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Full Length Research Paper

Inflorescence rot disease of date palm caused by *Fusarium proliferatum* in Southern Iraq

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Date palm is one of the important income sources for many farmers in different parts of several countries, including Iraq, Iran, Saudi Arabia, North Africa etc. Inflorescence rot is a serious disease of date palm which limits its yield. The identification of the causal organism is a key step to tackling this disease, and such studies are very scanty. The objective of this present study was to identify the causal agent of inflorescence rot disease occurring on date palms prevailing in Southern Iraq. The diseased date palm trees were observed in Shaat-Al-Arab and Al-Deer regions near Basrah in Iraq. The isolates were identified using morphological and molecular parameters. The internal transcribed spacer (ITS)/5.8S regions were amplified by polymerase chain reaction (PCR) and sequenced. The PCR method and phylogenetic relationship successfully identified that the causal organism of date palm inflorescence rot disease in southern part of Iraq is *Fusarium proliferatum*. Pathogenecity test confirmed the disease-causing ability of the fungus, and sequence comparison for similarity of ITS region. Identification of this new causal agent of inflorescence rot may help the plant pathologists to control this disease.

Key words: *Fusarium proliferatum*, ITS1, ITS4, pathogenecity, PCR, isolates, phylogeny.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is one of the important income sources for many farmers in different parts of Iraq, Iran and North Africa. Date palm trees are infected by several pathogens like fungi, bacteria and viruses, in addition to serious damage by different insect herbivores and mites. Inflorescences are vital part of date palm tree with respect to its date production and are also infected with different pathogens. One of the diseases of date palm is inflorescence rot (also known as Khamedj disease in North Africa). This disease and its causal fungus were reported for the first time by Cavara (1925a,b) in Libya.

Inflorescence rot is a serious disease and considered as a limiting yield factor. During the hot and humid season, this disease causes a crucial loss especially in heavy rains (up to two months) before emergence of the spathes. In favourable conditions, this disease can cause the infection on the same date palm tree repeatedly every year (Abdullah et al., 2005). This disease has been reported in Iraq by Hanford (1949) as severe outbreaks in 1948 to 1949 and 1977 to 1987, affecting male and female palms and caused 80% loss of the annual harvest (Al-Hassan and Waleed, 1977), and in United Arab Emirates (UAE) and Bahrain by Djerbi (1982). The disease transmission during the infection season occurs through the contamination of male inflorescences during the pollination period. The symptoms will be more apparent on the internal face of the spathes where the fungus has already begun the infection. The infections of the young inflorescence occur early when the spathe is still hidden in the leaf bases.

The fungus penetrates directly into the spathes and then reaches the inflorescences where the fungus sporulates abundantly. The first symptoms occur at the external surface of unopened spathes especially if emerges early in the spring season with typical brownish or rusty colour lesion (Abdullah et al., 2005; Djerbi, 1983). The fungal pathogen *Mauginiella scaettae* cavara is considered a major cause of inflorescence rot (Al-Ani et al., 1971). The primary infections by *M. scaettae* probably occur during the early stages of floral bud formation and prior to the envelope development of the spathes and their hardening (Al-Roubaie et al., 1987). Infected inflorescences remaining on palms from the previous

season or infected leaf bases may serve to spread the disease. The fungal pathogens isolated from the rotted inflorescence included *M. scaettae*, *Fusarium moniliform* Shed, *Fusarium solani* (Mart.), *Thielaviopsis paradoxa* (de Seynes) and *Fusarium oxysporum* (El-Behadili et al., 1977; Rattan and Al-Dboon, 1980).

Several causal agents of inflorescence were isolated and reported in literature like *T. paradoxa* in Libya (Edongali, 1969), *M. scaetta* in Spain (Abdullah et al., 2005), *M. scaetta* and *T. paradoxa* (*Ceratocystis paradoxa*) from inflorescence and fruits in Riyadh region (Cohen et al., 2010), *Fusarium proliferatum* from infected fruit stalks on date palm generated necrotic lesions in fruit stalk (Cohen et al., 2010), and from roots and leaves of declining date-palm trees in Saudi Arabia (Abdalla et al., 2000). The identification of causal organism is a key step to tackle this disease, and such studies are very scanty. The causal agent of inflorescence rot disease occurring on date palms at the Elx grove in South-East–Spain was identified through sequencing of the internal transcribed spacer (ITS) region of this fungus (Abdullah et al., 2005). The objective of our studies was to identify the causal agent of inflorescence rot disease occurring on date palms at Basrah, Iraq.

