Morphological and Molecular Identification Study of Rose and Lantana Fungal Leaf Spot Pathogens

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ABSTRACT

The fungi were isolated from diseased leaves of ornamental plants such as *Rosa hybrid* and *Lantana camara*. All symptomatic leaves with leaf spot infections were collected and transported to the lab for morphological and molecular diagnosis. The isolation findings revealed that each fungus, *Alternaria alternata*, *Fusarium chlamydosporum*, and *Botrytis cinerea*, was isolated from each studied plant. The genetic study revealed the identities of each fungus as follows: a 100% identity for both *A. alternata* and *B.* cinerea, and a proportion of 99.60% similarity with F. *chlamydosporum*. The pathogenicity test results revealed that the fungus *A. alternata* had the highest level of pathogenicity in terms of disease percentage and disease severity, which were 83.34 and 64.50% on the rose plant, respectively, and 83.34 and 66.25% on the lantana plant, respectively; followed by B. cinerea with 66.67 and 41.50% on the rose plant, respectively, and F. *chlamydosporum* with 50 and 31.25% on the rose plant The current study suggests looking for acceptable and effective therapies to prevent the spread of certain forms of fungus.

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1. Introduction

Ornamental plants represent the most significant part of the cultivated plants of great importance in agricultural activities worldwide because of their economic and aesthetic value. Ornamental plants belong to vascular plants or higher species. The species that belong to ornamental plants include shrubs that may be deciduous or evergreen [1]. Rose is one of the economically critical ornamental plants because of this crop's medical and cosmetic importance. The *Rosa hybrid* is considered as one of the most famous perennial ornamental plants [2]. In addition to the rose, there is the shrub *Lantana camara*, considered as one of the most important ornamental plants from an economic and medical point of view. Ornamental plants are represented by many types of plants that show a great diversity in terms of leaves and flowers, outdoor plants that grow directly under the sun's rays and plants that grow in the shade and plants that are annual or perennial. Ornamental plants give beauty in the places where they are; from all this, it has increased in use by humans in prehistoric times, as they have benefited from it in terms of medicine and cosmetics, so it occupies an important position in the cultivation of plants in the world. Ornamental plants, including roses, are among the plants that have significant commercial and economic value. Including perfumes, oils, rose syrup, and herbal tea has a significant role in the economic field [3,4]. Ornamental plants are exposed to many diseases resulting from infection with fungi or other microorganisms, including viruses, nematodes, bacteria and phytoplasma, which cause symptoms that lead to distortion of the plant's external appearance and as a result, this negatively affects the beauty of its appearance and sometimes leads to the death of the entire plant. Among the common diseases that affect ornamental plants are leaves spots. The fungi that cause such symptoms are *Alternaria alternata*, which causes spot diseases, *Botrytis cinerea*, which known as a gray mold; and *Fusarium chlamydosporum*, which causes wilt, yellowing and spotting disease on the shoots [5,6,7]. The current study aimed to isolate and diagnose some pathogenic fungi of rose and lantana, depending on phenotypic and molecular methods and test their pathogenicity on the plants under investigation.Materials and Methods

2.1 Isolation of fungal pathogens from ornamental plants

2.1.1 Sample collection

A field survey was conducted for many of the nurseries in Basrah Governorate on 15/10/2021; it continued for two months. Leaves infected with spot diseases were obtained from ornamental plants, rose and lantana, and brought to the laboratory for isolation procedure.

2.1.2 Fungal isolation

The leaves of the ornamental plants under study infected with diseases that had previously been collected were brought to the laboratory and were cut into small pieces the size of the piece 5 mm and placed in a solution of sodium hypochlorite NaOCl at a concentration of 1% for 3 minutes for superficial sterilization and then placed in sterile distilled water for 2 minutes for disposal from the remnants of the sodium hypochlorite solution. The leaves were dried by placing them on a Whatman No.2 filter paper to dry completely. Then, about three pieces were placed in a 9 cm Petri dish containing PDA medium, and incubated in the incubator at a temperature of 2 ± 25° C. The growths were followed daily. The growing fungi were purified by taking part of the developed colony; subsequently transferred into Petri dishes containing PDA medium, and incubated at the same conditions as above to complete the growth of pure fungal isolates [8].

