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Use of molecular markers to compare *Fusarium verticillioides* pathogenic strains isolated from plants and humans

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ABSTRACT. *Fusarium verticillioides* is a pathogen of agriculturally important crops, especially maize. It is considered one of the most important pathogens responsible for fumonisin contamination of food products, which causes severe, chronic, and acute intoxication in humans and animals. Moreover, it is recognized as a cause of localized infections in immunocompetent patients and disseminated infections among severely immunosuppressed patients. Several molecular tools have been used to analyze the intraspecific variability of fungi. The objective of this study was to use molecular markers to compare pathogenic isolates of *F. verticillioides* and isolates of the same species obtained from clinical samples of patients with *Fusarium* mycoses. The molecular markers that we used were inter-simple sequence repeat markers (primers GTG_5 and $GACA_4$), intron splice site primer (primer EI1), random amplified polymorphic DNA marker (primer OPW-6), and restriction fragment length polymorphism-internal

transcribed spacer (ITS) from rDNA. From the data obtained, clusters were generated based on the UPGMA clustering method. The amplification products obtained using primers ITS4 and ITS5 and loci ITS1-5.8-ITS2 of the rDNA yielded fragments of approximately 600 bp for all the isolates. Digestion of the ITS region fragment using restriction enzymes such as *Eco*RI, *Dra*I, *Bsh*I, *Alu*I, *Hae*III, *Hin*fI, *Msp*I, and *Pst*I did not permit differentiation among pathogenic and clinical isolates. The inter-simple sequence repeat, intron splice site primer, and random amplified polymorphic DNA markers presented high genetic homogeneity among clinical isolates in contrast to the high variability found among the phytopathogenic isolates of *F. verticillioides*.

Key words: *Fusarium verticillioides*; ISSR; Intron; RAPD

INTRODUCTION

Corn (*Zea mays* L.) is a crop of great socio-economic significance in Brazil. Every year, the country produces about 50 million tons of grain. In fact, in 2008, corn production reached more than 59 million tons, with the State of Paraná accounting for 26.5% of it (Ramos, 2008; IBGE, 2009).

Fusarium verticillioides Sacc. Nirenberg (= *Fusarium moniliforme* Sheldon) is a non-obligate parasite, which infects important crops such as sorghum, sugarcane, and maize. It occurs worldwide, both in the soils of tropical and subtropical regions, as well as humid and sub-humid temperate zones (Figueira, 2003; Meirelles, 2005; Bernd, 2006). *F. verticillioides* is probably the most common pathogen of maize crops throughout the world - it causes stalk, root, ear, and kernel rot, and seedling blight (Meirelles, 2005). This pathogen may cause damage during all stages of plant development by infecting the roots, stem, and grain, although in most cases, the infection is asymptomatic and can be characterized as an endophytic relationship (Pamphile and Azevedo, 2002; Mirete et al., 2004; Sartori et al., 2004; Bacon et al., 2008).

F. verticillioides has also been reported to be the etiological agent of superficial and disseminated infections in humans. Fungal infections are considered relevant, especially in immunosuppressed patients, and are frequently associated with high morbidity and mortality (Pamphile and Azevedo, 2002; Mirete et al., 2004; Sartori et al., 2004; Tezcan et al., 2009).

F. verticillioides has the ability to produce several types of mycotoxins, including moniliformin, fusariocin, fusarona C, fusaric acid, and fumonisin, and is considered to be one of the most important global sources of contamination in food products that are derived from maize and other types of grains (Marasas et al., 1986; Bacon and Hinton, 1996; Mirete et al., 2004).

Taxonomically, *F. verticillioides* belongs to the *Liseola* section of the *Gibberella fujikuroi* (Sawada) Wolenw. complex, which is a teleomorph that is associated with *Fusarium* isolates of this section. It is subdivided into at least 8 genetically distinct biological species or mating populations and is identified by the letters A to H. The mating populations A and D produce high levels of mycotoxins, while the others produce few or none (Schiabel, 2004; Meirelles, 2005). *F. verticillioides* corresponds to the biological population A. Patiño et al. (2006) demonstrated that taxonomic studies on this complex are highly controversial and that the complex totals approximately 36 species using only morphological tools.

Several techniques have been used to assess the intraspecific variability in *F. verticillioides*: restriction fragment length polymorphism-internal transcribed spacer (RFLP-ITS) from rDNA (Patiño et al., 2006; Dissanayake et al., 2009); random amplified polymorphic DNA (RAPD; Nagarajan et al., 2006; Singh et al., 2006; Bayraktar et al., 2008), inter-simple sequence repeat (ISSR; Luongo et al., 2007; Bayraktar et al., 2008); amplified fragment length polymorphism (AFLP; Reynoso et al., 2009); intron splice site primer (ISSP; Brasileiro et al., 2004).

Given the relevance of molecular methods in analyzing genetic variability, our study aimed to analyze and genetically compare *F. verticillioides* isolates that were obtained from plants and clinical samples using the RFLP of the ITS region, as well as ISSP (EI1), RAPD (OPW-6), and ISSR (GTG_5 and $GACA_4$) molecular markers.

MATERIAL AND METHODS

Fungal strains

Sixteen isolates of *F. verticillioides* (6 from clinical samples of patients with *Fusarium* mycoses of which 9 were phytopathogenic and 1 was endophytic) were provided by the Culture Collection, University of Recife - Mycology (URM) of the Mycology Department, Universidade Federal de Pernambuco (Table 1). *F. oxysporum* and *F. solani* strains were used as the outgroup for comparative analysis of the genetic variability of the 16 isolates.