MATERIALS AND METHODS

Survey area and sample collections

Shaat-Al-Arab and Al-Deer regions near Basrah in Iraq were surveyed for the disease. Five samples were collected from each area and stored at 5°C.

Isolation of the pathogen

Briefly, 10 small pieces from each infected inflorescence (5 cm long) were surface-sterilized with 5% sodium hypochloride for 10 min, rinsed three times in sterile distilled water and blotted dry on sterilized filter paper. 10 pieces were placed onto plates containing potato dextrose agar (PDA) supplemented with chloramphenicol 50 µg/ml. All plates were incubated at 25°C until the fungus started steady growth. A hyphal tip for each isolate was excised on to a PDA plate to establish its colony. Single spore technique was used to ensure the purity of cultures. Isolated fungi were identified based on the morphological characters in culture and at molecular level by phylogenetic characterization.

Pathogenecity test

Four monosporic cultures were grown on potato carrot agar (PCA) composed of 20 g potato, 20 g carrot, 20 g agar and 1 L distilled water. Conidial suspensions from one of the four monosporic cultures were prepared in sterile water. The concentration of conidia was quantified microscopically using a neuberger chamber and adjusted to 10^6 conidia/ml with sterile distilled water containing 0.02% Tween 20. Three opened healthy spathes were detached, the upper external surfaces removed and surface-sterilized with 95% ethanol. The flowers and strands of detached spathes were inoculated by spraying with either 20 ml of the conidial suspensions or water (controls) using an atomizer. Inoculated spathes were incubated in plastic boxes at 25°C in the dark. After four days of inoculation, spathes were examined for lesions.

Re-isolation of the pathogen

To satisfy Koch's postulates, we re-isolated the pathogen from the sites of disease symptoms following the method previously described.

Genomic DNA extraction

Contamination free monosporic isolates obtained from infected inflorescences of date palms were used for genomic deoxyribonucleic acid (DNA) study. The monosporic isolates were grown on V8 juice agar for one week at 25°C. The mycelia were scraped off from the culture plates (V8) with a sterile scalpel, placed in a sterile micro tube containing sand and ceramic bead. 5 ml of extraction buffer was added. DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Moller et al., 1992). Extracted DNA was stored at -20°C until further use.

PCR amplification

The ITS/5.8S regions were amplified using primer pair ITS1 (5**'** -TCCGTAGGTGAACCTGCGG-3**'**) hybridizes at the end of 18S recombinant deoxyribonucleic acid (rDNA) and ITS4 (5**'**-TCCTCCGCTTATTGATATGC-3**'**) and at the beginning of 28S rDNA (Ferrer et al., 2001). Amplification was carried out in 25 µL reaction mixtures containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.6 mM of each dNTPs, 0.25 µM of each primer, 1.25 U *Taq* polymerase (Promega) and 4 ng gDNA. PCR was performed in a thermal cycler (MJ research, PTC-100). PCR cycling profile: hot start at 95°C for 5 min, 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were resolved on 1% (w/v) agarose gel (with ethidium bromide, 10 ng/100 ml). The fingerprints were examined under ultra-violet transilluminator (SynGen, Synoptics Ltd, UK) at 300 nm).

DNA sequencing

Amplified DNAs from the PCR were purified using the QIAquick gel extraction kit (QIAGEN) following instructions specified by the manufacturer and directly cycle sequenced in both directions using the big dye terminators ready reaction kit (PE Applied Biosystems, Foster City, California) on an ABI prism automated DNA sequencer (model 377, version 2.1.1; Applied Biosystems Warrington, United Kingdom) with ITS1 and ITS4 primers.

