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Pathogenic and genetic characterization of Algerian isolates of *Fusarium oxysporum* f. sp. *lentis* by RAPD and AFLP analysis

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Thirty-two isolates of *Fusarium oxysporum* f. sp. *lentis* were isolated from wilted lentil plants collected from different lentil growing areas in north-west Algeria. A pathogenicity test was performed for all isolates. Results indicated that the *Fol* isolates represent a single race but differ in their aggressiveness on the susceptible lines. The amount of genetic variation was evaluated by polymerase chain reaction (PCR) amplification with a set of 6 RAPD primers and 3 AFLP selective nucleotide primer pairs. All amplifications revealed scorable polymorphisms among the isolates, and a total of 8 polymorphic fragments were scored for the RAPD primers and 93 for the AFLP primers. Genetic similarity between each of the isolates was calculated by using the Jaccard similarity coefficient and cluster analysis was used to generate a dendrogram showing relationship between them. The isolates could be grouped into two subpopulations based on RAPD and AFLP analysis. Results obtained indicate that there is little genetic variability among a subpopulation of *Fol* as identified by RAPD and AFLP markers and that there is no apparent correlation with geographical origin or aggressiveness of isolates. Also, the data suggest that *Fol* isolates are derived from two genetically distinct clonal lineages.

Key words: *Lens culinaris,* Fusarium wilt, molecular markers, pathogenicity.

INTRODUCTION

Lentil (*Lens culinaris* Med.) wilt caused by *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen f. sp. *lentis* Vasudeva and Srinivasan (*Fol*), is the most important disease affecting lentils in Algeria, causing significant economic losses (Setti and Bouznad, 1998; Belabid et al., 2000). The pathogen persists in the soil as chlamydospores that can remain viable for several seasons (Erskine and Bayaa, 1996), and is capable of colonizing crop residue and roots of most crops grown in rotation with lentil. It can enter the host through root tips, primarily in the area of elongation, a process which is aided by wounding (Bhalla et al., 1992). As a result of the persistence of the pathogen in the soil, the disease is best controlled through host plant resistance. No

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physiological races of the pathogen have been reported so far (Bayaa et al., 1997) although *Fol* isolates exhibit great variability in morphology and aggressiveness (Abbas, 1995).

Understanding the physiological races of the pathogen is essential to guide the development of appropriate strategies for disease management that will enhance durability of *Fusarium* wilt resistance. A better understanding of the inheritance of host plant resistance available in the new cultivars is also important to apply appropriate screening and breeding procedures (Bayaa et al., 1997). Determination of both host specificity and genetic diversity in *Fol* population are important in breeding for resistance. Assessment of genetic diversity in *Fol* is needed to determine whether isolates constitute genetically distinct groups. It might also enable the identification of diagnostic molecular markers to differentiate isolates (Assigbetse et al., 1994). Random Amplified Polymorphic DNA (RAPD) markers generated with single primers of arbitrary nucleotide sequence have been used in detecting intraspecific polymorphisms among fungi (Assigbetse et al., 1994). This technique can generate specific DNA fragments that can be used for the identification of isolates, and in molecular ecology (Hadrys et al., 1992). For plant pathogenic fungi, RAPD analysis provided markers to differentiate races A, 3 and 4 of *F. oxysporum* f. sp. *vasinfectum* (Assigbetse et al., 1994), races 0, 2 and 1,2y of *F. oxysporum* f. sp. *melonis* (Namiki et al.,1998) and races 0, 1B/C, 5, and 6 of *F. oxysporum* f. sp. *ciceris* (Jiménez-Gasco et al., 2001).

Amplified Fragment Length Polymorphism (AFLP) is a PCR-based fingerprinting technique (Vos et al., 1995) that has been used for the genetic mapping of *Mycosphaerella graminicola* (Kema et al., 2002). Based on its ability to generate large numbers of polymorphisms and the consistency of fragment amplification by PCR, the AFLP technique have been widely used for the characterization of individual isolates within complex genera such as *Colletotricum* (O'Neill et al., 1997), *Septoria tritici* (Schnieder et al., 1998) and *Leptosphaeria maculans* (Pongam et al., 1999). Recently, AFLP analysis was used to study the genetic relationships within and between natural populations of five *Fusarium* spp. (Abdel-Satar et al., 2003).

