Phytopath. medit., 1994, *33*, 223-229

Random Amplified Polymorphic DNA (RAPD) analysis: a tool for rapid characterization of *Fusarium oxysporum* f. sp. *albedinis* isolates?

D. Fernandez and A. Tantaoui *

Laboratoire de Phytopathologie, Institut Français de Recherche Scientifique pour le Développement en Coopération (ORSTOM), Montpellier, France
* Laboratoire de Phytopathologie, Institut National de la Recherche Agronomique (INRA), Marrakech, Maroc

Summary. The usefulness of Random Amplified Polymorphic DNA (RAPD) was examined as a potential tool to differentiate *Fusarium oxysporum* f. sp. *albedinis* from other *F. oxysporum* associated with wilted date palm. A molecular study was carried out on a set of 17 isolates of *F. oxysporum* from wilted palms or from the rhizosphere, collected in three palm groves. Three out of the twenty primers tested revealed scorable polymorphisms between the strains, indicating that isolates could be separated into three groups. The first one, RAPD1, only grouped isolates of *F. oxysporum* f. sp. *albedinis*. The two others, RAPD2 and RAPD3, contained all *F. oxysporum* isolates collected from date palm roots or the rhizosphere, regardless of their origin. These data showed that the RAPD technique allows differentiation of *F. oxysporum* f. sp. *albedinis* from other *F. oxysporum* associated with wilted date palms. DNA random amplification is thus a promising tool for evaluating genetic diversity within *F. oxysporum* populations in infested palm grooves.

Introduction

Bayoud is a vascular wilt of date palm caused by *Fusarium oxysporum* f. sp. *albedinis*, widely distributed in all date palm growing areas of Morocco and of the western parts of Algeria. Control is achieved by prevention measures to slow down the disease spread. The search for resistant cultivars is also in progress in Morocco. Part of this program deals with characterization and identification of the pathogen which is known to exhibit variation in colony morphology and pathogenicity (Djerbi *et al.*, 1985, 1985a).

Vegetative compatibility and RFLP (Restriction Fragment Length Polymorphism) analyses allowed differentiation between pathogenic and non-pathogenic strains of *F. oxysporum*. Using vegetative compatibility analysis, Djerbi and Den Braber (1990) showed that isolates of *F. oxysporum* f. sp. *albedinis* could be distinguished from isolates of other *formae speciales*. In addition, Tantaoui and Boisson (1991) gave evidence that isolates of *F. oxysporum* f. sp. *albedinis* from leaves of wilted trees belonged to a vegetative compatibility group (VCG1) distinct from those obtained with other *F. oxysporum* isolates collected from date-palm roots or rhizosphere (VCG2, 3 and 4). Such grouping was also confirmed by restriction fragment length polymorphism (RFLP) analysis that revealed the occurrence of a characteristic mitochondrial DNA (mtDNA) haplotype in *F. oxysporum* f. sp. *albedinis* isolates (Tantaoui and Fernandez, 1993).

Recent advances in genetic research have included the development of an adaptation of the polymerase chain reaction (PCR) known as random amplified polymorphic DNA (RAPD) technique (Williams *et al.*, 1990) or arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990). In phytopathogenic fungi, the RAPD technique has been successfully used to compare strains at the intraspecific level and the genetic variation was

related to the host specialization (Assigbetse *et al.*, 1994; Crowhurst *et al.*, 1991; Goodwin and Annis, 1991; Grajal-Martin *et al.*, 1993; Guthrie *et al.*, 1992; Haemmerli *et al.*, 1992; Hamelin *et al.*, 1993; Manulis *et al.*, 1994; Schäfer and Wöstemeyer, 1992).

The aim of this study was to investigate the usefulness of the RAPD technique to detect genomic DNA polymorphisms among *F. oxysporum* isolates from palm groves and to provide a rapid method for characterization of *F. oxysporum* f. sp. *albedinis*. We applied this technique to the same set of strains used in previous studies (Tantaoui and Boisson, 1991; Tantaoui and Fernandez, 1993). We report here the preliminary results obtained by screening twenty primers. RAPD analysis proved highly effective in separating *F. oxysporum* f. sp. *albedinis* from other *F. oxysporum* collected from infested palm groves.

