Pathogenicity of Toxigenic *Fusarium proliferatum* from Date Palm in Saudi Arabia

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ABSTRACT

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Fusarium proliferatum was isolated for the first time from roots and leaves of declining date-palm trees (*Phoenix dactylifera* L.) in the Al-Qassim and Al Medina Al Monawara regions in Saudi Arabia. The disease symptoms caused by *F. proliferatum*, which include wilt and dieback, were similar to those caused by *F. oxysporum* f. sp. *albedinis*, the causal agent of Bayoud, the most important disease of date palm found in North Africa. Koch's postulates were completed by stem injection of date-palm seedlings using two strains of *F. proliferatum*. The pathogenicity tests, performed on local cv. Succary using two strains of *F. proliferatum* isolated from roots, produced severe symptoms of disease. The identity of *F. proliferatum* was confirmed by comparing the data obtained by partial sequences of a large subunit of rDNA to American and European Gene Bank data. All nine *F. proliferatum* strains isolated from diseased plants were shown to belong to mating population D of *Gibberella fujikuroi*. Finally, the strains were also tested for the production of beauvericin, fumonisin B₁, fusaproliferin, fusaric acid, and moniliformin. Two strains out of nine were able to produce all five toxins and all strains produced at least three of them. This is the first time that toxigenic *F. proliferatum* strains known to belong to mating population D of *G. fujikuroi* were isolated from diseased date-palm plants.

Additional keywords: *Liseola* section, mating type, mycotoxins

Bayoud disease, caused by *Fusarium oxysporum* Schlechtend. emend. W. C. Snyder & H. N. Hans f. sp. *albedinis* (Killian & Maire) Gordon (20), is a vascular wilt of date-palm trees (*Phoenix dactylifera* L.), widely distributed in North Africa (7,20). Currently, strict phytosanitary rules are applied at the borders of date-palm countries that remain free of Bayoud, including the Middle East countries where most of the world date-palm cultivation is concentrated. In Saudi Arabia alone, there are more than 15 million trees which produce about 649,000 tons of fruit (1).

During an investigation in Saudi Arabia, and, in particular, the Al Qassim and Al Medina Al Monawara regions, date palm showed symptoms of wilt and dieback very similar to those caused by *F. oxysporum* f. sp. *albedinis*. However, surprisingly, the main fungal species isolated from the infected leaves and roots of the date palm trees was identified as *F. proliferatum* (T. Matsushima) Nirenberg. Strains of *F. oxysporum* and *F. solani* (Mart.) Appel & Wollenweb. emend. W. C. Snyder & H. N.

Hans were also isolated, but at very low frequency.

F. proliferatum is a well-known, worldwide pathogen of several plant crops, including maize (18), rice (5), and asparagus (8). It is a toxigenic species, producing a broad range of toxins, such as fumonisin B₁ (FB₁; 28), moniliformin (MON; 23), beauvericin (BEA; 18), fusaric acid (FA; 2), and the recently described fusaproliferin (FUP; 33). Some of these toxins have well-known phytotoxic activity; these include FA, which is implicated in the pathogenesis of tomato-wilt symptoms (10); MON, which is toxic toward tobacco plants (4), and FB₁, which has shown to be phytotoxic to maize and tomato (14).

F. proliferatum belongs to the *Liseola* section of the *Fusarium* genus (29) and its teleomorph belongs to the *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura complex, where at least eight different mating populations (MPs) were recognized (16). On the basis of fertility, strains of *F. proliferatum* belong to MPs C (syn. *F. fujikuroi* Nirenberg) and D. Several reports indicate that members of different MPs can be preferentially isolated from different hosts (16) and may differ in the spectrum of mycotoxins produced (16,24). Also, members of MPs C and D were reported to have a different spectrum of mycotoxins (5,24). Therefore, it is important to assess the MPs of the *F. proliferatum* strains isolated from date palm, because this could

also reflect differences in pathogenicity and ecology of this group of isolates.

The possible involvement of *F. proliferatum* as a main casual agent responsible for the symptoms on date-palm trees was investigated. Specific objectives of this study were to (i) evaluate the pathogenicity of *F. proliferatum* strains on date palm, (ii) investigate the toxicological profile of this group of strains, and (iii) study their possible fertility and assign them to a specific MP.

