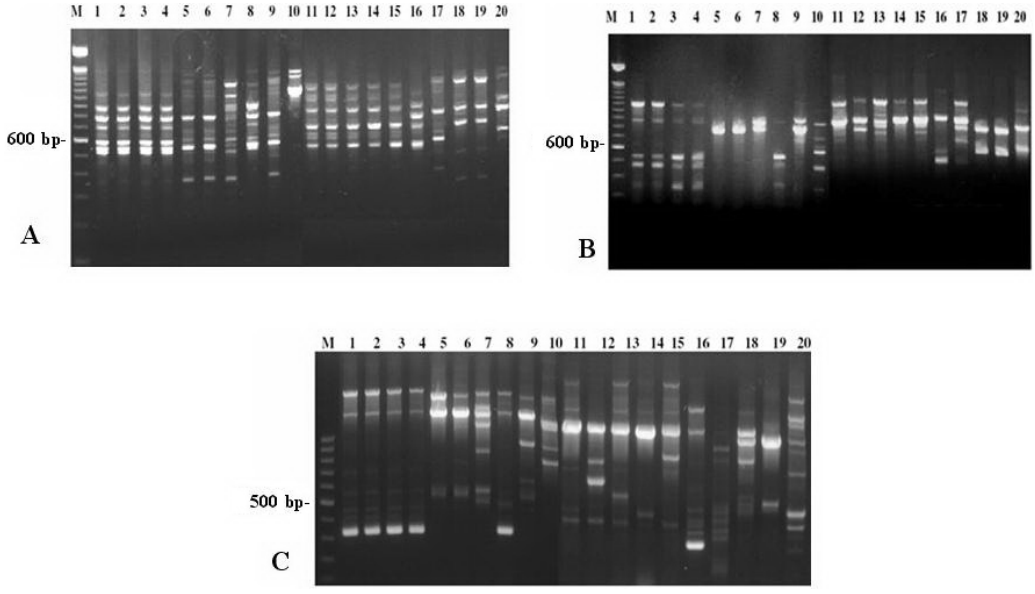

Figure 2. Amplification profiles of the ISSR regions with primers $(GTG)_5$ (A), $(GACA)_4$ (B) and M13 (C) of *Colletotrichum* isolates. *Lane M* = 1-kb molecular weight marker (A and B) or 100 bp (C). *Lanes 1* to *17* = DNA of *Colletotrichum gloeosporioides*: 4626, 4627, 4628, 4629, 4894, 4896, 4900, 4905, 4908, 4852, 4854, 4856, 4857, 4858, 4859, Ci, and PI13. *Lanes 18* and *19* = DNA of *C. acutatum* (PI15 and Acu, respectively). *Lane 20* = DNA of *C. sublineolum* (Sub).