Negative control

Two negative controls were included in amplification; a reagent control (sterile distilled water) and a sample extraction control. The sample extraction control consists of sterile distilled water subjected to the same extraction procedures as the specimens.

Phylogenetic data analysis

F. proliferatum isolates, for which the rDNA sequence is available in GenBank, were assayed for selected primers hybridization using PCGENE program that facilitates the positive or negative *in silico* binding of primers to the sequence target. After alignment of the selected sequences using clustalW software, fragment sizes were

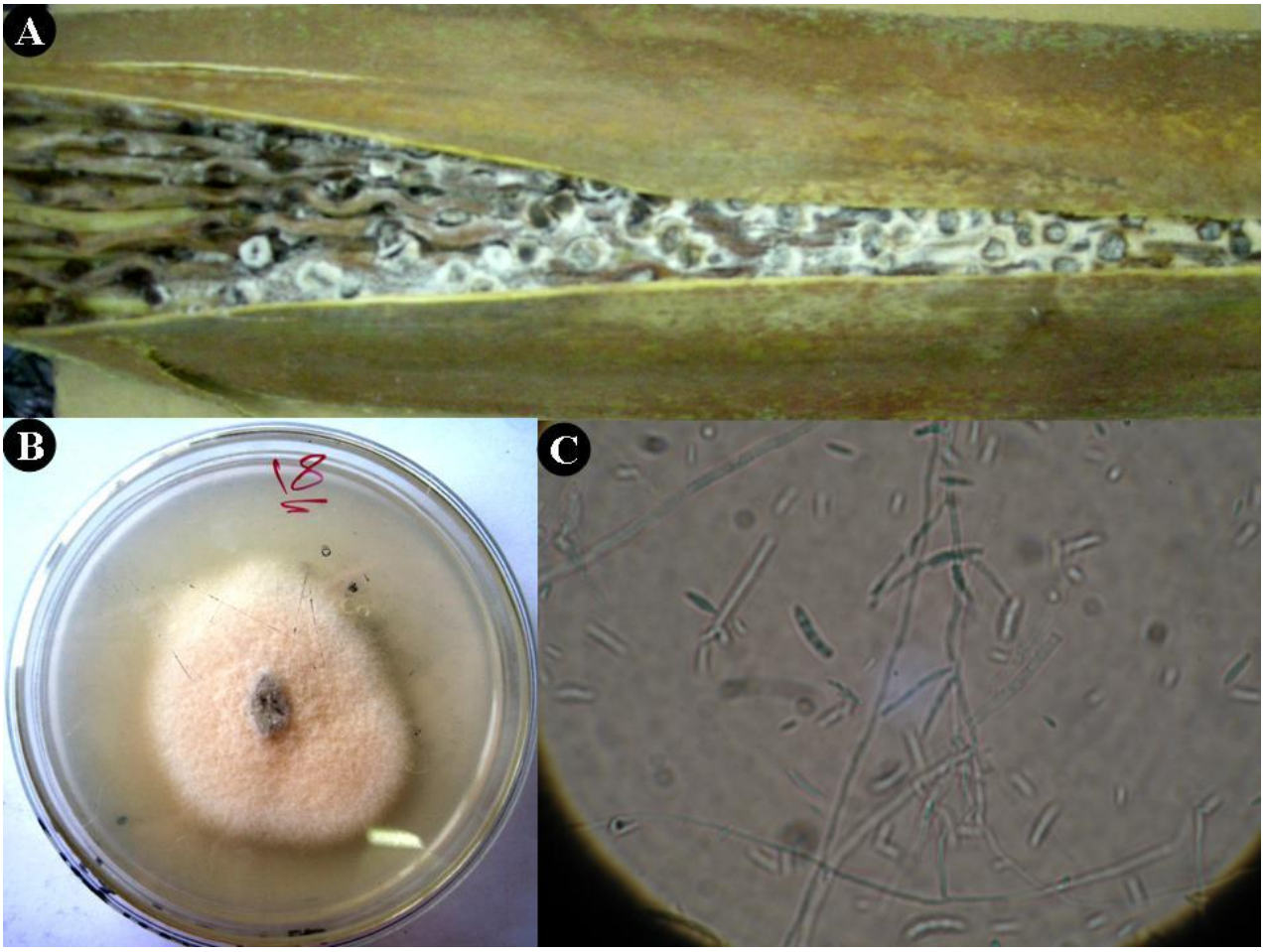

Figure 1. Inflorescence rot disease of date palm. A, symptoms of disease on the spathe; B, growth of fungus on potato carrot agar (PCA); C, hyphae and conidia of the fungus.