Material and methods

I s o l a t e s . Reference, origin, VCG and mtDNA RFLP groups of the isolates are listed in Table I. Geographic origin, colony morphology and pathogenicity of the isolates were previously reported by Tantaoui and Boisson (1991). The four *F. oxysporum* f. sp. *albedinis* isolates originated from three Moroccan palm groves and are highly virulent. The other thirteen *F. oxysporum* originated from a same palm grove and were shown to be weakly or not virulent on date palm tree. All cultures were single-spored, and maintained on potato dextrose agar slants.

D N A e x t r a c t i o n . Isolates were grown in 200 ml of GYP medium (glucose 2%, yeast extract 0,5% and peptone 0,5%) for 5 days at 25°C. The mycelium was harvested by filtration and freeze-dried for 48 h. Total DNA extraction was

TABLE I. - Reference, source, geographic origin, VCG, mtDNA RFLP group of the seventeen *F. oxysporum* isolates tested and RAPD group determined in this study.

Reference	Source of isolation	Geographic origin	VCG (*a*)	RFLP group (*b*)	RAPD group
Fusarium oxysporum f. sp. *albedinis* :					
127	palm tree's leaf	Ziz valley	1	1	1
BFG1	palm tree's leaf	Zagora	1	1	1
BSL1	palm tree's leaf	Zagora	1	1	1
SLY	palm tree's leaf	Tata	1	1	1
Fusarium oxysporum :					
ZL14	rhizosphere	Zagora	2	2	2
ZL29	rhizosphere	Zagora	2	2	2
ZL4	rhizosphere	Zagora	2	2	2
ZL19	rhizosphere	Zagora	2	2	2
IZ197	rhizosphere	Zagora	2	2	2
AZ4	rhizosphere	Zagora	2	2	2
A15	palm tree root	Zagora	3	2	2
A16	palm tree root	Zagora	3	2	3
H3	rhizosphere	Zagora	3	2	3
H4	rhizosphere	Zagora	3	2	3
ZC15	rhizosphere	Zagora	4	2	3
ZC17	rhizosphere	Zagora	4	2	3
ZC21	rhizosphere	Zagora	4	2	2

(*a*) Vegetative compatibility group determined by Tantaoui and Boisson (1991).
(*b*) RFLP group determined by Tantaoui and Fernandez (1993).

performed by the Lee *et al.* (1988) miniprep procedure and the DNA was dissolved in TE buffer (Tris HCl 10 mM pH 7,5; 0.1 mM EDTA) to a final concentration of 5 ng μl^{-1}. Quantification of DNA was done by using UV absorbance at 260 nm.

RAPD primers. The primers used are listed in Table II. All primers were random 10-base oligomers (Kit F, Operon Technologies, Alameda, California, USA).

RAPD assay. Amplification reactions were performed according to a modification of the protocol reported by Williams *et al.* (1990). Each 25 µl reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatin, 50 µM of each dATP, dCTP, dGTP and dTTP, 15 picomoles of primer, 25 ng of genomic DNA, and 1U of *Taq*DNA polymerase (Promega, France). Negative controls (no DNA template) were done in every experiment to test for the presence of contamination. The amplification was performed with a DNA thermal cycler (PHC-3, Techne, USA) programmed as follows: 1 cycle for 5 min at 95°C (before the addition of the *Taq*DNA polymerase), followed by 45 cycles of 1 min at 94°C, 1 min at 34°C, 2 min at 72°C. A cycle with 15 min at 72°C was conducted after the 45 cycles. After the reaction, 20 µl of the amplification products were separated by electrophoresis on 1.4% agarose gel stained with ethidium bromide and then photographed under UV light.

RAPD assays were performed at least twice for each isolate with each primer in order to ensure that amplification patterns were reproducible.

Cluster analysis. Comparison of each profile for each primer was done on the basis of the presence (1) versus absence (0) of RAPD products of the same length. Bands of the same length were scored as identical. Analyses were based on Simple Matching index (Sokal and Michener, 1958) which measures proportion of common discrete data (either 0 or 1) between the isolates. A dendrogram was derived from the distance matrix by using the UPGMA algorithm (Sneath and Sokal, 1973) contained in the computer program package Phylip 3.4 (developed by J. Felsenstein, Dept Genetics, University of Washington, U.S.A., in 1991).

TABLE II. - List and sequence of the twenty primers used in this study.