MATERIALS AND METHODS

Fungal isolation and identification. Infected tissues of date-palm trees (roots and leaves), collected from the Al Qassim and Al Medina Al Monawara regions, were washed carefully, cut into pieces 1 cm long, soaked for 1 to 2 min in 0.5% NaOCl solution, rinsed in sterile distilled water (SDW), and placed on acidified potato dextrose agar (APDA) with pH = 5. Wheat plants from the same area (Buriedah, Al Qassim region) were also analyzed for evaluating a possible *Fusarium* spp. contamination.

Plates were incubated at 25°C for 2 weeks. After incubation, the *Fusarium* spp. colonies were single spored and transferred to potato dextrose agar (PDA) and carnation leaf agar (CLA) for the identification that was made according with the taxonomic system of Nelson et al. (29). To preserve the cultures, mycelia and conidia from wild strains grown on CLA were transferred aseptically in 1 ml of a sterile water-18% glycerol solution and frozen at -75°C. The isolates were deposited with accession number in the collection of the Istituto Tossine e Micotossine da parassiti vegetali (ITEM), Bari, Italy. For further confirmation of the strain identity, representative isolates were deposited in the International Mycological Institute.

The *Fusarium* spp. strains used in this study were nine representative strains of *F. proliferatum*, one strain of *F. oxysporum*, and one strain of *F. solani* (Tables 1 and 2).

Sequence analysis. To confirm the morphological identification of the *F. proliferatum* strains, three isolates of *F. proliferatum* from date palm (ITEM 2337, 2341, and 2386) were analyzed by sequencing a fragment of around 500 base pairs (bp) of the 5' end of the region coding for the 28S rDNA. DNA for polymerase chain reaction was extracted by a modified version of the

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sodium dodecyl sulfate protocol of Raeder and Broda (31) and the amplification of the 5' end of the region coding for the 28S rDNA was performed as reported in Mulé et al. (26). Sequencing was carried out according to the fluorescent-labeled Big Dye Deoxy protocol (Perkin Elmer, Warrington, GB) on an Applied Biosystem model 310 automated sequencer.

Pathogenicity tests. The strains that were tested for pathogenicity (Table 1) were grown for 7 to 14 days at 25°C on APDA. We compared two representative strains of *F. proliferatum* to one strain each of *F. oxysporum* and *F. solani*. The inoculum was prepared for each strain by flooding the agar surface with 10 ml of SDW and scraping with a spatula. The resulting spore suspension was filtered through four layers of cheese cloth. The filtrate was diluted with SDW and conidial concentration was adjusted with a hemacytometer to 10^6 conidia/ml. Seeds of the local date palm cv. Succary were surface disinfected for 10 min in 0.5% NaOCl solution, soaked in tap water for 24 h, and then planted in seedling bags (pots) filled with steamed sandy loam soil and peat moss (1:1 vol/vol). One seedling was grown in each pot. Plants were maintained on the greenhouse bench at $25 \pm 3^\circ\text{C}$. Plants were inoculated 6 months after seedling emergence. Seven plants were inoculated with each isolate; consequently, 35 seedlings were used in each experi-

mental run. Each seedling was inoculated by injecting 2 ml of inoculum into the crown area using a hypodermic needle and syringe. The control set of seedlings were injected with SDW. All plants were watered and covered separately with plastic bags for 24 h to maintain high humidity. The pathogenicity test was conducted twice.

Disease rating was determined 3 months after inoculation. Individual plants were rated on a scale from 0 to 4 according to visible symptoms on each seedling, where 0 = no visible symptoms, 1 = small lesion (length of the lesion from 5 to 10 mm), 2 = medium lesion (length 10 to 30 mm), 3 = large lesion (length 30 to 70 mm), and 4 = whole leaf blighted. Scores were computed to determine the disease severity index (DSI), where $\text{DSI} = \Sigma(\text{seedlings/class} \times \text{class score})/\text{total seedlings}$. The experiment was designed using a randomized block design. Data were analyzed according to general linear models procedures of SAS (Statistical Analysis System, version 6.12; SAS Institute, Inc., Cary, NC) using the following linear model:

$Y_{ijk} = \mu + A_i + B_j + C_k + BC_{ij} + e_{ijk}$, where Y_{ijk} = the DSI on the ijk seedlings; μ = overall mean; A_i = the effect of the i^{th} replicate ($i = 1$ through 7); B_j = the effect of the j^{th} experimental run ($j = 1$ or 2); C_k = the effect of the k^{th} treatment (isolate + control) ($k = 1$ through 5); BC_{ij} = the interaction effect between B_j and C_k ; and e_{ijk} = random error.

