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Molecular fingerprinting of *Fusarium oxysporum* f. sp. *passiflorae* isolates using AFLP markers

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ABSTRACT: *Fusarium oxysporum* f. sp. *passiflorae* W.L. Gordon (FOP) is one of the most important fungal pathogens of passion fruits. Understanding molecular variation of isolates from different areas is of utmost importance. Molecular fingerprinting on 14 isolates of FOP were conducted using AFLP molecular markers (*Amplified Fragment Length Polymorphism*), and their genetic variability were estimated. Twenty-five AFLP primer combinations were selected for amplification of FOP isolates and one for *Fusarium oxysporum* f. sp. *cubense* W.C. Snyder & H.N. Hansen (FOC), resulting in 99% polymorphic fragments, with an average of 40 fragments per primer combination. Specific fingerprints could be generated for most of the isolates evaluated; we observed a high power of discrimination of the AFLP primer combinations, with the presence/absence of up to 26 specific fragments per isolate. Thus, specific fingerprinting was obtained for 10 of the 15 isolates analyzed. The values of the polymorphic information content, the index and the resolving power of the markers showed wide variation and reflected the high informative contents of the primers used in the characterization of the FOP isolates. The FOP isolates were divided into four groups, irrespective of their geographic origins, with the allocation of 5, 7, 1 and 1 FOP isolates into Groups II, III, IV and V, respectively. A wide genetic diversity was observed in FOP isolates, which should be taken into consideration when implementing strategies for the improvement of passion fruit in the search for cultivars with multiple resistance to different isolates.

Keywords: passion fruit, fusarium wilt, fingerprint, variability, molecular marker

Introduction

Brazil is the world's largest producer and consumer of passion fruit (*Passiflora edulis* Sims f. *flavicarpa* Deg.), with approximately 62,000 hectares cultivated and an annual production of 920,000 tons. The states of Bahia, Espírito Santo, and Ceará are responsible for approximately 73% of the national production. However, recently yield has decreased due to disease problems, especially with the occurrence of fusarium wilt caused by *Fusarium oxysporum* f. sp. *passiflorae* W.L. Gordon (FOP) (McKnight, 1951).

Knowledge of the genetic diversity of FOP has contributed to the development of disease control strategies (Kistler et al., 2001), as some of these pathogens have a high capacity of variation, and their apparent absence of a sexual stage is compensated by other processes that ensure permutation and maintenance of genetic diversity (Kistler et al., 1992; Leslie, 1993). Fungi with a high level of genetic diversity can be difficult to control, as they tend to adapt more quickly to any control measure, such as the introduction of a resistant cultivar.

FOP isolates obtained from infected plants in various production regions show some morphological differences in colony growth, although the degree of pathogen aggressiveness in relation to the appearance of disease symptoms in different varieties of passion fruit is not effectively known. Morphological and genetic differences between FOP isolates may be associated with their pathogenicity, and these observations must be taken into consideration when the passion fruit germplasm is screened for sources of resistance and, later, in the development of resistant cultivars.

In order to do so, it is vital to better understand the genetic variability of the FOP isolates, which are still unknown. Recently, molecular techniques have been increasingly used in studies of genetic diversity and species phylogeny, or for the characterization of populations of *F. oxysporum* within and between *formae speciales* (Baayen et al., 2000; O'Donnell, 2000; Abd-Elsalam et al., 2004; Bogale et al., 2006; Groenewald et al., 2006; Stewart et al., 2006). Among these techniques, AFLP (Amplified Fragment Length Polymorphism) is a class of marker frequently used (Zeller et al., 2003; Belabid et al., 2004).

Amplified Fragment Length Polymorphism (AFLP) markers are extremely powerful because of their high multiplex power, i.e., enabling the generation of a large number of bands per gel (amplification). Therefore, it has high potential for the development of specific primers in the identification of isolates. Given these needs and the molecular tools available, this study was conducted to assess the possibility of developing specific fingerprinting within the *formae speciales* of FOP to be able to trace the presence of these isolates in the main passion fruit-producing regions and to find the different ways of genetic structuring of FOP isolates, one of the principal infectious agents of passion fruit.