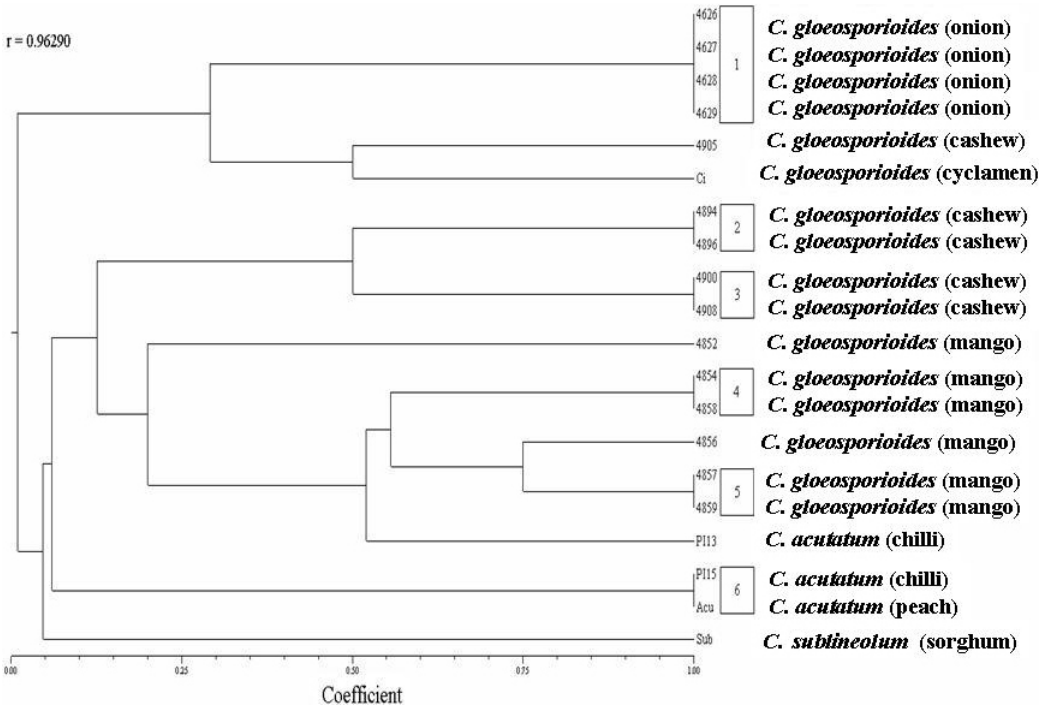

Figure 3. Dendrogram constructed by the UPGMA method, using the Jaccard coefficient (J) from the ISSR profiles with primer $(GTG)_5$ obtained from 17 isolates of *Colletotrichum gloeosporioides* (4626, 4627, 4628, 4629, 4894, 4896, 4900, 4905, 4908, 4852, 4854, 4856, 4857, 4858, 4859, Ci, and PI13), 2 isolates of *C. acutatum* (PI15 and Acu), and 1 of *C. sublineolum* (Sub).

The amplification profiles of the ISSR regions, using the primer $(GACA)_4$ are illustrated in Figure 4. The dendrogram (Figure 4) generated from the amplification profiles showed six groups, at the similarity level of fragment size of 85%. The first group is represented by four isolates of *C. gloeosporioides*: 4626 (onion peduncle/PE), 4627 (onion leaf/PE), 4628 (onion inflorescence/PE), and 4629 (onion leaf/PE). The second group consisted of *C. gloeosporioides* isolates: 4894 (cashew leaf/PE) and 4896 (cashew leaf/PE). The third is represented by isolates of *C. gloeosporioides*: 4900 (cashew leaf/PE) and 4908 (cashew inflorescence/PE). The fourth group is formed by representatives of *C. gloeosporioides*: 4854 (sword mango leaf/PE) and 4858 (pink mango leaf/PE). The fifth is represented by isolates of *C. gloeosporioides*: 4856 (pink mango leaf/PE), 4859 (pink mango leaf/PE) and 4857 (sword mango leaf/PE). The sixth group consists of the representatives PI15 (pepper/SP) and Acu (peach/SP) of *C. acutatum*. The first group showed 100% similarity in fragment size and around 34% similarity with the isolate 4905 of *C. gloeosporioides*, while the isolates from the other groups also showed 100% similarity in fragment size, and the isolate PI13 (pepper/SP) of *C. gloeosporioides* showed 45% similarity with the representatives of the fourth and fifth groups. Analyzing the dendrogram, the isolates 4905 (cashew leaf/PE), 4852 (sword mango leaf/PE), PI13 (pepper/SP), Ci (cyclamen/SP), and Sub (sorghum/SP) were distant from the other isolates of *C. gloeosporioides*, although this marker differentiated the three species studied.

