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Research Article

Efficiency Evaluation of Silver Nanoparticles in the Controlling of the Fungi Associated with the Date Palm Offshoots

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Abstract: Recently, Iraq has imported large numbers of tissue culture date palm offshoots from different countries. It is to build new orchards of date palm trees or plant them with the old orchards and some of them in the home's gardens. As a result of the widespread of many symptoms associated with these offshoots, this study was conducted in Basra Governorate, Iraq. To examine the capability of silver nanoparticles in controlling pathogens. The 36 fungi species were isolated from the shoot system of tissue culture date palm offshoots. *Alternaria* sp. was recorded at a high frequency compared to the *Cladosporium* spp. and *Ulocladium* spp. *Neodieghtonia phoenicum*, *Scytalidium lignicola*, and *Neoscytalidium dimidiatum* caused black scorch. Moreover, *Phoma costarricensis* has been recorded as causing the leaf spot disease. The roots infected by wilt disease have shown three various fungi, *Fusarium solani*, *Fusarium proliferatum*, and *Fusarium fujikuroi*. The study also illustrated that silver nanoparticles possessed a high ability to inhibit fungi growth in the laboratory

Keywords: Silver nanoparticle, leaf spot, black scorch, wilt disease.

1. INTRODUCTION

The Date palm (*Phoenix dactylifera* L.) is mostly found in the Middle East countries such as Iraq; dates are of great nutritional importance because they contain energy sources and vitamins as well as antimicrobial, antioxidant, anti-inflammatory, mutagenic, anticancer, and stomach and liver protection activities [1]. It can be infected by many diseases, whether the vegetative or root systems. Most of the reported date palms diseases are attributed to fungal pathogens such as Bayoudh disease wilt disease, black scorch, and leaf spot disease [2]. Nanotechnology can potentially decrease many challenges in disease control by reducing chemical inputs and enhancing the fast detection of pathogens [3]. Silver nanoparticles are the first to be used in plant disease control because of their antimicrobial activity [4].

This is because Iraq imported large numbers of tissue culture offshoots from different countries, complete orchards were formed from it, and others were planted overlapping with the old orchards and some home gardens. Because of the spread of many disease symptoms associated with these offshoots such as death and wilt of tissue culture offshoots, leaf spots, black scorch, and due to the lack of adequate studies on the fungi associated with the date palm offshoots produced by using the tissue culture technique [5, 6]. One of the modern strategies for controlling pathogens is the use of silver nanoparticles. This study aimed to detect the pathogenic fungi on date palm offshoots and secondly to evaluate the role of silver nanoparticles in its control.

2. MATERIALS AND METHODS

2.1. Isolation of Tissue Culture Offshoots Leaves and Roots

Samples were collected from the date palm offshoots produced from the tissue culture grown in the different areas of Basrah, Iraq showing symptoms of leaf spots and black scorch. The plant parts were taken from the leaves. These leaves were cut into smaller pieces and sterilized with 10 % sodium hypochlorite for two minutes. After that, these parts were washed with sterile distilled water

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to get rid of the chloride effect. Then, the samples were dried with sterilized filter paper. Every four pieces were transferred by sterile forceps to Petri dishes, containing Potato Dextrose Agar (PDA) which was sterilized with an autoclave instrument. The antibiotic Chloramphenicol 250 mg/L was added to the dishes and incubated at 25 ±1 °C for 5-7 days. The fungal growths were examined using a microscope. The fungi were purified on a PDA media for morphological diagnosis (genus and species level). The isolates were kept in test tubes containing a PDA culture medium at sloping. Then the samples were saved in the refrigerator until use. As for isolating from the roots, samples were taken from the roots of date palm tissue culture offshoots less than three years old showing symptoms of yellowing and wilting, and the same steps were taken for isolation from the leaves. Fungi were morphologically identified based on the following taxonomic keys [7-11].

2.2. Molecular Identification of Isolated Fungi

DNA of isolated fungi was extracted by using (gSYNC TM DNA Extraction Kit). Primers F: TCCGTAGGTGAACCTGCGG: and R: TCCTCCGCTTATTGATATGC were used to amplify the region of the ITS1-ITS4 gene. The amplification process, electrophoresis technique was to isolate the fungal DNA. The gel was checked with a gel documentation instrument to examine the quality of the bands in the gel and determine the success of the DNA amplification process [12]. A 20 µl of the amplification product for each isolate was sent to the Korean company Macrogen for the nitrogenous base sequences and investigated by the National Center for Biotechnology Information.