Materials and Methods

Biological material

Fourteen monosporic cultures of FOP from the Embrapa Cassava and Fruits fungus collection were used. The isolates were taken from passion fruit plants displaying typical symptoms of wilt disease and obtained from farms in the states of Bahia and Espirito Santo. After isolation, these were subjected to morphological studies to confirm the identity of the pathogen. In addition to this, one isolate of *Fusarium oxysporum* f. sp. *cubense* W.C. Snyder & H.N. Hansen (FOC) was used as an outgroup (Table 1).

Growth of the fungus in liquid medium, and extraction of DNA

To obtain the mycelial mass, the isolates were chopped up and incubated at 25 °C in potato-dextrose (PD) liquid medium (200 g of potatoes peeled and cut into small pieces, cooked in distilled water for 20 min, filtered through a muslin cloth and mixed with 5 g of unhydrated dextrose (Impex). The isolates were shaken continuously for a period depending on each isolate. The mycelial mass of each isolate was filtered and frozen in liquid nitrogen for the extraction of DNA.

The extraction of DNA from the isolates was performed using the CTAB method ($(C_{16}H_{33})N(CH_3)_3Br$, cetyltrimethylammonium bromide hexadecyltrimethylammonium bromide) described by Zolan and Pukilla (1986). DNA quantification was performed using visual comparison to the intensities of fragments of a series of known concentrations of Lambda DNA (Invitrogen), after electrophoresis (3 v cm^{-1}) in 1.0 % agarose gels containing ethidium bromide (1.0 µg mL^{-1}).

Evaluation of primer combinations

A preliminary study was performed with 64 primer combinations and two FOP isolates (013 and 071) to identify the more polymorphic combinations for definitive analyses with the marker. The best combinations were used for characterization of the 14 FOP isolates and the one FOC isolate (Table 2).

Table 1 – Isolates of *Fusarium oxysporum* f. sp. *passiflorae* and *Fusarium oxysporum* f. sp. *cubense*, used on the molecular analysis with Amplified Fragment Length Polymorphism (AFLP) markers.

Number	Isolate	f. sp.	Geographic origin
1	FOC143	*cubense*	Janaúba (MG)
2	FOP001	*passiflorae*	Cruz das Almas (BA)
3	FOP002	*passiflorae*	Cruz das Almas (BA)
4	FOP003	*passiflorae*	Cruz das Almas (BA)
5	FOP004	*passiflorae*	Cruz das Almas (BA)
6	FOP005	*passiflorae*	Cruz das Almas (BA)
7	FOP008	*passiflorae*	Ubaíra (BA)
8	FOP013	*passiflorae*	Ubaíra (BA)
9	FOP022	*passiflorae*	Ubaíra (BA)
10	FOP023	*passiflorae*	Ubaíra (BA)
11	FOP028	*passiflorae*	Ubaíra (BA)
12	FOP057	*passiflorae*	Ubaíra (BA)
13	FOP069	*passiflorae*	Linhares (ES)
14	FOP071	*passiflorae*	Porto Seguro (BA)
15	FOP072	*passiflorae*	Livramento de Nossa Senhora (BA)

Amplification with AFLP markers

Analyses were performed using the Vos et al. (1995) protocol with minor modifications, using the commercial AFLP® Core Reagent Kit (Invitrogen). Two combinations of endonucleases were employed. In the digestion reaction, 250 ng of genomic DNA were digested using *Eco*RI and *Mse*I for 2 hours at 37 °C. Reactions were then incubated at 70 °C for 15 min to deactivate the enzymes. For connection of the adapters, fragments of digested DNA were added to 5X buffer of *T4* DNA ligase (Invitrogen), 0.1 µM of each enzyme's adapter (*Eco*RI and *Mse*I), 1 U of *T4* DNA ligase (1 U μL^{-1}, Invitrogen) and 5.0 µL of ultra-pure sterilized water, and the reactions were incubated at 20 °C for 3 h. The mixture was diluted 1:5 in a 1:10 mixture of TE and ultra-pure water.

The pre-amplification was performed in a final volume of 20 µL, consisting of 3.0 µL of the solution containing the digested DNA linked to the adapter, 0.15

Table 2 – Characterization of the AFLP primers by the total number of fragments (NB), number of polymorphic fragments (NBP), polymorphic information content (PIC), marker index (MI) and resolving power of the marker (Rp).