Fertility tests. Tester strains for mating population tests were received from J. F. Leslie, Kansas State University. Strains were crossed on carrot agar as male parents with tester strains of mating population A through F as described by Klittich and Leslie (13). Mated cultures were considered interfertile if perithecia formed within 6 weeks. All strains were crossed twice with both testers from each mating population.

In vitro toxin production. Single-conidium strains of *F. proliferatum* were cultured on 100 g of autoclaved yellow maize kernels that were adjusted to about 45% moisture in 500-ml Erlenmeyer flasks. Kernels were inoculated with 2 ml of an aqueous suspension containing approximately 10^7 conidia/ml. Cultures were incubated at 25°C for 4 weeks. Each strain was cultured in three replicate flasks. Harvested cultures were dried in a forced-draft oven at 60°C for 48 h, finely ground, and stored at 4°C until use. Control kernels were treated the same way, except that they were not inoculated.

Toxin analysis. For BEA and FUP extraction, 10 g of each culture was homogenized by shaking in 100 ml of methanol (99.5%) for 1 h. Samples were then filtered through Whatman no. 4 filter paper and methanol was removed under reduced pressure. Each extract was eluted in 1 ml of methanol and filtered through an Acro-disk filter (pore size, 0.22 μm), before high performance thin layer chromatography (HPTLC) analysis, performed for BEA according to the procedure described by Logrieco et al. (19) and for FUP following the procedure described by Logrieco et al. (17).

For FA extraction and its derivatization, 2.5 g of each infected sample was extracted in an Ultraturax with 25 ml of MeOH-1% aqueous KH_2PO_4 (50:50 vol/vol) for 3 min at 13,500 rpm at pH 3. The homogenized material was centrifuged at 4,000 rpm for 5 min. The upper phase was extracted with CHCl_3 (3×35 ml) and organic extracts were collected, evaporated to dryness, and finally redissolved in 2 ml of CHCl_3 . A total of 250 ml of FA chloroformic solution was derivatized with 100

Table 1. Disease severity on 9-month-old seedlings of date palm (*Phoenix dactylifera* var. *Succary*) artificially inoculated with various *Fusarium* spp. isolates recovered from date palm

Fungal species	PPD ^w	ITEM ^x	Disease severity index ^y
<i>F. proliferatum</i>	1	2336	3.48 a
<i>F. proliferatum</i>	2	2337	3.53 a
<i>F. oxysporum</i>	3	...	1.90 b
<i>F. solani</i>	5	...	3.61 a
Control ^z	...	...	0.68 c

^w PPD = Plant Protection Department, Buraidah, Saudi Arabia.

^x ITEM = Istituto Tossine e micotossine da parassiti vegetali accession number, Bari, Italy.

^y Disease severity index = $\Sigma(\text{seedlings/class} \times \text{class score})/\text{total score}$. Individual plants were rated on a scale from 0 to 4 according to visible symptoms on each seedling, where 0 = no symptoms visible, 1 = small lesion (length of the lesion from 5 to 10 mm), 2 = medium lesion (length, 10 to 30 mm), 3 = large lesion (length, 30 to 70 mm), and 4 = whole leaf blighted. Values followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

^z Seedlings were injected with 2 ml of sterile distilled water.

Table 2. Toxin production by *Fusarium proliferatum* isolated from date palm in Saudi Arabia^a

Strain (ITEM) ^z	Region of origin	Plant part	Toxins ($\mu\text{g/kg}$)				
			BEA	FB ₁	FUP	FA	MON
2336	Al-Qassim (Bakireia)	Root	300	750	150	ND	70
2337	Al-Medina Al Monawara	Root	250	630	100	230	60
2339	Al-Qassim (Buriedah)	Leaves	20	trace	70	ND	70
2341	Al-Qassim (Buriedah)	Root	20	trace	80	ND	60
2342	Al-Qassim (Buriedah)	Root	120	880	90	ND	ND
2343	Al-Qassim (Buriedah)	Root	130	ND	120	ND	40
2383	Al-Qassim (Buriedah)	Root	100	900	300	ND	330
2386	Al-Qassim (Buriedah)	Root	400	1,000	200	1,300	200
2387	Al-Qassim (Buriedah)	Root	70	880	200	ND	270

^a Strains were grown on autoclaved yellow maize kernels at 25°C for 4 weeks. BEA = beauvericin, FB₁ = fumonisin B₁, FUP = fusaproliferin, FA = fusaric acid, MON = moniliformin; ND = not detected.