2.3. Pathogenicity Experiment of Fungi Isolated from the Shoots

The separated leaves method was used with some modifications [13]. This method involves a 20 cm long piece taken from the fronds of the third basement of the date palm Al-Sayer cultivar. At first, the leaves were washed with tap water after removing the leaflets, then sterilized with 70 % ethanol, washed with sterile distilled water, and dried with filter paper then three holes were punched in each piece using a sterile 0.5 cm cork borer to put in each hole. A 0.5 cm disc taken from the edge of a seven-day-old colony of fungi isolated and grown on PDA. Each hole was wrapped with cling film, for two days after inoculation with the pathogenic fungi. The pieces of leaves were placed in 1-Liter flasks containing 30 mL of sterile distilled water. The nozzles of the flask were blocked with sterile aluminum foil. The flasks were incubated in the incubator at a temperature of 25 ± 1 °C for 21 days. The development of the spot was observed on the rachis pieces every 7, 10, 15, and 21 days. The radius average of the damaged tissue around the site of injury was measured by the ruler, the experiment was carried out using three replications. The control treatment included a placing of 0.5 mm disc PDA in the holes. The development of symptoms was monitored and the diameters of resulting spots were measured.

2.4. Pathogenicity Experiment of Fungi Isolated from Roots

This experiment was carried out using date palm seedlings resulting from the cultivation of the seeds of the Hillawi cultivar. The soil and peat moss mixture (2:1) was sterilized by the autoclave at 121 °C for 15 minutes. After one day of sterilization, it was placed in plastic pots of 1 kg in equal quantities, the soil was contaminated with isolates of *Fusarium* spp., which grown on Millet with ratio of 1 % w/w [14]. The control treatment was sterilized. The millet seeds were added to the same ratio, and then the soil in the plastic pots was moistened for planting using the seeds of the cultivar Hillawi. The pots were planted at a rate of 10 seeds per pot. The Control treatment included the cultivation of seedlings of the Hillawi cultivar in the sterile soil that does not contain the previous fungi. The experiment lasted for two months, during which the percentage of germination and death of seedlings for all pots was recorded according to the following equations:

$$\text{Germination \%} = \frac{\text{number of germinated seeds}}{\text{Total number of seeds}} \times 100$$

$$\text{Seedling's death \%} = \frac{\text{Number of dead seedlings}}{\text{Total number of seedlings}} \text{ x } 100$$

The experiment was performed based on the complete randomized design (CRD) with three replications for each treatment.

2.5. Evaluation of the Efficiency of Silver Nanoparticles in Inhibiting of the Growth of Pathogenic Fungi

The silver nanoparticles AgNPs with a size of 20 nm were obtained from the Chinese company Hongwu International Group Ltd. A 1000 parts per million standard solution was prepared in 1-liter flasks containing 500 ml of potato dextrose Agar (PDA) to obtain the various concentrations (0, 25, 50, 75, and 100 ppm) of silver nanoparticles. The flasks were shaken well with the amount of Agar to PDA, and then the amount of the standard solution of silver nanoparticles was added. PDA was poured into petri dishes with a size of 9 cm. After solidification media, the center of each plate was inoculated with a 0.5 cm disc from the culture of each pathogenic fungus taken from the edge of a 7-day-old newly grown colony. The dishes were incubated at a temperature of 25±1 °C until the growth of the pathogenic fungi in the control treatment was completed. The experiment was carried out in three replications for each treatment, thereafter the radial growth of the fungi was measured according to the equation:

$$\text{Inhibition\%} = \frac{\text{the colony diameter in treatment} - \text{the colony diameter in cont}}{\text{the colony diameter in control}} \times 100$$

All laboratory experiments were carried out with a complete random design (CRD) procedure, and the averages were compared with the LSD. Test. Below the 0.01 probability level [15]. The data were analyzed statistically using the SPSS statistical program.