manually calculated. ITS2/5.8S rDNA sequences were analyzed by using the BLAST program of the GenBank database, National Institutes of Health. The alignment provides a list of matching organisms, ranked in order of similarity between the unknown/ observed sequence and the sequence of the corresponding organism from the database. The most similar 100 sequences were downloaded from NCBI and aligned using ClustalX2. The phylogenetic tree was viewed using MEGA5.0.

RESULTS

The inflorescence rot symptoms were obvious for the first time at the emerging spathes especially in early spring. It was on the external surface of unopened spathes with rusty lesions. Similar lesions were also seen inside the spathes and the flowers finally changed to a brownish color. The symptoms appeared mostly near the top of spathe and the disease invasion resulted in complete destruction of all flowers. Some of the infected spathes remained unopened and dried. The disease became more severe in high humidity especially if the spathe emerged earlier in the rainy season (Figure 1A).

Four isolates of *F. proliferatum* were isolated from the infected date palm spathes, two from Shaat-Al-Arab region and two from Al-Deer in Basra Iraq. Their classification was based on the morphological characters and phylogenetic similarity. The identification of the isolates was performed as described by Leslie and Summerell (2006). Four days post-incubation onto PDA plates which resulted to the abundant aerial mycelia initially were white and became purple violate, with violate pigments usually diffused into the agar medium (Figure 1B). On carnation leaf piece agar (CLA) the macroconidia are cylindrical almost with 3 to 5 septa and produced in chain (Figure 1C).

PCR specificity

The primers used in this study were ITS1 and ITS4, and successfully amplified the DNA from fungal isolates. The fragment obtained was about 280 bp (Figure 2). No amplification products were detected by using the ITS1-ITS4 and ITS86-ITS4 primer pairs with genomic DNA