^z Istituto Tossine e micotossine da parassiti vegetali accession number, Bari, Italy.

µl of CHCl₃ bis(trimethylsilyl)trifluoroacetamide (BSTFA) 1% chlorotrimethylsilane (TMCS) under nitrogen in conic vials at 70°C for 15 min. The solution was dried with nitrogen flow, the residue was dissolved in 2 ml of CHCl₃, and 2 µl were used for analysis by gas chromatography-mass spectrometry (GC-MS).

GC-MS was carried out using a Shimadzu 17A gas chromatograph equipped with a QP5000 mass spectrometer detector. The chromatography column used was a RTX (5% diphenyl, 30 m, 0.25 mm i.d. by 0.25 mm). The injector temperature was set at 250°C; the column temperature was kept at 100°C for 2 min, then raised to 200°C at 35°C/min, after which the temperature was held constant for 4 min. The temperature was then adjusted at 25°C/min to 250°C and maintained for 3 min. The mass spectrometer source was set at 280°C and 70 eV. The range's detector was fixed from 50 to 300 atomic mass units. The retention time observed for silylated FA was 9.67 min, the quantification limit was 1 ppm, and the detection limit was 0.01 ppm. In single-ion monitoring (SIM) mode, 193 and 135 m/z peaks were chosen and quantification limit and detection limit were 0.01 ppm and 0.01 ppt, respectively. The FA recovery obtained with spiked samples was comparable with values previously reported by Porter et al. (30).

FB₁ extraction and analysis were performed by HPTLC according to the procedure described by Munkvold et al. (27) and MON extraction and analysis as described by Botalico et al. (3).

RESULTS

Sequence analysis. The sequences were compared to European and American data bases (EMBL and GenBank) showing 100% similarity with a sequence derived from a *F. proliferatum* isolate (NRRL 22944) available in the EMBL data base. The sequences were submitted to EMBL as: item 2337 = AJ271213, item 2341 = AJ271214, and item 2386 = AJ271215.

Pathogenicity test. The two *F. proliferatum* strains tested and the *F. solani* strain caused the most severe symptoms of the disease, with DSI of 3.48, 3.53, and 3.61, respectively (Table 1), whereas a lower disease severity was observed in plants inoculated with the strain of *F. oxysporum*. All results were significantly different from the control, which showed a DSI of 0.68.

Fertility test. All nine strains of *F. proliferatum* from date palm tested for fertility belonged to mating population D of *G. fujikuroi* and were of the same mating type, D⁺. Strains of *F. proliferatum* from wheat grown in the same area (Buriedah, Al Qassim region) were also tested for fertility and were of mating types D⁺ and D⁻ (data not shown).

Toxin production. The results of toxin production by strains of *F. proliferatum*

grown on autoclaved maize kernels are summarized in Table 2. All nine strains from date palm produced BEA (from 20 to 400 µg/kg) and FUP (from 70 to 300 µg/kg). FB₁ was produced at high levels by six out nine strains from date palm (from 630 to 1,000 µg/kg), whereas two of them produced the toxin in traces and ITEM 2343 did not produce any detectable amount of FB₁. Finally, MON was produced by all strains from date palm (from 40 to 330 µg/kg) but ITEM 2342, and FA was produced by only two out of nine strains isolated from date palm (230 and 1,300 µg/kg, respectively).

DISCUSSION

F. proliferatum was the predominant species isolated from visibly diseased trees of date palm in the regions of Al Qassim and Al Medina Al Monawara, with symptoms very similar to Bayoud disease, the vascular wilt of date-palm trees widely distributed in North Africa (20). The casual agent of Bayoud disease is *F. oxysporum* f. sp. *albedinis* (20), which has not been reported as occurring in date palm trees in Saudi Arabia. In this investigation, in addition to *F. proliferatum* strains, *F. oxysporum* was also isolated from date-palm trees with disease symptoms, but at a very low frequency. Based on morphological criteria, *F. proliferatum* has often been misidentified with other *Fusarium* spp. (29). Therefore, we confirmed the identity of the strains by sequencing a fragment of around 500 bp of the 5' end of the region coding for the 28S rDNA. This fragment had 100% homology with a sequence derived from a *F. proliferatum* isolate (NRRL 22944) available in the EMBL data base. As far as we are aware, this is the first report of the occurrence on date palm trees of *F. proliferatum*, which is a well-known pathogen of several plant hosts, including asparagus (8), maize (18), and rice (5).

