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Analysis of Genetic Diversity of *Fusarium oxysporum* f. sp. *phaseoli* Isolates, Pathogenic and Non-pathogenic to Common Bean (*Phaseolus vulgaris* L.)

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Abstract

Twenty isolates of *Fusarium oxysporum* from Brazil, pathogenic and non-pathogenic to common bean, were analysed using random amplified polymorphic DNA (RAPDs) to study the genetic diversity. RAPD analysis using 23 oligonucleotides resulted in the amplification of 229 polymorphic and 7 monomorphic DNA fragments ranging from 234 to 2590 bp. High genetic variability was observed among the isolates, with the distances varying between 8% and 76% among pathogenic, 2% and 63% among the non-pathogenic and 45% and 76% between pathogenic and non-pathogenic isolates. The analysis of genetic distance data showed that the pathogenic isolates tended to group in one group and the non-pathogenic in another. The genetic distance values of 30% among the pathogenic isolates in cluster A are compatible with the genetic distance values observed within the physiological races, but the distance values among the pathogenic isolates in clusters B and G are not compatible with the distance values observed within the race. Although our results are preliminary, it was not possible to exclude the existence of more than one race of this fungus in Brazil.

Introduction

Species identification within the genus *Fusarium* can be performed through morphological characteristics; however, intraspecific differences, such as race and *formae speciales*, are more complicated to identify. By this way, the use of molecular marker techniques, associated with the traditional pathogenicity tests and vegetative compatibility groups (VCG), can facilitate the identification of pathogen populations (Coelho-Neto and Dhingra, 1999). Molecular markers can also be used for inter and intrapopulation genetic diversity studies of pathogenic and non-pathogenic fungi, to explain pathogen–host relationship, to differentiate species, to identify pathogenic races and to understand

pathotype evolution (Appel and Gordon, 1995; Gordon and Martin, 1997; Kerényi et al., 1997; Jiménez-Gasco et al., 2004; Wilson et al., 2004). Information regarding disease and genetic diversity mechanisms on interpathogen and intrapathogen populations have direct implications in the development of resistant varieties.

A tool commonly used for the determination of fungus genetic diversity is the analysis of random amplified polymorphic DNA (RAPD). This technique has already been used for the detection and characterization of phytopathogenic fungi (Wilson et al., 2004), as well as for the differentiation of *F. oxysporum* races in many *formae speciales*, such as the *formae speciales cubense* (Bentley et al., 1994), *dianthi* (Manulis et al., 1994), *pisi* (Grajal-Martin et al., 1993), *vasinfectum* (Assigbetse et al., 1994) and *ciceris* (Jiménez-Gasco et al., 2004).

Several races have been described in *F. oxysporum* f. sp. *phaseoli* (Ribeiro and Hagedorn, 1979; Woo et al., 1996), the causal agent of common bean vascular wilt and in most cases a relationship between race and geographic origin was observed. However, the results of pathogenicity and race characterization using the CIAT (Centro Internacional de Agricultura Tropical) differential cultivars system of *F. oxysporum* f. sp. *phaseoli* isolates from Spain and Greece indicated that isolates classified in the same race were not homogeneous with respect to virulence (Alves-Santos et al., 2002). All the pathogenic isolates already found in Brazil have been classified as race 2 (Woo et al., 1996; Jiménez-Gasco et al., 2002).

Besides the pathogenic population, *F. oxysporum* is commonly isolated from asymptomatic roots of crop plants (Gordon and Martin, 1997). In most of the cases non-pathogenic isolates could be distinguished from pathogenic ones (Gordon and Okamoto, 1992; Woo et al., 1996). Non-pathogenic strains isolated from bean (Coelho-Neto and Dhingra, 1999) were ana-

lysed for the presence of the transposable element *impala* (Langin et al., 1995) and they showed more diversity in the *impala* distribution than the pathogenic strains (Zanotti et al., 2005). A high level of diversity presented by non-pathogenic isolates seems to be a common characteristic of *F. oxysporum* strains (Alves-Santos et al., 2002).

The objective of this study was to analyse the genetic diversity of 20 *F. oxysporum* isolates from Brazil, pathogenic and non-pathogenic to common bean using the technique RAPD.

Materials and Methods

Fungal isolates and culture conditions

Fusarium oxysporum isolates pathogenic and non-pathogenic to common bean were supplied by Dr Onkar Dev Dhingra from the Departamento de Fitopatologia of the Universidade Federal de Viçosa (UFV), Viçosa, MG, by the Departamento de Biologia Celular of the UFV, Viçosa, MG and by the Centro Nacional de Pesquisa de Arroz e Feijão of the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Santo Antônio de Goiás, GO (Table 1).