2.2 Phenotypic diagnosis and description of fungi isolated from rose and lantana

The phenotypic characteristics of the developing pure isolates were recorded by measuring the lengths of the colonies, in addition to recording the morphological features of the fungal growths by recording the shape of the colony edges, whether they are circular or zigzag, and then making slides from each dish. They were taken from the colony's edge and examined under a light microscope to classify and diagnose them based on the phenotypic characteristics such as the colony's shape and edge, the conidia and the conidial sporangia in addition to the colour of the fungal hyphae. The fungi were classified depending on the taxonomic sources into the approved taxonomic sources [9,10,11,12,13,14,15].

2.3 Molecular classification

The fungi that were isolated and purified from the infected leaves of ornamental and lantana plants infected with spot diseases, which were identified and diagnosed by their phenotypic and microscopic characteristics, were used to prepare them for comparison with molecular diagnostic experiments using the global molecular code for the transcribbed spacer (ITS) internal trans-regions in the polymerase chain reaction. Chain Reaction (PCR) to amplify the digested pieces of DNA [16]. Deoxyri bonucleic acid (DNA) extraction from pure fungal samples of the fungi under study was carried out using [17] method, which was modified by [18,19]. The American company Junaid supplies the purification and extraction Genomic DNA Mini Kit.

2.3.1 PCR polymerase chain reaction

The method of [20] that modified by [21], was followed to conduct a chain reaction of polymers to pieces of DNA extracted from fungal samples. The materials used in this process were collected in Tables 1 and 2 and placed in Eppendorf tubes with a volume of 200 microliters, and the total volume of materials was 25 microliters. After confirming the success of the process of replication of ITS with the genome of the fungi prepared for the study, it was sent to the South Korean company Macrogene to determine the sequences of the nitrogenous bases of the fungi under study.

Table 1: Materials used in the PCR polymerase chain reaction

Material	Quantity (microliter)
Master Mix	12.5
primers ITS1	1
primers ITS4	1
DNA extracted pieces	5
Nuclease- Free Water	5.5
The final volume of solution	25

Table 2: Program for ITS1 and ITS4 primers using PCR technology

Stage	Celsius temperature	time per minute	number of cycles
Initial Denaturation	94	5:00	1
Denaturation	94	0:30	32
Annealing step	58	0:45	
Extension	72	1:00	
Final extension	72	5:00	1

2.3.2 Molecular diagnosis of the fungi

After obtaining the complete nitrogenous base sequences of the multiplicities segment of the fungi under study using the primer ITS4 and ITS2, the above data were processed using Ltd Chromas Ver Program 2.6.6. Then a partial matching process was performed with all sequences stored in

NCBI by the Basic Local Alignment Search Tool (BLAST), and the percentage of 99% and above was adopted as the degree of similarity of the Maximum score and the degree of Query cover in determining the diagnostic identity of the fungus. A phylogenetic tree with many sequences available at the National Center for Technical Information NCBI was drawn using https://www.ebi.ac.uk./Tools/msa/clustalwz, and https://tree.bio.ed.ur/ software/figtree [22].

2.4 Pathogenicity test on rose plants and lantana

The pathogenicity of the fungi *Alternaria alternata*, *Botrytis cinerea* and *Fusarium chlamydosporum* was tested on rose and lantana, where the two plants were selected about the same age and without any disease symptoms, and they followed the method of [8]. The percentage of infection and the severity of the condition were calculated two weeks after the inoculation.

2.5 Statistical analysis

The experiments were applied according to the unifactor experiment and the multifactorial experiment according to a complete randomized design (CRD), the data analysis according to ANOVA Analysis with the application of an (LSD) Least Significant Difference to determine the significant differences between the treatments. 3-6 replicates were applied for each treatment according to the type of experiment.

2. Results and discussion

The isolation results showed many of fungi from leaves infected with spotted rose and lantana. The highest isolation frequency of fungi was found with *Alternaria alternata*, and the high rate of isolation of this genus is due to its high efficiency in producing extracellular enzymes of different types [23,24,25,26,27]. The fungus *Fusarium chlamydosporum*, isolated from the lantana shrub plant, was observed to be effective on each examined plants with leaf spot symptoms and a high frequency and appearance among the isolated fungi. This fungus infects many plant species, including the ornamental plant orchid in addition to corn, wheat and barley [12]. In addition, *Botrytis cinerea* has been isolated, and this fungus is considered as one of the fungi that infect ornamental plants in greenhouses, causing great economic losses [11].

