Kirkuk University Journal for Agricultural Sciences
ISSN:2958-6585
https://kujas.uokirkuk.edu.iq

RESEARCH ARTICLE https://doi.org. 10.58928/ku26.17212

Morphological and Molecular Diagnosis of Fungi Causing Root Rot and Death of Date Palm Offshoots in Karbala

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Received:24/02/2026 **Revised: 01/05/2026** **Accepted: 20/05/2026** **Published: 08/06/2026**

ABSTRACT

Study aimed to isolate and identify the fungi causing root rot and death of date palm offshoots (*Phoenix dactylifera L.*) in orchards of the Al-Husseiniya area in Karbala Governorate. The field survey included 12 orchards, and its results showed a clear spread of the disease, with deterioration percentages ranging from 14–28% and death percentages from 13–42%, indicating the presence of influential pathological factors in the success of offshoot cultivation. Laboratory isolation resulted of the fungus *Alternaria alternata*, which caused a significant decrease in the germination percentages of radish seeds and palm seedlings to 15–17%. The molecular Identification of the most pathogenic isolate was confirmed using amplification of the ITS region and the Actin gene, and the genetic comparison via the GenBank database showed a high match with global isolates. The *A. alternata* sequence was deposited under the number PV803540, and the phylogenetic tree showed a clear evolutionary convergence with internationally registered isolates. The results confirm that fungal infection is a major factor in the deterioration of palm seedlings, highlighting the importance of early Identification and the adoption of integrated preventive management to minimize losses.

***Keywords*:** Date Palm; Root Rot; Alternaria alternata; Molecular Identification; Pathogenicity.

INTRODUCTION

The date palm, *Phoenix dactylifera* L., is considered one of the most important economic trees in Iraq and the world, because it is characterized by a high ability to tolerate various difficult environmental conditions such as drought, high temperatures, and soil salinity, in addition to its agricultural and historical status **[1]**. Iraq is considered one of the oldest homelands of date palm cultivation, as it includes about 600 cultivars grown on an area of 123,230 hectares, with a number of trees estimated at 17,348,741 palms, and an annual productivity reaching 735,353 thousand tons **[2,3].**

Vegetative propagation by offshoots is the most common method in date palm cultivation, because it provides genetic uniformity and high success percentages when suitable offshoots are selected in terms of age and weight **[4].** The size and weight of the offshoot affect growth characteristics, including the survival percentage and the number, length, and diameter of roots **[5].** Also, treating the separation area of offshoots with protective materials such as ash or chemical fungicides, and observing the timing of planting, can increase the success percentage to about 89% **[6].** However, deterioration and early loss of offshoots represent a major agricultural and economic challenge. Recent investigations suggested that pathogenic fungi, particularly soil-born pathogens, play a main role in decreasing root efficiency and affecting the absorption of water and nutrient elements and accelerating offshoot death **[7].**

This study aims to evaluate the prevalence of deterioration and death of saplings in orchards, isolate and identify the pathogenic fungi associated with the roots, and perform molecular characterization of the most virulent isolates.

Materials and Methods

Field survey

A survey was conducted in 12 orchards in the Al-Husseiniya area to determine the percentages of deterioration and offshoot death. The percentages were recorded for each area, and samples were placed in plastic bags with the information for each sample fixed, then transported to the laboratory and kept in the refrigerator under a temperature of 4° C for the purpose of isolating the fungi associated with roots. After that, fungi were isolated and diagnosed through washing the roots with running water for 30 minutes to remove soil, and then they were cut into small pieces (0.5–1 cm long) after surface sterilization in 1% sodium hypochlorite solution for two minutes, washing distilled water and then drying of a sterile blotting paper Then samples were transferred in Petri dishes with Potato Dextrose Agar (PDA) medium, submitted to at 121 °C and pressure of 15b/in² for 20 minutes, enriched with Amoxicillin antibiotic at the concentration of 125 mg / L to minimize bacterial contamination. The plates were incubated at 25 ± 2 °C for three days, the fungal isolates were then purified by re-culturing on fresh PDA medium. The isolated fungi were diagnosed depending on macroscopic and microscopic characteristics using the approved taxonomic keys **[8].** After that, the pathogenicity of the pathogenic isolates was tested using radish seeds in the laboratory on WA (Water Agar) culture medium.