3. RESULTS AND DISCUSSION

3.1. Isolation of Fungi from Date Palm Shoots

The results of fungal isolation from plant parts infected with leaves spot and black Scorch diseases on the shoots (Table 1) showed the isolation of 36

Table 1. Fungi Isolated from Leaf Spot, Black Scorch and Wilt Disease of Date Palm Tissue Culture Offshoots. Where: A=Leaf Spot Disease, B=Black Scorch Disease, C=Wilt Disease

Isolate name	A	B	C	*Isolate name*	A	B	C
Alternaria alternata	+*	+	**-	*Rhizopus sp.*	-	-	+
Alternaria aspera	+	+	-	*Thielaviopsis paradoxa*	-	+	-
Alternaria botrytis	+	-	-	*Aspergillus restructus*	-	+	-
Alternaria chartarum	+	+	-	*Aspergillus peniciliodes*	-	+	-
Alternaria chlamydospora	+	+	-	*Penicillium spp.*	-	+	+
Alternaria concatenata	+	+	-	*Chaetomium sp.*	-	+	-
Alternaria dianthicola	+	+	-	*Rhizoctonia solani*	-	+	-
Alternaria longipes	+	+	-	*Fusarium solani*	-	-	+
Alternaria penicillata	+	-	-	*Fusarium proliferatum*	-	-	+
Alternaria Petroselini	+	-	-	*Fusarium fujikuroi*	-	-	+
Alternaria radicina	+	+	-	*Nigrospora sphaerica*	+	-	-
Alternaria tenuissima	+	+	-	*Neoscytalidium dimidiatum*	+	+	-
Bipolaris australiensis	+	+	-	*Aspergillus spp.*	-	-	+
Cladosporium cladosporoides	+	+	-	*Ulocladium sp.*	-	+	-
Cladosporium elatum	+	+	-	*Ulocladium alternariae*	+	+	-
Cladosporium herbarum	+	+	-	*Ulocladium atrum*	+	+	-
Cladosporium oxysporum	+	-	-	*Stemphylium sp.*	+	+	-
Drechslera biseptata	+	+	-	*Scytalidium lignicola*	-	+	-
Fusarium chlamydospora	+	+	-	*phoma exigua*	-	+	-
Fusarium oxysporum	+	+	-	*Phoma costarricensis*	+	-	-
Neodeightonia phoenicum	-	+	-	*Trichoderma sp.*	-	-	+

*Isolated ** non-isolated

species of fungi, 12 of them belong to the genus *Alternaria*, four belong to the genus *Cladosporium*, and three species belong to the genus *Ulocladium*. Thirty-one (31) species were isolated from palms affected by leaf spot disease, and 32 species were associated with black Scorch symptoms [16]. Mentioned the above fungi as major genera associated with the symptoms of date palm leaf spot disease in Basrah. The results of the study also showed the isolation of new species of fungi associated with date palm leaf spots disease, such as *A. petroselini*, *A. penicillata*, *A. botrytis*, *Cladosporium oxysporum*, and *P. costarricensis*. Most of these fungi were registered as fungi accompanying disease states for many plants [17]. isolated *C. oxysporum* from tomato plants infected with leaf spots in greenhouses. Misawa and Kurose [18] showed that *A. petroselini* was isolated from parsley which showed symptoms of leaf blight and stem rot.

No studies refer to the isolation of the fungus *P. costarricensis* as a cause of date palm leaf spot disease, as it is one of the common fungi on the Arabica coffee plant (coffee) in South American countries such as Costa Rica and Brazil, and it causes spot and blight diseases [19]. As for Black Scorch Disease, *T. paradoxa* was isolated, and this is consistent with previous studies of this disease, as it was recorded by Klotz [20] as a cause of black scorch disease on date palms for the first time. After that, many researchers in Iraq mentioned this fungus as a cause of black Scorch, terminal bud rot, and deterioration of date palms [21]. In addition to the fungi above, many fungi were isolated, the most pathogenic of which was the fungus *N. phoenicum*, which was isolated and recorded for the first time in Iraq as a cause of black Scorch This is consistent with many studies that indicated the pathogenicity of this fungus on palms, as the genus *Neodeightonia* is an important fungus belonging to the family Botryosphaeriaceae, which is characterized by causing important and common diseases on plants. Abbas *et al* [22] recorded the fungus *N. Phoenicum* on date palms in Greece, *N. phoenicum* in Qatar was also found by Ligoxigakis *et al.* [23] on date palm trees. The results of fungi isolation from the roots of plants infected with wilt disease showed the isolation of several types of fungi, most of which belong to the genus *Fusarium*, namely *F. solani*, *F. proliferatum*, and *F. fujikuroi*. This result is consistent with previous studies in which it referred to the isolation of one or several species of the fungus *Fusarium* with disease states similar or close to the wilt cases studied such as *F. oxysporum*, *F. monliforme*, *F. proliferatum*, and *F. solani* [24-26]. These results are in agree with many previous studies in which it was mentioned the relationship between the *Fusarium* species with date palm wilt diseases such as *F. solani* [6]. Khazaal and Ameen [27] Isolated *F. proliferatum* and *F. fujikuroi*, in addition to other species of the same genus, from the roots of date palms affected by sudden Decline syndrome in Basrah Governorate. Alananbeh *et al*. [28] Indicated that *F. proliferatum* is associated with yellowing and death of date palms in Jordan. In the United Arab Emirates, it was reported that the fungus *F. solani* was isolated from shoots infected with wilt, death, and drying of palm fronds [29].

