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GENETIC DIVERSITY AMONG *Colletotrichum Sublineolum* PATHOTYPES ISOLATED FROM SORGHUM (*Sorghum bicolor*)

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ABSTRACT – Sorghum anthracnose, caused by *Colletotrichum sublineolum* is a serious disease in Brazil. The fungus is highly variable pathogenically with many physiological races. The present study was made to evaluate the usefulness of molecular methods (SDS-PAGE, RAPD, ARDRA and rDNA sequencing) for identifying *C. sublineolum* pathotypes isolated from sorghum. Although protein profile (SDS-PAGE) showed a relative low level of polymorphism, differences could be observed due to presence/absence of some polypeptides as well as to intensity of some bands among pathotypes. DNA profile of arbitrarily amplified sequences (RAPD) using sixteen random primers revealed a high degree of polymorphism while amplified rDNA (ITS region and 18S gene) digested with six different restriction enzymes (ARDRA) showed similar patterns. Nucleotide sequencing analysis of the complete ITS region and partial sequencing of 18S rRNA gene were useful for revealing genetic differences among the five pathotypes. In the present study, RAPD and rDNA sequencing were the most profitable methods for identifying *C. sublineolum* pathotypes.

Key words: *Colletotrichum sublineolum*, *Sorghum bicolor*, SDS-PAGE, RAPD, ARDRA, rDNA sequencing

DIVERSIDADE GENÉTICA ENTRE PATÓTIPOS DE *Colletotrichum Sublineolum* ISOLADOS DO SORGO (*Sorghum bicolor*)

RESUMO - A antracnose do sorgo, causada por *Colletotrichum sublineolum,* é séria doença, no Brasil. O fungo é altamente variável do ponto de vista patogênico e várias raças têm sido noticiadas. Este estudo foi realizado com o objetivo de avaliar a utilidade de métodos moleculares (SDS-PAGE, RAPD, ARDRA e seqüenciamento de rDNA) para identificar patótipos de *C. sublineolum* isolados do sorgo. Embora o perfil protéico (SDS-PAGE) tenha mostrado baixo grau de polimorfismo, foi possível observar diferenças devido à presença/ausência de alguns polipeptídios, bem como diferenças quanto à intensidade de algumas bandas. O perfil de DNA amplificado arbitrariamente (RAPD), usando dezeseis primers aleatórios, revelou elevado grau de polimorfismo entre os

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patótipos, enquanto que o produto da amplificação de rDNA (região ITS e gene 18S), digerido com seis enzimas de restrição diferentes (ARDRA), apresentou padrão similar. Análise de nucleotídeos da seqüência completa da região ITS e do seqüenciamento parcial do gene 18S rRNA revelaram diferenças genéticas entre os cinco patótipos. Entre os métodos testados no presente estudo, RAPD e seqüenciamento de rDNA foram mais efetivos para identificar patótipos de *C. sublineolum*.

Palavras-chave: *Colletotrichum sublineolum*, *Sorghum bicolor*, SDS-PAGE, RAPD, ARDRA, seqüenciamento de rDNA

Sorghum bicolor [(L.) Moench] has been one of the most important crops in arid and semi-arid regions of the world for more than three thousand years (Doggett, 1976). Nowadays, this crop is essential for human life on marginal lands along the poorest regions of the world (Frederiksen, 1986; ICRISAT, 1976). In the last few decades the importance of sorghum has increased as a feed crop that can be grown as part of sustainable agroecosystem (ICRISAT, 1976). However, diseases are serious problem limiting sorghum expansion to the new agricultural frontiers particularly in hot semi-arid tropical environments (ICRISAT, 1976). Sorghum anthracnose caused by the fungus *Colletotrichum sublineolum* (Henn.) is a serious epidemic disease in many countries. *C. sublineolum* pathotypes are highly variable and may change even while attempts are being made for classifying its virulence or efforts are being made for controlling the pathogen in the field (Casela *et al*., 1995; 2001). Whether pathogen changes are due to genetic, physiological or environmental events, they must be investigated. The development of efficient tools for promptly identifying new pathogen genotypes would help researchers follow the shift in genetic make-up of the pathogen population, thus providing a dynamic picture of the interactions between the host and pathogen genotypes.