Primer combination	NB	NBP	PIC	MI	Rp
E+CC/M+ATA	29	28	0.19	5.32	7.28
E+CC/M+ATT	28	24	0.17	4.08	5.30
E+CC/M+ATC	22	20	0.18	3.60	4.62
E+CG/M+AAA	55	50	0.24	12.00	16.58
E+CG/M+AAT	54	46	0.18	8.28	10.28
E+CG/M+AAC	40	39	0.21	8.19	10.74
E+CT/M+AAC	39	38	0.18	6.84	8.44
E+CT/M+AAG	43	43	0.25	10.75	15.52
E+CA/M+ATC	50	47	0.15	7.05	8.56
E+CG/M+ATG	26	25	0.17	4.25	5.66
E+CG/M+ATT	42	37	0.16	5.92	9.86
E+CC/M+AAC	38	38	0.21	7.98	11.66
E+CG/M+AAG	39	38	0.18	6.84	8.74
E+CT/M+ATA	30	28	0.28	7.84	12.46
E+CT/M+ATT	45	44	0.21	9.24	11.68
E+CT/M+ATC	32	30	0.16	4.80	6.36
E+CC/M+AAG	53	49	0.18	8.82	12.18
E+CT/M+AAA	61	58	0.21	12.18	15.84
E+CT/M+AAT	49	48	0.17	8.16	9.90
E+CA/M+AAA	51	47	0.23	10.81	14.76
E+CA/M+AAT	54	53	0.24	12.72	17.90
E+CA/M+ATA	34	30	0.20	6.20	8.54
E+AA/M+CTG	36	33	0.15	4.95	5.88
E+AT/M+CTG	43	42	0.21	8.82	11.42
E+AC/M+CTG	57	54	0.23	12.42	16.48
Minimum	22	20	0.15	3.6	4.62
Maximum	61	58	0.28	12.72	17.90
Average	42	40	0.20	7.92	10.66

mM dNTPs (Invitrogen), 1X PCR buffer without $MgCl_2$ (Fermentas), 1.5 mM $MgCl_2$ (Invitrogen), 1U of Taq DNA polymerase (Fermentas), 0.5 µM of the primer for the rare-cutting enzyme with a selective base (*Eco*RI + C or *Eco*RI + A) and 0.5 µM of the primer for the frequent-cutting enzyme (*Mse*I + A or *Mse*I + C). The PCR program consisted of 95 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 2 min. This was followed by a final extension step for five minutes at 72 °C. The pre-amplified material was diluted 1:60 in a 1:10 mixture of TE and ultra-pure water.

In the selective amplification stage, 2.5 µL of the pre-amplified products were used, with 0.15 µM dNTPs (Invitrogen), 1X PCR buffer (Fermentas), 2 mM $MgCl_2$ (Invitrogen), 8 µg mL^{-1} BSA (New England Biolabs), 1.5 U of *Taq* DNA polymerase (Fermentas), 0.5 µM of the primer for the rare-cutting enzyme (*Eco*RI + CN or *Eco*RI + CN), where N can be any nucleotide, and 0.5 µM of the primer for the frequent-cutting enzyme (*Mse*I + CNN or *Mse*I + ANN), with a final volume of 20 µL. The amplification program consisted of 95 °C for 2 min, followed by 12 cycles of 94 °C for 30 s, 65 °C for 30 s with a reduction of 0.7 °C per cycle, and 72 °C for 2 min; followed by 24 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 2 min; with a final incubation at 72 °C for 2 min.

The amplified fragments were separated by electrophoresis in a 6% polyacrylamide denaturing gel at 70 W for 3 h. The amplification products were visualized by silver staining according to Creste et al. (2001). The molecular masses of the fragments were determined by comparison with a 50-bp ladder (New England Biolabs).

Molecular analysis of the data

For each primer pair, the number of polymorphic fragments (NPF), polymorphic information content (PIC), marker index (MI) and primer resolving power (Rp) was calculated. The polymorphic information content (PIC) was calculated using $PIC_i = 2f_i(1-f_i)$, where i is the information of marker I, f_i is the frequency of the amplified allele (presence of fragments) and $(1 - f_i)$ is the frequency of the null alleles (Roldan-Ruiz et al., 2000). The PIC was taken as the average of each primer combination.

