Phenotypic and molecular identification of fungal contaminants of date palm (*Phoenix dactylifera* L.) tissue culture in Iraq

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Abstract

The study aimed to identify fungal contaminants of date palm (*Phoenix dactylifera*) tissue cultures in the laboratories of the Palm Research Center in Basrah University. Three types of fungi were isolated from the tissue cultures. The its1–its4 ribosomal RNA region was amplified using polymerase chain reaction to confirm the molecular matching of the fungi. The matching of fungi and identified the types as *Fusarium coffeatum*, *fusarium luffae* and *Paecilomyces formosus*, with a matching rate ranging from 97.81% to 99.32%. The registration numbers for the its1–its4 region sequences are reported in the NCBI gene bank (AMFC OL589161, AMF OL589160 and AMP OL589158) respectively. This study is the first to record these fungi as date palm tissue contaminants in Iraq.

Keywords: date palm tissue culture, phenotypic and molecular diagnosis, fungal contamination

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Introduction

The date palm *Phoenix dactylifera* L. is an important fruit palm tree worldwide, particularly in the Middle East. Dates are an important source of energy and essential nutrients, such as carbohydrates, and minerals, such as iron, potassium and calcium (Dayani et al., 2012; Abass, 2013).

Plant tissue culture is widely used to grow and produce genetically identical and disease-free plants in a small area and short time (Odutayo et al., 2007; Eziashi et al., 2014). However, plant tissue cultures are prone to contaminants (Rawal and Keharia, 2019) .

Fungal and bacterial contaminants contribute to the failure of plant tissue cultures; microbial contamination of excised plant parts for tissue culture is caused by poor sterilisation of the plant samples, tools and work surfaces (Omamor et al., 2007; Cobrado and Fernandez, 2016; Izarra et al., 2020).

Microbial contamination leads to an economic loss of time, effort and materials (Abass, 2013). Fungi and bacteria are common pollutants in plant tissue cultures because of their ubiquity. Common fungal contaminants include *Aspergillus niger*, *Alternaria tenius*, *Fusarium culmorum* and *Aspergillus fumigatus* (Msogoya et al., 2012; Cobrado and Fernandez, 2016).

Common bacterial contaminants include *Bacillus subtilis*, *Bacillus licheniformis*, *Pseudomonas syringae*, *Erwinia* spp., and *Corynebacterium* sp. (Cobrado and Fernandez, 2016; Rawal and Keharia, 2019).

The phenotypic diagnosis of tissue-contaminating fungi may be inaccurate, and only a few fungal species can be diagnosed despite their diversity (Pinto et al., 2012; Abass, 2017; Rawal and Keharia, 2019). Molecular technology has been used to explore fungal diversity within species and even within strains. Considering the different gene pools of organisms, molecular technology is an accurate and fast way to identify and detect even the slightest variation of fungal contaminants in tissue cultures (Adeyemo and Erilude, 2014).

Polymerase chain reaction (PCR) is an accurate and reliable technique for distinguishing fungal species and strains through genotype recognition using ribosomal DNA (rDNA) and internal transcribed spacer (ITS) sequences. ITS

sequences consist of multiple copies in the fungal genome, and they significantly differ in convergent species; hence, differences in these sequences can be used for the taxonomy and characterisation of fungal aggregates (Cobrado and Fernandez, 2016).

Phenotypic diagnosis of tissue-contaminating fungi is less sensitive and may take a long time (Hasan et al., 2016). Therefore, this study aimed to classify fungal contaminants in textile farms using molecular technology to provide the best fungal control and sterilisation strategies.

Materials and methods

Purification of fungal contaminants: Plant parts were inspected daily to detect possible contaminants and isolate them for identification. Planting pipes and calluses showing fungal contamination were washed with sterile distilled water to remove traces of the agricultural medium used to grow date palm tissues, dried with sterile filter paper, and then transferred using sterile forceps to 9 cm-diameter petri dishes containing potato dextrose agar (PDA) supplemented with 250 mg/L chloramphenicol. The petri dishes were sterilised with a steam steriliser at a temperature of 121 °C and a pressure of 1.5 bar for 20 min before use. The inoculated dishes were incubated at a temperature of 25ºC for 3–7 days, after which the phenotypic characteristics of the fungal colonies were examined (shape, size, centre, limbs, colour, transparency and fungal growth) and recorded. The developing fungi were also examined under a (Compound microscope, Olympus, Pilipinas) (shape and colour of fungal hyphae and conidia).