Pathogenicity Test

A laboratory experiment was carried out to assess the pathogenicity of the most frequently encountered fungal species in the isolates by investigating their impact on germination percentage of radish seeds. The test was performed by plating Petri dishes with a diameter of 9 cm containing Water Agar (WA) medium that prepared, using antibiotic Amoxicillin at 125 mg/L to autoclaved for 20 min at 121 °C and pressure15 lb/in² from the cold solution then dissolving 20 g agar in 1000 ml distilled water. The petri plates were inoculated using 5 mm diameter disc of fungal cut from the edges of the colonies of isolates aged seven days old growing on PDA medium and incubated at 25 ± 2 °C with three replicates. After 3 days, the radish seeds soaked in 1% sodium hypochlorite solution for 2 min as surface sterilized were sown, and placed around the edge of each plate at a rate of 10 seeds for each plate. The dishes were incubated for each treatment, in addition to the control treatment (sterilized seeds not inoculated with the fungus). The results were recorded after seven days from planting by calculating the percentage of seed germination according to the following equation:

$$\text{Percentage of seed germination} = \frac{\text{number of seeds germinated}}{\text{Total number of seeds}}\ x\ 100$$

Pathogenicity test of some pathogenic fungal isolates on 60-day-old palm seedlings in the plastic house

The trial was implemented using the same test isolates that showed the highest frequency percentages within the samples taken from the collection areas. The isolates were cultured on local millet seeds (*Panicum miliaceum*) as 50 g millet seeds were added to 250 mL glass flasks, washed with distilled water, autoclaved at a temperature of 121 °C and a pressure of 15 lb /in² for 20 minutes and the sterilization was done twice after every two days. Each flask was inoculated with five fungal discs taken from the edges of colonies of the isolates growing on PDA medium at seven days of age, then the flasks were incubated at 25 ± 2 °C for 14 days, with shaking every 48 hours to ensure distribution of the fungus on all seeds **[9].**

Soil mixtures of soil and peat moss at a ratio of 1:2 (v/v) were applied, autoclaved in the same way, and added to plastic pots (15 cm diameter) at the rate of 1kg pot^{-1}. The pot where the inoculum was added and mixed with soil served to infest the soil. The soil was infested with 10g of each fungal isolate (grown in millet medium) per pot. The pots were watered carefully, then covered with perforated polyethylene bags for three days. After that, 60-day-old seedlings were transferred to the pots at a rate of one seedling per pot, and placed in the plastic house with three replicates for each treatment. The pots were irrigated regularly, with addition of a nutrient solution once every 15 days.

After 60 days from infestation, the seedlings were uprooted and disease severity was estimated depending on the disease index mentioned in **[10]**, as follows:

0 = healthy plant

1 = discoloration of root hairs with a yellowish-brown color and no effect on the vegetative growth

2 = extension of discoloration to the main roots and its change to light brown, with slight yellowing in the leaflets extending from their tips

3 = the brown discoloration includes the root system and the extension of yellowing in the vegetative growth to half of the leaflets

4 = discoloration of the root system with a blackish-brown color, death of most of the leaflet's area, and stunting of growth.

5 = death of the seedlings

The percentage of disease severity was calculated according to the McKinney **[11].** equation as cited in Mashhadani **[12].**

$$\text{Infection severity \%} = \frac{\text{Total number of plants in grade x grade number}}{\text{Total number of plants x highest grade}} \text{x100}$$

Molecular diagnosis of the most pathogenic fungus:

The Polymerase Chain Reaction (PCR) test was adopted in this study to confirm the macroscopic and microscopic diagnosis of the pathogenic fungal isolate causing the root rot disease and death of offshoot seedlings, according to what was reported by Narayanasamy **[13].** Genomic DNA was extracted and purified from the most pathogenic fungal isolates using the commercial kit DNeasy Plant Kit, according to the manufacturer's instructions.