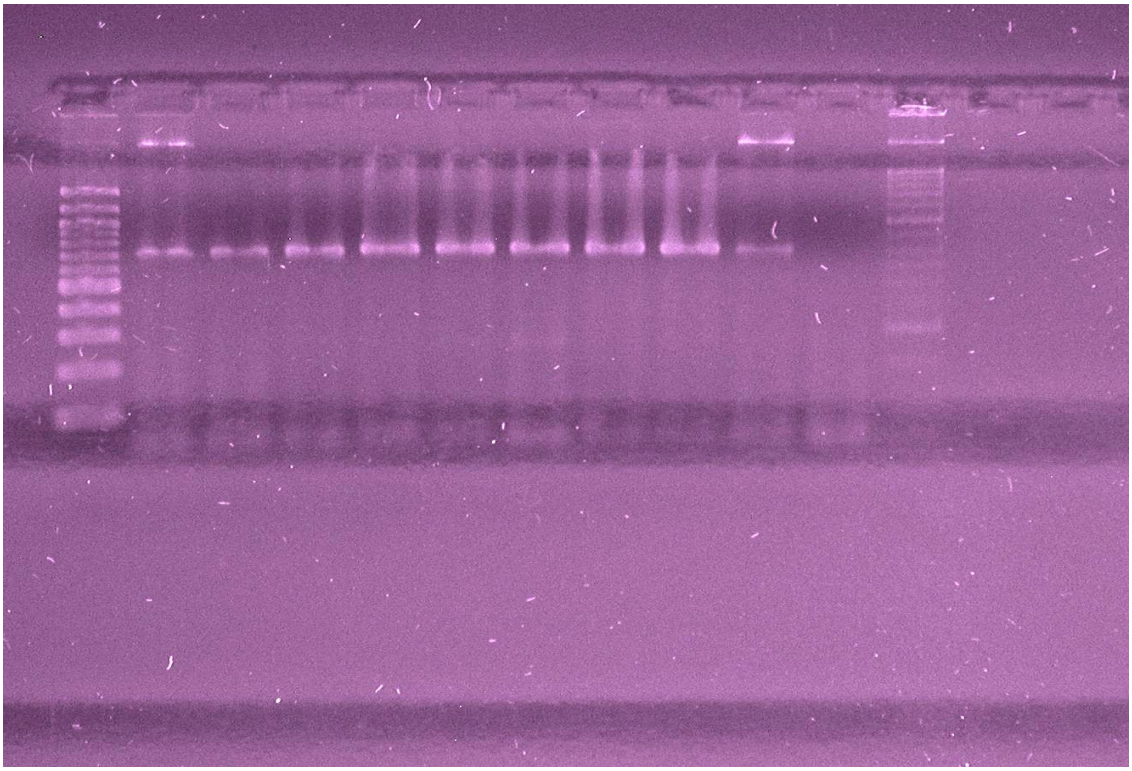

Figure 2. Electrophoresis pattern of ITS/5.8S regions amplified using primer pair ITS1 and ITS4.

isolated from date palm or from *Escherichia coli* genomic DNA. The fungal isolates were tested with the PCGENE program to ascertain the specificity of this method. The sizes of the fragments obtained were in agreement with those obtained by PCR.

Pathogenecity assessment

All the spathes previously inoculated with the spore suspensions of *F. proliferatum* showed disease symptoms and this test proved the ability of the pathogen to cause the inflorescence rot disease.

DNA sequencing and phylogenetic analysis

The obtained DNA fragments representing full-sequence ITS/5.8S rDNA was purified by gel extraction method and sequenced. The sequence results from all four inflorescence rot samples when compared to DNA database demonstrated that they were derived from the fungal ITS regions. All the sequenced samples were alike in their sequence. To identify the species, sequence similarity search was performed using basic local alignment search tool (BLAST) at NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The observed sequence neighbored the ITS1/ITS2 sequence of strain bxq33107 of *F. proliferatum* (Figure 3).

DISCUSSION

The PCR-based method is considered as a fast and more sensitive technique compared to the morphological identification. PCR based method has high discrimination ability to differentiate between morphologically similar species such as *F. proliferatum* and *F. moniliform*. Most of the previous studies classified *F. proliferatum* as *F. Moniliform* due to the high level of similarity between these two different species. In terms of morphology and biology, *Fusarium fujikuroi* and *F. proliferatum* have also been reported to be very similar (Leslie et al., 2007). The universal ITS regions are typically variable and quite informative (Diaz and Fell, 2004). The ability of the ITS region to differentiate within *Fusarium* species have been reported as a reliable and faster way to discriminate the isolates at both the genus and species level (Oechsler et al., 2009).

Our results show that the presence of inflorescence rot disease in date palms from Shaat-Al-Arab and Al-Deer areas in southern part of Iraq, several isolates collected in the present studies represented the new pathogen. These isolates were identified from the rotted inflores-cences of the two areas surveyed. The pathogenecity test proved the virulence of these isolates. The observed morphological description was similar to a previous report (Leslie et al., 2007). The identification of fungi was confirmed by using the ITS region amplification and sequencing. The sequence results demonstrated that

