Pathogenic Fungi A Companied with Sudden Decline Syndrome (wilting disease) of Date Palm Tree (*Phoenix dactylifera* L.)*

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Abstract

Sudden death syndrome is one of the precarious diseases that infect date palm tree in different growing areas of palms around the world, and this disease comes after Bayoud by its violence. *Fusarium* species considering as one of the most suspected fungal genera that cause this disease. In the present study, a nine species of *Fusarium* have been isolated from the date palm tree and the soil of palm cultivated orchards, these are: *F. annulatum, F. anthophilum, F. fujikuroi, F. nygamai, F. oxysporum, F. proliferatum, F. pseudocircinatum, F. sudanense* and *F. verticillioides*. However, these fungi were identified morphologically and molecularly using two types of primers. The internal transcribed spacer (ITS) and translational elongation factor 1-*a* (TEF1-*a*) regions of the *Fusarium* species were amplified sequenced. The results of pathogenicity test have been showed that the highest severity by*F. anthophilum* with percentage rate of 72.92%, while the lowest percentage of severity with 16.67% recorded with *F.sudanense*.A seven of *Fusarium* species which were isolated in this study, these are:*F. annulatum, F. anthophilum, F. fujikuroi, F. nygamai, F. pseudocircinatum, F. sudanense* and *F. verticillioides* have been first recorded on date palm trees as sudden decline pathogens in Iraq.

Keyword: sudden decline syndrome, Date palm, *Fusarium* specie, ITS, TEF1-α

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1. Introduction

Date palm the symbol of life in the desert is one of most tolerating fruit tree in the arid and semi-arid zones. And it is one important economic fruit tree due to the high nutritional value of its fruits and historical status. In 1960s, Iraq occupied the world-leading position due to the number of cultivated date palm tree which is reached more than 30 million [1]. But, unfortunately more than 50% of trees number was declined dramatically during the wars broke out in this region since the beginning of 1980s, which caused the neglected of orchard and lack of service [1].

In the modern era, date palm tree in Iraq is subjected to many agricultural biotic and abiotic constraints which make the Iraqi cultivars under endangered plants [2].

Sudden death syndrome is one of the precarious diseases infects this tree in different palms growing areas of the world. This illness status was first observed in 1951 by Darley and Wilbur [3], but unfortunately, they were not able to explain the real cause of this disease. Nevertheless, since 1982 and up to time of writing this paper, several scientists were able to isolate various fungal pathogens accompanied with this disease, like *Alternaria alternate*, *Botrodiplodia theorbromae*, *Chalaropsis radicicola*, *Chaetomium* sp., *Chatosphaeropsis* sp., *Diplodia phoenicum*, *Fusarium oxysporum*, *F. monliforme*, *F. proliferatum*, *F. solani*, *F. moniliforme*, *F. equesti*, *F. semitectum*, *Gliocladium* sp., *Gliocladium phoenics*, *Mauginiella scaettae*, *Mycospherella scaettae*, *Mycosphaerella* sp., *Omphalia tralucida*, *Phomopsis phoenicola*, *Phomopsis phoenicola*, *Phomopsis phoenicum*, *Paecilomyces* sp., and *Thielaviopsis paradoxa* [4-10]. However, the causal agents of this disease still uncertain and mysterious due to different fungi species associated with this lethal disease [10].In Iraq, this disease has been identified by [11]in the middle

area of Iraq. But, after a short time, this disease had been recorded at different parts of this country. Recently, this disease had been recorded at different date palm growing area in Basrah city, South-east of Iraq.

From our point of view, the sudden death syndrome is very serious and comes after Bayoud disease by its violence, where it can infect the date palm tree at different age stages. And Infection can happen at any time of the year. Also, the suspected pathogens have a selectivity behavior; where it attacks the selected individual trees in the orchard [12].

The last thing is no one recorded any susceptible cultivars for this lethal disease. Because this disease becomes a real threat to the date palm tree in Iraq, therefore our study aimed to isolate and identify the most lethal fungus pathogens that caused the wilt disease of this socio-economic tree in Basrah Province South East of Iraq.

2. Materials and Methods

2.1: Field survey study:

Three orchards for date palm cultivation were selected for this study, Abu-Al Khaseeb-Al Bahadria (orchard 1+2) and Al-Hartha Date Palm station run by Agricultural Directorate from September 2017 to December 2017. These fields locate in Basrah Governorate, South-east of Iraq, infected plants were counted and the disease incidence was calculated according to the following formula:

Disease incidence%= number of infected plant / total number of plants within the studied area ×100

2.2: Isolation of fungi

2.2.1: From soil samples

Samples used for this study were collected from the soil around the healthy and infected trees area during September 2017 to December 2017. Soil dilution

technique for isolating the suspected pathogenic fungi was achieved according to methods of [13]**.** One ml. of each diluted solutions was poured in Petri plates containing Potato Dextrose Agar medium (PDA) fortified with chloramphenicol (25mg) to inhibit bacterial growth. Three replicates for each dilution were made. The plates were incubated at 25°C [13].