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

Fusarium species	Accession No. (URM collection)	Substrate or host
F. verticillioides	URM5352	*Zea mays*
	URM3278	*Sorghum bicolor* L. Moench
	URM3007	Root of *Oryza sativa*
	URM5094	Endophytic of *Saccharum officinarum*
	URM3096	*Alibertia myrciifolia* leaf
	URM2388	*S. officinarum*
	URM2542	*Z. mays*
	URM2387	*S. officinarum*
	URM2495	*Sorghum bicolor* L. Moench
	URM2241	*S. officinarum*
	URM5390	Face lesion
	URM5284	Nose biopsy
	URM5354	Leg lesion
	URM4051	Nails
	URM5285	Face lesion
	FV	Face lesion
F. solani	URM/FS	Not advised
F. oxysporum	URM/FO	Not advised

Fungal growth and DNA extraction

Fusarium isolates were maintained in potato dextrose agar medium for 7 days at room temperature. The conidia of each isolate were suspended in 3 mL 0.1% Tween 80 (v/v; 10^6 conidia/mL) and transferred to Erlenmeyer flasks that contained 100 mL liquid potato dextrose agar medium. The flasks were shaken at 27 rpm for 120 h at 28°C to perpetuate fungal growth. Subsequently, the mycelia were collected by vacuum filtration and washed with distilled water that was autoclaved. The wet weights were determined and the samples were stored at -20°C.

DNA was extracted following the technique described by Kuramae-Izioka (1997) using liquid nitrogen until mycelia were completely pulverized. It was then transferred to microtubes that contained 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). After homogenization, the microtubes were incubated at 65°C for 30 min and gently shaken by inversion every 10 min. Next, 500 µL 5 M potassium acetate was added, followed by homogenization and centrifugation at 14,500 *g* for 10 min. Each supernatant was extracted with chloroform-isoamyl alcohol (24:1), and centrifugation was carried out at 14,500 *g* for 10 min. One volume of isopropanol was added to each recovered aqueous phase, and the mixture was cooled at 4°C for 3 h to precipitate the DNA. Then, DNA samples were centrifuged at 14,500 *g* for 10 min. The precipitates were washed with 70% ethanol, centrifuged for 10 min, dried at room temperature, resuspended in Tris-EDTA buffer, pH 8.0 (1 M Tris-HCl and 0.5 M EDTA), and stored in a freezer 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 a lambda phage DNA molecular weight marker (Invitrogen Life Technologies, Brasil). After electrophoresis, the gel was stained in ethidium bromide solution (1X TBE/0.5 µg/mL ethidium bromide; Sambrook et al., 1989) for 30 min, observed using an ultraviolet transilluminator, and photographed using a digital camera (effective resolution, 7.2 megapixels).

RFLP-ITS from rDNA

Amplification reactions were performed in a final volume of 25 µL that contained 1X Taq buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 mM each of ITS4 and ITS5 primers (Table 2), 0.04 U Taq DNA polymerase (Invitrogen Life Technologies), and 25 ng DNA, as described by White et al. (1990). A thermal cycler was used with the following schedule: initial denaturation at 95°C for 4 min; 40 cycles of 92°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and a final extension for 5 min at 72°C. Amplified products of the locus ITS1-5.8S-ITS2 of the rDNA were separated by electrophoresis on a 1.0% agarose gel at 3 V/cm in 1X TBE 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 by using an ultraviolet transilluminator, and photographed with a digital camera (effective resolution, 7.2 megapixels).

Table 2. Primers used to analyze the genetic diversity of *Fusarium* isolates.

Primer	Sequence (5'-3')	Molecular marker	Reference/Supplied
ITS4	TCCTCCGCTTATTGATATGC	ITS	White et al. (1990)
ITS5	GGAAGTAAAAGTCGTAACAA	ITS	White et al. (1990)
EI1	CTGGCTTGGTGTATGT	ISSP	de Barros Lopes et al. (1996)
$(GTG)_5$	GTGGTGGTGGTGGTG	ISSR	Lieckfeldt et al. (1993)
$(GACA)_4$	GACAGACAGACAGACA	ISSR	Meyer and Mitchel (1995)
OPW-6	AGGCCCGATG	RAPD	Operon Technologies, Inc.

Aliquots of 4 µL amplicons were subjected to enzymatic digestion with *Eco*RI, *Dra*I, and *Bsh*I, *Hae*III, *Msp*I, *Hin*fI, *Pst*I, and *Alu*I, separately, according to manufacturer instructions. Fragments were separated on 1.5% agarose gel (w/v) using a 100-bp molecular weight marker (Invitrogen Life Technologies)

ISSP and ISSR reactions

DNA samples were subjected to PCR with EI1, $(GTG)_5$, and $(GACA)_4$ primers (Table 2). For the first primer, amplification reactions were performed in a final volume of 25 µL under the following conditions: 1X buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 1.5 mM $MgCl_2$, 0.25 mM dNTP, 0.5 mM primer, 0.04 U Taq DNA polymerase (Invitrogen Life Technologies), and 25 ng DNA. The amplification proceeded as follows: an initial denaturation step at 94°C for 3 min; 40 cycles of 1 min at 94°C, 2 min at 45°C, 1 min and 30 s at 74°C; and a final extension of 5 min at 74°C. For the ISSR primers $(GTG)_5$ and $(GACA)_4$, amplification reactions were performed in a final volume of 25 µL under the following conditions: 1X buffer (20 mM Tris-HCl, pH 8.4 and 50 mM KCl), 0.75 mM $MgCl_2$, 0.25 mM dNTP, 0.25 mM primer, 0.04 U Taq DNA polymerase (Operon Technologies, Alameda, CA, USA), and 25 ng DNA. The amplification cycles consisted of an initial denaturation at 93°C for 5 min; 40 cycles of 20 s at 93°C, 45 s at 55°C, and 90 s at 72°C; and a final extension of 6 min at 72ºC. The 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). Then, the gel was stained in ethidium bromide solution for 30 min, visualized by using an ultraviolet transilluminator, and photographed with a digital camera (effective resolution, 7.2 megapixels).

