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Article

# Effect of some antibiotics and nanoparticles antibiotics and nanoparticles on fungi associated with date palm tissue cultures, *Phoenix dactylifera* L.

*Alaa Naser Ahmed*[1], *and Mohammed Hamza Abass*[2]
[1] *Department of Plant Protection, College of Agriculture, University of Basrah, Iraq*
[2] *Department of Plant Protection, College of Agriculture, University of Basrah, Iraq*
* *Correspondence: Emails: *alaa.ahmed@uobasrah.edu.iq; mohammed.alkinany@uobasrah.edu.iq*
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**ABSTRACT**
Tissue cultures faces significant challenges, the most important of which is the problem of microbial contamination. Therefore, the study aimed to evaluate the efficiency of some antibiotics (Amphotericin, Fluconazole, Nystatin) and nanoparticles (TiO2 and ZnS) in controlling fungal contamination of date palm tissue cultures. The study showed significant differences in the effect of antibiotics and nanoparticles on the radial growth of fungi associated with date palm tissue cultures with different concentrations. The results showed the effect of the antibiotic Nystatin and the TiO2 nanoparticles on the radial growth of the studied fungi. Nystatin and (TiO2) recorded the highest rate of inhibition of the radial growth of all the studied fungi, as they reached 30.025 and 17.951% at the concentration of 150 and 100 PPM, respectively, compared to the antibiotic (Amphotericin and Fluconazole) and the nanoparticles (Zns), the inhibition percentage was 5.258, 12.535 and 7.887% at concentrations of 40, 150 and 100 PPM, respectively. The results also showed the effect of the third concentration of all the studied antibiotics and nanoparticles, as (Nystatin at the concentration of 150 PPM and TiO2 at the concentration of 100 PPM) recorded the highest percentage of inhibition of radial growth for all studied fungi, which amounted to 72.811 and 55.957%, respectively. It appears from the results of this study; also, the fungus *Alternaria alternata* recorded the highest rate of radial growth inhibition, which amounted to 21.681% compared to the other studied fungi.
**Keywords:** Antibiotics; Nanoparticles; Contaminating fungi; Date palm tissue cultures.

## INTRODUCTION

The cultivation of the date palm (Phoenix dactylifera L.) is one of the main economic matters in the dry areas of the Middle East and North Africa [1].

The date palm reproduces by cuttings or the vegetative method in the propagation of offshoots. This method produces genetically identical plants to the mother plant True-to-type and gives fruits identical to the mother plant and of good quality [2, 3]. The offshoot is the growth that emerges From the area close to the base around the trunk, which results from the axillary buds of the leaves and forms its roots with its connection to the mother tree and can be separated and

planted as a separate plant [4]. Due to the small number of offshoots produced by each palm tree, studies have focused on propagation by tissue cultures. The limited offshoots produced by the palm each year and the increasing demand for some varieties and obtaining plants free from diseases prompted. The palm propagation workers use the tissue cultures technique and the possibility of obtaining the new plant very quickly compared to the traditional method of cultivating date palm offshoots [5, 6, 7, 8].

Despite the efficiency of the tissue cultures technology in terms of the abundance of plants that can be produced from one plant origin, tissue cultures faces great challenges, the most important of which is the problem of microbial contamination, which causes the rotting of the callus tissue cultures and the browning of the planted plant part and the destruction of its tissue cultures, which leads to tissue cultures death. The organism due to the effect of the secretion of toxic and growth-inhibiting substances and degrading enzymes by microbial contamination [5, 42]. Fungi are among the main groups that cause microbial contamination in date palm tissue cultures at very high rates, which means destroying a large amount of date palm tissue cultures [5, 9, 10].

Antibiotics were used in plant tissue cultures to obtain tissue cultures free from microbial contamination of various types of plants. Different antibiotics were used, including Griseofulvin, Nystatin, Amoxillin, Stryptomycin, Gentamycin, and Chloramphenicol, which were added to the culture media during the early stages of cultivation in date palms [11, 12]. [13] indicated in another study the use of low concentrations of the antibiotic Chloramphenicol instead of Gentamycin as prevention or decontamination agents in date palm tissue cultures.