3.1 Phenotypic and microscopic classification of *Alternaria alternata*

The fungus *Alternaria alternata* was isolated from the rose plant affected by leaf spots. The colony of pure fungus appeared in a dark gray to dark black colour to olive green, and the edges of the colony were white, while the back of the colony appeared in a dark brown colour (**Figure** 1). As for the microscopic observation, the spores appeared in the form of chains arranged from top to bottom and take the shape of a pear or be oval or conical and divided by longitudinal and transverse conidia barriers of brown or dark golden colour; in addition to the presence of the beak at the beginning of the spores. These results are in a good agreement with the results of Al-[14,15,28].

3.2 Phenotypic and microscopic classification of fungus *Botrytis cinerea*

Isolation of the fungus *Botrytis cinerea* from the leaves of the lantana plant, where this fungus is considered one of the pathogens that cause significant losses to ornamental plants, including rose [11]. The colony appears dark black (Figure 2). As for the microscopic form, the conidia appeared carried on a conidiophore of dark colour, straight, branched, and often double or triple-branched and take the form of grapes. This observation is in accordance with the results of [11,24].

3.3 Phenotypic and microscopic classification of fungus *Fusarium chlamydosporum*

Fusarium chlamydosporum was isolated from infected lantana plant leaves, grown on culture media, and then purified. The pure colony appeared in the form of cottony white with a yellow or purple colour in the old colonies, while the back of the colony seemed to be yellowish-golden as shown in **Fig.3**. Microscopic examination showed that each conidia carrier appeared short and branched, bearing individual conidia that were oval and contained conidia septums. This diagnosis was identical to that of [12].

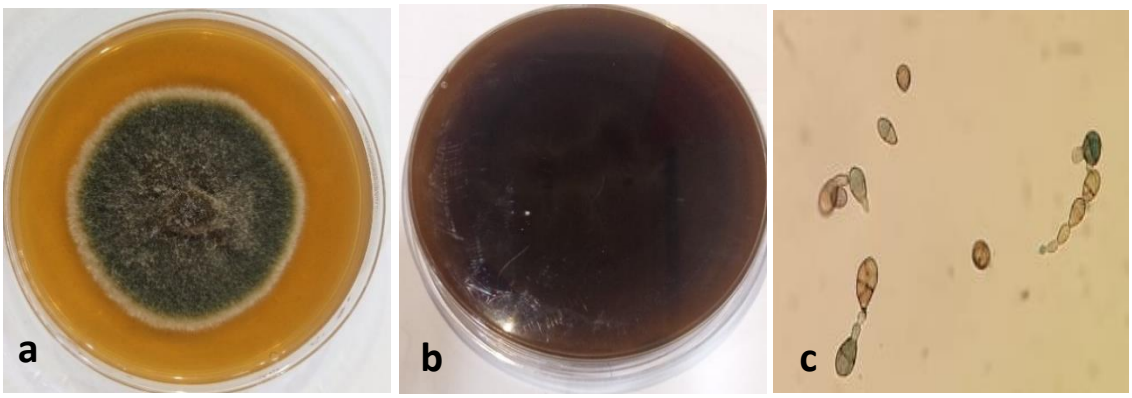

Figure (1) Phenotypic growth of the fungus on PDA medium and the microscopic shape of *Alternaria alternata* isolated from the rose plant.