Brazil is a tropical country with a Continental dimension and highly heterogeneous environment. Many physiological races of C. *sublineolum* infecting sorghum have been identified in Brazilian fields and new races appear every year (Casela & Frederiksen, 1994; Casela *et al*., 2001). In addition, phenotypic and physiological differentiation among *C. sublineolum* pathotypes are not always straightforward methods (Casela, 1992; Frederiksen *et al*., 1995; Pande *et al*., 1991). These effectively turn the breeding for new resistant varieties a fastidious compromise (Mek & Warren 1992; Guthrie *et al*., 1992). The knowledge concerning genetic diversity of *C. sublineolum* populations is crucial for disease management and for establishing the relationship among environmental variations and changes in pathotype aggressiveness (Casela, 1992; Casela *et al*., 2001; Wise *et al*., 1995). Even so, attempts are being made to define alternative protocols based on molecular techniques for quick and efficient identification of *C. sublineolum* pathotypes to be used as tool for helping phytopathologists achieve this objective.

Molecular methods based on protein profile (SDS-PAGE) and DNA analysis (RAPD and rDNA sequencing), have been widely used to address taxonomic tasks and genetic variation in plant pathogens (Amann *et al*., 1996; Clarkson,

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1992; Di Cello *et al.*, 1997; Gürtler & Stanisich, 1996; Khachatourians, 1996; Lane, 1991; Lane *et al*., 1985; Weisburg *et al.*, 1991). Protein markers (SDS-PAGE) are based in pattern of gene expression and, in many cases are able to reveal differences in gene products and gene expression among individuals. The random amplified polymorphic DNA (RAPD) is a powerful technique for fingerprinting individual organism and for estimating similarity within and between populations and species. (Bruijn *et al*., 1996; Guthrie *et al*., 1992; Honeycutt *et al*., 1995; Vilgalys *et al*., 1994). RAPD has been successfully used for addressing genetic variation in plant pathogens (Carbone & Kohn, 1993; Chen, 1992; Vaillancourt & Hanau, 1992; Welsh & McClelland, 1990; Williams *et al.,* 1990).

In the last two decades, ribosomal RNA genes have been chosen for taxonomic and phylogenetic studies in many groups of organisms. In a single cell, rRNA genes are present in multiple copies containing variable and conserved regions. Since ribosomes are ubiquitous in living organisms, rDNA analysis allows comparisons between widely different taxonomic groups and provides valuable information for phylogenetic studies examining relationships between higher taxa (Gerbi, 1986; Herion *et al*., 1992; Hibbett & Dixon, 1991; Hibbett, 1992; Lee & Taylor, 1992; O'Donnell, 1992). However, DNA sequencing is a laborious and expensive proposition if a great number of different related organisms have to be studied. Alternatively, restriction analysis of the intergenic spacer region of rDNA repeats (ARDRA) is a quick and inexpensive method and has been useful for examining genetic variability of multiple pathotypes of the same species or related species. ARDRA was successfully applied for strain identification in *Fusarium oxysporum* (Alves-Santos *et al*., 1999; Appel & Gordon, 1995; 1996; Chakrabarti *et al*., 2001; Latha *et al*., 2003), *Pyrenophora graminea* (Pecchia *et al*., 1998; 2004) and other eukariotic species (Clark *et al*., 1995; Medlin *et al*., 1988).

In the present study, molecular techniques based on protein (SDS-PAGE) and DNA markers (RAPD, ARDRA, rDNA sequencing) were applied for evaluating their usefulness for identifying *C. sublineolum* pathotypes isolated from sorghum cultivated in Brazilian fields. To achieve this, single spores of five *C. sublineolum* previously characterized as pathotypes 30C, 15E, 15C, 31B, and 29A (Casela & Ferreira, unpublished results) were used. Pathotypes were collected from Capinópolis (30C), Cravinhos (15E and 15C) and Sete Lagoas (31B and 29A) in fields of cultivated sorghum showing symptoms of anthracnose. After single spore isolation, each pathotype was cultured in a flask containing 250 mL of BCYE (4% L-Cysteine, 11.5 g Yeast Extract, 1.5 g charcoal activated, 6g ACES buffer, 1g a-ketoglutarate, 2.5% ferric pyrophosphate, 17g agar. L^{-1}, pH 6.8) medium for 72 h at 25 ^{o}C. Mycelial mass was harvested, washed three times with distilled water and briefly dried in a sterile filter paper. For long-term storage, fungus mycelia were harvested from Petri dishes and maintained in sterilized mineral oil at –80 ^{o}C.

