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Original Research Article

Pathogenic and molecular detection of *Fusarium oxysporum* f. sp. *albedinis* isolates from different areas in southwest Algeria

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Abstract

Purpose: *To investigate the intra-specific variations in eleven Fusarium oxysporum isolates from infected date palm using pathogenicity and molecular methods.*
Methods: *Eleven isolates of Fusarium oxysporum obtained from infected date palms in the south-west region of Algeria were subjected to confirmatory test using a specific polymerase chain reaction (PCR) technique with the primer pairs, TL3-FOA28 and BIO3-FOA1. Polymorphism in the 5' domain of the large subunit rRNA was investigated. Small libraries of the domain, amplified by the primer pair, LR3/LROR, were constructed and the inserts sequenced.*
Results: *The 11 isolates of Fusarium oxysporum collected from the infected date palm were confirmed as Fusarium oxysporun f. sp albedinis. Results from the investigation of polymorphism in the 5' domain of the large subunit rRNA revealed that the sequences were 100 % homologous or extremely close (> 99.4 %, differing by no more than one to three nucleotides) to several Fusarium oxysporum sequences. In addition, F. inflexum (U34548.1) was highly homologous to one of the F. oxysporum f. sp. albedinis.*
Conclusion: *The sequences of the 11 isolates are almost 100 % homologous to several F. oxysporum species. It is noteworthy that a sequence highly homologous to one of the F. oxysporum f. sp. albedinis is obtainable from a different species, F. inflexum (U34548.1).*

Keywords: *Fusarium oxysporum f. sp. albedinis, Date palm, rRNA gene polymorphism*

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INTRODUCTION

The date palm, *Phoenix dactylifera* L. plays an essential ecological role on oasis development, and also in life in the desert [1]. It has appreciable socio-economic importance because dates occupy a prominent place in human and animal food, and are good sources of foreign exchange earnings. Moreover, dates offer significant agro-food opportunities [2]. Palm

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vascular wilt, known as Bayoud disease, is one of the most serious fungal pathogens of date palms in North Africa, and the highest economically severe disease of date palm in that region [3]. The disease was first reported in Morocco in 1870 [4]. Subsequently, it spread to Algeria and was discovered in Mauritania in 1999 [5].

The "Bayoud" disease is due by *Fusarium oxysporum* f. sp. *albedinis* (Foa.). Since the discovery of date palm vascular wilt disease, various control strategies such as chemical fumigation of the soil and resistant cultivars have been developed and attempted. *Fusarium* species possess high levels of phenotypic and genotypic diversity [6]. Thus, successful control of Bayoud disease depends largely on a good knowledge of the species, including levels and nature of genetic variations. The use of polymerase chain reaction as molecular marker for species identification, and as a diagnostic tool has become very popular during the last decade [7].

Nuclear ribosomal genes are among the most frequently used genes for sequence-based identification. Yet, nuclear ribosomal genes are present in multiple copies in most eukaryotes, and intragenic variation of ribosomal genes has been shown in fungi [8]. Single sequences of the 5'end of the large subunit rRNA were obtained previously from several *Fusarium* isolated from infested palm groves in the south-west region of Algeria. Some were fairly different from the *F. oxysporum* sequences deposited in the Genbank.

The purpose of this research was to investigate intra-specific variations in the large subunit rRNA in *F. oxysporum* strains isolated from different infested palm groves in south-western Algeria.

EXPERIMENTAL

Fungal isolates

A total of 11 isolates of *Fusarium oxysporum* from the rachis of date palms infected from infested palm groves in different regions in the south-west of Algeria were used in this research (Table 1). The isolates were deposed in the Laboratory of Valuation of Vegetal Resource and Food Security in Semi-arid Areas, Southwest of Algeria, at the Tahri Mohamed University, Bechar. Algeria. All the cultures were derived from a mono spore culture and preserved on *potato dextrose agar* (PDA) prior to use.

