MORPHOLOGY, MOLECULAR IDENTIFICATION, PATHOGENICITY AND FIRST REPORT OF *FUSARIUM EQUISETI* ASSOCIATED WITH ROOT ROT AND WILT DISEASE OF *CATHARANTHUS ROSEUS* IN BAGHDAD, IRAQ

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ABSTRACT

This study was conducted in the Plant Protection Department-College of Agricultural Engineering Sciences - University of Baghdad for the period 2021-2022 with the aim of isolating and identification the pathogens that cause root rot and wilt disease on the *Catharanthus roseus* plant in different regions of Baghdad by morphological and molecular methods using PCR technique dependence on the ITS region. The results of sampling collection showed the spread of root rot and wilt disease on *C. roseus* plant in all the regions covered by the study in Baghdad (Al-Gurayat, Al-Sidiyah, Al-Khadhra, Al-Ghazaliya and Al-Dura) and the incidence rate between 15-85%.The results of isolation and identification showed the presence of 4 types of fungi associated with diseased plant: *Fusarium oxysporum*, *F. solani*, *F. equiseti* and *Rhizoctonia solani*. All the isolates were differed in their pathogenicity on the dwarf and shrub cultivars of *C. roseus*, the FeL1 isolate recorded the highest incidence & diseases severity 100 & 65% respectively both cultivars of *C. roseus*, meaning that there are no significant differences between two cultivars, also the infection intensity of FeL2 isolate was 75% in shrub cultivar, while the control treatment recorded the lowest infection rate and infection intensity reaching 0%. Also, these fungi were diagnosed based on the microscopic and molecular characteristics. The results of the molecular identification showed that the three isolates FeL1, FeL2 and FeL9 belong to the *F. equiseti* and they showed a conformity ratio of 99.60, 99.39 and 95.97 respectively when compared with the nucleotide sequences of isolates of *F. equiseti* gene bank of Brazil JQ936262, Ghana OL998428 and China GU586830 respectively. All isolates were recorded in the gene bank and the bank code was given for each isolate. Also, the results confirmed that the sequences belonging to the two isolates (FsL8 and FsL10) gave a conformity ratio of 99.62 and 99.63 respectively when compared with the sequences of KU528858 from Tunis and OM936032 from Pakistan and confirmed their identification as *F. solani*, while the results of the FoL7 isolation, its identification was confirmed to be related to the *F. oxysporum* after it showed an conformity ratio of 99.98 with the isolate from China with the number MN96002.

Key words: Ornamental plants, *Catharanthus roseus*, Medagascar Periwinkle, *Fusarium*, *Fusarium equiseti*, PCR.

التشخيص المظهري والجزيئي والامراضية واول تسجيل للفطر *Fusarium equiseti* المسبب مرض تعفن الجذور والذبول على نبات عين البزون *Catharanthus roseus* في محافظة بغداد– العراق

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المستخلص

اجريت هذه الدراسة في جامعة بغداد كلية علوم الهندسة الزراعية / قسم وقاية النبات للفترة 2021–2022 بهدف عزل وتشخيص المسببات المرضية المسبب مرض تعفن الجذور والذبول على نبات عين البزون *Catharanthus roseus* في مناطق مختلفة من بغداد مظهريا وجزيئيا باستعمال تقنية (PCR)اعتماد على منطقة ITS. أوضحت النتائج إنتشار المرض على نباتات عين البزون في جميع المناطق التي شملتها الدراسة في بغداد (الكريعات،السيدية ،الخضراء ،الغزالية والدورة) وتراوحت نسبة الإصابة بين 15–85% . بينت نتائج العزل والتشخيص وجود 4 أنواع من الفطريات المرافقة للنباتات المصابة هي *Fusarium oxysporum* و *Fusarium solani* و *Fusarium equiseti* و *Rhizoctonia solani* تباينت في الظهور بأختلاف المناطق كما وجد أن جميع العزلات مختلفة في مقدرتها الأمراضية على نباتات عين البزون للصنفي القزمي والشجيري. وسجلت العزلة FeL1 أعلى معدل لنسبة وشدة الاصابة إذ بلغت 100 و 75% على الصنفين . تم تاكيد تشخيص العزلات جزيئيا، إذ وجد أن العزلات FeL1 ، FeL2 و FeL9 تعود الى النوع *F equiseti* وقد اظهرت نسبة تطابق بلغت 99.60 ، 99.39 و 95.97 على التتابع عند مقارنتها مع التسلسلات النيوكليوتيدية المكافئة لعزلات المسبب *F. equiseti* المسترجعة من بنك الجينات . كما تم تسجيل العزلات الثمانية في بنك الجينات واعطيت الرمز البنكي (Accession No.) لكل عزلة. كما اكدت النتائج ان التتابعات العائدة للعزلتين (FsL8 وFsL10) اعطت نسبة تطابق بلغت 99.62 و 99.63على التتابع عند مقارنتها مع مع تسلسلات العزلات المسترجعة من بنك الجينات من تونس KU528858 و OM936032 من باكستان واكد تشخيصها على انها تعود للفطر *F.solani*، , العزلة FoL7 فقد تم تاكيد تشخيصها انها تعود الى الفطر *F.oxysporum* بعد ان اظهرت نسبة تطابق بلغت 99.98 مع العزلة من الصين ذات الرقم (MN96002) ،