SDS-PAGE was performed according to Laemmli (1970) and Jackman (1985). Freshly cultured mycelium was washed three times with 10 mL of TE buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA) and stored for several months at –80 ^{o}C until used. Mycelial mass (200 mg) of each isolate were powdered with liquid nitrogen, using a pestle and a mortar, and transferred to a 1.5 mL microcentrifuge tubes containing 100 mL of sample buffer (62.5 mM Tris-HCl pH 8.0, 20%

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(v/v) glycerol, 2% SDS, 5% b-mercaptoethanol, 0.02% bromophenol blue). Samples were heated for 10 minutes in boiled water and immediately placed on ice for five minutes. In the next step, samples were centrifuged for 10 min at 3000 rpm and 15 mL of supernatant were loaded onto a 12% acrylamide gel. Electrophoresis was performed in 10% Tris-Glicine buffer (0.025 M Tris base, 0.192 M glicine, 0.1% SDS pH 6,8) with Protean II minigel electrophoretic system (Bio-Rad Laboratories, Mississagua, ON) at 60V for 1h. After electrophoresis, protein were visualized by coomassie blue staining method (Laemmli, 1970) and photographed with Eagle Eye System (Stratagene, La Jolla, CA).

Genomic DNA extraction was performed by a modified extraction protocol (Guthrie *et al.*, 1992). Briefly, 500 mg of dried mycelial mass of each isolate were powered in liquid nitrogen using a mortar and pestle, and transferred to 50 mL propilene tubes containing 5 mL of extraction buffer (0.1 M Tris-HCl pH 8.0; 0.2 M NaCl; 0.02 M EDTA; 1,0% SDS; 0.1% b-mercaptoethanol). Each tube was vigorously agitated for obtaining a uniform suspension without lumps and then incubated for 15 min at room temperature. Afterwards, an equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added to each sample, vigorously agitated, and incubated 10 min at room temperature. Cell debris was removed by centrifugation at 4 ºC with 8000 rpm for 10 minutes. Aliquots of 5 mL of the supernatant layer were transferred to a 50 mL propilene tube and an equal volume of ice cold ethanol was added to each sample and gently inverted several times to precipitate nucleic acids. Nucleic acids were spooled out on a glass hook, briefly washed in 70% ethanol and dissolved with 0,5 mL of TE buffer containing 40 mg/mL RNAse H. The quality of DNA was checked by spectrophotometer measures (OD 260/280) and by electrophoresis in 1% agarose gel stained with ethidium bromide. The final concentration of DNA was adjusted to 25 ng/mL.

RAPD reaction was performed in a final volume of 25 mL containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM of each deoxynucleotide triphosphate, 0.4 mM primer, 1 U of Taq DNA polymerase (*Phoneutria*, Belo Horizonte, Brazil) and 25 ng of DNA template. The following sixteen 10-mer primers (Operon Technologies, Alameda, CA) were used: OPA 5; OPA 6; OPA 8; OPA 12; OPA 14; OPA 15; OPA 17; OPA 18; OPA 19; OPA 20; OPT 1; OPT 2; OPT 3; OPT 4; OPT 5; OPT 6. DNA amplifications were made in thermocycler (PTC-100, MJ Research MS, USA) programmed for an initial melt of 1min for denaturation at 94 ºC, followed by 30 cycles of 94 ºC for 15 s; 35 ºC for 30 s (annealing); 72 ºC for 30 s (extension). An additional step at 72 ºC for 10 min was performed in the final of 30 cycles and fast ramp was applied in all steps. Control tubes containing master mix without template DNA were included in each run and reactions were repeated at least three times to check the reproducibility of amplified DNA (Dassanayake & Samaranayake, 2000). The PCR products were electrophoresed in agarose gel (1.2%) immersed in 0.5 TBE buffer (89 mM Tris-borate, 2.5 mM EDTA, pH 8.0) containing 10 mg/mL of ethidium bromide. The gel was run for 4 h with an initial step of 15 min at 100 V followed by 75 V, and visualized with UV light and photographed with Eagle Eye System (Stratagene, La Jolla, CA).

Amplifications of ribosomal RNA genes (rDNA) were performed using universal primers for ITS region (Internal Transcribed Spacer) and for 18S rRNA gene. All primers represent conserved regions of fungi species