Little is known about the genetic complexity of the *Fol* populations in Algeria or about the origin and spread of the pathogen, i.e., whether it was introduced as a single lineage in one location or in several locations or arose independently. The objective of this study was to determine the utility of two rapid PCR-based approaches, RAPD and AFLP markers to determine the genetic structure of a collection of isolates obtained from diseased lentils in Algeria, their relation to pathogenicity (aggressiveness and virulence), considering that genetic variation could indicate variability of the local population of *Fol*. A partial account of this work has previously been published (Belabid and Fortas, 2002).

MATERIALS AND METHODS

Fungal isolates, plant cultivars and pathogenicity test

Thirty-two isolates of *Fol* were obtained from wilted lentil plants collected from major lentil growing areas in north -western region of Algeria (Belabid et al., 2000). The fungus was isolated from the stem of the wilted plant as described by Bayaa et al. (1994). The geographical origin of the isolates is presented in Table 1. The isolates collected were identified according to identification keys of *F. oxysporum* (Nelson et al., 1983). The *formae speciales lentis* was defined by inoculation of the isolates on a very susceptible lentil line (ILL 4605). All isolates were single-spored and stored at 5°C in the dark in tubes containing Difco potato dextrose agar (PDA). Inoculum was prepared as described by Belabid and Fortas (2002) and spore suspensions were adjusted to 2.5×10^6 microconidia/ml.

The *in vitro* pathogenicity test was carried out in the laboratory on five lentil lines (ILL 5588, ILL 5883 – resistant and ILL 1939, L692-16-1 – susceptible) differing in susceptibility to the wilt fungus. These were obtained from the Lentil International *Fusarium* Wilt Nursery of the International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo-Syria. In addition, a local lentil line (Métropole-moderately susceptible) was obtained from the Technical Institute of Greats Cultures (I.T.G.C.) Tiaret-Algeria. Seeds were surface–sterilized using 2% sodium hypochlorite for 3 min, rinsed in sterile water and germinated (10 seeds/pot) for 2 weeks in plastic pots containing 500 g of sterilized sand. Pots were then placed in an incubator set at 20°C and 12/12h light/dark; and irrigated weekly. The 15-days old seedlings were carefully uprooted and the roots washed under running tap water to remove excess sand. Root tips around 0.5 cm long were cut off with sterilized scissors to facilitate the entry of the pathogen into the roots. The roots of the seedlings were then dipped into 10 ml of the inoculum for 10 min and introduced in 70 ml of Hoagland liquid medium in tubes. The seedling stems were wrapped with cotton and parafilm to plug the tube. As a control, seedlings with similarly prepared root tips were dipped in sterilized distilled water for 10 min prior to their introduction in tubes containing Hoagland solution. The tubes were incubated under similar conditions as previously stated. The experiment was a split plot design with 5 replications.

The disease severity was recorded starting from the 5^{th} day after inoculation and continued for three weeks at 2-days interval using a 1-9 scale (Bayaa et al., 1995). After the final score, re-isolation of the fungus from the stem just above the crown was performed and the fungus was maintained on PDA. ANOVA of the disease scores (number of days between inoculation and death of seedlings) was carried out using the Newman-Keuls test (Dagnelie, 1975).

Mycelium production and DNA extraction

Fungal isolates were grown in a 125 ml Erlenmeyer flasks containing 75 ml liquid culture of Lentil-dextrose. Each Erlenmeyer was inoculated with one mycelial plug (5 mm diameter) taken from a 5-day old culture raised on PDA medium. The cultures were incubated at 22°C for 10 days in the dark. The mycelium was washed in deionized sterile water, dried with filter paper, freeze-dried for 48 h, and stored at - 45°C until use. DNA was extracted following the procedure described by Saghai-Maroof et al. (1984), dissolved in 0.5 ml TE buffer, treated with RNAase to degrade the RNA and stored at + 4°C until use. DNA was quantified using a spectrophotometer (Beckman DU-65). The quality of the extracted DNA was visually checked on 1.6% agarose gels.