2.2.2: From date palm parts

A total of 50 leave and roots parts were washed in running tap water for 10 minutes. Leaves and roots samples were cut into pieces 1×1 cm, then soaked in 10% NaOCl solution for 1 to 2 minutes. After that the samples were rinsed in sterile distilled water, then soaked in 70% alcohol for 1 min, and placed on culture media (either potato dextrose agar (PDA) or potato carrot agar (PCA)that contained 250 mg chloramphenicol antibiotic. Plates were incubated at 25°C in a moist chamber. After 7-14 days of incubation, a single spore technique was used to obtain pure cultures for identification.(PDA) medium was used to assess colony characteristics such as pigmentation and growth rate, while carnation leaf agar (CLA) and SNA-Synthetic Nutrient Agar medium (1.0g KH_2PO4, 1.0gKNO_3, 0.5g$MgSO_4x7H_2O$, 0.5g KCl,0.2gglucose, 0.2gsucrose, 1liter distilled water,20g agar). To examine conidial morphology and detect the presence of chlamydospores, a piece of autoclaved filter paper was placed onto the surface of synthetic nutrient agar medium. Morphological identification was based on [14- 16].

2.3: Calculate the percentage of frequency and occurrence:

Percentage of frequency and occurrence were calculated according to the following formulas:

Frequency %= Number of species colonies/Total number of colonies ×100

Occurrence %= Number of samples which occurrence species/ Total number of Samples ×100

2.4: DNA extraction:

Pure fungi isolates were diagnosed by molecular techniques depending on large subunit (LSU) rRNA gene sequences [17]. First, genomic DNA of each isolate was extracted according to [18] as following steps: Single colony of freshly grown fungal mycelium was dropped in 2 ml in Eppendorf tube (microcentrifuge tube) contains 400µl of DNA extraction buffer (Triton X- 100, SDS, NaCl , Tris – HCl and EDTA) and 400 µl of Phenol: Chloroform: Isoamyl alcohol (25: 24: 1, v: v). Cell was lysis by adding glass beads and vigorously vortex for 15 min. Thereafter, the lysate cell had been centrifuged at 10000 rpm and 4◦C for 10 min. The upper layer (representing DNA) was re-mixed by vortex with cooled 1 ml of absolute ethanol and precipitated by centrifugation as mentioned previously. Nucleic acids pellet was resuspended in a mixture of 400 µl (1x) TE buffer (Tris – HCl, EDTA) and 10µl of (4M) NH4OAc and 1 ml of cooled ethanol were added and the mixture was vortexed for few seconds. Finally, pure DNA pellets were obtained after centrifugation as mentioned previously, and resuspended by 50µl of (1x) TE buffer. Samples were stored at - 20 C° until use. Genomic DNA samples were examined by gel electrophoresis for 30 min using 0.8% TBE agarose gel and 60 voltages.

2.5: Polymerase Chain Reaction (PCR):

Two regions were amplified and sequenced, ITS-rDNA and translation elongation factor 1 alpha (tef1α) genes, with the following primers: ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) or EF1T (5′ -ATG GGT AAG GAA GAC AAG AC -3′) and EF2T (5′- GGA GGT ACC AGT GAT CAT GTT-3′), respectively [17],[19].

The PCR reaction contained7µl genomic DNA and PCR buffer (25 µl Master Mix , 2 µl Primer Forward ,2 µl Primer Revers and 14 µl Nuclease free water).

The PCR program was: for (ITS-rDNA) one cycle at 95 °C for 1 min, 35 cycles of 95 °C for 1 min, 55 °C for 45 sec, and 72 °C for 1 min, and a terminal incubation at 72 °C for 10 min while for (tef1α) one cycle at 94 °C for 3 min, 30 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min, and a terminal incubation at 72 °C for 5 min. All the PCR amplifications were carried out in a bioneer thermocycler. The amplification products were electrophoretically separated on 2 % agarose gel at 60 volts for 1 hr. PCR products of amplified ITS1-ITS 4 regions and tef1α were sent to Macrogen company (Korea) for DNA sequencing.

2.6: **Pathogenicity test:**

The pathogenicity test of suspected pathogenic fungi was conducted according to [20] as follows the isolates that were tested for pathogenicity (Table 2) were grown for 7 to 14 days at 25°C on A PDA. The inoculums was prepared for each isolate by flooding the agar surface with10 ml of distilled water and scraping with a spatula. The resulting spore suspension was filtered through four layers of cheese cloth. The filtrate was diluted with distilled water and conidial concentration was adjusted with a hemocytometer to 10^{6} conidia/ml. Seeds of the local date palm 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 ± 3°C. Plants were inoculated 6 months after seedling emergence. four plants were inoculated with each isolate. 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 distilled water. All

plants were watered and covered separately with plastic bags for 24 h to maintain high humidity.

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 DSI = ∑ (seedlings/class × class score)/total seedlings.

3- Results and Discussion

3.1: Symptomatology:

The Symptoms of sudden decline disease started with the change of the color of leaves to yellow to yellowish-orange and then turned into pale yellow, and yellowing begins in the palm of the outer palm of the top and go down on the wicker in the ring, towards the heart of the palm and ultimately leads to its death. In some cases, yellowing appears to random sites on the ring at the top, center, or base on the side or sides of the ring the emergence of the disease is random on palm trees within the same orchard as the disease takes the dodgy way of infection (Figure 1). At the time of dissection of the developing summit of one of the affected palm trees, the rotting and decomposition of the area near the crown were observed and turned into a creamy color. It was transformed into a loose texture. Some larvae of the flaming flies reached the depth of the developing summit(Figure2). In the present study, the calculation infection rates for the stations understudy were: 50% in the first field, 20% in the second field and 55% in the third field. The symptoms which observed in current study were similar for the other studies in Iraq as [9], [4],[21], and [11]as well in Iran [23] had recorded same symptoms too.