The marker index (MI) was calculated by $MI = PIC \times EMR$, where *EMR* is the "effective multiplex relationship" given by the product of the total number of fragments per primer (n) and the fraction of polymorphic fragments (β) (Varshney et al., 2007). The resolving power (Rp) was calculated using the formula $Rp = \sum I_b$, where $I_b = 1-[2x|0.5-p|]$ and p is the proportion of accessions with fragments (Prevost and Wilkison, 1999).

AFLP polymorphic markers were computed as the absence (0) and presence (1) of fragments. The dissimilarities between the genotypes were calculated by the complement of simple matching, using the Genes program (Cruz, 2006). From the dissimilarity matrix, the dendrogram was generated with the UPGMA method (Unweighted Pair Group Method with Arithmetic Mean), using Statistica 7.1 software (Statsoft, 2005). Fusion point criteria were used as cutting point to define clusters (Mingoti, 2005), where a graph with the number of groups versus the distance at each stage (fusion) is made, and jumping points indicate the optimal number of clusters. The consistency between the dissimilarities of the clusters and of the matrices was verified using cophenetic correlations with the Genes 1.0 program (Cruz, 2006). The presence of private alleles was determined using the GenAlEx program (Peakall and Smouse, 2006).

Results

Informative content of the AFLP marker

Consistent molecular profiles were obtained for all of the isolates evaluated. The 25 AFLP primer pairs produced 1,050 fragments (ranging from 30 to 916 bp). Of this total, 989 (94.0 %) fragments were polymorphic (Table 2). On average, each primer pair produced 40 polymorphic fragments. The lowest number of fragments was detected for the *Eco*RI + CC / *Mse*I + ATC pair and the highest for *Eco*RI + CT / *Mse*I + AAA, with 20 and 58 fragments, respectively. Figure 1 illustrates three primer combinations that were evaluated (E + CT / M + AAC, E + CT / M + AAG and E + CT / M + ATA).

The polymorphic information content for dominant markers such as the AFLP ranged from 0 to 0.50 (Roldan-Ruiz et al., 2000). Thus, on average for the primers pairs evaluated, the polymorphic information content (PIC) varied from 0.15 (primers E + CA / M + ATC and E + AA / M + CTG) to 0.28 (primers E + CT / M + ATA), with an average of 0.20, which reflects the informative content of the primers used (Table 2).

The marker index (MI), which incorporates the informative content of the marker (PIC), the number of fragments per primer pair and the fraction of polymorphic fragments, varied from 3.60 (*Eco*RI + CC / M + ATC) to 12.72 (*Eco*RI + CA / *Mse*I + AAT), with an average of 7.92. In the isolates evaluated, Rp varied from 4.62 to 17.90 for the combinations *Eco*RI + CC / *Mse*I + ATC and *Eco*RI + CA / *Mse*I + AAT respectively, following the same tendency as the MI. This fact is shown by the high correlation between these two indices ($r = 0.97$, $p < 0.01$).

Discriminatory capacity of AFLP markers

The evaluation of the discriminatory capacity of the AFLP primers was also evaluated on the basis of the presence of unique fragments (+) and on the absence of specific fragments (-) in certain isolates. High discriminatory powers of the combinations of AFLP primers were observed, with a range of 2 to 12 presences of unique fragments (+) for the combinations *Eco*RI + CG / *Mse*I

+ AAA and *Eco*RI + CT / *Mse*I + AAT, respectively; and a range of 8 to 27 absences (-) for the combinations *Eco*RI + CC / *Mse*I + ATT/ATC and *Eco*RI + CA / *Mse*I + ATC, respectively, with an average of 23.0 -/+ per combination (Table 3). Considering presence and absence of unique fragments, 575 differences were observed among the isolates evaluated. Only isolates FOP003, FOP004, FOP022, FOP057 and FOP069 had no unique fingerprinting. However, a large number of exclusive FOC fragments were observed, highly differentiated from the *passiflorae* form.