Code	Sequence 5' to 3'
OPF-01	ACGGATCCTG
OPF-02	GAGGATCCCT
OPF-03	CCTGATCACC
OPF-04	GGTGATCAGG
OPF-05	CCGAATTCCC
OPF-06	GGGAATTCGG
OPF-07	CCGATATCCC
OPF-08	GGGATATCGG
OPF-09	CCAAGCTTCC
OPF-10	GGAAGCTTGG
OPF-11	TTGGTACCCC
OPF-12	ACGGTACCAG
OPF-13	GGCTGCAGAA
OPF-14	TGCTGCAGGT
OPF-15	CCAGTACTCC
OPF-16	GGAGTACTGG
OPF-17	AACCCGGGAA
OPF-18	TTCCCGGGTT
OPF-19	CCTCTAGACC
OPF-20	GGTCTAGAGG

Results

Screening of the primers revealing polymorphisms. Primers were first screened for usefulness in detecting variability between strains. In a first experiment, two strains were randomly chosen: 127 (*F. oxysporum* f. sp. *albedinis*) and ZL14 (*F. oxysporum* from rhizosphere). Amplification reactions were performed with nineteen primers and 15 of these gave amplification products (Fig. 1). The size of the amplified DNA fragments ranged from 0.4 to 2.1 kb. The number of bands in the profiles varied, depending on the primer and the isolate tested. It ranged from 1 (primer OPF-03) to 7 (primer OPF-09). Nine of the 15 amplifying primers revealed polymorphisms between strains 127 and ZL14,