Figure (2)A- the rotting and decomposition of the area near the crown,**B-** Some larvae of the flaming flies reached the depth of the developing summit

3.2: Isolation and identification of the pathogens:

In the present study, nine *Fusarium* species have been isolated from date palm parts and soil samples table (1), seven of them has first recorded on date palm trees as sudden decline pathogens: *F. annulatum*, *F. anthophilum*, *F. fujikuroi*, *F. nygamai*, *F. pseudocircinatum*, *F. sudanense* and *F. verticillioides*. However, the highest frequency species of *Fusarium* was *F.fujikuroi* recovered from almost all the surveyed regions with frequency percentage of 32.31%

while the lowest was 3.08% for *F. anthophilum*. The highest occurrence percentage was 4% for *F.fujikuroi*.

Table (1) Fungi that appeared during the study with frequency and appearance Percentages

Fusarium species	No.of samples in which they appeared	Occurrence percentage	Isolated from			No.colonies	Frequency percentage
			Orch.1	Orch.2	Orch.3		
F.annulatum	1	1	1	0	0	6	9.23
F. anthophilum	1	1	0	0	1	2	3.08
F. fujikuroi	4	4	1	1	2	21	32.31
F. nygamai	1	1	1	0	0	4	6.15
F. oxysporum	1	1	1	0	0	4	6.15
F. proliferatum	3	3	1	1	1	12	18.46
F. pseudocircinatum	1	1	1	0	0	10	15.38
F. sudanense	1	1	0	0	1	2	3.08
F. verticilloides	1	1	1	0	0	4	6.15
No.of colonies	65						

*samples are of date palm parts,except with *F. fujikuroi* which is soil as well

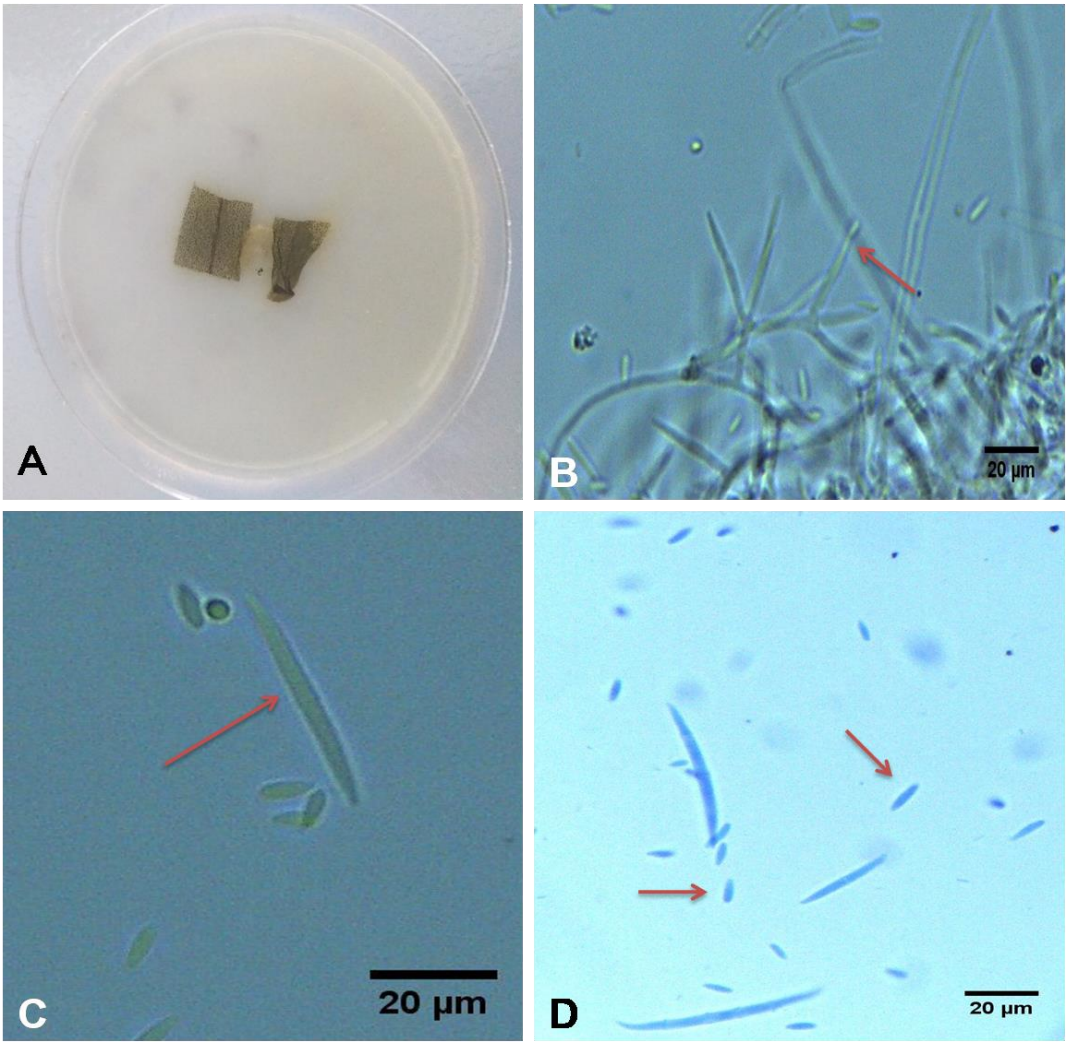
Many studies have shown that *Fusarium* species causing sudden decline syndrome is *F.solani*, *F.proliferatum* ,*F.oxysporum* ,and *F.moniliforum* [24-28].

