Reliable Detection of the Fungal Pathogen *Fusarium oxysporum* f.sp. *albedinis*, Causal Agent of Bayoud Disease of Date Palm, Using Molecular Techniques

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Bayoud, caused by the soilborne fungus *Fusarium oxysporum* f.sp. *albedinis* (FOA), is the most serious disease of date palm. Since the disease is located in the North African countries of Morocco and Algeria, and advancing steadily eastwards, the ultimate goal is to prevent spread of the pathogen to other date-growing areas in the region and farther afield. Molecular diagnostic techniques have been developed for detection of FOA. In view of the fact that the fungus does not exist in Israel, DNA of FOA was obtained to determine the reliability of these methods for diagnostic purposes. Random amplified polymorphic DNA was not reliable enough for differentiation between FOA and various pathogenic and saprophytic *Fusarium* isolates. However, the polymerase chain reaction utilizing FOA-specific primers was accurate and enabled amplification of a unique band specific to FOA DNA alone, and not that of the other tested pathogenic and saprophytic *Fusaria*. The availability of a rapid and reliable diagnostic tool for detection of FOA will enable the Plant Protection and Inspection Services of the Israel Ministry of Agriculture to test date palm tissue for the presence of the pathogen.
KEY WORDS: Bayoud disease; date palm; *Fusarium oxysporum* f.sp. *albedinis*; *F.o.* f.sp. *canariensis*; molecular diagnostics; RAPD-PCR; species-specific primers.

INTRODUCTION

Bayoud of date palm (*Phoenix dactylifera* L.), caused by the fungus *Fusarium oxysporum* f.sp. *albedinis* (FOA), is the most important disease of this crop. It is currently confined to Morocco and the western and central regions of Algeria, having destroyed approximately 20 million trees since it was first discovered, sometime before 1870 (3). Although preventive measures and eradication programs have been undertaken to curb the spread of the pathogen, no cure for the disease exists. FOA continues to advance relentlessly eastwards within Algeria; therefore, Mediterranean date-growing areas in particular and other regions of cultivation worldwide, remain threatened by this fungus.

FOA is soilborne and host-specific; however, the henna bush, when intercropped with date palm, may serve as a symptomless carrier of the fungus (2). FOA produces typical micro- and macroconidia, as well as chlamydospores, allowing the pathogen to survive in soil under adverse environmental conditions. The fungus enters palm trees *via* roots, eventually causing wilting and death of plants. The pathogen is dispersed by water, wind, diseased offshoots which move soil from diseased areas, and in infected wood or rachis

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of date tissues (2,3). Spread of FOA from one oasis to another has been attributed to movement of the pathogen in artifacts made of infected date tissue such as baskets, ropes and saddles. The search for resistant cultivars is in progress in Morocco. The high quality 'Medgool' variety, predominantly cultivated in Israel, is considered highly susceptible to FOA (3,4). No curative control methods exist for diseased palm trees, but eradication of the pathogen in soil is achieved using methyl bromide and other soil fumigants such as chloropicrin (3,13).

Due to the fact that FOA is restricted to Morocco and Algeria, an early detection method is imperative for preventing further spread of the pathogen to uninfected date-growing regions. It has been shown that FOA varies in colony morphology and cannot be distinguished from other *formae speciales* of *F. oxysporum.* Micro- and macroscopic criteria, such as conidial shape, size and growth rate, therefore are inadequate for detection and identification of the pathogen (8,12). However, it was reported that random amplified polymorphic DNA (RAPD) tests and mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP), could differentiate reliably between FOA and certain saprophytic and other pathogenic *formae speciales* of *F. oxysporum* (6,12). Additional studies have utilized primer sets to amplify specific fragments of FOA DNA by the polymerase chain reaction (PCR) (5). The FOA species-specific primers were synthesized from regions flanking the insertion sites of the *Fot* 1 transposable element in clones of an FOA genomic library (5). This technique seemed reliable enough to differentiate between FOA from various saprophytic and pathogenic isolates of *Fusarium* from a wide host range (5), but did not include isolates from Israel.