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(Bruns *et al*, 1992; Gargas & DePriest, 1996; White *et al*., 1990). The ITS DNA was amplified with primers ITS1 (forward) (5'-TCCGTAGGTGAACCTGCGG-3') complementary to 18S gene and ITS4 (reverse) (5'-TCCTCCGCTTATTGATATGC-3') that anneal with the 28S gene (Gargas & DePriest, 1996; Hibbett, 1992). The 18S rRNA gene was amplified by using NS1F (5'-GTAGTCATATGCTTGTCTC-3') and NS8R (5'-TCCGCAGGTTCACCTACGGA-3') primers (Gargas & DePriest, 1996; Hibbett, 1992). PCR reactions were made with 10-20 ng of *C. sublineolum* genomic DNA plus 2.5 mL 10X PCR buffer (200 mM Tris-HCl (pH 8.4) and 500 mM KCl), 2.0 mM of each primer, 25 mM dNTP, 2,5 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase (*Phoneutria*, Belo Horizonte, Brazil) in a total volume of 25 mL. Thermocycling was performed in a model PTC-100 thermalcycler machine (MJ Research, MS, USA) with the following conditions: one cycle for denaturation of DNA samples at 94 °C for 1 min, 30 cycles of 1 min at 94 °C, 1 min at 50 °C (annealing) and 2 min at 72 °C (extension). Finally, reactions were incubated for 10 min at 72 °C. Control tubes containing master mix without template DNA were included in each run and reproducibility was checked out by replicas for each reaction. Aliquots of 500 ng of ITS and 18S rDNA amplified products were digested for 4 h with 5 U of tetrameric (*Alu* I, *Mbo* I, *Msp* I and *Sau*3A I) and hexameric (*Eco*RI) restriction endonucleases (Invitrogen™, Carlsbad, CA) containing the appropriated buffer. DNA digests were analyzed by horizontal gel electrophoresis at 6 V/cm^2 in 1.5 % agarose gel (wt/v) for ITS or 1.0 % agarose gel for 18S in 1X TAE buffer (0.04M Tris-acetate, 0.001M EDTA, pH 8.0) containing ethidium bromide (0.5 mg/L). Gels were visualized under UV light, photographed and the fingerprints were compared visually with the overview gels. Alternatively, digested PCR products of ITS region were resolved in 10% poliacrylamide gel and silver stained (data not shown).

Sequencing reactions of rDNA (ITS and 18S) were performed in a total volume of 25 ml containing 200-300 ng of amplified rDNA, 20 pmol of primer (ITS1, ITS4, NS1F, or NS8R) and 8.0 mL reaction premix (Applied Biosystems, Lincoln Centre Drive Foster City, USA). Reaction conditions were established with an initial step of DNA denaturation at 96 °C for 30 s, followed by 25 cycles of 30 s at 96 °C, annealing for 15 s at 50 °C and extension for 4 min at 60 °C. The reaction products were precipitated with 2,5 mL of 3 M sodium acetate, pH 4.6 plus 50 mL 95% cold ethanol for 10 min on ice, centrifuged for 30 min at 16000 rpm, and washed with 250 mL of 70% ethanol. Sequencing was performed in an automatic sequencer (ABI-377, Applied Biosystems, Lincoln Centre Drive Foster City, USA) and repeated at least three times. *C. sublineolum* rDNA sequences generated in the present study were deposited in Genbank database and their respectively accession numbers are shown in Table 1.

DNA patterns of RAPD were recorded in a binary form (presence or absence) for constructing a dendrogram. Amplified fragments lower than 200 bp and faint bands were neglected. Dendrogram was constructed from the distance matrix by using the unweighted-pair group method with arithmetic mean (UPGMA). Both procedures were done with the Statistica software version 4.2 for windows (StatSoft, Inc. USA). DNA sequences of 18S rRNA gene and internal transcribed spacer region of 18-28S of *C. sublineolum*, as well as other fungi sequences

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TABLE 1. GenBank accession numbers generated by DNA sequencing of ITS region spacer, 5.8S rRNA and partial sequencing of 5´- and 3´- end of 18S rRNA gene of five *C. sublineolum* pathotypes isolated from Brazilian sorghum.

C. sublineolum **pathotypes**	**Primers* and annealing position**	**rDNA Sequences**	**Accession numbers**
30C	NS1F (18S 5'- end)	5´- partial 18S	AY622774
30C	NS8R (18S 3'- end)	3´- partial 18S	AY622779
30C	ITS1 (18S 3'- end) and ITS4 (28S 5' end)	ITS region **	AY622784
15E	NS1F (18S 5'- end)	5´- partial 18S	AY622775
15E	NS8R (18S 3'- end)	3´- partial 18S	AY622780
15E	ITS1 (18S 3'- end) and ITS4 (28S 5'- end)	ITS region **	AY622785
15C	NS1F (18S 5' end)	5´- partial 18S	AY618464
15C	NSR8 (18S 3'- end)	3´- partial 18S	AY622778
15C	ITS1 (18S 3'- end) and ITS4 (28S 5'- end)	ITS region **	AY622783
31B	NS1F (18S 5'- end)	5´- partial 18S	AY618463
31B	NS8R (18S 3'- end)	3´- partial 18S	AY622777
31B	ITS1 (18S 3'- end) and ITS4 (28S 5'- end)	ITS region **	AY622782
29A	NS1F (18S 5'- end)	5´- partial 18S	AY618462
29A	NS8R (18S 3' end)	3´- partial 18S	AY622776
29A	ITS1 (18S 3'- end) and ITS4 (28S 5'- end)	ITS region **	AY622781