Pathogenicity test

Pathogenicity test was carried out by inoculation of the roots of young date palm roots (at the two to three-leaf stage) with the fungal isolates. In this process, date palm seedlings (3 to 4 months old) were obtained from disinfected seeds and cultivated in plastic containers filled with a combination of sterile sand and peat. The plant roots were immersed for 24 h in 200 mL of a suspension of conidia (10^7 conidia/mL), transplanted back, and kept in a 16-h light regime at 25 °C for 3 weeks [9]. Confirmation of pathogenicity was recognized by the death of the plants after 1 - 2 months as outlined earlier [10].

DNA extraction

Cultures were cultivated in 100 ml of potato dextrose broth medium on an orbital shaker (150 rpm) at 25 °C for 7 days. Genomic DNA was extracted by thermal lysis [11].

Table 1: Geographical origin of the *Fusarium* isolates in this study

N°	Isolate code	Region	Oasis	Date palm
1	FO6	Touat	Waina	Tgaza
2	FO4	Touat	Oulad Aroussa	Tilmssou
3	FO36	Touat	Mehdia	Tgaza
4	FO4b	Touat	Tililane	Adam
5	FO3	Saoura	Mazer	Khalet
6	2Fb	Saoura	Beni Abbes	Hemira
7	F4b2	Saoura	Mazer	Feggous
8	F2Pd	Saoura	Beni Abbes	Toumliha
9	F2Pd2	Touat	Mehdia	Tinaceur
10	FTin	Touat	Oulad Aroussa	Degla Baida
11	FO3b	Gourara	Oulad Aissa	Tgaza

PCR amplification

All PCR reactions were performed with DreamTaq Green polymerase (ThermoFisher). The primers that were used for the different amplifications are listed and referenced in Table 2. The thermocycler was programmed as follows: first step at at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 55 °C during 30 s., 72 °C for 1 min, and a final extension step at 72 °C for 5 min. The PCR products were resolved by electrophoresis on a 1.6 % agarose gel containing Midori Green Advance DNA Strain (Nippon Genetics Europe GmbH), and visualized on a UV trans-illuminator [12].

Construction of clone libraries, DNA sequencing, and sequence analysis

PCR amplification products obtained with the primers LROR/LR3) were cloned in the commercial plasmid pJET1.2, using the CloneJET PCR cloning kit (ThermoScientific) in line with the manufacturer's protocol. The ligation mix was used to transform chemically competent cells of *Escherichia coli* XL1blue by thermal choc. The transformed cells were selected on L-plates with 100 µg/mL of Ampicillin. Colonies were picked and streaked on the same medium. A simple procedure of PCR on colony was used to control for the presence of the expected insert. The PCR reaction was performed with DreamTaq Green PCR mix (ThermoFisher) in which a little of the colony from the plate was mixed with the plasmid-specific primers pJET1.2 fd and pJET1.2 rv. The PCR conditions were consistent with those recommended by the CloneJET cloning kit. The insert size was verified by electrophoresis on agarose gel. After purification, PCR products of the expected length were sent to Genewiz (England) to be sequenced on both strands with the primers pJET1.2fd and pJET1.2rv. Sequences obtained from the clones were assembled and aligned in MEGA7 [16]. The same package was used for further phylogenic analyses.

The sequences and their GenBank accession numbers (in brackets) were: L2Fb1 (MG209822.1), L2Fb2 (MG209823.1), L2Fb4 (MG209824.1), L2Fb6 (MG209825.1), L2Fb10 (MG209826.1), L2Fb15 (MG209827.1), L2Fb18 (MG209828.1), LAFO1 (MG209829.1), LAFO2 (MG209830.1), LAFO3 (MG209831.1), LAFO4 (MG209832.1), LAFO5 (MG209833.1), LAFO 10 (MG209834.1), LAFO 12 (MG209835.1), LAFO 15 (MG209836.1), LAFO 16 (MG209837.1).

RESULTS

Pathogenicity of the isolates

The results obtained showed that *F. oxysporum f. sp. albedinis* was identified among the *F. oxysporum* isolates obtained from date palms, symptomless carriers and soil after inoculating the roots of young date plants at the two-leaf stage. Inoculation *F. oxysporum f. sp. albedinis* caused the death of the plants after 1 - 2 months. Tests on plantlets confirmed the pathogenicity of all 11 isolates.