Nanotechnology has received great interest in research for recent applications in agriculture, antimicrobials and medicine. Nanoparticles have a wide range of applications in the agricultural field due to their unique properties, including high penetration capacity, large surface area and high chemical activity [14]. Particles in nanometer size show different physical properties from each original material [15]. The small size of the nanoparticles leads to an efficient surface reaction of the particles in microbial contamination due to the increased coverage area of the treated surface [16, 17]. Studies have mentioned the role of nanoparticles in reducing fungal contamination of date palm tissue cultures. [18] showed the efficiency of ZnO NPson in reducing microbial contamination and non-toxicity of plant tissue cultures and stimulating the growth of date palm tissue cultures.

The study aims to evaluate the efficiency of using some antibiotics (Amphotericin, Fluconazole, Nystatin) and nanoparticles (TiO2 and ZnS) in controlling fungal contamination of date palm tissue cultures

## MATERIALS AND METHODS

*Isolation and purification of contaminated fungi of date palm tissue cultures*

Daily follow-ups of the plants were conducted to detect contamination of plant parts grown inside the cultivation tubes and to isolate them to diagnose. Contaminated cultivation tubes and the appearance of fungi on the callus were identified. The process of isolating and diagnosing the fungi after the appearance of cases of fungal contamination was done by washing the pieces with sterile distilled water to remove traces of the culture medium used to grow date palm tissue cultures and several times drying them with sterile filter paper. Then, those pieces were transferred by sterile forceps to Petri dishes with a diameter of 9 cm containing potato extract, dextrose, and agar (PDA) in the culture medium, supplemented with the antibiotic Chloramphenicol at a concentration of 250 mg l sterilized by a steam sterilizer at a temperature (121 ° C) and a pressure (1.5 bar). For 20 minutes, the dishes were incubated at 25°C for 3-7 days.

*Phenotypic diagnosis of contaminated fungi of date palm tissue cultures*

The fungi were morphologically diagnosed according to the phenotypic characteristics of the colony of fungi (shape, size, center, edges, color, transparency, and growth of the fungus on the culture medium), and these characteristics were recorded. The developing fungi were also examined under the light microscope (the shape and color of the fungal hyphae, conidiophore, the shape, color, and measurements of the spores (Conidia)) and some of the necessary microscopic characteristics in the microscopic classification process. Then the fungi were examined, isolated, and purified for diagnosis [19]. The developing fungi were diagnosed based on: [20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31].

*Molecular diagnosis of fungi contaminating date palm tissue cultures*

DNA extraction and agarose gel electrophoresis

DNA was extracted from pure fungal isolates according to [32] by using a (Genomic Purification Kit DNA) from Promega, USA, and total DNA was extracted from cells according to the manufacturer's instructions. This experiment was carried out 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 liquid nitrogen to crush them into a fine powder. The concentration of the extracted DNA was determined using Nano Drop at wavelengths of 260 and 280 nm, electrophoresis was carried out after 0.5 g of agarose gel was prepared with 25 ml of buffer solution, and the detection of the amplified beams was performed by a UV device and images with a digital camera.

PCR polymerase chain reaction test

25 mg of powder was taken and then transferred to 1.5 Eppendorf tubes for DNA extraction using a polymerase chain reaction (PCR) test. The polymerase chain test was carried out according to [33] method by mixing the reaction materials in a 1.5 µl Eppendorf tube and according to the leaflet provided with Green Master Mix manufactured by Promega ITS1 and ITS4 primers. Then the samples were centrifuged a 10,000 Microfuge rpm for 30 sec to ensure that all materials were homogeneous in the Eppendorf tube. The pieces were placed in a PCR sprint thermal cycler, and the device was run according to the program (Table 1). PCR amplification of the ITS1-ITS4 ribosomal RNA region was performed to molecularly identify the fungi [9] (Table 2).

| 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** |

**Table 1. Primer sequences that adopted in PCR technology**

*Determination of the nucleotides sequences of the products of the ITS replication*

After confirming the success of obtaining Amplicons' multiplication products for the ITS primers with the genome of the fungi under study, the samples were sent to the South Korean company Macrogen to determine the sequences of the nucleotides of the fungi under study by sending a volume of the product of the polymerase chain reaction (PCR) 25 microliters to 10 samples; the samples were marked to distinguish them from each other.*Genetic analysis of polymerase products*

After obtaining the results of the sequences of nucleotides from the replication of the ITS primers for the fungi under study and processing the data using Chroma's program, matching with the sequences stored in the NCBI (National Center for Biotechnology Information) was performed by applying the Blast (Basic Local Alignment Search Tool). It was a Determination of the fungal species by the degree of similarity, Maximum score and Query cover.