RAPD reactions

Initially, a selection was made from 12 arbitrary oligonucleotide primers from Operon Technologies (OPW, OPA, and OPX kits) with the total DNA of the isolate URM3278 to amplification quality with each of them. From these, primer OPW-6 was selected (Table 2). The amplification reactions were conducted in a final volume of 25 µL under the following conditions: 1X buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 3.4 mM $MgCl_2$, 0.25 mM dNTP, 0.5 mM primer, 0.5 U Taq DNA polymerase (Operon Technologies), and 25 ng DNA. The amplification cycles consisted of an initial denaturation at 94°C for 5 min; 40 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C; and a final extension of 5 min at 72°C. The 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).

Statistical analysis

The data obtained were analyzed by the Numerical Taxonomy System of Multivariate Programs - NTSYSpc 2.1 (Rohlf, 1988; Bussab et al., 1990) using the Jaccard coefficient. A dendrogram was generated by using the UPGMA clustering method.

RESULTS

Amplification with primers ITS5 and ITS4 produced one fragment of approximately 600 bp in size for all the clinical and phytopathogenic *F. verticillioides*, *F. oxysporum*, and *F. solani* isolates (Figure 1).

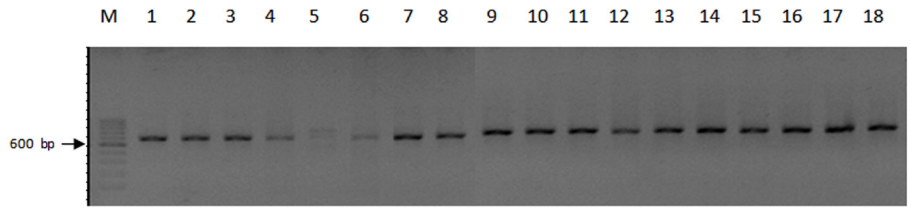

Figure 1. Profiles of amplification of rDNA-internal transcribed spacer (ITS) region of the *Fusarium* isolates obtained with ITS4 and ITS5 primers. *Lane M* = 100-bp ladder; *lanes 1-8* = phytopathogenic strains of *F. verticillioides*: URM5352, 3728, 3007, 5094, 3096, 2388, 2542, 2387; *lanes 9-14* = clinical strains of *F. verticillioides*: 5390, 5284, 5354, 4951, 5285, FV; *Lanes 15* and *16* = phytopathogenic strains of *F. verticillioides*: 2495, 2241; *lanes 17* and *18* = strains of *F. oxysporum* and *F. solani*.

RFLP-ITS from rDNA

Digestion with *Eco*RI produced two DNA fragments (possibly overlapping) of approximately 300 bp for all the isolates (Figure 2). No digestion was observed for *Dra*I (Figure 2). Digestion with *Alu*I resulted in fragments of approximately 450 and 150 bp for isolates 1, 2, 3, 5, 8, 11, 12, 13, 15, 16, and 17 and fragments of 150 and 380 bp for isolates 4, 6, 7, 14, 9, 10, and 18 (Figure 2). Digestion with *Hae*III produced fragments of 90, 180, and 300 bp for isolates 1, 2, 3, 5, 8, 11, 12, 13, 15, and 16; fragments 90, 180, and 380 bp for isolates 4, 6, 7, 14, 9, and 18; fragments of 90, 150, and 380 bp for isolate 10; and fragments of 180 and 280 bp for isolate 17 (Figure 2). Digestion with *Hin*fI produced two fragments (possibly overlapping) of 300 bp for isolates 1, 2, 3, 8, 11, 12, 13, 14, 15, and 16; fragments of 100, 200, and 300 bp for isolates 4, 5, 6, 7, 9, 10, and 18; and fragments of 280 and 300 bp for isolate 17 (Figure 3). Digestion with *Msp*I resulted in fragments of 120, 180, and 280 bp for isolates 1, 2, 3, 5, 8, 11, 12, 13, 15, and 16; fragments of 100 and 500 bp for isolates 4, 6, 7, 14, 9, 10, and 18; and fragments of 400 and 200 bp for isolate 17 (Figure 3). The restriction profile for *Pst*I produced fragments of 150 and 450 bp for isolates 1, 2, 3, 5, 8, 11, 12, 13, 15, and 16 and fragments of 500 bp for isolates 4, 6, 7, 14, 9, 10, 18, and 17 (Figure 3).