3.2. Pathogenicity of Fungi isolated from Date Palm Shoots

The results of the pathogenicity test (Figures 1 and 2) showed the ability of the tested fungi to cause infection and the appearance of disease spots after 21 days of inoculation. The fungus *N. phoenicum* recorded the highest infection rate of 2.8 cm, an increased rate of 0.2 cm/day, with a statistically significant difference for all tested fungi except for *P. costarricensis*, which achieved an infection rate of 2.65 cm, with an increase of 0.189 cm/day, and *A. concatenata* and *S. lignicola* each achieved an infection rate of 2.55 cm and 2.48 cm and an increased rate of 0.182 cm/day and 0.177 cm/day for both fungi, respectively. As for the rest of the tested fungi, the rates of infection spread on the leaves (spots size) ranged between 1.5 to 1.1 cm. *A. restructus*, *A. peniciliodes*, *Penicillium spp*., *Chaetomium spp.*, and *R. solani* have recorded no infection on date palm leaves. Many fungi that have proven their ability to cause infection and the emergence of disease spots were recorded in previous studies as pathogens on date palms [16]. Recorded the fungi *A. alternata*, *B. australiensis*, *C. herbarum*, *F. oxysporum*, and *T. paradoxa* as the causative fungi for date palm leaf spot disease. Alasadi and Alnajim [30] Recorded the fungus *A. dianthicola* as the cause of leaf spot disease on date palm and canary palm. The fungus *A. radicina* was recorded as a cause of black spot disease on date palm leaves in Basrah [31].