* Oligonucleotide primer sequences were described in material and methods.
** Internal spacers and 5.8S rRNA gene

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available in the GenBank database were used for comparison (data not shown). rDNA sequences were aligned using the CLUSTAL W Multiple Sequences Alignment program (Thompson *et al*., 1994) and dendrograms were constructed from the distance matrix by using the unweighted-pair group method with arithmetic mean (UPGMA). Both procedures were done with software Statistica, version 4.2 for windows (StatSoft, Inc. USA).

SDS-PAGE of total protein extract from mycelial mass of five *C. sublineolum* pathotypes showed a relative homogenous profile and most of the bands were shared by all pathotypes. The main differences among pathotypes were commonly related to intensity of certain bands (Figure 1). Although the existing differences allow discriminating each of the five *C. sublineolum* phenotype, it was stated that total

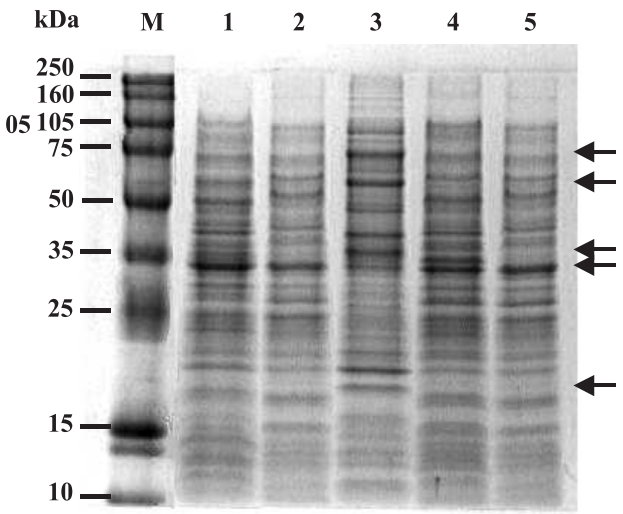

FIGURE 1. Electrophoretical profile (SDS-PAGE) of total protein extract from mycelial mass of five *C. sublineolum* pathotypes. Some differences observed between pathotypes were indicated by arrows. M = molecular marker (Rainbow Molecular Weight Markers, New England Biolabs) while numbers indicate the pathotypes 29A, 31B, 15C, 30C and 15E, respectively.

protein profile may not be an effective method for fingerprinting when a high number of pathotypes must be identified. Nevertheless, different results were observed with random amplified polymorphic DNA (RAPD). After previous screening, sixteen random primers were selected to assess genetic diversity among the five pathotypes. A high level of DNA polymorphism was detected, yielding a typical genomic fingerprint for each isolate. Fragment sizes of amplified DNA ranged from 0.2 to 2.5 kb and some bands were unique while others were present in all pathotypes (Figure 2). The number of polymorphic bands scored per primer varied from 5 to 8 and a total of 88 bands were used for cluster analysis. UPGMA algorithm generated a dendrogram with the five *C. sublineolum* pathotypes been clustered in two groups. Although the pathotypes from Cravinhos (15E and 15C) and Sete Lagoas (29A and 31B) were separated, the pathotype from Capinópolis (30C) was grouped with the Sete Lagoas pathotypes (Figure 3). In a recently study applying RAPD and RFLP-PCR markers for identifying