A volume of 2 µL of genomic DNA was used as a template in the PCR reaction, with Ready-To-Go PCR Beads that contain the basic components of the reaction. The internal transcribed spacer (ITS) region was amplified with ITS1 and ITS4 primers, which allowed targeting of regions flanking small and large subunit rRNA genes in fungi **[14].** Amplification of the Actin gene with primers ACT-512F and ACT-783R was performed for confirmation of molecular diagnosis

A thermal program was used to conduct PCR reactions for ITS region. The program included an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation (95 °C/40 s), annealing (55 °C/40 s), and extension (72 °C/1 min). The final extension was done at 72 °C for 5 minutes. For amplification of the Actin gene, an alteration in the thermal program was used. This involved 40 cycles along with suitably timed heating stages for each stage **[15].** The electroplating products were carried over on the agarose gel at a concentration of 1.5%, dyed with ethidium bromide, and then examined using ultraviolet light. PCR products for its region and Actin gene were sent to Macrogen (South Korea) to determine the sequence of nitrogenous bases, after receiving the results, analyzed and purified using Chromas and ApE software and then compared the sequences using the BLAST software within the GenBank database of the National Center for Biotechnology Information (NCBI) for the genetic identification of isolates, the approved sequences were used in the construction of the phylogenetic tree using Bio Edit and Mega software, and the final sequences were deposited in the GenBank repository after obtaining their deposit numbers **[16].**

Results and Discussion

Field survey

The survey showed the prevalence of deterioration and death of offshoots in all orchards (Table 1). The percentage of deterioration ranged between 14-28%, and the highest percentage was recorded in the area of the Al-Sharfa (28%), and the lowest in the Al-shahir (13%). This discrepancy is due to different agricultural factors such as the cleanliness of the orchard, the separation of offshoot, the use of pesticides or growth regulators in addition to environmental conditions such as high humidity on the banks of riverbanks **[17].**

These results are consistent with what previous studies indicated that poor agricultural practices, especially the failure to remove infected palmate residues and the failure to apply preventive treatments before planting the offshoots, contribute significantly in the increase the severity of fungal diseases **[18].**Other studies also clarified that wounds resulting from improper separation of offshoots, in addition to high humidity in orchards close to water sources, provide suitable conditions for the growth of pathogenic fungi and increasing fungal inoculum density, which leads to higher percentages of deterioration and death in newly planted offshoots **[19].**

Table 1: infection rate of date palm offshoots in the orchards included in the survey

Sample no.	Location	Dead Offshoots (%)	Deteriorated Offshoots (%)
1	Al-Husseiniya / Al-Sada Al-Sharfa	42	28
2	Al-Husseiniya / Atrah	20	16
3	Al-Husseiniya / Al-Atishi	28	22
4	Al-Husseiniya / Al-Taff	16	14
5	Al-Husseiniya / Al-Wand	26	24
6	Al-Hasaniyah / Awn	15	17
7	Al-Husseiniya / Bab Baghdad	15	18
8	Al-Husseiniya / Al-Salamiyah	38	16

9	Al-Husseiniya / Al-Ibrahimiyah	39	19
10	Al-Husseiniya / Al-Shahir	13	15
11	Al-Husseiniya / Albu Ghanim	36	22
12	Al-Husseiniya / Al-Shami	24	20

Isolation and Diagnosis of Fungi

Four major types of *Alternaria alternata, Fusarium graminearum, Pythium aphanidermatum, and Rhizoctonia solani* roots were isolated (Table 2). The fungal prevalence was higher in degraded and dead roots, reflecting their negative effect on water and nutrient uptake, and accelerating the death of the roots.