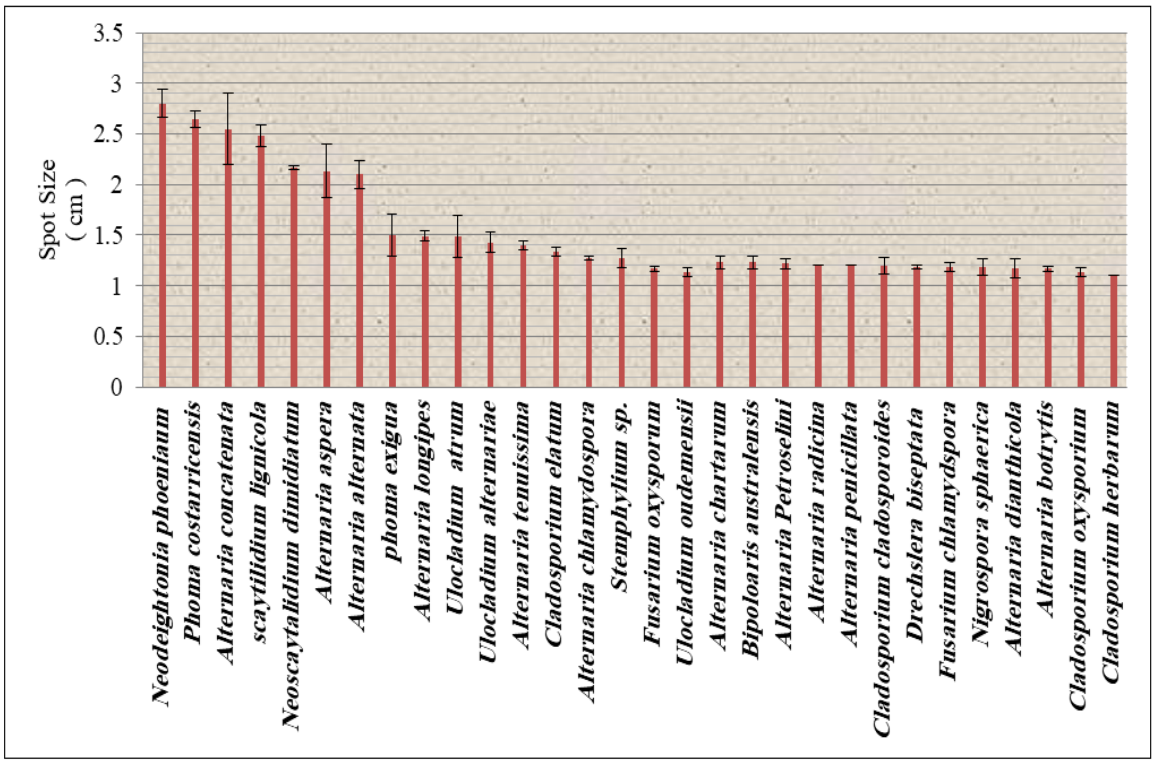

Fig. 1. The size of the diseased spot in centimeters after 21 days of inoculation with fungi isolated from the shoot system.

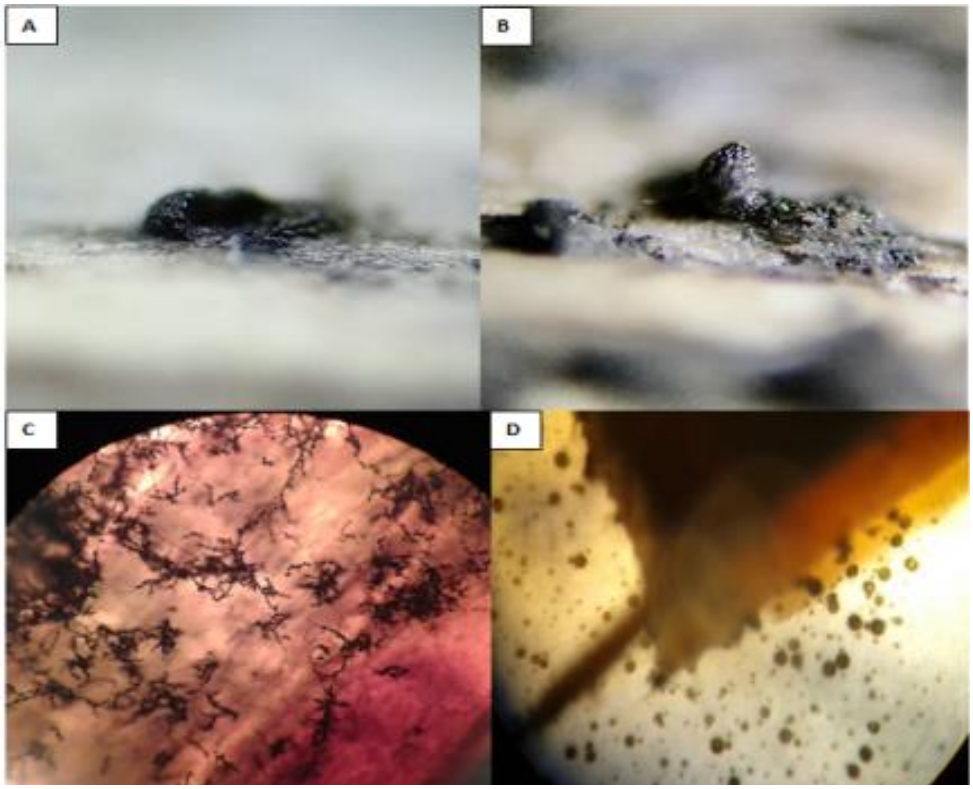

Fig. 2. The structures formed by some fungi on the surface of the plant tissue (leaf) after conducting a pathogenicity experiment. A. The pycnidium of *N. phoenicum*, *B. Aggregation* of conidia after releasing from pycnidium of *N. phoenicum*, *C. Conidia* of *A. alternata*. D. Pycnidia of *P. costarricensis*.