Indeed, typical symptoms of date palm vascular wilt were observed in seedlings inoculated with conidia suspension of each *Fusarium* isolate.

PCR-based identification

In this study, PCR amplification with the fungal-specific universal primer pairs (ITS1/ITS4) was obtained from DNA preparations of DNA of all 11 *Fusarium* isolates and clear bands were seen on the gel (Figure 1). The amplification yielded a product about 600 bp, as expected. In addition, PCR analysis using primers LROR/LR3 was successful for all isolates and showed a product of the desired size (550 bp). These primers amplify a fragment of about 550 bp (primers not included) in the Fungi 28S RNA gene [22].

Table 2: Primers used for the molecular characterization of *Fusarium oxysporum* f. sp. *albedinis* analysis

Primer name	Primer sequence	Size of the amplified DNA fragment (bp)	Reference
ITS1	*5'- TCCGTAGGTGAACCTGCGG*	600	[13]
ITS4	*5'- TCCTCCGCTTATTGATATGC*		
LROR	*5'- ACCCGCTGAACTTAAGC*	550	[14]
LR3	*5'- CCGTGTTTCAAGACGGG*		
FOA1	*5'- CAGTTTATTAGAAATGCCGCC*	204	[15]
BIO3	*5'- GGCGATCTTGATTGTATTGTGGTG*		
FOA28	*5'- ATCCCCGTAAAGCCCTGAAGC*	400	[15]
TL3	*5'- GGTCGTCCGCAGAGTATACCGGC*		

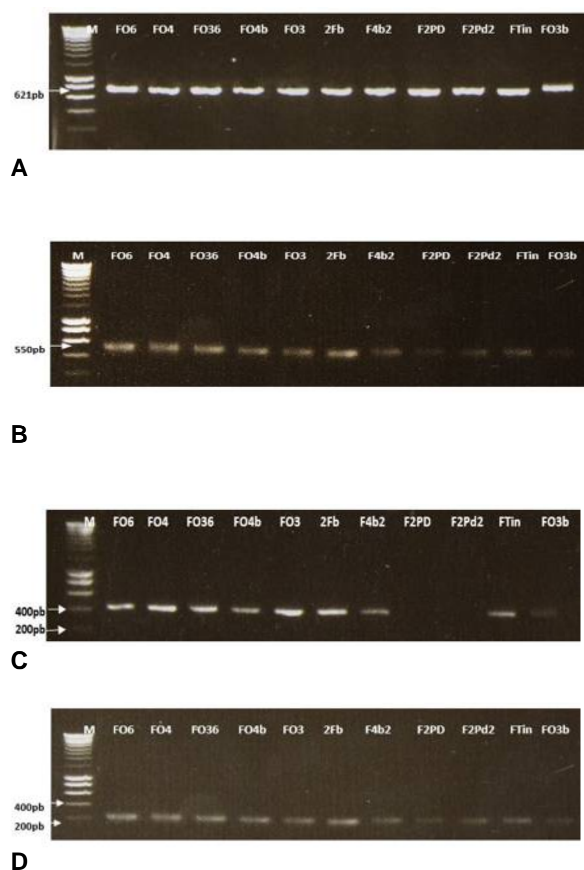

Figure 1: Agarose 1.5 % gels showing the PCR products of *Fusarium* isolates DNA. A: PCR products obtained with the universal primers ITS1/ITS4 (positive control); B: PCR products with primer pair LROR/LR3; C: PCR products with the Foa specific primers (TL3-FOA28) and D: (BIO3-FOA1).

rRNA polymorphism

Two isolates, 2Fb and FO36, were chosen for further analysis. The two isolates come from two different locations and were independently isolated. Small libraries of the 5'domain of the large subunit rRNA were constructed by cloning the 550 bp-long PCR product obtained with primers LROR/LR3 into the commercial plasmid pJET1.2 (Thermofisher).