**RESULT**

*Study the effect of different concentrations of antibiotics and nanoparticles on fungi associated with date palm tissue cultures.*

Antibiotics were used in the experiments, which are (Amphotericin, Fluconazole and Nystatin) Table (3) and Nanoparticles (TiO2 and ZnS) shown in Table (4).

| Name | Chemical formula | Common name | Used concentration PPM | | |
|---|---|---|---|---|---|
| | | | First concentration | Second concentration | Third concentration |
| **Amphotericin** | $C_{47}H_{73}NO_{17}$ | Amphotericin | 10 | 20 | 40 |
| **Fluconazole** | $C_{13}H_{12}F_2N_6O$ | Fluconazole | 50 | 100 | 150 |
| **Nystatin** | $C_{47}H_{75}NO_{17}$ | Nystatin | 50 | 100 | 150 |

**Table 3. The antibiotics used, their active groups, and the concentrations used**

| Name | Chemical formula | Common name | Used concentration PPM | | |
|---|---|---|---|---|---|
| | | | First concentration | Second concentration | Third concentration |
| **TiO2** | TiO2 | Nano titanium dioxide | 50 | 75 | 100 |
| **ZnS** | ZnS | zinc sulfide | 50 | 75 | 100 |

**Table 4. The nanoparticles used, their active groups and the concentrations used**

The media of dextrose, potato and agar (Potato Dextrose Agar) (PDA) was prepared as previously mentioned, and antibiotics and nanoparticles were added to the medium after the sterilization process, with several concentrations as shown in Tables (3,4).

The culture media was poured into 9 cm Petri dishes for each treatment (3 concentrations for each substance in 3 replications) with the control treatment without addition. A 0.5 cm disc of a fresh colony of the studied fungi was placed in the middle of each dish of the treatments and incubated the studied concentrations with the control treatment without addition. The plates were kept at a temperature of 25 ± 2°C until the fungus growth reached the edge of the Petri dish in the control treatment. The fungal radial growth inhibition percentage was calculated, and the discs were taken using a sterile Cork Borer. The discs were placed in the center of each dish in an inverted shape, the size of 0.5 cm from each fungi separately from a culture of newly grown fungi from the studied materials and concentrations with the control treatment without the addition mentioned previously. The dishes were incubated at 25 ± 2 °C until the fungus growth reached the edge of the Petri dish in the control treatment. The percentage of fungal radial growth inhibition was calculated by taking the average of two perpendicular diameters passing from the center of the Petri dish as in the following formula:

Percentage of radial growth inhibition = control growth rate - treatment growth rate/control growth rate x 100

*Molecular diagnosis of contaminated fungi of date palm tissue cultures*

Table (6) summarises the results of BLASTn for sequencing the ITS1-ITS4 region of the ribosomal DNA of the fungi isolated in this study, and the database confirmation of the fungi matching within the matching ratio ranged between 95.30%-100%. And registration numbers for the ITS1-ITS4 region sequences in the NCBI GenBank.