3.3: Morphological and Molecular identification:

The morphological identification of *Fusarium* species was performed using the relevant publications. Based on the morphological characters, *Fusarium* species which isolated from plants parts and soil were identified as *Fusarium annulatum*, *F. anthophilum*, *F. fujikuroi*, *F. nygamai*, *F. oxysporum*, *F. proliferatum*, *F. pseudocircinatum*, *F. sudanense* and *F. verticillioides*.

1- ***F.anthophilum*(A. Braun) Wollenweber:**

On PDA Aerial mycelia are white and grow rapidly. Sporodochia, if present, are tan to orange in color and maybe discrete or confluent. The agar may be colorless or contain violet pigments, sporodochia orange to tan color, when present on CLA, macroconidia a relatively slender with no significant curvature thin walled(3-4) septate, Apical Cell curved, Basal cell usually relatively poorly developed, but foot shaped when clearly developed, microconidia pyriform, globose and ovoid. Usually 0-septate, but occasionally 1-septate on false heads. No chains. Conidiogenous cells monophialides and branched and unbranched polyphialides. Chlamydospores absent (Figure 3)

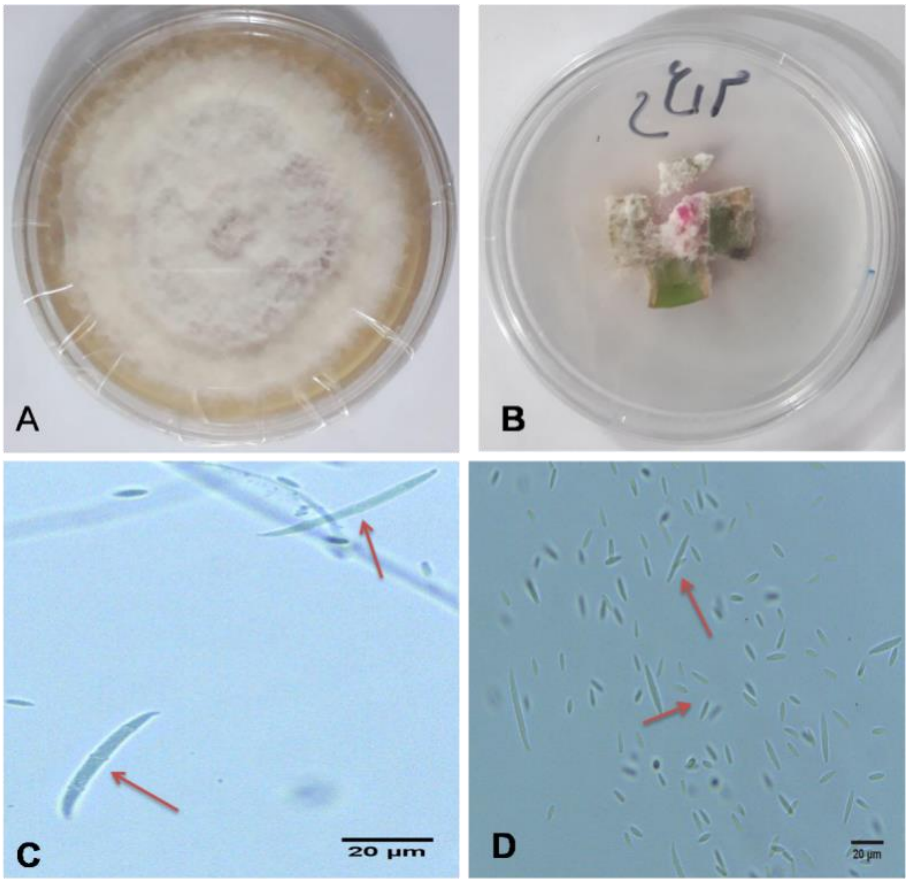


Fig(3):*Fusarium anthophilum* (A) CLA media (B) mycelium and Conidiogenous cell (C) Macroconidia (D) Microconidia.

2-***F. annulatum*** **Bugnicourt:** On PDA Aerial mycelia are white, microconidia produced in chains and false heads on monophialides and polyphialides. Macroconidia are thin walled strongly curved shaped on CLA, with the basal cell distinctly foot-shaped. Chlamydospores are absent.