Table 1. Isolates of *Fusarium oxysporum* f. sp. *lentis* used in this study, their origin and aggressiveness on lines L692-16-1 and ILL 1939.

Isolates	Department	Geographic origin	Host Genotype	Disease Development (days) *
1	Mascara	Tliouanet 1	Unknown	13.8 b
2	Mascara	Tliouanet 2	Unknown	13.2 b
3	Tiaret	Ain Meriem 1	Métropole	8.4 a
4	Tiaret	Ain Meriem 2	Métropole	13.2 b
5	Tiaret	Ex ITGC	Belkan 755	8.1 a
6	Tiaret	Ex ITGC	Belkan 755	7 a
7	Tiaret	Domaine Bouakez 1	Belkan 755	13.2 b
8	Tiaret	Domaine Bouakez 2	Belkan 755	8.2 a
9	Tiaret	Sebbaine 1	Syrie 229	14 b
10	Tissemsilt	Amouri 1	Belkan 755	8.8 a
11	Tissemsilt	Amouri 2	Belkan 755	8.6 a
12	Tissemsilt	Amouri 3	Belkan 755	8.3 a
13	Tissemsilt	Ouled Bessam 1	Métropole	8.6 a
14	Tissemsilt	Ouled Bessam 2	Métropole	14.3 b
15	Tissemsilt	Ouled Bessam 3	Métropole	8.6 a
16	Sidi-belabes	Tirmen	Unknown	19.4 c
17	Sidi-belabes	ITGC 1	Flip 88	21.4 c
18	Sidi-belabes	ITGC 2	Flip 88	24 c
19	Ain temouchent	Oulhassa	Unknown	20.3 c
20	Ain temouchent	Tedmaya	Unknown	22.6 c
21	Sidi-belabes	Lamtar	ILL 4400	15.8 b
22	Sidi-belabes	Tellagh	ILL 4400	16.6 b
23	Sidi-belabes	Sfizef	Syrie 229	10.5 a
24	Mascara	El-bordj	Syrie 229	10.4 a
25	Mascara	Mamounia	Syrie 229	10.1 a
26	Mascara	Sidi kada	Syrie 229	15.8 b
27	Mascara	F. expérimentale	ILL 1939	10.2 a
28	Mascara	Froha	ILL 4400	15.8 b
29	Tissemsilt	Tasslamt 1	Métropole	8.8 a
30	Tissemsilt	Tasslamt 2	Métropole	8.8 a
31	Tiaret	Domaine Bouhadjar	Métropole	8.6 a
32	Tiaret	Sebbaine 2	Syrie 229	13.8 b

The two susceptible lines (L692-16-1 and ILL 1939) were scored 9 (susceptible) with all isolates and the resistant lines (ILL 5883 and ILL 5588) were score 1 (resistant) with all isolates.
*Means of days between inoculation and death of seedlings obtained on the two susceptible lines (L692-16-1 and ILL 1939). Means columns followed by the same letter are not significantly different ($p < 0.05$) according to Newman and Keuls test.

RAPD analysis

The protocol of Williams et al. (1990) was employed with minor modification. RAPD and AFLP analysis was conducted using a Perkin Elmer 9600 thermocycler. The reaction mixture (23 µl) containing 1x PCR buffer (10mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50mM KCl, 0.1mg/ml gelatine), 200 µM each of dNTPs, 2.5 units/100µl *Taq* DNA polymerase (all reagents were from Boehringer Mannheim), 0.2 µM of primer (Operon Technologies, Inc. Du Pont) and 25 ng of extracted DNA. The mixtures were subjected to the following conditions: hold at 94°C for 2 min for starting, followed by 40 cycles of 92°C for 1 min, 36°C for 1 min and 72°C for 2 min, and a final hold for 72°C for 5 min. A total of six decamer primers that gave robust amplification were selected for this study (Table 2). The PCR products were electrophoretically separated in a 1.6% agarose gel in Tris-Acetate-EDTA buffer, visualized by ethidium bromide and photographed under UV light (Eagle Eye II, Still Video System).