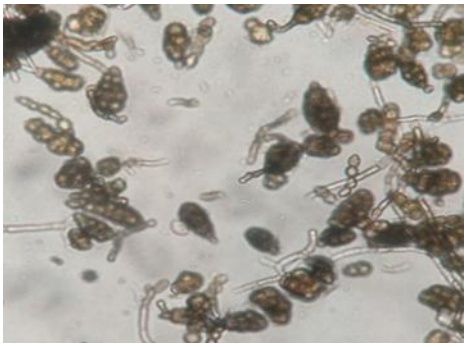
A. alternata recorded the highest frequency in the orchards of al-Atishi, Al-Taf, Bab Baghdad and Al-Shami, with a rate of 15.60% and the highest frequency of 42% in sample 1, which is known to secrete alternariol and tenuazonic acid toxins, and cytolytic enzymes **[20,7].** In contrast, *R. solani* recorded the lowest incidence (9%), consistent with areas with the lowest degradation and death.

Table 2: types of fungi associated with the roots of date palm offshoots

Fungi	Sample number*
Alternaria alternata	3-112-7-4-
Fusarium graminearum	11-6-2
Pythium aphanidermatum	7-6-4
Rhizoctonia solani	10-4

*The numbers represent the locations of all samples (Table 1).

Figure 1: Alternaria alternata on W.A medium



Detection of pathogenic isolates using red radish seeds in the laboratory

All isolates showed a pathogenic effect with a significant decrease in germination compared to (100%) (Table 3) as the first isolation (*Alternaria alternata*) recorded the lowest germination rate (17%), followed by the second and third isolates, while the fourth isolation (*R. solani*) was the weakest (30%), consistent with Al-Jubouri **[21].** on the impact of different isolates on the severity of pathogenesis **[22,23].**

Table 3: pathogenicity test on radish seeds

Isolate no.	Fungus	Germination percentage*
Control	Without fungus	100
1	*Alternaria alternata*	17
2	*Fusarium graminearum*	24
3	*Pythium aphanidermatum*	26
4	*Rhizoctonia solani*	30
L.S.D at 5%		3.1

Second - Detection of nursing isolates on palm seedlings

In the greenhouse experiment, germination was further reduced under the influence of *A. alternata* (15%), followed by *F. graminearum* (25%) and *P. aphanidermatum* (27%), while *R .solani.* (33%) is the least influential (Table 4). The strong effect of isolates is due to the secretion of secondary toxins and phenolic compounds that disrupt the outer seed layer and inhibit enzymatic activity, which Studies have confirmed such as **[20].**

Table 4: pathogenicity test on 60-day-old date palm seedlings

Isolate no.	Fungus	Germination percentage*
Control	Without fungus	100
1	Alternaria alternata	15
2	Fusarium graminearum	25
3	Pythium aphanidermatum	27
4	Rhizoctonia solani	33
L.S.D at 5%		3

Molecular diagnosis:

The rDNA-ITS region was amplified using the ITS1-ITS4 primers, producing amplification along 500–1000 nitrogen bases. The analysis via BLAST confirmed that the most pathogenic isolation is *Alternaria alternata*, and the sequence was deposited in GenBank under the accession number PV803540 (Table 5). A phylogenetic tree was also plotted to compare isolation to previously recorded isolates.