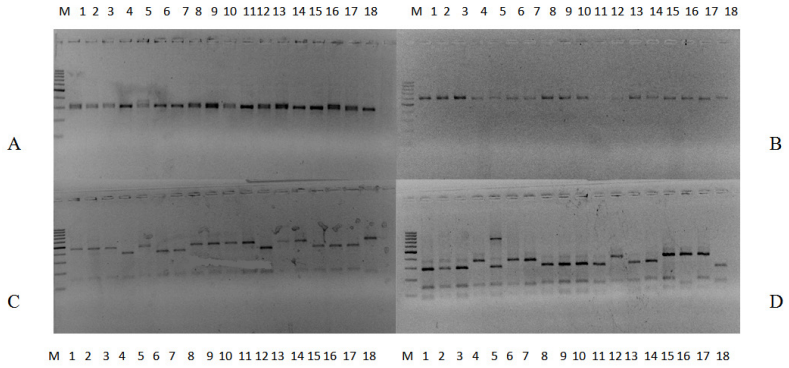

Figure 2. Restriction profiles of the internal transcribed spacer region of the rDNA of *Fusarium verticillioides*, *F. oxysporum*, and *F. solani* isolates with *Eco*RI (A), *Dra*I (B), *Alu*I (C), *Hae*III (D) enzymes. *Lane M* = 100-bp ladder; *lanes 1-8* = phytopathogenic strains of *F. verticillioides*: URM5352, 3728, 3007, 5094, 3096, 2388, 2542, 2387; *lanes 9-14* = clinical strains of *F. verticillioides*: 5390, 5284, 5354, 4951, 5285, FV; *lanes 15* and *16* = phytopathogenic strains of *F. verticillioides*: 2495, 2241; *lanes 17* and *18* = strains of *F. oxysporum* and *F. solani*.

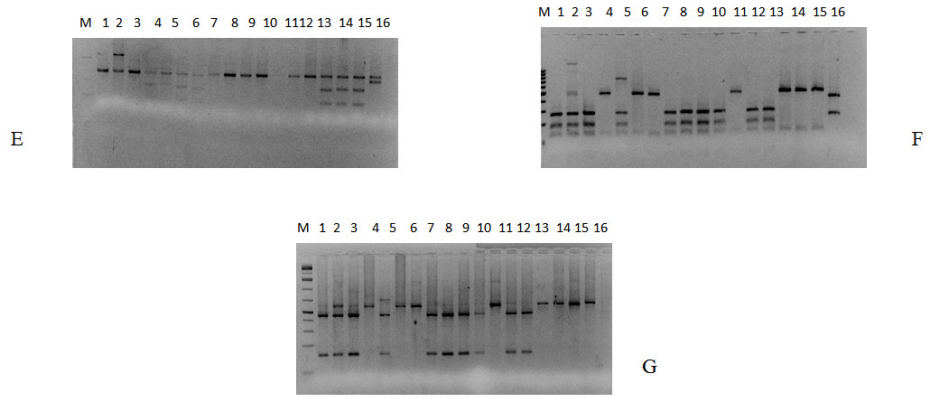

Figure 3. Restriction profiles of the internal transcribed spacer region of the rDNA of *Fusarium verticillioides*, *F. oxysporum*, and *F. solani* isolates with the *Hin*fI (E), *Msp*I (F), *Pst*I (G) enzymes. *Lane M* = 100-bp ladder; *lanes 1-8* = phytopathogenic strains of *F. verticillioides*: URM5352, 3728, 3007, 5094, 3096, 2388, 2542, 2387; *lanes 9-14* = clinical strains of *F. verticillioides*: 5390, 5284, 5354, 4951, 5285, FV; *lanes 15* and *16* = phytopathogenic strains of *F. verticillioides*: 2495, 2241; *lanes 17* and *18* = strains of *F. oxysporum* and *F. solani*.

Digestion with *Bsh*I produced a 500-bp fragment for isolates 1, 2, 3, 5, 8, 11, 12, 13, 15, 16, and 17 and a 400-bp fragment for isolates 4, 6, 7, 14, 9, 10, and 18 (Figure 4).

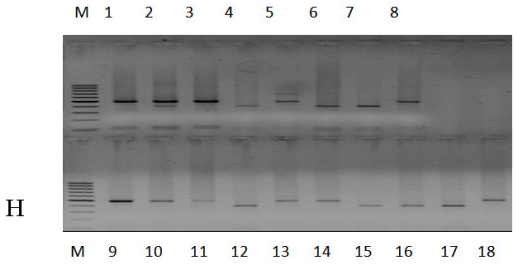

Figure 4. Restriction profiles of the internal transcribed spacer region of the rDNA of *Fusarium verticillioides*, *F. oxysporum*, and *F. solani* isolates with the *Bsh*I enzyme (H). *Lane M* = 100-bp ladder; *lanes 1-8* = phytopathogenic strains of *F. verticillioides*: URM5352, 3728, 3007, 5094, 3096, 2388, 2542, 2387; *lanes 9-14* = clinical strains of *F. verticillioides*: 5390, 5284, 5354, 4951, 5285, FV; *lanes 15* and *16* = phytopathogenic strains of *F. verticillioides*: 2495, 2241; *lanes 17* and *18* = strains of *F. oxysporum* and *F. solani*.

ISSP

The amplification profile of the ISSP region using the EI1 primer is illustrated in Figure 5. The dendrogram that was generated from the amplification profile showed 3 groups at a similarity level of 100% for fragment size. The first group that was formed was represented by 2 isolates of phytopathogenic *F. verticillioides* (isolates 4 and 7). The second group consisted of 2 phytopathogenic *F. verticillioides* isolates (isolates 3 and 8) and 5 from clinical samples (numbers 11, 12, 13, 16, and 15). Finally, the third group was represented by 2 isolates of *F. verticillioides*, 1 obtained from a clinical sample (number 14) and 1 that was considered phytopathogenic (number 10). Isolates 1, 2, 6, 9, and 5 of *F. verticillioides*, and the *F. oxysporum* (number 18) and *F. solani*

(number 17) isolates did not form groups, which indicated that they were genetically distant from the other analyzed isolates, and presented similarity of fragment sizes between 35 and 80%. The use of this marker for isolates of *F. verticillioides* has not been reported in the literature to date.