AFLP analysis

The AFLP protocol was carried out as reported by Vos et al. (1995) with slight modifications. Aliquots of 1.5 µg DNA were digested with a combination of 5U *Mse* I and 5U *Pst* I in 40 µl of restriction buffer (10 mM Tris-acetate pH 7.5, 10 mM $MgCl_2$, 50 mM potassium acetate, 5 mM dithiothreitol (DTT), 1 mM ATP and 50 ng/µl BSA) and incubated for 3 h at 37°C. The digested DNA was ligated by

Table 2. Polymorphism and genetic similarity obtained by RAPD and AFLP primers.

Markers	Primers	Sequences 5'- 3'	Amplified fragments	Polymorphic Fragments	Genetic Similarity (%)
RAPD	OPC-14	TGCGTGCTTG	6	2	92.0
	OPC-20	ACTTCGCCAC	4	1	92.2
	OPF-11	TTGGTACCCC	5	2	92.2
	OPF-16	GGAGTACTGG	2	1	93.8
	OPG-18	GGCTCATGTG	3	1	95.3
	OPY-10	CAAACGTGGG	2	1	96.1
AFLP	P16/M11	P-cc /M-aa	106	33	84.5
	P11/M13	P-aa /M-ag	87	28	82.2
	P13/M11	P-ag/M-aa	94	32	82.7

adding 10 µl of ligation mixture (50 pmol of *Mse* I-adapter, 5 pmol of *Pst* I-adapter, 1U of T4 DNA-ligase, 1mM ATP, 1 x ligation buffer) and incubated as described before. The ligated DNA was diluted 1:5 with distilled water. 2 µl of ligated DNA was added to a reaction mixture containing 50 ng primer *Mse* I (M), 50 ng primer *Pst* I (P), 1 x PCR buffer, 0.2 mM of dNTPs and 1U of *Taq* DNA polymerase. Three primer combinations were tested for the amplification: P-cc/M-aa, P-aa/M-ag, P-ag/M-aa (Boehringer Mannheim). The PCR amplification temperature profile was one cycle at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min and then the annealing temperature was lowered during each cycle by 0.7°C for 12 cycles. The remainder of the amplification was 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. Each sample was diluted 1:1 with loading buffer, the amplified fragment were analysed on 6% polyacrylamide sequencing gel in Tris-Borate-EDTA buffer. Gels were visualized by silver staining method (Bassam et al., 1991) and then photographed.

Data analysis

Comparison of each profile for each primer was done on the basis of the presence versus absence (1/0) of RAPD and AFLP fragments of the same molecular weight. Fragments of the same molecular weight were scored as identical. For the AFLP, bands between 517 and 51 bp were scored. A binary matrix combined all the data records for all isolates. The numerical taxonomic software package NTSYS-pc, version 1.80 (Rohlf, 1993) was used to order the isolates by unweighted paired group method with arithmetic averages (UPGMA) based on Jaccard's similarity coefficient (Sneath and Sokal, 1973).

RESULTS

Pathogenicity test

Typical symptoms of lentil *Fusarium* wilt were observed when seedlings of L692-16-1 and ILL 1939 (susceptible lines) when inoculated. Foliage alterations of the inoculated seedlings included sudden drooping of leaflets starting from the apical part and progressing downward and final wilting of the whole plant. The leaflets breaches did not fall prematurely and remained fixed on the stem. Yellowing and drying of the stem began at stage 5 and progressed from the apex to the base. Complete death of the susceptible seedlings (L692-16-1 and ILL 1939) occurred during the 3rd week. On the other hand, no symptom was recorded on seedlings of the resistant lines (ILL 5883 and ILL 5588) . The line Métropole was moderately susceptible, with a rating between 4.6 and 5.4 with the different isolates tested.