Table 5: fungal isolate identified in GenBank

ت	Fungi	Accession number
1	*Alternaria alternata*	PV803540

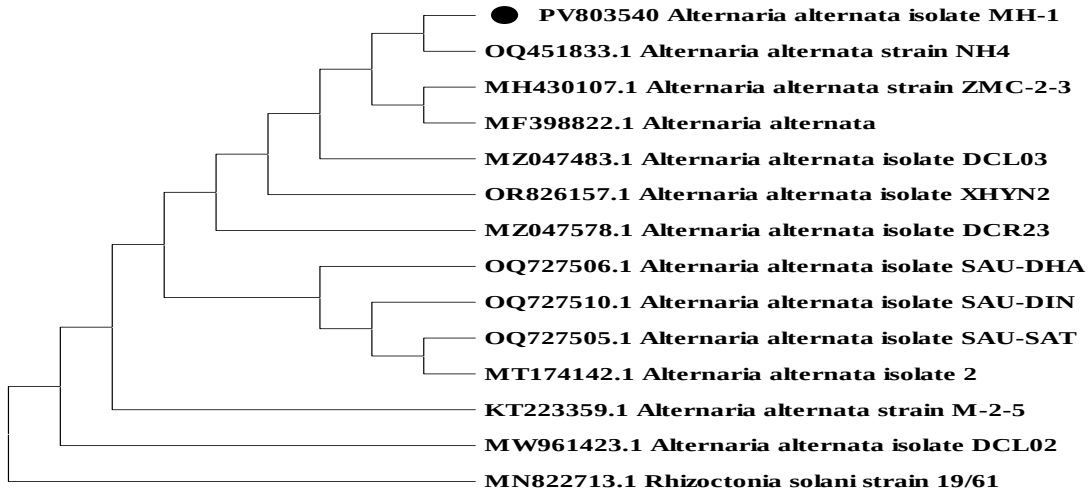


Figure 3: phylogenetic tree of Alternaria alternata isolate MH-1 (marked with a black circle: PV803540), constructed based on its nucleotide sequences of the ITS-rDNA region, together with sequences of global strains of the same pathogenic fungus obtained from the GenBank database. Genetic distances were calculated using the neighbor-joining method.

Conclusion

The spread of the deterioration and death of date palm seedlings is strongly linked to the infection of pathogenic agents. One of these pathogens is *Alternaria alternata* is the most pathogenic fungus, and it caused the largest decrease in germination and the growth of palm seedlings. The severity of pathogenesis varies between isolates and depends on the type of fungus, the amount of toxins produced and environmental conditions. Molecular diagnosis confirmed the identity of the most pathogenic isolation, allowing follow-up of its use in future studies for prevention and early

diagnosis

Supplementary Materials:

No Supplementary Materials.

Funding:

This research received no external funding.

Author contributions Mariam Hussain Chaffat: conceptualization; methodology; field survey and sampling; laboratory isolation and morphological identification; pathogenicity assays on radish seeds and date palm saplings; molecular work (DNA extraction, PCR, sequence analysis, phylogenetic analysis); data analysis and interpretation; writing—original draft; writing—review and editing.

Acknowledgment

The author thanks the Plant Protection Department, College of Agriculture, University of Kerbala (Karbala, Iraq) for providing facilities and support, and thanks the orchard owners in the Al-Husseiniya area for allowing sample collection. The author also acknowledges Macrogen (South Korea) for sequencing services.

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Conflict of interest

The authors declare no conflicts of interest associated with this manuscript.

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التشخيص المورفولوجي والجزيئي للفطريات المسببة لتعفن الجذور وموت فسائل نخيل التمر في كربلاء

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الخلاصة

هدفت هذه الدراسة إلى عزل وتحديد الفطريات المسببة لتعفن الجذور وموت شتلات نخيل التمر (***Phoenix dactylifera L***.) في بساتين منطقة الحسينية بمحافظة كربلاء ، شمل المسح الميداني 12 بستانًا، وأظهرت نتائجه انتشارًا واضحًا للمرض، حيث تراوحت نسب التلف بين 14 و28%، ونسب الموت بين 13 و42%، مما يشير إلى وجود عوامل مرضية مؤثرة في نجاح زراعة الشتلات. أسفر العزل المختبري عن تحديد فطر ***Alternaria alternata***، الذي تسبب في انخفاض ملحوظ في نسب إنبات بذور الفجل وشتلات النخيل إلى 15-17%. تم تأكيد التحديد الجزيئي للعزلة الأكثر إمراضًا باستخدام تضخيم منطقة ***ITS*** وجين الأكتين، وأظهرت المقارنة الجينية عبر قاعدة بيانات ***GenBank*** تطابقًا عاليًا مع العزلات العالمية. تم إيداع تسلسل ***A. alternata*** تحت الرقم ***PV803540***، وأظهرت الشجرة التطورية تقاربًا تطوريًا واضحًا مع العزلات المسجلة دوليًا. وتؤكد النتائج أن العدوى الفطرية عامل رئيسي في تدهور شتلات النخيل، مما يسلط الضوء على أهمية التشخيص المبكر واعتماد الإدارة الوقائية المتكاملة للحد من الخسائر.

الكلمات المفتاحية: نخيل التمر، تعفن الجذور، فطر *Alternaria alternata*، التحديد الجزيئي، القدرة على إحداث المرض.