3-***F. fujikuroi*** **Nirenberg:** Is very similar in colony morphology to *F. verticillioides* and almost in distinguishable from *F.proliferatum*. None the less, *F. fujikuroi* forms white mycelia that become gray, violet or magenta with age. Sporodochia normally are absent but, if present, they are pale

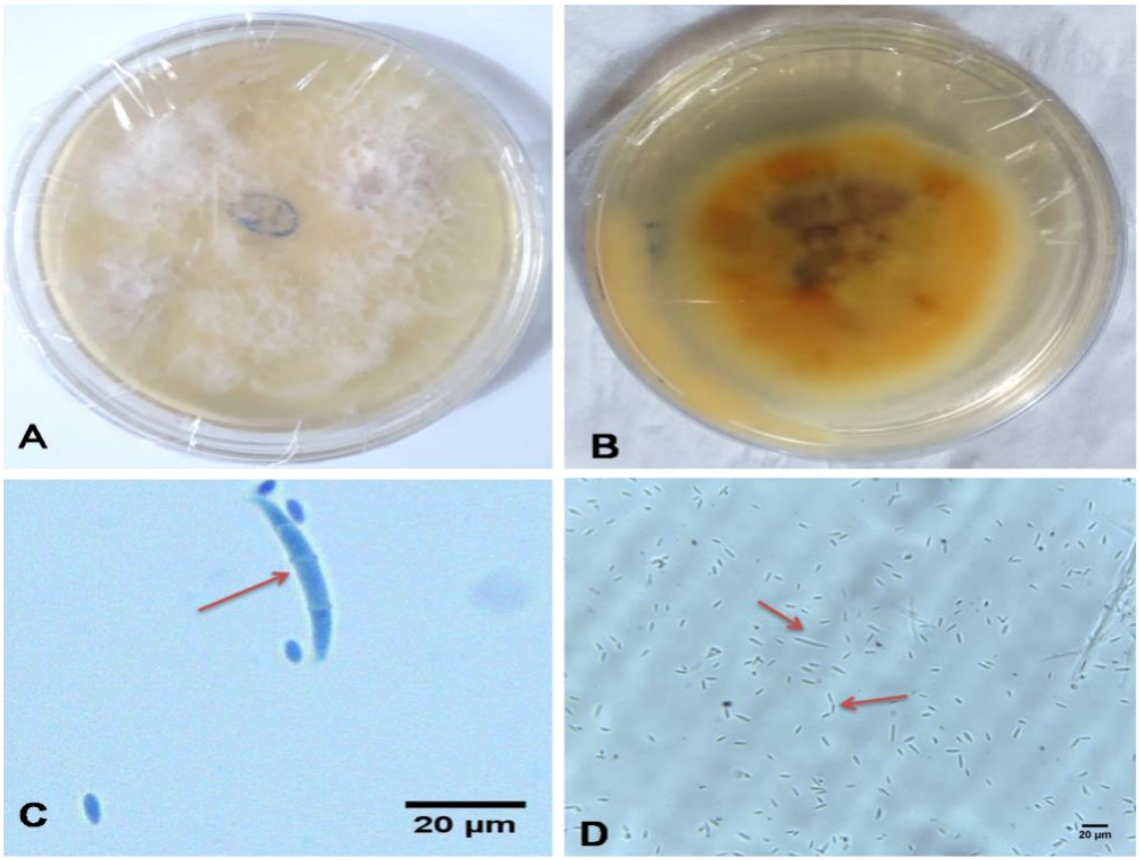
orange. Dark colored sclerotia may develop in some isolates.Pigmentation in the agar varies and ranges from no pigmentation or grayish orange in some is olive to violet grey, dark violet or dark magenta (almost black).In others, produce orange sporodochia. Macroconidia relatively slender, medium length with no significant curvature, apical cell tapered, basal cell poorly developed(3-5) septate. Oval or club shaped microconidia with a flattened base has 0- to 1-septate. A few pyriform microconidia(short to medium in length) with false heads and chains of may be present in some isolates. Conidiogenous cells polyphialides (commonly) and monophialides. Chlamydospores are absent(Figure 4).



Fig(4):*F. fujikuroi* (A) Mycelium on PDA (B) Fungus on CLA (C) Macroconidia (D) Microconidia

4-***F. nygamai* Burgess & Trimboli:** The mycelium initially is white becoming violet with age, with violet pigments produced in the agar. Sporodochia