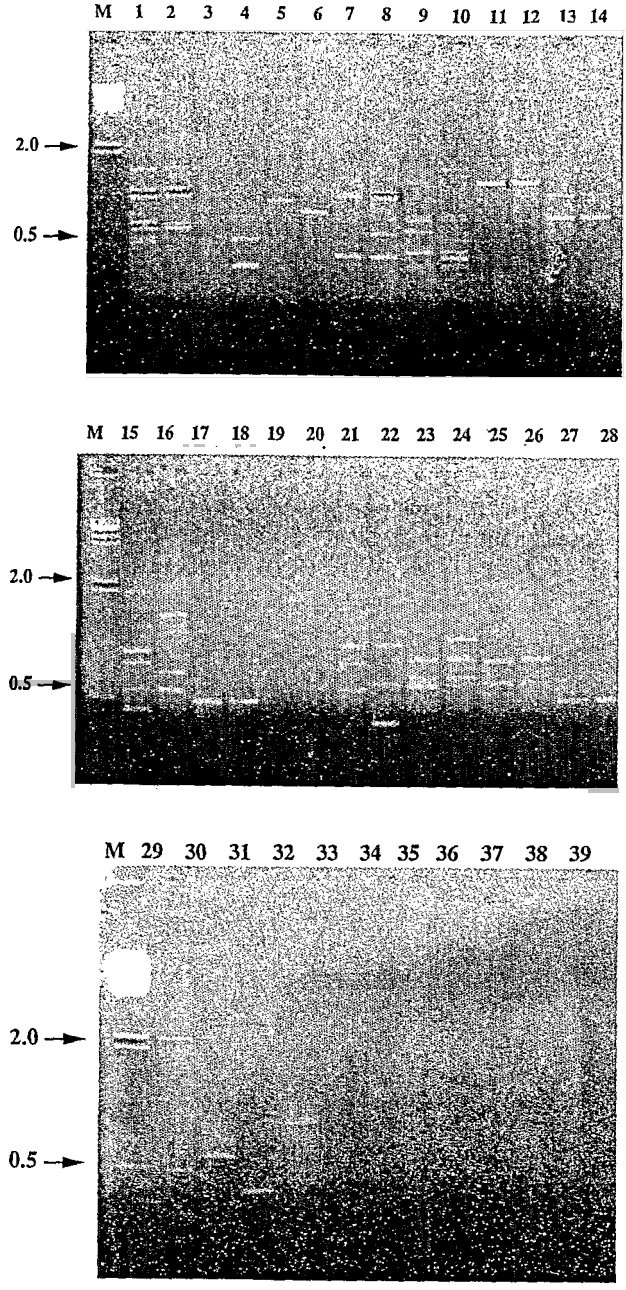

Fig. 1 - Electrophoretic patterns of amplified DNA fragments obtained in RAPD experiment with 19 primers on isolates 127 (*F. oxysporum* f. sp. *albedinis*, impair lanes) and ZL14 (*F. oxysporum*, pair lanes). Key to primers: (1-2) OPF-01, (3-4) OPF-02, (5-6) OPF-03, (7-8) OPF-04, (9-10) OPF-06, (11-12) OPF-07, (13-14) OPF-08, (15-16) OPF-09, (17-18) OPF-10, (19-20) OPF-11, (21-22) OPF-12, (23-24) OPF-13, (25-26) OPF-14, (27-28) OPF-15, (29-30) OPF-16, (31-32) OPF-17, (33-34) OPF-18, (35-36) OPF-19, (37-38) OPF-20. Lane 39: no DNA template, OPF-01. M = molecular-weight marker (lambda phage cleaved with *Eco*RI). Arrows indicate molecular length in kb.

and at least 3 of them (OPF-03, OPF-12 and OPF-13) unambiguously distinguished the two strains. The other primers gave rise to complex bands profiles with varying intensity (Fig. 1).

In order to assess whether polymorphisms detected by primers OPF-03, OPF-12 and OPF-13 were due to real changes in DNA sequences rather than to variations in DNA template concentration, we tested various DNA amounts (from 1 ng to 50 ng) of one strain with each of the three primers. Data showed that DNA template concentrations affected only DNA band intensity and not the amplified product number (data not shown). These three primers (OPF-03, OPF-12 and OPF-13) were then chosen for further analysing our set of strains.

RAPD analysis with primers OPF-03, OPF-12 and OPF-13. Two or three distinct patterns of amplified products were obtained for each primer among the seventeen isolates. Primers OPF-03 and OPF-12 displayed the same polymorphic patterns between *F. oxysporum* f. sp. *albedinis* and *F. oxysporum* isolates as previously observed (Fig. 1), whereas primer OPF-13 revealed polymorphisms among *F. oxysporum* isolates (Fig. 2). Amplified DNA fragments were recorded for each isolate with each primer and by combining the 3 primers, 13 distinct amplified products were obtained. Table III shows the RAPD phenotype assigned to each isolate. The dendrogram obtained by analysis of combined data by simple matching coefficient (Sneath and Sokal, 1973; Sokal and Michener, 1958) clearly separated

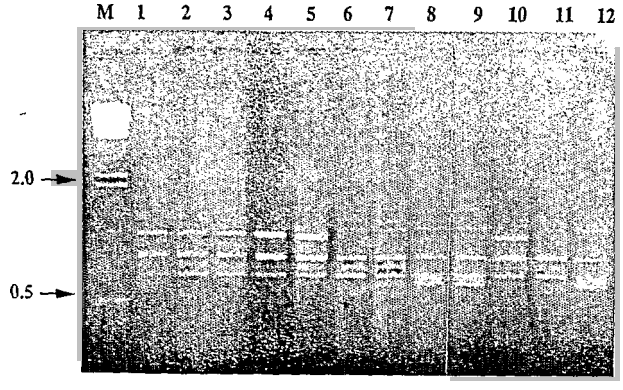

Fig. 2 - Three distinct electrophoretic patterns of amplified DNA fragments in RAPD experiments with primer OPF-13. *F. oxysporum* f. sp. *albedinis:* RAPD group 1 (lanes 8, 9 and 12); *F. oxysporum:* RAPD group 2 (lanes 1, 2, 3, 4, 5 and 10) and RAPD group 3 (lanes 6, 7 and 11). Arrows indicate molecular length in kb.

TABLE III. - RAPD phenotype obtained for each isolate with primers OPF-03, OPF-12 and OPF-13, and RAPD group consequently determined. Amplified DNA fragments were recorded for each isolate with each primer and coded either as present (1) or absent (O).

Isolate	RAPD phenotype			RAPD group
	OPF-03	OPF-12	OPF-13	
127	1O	11OO	1O1O111	1
BFG1	1O	11OO	1O1O111	1
BSL1	1O	11OO	1O1O111	1
SLY	1O	11OO	1O1O111	1
ZL14	O1	1O11	O11O1OO	2
ZL29	O1	1O11	O11O1OO	2
ZL4	O1	1O11	O11O1OO	2
ZL19	O1	1O11	O11O1OO	2
IZ197	O1	1O11	O11O1OO	2
AZ4	O1	1O11	O11O1OO	2
A15	O1	1O11	O11O1OO	2
A16	O1	1O11	OO111OO	3
H3	O1	1O11	OO111OO	3
H4	O1	1O11	OO111OO	3
ZC15	O1	1O11	OO111OO	3
ZC17	O1	1O11	OO111OO	3
ZC21	O1	1O11	O11O1OO	2