The main goal of this study was to determine whether the available molecular tools, RAPD analysis and a species-specific primer pair for FOA, would facilitate reliable and accurate differentiation between the true date palm pathogen, and the related *F.o. canariensis* species causing wilting of the Canary Island date palm, and various pathogenic and nonpathogenic *formae speciales* of *F. oxysporum* from Israel and the neighboring regions. Detection of FOA DNA in a root fungal DNA mixture was also assessed, since infected plant material was unavailable. Reliable molecular diagnostic tools, implemented by the appropriate quarantine authorities, are paramount for avoiding establishment and spread of such pathogens into new, uninfected areas. Since FOA is considered a quarantine organism in Israel, and date and ornamental palms as well as date palm products are frequently imported, accurate and reliable detection methods are imperative for preventing entry of this destructive pathogen into the country.

MATERIALS AND METHODS

Fungal cultures and growth conditions, plant material and DNA source The various *formae speciales* of *Fusarium* used in this study (Table 1) were initiated from a single-spore culture and maintained on PDA (potato dextrose agar) growth medium at 25°C. The fungal cultures included pathogenic isolates from various crops grown in Israel, and undefined isolates of *Fusarium* collected during field trips to date palm plantations in El-Arish, Egypt in 1998, and Gaza, Palestinian Authority in 1999. Five samples of date palm roots, cv. 'Medgool', were collected from Hazeva in the Arava desert for DNA extraction and analysis (see below).

Fungal DNA of three isolates (F3, F60 and F81) of FOA was received from Dr. D. Fernandez (Orstom, France); DNA of two isolates (1042 and 1148) of FOA was received

from Dr. M.B. Dickman (Univ. of Nebraska, USA) *via* Ms. J. Juba (Fusarium Research Center, Pennsylvania State Univ., PA, USA); DNA of three isolates (FO53, 95-1009 and 95-456) of *F.o. canariensis* (FOC) was received from Ms. T. Plyler (Univ. of Florida, FL, USA); and DNA of one isolate (Raguna 2) of FOC was received from Dr. Q. Migheli (Univ. of Torino, Italy).

TABLE 1. *Fusarium* isolates used in this study

Fusarium species	Isolate	Origin	Host
F.o. albedinis (FOA)	F3	Algeria	date palm leaf
F.o. albedinis (FOA)	F60	Algeria	date palm leaf
F.o. albedinis (FOA)	F81	Morocco	date palm leaf
F.o. albedinis (FOA)	1042	unknown	date palm
F.o. albedinis (FOA)	1148	Algeria	date palm
F.o. canariensis (FOC)	95-1009	FL, USA	ornamental palm
F.o. canariensis (FOC)	95-456	FL, USA	ornamental palm
F.o. canariensis (FOC)	FO53	CA, USA	ornamental palm
F.o. canariensis (FOC)	Ragusa 2	Italy	ornamental palm
F.o. basilici (FOB)	B74T	Israel	basil
F.o. lycopersici (FOL)	1098L	Israel	tomato
F.o. lycopersici (FOL)	LEV 1642	Israel	tomato
F.o. lycopersici (FOL)	2GR	Israel	tomato
F.o. radicis (FORL)	C809L	Israel	tomato
F.o. dianthi (FOD)	E112	Israel	carnation
F.o. melonis (FOM)	KKI	Israel	melon
F.o. melonis (FOM)	FOM 1,2	France	melon
F. subglutinans	F.SUB 506	Israel	mango
F. oxysporum (FUS)[z]	FUS 3I	Gaza, PA	date palm (np)[y]
F. oxysporum (FUS)	FUS 21/30	El-Arish, Egypt	date palm (np)
F. oxysporum (FUS)	FUS 31/40	El-Arish, Egypt	date palm (np)

[z] An additional 11 saprophytic *F. oxysporum* isolates from date palm originated from Gaza, Palestinian Authority.
[y] np, nonpathogenic isolates.