Fungi were isolated and purified for diagnostic purposes (Almayahi et al., 2010). The developing fungi were identified following the methods described by Ellis (1971), Samson (1974), Domsch et al. (1980), Barnett and Hunter (1998), Leslie and Summerell (2006) and Samson et al. (2009).

The isolates were transferred to a slant culture medium in sterile 50 mL tubes with a tight lid and then incubated at 25 °C. The growth of the fungi was observed, after which the tubes were stored in the refrigerator at 4 °C for use in subsequent experiments.

Molecular identification

DNA extraction and acarose gel electrophoresis

Total DNA was extracted from the cells using a DNA Geneaid extraction kit (Promega Genomic DNA Purification kit A1120, USA) in accordance with the manufacturer's instructions. This experiment was conducted using a pure culture of fungi incubated on a PDA medium at 25 ± 2 °C for 7 days. The fungi were scraped from the dishes and then placed in a ceramic mortar with the addition of liquid nitrogen to crush them into fine powder. The concentration of the extracted DNA was measured using NanoDrop, and the genomic DNA was stored at -20 °C until use. Electrophoresis was performed using 0.5 g of acarose, which was prepared as follows. In brief, 25 mL of structured solution was heated on a hot plate, allowed to cool down to 40–50 °C and then added with 0.5 µL of ethidium bromide. A mixture of 1 µL of bromophenol blue dye and 5 µL of DNA ladder was placed in the first hole of the acarose gel, and 9 µL of the polymerisation reaction mixture was placed in the second hole. This step was performed for the rest of the samples, and then an electric current was passed for the hourly detection of beams resulting from amplification using an ultraviolet device. Images were obtained using a digital camera.

PCR test

The powder (25 mg) was transferred to a 1.5 µL Eppendorf tube for DNA extraction through PCR following the White method (1990). The PCR was conducted using the Green Master Mix (Promega) with the prefixes ITS1 and ITS4. The samples were centrifuged at 10,000 rpm for 30 s to ensure the homogeneity of all materials in the Eppendorf tube. Then, they were placed in the PCR sprint thermal cycler amplifier, and the device was operated following the program conditions in Table 1.

PCR amplification of the its1–its4 ribosomal RNA region was performed for molecular identification of fungi (Table 2).

Table (1) The sequence of nitrogenous bases in the ITS primer used in the amplification process

Primer	Primer Sequences(5´-3´)	Length	Tm	Ta
ITS1	F-5-TCC GTA GGT GAA CCT GCG G-3	19base	62 C°	57 C°
ITS4	R-5-TCC TCC GCT TAT TGA TAT GC-3	20base	58 C°	53 C°

Table (2) The program of the PCR process used in the current study using the ITS primer

Sr .No	Steps	Temperature/C°	Time	No. of cycles
I	Denaturation1	95	1 min	1
II	Denaturation2	95	1 min	35
III	Annealing	55	45 Sec	
IV	Extention	72	1 min	
V	Final Extention	72	10 min	1

sample preparation

The samples were prepared in accordance with the instructions of the Korean company Macrogen. by sending the volume of the product of the PCR 25 microliters to 3 samples and taught the samples to distinguish them from each other. The samples were preserved in a cold box containing ice.

Genetic analysis of polymerisation products

The resulting samples from PCR polymerisation were analysed at the South Korean company Macrogen company.

Results and discussion

Isolation of fungi associated with date palm tissue culture

Three fungal species isolated from the date palm tissue cultures were identified as *Fusarium coffeatum*, *F. luffae* and *P. formosus* according to their phenotypic traits (Table 3, Fig. 1). Microscopic identification was performed by examining fungal slices under a compound light microscope after applying a drop of lactophenol blue dye (Aina et al., 2011).