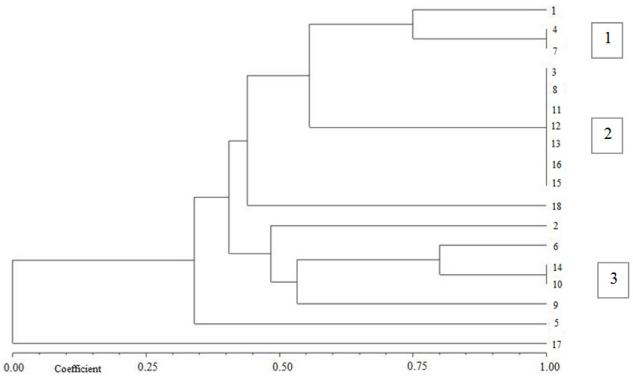

Figure 5. Dendrogram constructed by the UPGMA method, using the Jaccard coefficient from the intron splice site primer profiles with primer EI1 obtained from 16 isolates of *Fusarium verticillioides* (URM5352/1, 5094/4, 2542/7, 3007/3, 2387/8, 5390/11, 5284/12, 5354/13, FV/16, 5285/15, 3278/2 2388/6, 4051/14, 2241/10, 2495/9, 3096/5), *F. oxysporum* (URM/FO/18), and *F. solani* (URM/FS/17).

ISSR

Amplification profiles of the ISSR regions of *Fusarium* isolates using $(GTG)_5$ and $(GACA)_4$ primers are illustrated in Figures 6 and 7, respectively. The dendrogram showed 2 groups with 100% similarity. The first group that was formed was represented by 3 phytopathogenic *F. verticillioides* isolates (numbers 1, 3, and 5) and 5 from clinical samples (numbers 11, 12, 13, 16, and 15), which showed high homogeneity among the clinical samples when compared with the phytopathogenic samples. The second group was represented by 2 phytopathogenic *F. verticillioides* isolates (2 and 6).

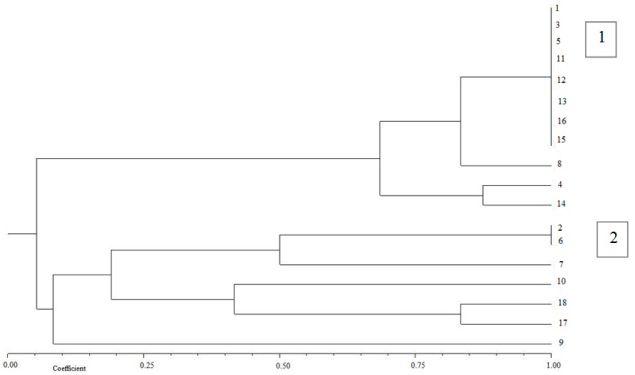

Figure 6. Dendrogram constructed by the UPGMA method, using the Jaccard coefficient from the inter-simple sequence repeat profiles with primer $(GTG)_5$ obtained from 16 isolates of *Fusarium verticillioides* (URM5352/1, 3007/3, 3096/5, 5390/11, 5284/12, 5354/13, FV/16, 5285/15, 2387/8, 5094/4, 4051/14, 3278/2, 2388/6, 2542/7, 2241/10, 2495/9), *F. oxysporum* (URM/FO/18), and *F. solani* (URM/FS/17).

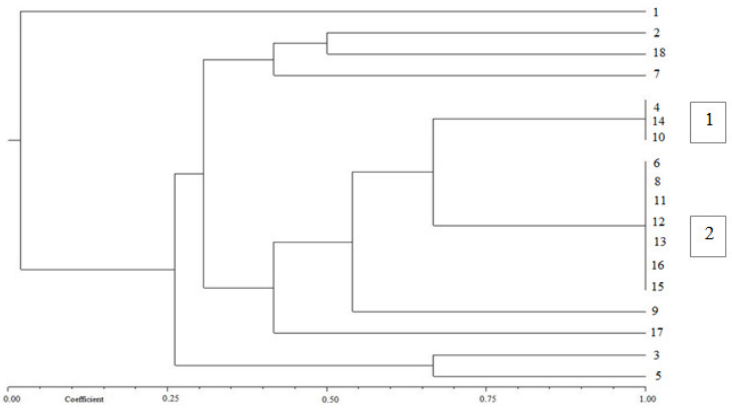

Figure 7. Dendrogram constructed by the UPGMA method, using the Jaccard coefficient from the inter-simple sequence repeat profiles with primer $(GACA)_4$ obtained from 16 isolates of *Fusarium verticillioides* (URM5352/1, 3278/2, 2542/7, 5094/4, 4051/14, 2241/10, 2388/6, 2387/8, 5390/11, 5284/12, 5354/13, FV/16, 5285/15, 2495/9, 3007/3, 3096/5), *F. oxysporum* (URM/FO/18), and *F. solani* (URM/FS/17).

RAPD

The dendrogram that was generated from amplification profiles using the RAPD OPW-6 primer (Figure 8) showed 3 groups with similarity levels of 100%. The first group consisted of 2 phytopathogenic *F. verticillioides* isolates (numbers 1 and 3) and 3 isolates that were obtained from clinical samples (numbers 11, 12, and 15). The second group was represented by 2 isolates of *F. verticillioides* plant pathogens (numbers 2 and 9) and 2 isolates from clinical samples (numbers 13 and 16). The third group was represented by 2 phytopathogenic *F. verticillioides* isolates (numbers 4 and 10).