The disease development on L692-16-1 and ILL 1939 revealed variability in aggressiveness amongst isolates. The period between inoculation and death of seedlings varied from 7 to 24 days, showing significant differences at $P < 0.05$. Based on this character (aggressiveness), the isolates were classified in 3 groups; highly, moderately and weakly aggressive (Table 1). The highly aggressive isolates (50%) caused drying and death of seedlings in 7 to 11 days period. They were mainly collected from Tissemsilt and Tiaret. The moderately aggressive isolates (34.3%) lead to seedling mortality in 12 to 17 days. All isolates in this group were from Mascara and Tiaret. The weakly aggressive isolates (15.6%) caused seedling death within 18 to 24 days. These isolates were all from Ain Témouchent and Sidi-Belabbes.

Polymorphism and genetic similarity between *Fol* isolates revealed by RAPD and AFLP combined markers

RAPD analyses of DNA from all 32 *Fol* isolates using the six oligonucleotides primers listed in Table 2 produced a total of 22 bands. 8 RAPD fragments showed polymorphism. The number of bands scored using the 6 primers ranged between 6 (OPC-14) and 2 (OPF-16, OPY-10). The number of polymorphic fragments per primer was 1.3. The size of amplification DNA fragments generated with the RAPD primers ranged from 0.2 to 2.1 kb. The similarity percent revealed by each RAPD primer oscillated between 92 (OPC-14) and 96 (OPY-10).

The AFLP primers produced a total of 287 bands for the 32 *Fol* isolates. Among them, 93 AFLP fragments were