F. oxysporum f. sp. *albedinis* isolates from the other *F. oxysporum* analysed (Fig. 3). Three groups were differentiated in our set of strains: RAPD1 was restricted to the four *F. oxysporum* f. sp. *albedinis* isolates, RAPD2 included the six isolates from VCG2, A15 (VCG3) and ZC21 (VCG4), whereas RAPD3 contained isolates A16, H3 and H4 from VCG3 and isolates ZC15 and ZC17 from VCG4.

Discussion and conclusion

This preliminary study was done to investigate the potential of the RAPD technique to detect polymorphisms among *F. oxysporum* isolated in Morocco's wilt diseased palm groves, in order to estimate the amount of genetic variability within the sampled population and to provide diagnostic markers for differentiating *F. oxysporum* f. sp. *albedinis* isolates.

By screening twenty primers and by testing three of them which displayed scorable polymorphisms, we showed that RAPD analysis allowed differentiation of *F. oxysporum* f. sp. *albedinis* isolates from *F. oxysporum* isolated from either palm roots or rhizosphere. Three distinct RAPD groups were obtained among the seventeen isolates, the first one (RAPD1) grouping only *F. oxysporum* f. sp. *albedinis* isolates, RAPD2 and RAPD3 groups including others *F. oxysporum* independently of their respective isolation source.

RAPD analysis allowed us to detect a level of polymorphism among the strains higher than that obtained by the analysis of mtDNA RFLP (Tantaoui and Fernandez, 1993). However, this approach did not confirm the genetic grouping obtained by vegetative compatibility (Tantaoui and Boisson, 1991). The first RAPD group contained *F. oxysporum* f. sp. *albedinis* isolates which were