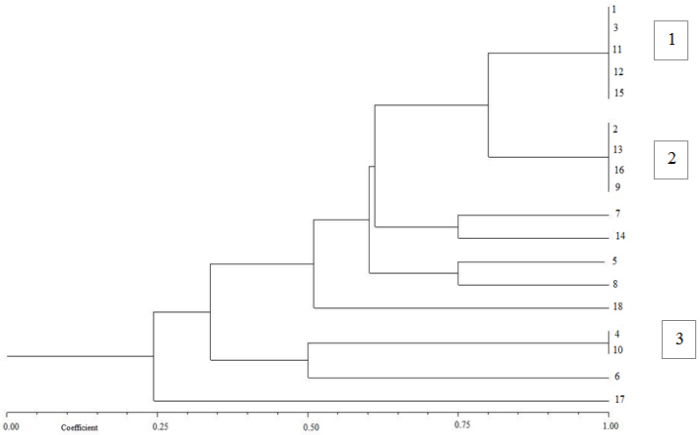

Figura 8. Dendrogram constructed by the UPGMA method, using the Jaccard coefficient based on the RAPD profiles obtained with primer OPW-6 obtained from 16 isolates of *Fusarium verticillioides* (URM5352/1, 3007/3, 5390/11, 5284/12, 5285/15, 3278/2, 5354/13, FV/16, 2495/9, 2542/7, 4051/14, 3096/5, 2387/8, 5094/4, 2241/10, 2388/6), *F. oxysporum* (URM/FO/18), and *F. solani* (URM/FS/17).

DISCUSSION

Molecular techniques, especially those that involve ribosomal genes and their ITS regions, have contributed greatly to the identification of fungal species, improving taxonomic studies, and phylogenetic analysis. These in turn help considerably with carrying out morphological studies for identifying species and segregating strains, thus facilitating and enriching the study of pathogenic fungi (Driver et al., 2000; Martínez-Culebras et al., 2000; Hajek et al., 2003; Enkerli et al., 2005).

Visentin et al. (2009) treated RFLP-ITS from rDNA with *Alu*I, *Mbo*I, *Hin*fI, *Taq*I, and *Hae*III to differentiate isolates of *F. proliferatum* from isolates of *F. verticillioides*. They obtained one fragment that was approximately 600 bp in size for the *F. verticillioides* isolates and were able to differentiate the 2 species with respect to the number of base pairs after their rDNA had been digested. Dyssanayake et al. (2009) also amplified this region with primers ITS1 and ITS4 using digestion products by *Rsa*I *Scr*fI, *Hin*fI, *Hae*III, and *Msp*I. These authors obtained fragments of approximately 570 bp for the 32 isolates of *Fusarium* (*F. oxysporum*, *F. solani*, and *F. verticillioides*).

No digestion was observed for *Dra*I in this study, possibly because this is a rare-cutting enzyme. Using the same enzyme restriction, Brasileiro et al. (2004) did not observe a cutting site for *F. solani* isolates. None of the enzymes that were used in this study was effective for separating clinical isolates from phytopathogenic isolates of *F. verticillioide*s, and only *Hae*III, *Hin*fI, and *Msp*I could be used to distinguish *F. verticillioides* from the *F. solani* isolate. However, the formation of distinct groups with respect to the number of fragments that was generated was observed: isolates 1, 2, 5, 8, 11, 12, 13, 15, 16, and 17 often appeared in the same group, and isolates 4, 6, 7, 14, 9, 10, and 18 often formed a second group. There was no relationship between the groups and the source (clinical or phytopathogenic). The RFLP-ITS technique implies the use of only a single region of the genome and cannot be used to distinguish between pathogenic and clinical isolates of *F. verticillioides*. Martins (2005) could not distinguish phytopathogenic from non-pathogenic isolates of *Fusarium* species that were obtained from different host plants using the same technique.

The amplification profile of the ISSP regions of *Fusarium* isolates using the EI1 primer showed low genetic diversity among the clinical isolates in contrast to the high variability that was found among the phytopathogenic isolates of *F. verticillioides*. The presence of phytopathogenic isolates of *F. verticillioides* in groups where clinical isolates prevailed (group 2) and a co-occurrence of clinical and phytopathogenic isolates in the same group (group 3) showed that there was no genetic difference between the clinical and phytopathogenic isolates in these groups. This can be explained by the supposition raised by several authors that the isolates of *F. verticillioides* that cause human mycoses may have been acquired in the field after traumatic exposure to plants that were decomposing in the soil during tillage or other such field operations (Montiel, 2004).

Brasileiro et al. (2004) used the EI1 primer to detect intraspecific polymorphisms among isolates of *F. solani* from different hosts and reported that some isolates may have represented a clonal lineage by means of forming a group, and that other isolates of the same species showed genetic differences based on the use of this primer.

To date, this is the first report of using the EI1 primer for the genetic analysis of isolates of *F. verticillioides*.

In contrast with the results obtained with the ISSR primers $(GTG)_5$ and $(GACA)_4$, Brasileiro (2003) showed the existence of large intraspecific molecular diversity within the *F. solani* isolates, with no coincidence between the 4 groups and the geographical origin of the isolates. Barve et al. (2001) used 13 complementary ISSR oligonucleotides for the loci of microsatellites $(AT)_{10}$, $(CT)_{10}$, $(TG)_{10}$, $(ACA)_5$, $(ACC)_5$, $(AGC)_5$, $(ACT)_5$, $(AGC)_5$, $(AGG)_5$, $(AGT)_5$, $(ATC)_5$, $(GACA)_4$, and $(GATA)_4$ to analyze the genetic variability of 4 strains of *F. oxysporum* f. sp *ciceris*. They found that only primers $(AGT)_5$, $(ATC)_5$, and $(GATA)_4$ generated polymorphisms. Bayraktar et al. (2008) analyzed 74 isolates of *F. oxysporum* f. sp *ciceris* using 20 ISSR primers and confirmed that the genetic variability among the isolates was higher with respect to the different regions that were studied.