The isolates were grown at 28°C for 4 days in enriched BDA (Oxoid Basingstoke, UK) medium (casein 1.5 g/l, yeast extract 2.0 g/l, peptone 2.0 g/l, pH 6.8).

DNA extraction and RAPD amplification

After 4 days of growth aliquots of 250 µl of a conidial suspension prepared in 0.1% Tween-80 were spread on plates containing enriched BDA covered with cling film. After 72 h of growth, the mycelium was used for DNA extraction according to Speacht et al. (1982).

Table 1
Description of *Fusarium oxysporum* isolates used

Isolates	Origin	Pathogenicity
D2	DBG-UFV	Pathogenic
D2/2	DBG-UFV	Pathogenic
D2/3	DBG-UFV	Pathogenic
Fus 1	DBG-UFV	Pathogenic
Fus 4	DBG-UFV	Pathogenic
Las	DBG-UFV	Pathogenic
Fop 53	EMBRAPA-GO	Pathogenic
Fop 4005	EMBRAPA-GO	Pathogenic
Fop 46	EMBRAPA-GO	Pathogenic
Fop 4007	DBG-UFV	Pathogenic
Fop 59	EMBRAPA-GO	Pathogenic
Fo Br-H	DFP-UFV	Non-pathogenic
Fo Br-I	DFP-UFV	Non-pathogenic
Fo 1-6	DFP-UFV	Non-pathogenic
Fo 3-4	DFP-UFV	Non-pathogenic
Fo 5-4	DFP-UFV	Non-pathogenic
Fo 5-16	DFP-UFV	Non-pathogenic
Fo 7-18	DFP-UFV	Non-pathogenic
Fo 10	DFP-UFV	Non-pathogenic
Fo 15-17	DFP-UFV	Non-pathogenic

DBG-UFV, Departamento de Biologia Geral, Universidade Federal de Viçosa, MG; DFP-UFV, Departamento de Fitopatologia, Universidade Federal de Viçosa, MG; EMBRAPA-GO, Empresa Brasileira de Pesquisa Agropecuária, Santo Antônio de Goiás, GO.

Amplification reactions were performed according to Williams et al. (1990) with 23 random primers (Operon Technologies Inc., Alameda, CA, USA). The amplified products were analysed by electrophoresis in 1.5% agarose gel and stained with ethidium bromide. The bands were visualized on an ultraviolet transilluminator, and photodigitalized using an Eagle Eye™ video system (Stratagene, La Jolla, CA, USA).

Data analysis

RAPD bands were scored as (1) for presence, or (0) for absence. The presence of bands with the same size (1,1) was indicative of similarity among the isolates. The patterns (0,1) and (1,0) were indicative of dissimilarity. Only strong DNA bands were scored. The data were analysed with the aid of the statistical software GENES (Cruz, 1997). The genetic distance values were calculated using Nei & Li coefficient (Cruz and Carneiro, 2003) according to the formula:

$$d_{ii'} = 1 - S_{ii'} \times 100$$

in which, $d_{ii'}$ is the genetic distance value between genotypes i and i'

$S_{ii'}$ is the Nei & Li coefficient:

$$S_{ii'} = \frac{2a}{2a + b + c},$$

where a bands or the same allelic form present in genotypes i and i' ; b bands or the same allelic form present in genotype i ; c bands or the same allelic form present in genotype i' .

Cluster analysis was carried out using Unweighted Pair Group Method Using Arithmetic Averages (UPGMA) method with the aid of STATISTICA version 4.2. Tocher cluster analysis was performed as described by Cruz and Carneiro (2003).

Results and Discussion

Analysis of genetic variability by RAPD

Of a total of 23 oligonucleotides tested, 16 (OPG03, OPG13, OPG14, OPG16, OPH07, OPI14, OPI19, OPK12, OPK16, OPL05, OPL16, OPM01, OPP02, OPV19, OPY14 and OPY16) generated polymorphic products among the isolates of *F. oxysporum*.