3.3. Pathogenicity Test for the Tungi Root System

The results of this study in Figures (3, 4, and 5) indicated that *Fusarium spp*. Have a negative effect on the germination of date palm seeds. The percentage of germination was 53.33 % for isolate *F. fujikuroi* F3 and *F. solani*, and it reached 56.67 % for isolate *F. proliferatum* F1, *F. fujikuroi* F4, and *F. proliferatum* F7, while it reached 93.33 % in the control. The results revealed significant differences in the percentage of seedling death of date palms, which amounted to 76.67, 76.67, 80.00, 76.67 and 83.33 % for the fungus *F. proliferatum* F1, *F. fujikuroi* F3, *F. fujikuroi* F4, *F. solani* and *F. proliferatum* F7. respectively, while the control treatment (free from pathogens) amounted to 16.67 %. These results were consistent with previous studies that confirmed the role of the fungus *Fusarium* spp, in reducing the germination of seeds of many different plants, including date palm seeds [32-34]. The variation in the percentage of seed infection with *Fusarium* species may be due to the genetic variance among the species of the genus *Fusarium* spp. or to the ability of fungal isolates to produce various enzymes or to secrete many different toxic substances. The ability of the fungus *Fusarium* spp. to cause the death and rotting of seedlings in many different plants may be due to its ability to the production of cell wall-degrading enzymes such as Chitinase, Polygalacturonas, and

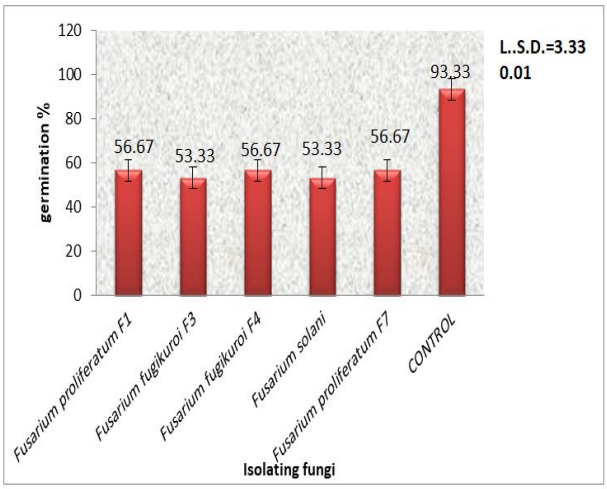

Fig. 3. Effect of fungi isolated from the roots on the percentage of germination of date palm seeds.

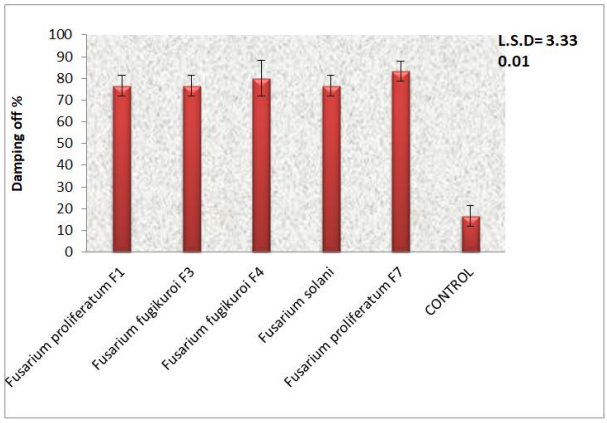

Fig. 4. Effect of fungi isolated from roots on Damping off percentages of date palm seedlings.

Fig. 5. Symptoms of infection with *Fusarium* species on palm seedlings. 1=Control, 2=*F. proliferatum* F1, 3=*F. fujikuroi* F3, 4=*F. fujikuroi* F4, 5=*F. solani*, 6= *F. proliferatum* F7.

Cellulase [35] in addition to its production of many toxins such as Fusaric acid, Dehydro Fusaric acid and Lycomarsmin, which are among the main pathogenicity factors of this fungus [36].

3.4. Molecular Identification of Pathogenic Fungi of the Vegetative and Root System

The results of molecular identification were in agreement with the phenotypic identification Figure (6). Molecular identification is based on the amplification of the gene region ITS1-ITS4 showed that the fungal isolates F1 and F7 belong to the *F. proliferatum*, the isolate F1 was recorded in the NCBI under accession number OM535259.1, and the isolate of this fungus matched with the isolate registered under accession number MN871570.1, and the similarity percentage was 100 % between the two isolates. The second isolate F7 of the fungus *F. proliferatum* was recorded with the accession number OM535261.1 and it was identical to isolate MT509801.1 with a similarity percentage of 98.41 %. The results also showed that isolate F3 and F4 belong to *F. fujikuroi* and were recorded in the gene bank with accession number OM535264.1 and OM535265.1 respectively. The isolate of the *F. solani* was recorded with accession number OM535266.1, Molecular diagnosis of other fungi and their registration numbers in the gene bank are shown in (Table 2) Several previous studies indicated the pathogenicity of this fungus to the date palm [6, 22, 37, 38].