The amplification of random genomic sequences by using RAPD markers has been used to differentiate *Fusarium* species and to assess their genetic relationships (Assigbetse et al., 1994; Nelson et al., 1997). Kuramae and Souza (2002) estimated the genetic variability of 4 *formae speciales* of *Fusarium oxysporum* (*F. oxysporum* f. sp *cubense*, *F. oxysporum* f. sp *lycopersici*, *F. oxysporum* f. sp *phaseoli*, and *F. oxysporum* f. sp *vasinfectum*) and between 2 strains/races of *F. oxysporum* f. sp *lycopersici* by using RAPD. The genetic variability using RAPD was up to 50% among *formae speciales*, and between the races 1 and 2 of *F. oxysporum* f. sp *lycopersici*, it was 7%. This is an evidence of the high capacity of the technique to display genetic differences even among isolates of the same *forma specialis*. The dendrogram that was generated from the amplification profile using the OPW-6 primer demonstrated high homogeneity among the clinical isolates. However, when compared with phytopathogenic *F. verticillioides*, this similarity was less marked than that observed by analyzing the dendrograms that were generated by the other markers (EI1, GTG_5, and $GACA_4$). This was because the clinical isolates (11, 12, 13, 15, and 16) had 75% similarity despite the co-occurrence of clinical and phytopathogenic isolates in the same group having been more balanced than in previous analyses.

The results of this study revealed the existence of a high degree of homogeneity among clinical isolates when compared to phytopathogenic isolates of *F. verticillioides* for most of the markers that were tested. Our findings also indicated that the co-occurrence of some clinical and phytopathogenic groups can be used to reinforce the hypothesis that mycoses, in which the etiological agent is *F. verticillioides*, may be acquired in the field after traumatic exposure to plants.

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REFERENCES

Assigbetse KB, Fernandez D, Dubois MP and Geiger J-P (1994). Differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* races on cotton by random amplified polymorphic DNA (RAPD) analysis. *Phytopathology* 84: 622-626.

Bacon CW and Hinton DM (1996). Fusaric acid and pathogenic interactions of corn and non-corn isolates of *Fusarium moniliforme*, a nonobligate pathogen of corn. *Adv. Exp. Med. Biol.* 392: 175-191.

Bacon CW, Glenn AE and Yates IE (2008). *Fusarium verticillioides*: managing the endophytic association with maize for reduced fumonisins accumulation. *Toxin Rev.* 27: 411-446.

Barve MP, Haware MP, Sainani MN, Ranjekar PK, et al. (2001). Potential of microsatellites to distinguish four races of