[a] colony surface [b] colony reverse [c] fungus under the microscope

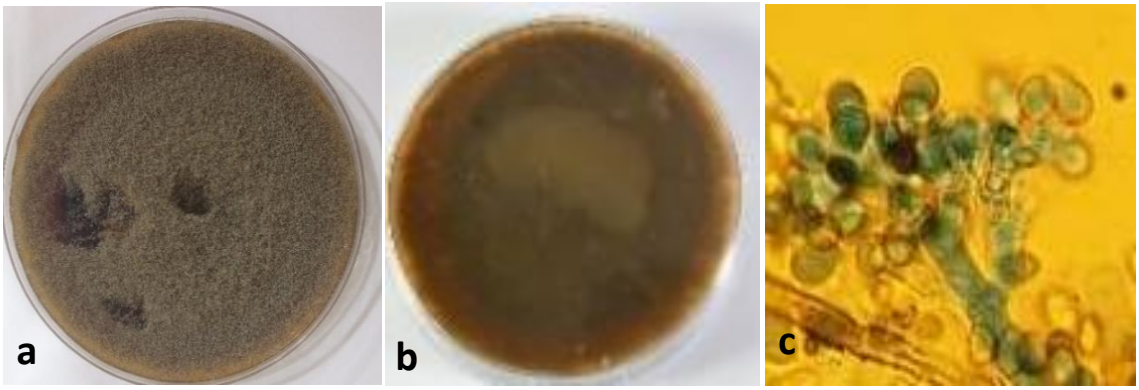

Figure 2: Phenotypic growth of the fungus on PDA medium and the microscopic shape of the isolate of the fungus *Botrytis cinerea* from the lantana plant.
[a] colony surface [b] colony reverse [c] fungus under the microscope

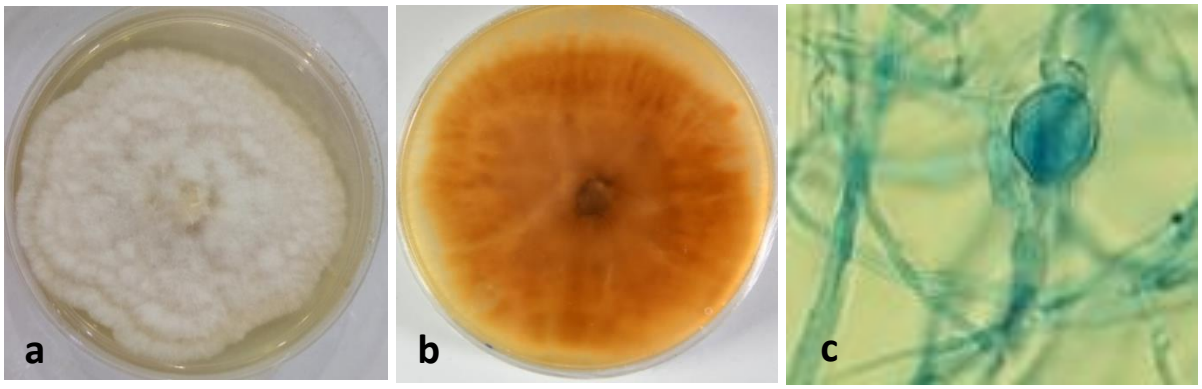

Figure 3: Phenotypic growth of the fungus on PDA medium and the microscopic shape of the isolate of the fungus *Fusarium chlamydosporum* from the lantana plant.
[a] colony surface [b] colony reverse [c] fungus under the microscope

3.4 Pathogenicity test of fungi isolated on rose plants and lantana

The results of the experiment of pathogenicity of the fungi *Alternaria alternata*, *Botrytis cinerea*, and *Fusarium chlamydosporum* conducted in a greenhouse on rose and lantana using spores suspension (1×10^6 spore/ml) for the above fungi showed the superiority of *A. alternata* in the pathogenicity events of the two plants with disease percentage and the severity of the infection. Which amounted to 83.34 and 64.50 % for the rose plant, respectively, and 83.34 and 66.25% for the lantana plant, respectively. Significant differences were recorded, P> 0.05, followed by the fungus *B. cinerea*, with a percentage of 66.67 and 41.50% as a disease percentage and severity, respectively and with a significant difference from the fungus *F. chlamydosporum*. The results did not differ among them in the lantana plant, as shown in Table 3.

Table 3: Test of the pathogenicity of fungi isolated on rose plants and lantana

Fungus	Rose		Lantana	
	Disease %	Disease severity	Disease %	Disease severity %
Alternaria alternata	83.34 a	64.50 a	83.34 a	66.25 a
Botrytis cinerea	66.67 b	41.50 b	50.00 b	30.50 b
Fusarium chlamydosporum	50.00 c	31.25 c	50.00 b	30.50 b
L.S.D.(0.05)	10.50	8.25	10.50	10.25

It is worth noting that the disease symptoms began to appear ten days after the inoculation on both plants under study, beginning with the fungus *A. alternata* followed by *B. cinerea*, while symptoms began to appear with a long time with *F. chlamydosporum*. The superiority of the pathogenic fungus *A. alternata* in causing disease and the emergence of disease symptoms on each of the plants under study (rose and lantana) is due to the action of enzymes degrading cellulose, pectin, fats lipids and others, as well as the important role of mycotoxin in the occurrence of symptoms on plants [27,30].