Table (3) phenotypic characteristics of fungi isolated from the primary callus of date palm tissues

fungi	Colony shape	Colony color	Colony appearance	Growth period	Conidia shape	conidia Color	conidia Diameter	myceliu m Shape	mycelium Color	Conidiophores
Fusarium coffeatum	The fungal hyphae is spread with radial growth in the form of rings, the center is brown, surrounded by yellow color, and the edges are in the form of white fluffy growth	The center is brown surround ed by yellow color and the edges are in the form of white fluffy growth	The center is brown and the edges are creamy	9 days	Elongated in shape, and there are two types, the macroconidia one is divided into 2-4 septate, the microconidia one is in size, and the ends are thin	light gray to transpa rent	macrocon -21.6 idia x 33.6 4.8-3.6 microcon idia x 24-14.4 4.8-2.4	Thin and curvy with spaced septates	transparent to light gray	Short, branched, translucent
Fusarium luffae	The fungal hyphae is diffuse with radial growth in the form of rings, the center is yellow, surrounded by a pink color, and the edges are in the form of white fluffy growth	The center is yellow surround ed by a pink color and the edges are in the form of white fluffy growth	orange to yellow	15 day	Spindle-shaped and there are two types, the large one divided into one or more, and the small one by size	light gray to transpa rent	macrocon -24 idia x33.6 4.8-3.6 microcon - 9.6 idia x 16.8 7.2-4.8	Thin and curvy with spaced septates	transparent to light gray	Short, branched, translucent
Paecilomyces formosus	The fungal hyphae is diffuse with a radial growth in the form of a white fluffy growth under it greenish-yellow growth	A fluffy white growth under it is a greenish-yellow growth	The center is gray surrounded by a gray-yellow ring	4 days	Spherical and oval, elongated, with a pointed end, in a light yellow color, and the elongated ones are in the form of a chain	Short and wider than the conidia in brown color	x 6-4.8 4.8-2.4	Branched , weak, twisted with spaced septates	light yellow	transparent to light gray

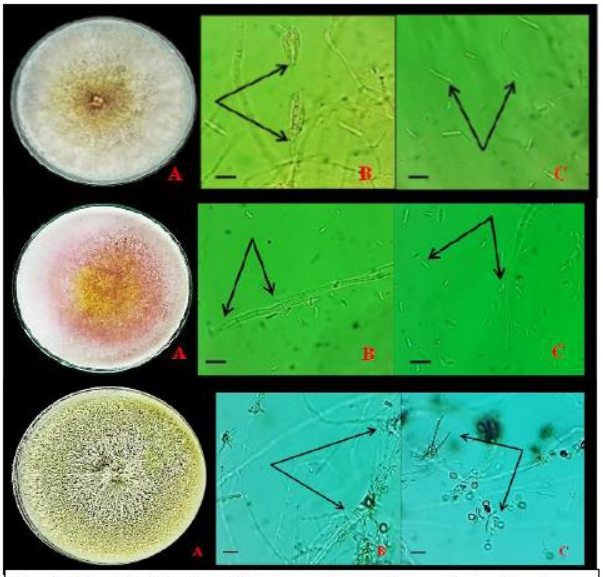


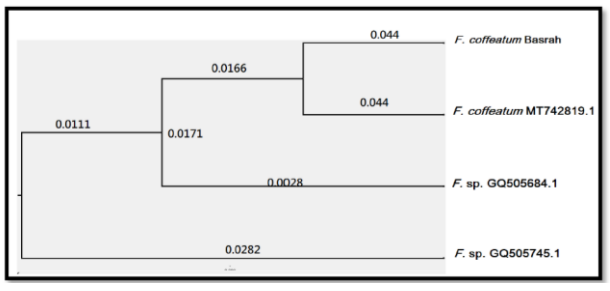
Fig. (1) Morphological traits of *Fusarium coffeatum*, *Fusarium luffae*, *Paecilomyces formosus*
(A) colony morphology on PDA after 9 days, (B) Conidiophores (C) macroconidia and microconidia surface decorations with a magnification of 40 x. Scale bar= 10 µm.