All fungi were cultured in the dark on potato dextrose agar (PDA; Difco, France) and maintained at 25°C. For DNA extraction, liquid cultures comprising 100 ml potato dextrose broth (PDB; Difco, France) in 250 ml Erlenmeyer flasks were inoculated with five mycelial disks derived from colony margins. The cultures were agitated for 5–6 days on a rotary shaker at 150 rpm and maintained at 25°C. Twelve hours before harvesting mycelia, the cultures were fragmented by blending for 10 sec at 24,000 rpm with a tissue homogenizer (Ultra Turrax T25, Janke & Kunkel, IKA Labortechnik, Germany) under sterile conditions and returned to the shaker. To avoid contamination, the homogenizer was washed in sterile water before blending each culture.

Isolation and purification of fungal DNA and DNA from palm roots Mycelia from 100 ml PDB cultures were collected by vacuum filtration and lyophilized until dry. DNA was extracted and purified as described previously (7). The DNA was dissolved in 0.5 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) to an approximate concentration of 200–500 μg/ml and diluted to a concentration of 10–100 ng/μl for PCR reactions. For palm root DNA extraction, 100 mg root segments were weighed and DNA was extracted and adjusted to a concentration of 100 ng/μl, using the same protocol as for fungal DNA extraction.

PCR amplification of fungal and palm root DNA For RAPD PCR, 10-base oligomer primers used were: OPF-04 (GGTGATCAGG), OPF-05 (CCGAATTCCC), OPF-08 (GGGATATCGG), OPF-12 (ACGGTACCAG) and OPF-13 (GGCTGCAGAA) of Kit F, purchased from Operon Technologies (Alameda, CA, USA). PCR primers for FOA-specific amplification included two primer pairs: FOA1 (CAGTTTATTAGAAATGCCGCC) coupled with BIO3 (GGCGATCTTGATTGTATTGTGGTG), and FOA28 (ATCCCCGTAAAGCCCTGAAGC) coupled with TL3 (GGTCGTCCGCAGAGTATACCGGC) (5). PCR reactions were performed in a total volume of 20 μl, containing 10–100 ng genomic DNA, 50 mM KCl, 10 mM Tris-HCl, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, 1 unit *Taq* DNA polymerase (Promega) and 1 μM primer, as previously described (6). The reactions were incubated in a PTC-100 thermocycler (MJ Research, Inc., MA, USA) starting with 5 min of denaturation at 95°C. For RAPD-PCR, this was followed by 45 cycles consisting of 1 min at 94°C, 1 min at 34°C and 2 min at 72°C. One cycle for 15 min at 72°C was conducted after the 30 cycles. FOA-specific PCR reactions were performed according to Fernandez *et al.* (5) as follows: 1 cycle for 4 min at 95°C followed by 30 cycles for 30 sec at 92°C, 30 sec at 60°C and 30 sec at 72°C for the FOA1-BIO3 primer pair; and 30 cycles for 30 sec at 92°C, 30 sec at 62°C and 45 sec at 72°C for the FOA28-TL3 primer pair. Thereafter, a cycle of 15 min at 72°C was conducted.

FOA DNA was diluted from 100 ng to 10 pg to determine the threshold levels of detection using the specific primer reaction. Alternatively, palm root DNA at a concentration of 100 ng was mixed with different concentrations of FOA DNA as above, since FOA-infected roots were not available in this study.

All amplification products were separated in agarose gels (1.5% w/v; 15×10 cm, W$\times$L) in TAE buffer (9) electrophoresed at 80 V for 2 h.

RESULTS

RAPD detection of FOA Various *formae speciales* of *Fusarium*, from Israel, Gaza and El-Arish, were PCR-amplified using the primers OPF-04, OPF-05, OPF-08, OPF-12 and OPF-13. In two FOA DNA samples of isolates F-60 and 1042, polymorphisms were detected using primer OPF-13 (Fig. 1). Polymorphisms were also observed among DNA from the three other isolates using primer OPF-13 (data not shown). However, based on RAPD-PCR, most pathogenic and saprophytic *Fusaria* could be distinguished from the FOA isolates (Figs. 1 and 2). With primer OPF-08 the saprophytic isolate FUS 3I possessed banding patterns similar to the FOA isolates, F60 and 1042 (Fig. 2). In most cases the saprophytic (FUS 3I, FUS 21/30 and FUS 31/40) and pathogenic *Fusarium* isolates could be distinguished from FOA using primers OPF-13 (Fig. 1) and OPF-08 (Fig. 2), OPF-04, OPF-05 and OPF-12 (data not shown). It should be emphasized that distinct differences were observed between amplified products of FOA DNA and those from the ornamental Canary Islands date palm, FOC isolate FOC FO53, originating from California (Figs. 1 and 2). Amplified polymorphic DNA of three additional FOC isolates, two originating from Florida (95-1009 and 95-456) and one from Italy (Ragusa 2), was identical to that of isolate FOC FO53 (data not shown).