3.5 Molecular diagnosis of fungi isolated from plants under study and drawing of the genetic tree

Molecular diagnosis was carried out on pure culture of each fungi isolated from ornamental plants, rose and lantana, which proved highly pathogenic in pathogenicity tests experiments, as shown in the picture (4). The diagnosis process was carried out by relying on the universal

molecular code type ITS. After determining the sequences of the nitrogenous bases and matching the results using BLAST for nucleotides, a high percentage of matches was found between the species under study and some species registered in the Gen Bank. Matching results with *Alternaria* were 100% similar to *A. alternata* in the 545 base pair sequences with the sequences deposited in the gene bank with a registered gene access code Gen Bank accession Number: OW986459.1. As for the fungus, *Botrytis* under study, a 100% match rate was recorded with the fungus *B. cinerea* with the sequences recorded with the code MH370075.1 for sequences amounting to 554 base pairs, and a matching percentage of 99.60% was recorded for the fungus *Fusarium* with the type *F. chlamydosporum* with the recorded genetic access code: MK271103.1 (Figure 4, Table 4).

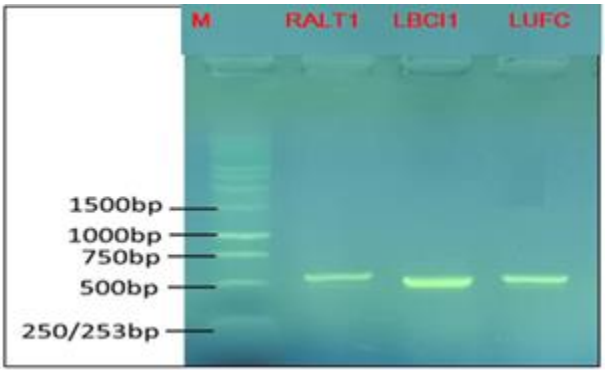

Figure 4: Results of agarose gel electrophoresis: M is the molecular weight index (100bp), and the numbered letters represent the fungi used in the current study.

Table 4: Match percentage, coverage and gene accession code of pathogenic fungi of rose and lantana plant.

No.	Isolation	Isolation code	Identity %	Query cover %	Gene accession code
1	*A. alternata*	RALT1	100	100	OW986469.1
2	*B. cinerea*	LBCI1	100	100	MT57370.1
3	*F. chlamydosporum*	LFUC	99.60	99	MH377075.1

The phylogenetic tree was drawn using the Neighbor-Joining method of genetic convergences using the nitrogenous bases of the fungi matching with the species under study and the fungi deposited in the NCBI for fungi as in Figures (5, 6 and 7). The high matching rate of *A. alternata* was similar to what was reached in several similar studies by [14,15,31], as well as what was reached by researchers [11] with the high matching of *B. cinerea* and *F. chlamydosporum* [12].