Molecular identification

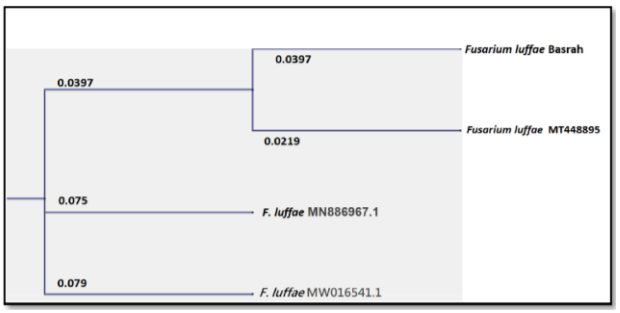
Table 4 shows a summary of the BLASTn results for sequencing the its1–its4 region of the ribosomal DNA of the fungi isolates and the phylogenetic subtrees for the fungal species (Fig. 2.A.B.C).

Fungal isolate	Base pair	GenBank accession number	Query coverage	Percentage of sequence identity	GenBank accession number of organism with the highest sequence identity
Fusarium coffeatum	543	AMFC OL589161	100%	97.81%	(MT742819.1)
Fusarium luffae	517	AMF OL589160	99%	98.06%	(MT448895.1)
Paecilomyces formosus	560	AMP OL589158	83%	99.32%	(JX406544.1)

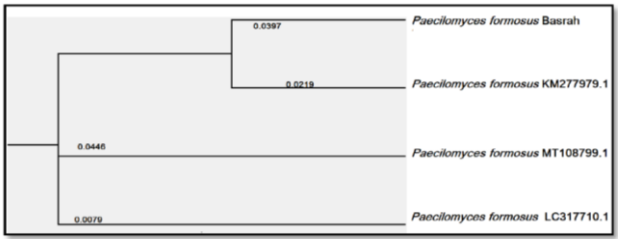
Table (4) Summary of the isolates blast sequence on the NCBI nucleotide database and the fungi identified from the NCBI based on internal space region markers



(Fig. 2.A) Phylogenetic subtrees for the fungal species *Fusarium coffeatum* generated by maximum likelihood technique depicted the branch lengths values between the identifier fungi and reference fungi from GenBank (https://www.ebi.ac.uk/Tools/msa/clustalw2/ ; http://tree.bio.ed.ac.uk/software/figtree/).



(Fig. 2. B) Phylogenetic subtrees for the fungal species *Fusarium luffae* generated by maximum likelihood technique depicted the branch lengths values between the identifier fungi and reference fungi from GenBank (https://www.ebi.ac.uk/Tools/msa/clustalw2/ ; http://tree.bio.ed.ac.uk/software/figtree/).



(Fig. 2.C) Phylogenetic subtrees for the fungal species *Paecilomyces formosus* generated by maximum likelihood technique depicted the branch lengths values between the identifier fungi and reference fungi from GenBank (https://www.ebi.ac.uk/Tools/msa/clustalw2/ ; http://tree.bio.ed.ac.uk/software/figtree/).

The database confirmed the matching of fungi, which were identified as *F. coffeatum*, *F. luffae* and *P. formosus*, with a matching rate ranging from 97.81% to 99.32%. The registration numbers for the its1–its4 region sequences are reported in the NCBI gene bank (AMFC OL589161, AMF OL589160 and AMP OL589158)
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Several studies have isolated fungal contaminants from date palm tissue cultures. Abass (2013) isolated and identified fungal contaminants, including *A. niger*, *Alternaria alternata* and *Penicillium* spp., from various date palm tissues and tissue culture laboratories in Iraq. Abass (2017) also found through molecular technology that bacteria belonging to the genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Epicoccum* and *Penicillium* are the dominant fungal contaminants in date palm tissue cultures.

Identification of contaminants is important to determine the best strategies to provide the best fungal control and sterilisation strategies. Abeer and Abdel (2017) confirmed that fungal contaminants negatively affect date palm reproduction in the laboratory and that the dominant fungi in date palm tissue cultures are *Alternaria* sp. *A. niger* and *Penicillium* sp.). Meanwhile, Jasim et al. (2021) reported *Alternaria* sp., *Fusarium* sp., *Aspergillus* sp. and *Penicillium* sp. as the dominant fungal contaminants in date palm tissue cultures.

The present study is the first to identify *F. coffeatum*, *F. luffae* and *P. formosus* as fungal contaminants of date palm tissue cultures in Iraq.

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