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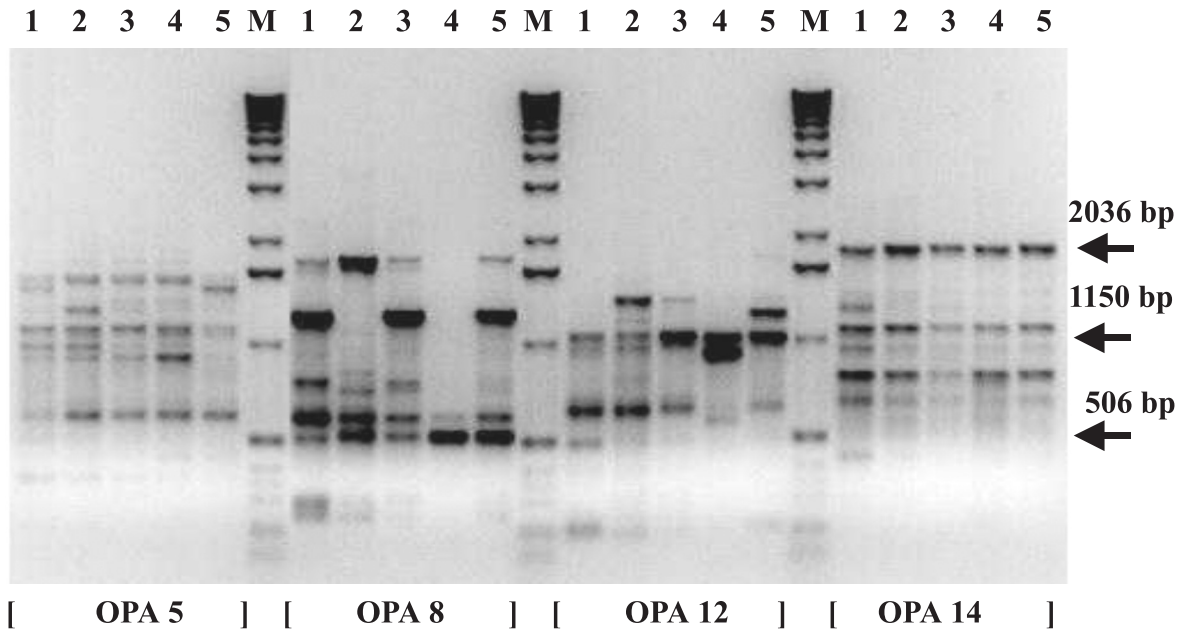

FIGURE 2. RAPD amplified products of five *Colletotrichum sublineolum* pathotypes resolved in an 1,2% agarose gel. M = 1 kb molecular size marker Ladder (Invitrogen™, Carlsbad, CA). Lanes 1 through 5, RAPD patterns of fungus pathotypes 29A, 31B, 15C, 30C and 15E, respectively. Random primers (OPERON™) are indicated bellow the figure.

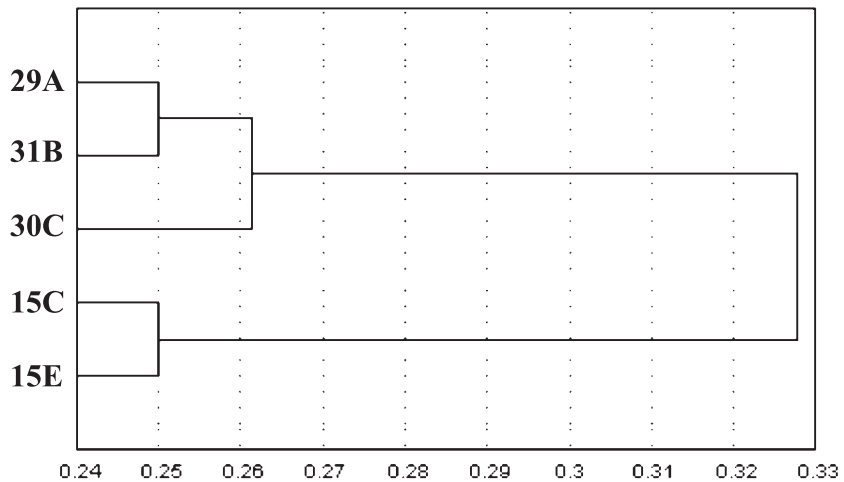

FIGURE 3. Dendrogram showing genetic relationships among five *C. sublineolum* pathotypes, based on RAPD patterns produced with sixteen random primers (OPERON, Technologies). The dendrogram was constructed from the distance matrix by using the unweighted-pair group method with arithmetic mean (UPGMA). Both procedures were done with the Statistica software for windows, version 4.2 (StatSoft, Inc. USA). Scale bar indicates dissimilarity between the five different pathotypes in percentage.

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Colletotrichum sublineolum isolated from four geographically distinct regions of Brazil revealed a high level of polymorphism for RAPD markers and all pathotypes were grouped according to place they were collected (Valério *et al*., 2005).