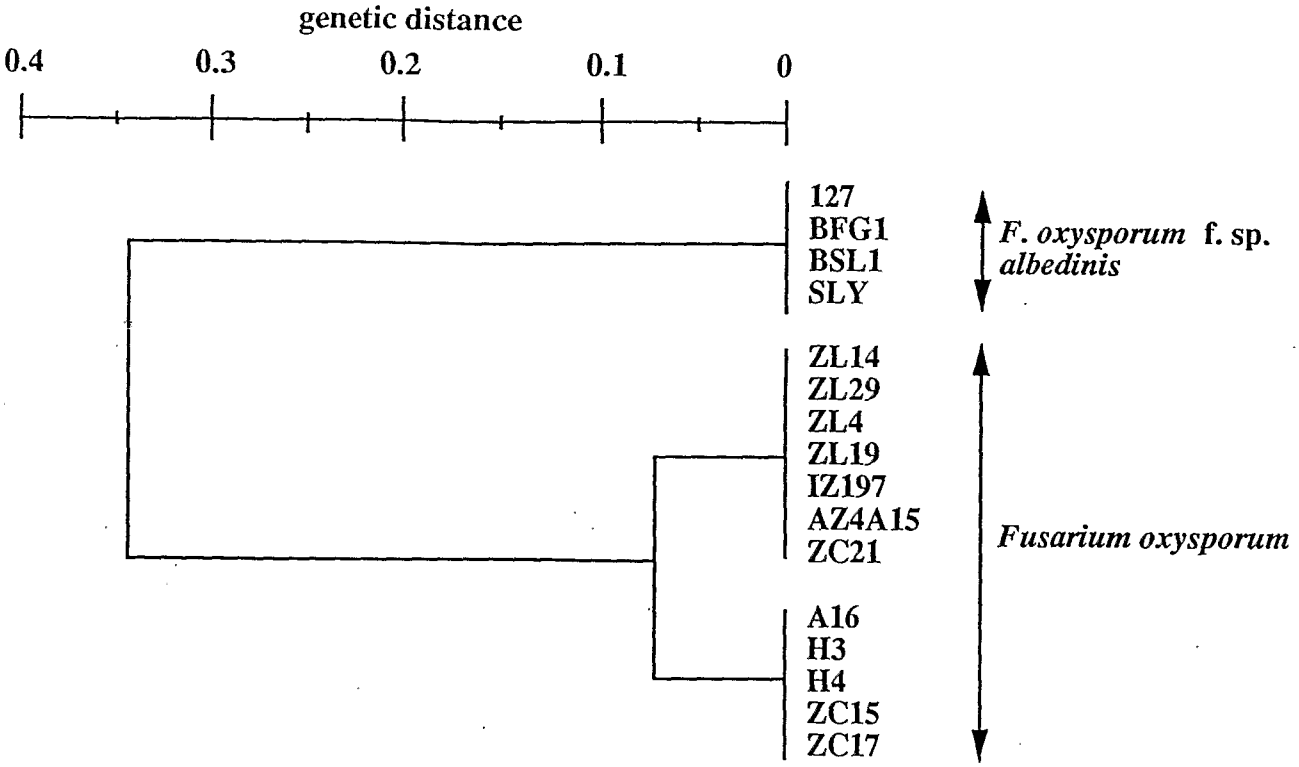

Fig. 3 - Dendrogram showing relationships among the 17 *Fusarium oxysporum* isolates. Genetic distances were obtained by random amplified polymorphic DNA analysis with 3 primers.

previously classified into a single VCG and gave the same mtDNA haplotype. The two other groups, RAPD2 and RAPD3, corresponded to *F. oxysporum* isolates previously separated into three vegetative compatibility groups (VCGs 2, 3 and 4) but only displaying a single mtDNA haplotype.

In other phytopathogenic fungi, RAPD analyses have proved useful for detecting genomic polymorphisms directly related to host specialization. Races of a pathogen were distinguished in *F. solani* f. sp. *cucurbitae* (Crowhurst *et al.*, 1991), in several formae speciales of *F. oxysporum* (Assigbetse *et al.*, 1994; Grajal-Martin *et al.*, 1993; Manulis *et al.*, 1994) as well as in *Gremmeniella abietina* (Hamelin *et al.*, 1993). In *F. oxysporum* f. sp. *vasinfectum*, the genetic variation revealed by RAPD technique among a strain collection of worldwide origin (Assigbetse *et al.*, 1994) was comparable to that obtained by examination of ribosomal and mitochondrial DNA RFLPs (Fernandez *et al.*, 1993). In the same way, RAPD amplification profiles allowed differentiation of aggressive and non-aggressive isolates of *Leptosphaeria maculans* (Goodwin and Annis, 1991; Schäfer and Wöstemeyer, 1992) which were previously separated by RFLP analysis (Koch *et al.*, 1991).

Thus, although the potential of the RAPD technique to detect intra-specific variation is at least equivalent to that of RFLP analysis in pathogenic fungi, less genomic material is needed, the technique is fast and no radioactivity is required. Furthermore, the technique can be improved by sequencing diagnostic DNA fragments and synthesizing oligonucleotides to specifically amplify them. This would allow detection and identification of a pathogen from any sample (infected palm tree or soil extract) without the need to purify and grow the fungus.

Data presented here resulted from preliminary analyses on the potential of three distinct techniques to study the genetic structure of *F. oxysporum* population from infected palm grooves. An extensive collection of isolates has been constituted and will be subjected to VCG, mtDNA RFLP and RAPD analyses.

Literature cited

ASSIGBETSE K.B., D. FERNANDEZ, M.P. DUBOIS and J.P. GEIGER, 1994. Differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* races on cotton by random amplified polymorphic DNA (RAPD) analysis. *Phytopathology, 84,* 622-626.

CROWHURST R.N., B.T. HAWTHORNE, E.H.A. RIKKERINK and M.D. TEMPLETON, 1991. Differentiation of *Fusarium solani* f. sp. *cucurbitae* races 1 and 2 by random amplification of polymorphic DNA. *Current Genetics,* 391-396.

DJERBI M. and K. DEN BRABER, 1990. A new method of identification of *Fusarium oxysporum* f. sp. *albedinis* on the basis of vegetative compatibility. *Proc. 8th Congress Mediterranean Phytopathological Union.* Agadir, Morocco, p. 513 (*abstract*).

DJERBI M., M.A. EL GHORFI and M.A. EL IDRISSI AMMARI, 1985. Etude du comportement du Henné (*Lawsonia inermis* L.) et de la Luzerne (*Medicago sativa* L.) et de quelques espèces de palmiers à l'égard du *Fusarium oxysporum* f. sp. *albedinis* agent du bayoud. *Annales Institut National Recherche Agronomique Tunisie, 58,* Note de recherche n° 3, 1-11.