Among the FOP isolates studied, FOP002 produced the highest number of differences (137), distributed among 24 combinations of AFLP primers; followed by FOP028, with 25 different fragments (presence/absence) distributed among 17 primer combinations; FOP008, with 12 different fragments (presence/absence) distributed among 11 primer combinations; and FOP005, with 11 different fragments (presence/absence) distributed among eight primer combinations. On the other hand, two unique fragments were found for isolate FOP023 (*Eco*RI + CG / *Mse*I + AAT - 234 bp and *Eco*RI + CG / *Mse*I + ATT - 115 bp) and three combinations (*Eco*RI + CA / *Mse*I + AAA - 150 bp, *Eco*RI + CA / *Mse*I + AAT - 626 bp and *Eco*RI + CA / *Mse*I + ATA – 249 bp) for isolate FOP001. For isolates FOP013, FOP071 and FOP072, a single specific fragment was generated for the combinations *Eco*RI + CC / *Mse*I + AAG - 215 bp, *Eco*RI + CT / *Mse*I + AAT - 260 bp and *Eco*RI + CT / *Mse*I + AAA - 775 bp, respectively. In FOP, the absence of fragments in just one specific isolate was observed in FOP002 with 9 absences (Table 3).

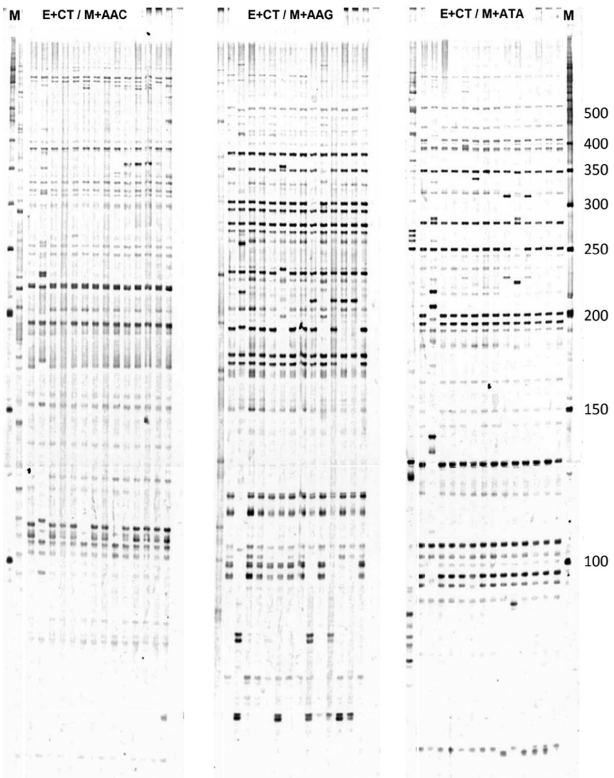

Figure 1 – Amplification pattern of three AFLP primer combinations. (M): 50-bp ladder. The combinations are indicated in the gel, as well as the first and the last individual in each combination, in accordance with Table 1. The orders are FOC143, FOP001, FOP002, FOP003, FOP004, FOP005, FOP008, FOP013, FOP022, FOP023, FOP028, FOP057, FOP069, FOP071 and FOP072.

Molecular characterization of the FOP

The genetic dissimilarity varied from 4% to 48%, with an average of 25%. Although the isolates had different geographical origins, the most similar isolates were FOP004 and FOP022, with only 4% dissimilarity. The cophenetic correlation was 0.95, indicating a high level of accuracy between the original data of the genetic distance matrix and the data represented in the dendrogram (Figure 2).

Using the cutting point defined by the fusion point criteria (Mingotti, 2005), five groups were formed (Figure 2). Isolate FOC143 remained separate from the others in Group I, showing broad molecular differences in relation to those of FOP isolates. Group II consisted of isolates from Cruz das Almas (FOP001), Ubaira (FOP013, FOP023 and FOP057) and Linhares (ES) (19°23'27" S, 40°4'17" W) (FOP069).