FOA-specific primers FOA species-specific primer pairs FOA28 and TL3, and FOA1 and BIO3 (5), were used to verify that all five representative FOA DNA samples were indeed from the pathogen responsible for Bayoud disease of date palm. The predicted

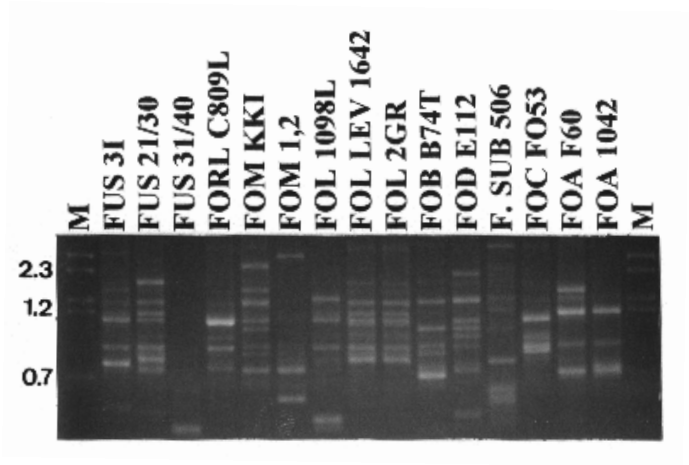

Fig. 1. RAPD-PCR of genomic DNA of *Fusarium* isolates (FUS 3I from date palm, Gaza; FUS 21/30 and FUS 31/40 from date palm, Egypt), *F. oxysporum radicis* (FORL C8903 from tomato, Israel), *F.o. melonis* (FOM KKI from melon, Israel; FOM 1,2 from melon, France), *F.o. lycopersici* (FOL 1098L, FOL LEV 1642 and FOL 2GR from tomato, Israel), *F.o. basilici* (FOB B74T from basil, Israel), *F.o. dianthi* (FOD E112 from carnation, Israel), *F. subglutinans* (F. SUB 506 from mango, Israel), *F.o. canariensis* (FOC FO53 from Canary Islands date palm, USA), and *F.o. albedinis* (FOA F60 and FOA 1042 from date palm, Algeria) using primer OPF-13. Lane M contains DNA markers with sizes in kb.

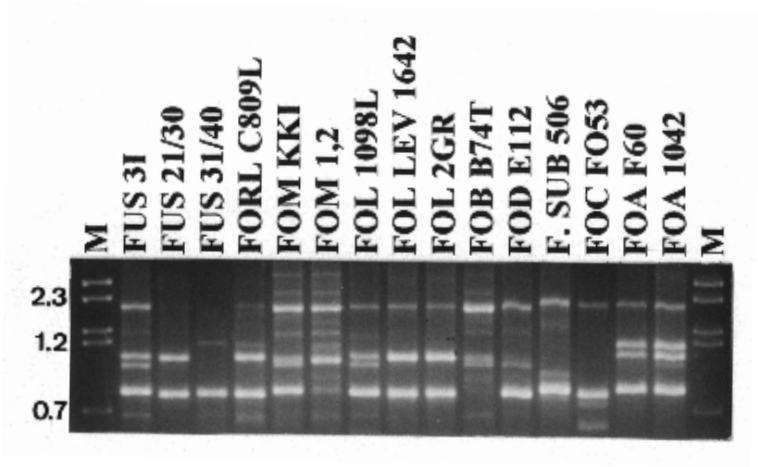

Fig. 2. RAPD-PCR of genomic DNA of *Fusarium* isolates (isolate designation according to Fig. 1) using primer OPF-08. Lane M contains DNA markers with sizes in kb.

