First molecular identification of *Fusarium fujikuroi* causing pollen rot of palm trees (*Phoenix dactylifera* L.) in Iraq and evaluation efficacy of some nanoparticles against it

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Abstract. This study was carried out to identify the causal agent of root rot on date palm in major growing areas in Iraq. As well as, the efficiency of ZnO and MgO NPs were assessed against the pathogen. Microscopic examination and morphological characterization revealed that *Fusarium* sp. was present in all examined samples and was the most pathogenic fungi. The PCR specificity identification based on the internal transcribed spacer (ITS)/5.8S regions showed that the pathogenic fungus was *Fusarium fujikuroi*. The *F. fujikuroi* inhibited seed germination 53.55% compared with seed germination of 81% in control. All concentrations (1,2,4 g/l) of ZnO NPs proved to be effective against *F. fujikuroi* particularly at concentrations 4 g/l with inhibition percentage exceeded 74.05%. Although, the same concentration of MgO NPs did not show same inhibition capacity against the same pathogen comparing with ZnO NPs, they were significantly better than in control. The results indicate clearly to possibly to use the fungicides with ZnO and MgO NPs particularly at concentrations 2,4g/l in management of pollen rot of date palm trees. Information from this study might assist scientists to design effective strategies in controlling date palm diseases.

1. Introduction

Date palm (*Phoenix dactylifera* L.) is an important fruit crop in Iraq includes eating fresh fruit and benefiting from its high nutritional value and its richness of carotenoids, citric acid, folic acid, and provitamins; this in addition to the antiviral, antibacterial, antifungal carotenoids antilucer, antitumor and immunomodulatory properties of phenolic compounds detected in dates [1]. Moreover, this crop has a great potential as a source of renewable energy by producing biofuel due to the content in the fruits. Also, seeds are used in animal feeding high carbohydrates, cosmetics, source of oxalic acid and charcoal, besides using them as a paste to relieve ague. Unchecked usage of fungicides has kindled many problems such as injurious consequences on human health, detrimental impact on insects involved in pollination and also imposed negative effects on domestic animals. Thus, introduction of chemical agents into the soil and water is affecting the stability of whole ecosystems [2]. Fungal pathogens isolated from the rotted pollen...
included _Mauginiella scaettae, Fusarium moniliforme_ Shed, _Fusarium solani_ (Mart.), _Thielaviopsis paradoxa_ (de Seynes) and _Fusarium oxysporum_ [3].

The disease is caused primarily by _Mauginiella scaettae_ an anamorphic fungus that reproduce by 1 – 6 celled arthroconidia. The disease was reported in Iraq [4]. Other fungi have been found associated with the diseased spathes include _F.oxysporum, F.moniliforme, F.solani Trichothecium roseum, Botrytis aclada, Thielaviopsis paradoxa, Acremonium strictum and Memnoniella_ sp. These fungi are initiated by hyphae growing in early season at leaf bases that penetrate flower bud before formation of spathes under suitable conditions, chlamydospores germinate and enter the vascular tissues [5]. Nanotechnology is one of the most fascinating and rapidly advancing sciences and possess potential to revolutionize many disciplines of science, technology, medicine and agriculture. Conversion of macromaterials in to nano size particles (1-100 nm) gives birth to new characteristics and the material behaves differently [6]. Nanosized forms of different metals are a part of the nanoparticles that have been enlisted for controlling plant diseases. Unchecked usage of fungicides has kindled many problems such as injurious consequences on human health, detrimental impact on insects involved in pollination and also imposed negative effects on domestic animals. Thus, introduction of chemical agents into the soil and water is affecting the stability of whole ecosystems [2]. Nanoparticles have attracted considerable attention in nanoscience and nanotechnology due to their excellent optical and electronic properties as well as their wide applications in various fields nanosized forms of different metals are a part of the nanoparticles that have been enlisted for controlling plant diseases. Nanoparticles synthesized from many element have been used as a potent pesticidal agent among different nanoparticles against a huge range of pathogens, fungi, and viruses differently [7]. The main objective of this work is to diagnose the causal agent of inflorescence rot and its control using some nanocomposites.

2. Materials and Methods

2.1. Isolation of the fungus associated with symptomatic tissues

Samples were collected and brought to the laboratory of plant diseases/ College of Agriculture/ University of Karbala. Samples were washed with distilled water to remove any suspended soil, than cut in to small pieces (0.5-1 cm) using a sterile sharp scalpel. The pieces were surface sterilized in sodium hypochlorite (1% NaOCl) for two min, rinsed in two changes of sterile distilled water and dried with filter papers to remove any excess water. The pieces were then transferred to petri-dishes containing potato dextrose agar (PDA), supplemented with chloramphenicol antibiotic at a concentration of 200 mg/L. petri-dishes were incubated at temperature of 25±2 °C for four days identified on the basis of the morphological characteristics.