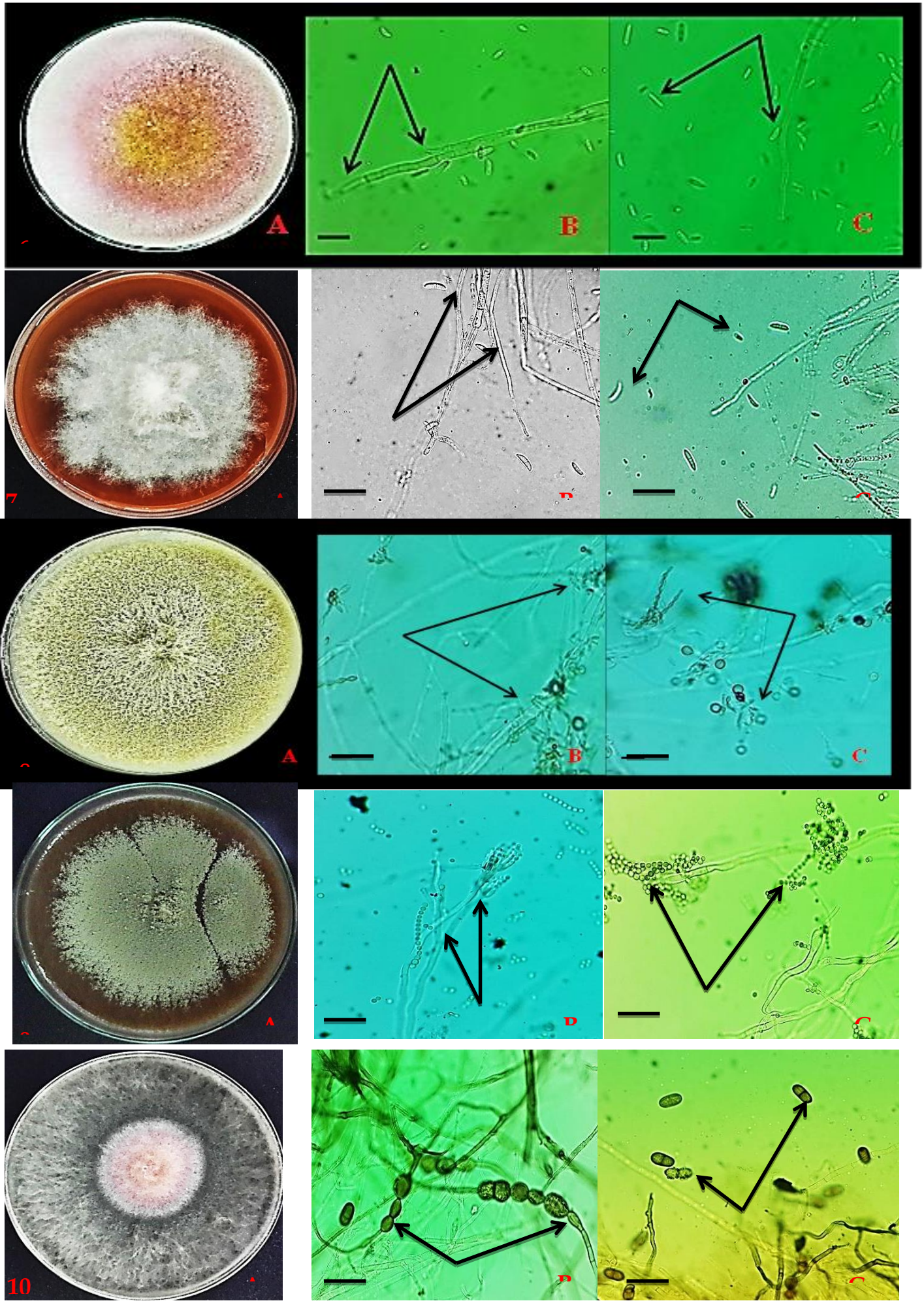


**Figure (2) Fungi isolated from the primary callus of date palm tissues.**

*6-Fusarium luffae,7-Fusarium solani,8-Paecilomyces formosus,9-Penicillium expansum,10-Neodeightonia phoenic*

| Fungal isolate | Base pair | GenBank accession number | Query coverage | Percentage of sequence identity | GenBank accession number of an organism with the highest sequence identity |
|---|---|---|---|---|---|
| *Alternaria alternata* | 547 | UBAMA1 OP090358 | 98% | 99.45% | **(KU936229.1)** |
| *Aspergillus fumigatus* | 599 | UBAMAF OP090360 | 82% | 100% | **(MT267795.1)** |
| *Chaetomium globosum* | 599 | UBAMC OP090361 | 85% | 99.31% | **(MT742687.1)** |
| *Cladosporium ramotenellum* | 518 | AMC OL589159 | 100% | 95.30% | **(MF473247.1)** |
| *Fusarium coffeatum* | 543 | AMFC OL589161 | 100% | 97.81% | **(MT742819.1)** |
| *Fusarium luffae* | 517 | AMF OL589160 | 99% | 98.06% | **(MT448895.1)** |
| *Fusarium solani* | 536 | UBAMF OP090359 | 98% | 99.43% | **(MG211160.1)** |
| *Neodeightonia phoenicum* | 583 | AMN OL589157 | 98% | 99.48% | **(KF766198.1)** |
| *Paecilomyces formosus* | 560 | AMP OL589158 | 83% | 99.32% | **(JX406544.1)** |

**Table 6. Summary of BLASTn results for sequencing the ITS1-ITS4 region of ribosomal DNA of fungi isolated from date palm tissue cultures.**

*Effect of different concentrations of antibiotics and nanoparticles on radial growth of fungi associated with date palm tissue cultures.*

Table (7) indicates significant differences in the effect of antibiotics and nanoparticles on the radial growth of fungi associated with date palm tissue cultures and in different concentrations. (Nystatin and TiO2) recorded the highest rate of inhibition of radial growth for all studied fungi, reaching 30.025 and 17.951% at concentrations of 150 and 100 PPM, respectively, compared to antibiotic (Amphotericin and Fluconazole) and nanoparticles (Zns), where the percentage of inhibition was 5.258, 12.535 and 7. 887% at 40, 150, and 100 PPM concentrations, respectively