Group III contained many isolates (FOP003, FOP004, FOP005, FOP008, FOP022, FOP071 and FOP072). The isolates of this group had quite diverse origins, including the municipalities of Cruz das Almas (12°48' 38" S, 39°6'26" W), Ubaira (13°15'7" S, 39°39'15" W), Porto Seguro (16°27'4" S, 39°3'53" W) and Livramento de Nossa Senhora (13°38'36" S, 41°50'32" W)", although all are within the state of Bahia. On the other

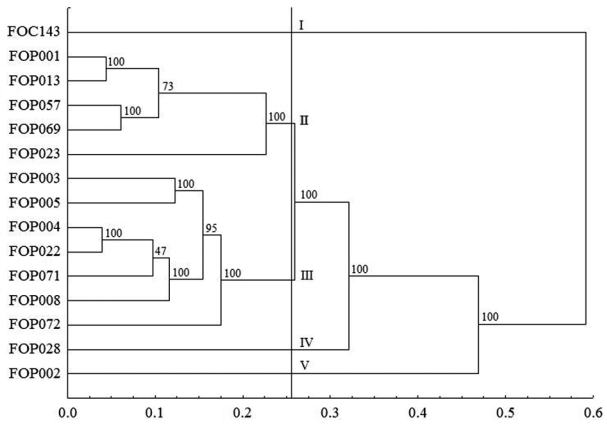

Figure 2 – Dendogram of the genetic relationships among 15 isolates (14 FOP and one FOC) obtained with the use of the dissimilarity matrix with simple matching coefficient. The grouping method was UPGMA. The vertical bar represents fusion point. Bootstrap values are shown in the branches nodes, and 0.95 of cophenetic value.

Table 3 – Number of unique fragments per FOP (*Fusarium oxysporum* f. sp. passiflorae) and FOC (*Fusarium oxysporum* f. sp. cubense) isolates in each AFLP primer combination.

Combination	No. of fragments absent	No. of fragments present	FOC143[1]	FOP001	FOP002	FOP005	FOP008	FOP013	FOP023	FOP028	FOP071	FOP072
E+CC/M+ATA	7	9	-(6) / +(5)		-(1) / + (2)					+(2)		
E+CC/M+ATT	10	8	-(10) / +(6)		+(2)							
E+CC/M+ATC	6	8	-(6) / +(5)		+(3)							
E+CG/M+AAA	2	16	-(2) / +(10)		+(6)							
E+CG/M+AAT	11	19	-(10) / +(9)		-(1) / +(6)	+(1)	+(1)		+(1)	+(1)		
E+CG/M+AAC	6	14	-(5) / +(7)		-(1) / +(4)		+(2)			+(1)		
E+CT/M+AAC	12	16	-(12) /+ (9)		+(6)	+(1)						
E+CT/M+AAG	7	13	-(7) / +(6)		+(5)		+(1)			+(1)		
E+CA/M+ATC	8	27	-(7) / +(13)		-(1) / +(10)	+(2)	+(1)			+(1)		
E+CG/M+ATG	4	12	-(4) / +(8)		+(4)							
E+CG/M+ATT	5	16	-(5) / +(9)		+(5)		+(1)		+(1)			
E+CC/M+AAC	9	17	-(9) / +(10)		+(6)					+(1)		
E+CG/M+AAG	9	14	-(9) / +(8)		+(5)					+(1)		
E+CT/M+ATA	2	9	-(2) / +(6)			+(1)	+(1)			+(1)		
E+CT/M+ATT	7	15	-(6) / +(9)		-(1) / +(5)					+(1)		
E+CT/M+ATC	8	14	-(8) / +(8)		+(3)	+(1)				+(2)		
E+CC/M+AAG	11	22	-(9) / +(11)		-(2) / +(6)		+(1)	+(1)		+(3)		
E+CT/M+AAA	6	26	-(6) / +(14)		+(6)	+(2)	+(1)			+(2)		+(1)
E+CT/M+AAT	12	20	-(11) / +(11)		-(1) / +(5)		+(1)			+(2)	+(1)	
E+CA/M+AAA	6	16	-(6) / +(5)	+(1)	+(9)					+(1)		
E+CA/M+AAT	11	16	-(10) / +(5)	+(1)	-(1) / +(8)					+(2)		
E+CA/M+ATA	6	16	-(6) / +(7)	+(1)	+(4)	+(1)	+(1)			+(2)		
E+AA/M+CTG	6	19	-(6) / +(11)		+(5)	+(2)	+(1)					
E+AT/M+CTG	6	15	-(6) / +(9)		+(5)					+(1)		
E+AC/M+CTG	3	18	-(2) / +(10)		+(8)							

[1]+ = presence of fragment and - = absence of fragment, numbers between brackets indicate the number of marks absent or present.

hand, isolates from this state were also found in two other groups, with FOP028 (Group IV) and FOP002 (Group V) from Ubaira and Cruz das Almas, respectively.