2.2. Pathogenicity test

The pathogenicity test was performed by mixing the fungal colony growing on the PDA medium at 10 days of sterile distilled water (20 g / L). The radish seeds were sown in a circular manner in the dishes after surface sterilization with sodium hypochlorite solution 1% for 1 minute and dried on filter paper. 20 seeds were transferred to each dish and three replicates were treated with the control treatment without fungus. The dishes were incubated in the incubator 25 ±1. Results were recorded after 10 days to calculate the number of seeds grown in each dish. The percentage of germination was calculated according to the following equation.

2.3. Molecular identification of the fungal pathogen

The partial ITS region of each DNA extracted from each _Fusarium_ isolate was PCR-amplified using the universal primer pair: ITS1 (TCCGTTCGGTGAACCAGCGG) and ITS4 (TCCTCCGCTATGATATGC) [8]. PCR amplification was done using Taq DNA polymerase (Roche, Cat. No. 11 146 173 001) in a final volume of 20 μl PCR reaction mixture containing 2 μl 10X PCR buffer, 1
μl each primer (10 pmol), 2 μl dNTPs (2 mM), 3 μl template DNA (30 ng/μl), and 1 unit Taq polymerase. Each sample volume was then completed to 20 μl by adding nuclease-free water.

PCR amplification was performed using the following conditions Initial denaturation at 94 °C for 1 min followed by 35 cycles each consisting of final denaturation at 94 °C for 30 sec, annealing temperature at 55 °C for 30 sec, initial extension for 1 min, and final extension at 72°C for 5 min [9]. PCR-amplified products were electrophoretically separated on a 1% agarose gel for 140 min at 80 V, 400 mA and visualized with ethidium bromide staining under UV illumination and images were captured using Vilber Lourmat, Taiwan gel documentation system. For DNA sequencing, the PCR-amplified products were gel-purified using the favorpprep PCR purification kit (Cat. No. FAGCK 001, Favorgen, Taiwan) and sent along with the primer pair (ITS1 and ITS4) to the Macrogen DNA sequencing service in Korea. PCR products were directly sequenced in both directions using the respective forward and reverse primers. The obtained nucleotide sequences were then aligned and compared with the sequences belonged to the other isolates and previously published in NCBI database using the Basic Local Alignment Search Tool (BLAST). Multiple sequence alignment of the nucleotide sequences were carried out and phylogenetic trees were constructed by the MEGA6 software [10], using the Neighbor-joining method.

2.4. In vitro evaluation of MgO and ZnO nanoparticles effect against F. fujikuroi

Different concentration of nanoparticles of MgO and ZnO NP (1, 2, 4 g/L) were evaluated against F. fujikuroi The fungal inoculates were prepared on potato dextrose agar (PDA) media at 28°C in petri-plates spore suspension of each isolate of fungal containing at least 20-30 spores per microscopic field was prepared from 10 days old fungal culture. One drop about 0.1ml of spore suspension was put in a cavity glass slide containing a drop (about 0.1ml) of different concentration of nanoparticles. These slides were kept in moist chamber prepared by putting two folds of filter paper in both sides of Petri-plates. These Petri plates were incubated at 24±2°C for 24 hours. Each treatment was replicated three times. Beltanol (8-Hidroxiquinoleine 37.5% (Sulfate) w/v) was used at concentration (1 ml/L) as a control against F. fujikuroi. The inhibition percentage was measured based on the following equation:

\[
\text{Inhibition} = \frac{\text{Average radial growth in control} - \text{Average radial growth in treatment}}{\text{Average growth in control}} \times 100
\]

3. Results and Discussion
3.1. Molecular identification of the fungal pathogen

PCR amplification of DNA extracted from these fungal isolates showed the possibility of amplifying ITS-rDNA region producing amplicons with sizes ranging between 600 and 700bp using the universal ITS1-ITS4 primers (Figure 1). The PCR product (ITS1, 5.8S rDNA and ITS4) amplified from each fungal isolate was sequenced with both directions and the generated nucleotide sequences were subjected to a BLAST search. This results confirmed the morphological identification, all obtained sequences belonged to Fusarium fujikuroi. PCR test was used in this study to diagnose different isolates of Fusarium because of its high due to its high accuracy in the diagnosis of many organisms, including pathogenic and non-pathogenic fungi such as F. solani, R. solani, Alternaria alternata and Aspergillus spp. [12 ; 13 ; 14]. In previous studies, identification of some fungi such as Fusarium verticillioides and Fusarium subglutinans mainly depending on morphological characters may lead to incorrect species identification after their re-diagnosis by PCR [11]. The results also showed that number of fungi associated with pollen rot Mauginiella scaetae, Cladosporium sp. Thus, this can eliminate the diagnostic problems that associated with depending on the morphological characters only.
3.2. Pathogenicity test