The insert of 18 clones from 2Fb and 16 from FO36 were sequenced. The number of clones was probably too small for a complete analysis of the polymorphism of the domain. Yet, the alignment of the sequences and the subsequent construction of phylogenetic trees gave valuable information about the diversity of the domain. Seven clones from FO36, and 11 from 2FB had the same sequences (less than 3 differences between 2 sequences):

LAFO 3, LAFO 4, LAFO 6, LAFO 7, LAFO 8, LAFO 10, LAFO 11, L2FB 1, L2FB 3, L2FB 5, L2FB 7, L2FB 8, L2FB 9, L2FB 11, L2FB 12, L2FB 13, L2FB 16, L2FB 17.

These sequences were 100 % homologous or extremely close (more than 99.4 %, differing by no more than one to three nucleotides) to the sequences of several *Fusarium oxysporum* such as *F. oxysporum f. sp. cumini* F11*, F. oxysporum f. sp. dianthi Fod001,* and *F. oxysporum f. sp. lycopersici* 4287 (Figure 2). Only two representatives of this group of homologous sequences are shown in the phylogenetic tree: LAFO10 (MG209834.1), and L2FB1 (MG209822.1). The majority sequence of the two isolates was completely consistent with their affiliation to the species *Fusarium oxysporum*. The remaining nine FO36, and five 2Fb sequences were all unique and diverged from the first group of sequences. L2FB10 (MG209826.1), L2FB2 MG209823.1), L2FB4 (MG209824.1), LB6 (MG209825.1), L2FB18 (MG209828.1), L2FB15 (MG209827.1), LFO15 (MG209836.1), LFO16 (MG209837.1), LFO1 (MG209825.1), LFO12 (MG209825.1), LFO2 (MG209825.1), LFO5 (MG209825.1), and LFO9 (MG209825.1) were very close to the first 18 sequences, but with a slightly less conserved sequence. None of those sequences were identical. Consequently, there is a clear polymorphism in the marker.

No comparable *F. oxysporum f. sp. albedinis* sequences are available in GenBank. Yet, several *F. oxysporum* sequences are available in the data banks. The diversity revealed by the two *F. oxysporum f. sp. albedinis* isolates followed the diversity of other *formae speciales* of *F. oxysporum*. For instance, two sequences of *F. oxysporum f. sp. lycopersici* 4287 were found between the FO36 and 2Fb sequences. On the other hand, one sequence of a distinct species *F. inflexum* (U34548.1) clustered with one of the groups of *F. oxysporum* sequences.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-1024,7729) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 60 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 418 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