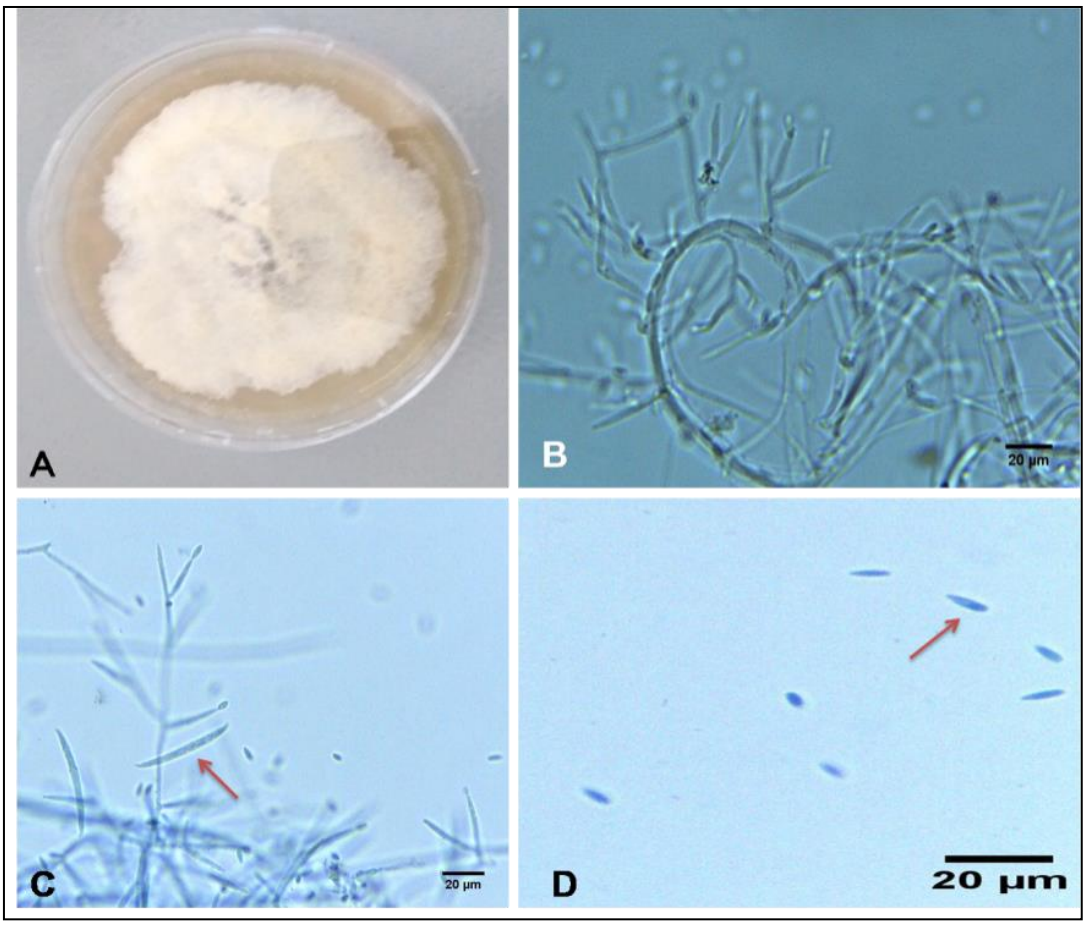
abundant orange sporodochia found on CLA. Macoconidia slender, thin walled, hyaline, straight to slightly curved and medium length, apical cells hort and tapered, basal cell notched or foot shaped (3-5) septate. Microconidia small, oval or club shaped, usually 0-septate, but occasionally 1-septate on false heads and chains, false heads are common, chains are usually short. Conidiogenous cells monophialides and occasional polyphialides. Chlamydospore formation is more consistent on SNA after 4-6 weeks(Figure 5).



Fig(5):*F. nygamai* (A) PDA (B) pigment form in the media that be orange to gray (C) Macroconidia (D) Microconidia

5-***F. pseudocircinatum*** **O'Donnell & Nirenberg**:On PDA white feathery mycelium produced orange to violet pigments in the agar, especially in the center of the culture. Coiled sterile hyphae are produced and are very important characters. Sporodochia sparse is hard to find. Macroconidia slender, slightly falcate and thin walled, apical cell beaked, basal cell foot shaped3 septate.

Microconidia oval to obovoid and 0-1septate, but usually0-septate.Chlamydospores are absent (Figure 6).