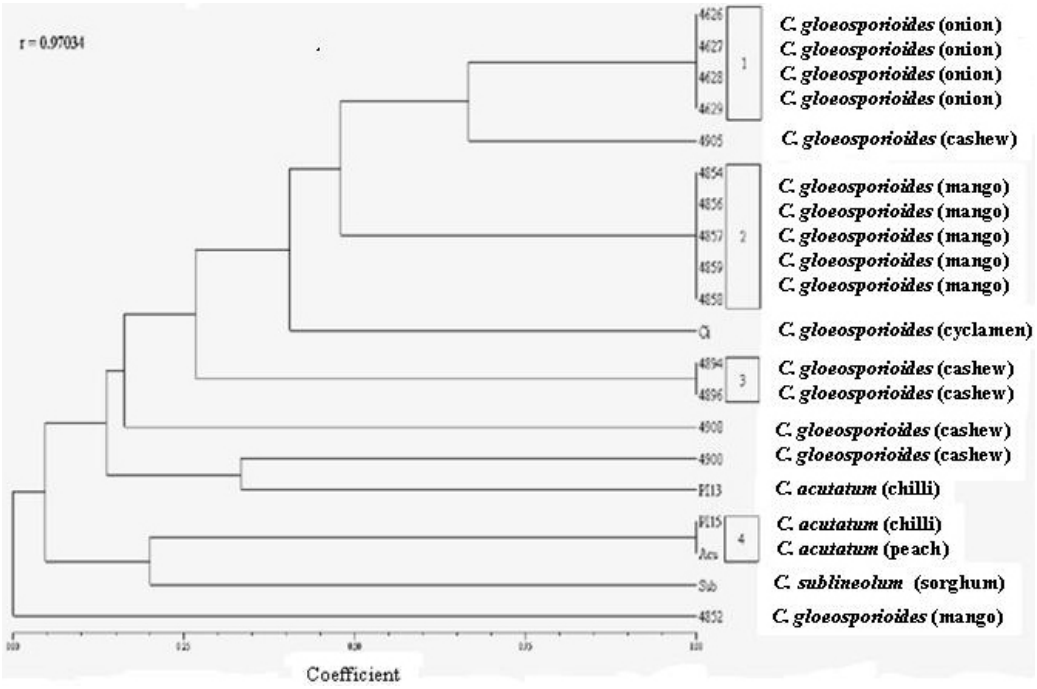

Figure 4. Dendrogram constructed by the UPGMA method, using the Jaccard coefficient (J) from the ISSR profiles with primer $(GACA)_4$ obtained from 17 isolates of *Colletotrichum gloeosporioides* (4626, 4627, 4628, 4629, 4894, 4896, 4900, 4905, 4908, 4852, 4854, 4856, 4857, 4858, 4859, Ci, and PI13), 2 isolates of *C. acutatum* (PI15 and Acu), and 1 of *C. sublineolum* (Sub).

The amplification profiles of the ISSR regions, using the primer M13 in 20 isolates of the genus *Colletotrichum* are illustrated in Figure 5. The dendrogram (Figure 5) generated from the amplification profiles showed three groups, at the level of similarity of fragment size of 85%. The first group is represented by four isolates of *C. gloeosporioides*: 4626 (onion peduncle/PE), 4627 (onion leaf/PE), 4628 (onion inflorescence/PE) and 4629 (onion leaf/PE). The second group is formed of *C. gloeosporioides* isolates: 4894 (cashew leaf/PE), 4896 (cashew leaf/PE) and 4905 (cashew leaf/PE). The third group is represented by isolates of *C. gloeosporioides*: 4854 (sword mango leaf/PE) and 4857 (sword mango leaf/PE). The three groups showed 100% similarity in fragment size; the isolate 4900 (cashew leaf/PE) of *C. gloeosporioides* showed 67% similarity to the representatives of the second group and the isolate 4859 (pink mango leaf/PE) showed 67% similarity to the third group. Analyzing the dendrogram, the isolates 4900 (cashew leaf/PE), 4908 (cashew inflorescence/PE), 4852 (sword mango leaf/PE), 4854 (sword mango leaf/PE), 4856 (pink mango leaf/PE), 4858 (pink mango leaf/PE), 4859 (pink mango leaf/PE), PI13 (pepper/SP), PI15 (pepper/SP), and Ci (cyclamen/SP), Acu (peach/SP) and Sub (sorghum/SP) were distant from the other isolates of *C. gloeosporioides*, although this marker differentiated the three species studied.