DJERBI M., M.H. SEDRA and M.A. EL IDRISSI AMMARI, 1985a. Caractéristiques culturales et identification du *Fusarium oxysporum* f. sp. *albedinis,* agent causal du bayoud. *Annales Institut National Recherche Agronomique Tunisie, 58,* Note de recherche n°1, 1-8.

FERNANDEZ D., K.B. ASSIGBETSE, M.P. DUBOIS and J.P. GEIGER, 1993. Differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* races on cotton by RFLP and RAPD analysis. *6th International Congress Plant Pathology,* Montreal, p. 134.

GOODWIN P.H. and S.L. ANNIS, 1991. Rapid identification of genetic variation and pathotype of *Leptosphaeria maculans* by random amplified polymorphic DNA assay. *Applied Environmental Microbiology, 57,* 2482-2486.

GRAJAL-MARTIN M.J., C.J. SIMON and F.J. MUEHLBAUER, 1993. Use of random amplified polymorphic DNA (RAPD) to characterize race 2 of *Fusarium oxysporum* f. sp. *pisi. Phytopathology, 83,* 612-614.

GUTHRIE P.A.I., C.W. MAGILL, R.A. FREDERIKSEN and G.N. ODVODY, 1992. Random amplified polymorphic DNA markers: a system for identifying and differentiating isolates of *Colletotrichum graminicola. Phytopathology, 82,* 832-835.

HAEMMERLI U.A., U.E. BRÄNDLE, O. PETRINI and J.M. MCDERMOTT, 1992. Differentiation of isolates of *Discula umbrinella* (teleomorph: *Apiognomonia errabunda*) from beech, chestnut, and oak using random amplified polymorphic DNA markers. *Molecular Plant-Microbe Interactions, 5,* 479-483.

HAMELIN R.C., G.B. OUELLETTE and L. BERNIER, 1993. Identification of *Gremmeniella abietina* races with random amplified polymorphic DNA markers. *Applied Environmental Microbiology, 59,* 1752-1755.

KOCH E. K. SONG, T.C. OSBORN and P.H. WILLIAMS, 1991. Relationship between pathogenicity and phylogeny based on restriction fragment length polymorphism in *Leptosphaeria maculans. Molecular Plant Microbe Interactions, 4,* 341-349.

LEE S., M. MILGROOM and J. TAYLOR, 1988. A rapid, high yield miniprep method for isolation of total genomic DNA from fungi. *Fungal Genetics Newsletter, 35,* 23-24.

MANULIS S., N. KOGAN, M. REUVEN and Y. BEN-YEPHET, 1994. Use of the RAPD technique for identification of *Fusarium oxysporum* f. sp. *dianthi* from carnation. *Phytopathology, 84,* 98-101.

SCHÄFER C. and J. WÖSTEMEYER, 1992. Random primer dependent PCR differentiates aggressive from non-aggressive isolates of the oilseed rape pathogen *Phoma lingam* (*Leptosphaeria maculans*). *Journal Phytopathology, 136,* 124-136.

SNEATH P.H.A. and R.R. SOKAL, 1973. Numerical Taxonomy. Freeman ed., San Francisco. 573 pp.

SOKAL R.R. and C.D. MICHENER, 1958. A statistical method for evaluating systematic relationships. *University Kansas Science Bulletin, 38,* 1409-1438.

TANTAOUI A. and C. BOISSON, 1991. Compatibilité végétative d'isolats du *Fusarium oxysporum* f. sp. *albedinis* et des *Fusarium oxysporum* de la rhizosphère du Palmier dattier et des sols de palmeraies. *Phytopathologia mediterranea, 30,* 155-163.

TANTAOUI A. et D. FERNANDEZ, 1993. Comparaison entre *Fusarium oxysporum* f. sp. *albedinis* et *Fusarium oxysporum* des sols de palmeraies par l'étude du polymorphisme de longueur des fragments de restriction (RFLP). *Phytopathologia mediterranea, 32,* 235-244.

WELSH J. and M. MCCLELLAND, 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research, 18,* 7213-7218.

WILLIAMS J., A. KUBELIK, K. LIVAK, J. RAFALSKI and S. TINGEY, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research, 18,* 6531-6535.

Accepted for publication: July 6, 1994