Amplified products of ITS region (560 bp) and 18S rRNA gene (2.0 kb) of the five *C. sublineolum* pathotypes were digested with five different restriction enzymes (Figure 4). ARDRA profile was very poor and little information about

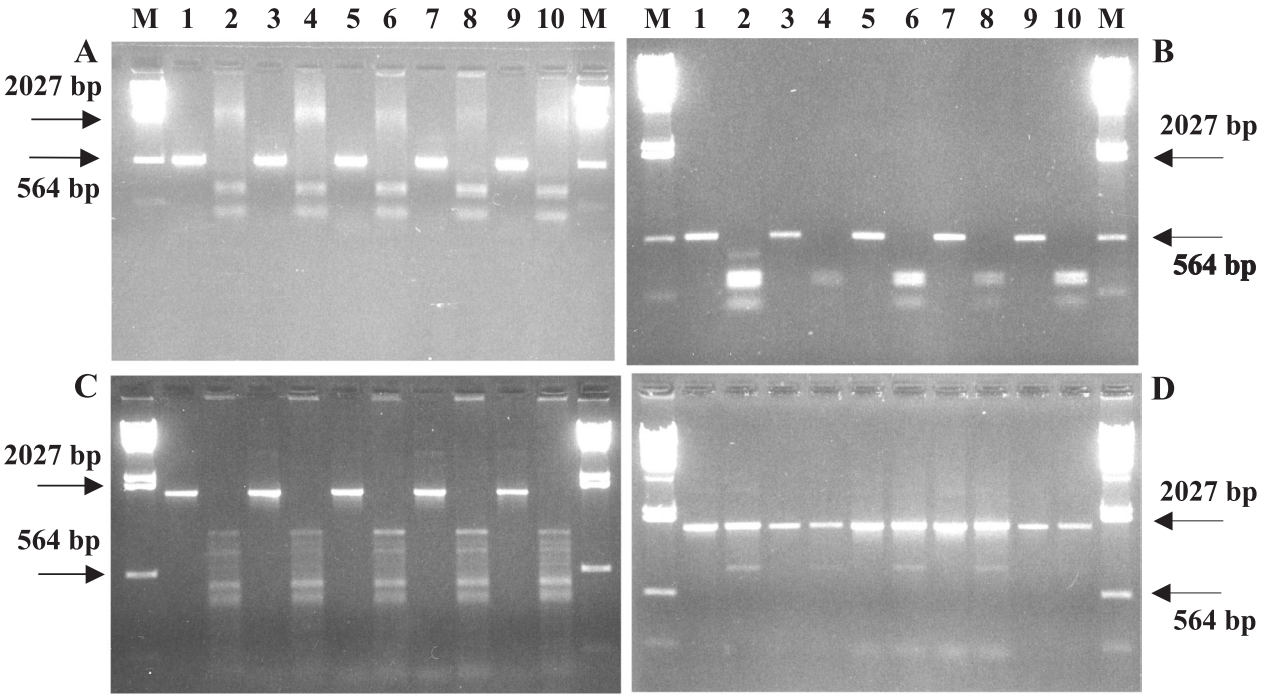

FIGURE 4. Agarose gel electrophoresis of PCR-amplified rDNA products from the internal spacer region (A and B) and 18S rRNA gene (C and D) of five *Colletotrichum sublineolum* pathotypes digested with endonucleases *Sau*3A I (A and C), *Mbo* I (B), and *Eco*R I (D). Lanes M, lambda *Hin*d III molecular size marker ladder (Invitrogen™, Carlsbad, CA) . Lanes 1, 3, 5, 7 and 9 contain non-digested PCR products and lanes 2, 4, 6, 8 and 10 are ARDRA patterns of pathotypes 29A, 31B, 15C, 30C and 15E, respectively.

pathotype identity could be obtained. Similar patterns of restricted DNA were observed for *Alu* I and *Msp* I (data not shown) and *Sau*3A I restriction enzymes (Figure 4). *Mbo* I and *Eco*R I restriction patterns were very similar and they were not effective to discriminate the five *C. sublineolum* pathotypes (Figure 4). In another study, RFLP over ITS domains and 5.8 rDNA genes of *C. sublineolum* did not show differences among the isolates (Valério *et al*., 2005). Although the high level of nucleotide conservation for restriction sites observed for ITS region, 5.8S rDNA and 18S rDNA genes of *C. sublineolum* in the present study, and for twelve restriction enzymes over ITS domain and 5.8S rDNA genes in another study (Valério *et al*., 2005), ARDRA profiles have been successfully used for species and strain

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differentiation in many fungi, prokaryotes and other eukaryotic species (Alves-Santos *et al*., 1999; Appel & Gordon, 1995; 1996; Atkins *et al*., 2005; Chakrabarti *et al*., 2001; Clark & Pung, 1994; Clark *et al*., 1995; Latha *et al*. 2003; Pecchia *et al*., 1998; 2004). Although ARDRA results in the present study and RFLP analysis of rDNA genes (Valério *et al*., 2005) were not suitable for genotiping *C. sublineolum* isolates, screening for RFLP variation using anonymous sized DNA fragments revealed a high level of variation at the DNA level in this specie (Rosewich *et al*., 1998).