The results of Table (7) showed the effect of the third concentration of all the studied antibiotics and nanoparticles, as (Nystatin at the concentration of 150 PPM and TiO2 at the concentration of 100 PPM) recorded the highest percentage of inhibition of radial growth for all studied fungi, which amounted to 72.811 and 55.957%, respectively, compared to With the control treatment (without addition), the rate of inhibition of radial growth of the studied fungi was 0.000%. The Table also shows that Alternaria alternata recorded the highest rate of radial growth inhibition, reaching 21.681% compared to the other studied fungi Many antibiotics have been used in plant tissue cultures to reduce microbial contamination of those tissue cultures. The effect of antibiotics in reducing microbial contamination and inhibiting their growth may be due to the presence of active substances in those antibiotics, which can bind to ergosterol found in the

cell membranes of fungi. The effect of Nystatin on reducing the radial growth of fungi associated with the cultivation of date palm tissue cultures in this study may be attributed to the binding of the active substance Nystatin to ergosterol, the main component of the cytoplasmic membrane in fungi, which leads to holes in this membrane, which leads to cytoplasm leakage outside the cell and thus cell death.

As for nanoparticles, the small size of the nanoparticles and the large surface area lead to the efficient interaction of the surface of the particles with microbial contaminate, chemical activity and high penetration ability, The effect of nanoparticles used in the study may be attributed to the ability of TiO2 to analyze organic compounds and its effect on the radial growth of the fungi studied. As for Zns nanoparticles, because they are preservatives for wood and leather, they are decomposing fungi, and they are also used as a fungicide.

| **No.** | **Materials** | **concentrations** | **percentage radial growth inhibition%** | | | | | | | | | | **Concentrations average** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Alternaria alternata* | *Aspergillus fumigatus* | *Chaetomium globosum* | *Cladosporium ramotenellum* | *Fusarium coffeatum* | *Fusarium luffae* | *Fusarium solani* | *Neodeighton ia phoe-* | *Paecilomyces formosus* | *Penicillium expansum* | |
| **1** | Amphotericin | control | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| | | 10 | 0.000 | 0.000 | 0.000 | 3.330 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.370 | 0.370 |
| | | 20 | 4.440 | 0.000 | 0.000 | 13.700 | 0.000 | 0.000 | 38.883 | 1.110 | 0.000 | 1.850 | 5.998 |
| | | 40 | 15.550 | 32.220 | 0.000 | 20.737 | 0.000 | 1.850 | 46.290 | 1.110 | 25.920 | 2.960 | 14.664 |
| | material average | | | | | | | | | | | | 5.258 |
| **2** | Fluconazole | control | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| | | 50 | 5.920 | 0.000 | 0.000 | 4.810 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 3.330 | 1.406 |
| | | 100 | 37.400 | 0.000 | 0.000 | 13.700 | 0.000 | 1.110 | 1.110 | 5.550 | 0.000 | 44.070 | 10.294 |
| | | 150 | 65.550 | 67.770 | 1.850 | 60.367 | 29.253 | 23.330 | 37.030 | 8.883 | 44.810 | 45.550 | 38.439 |
| | material average | | | | | | | | | | | | 12.535 |
| **3** | Nystatin | control | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| | | 50 | 16.660 | 0.000 | 0.000 | 4.440 | 0.000 | 13.330 | 0.000 | 0.000 | 0.000 | 1.110 | 3.554 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 100 | 65.180 | 71.850 | 54.440 | 59.997 | 1.480 | 25.920 | 7.400 | 54.440 | 51.480 | 45.180 | 43.737 |
| | | 150 | 76.290 | 81.480 | 76.660 | 81.850 | 51.110 | 85.550 | 59.997 | 61.850 | 76.660 | 76.660 | 72.811 |
| | material average | | | | | | | | | | | | 30.025 |
| **4** | TiO2 | control | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| | | 50 | 5.550 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 5.180 | 0.000 | 0.000 | 1.110 | 1.184 |
| | | 75 | 12.960 | 64.440 | 1.110 | 0.740 | 1.110 | 4.070 | 42.960 | 18.883 | 0.000 | 0.370 | 14.664 |
| | | 100 | 76.290 | 75.550 | 47.770 | 54.440 | 3.700 | 54.070 | 77.400 | 46.290 | 65.180 | 58.883 | 55.957 |
| | material average | | | | | | | | | | | | 17.951 |
| **5** | ZnS | control | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| | | 50 | 0.000 | 0.000 | 0.000 | 1.480 | 0.000 | 0.000 | 1.110 | 0.000 | 0.000 | 0.740 | 0.333 |
| | | 75 | 4.810 | 0.000 | 0.740 | 2.220 | 0.000 | 2.220 | 25.550 | 1.850 | 9.997 | 3.700 | 5.109 |
| | | 100 | 47.030 | 37.770 | 1.110 | 2.590 | 2.960 | 35.180 | 36.290 | 15.550 | 37.030 | 45.550 | 26.106 |
| | material average | | | | | | | | | | | | 7.887 |
| | Fungi average | | 21.681 | 21.554 | 9.184 | 16.220 | 4.481 | 12.331 | 18.960 | 10.776 | 15.554 | 16.572 | |
| | R.L.S.D | Fungi = 0.3160 | Materials = 0.2234 | Concentrations = 0.1998 | Fungi* Materials = 0.7065 | | Fungi* Concentrations = 0.6319 | | | Materials * Concentrations = 0.4468 | | Fungi* Materials * Concentrations = 1.4130 | |