Discussion

AFLP markers have been used to access the genetic variation of intimately related breeds or species, which would be impossible to discover using morphological traits or other molecular mechanisms (Abdel-Satar et al., 2003). In this study, these markers served to analyze the genetic variation of 14 isolates of FOP, representative of different passion fruit–producing fields. In general, the haplotypes generated by the AFLP markers showed high informative capacity and indicated high genetic variability present in FOP isolates.

For the selection of combinations of more informative markers, the polymorphic information content (PIC), marker index (MI) and resolving power (Rp) have traditionally been used. The Rp takes into consideration the capacity of a given primer combination to discriminate the genotype. In the isolates evaluated, the Rp varied in the same manner as the marker index (MI), which is shown by the high correlation between these two indices ($r = 0.97$ $p < 0.01$). This reinforces the fact that both indices can be used as criteria for selection of the best primers. Thus, adopting the principle of selection on the basis of the average values of the MI (7.92) and Rp (10.66), the primer combinations E + CA/M + AAT, E + CG/M + AAA, E + AC/M + CTG, E + CT/M + AAA, E + CT/M + AAG, E + CA/M + AAA, E + CC/M + AAG, E + CT/M + ATT, E + CC/M + AAC, E + AT/M + CTG and E + CG/M + AAC are the most promising for analysis of variability of the FOP (Table 2).

The highly informative nature of these markers contributes to the observation of polymorphisms between the FOP isolates, as well as contributes to identify specific marks for each isolate. The 395 differences of fragments among the 14 FOP isolates and one FOC isolate allow the identification of specific fingerprinting for 10 isolates. Of these isolates, FOP002 and FOP028 have particularly high numbers of polymorphic and specific fragments. As a consequence of the higher number of unique fragments; these isolates were grouped separately (Figure 3).

The five isolates that had no unique fragments were placed in Groups II and III, as these probably had only slight differences in relation to the others in the cluster, using AFLP markers. However, isolate FOC143 had completely different standard fingerprinting from the FOP isolates, separate from the others in the group,

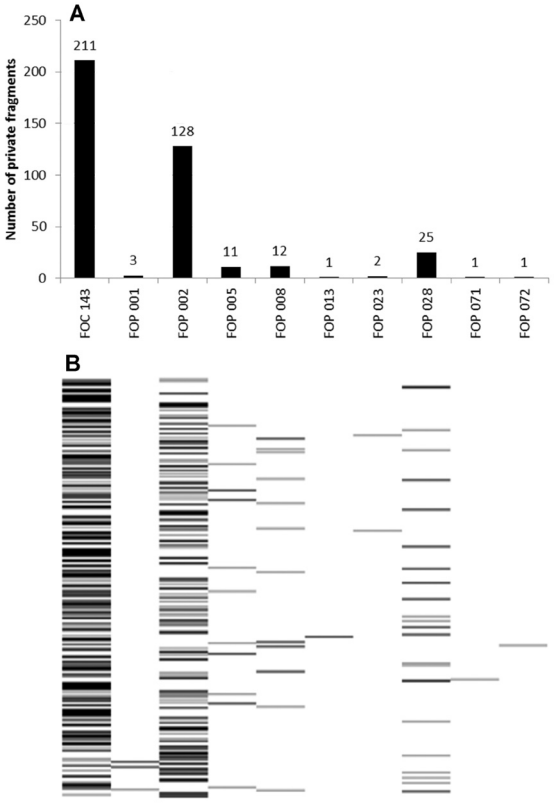

Figure 3 – Distribution of unique fragments (presence) for nine isolates of FOP and one of FOC using AFLP markers. A: graphic of the number of unique fragments in the isolates analyzed. B: fingerprinting of the isolates on the basis of the unique fragments.

which emphasizes the highly diverse natures of these two pathogens of the same species.