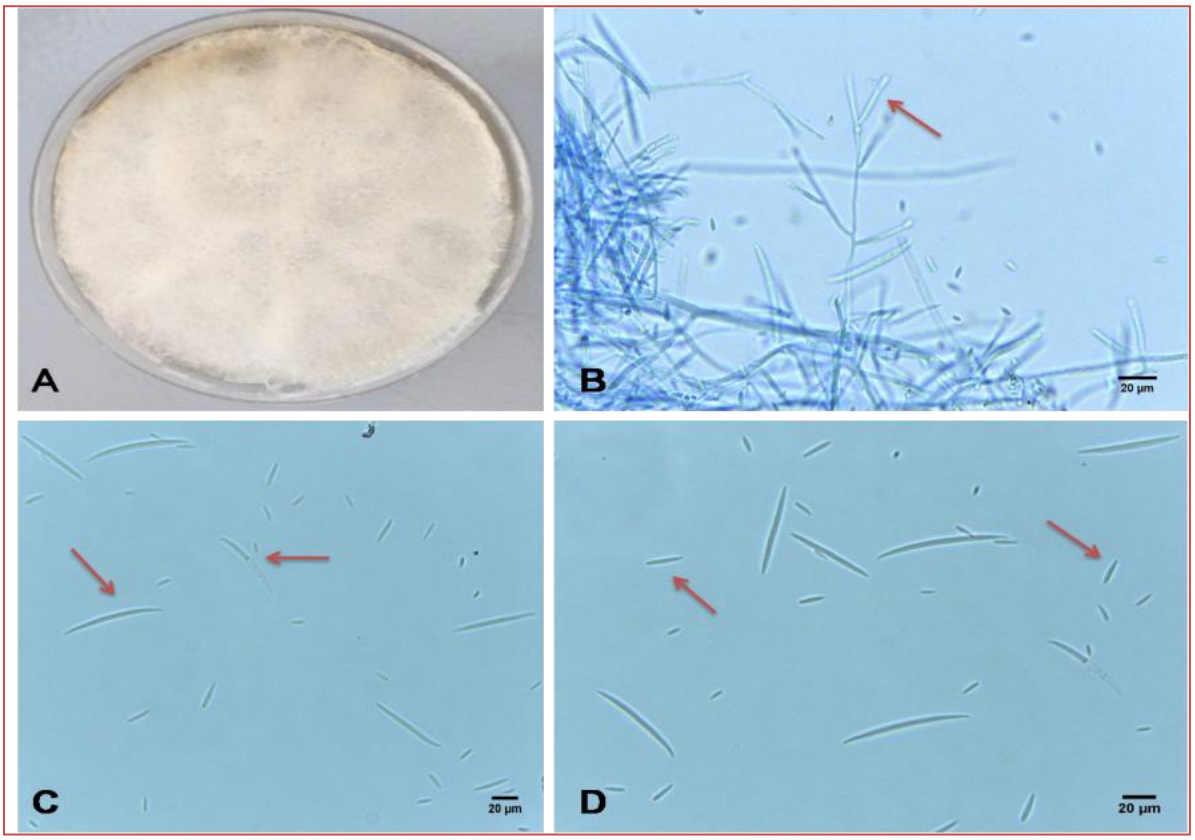


Fig(6):*F. pseudocircinatum* (A) PDA (B) Coiled mycelium (C) Macroconidia (D) Microconidia

6-*F. sudanense*Ahmed,Al-Hatmi&Hoog :The morphological characteristics of this fungus are very similar to *F. fujikuroi*, the differences between them are within the genetic classification through certain genes responsible for certain functions such as different growth at different temperatures and growth in different water potential.

7-*F. verticilliodes* (Saccardo) Nirenberg: On PDA Initially cultures have white mycelia but may develop violet pigments with age. Pigmentation in the agar varies, ranging from no pigmentation or grayish orange to violet grey, dark

violet or dark magenta (almost black) in others. Sporodochia may is tan or orange in color. Macroconidia relatively long and slender, slightly falcate or straight, and thin walled, apical cell curved and often tapered to a point**,** basal cell notched or foot shaped (3-5) septate. Microconidia oval to club shaped with a flat end base and usually 0-septate.Chlamydospores are absent (Figure 7).



Fig(7):*F. verticilloides* : (A) PDA (B) conidiogenus cell (C) Macroconidia (D) Microconidia

3.4: Molecular identification:

The identity of the *Fusarium* species recovered in this study was confirmed by PCR-based method is considered as a quick and more sensitive technique as compared to morphological identification, with high discriminative ability between morphologically similar species. Successful amplification with primer targeted to the genomic regions of the internal transcribed spacer (ITS) and translational elongation factor 1-*a* (TEF1-*a*) was obtained from all *Fusarium* species. The sequence results demonstrated that they were derived from the

fungal ITS and TEF1-*a* regions when compared with the database of sequences on GenBank. BLAST results showed that the most Identical sequence was ITS and TEF1-*a* regions of *Fusarium annulatum*, *F. anthophilum*, *F. fujikuroi*, *F. nygamai*, *F. oxysporum*, *F. proliferatum*, *F. pseudocircinatum*, *F. sudanense* and *F. verticillioides* 100,99.2,100,99.8,100,99.85,99.54,99.56 and 99.8% respectively. DNA sequences of different fungal nuclear regions including, rDNA, and tef-1α,have been used to confirm species level of *Fusarium* species isolated from date palms [20], [29], [30].

3.5: Pathogenicity tests

Date palm seedlings that were inoculated with spore suspensions of *Fusarium* species,(table 2) developed clear sudden decline symptoms after approximately 2 weeks(Figure8). No symptoms were observed on un-inoculated plants (control) (Figure8). *F. anthophilum* was the highest severity with 72.92%, while *F.sudanense* was the lowest percentage of severity with 16.67% (table2).