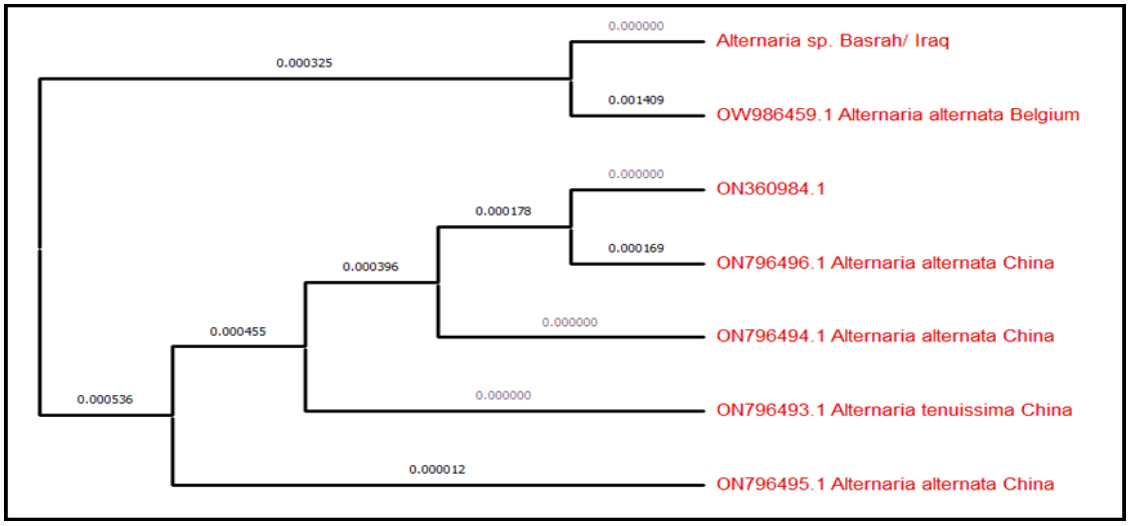

Figure 5: Neighbor-joining phylogenetic tree of the genetic similarity of *Alternaria alternata* compared to global sequences of nitrogenous bases recorded in NCBI/GenBank. https://www.ebi.ac.uk/Tools/msa/clustalw2/; http://tree.bio.ed.ac.uk/software/figtree/

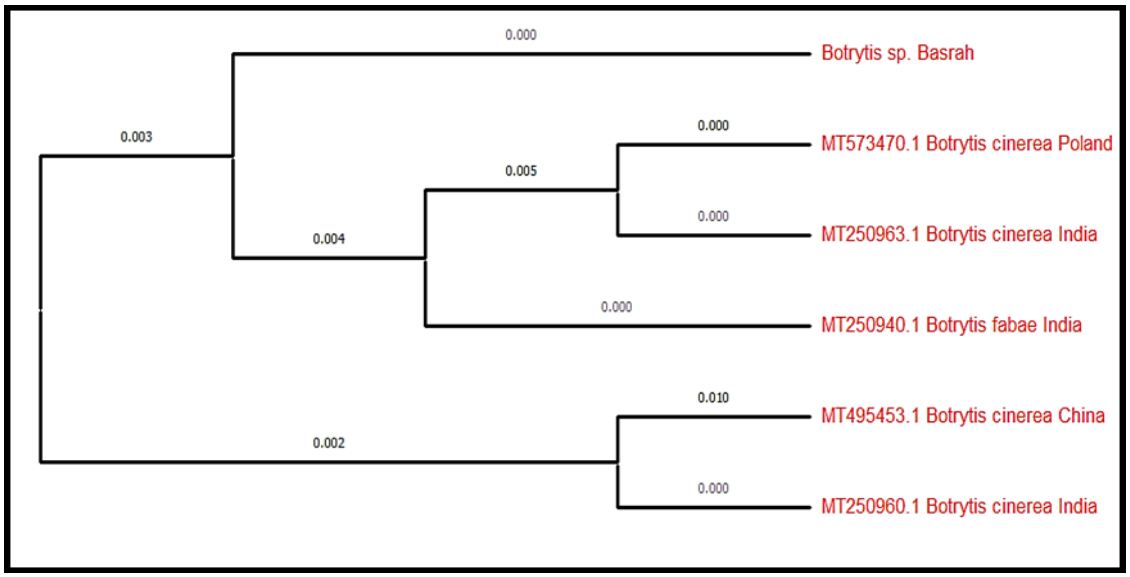

Figure 6: Neighbor-joining phylogenetic tree of the genetic similarity of *Botrytis cinerea* compared to global sequences of nitrogenous bases recorded in NCBI/GenBank. https://www.ebi.ac.uk/Tools/msa/clustalw2/ http://tree.bio.ed.ac.uk/software/figtree/