Studies of the diversity among pathogenic isolates of *Fusarium* have been done using mitochondrial DNA, ITS (Internal Transcribed Spacer) regions, PCR and real-time PCR techniques to determine genetic relationships between *formae speciales* (Kim et al., 1993; Oliveira and Costa, 2002) and to differentiate them into pathogenic races (Hirano and Airie, 2006; Inami et al., 2010). However, the success in the differentiation of pathogenic isolates between different *formae speciales* has given rise to contradictions, for example, in not distinguishing isolates of *F. solani* that are pathogenic to either soybeans or beans from those that are pathogenic to both crops with the use of the ARDRA technique (Amplified Ribosomal DNA Restriction Analysis) (Oliveira and Costa, 2002). On the other hand, Werner and Irzykowska (2007) demonstrated the applicability of RAPD in differentiating between pathogenic and non-pathogenic isolates of *F. oxysporum*, and Hirano and Arie (2006) used molecular differences in the endo-polygalacturonase (*pg1*) and exo-polygalacturonase (*pgx4*) genes of *F. oxysporum* f. sp. *lycopersici* and *radicis-lycopersici* isolates to design a set of primers able to differentiate between the pathogenic types of *F. oxysporum* in tomato.

Although the number of FOP isolates is reduced, the 14 analyzed in this study are representative of the principal passion fruit-producing State in Brazil, and therefore, the molecular results demonstrate that the AFLP technique has high potential for the identification of specific marks among the *forma specialis passiflorae*. The specific fragments could be cloned and transformed into SCAR (Sequence-Characterized Amplified Region) markers, enabling molecular analyses to be conducted more rapidly, as SCAR technique has fewer stages. The use of this information may help in the rapid identification of FOP, even within this *forma specialis*.

This is one of the first studies conducted to characterize FOP isolates from producing regions with a high rate of infestation of the disease. The results show that there are no differences in the gene pool in relation to geographic locations, as there is no grouping according to these criteria. Thus, the drawing up of a genetic improvement program for the selection of genotypes resistant to wilt disease should focus on the genetic diversity of the pathogen, verifying whether this standard does or does not reflect the aggressiveness and pathogenicity of the FOP isolates.

The isolates from the same geographic origin were found not to have identical fingerprinting. This observation corroborates the work of other authors showing that molecular information does not detect a strong relationship between genetic variation and geographic distribution or in the groupings generated (Abdel-Satar et al., 2003).

Group III included half of the isolates analyzed (Figure 2), showing genetic similarities between isolates from quite geographically distant areas in the state of Bahia, although significant molecular diversity was found. These considerations can also apply to Group II, including isolates with even higher genetic divergence, even from different states such as Bahia and Espirito Santo.

The information on the specificity of fragments is directly or indirectly related to the dissimilarity matrix between the FOP isolates, which is also shown by the dendrogram formed (Figure 2). Isolates FOP003, FOP004 and FOP022 had no specific fragments belonging to the same group. Furthermore, isolates FOP004 and FOP022 showed the lowest dissimilarity. Similarly, FOP069 from Linhares (ES), which also showed no specific fingerprinting, was allocated to Group II, along with isolates from different geographic areas of the Bahia.

As *F. oxysporum* has no known sexual stage, one hypothesis that has been suggested is that the genetic variation observed is in part generated by parasexuality (Kistler, 1997), amplifying the variability created by high mutations rates. Parasexuality occurs within heterokaryon fungi by the occasional fusion of two nuclei and the resulting formation of a diploid nucleus. During multiplication, there must be crossing-over in some mitotic divisions, resulting in the appearance of genetic

recombinants with diploid nuclei, which quickly and progressively loose individual chromosomes to revert to their haploid state (Agrios, 2005). Earlier research suggested genetic exchanges between and within individual lineages. Fourier et al. (2009), studying the relationships between VCG of FOC, demonstrated recombination of genetic material within some lineages of this pathogen, which must be due to parasexuality or heterokaryosis between individuals with very similar genomes.

The results found here are relevant to the study of passion fruit-FOP interactions, as the separation of the FOP isolates into different groups will help in screening for sources of resistance to this pathogen. The selection of the genotypes must be performed by inoculation of the plants with isolates from different groups. Thus, the genotypes with resistance to multiple isolates from different groups will have a greater chance of having longer-lasting resistance under field conditions. Understanding the diversity of this pathogen adds value to research on resistance to wilt disease, allowing the identification of future races and helping in the identification of sources of genetic resistance that are consistent in the various regions of the country.

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