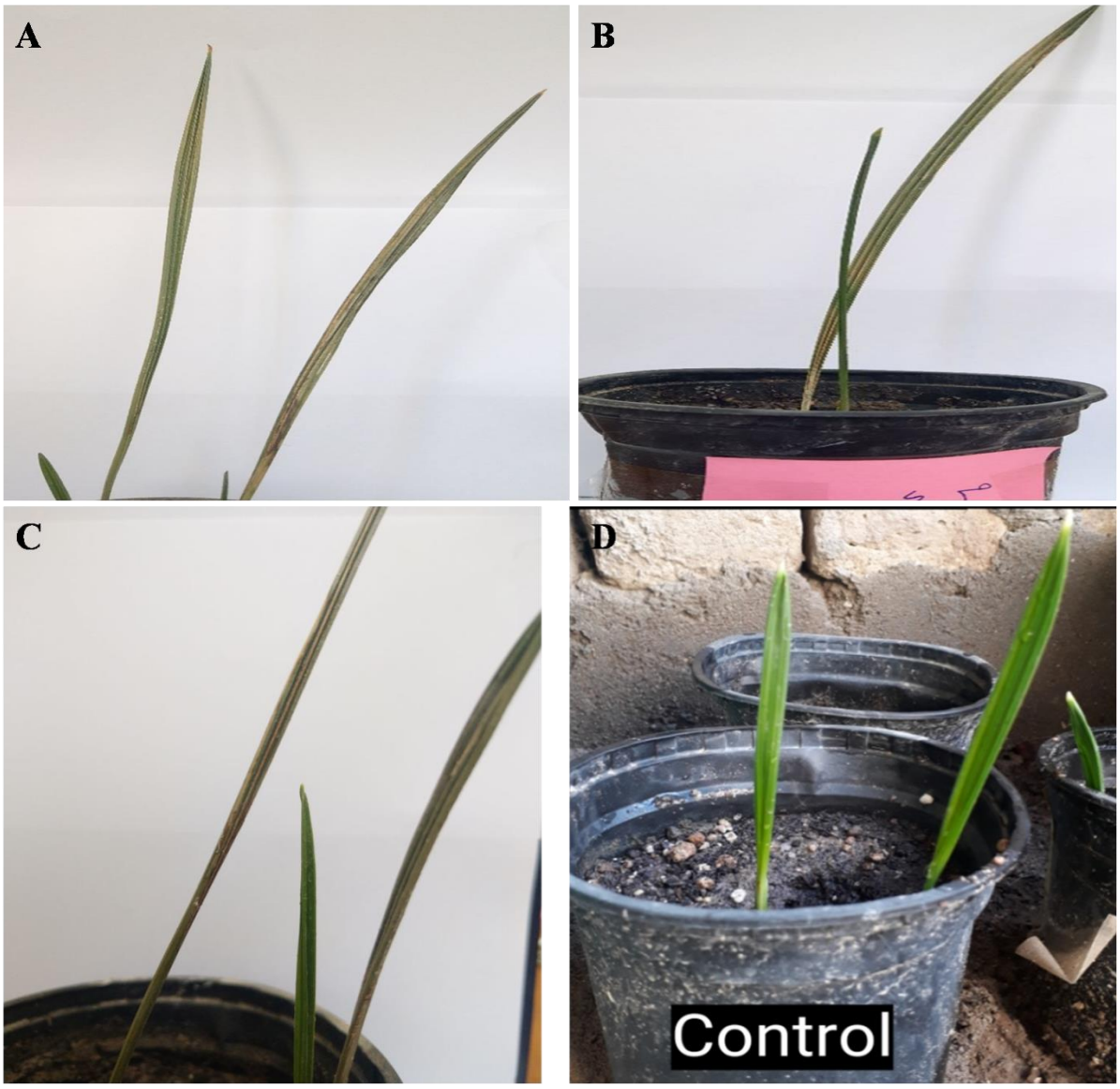
Table(2) Severity of *Fusarium* species on date palm seedling

No.	*Fusarium* species	Severity of disease %
1	*F. annulatum*	22.92
2	*F. anthophilum*	72.92
3	*F. fujikuroi*	54.17
4	*F. nygamai*	56.25
5	*F. oxysporum*	35.5
6	*F. proliferatum*	39.58
7	*F. pseudoeircinatum*	18.75
8	*F. sudanense*	16.67
9	*F. verticillioides*	20.83

R.L.S.D =4.353 ($p \leq 0.05$)

This agrees with previous studies conducted in the United Arab Emirates, Egypt, Saudi Arabia, and Iran tested when three species of *Fusarium* (*F.solani*,

F.oxysporum, and F.proliferatum) and was *F.solani* highest severity on date palm seedlings after 10 days[10].



Fig(8): pathogenicity test of *Fusarium* spp (A) *F. proliferatum* (B) *F. fujikuroi* (C) *F. anthophilum* (D) Control

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الفطريات الممرضة المصاحبة لمتلازمة التدهور المفاجيء على أشجار نخيل التمر *Phoenix dactylifera* L.

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الملخص :

تعد متلازمة الموت المفاجئ احد الأمراض الخطيرة التي تصيب أشجار النخيل في مختلف مناطق زراعة النخيل في العالم،وأن أنواع الجنس *Fusarium* تعد من الممرضات المهمة والخطيرة على نخيل التمر،في هذه الدراسة عزلت تسعة أنواع من الجنس *Fusarium*من أجزاء لأشجار نخيل التمر المصابة والتربة وهي : *F. annulatum* و *F. anthophilum* و*F. Fujikuroi* و*F. nygamai* و *F. oxysporum* و *F. proliferatum* و*F. pseudocircinatum* و*F. sudanense* و*F. verticillioides* وشخصت هذه الفطريات مظهرياً و جزيئياً باستخدام نوعين من البواديء لتضخيم المنطقتين ITS و TEF1-α وأظهرت نتائج اختبار القدرة المرضية للفطريات أن أعلى معدل نسبة شدة

إصابة 72.92 ٪ كانت لمعاملة الفطر *F.anthophilum* بينما اقل معدل نسبة شدة إصابة في معاملة الفطر *F.sudanense*، وسجلت سبع أنواع للفطر *Fusarium*لأول مرة على أشجار النخيل كممرضات موت مفاجيء في العراق وهي : *F. annulatum* و *F. anthophilum* و*F. fujikuroi* و *F. nygamai* و*F. pseudocircinatum* و *F. sudanense* و *F. verticillioides*