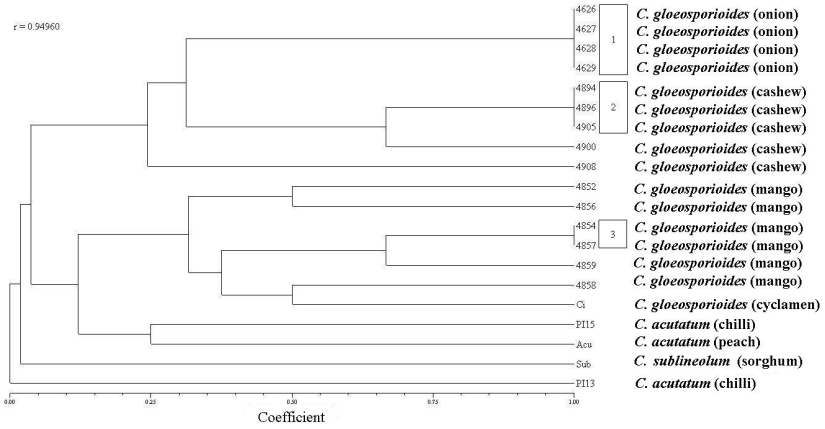

Figure 5. Dendrogram constructed by the UPGMA method, using the Jaccard coefficient (J) from ISSR profiles with primer M13 obtained from 17 isolates of *Colletotrichum gloeosporioides* (4626, 4627, 4628, 4629, 4894, 4896, 4900, 4905, 4908, 4852, 4854, 4856, 4857, 4858, 4859, Ci, and PI13), 2 isolates of *C. acutatum* (PI15 and Acu), and 1 of *C. sublineolum (*Sub).

*Pel*B gene amplification and analysis of amplification fragments digestion with restriction enzyme

The amplification of *pel*B gene fragments, using the specific primers P1 and P6 in 20 isolates of *Colletotrichum* is illustrated in Figure 6A. There was *pel*B gene amplification for all isolates of *C. gloeosporioides*, *C. acutatum* and *C. sublineolum*, with amplification products of approximately 600 bp, as expected.

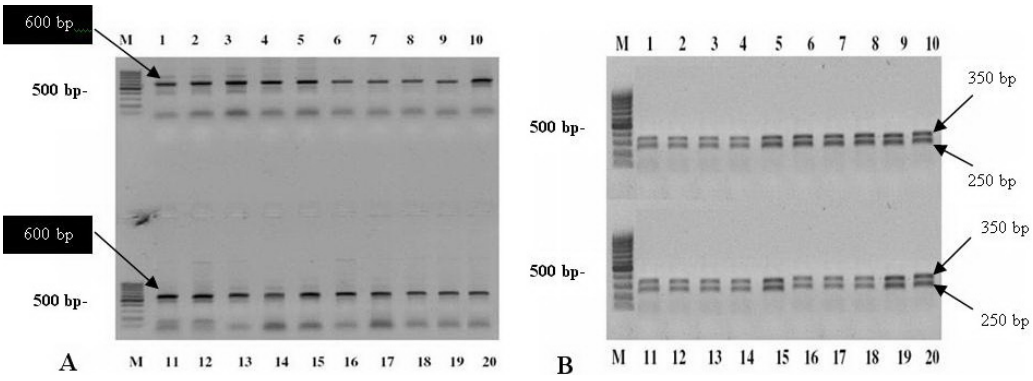

Figure 6. Amplification profiles of the restriction products of the *pel*B gene with restriction enzyme *Msp*I (A), amplification of the *pel*B gene of 20 isolates of the genus *Colletotrichum* obtained with the primers P1 and P6 (B). *Lane M* = Molecular weight marker, 100 bp. *Lanes 1* to *17* = DNA of *Colletotrichum gloeosporioides* (4626, 4627, 4628, 4629, 4894, 4896, 4900, 4905, 4908, 4852, 4854, 4856, 4857, 4858, 4859, Ci, and PI13). *Lanes 18* and *19* = DNA of *C. acutatum* (PI15 and Acu, respectively). *Lane 20* = DNA of *C. sublineolum* (Sub).