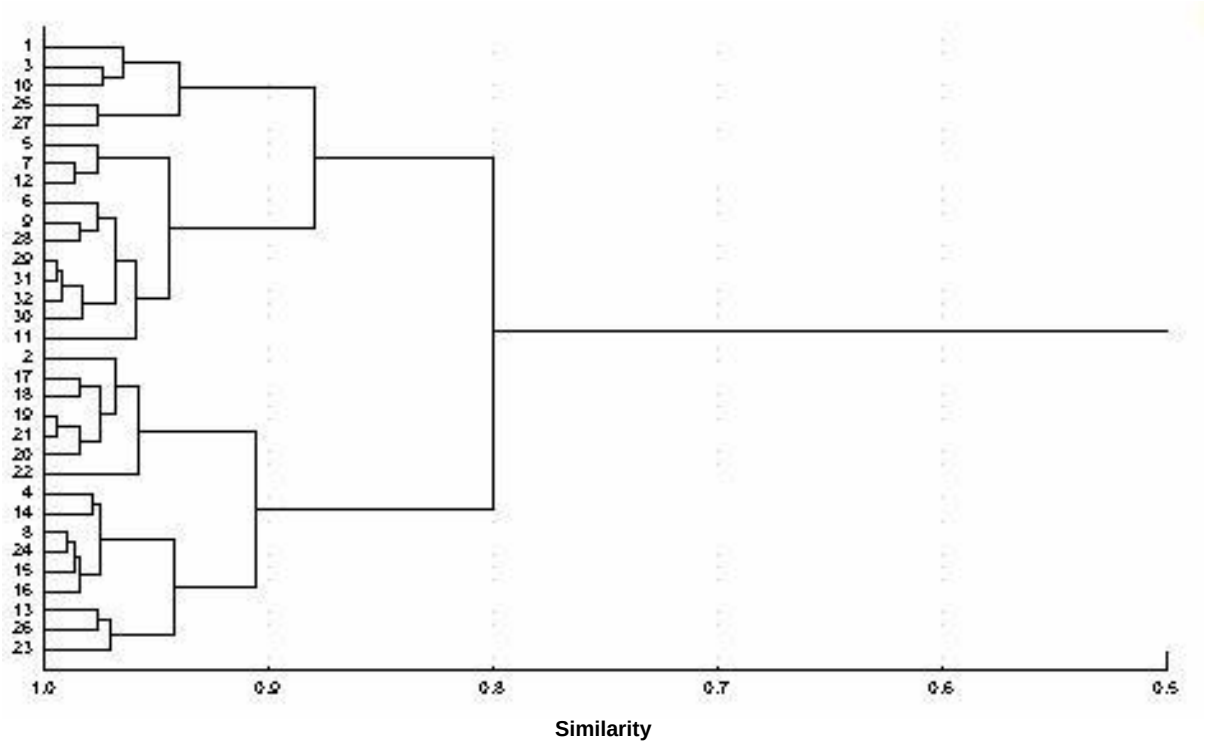

Figure 1. Dendrogram derived from cluster analysis (UPGMA) showing relationship among the 32 *Fusarium oxysporum* f. sp. *lentis* isolates listed in Table 1. Genetic similarity was obtained by RAPD and AFLP markers combination, using the Jaccard similarity coefficient.

polymorphic, with an average of 31 polymorphic bands per primer combinations. The similarity percent revealed by AFLP primer pair oscillated between 82 and 84% (Table 2).

DNA fingerprinting of 32 *Fol* isolates with RAPD and AFLP-primers combined were analyzed by Jaccard similarity coefficient to produce a dendrogram. At a genetic similarity of 0.8, UPGMA analysis of the AFLP and RAPD banding patterns separated the isolates into two distinct clusters (Figure 1).

The first group, AFLP-RAPD I, included 16 isolates (27, 12, 7, 32, 31, 30, 29, 11, 6, 9, 28, 5, 3, 10, 25 and 1). Isolates of this group originated from: Tiaret (7 isolates), Tissemsilt (5 isolates) and Mascara (4 isolates). The genetic similarity between the isolates of this group was between 0.98 and 0.88. The isolates of this group were aggressive (11 isolates) or moderately aggressive (5 isolates).

The second group, AFLP-RAPD II, included another 16 isolates (2, 23, 26, 16, 21, 24, 17, 19, 20, 18, 22, 4, 8, 15, 14 and 13). Isolates of this group originated from: Mascara (3 isolates), Ain Témouchent (2 isolates), Sidi-Belabbes (6 isolates), Tissemsilt (3 isolates) and Tiaret (2 isolates). The genetic similarity between the isolates of this group was between 0.98 and 0.90. Isolates of this group were aggressive (5 isolates), moderately aggressive (6 isolates) or weakly aggressive (5 isolates), all weakly aggressive isolates studied clustered in this group.

DISCUSSION

The pathogenicity study showed that the behaviour of *Fol* isolates was homogeneous, with no variations in virulence. It appeared therefore that the isolates studied were homogeneous for the character considered. It was concluded that there is only one race amongst Algerian isolates of *Fol*. Similarly, Erskine and Bayaa (1996) and Bayaa et al. (1997) have demonstrated that no physiological races of *Fol* exist in Syria. Nevertheless, the variability of aggressiveness of *Fol* has been determined in this study by conventional pathogenicity test. The population studied exhibits different aggressiveness levels. Similar results on the same *formae speciales* were previously obtained by Abbas (1995) in Syria. This study also showed that 50% of isolates are highly aggressive and 34% are moderately aggressive, distributed almost in all Algerian lentil producing areas (north-west). This represents an important risk to the planting of local lentil lines, because of their moderately susceptible (cases of Métropole) or susceptible reaction (cases of Syria 229) to *Fol*. The totality of aggressive isolates studied originates from Tissemsilt (44%) and Tiaret (31%). The most aggressive isolates likely evolved through a process of adaptative mutation (Hall, 1990).