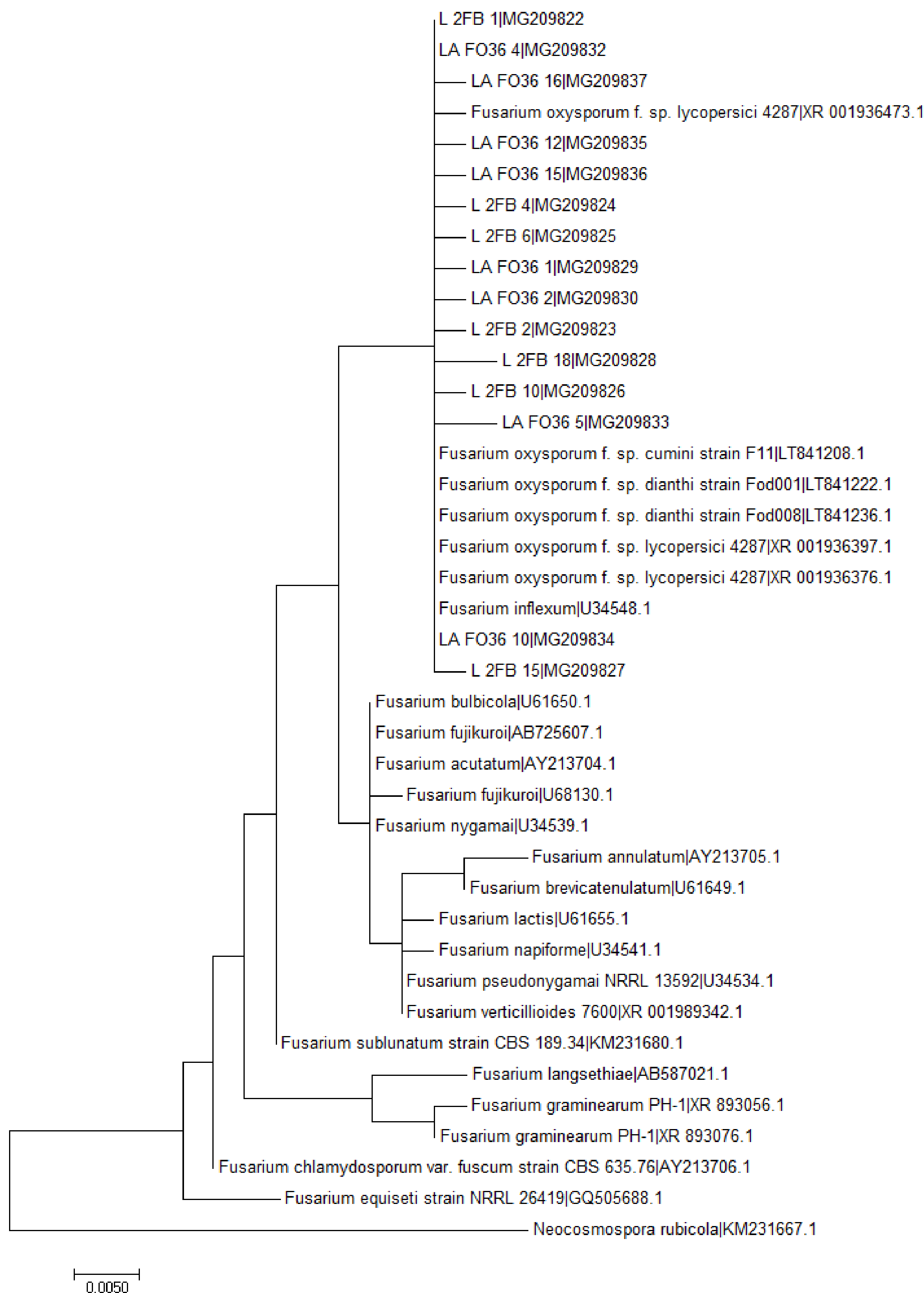

Figure 2: Molecular phylogenetic analysis by the Maximum Likelihood method

DISCUSSION

The pathogenicity test revealed that the inoculated seedlings showed symptoms of root browning, followed by rolling of the leaves, wilting and death of the seedlings. Thus, the test validated the isolation procedures and selection criteria. Therefore, the isolates were most likely

Foa [10,18]. DNA-based techniques have been developed for understanding the genetic diversity and phylogeny of *Fusarium* species [19]. The presence of many *formae speciales* of *F. oxysporum* can be detected by PCR [20,21]. Specific oligonucleotides have been developed for rapid identification of pathogens with PCR assay [15]. PCR base identification confirmed that the isolates were *F. oxysporum var. albedinis*.

The DNA samples were also amplified using the Foa specific primer pairs (TL3-FOA28) and (BIO3-FOA1) [15]. These primer pairs give products of 400 bp and 204 bp, respectively. Two isolates were not amplified with primer pair TL3-FOA28 (F2PD and F2Pd2). All gave amplification with the other Fao-specific primer pairs. This confirmed the identification of the 11 isolates as *F. oxysporum var. albedinis*. However, only two representatives of this group of homologous sequences are shown in the phylogenetic tree: LAFO10 (MG209834.1), and L2FB1 (MG209822.1). The majority sequence of the two isolates was completely consistent with their affiliation to the species *Fusarium oxysporum*.

CONCLUSION

The results based on 5'domain of the large subunit rRNA gene indicate similar polymorphism in two isolates from different areas in southwestern region of Algeria. Analysis of small gene libraries revealed that the degree of polymorphism is higher than expected from the sequences available in GenBank: 12 unique sequences and one principal sequence. This is far more than the number of sequences introduced in GenBank for other individual *F. oxysporum* strains. This can lead to some confusion regarding the identification of *Fusarium* strains when based on this limited domain. It is noteworthy that a sequence highly homologous to one of the *F. oxysporum f. sp. albedinis* is obtainable from a different specie, i.e., *F. inflexum* (U34548.1).

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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