3.5. Effect of Different Concentrations of Silver Nanoparticles on the Radial Growth of Fungi Causing Date Palm Wilt Disease

Figure 7 showed that the percentage of growth inhibition of the tested fungi increased with increasing concentration of silver nanoparticles. The percentage of growth inhibition of the fungus *F. proliferatum* F1 at the concentration (25, 50, and 100 ppm) was 62.20, 65.86, 67.73, and 72.56 % respectively. While the percentage of growth inhibition of *F. fujikuroi* F3 was 48.50, 50.33, 64.40, and 66.60 %, respectively. It was 53.67, 54.40, 57.70 and 65.50 %, respectively, for *F. fujikuroi* F4, while the percentage of growth inhibition of *F. solani* was 53.30, 52.90, 70.57 and 77.70 %, respectively. While the percentage of growth inhibition of *F. proliferatum* F7 was 48.13, 63.26, 66.23, and 69.23, respectively. The results of this test were similar to the results of previous studies that indicated the ability of silver nanoparticles to

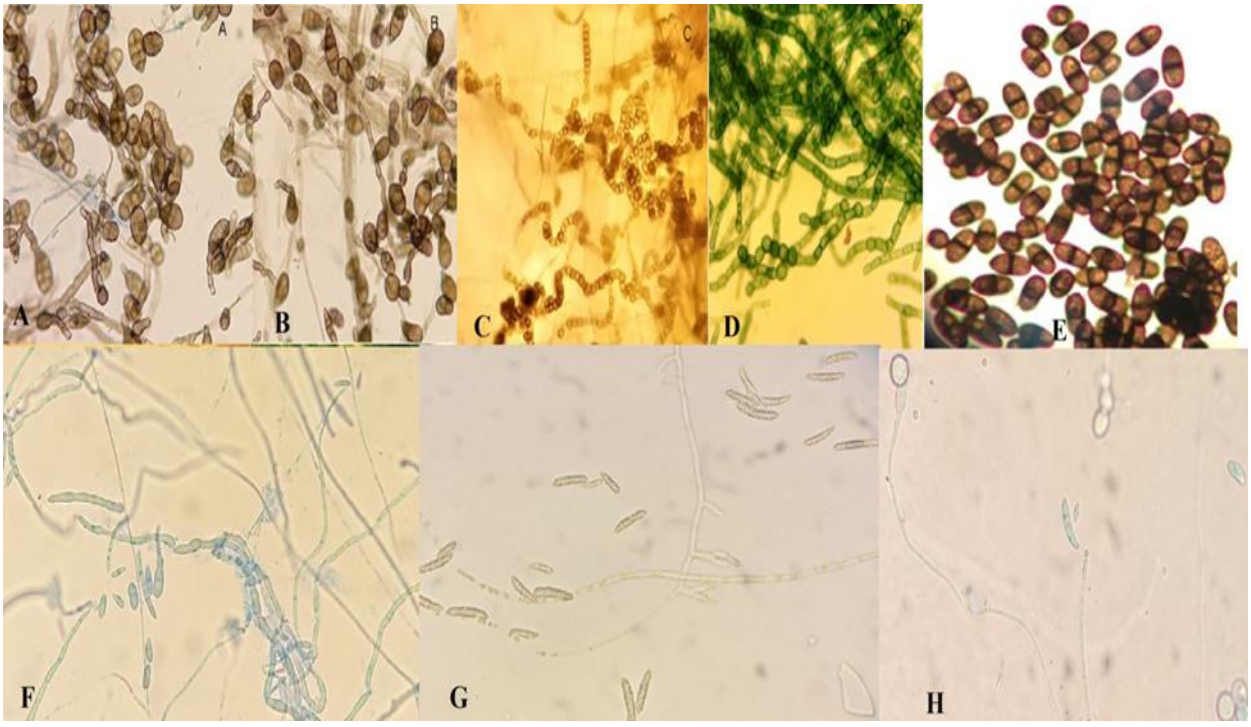

Fig. 6. Morphology of some fungi isolated from leaves and roots of date palm offshoots. *A: Alternaria tenuissima, B: Alternaria alternata, C: Neoscaytalidium dimidiatum, D: Scytalidium lignicola, E: Neodeightonia phoenicum, F: F. proliferatum G: F. fujikuroi H: F. solani*

Table 2. Molecularly identification of fungi that are registered in the gene bank (NCBI).