Amplification with these oligonucleotides generated 236 DNA fragments, of which 229 were polymorphic and seven monomorphic. In average 14.7 DNA fragments were amplified per oligonucleotide ranging from 8 to 21 bands per oligonucleotide. The size of the amplified fragments ranged from 234 to 2590 bp, as depicted in Fig. 1a,b. Wang et al. (2001) selected seven oligonucleotides, from a total of 132 screened, when evaluating the diversity among 14 *formae speciales* of *F. oxysporum* originated from China, and found 2–9 DNA fragments amplified per oligonucleotide. The highest genetic diversity among the isolates was reported by Pasquali et al. (2003), who evaluated 11 *formae speciales* of *F. oxysporum*, and obtained an average of

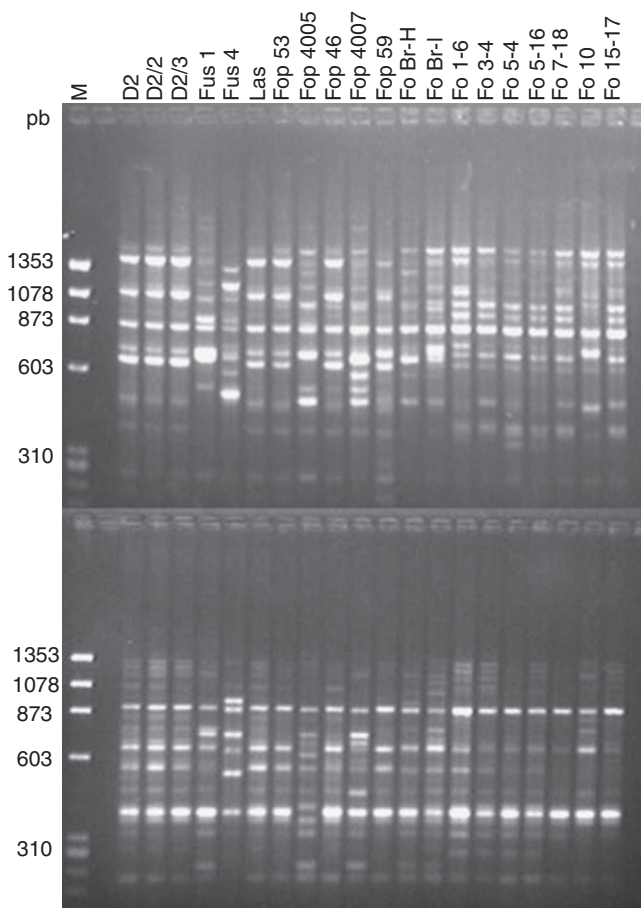


Fig. 1 Random amplified polymorphic DNA (RAPD) pattern of *Fusarium oxysporum* isolates generated by primers OPM01 (a) and OPL16(b). M corresponds to Φ X174 phage DNA cleaved with the enzyme *Hae*III

11.6 DNA fragments amplified per oligonucleotide. The average of 14.7 DNA fragments amplified per oligonucleotide shows a high genetic diversity among *F. oxysporum* isolates in Brazil.

Twelve of the 16 oligonucleotides produced similar amplification patterns among the pathogenic isolates Fop D2, Fop D2/2, Fop D2/3, Fop Las, Fop 53, Fop 46 and Fop 59. However, the equally pathogenic isolates Fus 1, Fus 4, Fop 4005 and Fop 4007 presented amplification patterns dissimilar among themselves with most of the tested oligonucleotides (Fig. 1a,b). The non-pathogenic isolates had a polymorphic RAPD amplification pattern with most of the oligonucleotides used, except for the isolates Fo 5-4, Fo 5-16, Fo BR-H and Fo BR-I, which presented similar patterns with approximately 10 of the 16 selected oligonucleotides (Fig. 1b).

In order to quantify the genetic divergence among the isolates, the genetic distances were calculated using the Nei & Li coefficient (Cruz and Carneiro, 2003). The Nei & Li coefficient is more indicated to compare individuals from populations or from origin knowingly different (Cruz and Carneiro, 2003). The distance values varied between 8% and 76% among the pathogenic isolates; 2% and 63% among the non-pathogenic

and 45% and 76% among pathogenic and non-pathogenic isolates. These values indicate a large genetic variability among the isolates, mainly between the pathogenic and non-pathogenic.

Hierarchical grouping methodologies and the Tocher's optimization method (Cruz and Carneiro, 2003) were used to cluster the genetically less divergent isolates. The Tocher method prioritizes the dissimilarity among groups allowing the formation of mutually exclusive groups using the genetic distance data among isolates. The Tocher's grouping separated the isolates into six different clusters and indicated a tendency for separation between pathogenic and non-pathogenic isolates (Table 2).

The dendrogram analysis, also based on the genetic distance matrix, separated the isolates into seven clusters (Fig. 2). The first cluster (A) contains pathogenic isolates that gave a similar amplification pattern; the second cluster (B) concentrates the pathogenic isolates Fop 4005 and Fop 4007 and the third cluster (C) contains the non-pathogenic isolates Fo BR-H and Fo BR-I. The other non-pathogenic isolates were positioned in clusters (C), (D) and (E), while clusters (F) and (G) contained the pathogenic isolates Fus 1 and Fus 4 respectively. These two isolates showed amplification patterns quite distinct from the others.