The occurrence of a single genotype over a large area, with increasing frequency may suggest that the aggressive genotype has migrated, most probably through infected seed. *Fol* inoculum can spread from one

region to another via external contamination of the lentil seeds (Setti and Bouznad, 1998). The exchange of contaminated seeds between farmers has probably contributed, to the creation of a homogeneous population of *Fol*. Wider geographical distribution and stability of the most predominant genotype further supports the view that the genotype of this fungus is more stable, and that parasexual recombination plays apparently no or only a minor role in the evolution of this pathogen. In addition, it has to be added that selection also influence genotype diversity because genotype that have highest fitness increase in frequency over time. No sexual stage is yet known for *F. oxysporum* (Nelson et al., 1983).

Results obtained demonstrate that genetic variability exists among Algerian *Fol* isolates as identified by RAPD and AFLP markers. The pathogen can be divided in two genetic subpopulations and can be characterized by a low polymorphism marker among isolates of the subpopulations. The data suggest that the isolates of *Fol* are derived probably from two genetically distinct clones. Similar results were also obtained with *F. oxysporum* f. sp. *erythroxyli* (Nelson et al., 1997). The limited genetic variability observed among isolates of *Fol* as indicated by RAPD analysis would be expected for a pathogen that became widespread relatively quickly as a result of an increase in production of the host plant (i.e. distribution by seed). This also assumes that the DNA markers used are sufficiently variable to distinguish between clonal lineage of *Fol*. RAPD and AFLP markers were diagnostic for all two clones in north western Algeria, supporting the view that pathotype are stable and composed of discernible clonal lineages.

Two PCR-based systems indicated that the isolates exist in two subpopulations in Algeria independent of geographic location, our result in harmony with these obtained by Namiki et al., (1998), Abd-Elsalam et al. (2002) and Kiprop et al. (2002). This is in contrast to that was reported for *F. oxysporum* f. sp. *vasinfectum* in which a close correlation was observed between RAPD pattern and geographic location (Assigbetse et al., 1994). However, a similar lack of correlation between RAPD pattern and geographic location was reported for *F. oxysporum* f. sp. *pisi* (Pomazi et al., 1994).

Analysis of the same isolates from north-west Algeria by vegetative compatibility grouping (VCG) revealed that all the isolates form a unique VCG-0471 and no incompatibility has been observed between them. This is an indication of the homogeneity of the Algerian *Fol* populations (Belabid and Fortas, 2002). The RAPD-AFLP analysis and VCG analysis are not corroborative. On the other hand, some correlation between RAPD data and VCG data was found for *F. oxysporum* f. sp. *phaseoli* (Woo et al., 1996) and *F. oxysporum* f. sp. *pisi* (Pomazi *et* al., 1994). For *F. oxysporum* f. sp. *cubense*, several VCGs were found within each RAPD group, indicating that although RAPD analysis and VCG analysis may both be a good indication of genetic variability within *formae speciales* of *F. oxysporum,* the same grouping of isolates may not always be achieved with the two methods of analysis (Bentley et al., 1995). The RFLP and RAPD banding patterns of *F. oxysporum* f. sp. *phaseoli* showed a correspondence to the VCGs, but not to the pathogenic races (Woo et al., 1996).

No apparent clustering could be observed between the RAPD and AFLP markers analysis (data not shown) of the *Fol* isolates. Similar results were obtained by Pejic et al. (1998). According to Bohn et al. (1999) correlation between different marker estimates is observed only for genetically related genotypes. Accordingly, correlations between genetic similarities estimates determined with RAPD and AFLP markers and the *Fol* isolates can only be expected if linkage disequilibrium exists between the markers loci in the isolates examined. Results of Namiki et al. (1998) previously mentioned that aggressiveness within races 0 and 2 could not be differentiated by cluster analysis based on DNA fingerprinting and, in some cases, strains of different pathogenic variants were more similar genetically than strains of the same pathogenic variant.

On the basis of our data we conclude that RAPD and AFLP have excellent potential for resolving the population dynamic and the evolution of the *Fol* population. Two fungal clones can be discriminated by DNA fingerprinting, this is not possible by using conventional techniques such as pathogenicity test or evaluation of morphologic or biometric characters. The two PCR-based procedure described in this paper appear to be well adapted for large scale characterization of *F. oxysporum* strains as required by studies of diversity of *Fol* population.

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