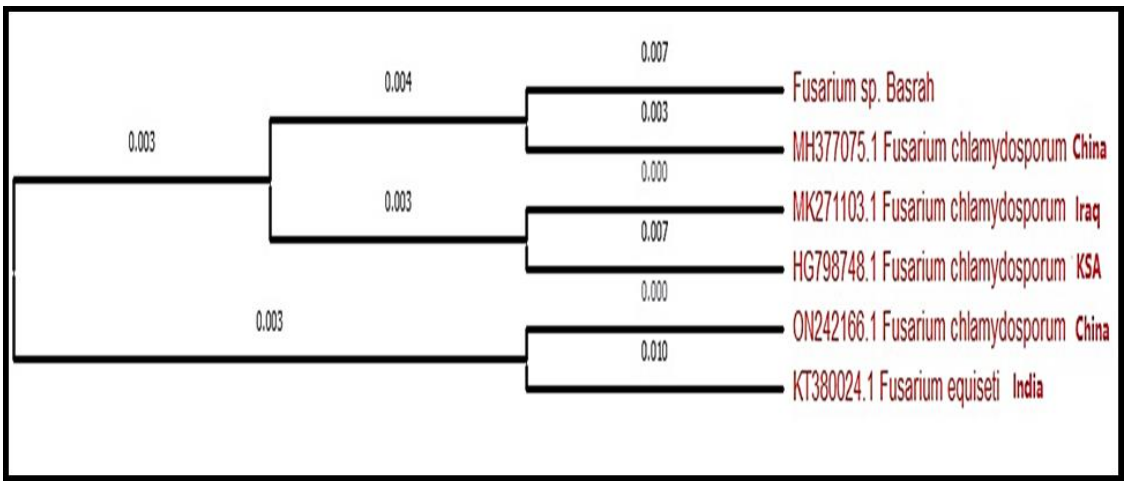

Figure 7: Neighbor-joining phylogenetic tree of the genetic similarity of *Fusarium chlamydosporum* compared to global sequences of nitrogenous bases recorded in NCBI/GenBank.https://www.ebi.ac.uk/Tools/msa/clustalw2/; http://tree.bio.ed.ac.uk/software/figtree/

3. Conclusions

In the present study; three different fungal pathogens were isolated from symptomatic leaves of rose and lantana plants as *A. alternata; B. cinerea* and *F. chlamydosporum*. Each examined fungi were identified on the bases of morphological; ,microscopic and molecular levels. High similarity of ITS sequences were observed on NCBI/ BLAST as OW986469.1; MT57370.1 and MH377075.1, respectively. Results of the pathogenicity test showed high and significant levels of effect on each examined plants; more detailed that the fungus *A. alternata* showed the highest level of pathogenicity in terms of disease percentage and disease severity which were 83.34 and 64.50% on the Rose plant, respectively, and 83.34 and 66.25% on the lantana plant, respectively; followed by *B. cinerea* whereas with *F. chlamydosporum* the percentages were 50 and 31.25% on Rose plant, respectively. The current study recommend the search of finding suitable and appropriate treatments to prevent the spread of these types of fungi.

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دراسة تصنيفية مظهرية وجزيئية لبعض ممرضات التبقع الفطرية على نباتي ورد الجوري والمينا الشجيري

حنين شاكر ياسر محمد حمزة عباس

قسم وقاية النبات/ كلية الزراعة/ جامعة البصرة/ البصرة/ العراق

المستخلص

تم عزل الفطريات المسببة لامراض تبقع الاوراق على نباتي ورد الجوري والمينا الشجيري، وتحديد الهوية الفطرية اعتماداً على المؤشرات المظهرية والجزيئية. بينت نتائج عزل الفطريات سيادة الفطريات التالية *Alternaria alternata* و *Botrytis cinerea* و *Fusarium chlamydosporum* من كلا نباتي الدراسة. أثبتت نتائج التشخيص الجزيئي المعتمدة على البادئات الجينية ITS (Internal Transcribed Spacer) الهوية الفطرية على مستوى النوع وبنسب تطابق بلغت 100% مع الفطرين *Alternaria 356alternata* و *Botrytis cinerea* ، و 99.60% مع الفطر *Fusarium chlamydosporum*. كما اثبتت نتائج اختبار الإمراضية على نبات ورد الجوري والمينا الشجيري الإمراضية العالية للفطر *A. alternata* لمؤشري النسبة المئوية للامراضية وشدة الاصابة على نباتي البحث واللتان بلغتا 83.34 و 64.50%, على نبات ورد الجوري، و 83.34 و 66.25%، على نبات المينا الشجيري، على التوالي. تلاه من ناحية التأثير الفطر *B. cinerea* بمتوسطات اصابة بلغتا 66.67 و41.50%، كنسبة اصابة وشدة اصابة على نبات ورد الجوري، على التوالي، وبفارق معنوي ($P\leq0.05$) عن الفطر *F. chlamydosporum* الذي سجل 50 و 31.25% على نبات ورد الجوري، على التوالي. وتوصي الدراسة الحالية بتركيز البحوث على ايجاد وسائل المقاومة المناسبة للحد من خطورة الممرضات على نباتات الزينة.