PCR-amplified products comprising the ITS region of rDNA and both, 5´- and 3´- ends of 18S ribosomal gene of the five C. *sublineolum* pathotypes were sequenced and aligned using CLUSTAL W program (Thompson *et al*., 1994). Nucleotide sequences of ITS region generated by ITS1 (forward) and ITS4 (reverse) primers were overlapped and the entire ITS region with 5.8S ribosomal gene of the five *C. sublineolum* pathotypes comprising 560 base pairs in length could be determined. Nucleotide sequencing alignment of ITS region of the five *C. sublineolum* pathotypes revealed a high degree of similarity, and of the 560 bp only 53 nucleotides were different (data not shown). In spite of the high level of nucleotide conservation for ITS region, the five *C. sublineolum* pathotypes could be clearly separated in five different races.

PCR-amplified 18S rRNA gene of the five *C. sublineolum* pathotypes were approximately 2,0 kb in length without detectable polymorphism. NS1F and NS8R primers were used for partial sequencing the 18S rRNA gene and sequence alignments of 5'- and 3´- end of 18S gene were useful for identifying each C. *sublineolum* pathotypes. Nucleotide comparison between the two 18S partial sequences of each isolate showed that 18NS8R sequence was more variable than 18NS1F sequence (data not shown). In addition, both 18S partial sequences (5'- and 3'- end) were more polymorphic than sequences obtained for ITS region.

Nucleotide sequence divergence for ITS region and 18S partial sequences (NS1F and NS8R) was estimated using the Jaccard method. A dendrogram was constructed from the distance matrix by using the UPGMA. Both procedures were done with the Statistica software, version 4.2 for windows (StatSoft, Inc. USA). Pathotypes from Sete Lagoas and Cravinhos were grouped in the same branch of dendrogram while the pathotype 30C, collected from Capinópolis, was separated from the others (Figure 5).

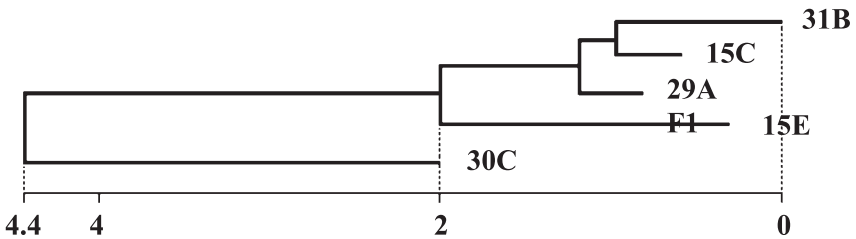

FIGURE 5. Dendrogram showing genetic relationships among *C. sublineolum* pathotypes, based on rDNA sequencing of ITS region and 18S gene. The dendrogram was constructed from the distance matrix by using the unweighted-pair group method with arithmetic mean (UPGMA). Both procedures were done with the Statistica software for windows, version 4.2 (StatSoft, Inc. USA). Scale bar indicates dissimilarity between the five different pathotypes in percentage.

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Dendrograms generated by RAPD (Figure 3) and rDNA sequencing approaches (Figure 5) are not comparable since clustering obtained by one method was disrupted when another method was used. Thus, results concerning genetic relationships among the five *C. sublineolum* pathotypes based in RAPD and rDNA sequencing analysis might be interpreted with caution. In the other hand, bootstrap analysis showed that 38% of RAPD scored DNA bands and 46 % of nucleotide differences of rDNA were sufficient to produce nearly 98 % of trustworthiness (data not shown).

In conclusion, among different molecular techniques (SDS-PAGE, RAPD, ARDRA and rDNA sequencing analysis) used in the present study for *C sublineolum* identification, RAPD was the most reliable technique for revealing genetic diversity among *C. sublineolum* pathotypes while direct comparison of nucleotide sequences of amplified rDNA was the most informative technique and thus, the best method for determining taxonomic relationships among *C. sublineolum* pathotypes.

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