**Table 7. Effect of different concentrations of antibiotics and nanoparticles in inhibiting radial growth of fungi associated with date palm tissue cultures**

*Each number represents 3 replicates*

It was reported by [35] that particles at nano size exhibit antimicrobial effects against a wide range of bacteria, fungi and other microbes and among the different types of nanoparticles, the most common are silver nanoparticles (AgNPs), especially in the agricultural sector.

## DISCUSSION

The results of the current study were in agreement with the results of [11] and [12], which stated that the use of the antibiotics Griseofulvin, Nystatin, Streptomycin or Gentamycin, and Chloramphenicol with the culture medium for tissue cultures in date palm has a positive role in eliminating microbial contamination in the early stages of date palm tissue cultures.[19] indicated the use of low concentrations of the antibiotic Chloramphenicol instead of Gentamycin as prevention or decontamination agents in date palm tissue cultures. It agreed with the results of El-[34] in the safe and effective use of the antibiotics Gentamycin and Chloramphenicol with the culture medium for tissue cultures in date palms, which led to the reduction of microbial contamination from bacteria and fungi.

Also, the results of the current study agreed with many studies, including what was indicated by [16,17] concluded that the small size of nanoparticles leads to an efficient surface interaction of particles in microbial contamination due to the increase in the surface area of the treated surface.

[36] indicated that adding TiO2 NPs in the culture media for plant tissue cultures and ornamental plant production had good potential in removing microbial contaminants from plant tissue cultures grown in the laboratory.

As the results of the current study agreed with [37, 38], silver nanoparticles can reduce bacterial and fungal contamination of date palm primary callus and have a positive role in the growth and development of primary callus and embryonic callus in date palm tissue cultures.

What was proven by [34, 39] demonstrated the ability of silver nanoparticles to reduce bacterial and fungal contamination of the primary callus and stimulate the growth and development of the primary callus and embryonic callus of date palm when added to the culture medium at a concentration of 5 mg l, increasing the number of embryos and plant growth in laboratory conditions and not Toxic effects on date palm tissue cultures.

It was pointed out by [18] that the efficiency of ZnO NPs in reducing microbial contamination and non-toxicity of plant tissue cultures and stimulating the growth of cultivated tissue cultures of date palm when added to the culture medium at a concentration of 150 mg liter[43,44].

## CONCLUSIONS

Microbial contamination has significant and negative risks in the growth and development of plant tissue cultures, including date palm tissue cultures, so the current study aimed to identify the most important fungi contaminating tissue cultures and to identify the dominant fungal species in them through phenotypic and molecular diagnosis, which proved the isolation and identification of ten different fungi. Multiple antibiotics were tested: Amphotericin, Fluconazole, and Nystatin with different concentrations, as well as two nanoparticles, TiO2 and Zns.

The effectiveness of the chemicals was significant in terms of their effect on the percentage of radiotoxicity of the fungi under study, and the treatments of the antibiotic Nystatin and the TiO2 nanoparticles were superior in limiting the

growth of contaminated fungi. Thus, the current study recommends to benefit from these compounds in reducing microbial contamination and its negative effects on date palm tissue cultures

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