The digestion of amplification fragments of the *pel*B gene of isolates of *C. gloeosporioides*, *C. acutatum* and *C. sublineolum* (Figure 6B) with the enzyme *Msp*I yielded two fragments of 350 bp and another of 250 bp for all isolates, not showing variation in sequence among the isolates of *C. gloeosporioides* or between the species *C. acutatum* and *C. sublineolum*.

DISCUSSION

The results show that the use of species-specific primers confirmed the identity of *C. gloeosporioides* isolates stored in the fungal collection-RMU and identified by classical methodology.

The primer CgInt, which is part of the ITS1, designed to identify isolates of *C. gloeosporioides*, was used with ITS4 by Mills et al. (1992) and Freeman et al. (2000). These studies resulted in the formation of 450-bp fragments for the isolates of *C. gloeosporioides* analyzed, where these results were similar to those found here.

The same specific primers were used by Ureña-Padilla et al. (2002) to confirm the identification of *Colletotrichum* species from strawberry, which were analyzed to determine the disease etiology. Isolates were identified by PCR using the species-specific primers Cg/fInt1 or CaInt2 and ITS4 region of rDNA. All 176 isolates from strawberry were identified as *C. gloeosporioides* or *C. acutatum* by amplification of DNA fragment from 450 to 490 bp. The primers Cg/fInt1/ITS4 confirmed the identity of 132 isolates of *C. gloeosporioides* obtained from the strawberry crown, and the primers CaInt2/ITS4 confirmed the identity of 46 isolates of *C. acutatum* obtained from fruit rot. Six isolates of *C. fragariae* produced amplification products with the primer Cg/fInt1/ITS4.

The primers $(GTG)_5$, $(GACA)_4$ and M13 showed the intraspecific variability of isolates from different hosts of *C. gloeosporioides* and also distinguished the isolates of *C. acutatum* and *C. sublineolum*.

Indeed, the three ISSR primers used showed grouping of isolates of *C. gloeosporioides* from the same host, with the groups containing onion isolates, mango isolates (except isolate 4852 from the city of Garanhuns using the marker $(GTG)_5$ and cashew isolates, all of which originated from different cities in Pernambuco State.

In order to determine host specificity and genetic diversity of different isolates of *C. gloeosporioides*, Freeman and Shabi (1996) used the ISSR primers $(CAG)_5$, $(TGTC)_4$, $(GACA)_3$, and $(GACA)_4$ for genomic DNA amplification of *C. gloeosporioides* (65 isolates from avocado and 63 from almond) and obtained uniform fragment patterns for all almond isolates from different geographical positions of Israel and also found that almond isolates from the United States were distinct from those from Israel. Avocado isolates from Israel and from the United States were the most diverse. Talhinhas et al. (2005) analyzed isolates from the genus *Colletotrichum* obtained from olives (*Olea europea* L.) from Portugal, using the microsatellite primers $(CAC)_5$, $(CAG)_5$, $(CAG)_5$, $(GACG)_4$, $(GCA)_5$, $(TCC)_5$, and MR and showed intraspecific diversity in *C. acutatum* and three isolates of *C. gloeosporioides* from different hosts. Afanador-Kafuri et al. (2003) analyzed 95 species of the genus *Colletotrichum* isolated from pitomba (*Talisia esculenta* Radlk.), passion fruit (*Passiflora edulis* Sims.) and mango (*Mangifera indica* L.) using the primers $(CAG)_5$, $(AGG)_5$, $(GACA)_3$, and $(GACA)_4$, and there was indication that the species *C. acutatum* from pitomba showed little genetic variability, while *C. gloeosporioides* isolates from passion fruit and mango were more heterogeneous. Vila Nova (2004) analyzed the genetic variability of 15 isolates of *C. gloeosporioides*