Fusarium oxysporum f. sp. *ciceri* prevalent in India. *Theor. Appl. Genet.* 102: 138-147.
Bayraktar H, Dolar FS and Maden S (2008). Use of RAPD and ISSR markers in detection of genetic variation and population structure among *Fusarium oxysporum* f. sp. *ciceris* isolates on chickpea in Turkey. *J. Phytopathol.* 156: 146-154.
Bernd LP (2006). Modelagem com Ênfase no Crescimento de *Fusarium verticillioides* e Produção de Fumonisinas na Perda de Qualidade do Milho. Master's thesis. Universidade Estadual de Londrina, São Paulo.
Brasileiro BTRV (2003). Variabilidade Genética em Isolados de *Fusarium solani* Detectada com a Utilização de Marcadores Moleculares. PhD thesis. Universidade Federal de Pernambuco, Recife.
Brasileiro BTRV, Coimbra MRM, Morais Jr MA and Oliveira NT (2004). Genetic variability within *Fusarium solani* specie as revealed by PCR-fingerprinting based on PCR markers. *Braz. J. Microbiol.* 35: 205-210.
Bussab WO, Miazaki ES and Andrade DF (1990). Introdução à Análise de Agrupamentos. Associação Brasileira de Estatística, São Paulo.
de Barros Lopes M, Soden A, Henschke PA and Langridge P (1996). PCR differentiation of commercial yeast strains using intron splice site primers. *Appl. Environ. Microbiol.* 62: 4514-4520.
Dissanayake ML, Tanaka S and Ito S (2009). Fumonisin B(1) production by *Fusarium proliferatum* strains isolated from *Allium fistulosum* plants and seeds in Japan. *Lett. Appl. Microbiol.* 48: 598-604.
Driver F, Milner RJ and Trueman JWH (2000). A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. *Mycol. Res.* 104: 134-150.
Enkerli J, Kölliker R, Keller S and Widmer F (2005). Isolation and characterization of microsatellite markers from the entomopathogenic fungus *Metarhizium anisopliae*. *Mol. Ecol. Notes* 5: 384-386.
Figueira ELZ (2003). Purificação e Caracterização dos Inibidores de Amilase em Milho (*Zea Mays* L.) Visando o Controle de *Fusarium verticillioides*. Master's thesis. Universidade Estadual de Londrina, São Paulo.
Hajek AE, Jensen AB, Thomsen L, Hodge KT, et al. (2003). PCR-RFLP is used to investigate relations among species in the entomopathogenic genera *Eryniopsis* and *Entomophaga*. *Mycologia* 95: 262-268.
IBGE - Instituto Brasileiro de geografia e estatística (2013). Available at [http://www.ibge.gov.br]. Accessed August 26, 2009.
Kuramae-Izioka EE (1997). A rapid, easy and high yield protocol for total genomic DNA isolation of *Colletotrichum gloeosporioides* and *Fusarium oxysporum*. *Rev. Unimar* 19: 683-689.
Kuramae EE and Souza NL (2002). Variabilidade genética entre *formae speciales* de *Fusarium oxysporum* e raças 1 e 2 de *F. oxysporum* f. sp. *lycopersici* através de RAPD e sequências de regiões ITS e rDNA. *Acta Scientiarum* 24: 1481-1485.
Lieckfeldt E, Meyer W and Börner T (1993). Rapid identification and differentiation of yeasts by DNA and PCR fingerprinting. *J. Basic Microbiol.* 33: 413-425.
Luongo L, Maccaroni M, Ferrarini A, Vitale S, et al. (2007). Molecular characterization of *Fusarium oxysporum* f. sp. *melonis*. *J. Plant Pathol.* 89: 46.
Marasas WFO, Thiel PG, Rabie CJ, Nelson PE, et al. (1986). Moniliformin production in F*usarium* section *Liseola*. *Mycologia* 78: 242-247.
Martínez-Culebras PV, Barrio E, García MD and Querol A (2000). Identification of *Colletotrichum* species responsible for anthracnose of strawberry based on the internal transcribed spacers of the ribosomal region. *FEMS Microbiol. Lett.* 189: 97-101.
Martins MK (2005). Variabilidade Genética de Isolados de *Fusarium* spp. e Estudo da Interação com a Planta Hospedeira. PhD thesis. Escola Superior de Agricultura Luiz de Queiroz, ESALQ, Piracicaba.
Meirelles PG (2005). *Fusarium verticillioides*: Caracterização Molecular e Detecção em Milho Através de Ensaio Imunoenzimático. Master's thesis. Universidade Estadual de Londrina, Londrina.
Meyer W and Mitchell TG (1995). Polymerase chain reaction fingerprinting in fungi using single primers specific to minisatellites and simple repetitive DNA sequences: strain variation in *Cryptococcus neoformans*. *Electrophoresis* 16: 1648-1656.
Mirete S, Vázquez C, Mulè G, Jurado M, et al. (2004). Differentiation of *Fusarium verticillioides* from banana fruits by IGS and EF-1α sequence analyses. *European J. Plant Pathol.* 110: 515-523.
Montiel HV (2004). Patógenos emergentes en micosis cutáneas y sistémicas. *Dermatol. Venezolana* 42: 4-18.
Nagarajan G, Kang SW, Nam MH, Song JY, et al. (2006). Characterization of *Fusarium oxysporum* f. sp. *fragariae* based on vegetative compatibility group, random amplified polymorphic DNA and Pathogenicity. *Plant Pathol. J.* 22: 222-229.
Nelson AJ, Elias KS, Arévalo GE, Darlington LC, et al. (1997). Genetic characterization by RAPD analysis of isolates of *Fusarium oxysporum* f. sp. *erythroxyli* associated with an emerging Epidemic in Peru. *Phytopathology* 87: 1220-1225.
Pamphile JA and Azevedo JL (2002). Molecular characterization of endophytic strains of *Fusarium verticillioides* (=*Fusarium moniliforme*) from maize (*Zea mays* L). *World J. Microbiol. Biotechnol.* 18: 391-396.
Patiño B, Mirete S, Vázquez C, Jiménez M, et al. (2006). Characterization of *Fusarium verticillioides* strains by PCR-RFLP analysis of the intergenic spacer region of the rDNA. *J. Sci. Food Agr.* 86: 429-435.

Ramos ATM (2008). Levantamento da Micoflora de Grãos Ardidos de Milho e Avaliação da Resistência Genética à *Fusarium verticillioides*. Master's thesis. Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba.

Reynoso MM, Chulze SN, Zeller KA, Torres AM, et al. (2009). Genetic structure of *Fusarium verticillioides* populations isolated from maize in Argentina. *Eur. J. Plant Pathol.* 123: 207-215.

Rohlf FJ (1988). NTSYS-PC. Numerical Taxonomy and Multivariate Analysis System. Version 2.1. Exeter Software, New York.

Sambrook J, Fristsch EF and Maniats T (1989). Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, New York.

Sartori AF, Reis EM and Casa RT (2004). Quantificação da transmissão de *Fusarium moniliforme* de sementes para plântulas de milho. *Fitopatol. Bras.* 29: 456-458.

Schiabel VC (2004). Genética e Toxicidade de *Fusarium verticillioides* em Grãos de Milho (*Zea Mays* L.) Sob Plantio Direto e Convencional. Master's thesis. Universidade Estadual de Londrina, Londrina.

Singh BP, Saikia R, Yadav M, Singh R, et al. (2006). Molecular characterization of *Fusarium oxysporum* f. sp. *ciceri* causing wilt of chickpea. *Afr. J. Biotechnol.* 5: 497-502.

Tezcan G, Ozhak-Baysan B, Alastruey-Izquierdo A, Ogunc D, et al. (2009). Disseminated fusariosis caused by *Fusarium verticillioides* in an acute lymphoblastic leukemia patient after allogeneic hematopoietic stem cell transplantation. *J. Clin. Microbiol.* 47: 278-281.

Visentin I, Tamietti G, Valentino D, Portis E, et al. (2009). The ITS region as a taxonomic discriminator between *Fusarium verticillioides* and *Fusarium proliferatum*. *Mycol. Res.* 113: 1137-1145.