Scientific Name	Accession number	Accession Number of Matching Isolates	Percent Identity %	Query cover %
F. proliferatum F1	OM535259.1	MN871570.1	100	100
F. fujikuroi F3	OM535264.1	MG543727.1	99.80	100
F. fujikuroi F4	OM535265.1	MT603294.1	100	100
F. solani F6	OM535266.1	MG932644.1	94.85	100
F. proliferatum F7	OM535261.1	MT509801.1	98.41	99
A. alternata	OK235483.1	MW008974.1	100	100
A. tenuissima	OM562280.1	MK534954.1	100	100
N. dimidiatum	OM562604.1	MK480470.1	100	93
N. phoenicum	MZ675601.1	NR_111325.1	99.62	100
S. lignicola	OM585626.1	MG672013.1	97.99	87
P. costarricensis	OK255499.1	KT881552.1	95.34	99

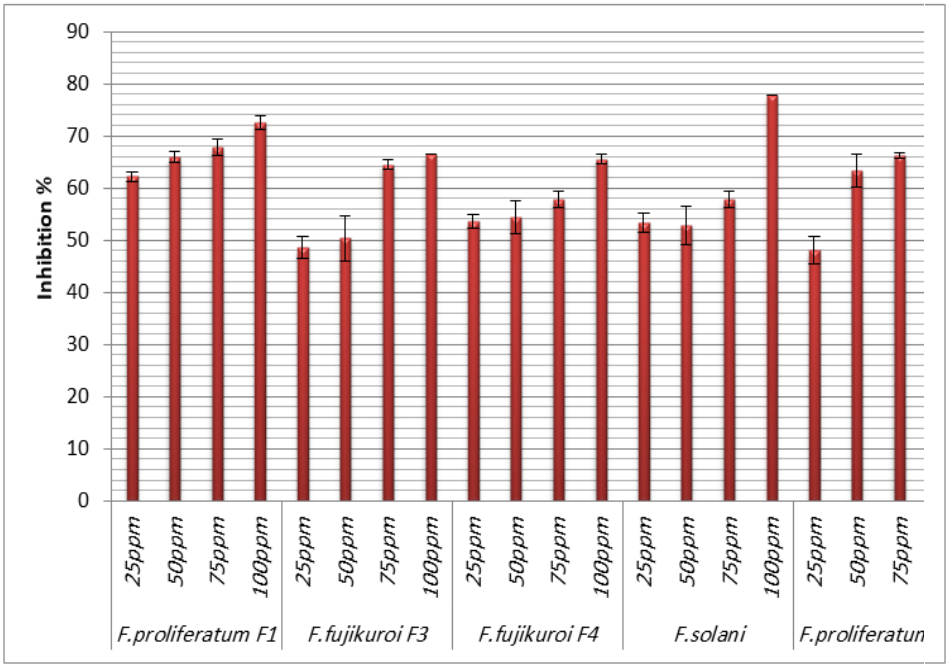

Fig. 7. Effect of different concentrations of silver nanoparticles on the radial growth of fungi causing date palm wilt disease.

inhibit the growth of different pathogenic fungi, such as *F. oxysporum* f.sp. radicis-lycopersici, *A. alternata*, *Botrytis cinerea*, and *Macrophomina phaseolina* [39, 40]. The results also showed that the effect of silver nanoparticles in inhibiting the growth of fungi increases with the increase in the concentration of Silver Nanoparticles used.

The effectiveness of silver nanoparticles is due to their ability to penetrate cells of microorganisms and disrupt the transport systems in cells, including ion exchange, which in turn affect important vital processes such as respiration and metabolism [41]. Silver ions may interact with oxygen, damaging cells and causing damage to proteins and fats, and nucleic acids [42]. Silver nanoparticles also cause an increase in the permeability of cell membranes by inhibiting the action of enzymes associated with cell membranes and affecting the process of gene expression [43-45] It is also found that Silver Nanoparticles disrupt the nucleic acid replication, which leads to disruption of the gene expression [46].

4. CONCLUSION

Most of the fungi recorded on the shoot and root systems of date palm trees resulting from tissue culture are similar to those isolated from date palm trees resulting from other methods of reproduction, such as propagation by offshoots. *P. costarricensis*, *N. phoenicum*, *S. lignicola*, and *N. dimidiatum* as new pathogens for the first time on date palm in Basra-Iraq. The silver nanoparticles in the laboratory played a major role in inhibiting the growth of pathogens.

5. CONFLICT OF INTEREST

There is no conflict of interest among the authors for publishing this manuscript.

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