using RAPD molecular markers and the ITS region of rDNA. In this study, isolates from onion (*Allium cepa* L.) included 5 from different regions of Pernambuco and 1 from Amazonas, and 9 isolates of the fungus obtained from different hosts. The author found a group of four isolates from onion plants in the dendrogram generated by profiles in the first technique and three of them in the second technique, showing greater homology between isolates from this host. Cavalcante (2005) used the RAPD technique and the ITS1 region of rDNA to analyze the genetic variability of *C. gloeosporioides* isolates, five isolates being from onion and 10 from mango trees of different regions of Pernambuco. In the dendrogram generated by the RAPD analysis, two groups were delineated separating all the mango tree isolates from those obtained in onion. Notably in this study, the isolates from onion and mango in different regions of Pernambuco also formed distinct groups when using the three ISSR primers. Vila Nova (2004) and Cavalcante (2005) using the RAPD technique showed a great genetic diversity among isolates of *C. gloeosporioides* from different hosts of Pernambuco. The results reported here show that clustering based on the host of isolates from different municipalities of Pernambuco State, reinforce the findings of other authors who suggest specialization of the pathogen *C. gloeosporioides* in relation to the host.

In this study, the presence of the *pe*lB gene was demonstrated in isolates of *C. gloeosporioides*, *C. acutatum* and *C. sublineolum* obtained from the hosts onion, cashew, mango, pepper, peach, cyclamen, and sorghum, and the restriction digestion of the amplified fragment with the enzyme *Msp*I did not show variation in the gene structure of these three species or between isolates of *C. gloeosporioides*.

C. gloeosporioides produces endopolygalacturonase (Prusky et al., 1989; Yakoby et al., 2000a), pectin lyase A (Templeton et al., 1994; Bowen et al., 1995), pectin methyl esterase (Ortega, 1996) and pectate lyase B (*pel*B) (Wattad et al., 1997) during the colonization of infected tissue in avocado. Additionally, it was reported that the species of *C. gloeosporioides* f. sp *malvae* produce *pel-1* and *pel-2* (Shih et al., 2000). So far, in the case of *C. gloeosporioides*, only the *pel*B gene was found as the true virulence factor, as demonstrated by the reduction in virulence after deletion of the *pel*B gene (Yakoby et al., 2001) in pathogen isolates of avocado. The *pel*B genes that encode pectate lyases have also been identified in other species. Lei et al. (1987) identified and characterized a *pel*B gene from *Erwinia carotovora*. The pectate lyase B of *E. carotovora* and *E. chrysanthemi* consist of 352 and 353 amino acids, respectively. The two proteins show 72% homology based on data from DNA sequence. Guo et al. (1995) isolated a *pel*B gene from *Fusarium solani* f. sp. *pisi*, which had 65% homology with pectate lyase A from *F. solani* f. sp. *pisi* species and no homology with other pectinolytic enzymes.

Studies of this gene were also performed by Prusky et al. (2001) who reported that *C. gloeosporioides* produces pectate lyase, which is a key factor in the development of disease virulence. During the growth of *C. gloeosporioides*, *C. acutatum* and *C. coccodes* in acidified medium of yeast extract, the fungus secretes ammonia and thereby increasing the pH. Pectate lyase secretion by *C. gloeosporioides* increased correspondingly as the pH in the medium increased. The *pel*B mutant *C. gloeosporioides* was able to increase ammonia accumulation and pH in the medium as in the wild type. *C. gloeosporioides* in avocado, *C. coccodes* in tomato and *C. acutatum* in apple (*Malus domestica* Borkh.) showed ammonia accumulation in the infected area, where the pH increased to 7.5 or 8 and the pectate lyase activity was optimum. In the non-host interactions, in which apples were inoculated with *C. gloeosporioides*, the addition of ammonia compounds significantly enhanced pathogenicity to levels similar to those caused by the

compatible interaction of *C. acutatum* and apple. The results therefore suggest the importance of ammonia secretion as a virulence factor, increasing the environmental pH and the pathogenicity of *Colletotrichum* species. The ability of *Colletotrichum* species to change pH at the infection site enhances virulence because ammonia secretion and the resulting increase in pH leads to a higher expression of *pel*B and hence a higher pectate lyase secretion (Yakoby et al., 2000b). Prusky et al. (2001) emphasize that the effect of environmental pH modulation in pectate lyase secretion could be used in programs of selective breeding of plants to control fruit deterioration.

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