The data of the pathogenicity tests on the date-palm seedlings showed that *F. proliferatum* should be regarded as a potentially dangerous pathogen of date palm in Saudi Arabia. In contrast, the *F. oxysporum* strain tested showed limited virulence on the date-palm seedlings. This shows that this strain may represent a saprophytic population in the soil. Although *F. solani* was highly pathogenic on seedlings of date palm, we considered it less important than *F. proliferatum* in the regions investigated because it was isolated rarely.

Moreover, we also assessed the ability of all *F. proliferatum* strains isolated from diseased plants to produce fertile perithecia in vitro crosses. Although just one mating type occurred among the isolates, the in vitro fertility of all the strains suggests that a potential for a high recombination in the date-palm fields exists, because the occurrence of the opposite mating type among the strains of *F. proliferatum* isolated from wheat plants grown in the same

areas of date palm plants was observed (data not shown). This is important because a high possibility of genetic recombination in the field could improve the genetic pool available for the pathogenic population of *F. proliferatum*. Mating population D of *G. fujikuroi* has been reported as occurring on several host plants (16), including asparagus (8) and rice (5), where the most common MP is C. *F. proliferatum* is considered the anamorph of both C and D MPs, which differ significantly in toxin production (5,24). This is the first report on the occurrence of *F. proliferatum* on date palm; therefore, it was of relevance to assess that MP D was the biological species of this group of isolates, this being, consequently, the first report of MP D on date palm. Moreover, it is interesting to observe that *F. proliferatum* strains from date palm belong to MP D together with many other populations of this species isolated from a very broad range of plant hosts, on which they show a high level of pathogenicity (e.g., asparagus, maize, rice, sorghum, and tomato; 5,9,16,18,25).

Among the toxins studied here, some of them have well-known phytotoxic activity, including FA, implicated in the pathogenesis of tomato wilt symptoms (10); MON, toxic toward tobacco plants (4); and FB₁, shown to be phytotoxic to maize and tomato (14). The *F. proliferatum* strains tested for pathogenicity on date-palm seedlings (ITEM 2336 and ITEM 2337) differed in the in vitro toxin production regarding FA alone, but no significant differences in the symptoms were observed in the pathogenicity assay. All of these secondary metabolites have phytotoxic activity and all tested strains proved to be producers; therefore, we suspect their involvement in the expression of symptoms on date palm plants. However, before drawing any conclusions about the role played by FA, MON, and FB₁ in date-palm wilt, it would be necessary to study the in vivo toxin production of the strains in pathogenic tests and compare the results with tests using pure toxins, also evaluating possible synergistic effects of the toxins on the plants.

In addition to the phytotoxic effects, all five toxins analyzed have mycotoxic activity. In particular, FB₁ was related to human esophageal cancer (32) and involved in several mycotoxicoses (12,35); FA affected both brain and pineal neurotransmitters of rats (30); MON was reported to cause hematological disorders, myocardial hypertrophy, and mortality in farm animals (11,15); BEA was toxic to several human cell lines and induces apoptosis in mammalian cells (21,22); and FUP showed cytotoxic activity and caused apoptosis on human B-lymphocytes (6,19) and teratogenic effects on chicken embryos (34).

It is worrisome that all strains of *F. proliferatum* we studied can produce at least

three of these toxins and that one strain (ITEM 2386) was able to produce all of them. *F. proliferatum* strains were also isolated from leaves of date-palm trees. In preliminary experiments on the virulence of *F. proliferatum* as a pathogen causing date-fruit rot, *F. proliferatum* was able to cause fruit rot at the Khalal and the Rutab stages (M. Y. Abdalla, unpublished). Similar results were also obtained by Elarosi (7), working with *F. lateritium* Nees:Fr. and *F. verticillioides* (Sacc.) Nirenberg (= *F. moniliforme* Sheld.) at the Eastern Province of Saudi Arabia. Therefore, the toxigenic data reported here warrant further investigations on the occurrence of *F. proliferatum* strains and some of these metabolites in date-palm fruit to evaluate the risk of consumption of contaminated date-palm fruit.

Finally, more attention should be given to the identification of *F. proliferatum*, having morphological traits similar to *F. oxysporum*, using tools other than morphological identification (e.g., molecular approaches) and further investigation is needed on the distribution of *F. proliferatum* in other geographical areas where date palm is cultivated.

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