single fragment for each primer pair was amplified for each FOA sample, namely, a 400-bp product with primer pair FOA28 and TL3 (Fig. 3A), and a 204-bp product with primer pair FOA1 and BIO3 (data not shown). The primer pair FOA28 and TL3 was further tested on DNA extracted from the *Fusarium* isolates listed in Table 1. No amplification was

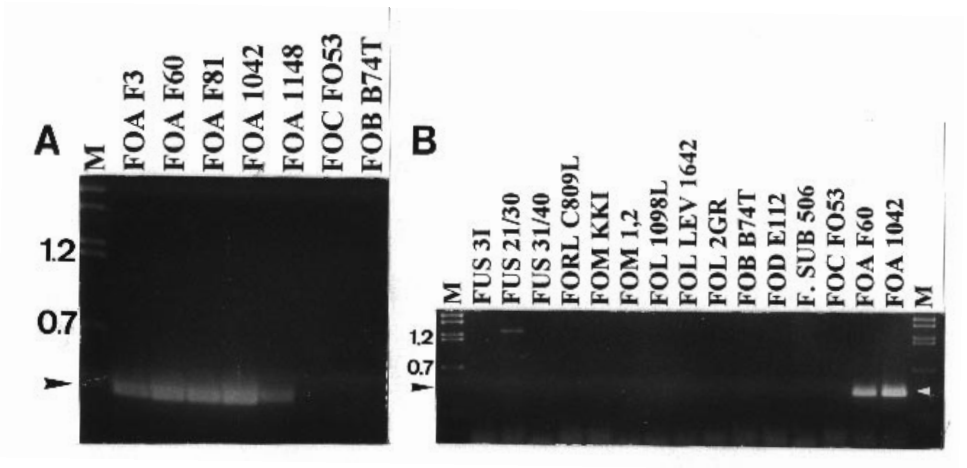

Fig. 3. *Fusarium oxysporum* f.sp. *albedinis* species-specific PCR amplification using primer pair FOA28 and TL3 of isolates of *F.o. albedinis* from date palm (FOA F3, -F60, -F81, -1042 and -1148), *F.o. canariensis* from Canary Islands date palm (FOC FO53), and *F.o. basilici* from basil (FOB B74T) (A) and additional isolates as designated in Figure 1 (B). Arrows denote the unique 400 bp FOA-amplified band. Lane M contains DNA markers with sizes in kb.

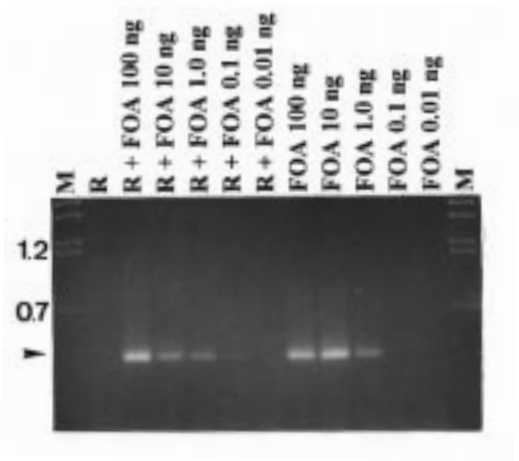

Fig. 4. *Fusarium oxysporum* f.sp. *albedinis* species-specific PCR amplification using primer pair FOA28 and TL3 with decreasing amounts of *F.o. albedinis* isolate FOA F60 DNA (100,000 to 100 pg), with or without DNA (100 ng) from date palm roots (R). An arrow denotes the unique 400 bp FOA-amplified band. Lane M contains DNA markers with sizes in kb.

observed with DNA originating from these isolates, whereas the specific 400-bp product was detected using template DNA from the FOA isolates F60 and 1042 (Fig. 3B). Similar results were obtained using primer pair FOA28 and TL3 (data not shown). With both primer pairs, FOC DNA from the USA (isolate FO53; Fig. 3) and Italy (isolate Ragusa 2, data not shown) did not react to the FOA-specific amplification.