Results of pathogenicity test showed that all *F. fujikuroi* isolates were highly pathogenic to radish seeds. It inhibited seed germination to 53.55, compared to seed germination of control 81 % (Table 1). It was found that multiple members if Fusarium genus produce different type of toxic compounds such as Fumosisin, Fusaric acid, Fusaproliferin, Fusarin, Zearalenone and others that assist in attack and parasite of plant hosts (5).
Tabel 1. Effect of *Fusarium fujikuroi* isolates on radish seed germination

| Isolates       | Namders of seeds | Percentage of germination |
|----------------|------------------|--------------------------|
| *Fusarium fujikuroi* | 8.66*            | 53.55                    |
| Control        | 19.00            | 81.00                    |
| L.S.D. (0.05)  | 2.9266           | 13.798                   |

*Each number in the table represents three replicates*

3.3. *In vitro evaluation of MgO and ZnO nanoparticles effect against F. fujikuroi*

Date presented (Table 1) showed that the tested nanoparticle of ZnO and MgO at the different tested concentration (1,2,4 g/L) significantly inhibited the fungal radial growth. The highest inhibition effects (74.05%) of nanoparticles tested was achieved by ZnO NPs at concentration 4 g/L. However, a complete inhibition of colony growth of *F. fujikuroi* was observed via the fungicides (Benlate) (Table 3). This in agreement with results of previous report [15] that showed Benlate has been most effective for checking the mycelial growth of *F. fujikuroi*.

Table 2. Inhibition effects of nano particles on growth of *Fusarium fujikuroi*

| Treatment       | Average rate of colony | Percentage of inhibition |
|-----------------|------------------------|--------------------------|
|                 | 1g 2g 4g  Average      | 1g 2g 4g  Average        |
| ZnO NPs         | 5.333 3.33 2.33 3.66   | 40.72 62.39 74.05 59.23  |
| MgO NPs         | 7.00 5.00 3.00 5.00    | 2.22 44.43 66.62 44.42   |
| Benlate         | 9.00 8.66 9.00 8.88    | 0.00 0.00 0.00 0.00      |
| Combined        | 3.66 2.33 1.00 2.33    | 59.20 77.73 100.00 78.9  |
| Average         | 6.25 4.83 3.83         | 3053 46.27 30.53         |
| L.S.D. (0.05)   | 1.0509 0.9101 1.822    | 12.215 10.578 21.157     |

*Each number in the table represents three replicates*

In the present study the fungicide (Benlate) and the nanoparticles of ZnO and MgO were applied against *F. fujikuroi*. Fungicides (Benlate) completely inhibit the colony growth of *F. fujikuroi*. It was also found that using nanoparticle (ZnO, MgONPs) at the concentration 4g of higher inhibited the growth of *F. fujikuroi*. The use of nanoparticles (ZnONPs, MgONPs) with fungicides preventing microbial growth and maintaining their mechanical properties. Available literature has established the fact that nanoparticles strongly inhibit fungal growth and germination. It is a proven fact that nanoparticles can permeate inside the cells of microbes at significantly smaller concentrations, thus, indicating that nanosized would be appropriate for their restriction. surface atoms, thus possessing immense antimicrobial effects in relation to bulk nanoparticles may be effect in direct in induces systemic resistant plant [16]. Available literature has established the fact that nanoparticles strongly inhibit fungal growth and germination. It is a proven fact that nanoparticles can permeate inside the cells of microbes at significantly smaller concentrations, thus, indicating that nanosized would be appropriate for their restriction. nanoparticles, which have a large surface area to volume ratio and high fraction of surface atoms, thus possessing immense antimicrobial effects in relation to. Available literature has established the fact that nanoparticles strongly inhibit fungal growth and sclerotial germination. It is a proven fact that nanoparticles can permeate inside the cells of microbes at significantly smaller concentrations, thus, indicating that nanosized silver would be appropriate for their restriction. Silver nanoparticles, which have a large surface area to volume ratio and high
fraction of surface atoms, thus possessing immense antimicrobial effects in relation to available literature has established the fact that nanoparticles strongly inhibit fungal growth and sclerotial germination. It is a proven fact that nanoparticles can permeate inside the cells of microbes at significantly smaller concentrations, [17].

4. Conclusion
In this study first molecular identification of F. fujikuroi causing pollen rot of date palm trees was achieved. Additionally, all concentrations of ZnO NPs demonstrated a high effectiveness against the pathogen particularly at concentrations 4 g/l. Although, the same concentrations of MgO NPs did not show same inhibition capability comparing with ZnO NPs, they were significantly better than in control treatment. The results suggest to possibility of application ZnO and MgO NPs particularly at concentrations 4g/l in management of root rot of date palm trees.

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