Although no DNA fragment was found that could specifically identify the pathogenic isolates, the grouping

Table 2 Identification of the clusters by the Tocher optimization method based on the RAPD data

Groups	Isolates
< 1.1 >	(NP) Fo 5-4, (NP) Fo 5-16, (NP) Fo 3-4, (NP) Fo 7-18, (NP) Fo 15-17, (NP) Fo 1-6
< 1.2 >	(P) D2/2, (P) D2/3, (P) Las, (P) Fop 53, (P) Fop 46, (P) D2, (P) Fop 59
< 1.3 >	(NP) Br-H, (NP) Br-I, (P) 4007
< 1.4 >	(NP) Fo 10
< 2 >	(P) Fus 1, (P) Fus 4
< 3 >	(P) 4005

NP, non-pathogenic; P, pathogenic; RAPD, random amplified polymorphic DNA.

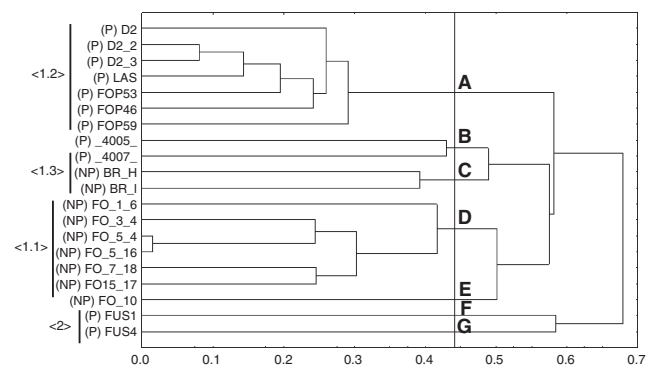


Fig. 2 Cluster analysis using the average of the genetic distances among genotypes of *Fusarium oxysporum* pathogenic and non-pathogenic isolates

trend among the pathogenic and non-pathogenic isolates indicates the existence of genome regions specific to pathogenic isolates. Milchemore et al. (1991) suggested the use of genetic differences among populations to isolate specific genes using genome DNA bulks. The results obtained indicate that DNA bulks and RAPDs can allow the isolation of genome regions specifically related to pathogenicity in *F. oxysporum* f. sp. *phaseoli*.

The occurrence of different band profiles in pathogenic and non-pathogenic isolates was reported in other *formae speciales*, such as *albedinis* (Tantaoui et al., 1996), *dianthi* (Manulis et al., 1994) and *erythroxyli* (Nelson et al., 1997). However, in *F. oxysporum* f. sp. *lycopersici* (Manulis et al., 1994; Suleman et al., 1994) an identical profile was reported. Several authors reported correlations between RAPD amplification pattern and race. Manulis et al. (1994) obtained identical band pattern with 22 oligonucleotides for isolates of *F. oxysporum* f. sp. *dianthi* belonging to the race same in Israel, confirming the existence of just one race in that country. In *F. oxysporum* f. sp. *vasinfectum* from different geographical regions, 11 oligonucleotides amplified genetic patterns, which divided the isolates into three clusters corresponding to the races (Assigbetse et al., 1994). On the other hand, isolates of *F. oxysporum* f. sp. *phaseoli* belonging to different physiological race were analysed using VCGs, RAPD and RFLP by Woo et al. (1996) did not show correlation between band pattern and race. However, the authors observed divergent patterns between pathogenic and non-pathogenic isolates. Bentley et al. (1995) observed the same with physiological races of *F. oxysporum* f. sp. *cubense* originated from different countries, where the amplification pattern did not show correlation with the races.

The genetic distance value of 30% among the pathogenic isolates in cluster (A) is compatible with the genetic distance values observed within the physiological races, as reported for cotton *F. oxysporum* isolates, which varies between 10% and 30% (Assigbetse et al., 1994). However, the distance values of the pathogenic isolate in clusters (B) and (G) shown by the dendrogram are not compatible with the distance values observed within the race. Ribeiro and Hagedorn (1979) and Woo et al. (1996) described only one race of *F. oxysporum* f. sp. *phaseoli* in Brazil. The former is a long-standing report and the latter analysed only two bean *F. oxysporum* isolates from Brazil. As race identification using differential cultivar can be strongly influenced by different factors (Windels, 1991; Cramer et al., 2003), it is not possible to exclude the existence of more than one *F. oxysporum* race in Brazil.

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