Specific amplification of FOA DNA combined with DNA from date palm roots FOA DNA was diluted to different concentrations and mixed with 100 ng palm root DNA to determine the threshold levels of detection using FOA-specific primers, since FOA-infected roots were not available in this study. FOA DNA alone or mixed with root DNA at concentrations ranging from 100 ng to 100 pg was readily detected using the FOA-specific primers (Fig. 4).

DISCUSSION

The main goal of this research was to determine the reliability of RAPD-PCR and FOA-specific PCR analyses for accurate and reliable differentiation between the Bayoud pathogen in date palm and FOC, saprophytic and pathogenic isolates of *Fusarium* from other hosts in Israel and the neighboring regions. The long-term aim is to use these tools to prevent the introduction of FOA into the region. This is of utmost importance since FOA poses a serious threat to the date palm industry in Israel, due to the continuous spread of the pathogen from Morocco to Algeria, moving eastwards. In addition, there has been a recent opening of borders in the region, allowing the flow of tourists, which may assist in uncontrolled dispersal of FOA *via* articles made from palm tissues, such as woven baskets, harboring resting structures of the pathogen. Therefore, strict phytosanitary regulations need to be enforced in date palm growing areas which are Bayoud-free, and rapid, reliable identification of FOA is imperative in attaining this goal.

Morphological criteria are insufficient for the detection of FOA and other *formae speciales* of *Fusarium* and inoculation trials for determining pathogenicity of FOA in date palm may not be reliable enough for verification of the causal agent of disease, since an additional *forma specialis*, such as FOC (4,8), has been reported to cause mortality of the true date palm. Furthermore, FOA-inoculation experiments are tedious and time-consuming, requiring a period of between 3 and 6 months before symptoms appear (3,11). In addition, no well-defined differential series of host genotypes are available. Since the FOA pathogen has not yet been detected in Israel, we resorted to obtaining DNA of the fungus, a safety precaution which on the one hand restricted our research, but on the other hand was the best way to prevent introduction of the pathogen into the country.

RAPD-PCR and FOA-specific PCR analyses have been reported to be reliable for identification of the Bayoud pathogen (5,6). Various pathogenic isolates of *Fusarium*, appearing on selected crops in Israel, were used as negative controls for the molecular detection methods. The FOA-specific primers were able to differentiate FOA DNA from that of other pathogenic *Fusarium* species (Fig. 3). However, the RAPD-PCR technique appeared to be less accurate, with polymorphisms observed among FOA DNA and similar banding patterns detected among amplified DNA of certain saprophytic *Fusaria* and FOA (Figs. 1 and 2). A number of field trips were conducted to date palm growing areas in Gaza and El-Arish with a view to importing offshoots to Israel, and to determine whether symptoms of Bayoud disease were present in surrounding plantations. Samples of wilted leaves and other plant tissue revealed the presence of *Fusarium* when cultured on a selective growth medium for this species. RAPD-PCR and the FOA-specific primer analyses failed to detect the Bayoud pathogen in these groves, indicating that these *Fusarium* isolates are most likely saprophytes.

In an effort to determine sensitivity of the FOA-specific primers, FOA and palm root DNA were mixed, since infected roots were not available. Fungal DNA was detected at

levels of 100 ng to 100 pg in the mixture, which was similar to the level (250 ng to 25 pg) of a fungal pathogen of wheat, *Stagonospora nodorum*, in naturally infected tissue, amplified by specific primers (1). Similarly, *F. culmorum*-specific primers were able to detect the presence of this cereal pathogen at levels of 50 pg DNA, when spiked with 10 ng of host DNA (10). Therefore, we are confident that the presence of FOA in infected date palm tissue can be detected using the FOA-specific primer PCR analysis.

In summary, it was demonstrated that FOA-specific primer analysis is reliable for identification of the Bayoud pathogen, *F.o. albedinis*. This method will be implemented in the Plant Protection Services and Inspection Department of the Israel Ministry of Agriculture for future diagnosis, in the event that FOA may be present in imported or other date palm material.

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