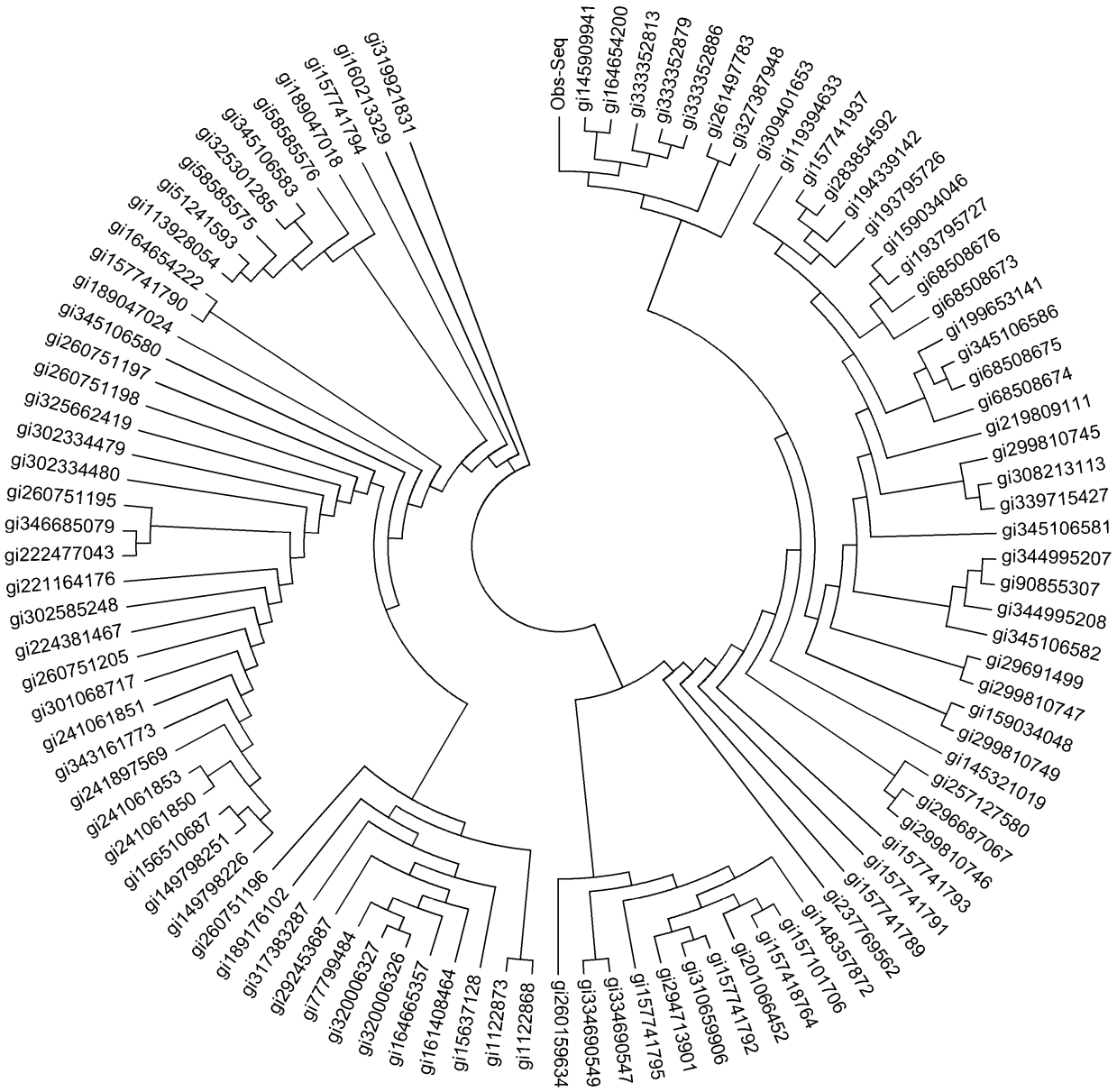

Figure 3. Phylogenetic relationship of fungus sequence with most related 100 sequences in GenBank.

they were derived from the fungal ITS regions when compared to the database. BLAST results showed the most similar sequence was ITS/5.8S rDNA region of *F. proliferatum* strain bxq33107 showing 100% identity. Result from sequence similarity established that the isolated strains belonged to *F. proliferatum* species.

Conclusion

This is the first report that the *F. proliferatum* is the causal agent of date palm inflorescence rot disease in southern part of Iraq. PCR amplification followed by sequencing is a successful method of detection and identification of the fungal pathogen through amplifying, sequencing and sequence comparison for similarity of ITS region. Pathogenecity test confirmed the disease-causing ability of the isolate. Detection of this new causal agent of inflorescence rot in Iraq will encourage plant pathologists to conduct further investigations to find out the best way for controlling this disease.

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