الكلمات المفتاحية : عين البزون, Medagascar Periwinkl , *Catharanthus roseus* ، *Fusarium* ، *Fusarium equiseti* ، PCR

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INTRODUCTION

Catharanthus roseus L. is one of the ornamental plants belonging to the family Apocynaceae. One of the common names of this plant is Medagascar Periwinkle in relation to its original region on the African island of Madagascar, from which it spread to different parts of the world, especially the tropical and semi-tropical areas (32). It is a perennial herbaceous plant, and it is usually planted in Iraq in gardens for ornamental purposes because it has a high coordinating value due to its multiplicity of forms (9). The economic importance of this plant has increased in recent years after the identification of its active ingredients (22). Research has proven that the *C. roseus* L. plant is medically famous as a source of the production of more than 130 alkaloid compounds from Terpenoid Indole Alkaliods of medical and pharmaceutical importance (7; 31). *C. roseus* L. plant is exposed to a number of pathogens that limit its production as it is considered a host for a large number of pathogenic fungi (19;23), and that the majority of the fungal diseases recorded on this plant are due to sudden wilt and root rot diseases caused by many pathogenic fungi: *Rhizoctonia solani*, *Fusarium oxysporum, F. solani*, *Thielaviopsis* sp., *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *Pythium aphanidermatum* and leaf blight caused by the *Phytophthora parasitica, P. tropicalis, Colletotrichum dematium* and *Botrytis cinerea* fungi (26*;* 28) . (5) was able to distinguish between 47 isolates of the *F. oxysporum* f. sp. *lycopersici* fungus causing fusarium wilt disease on tomato using PCR technique. (6) succeeded by using PCR technology in diagnosing 12 isolates of *F. solani* fungus that causes rotting of the roots and stems of broad bean in the agricultural fields of Babylon Governorate. (21) recorded *F. equiseti* for the first time as a cause of cauliflower wilt when isolated from plants that showed symptoms of infection such as leaf wilting, stem browning, root rot, and then plant wilt and death after its morphological and molecular diagnosis. In Iraq, (8) showed the molecular characteristics of the *F. equiseti* fungus that causes soybean seedling death disease by using PCR technology. In India, (15) studied the molecular characteristics of the *F. equiseti* pathogen that causes sudden wilt, which was recorded for the first time on hot peppers using PCR technology using the interfacial transcriptome of the ITS1-5.8S-ITS2 gene. Recently, it has been observed the emergence of disease symptoms on the *C. roseus* L. plant in some nurseries as well as home gardens, represented by the sudden and rapid wilting of the vegetative growth of seedlings and plants at the flowering stage, and the rotting of the root system of the plant.

Due to the economic importance of the plant as an ornamental and as a medicinal plant, the study aimed to:

1. Investigation of infection with the disease in some nurseries in Baghdad governorate.
2. Isolation of the pathogen and its morphological and molecular identification.

MATERIALS AND METHODS

Sample collection: Samples of infected *C. roseus* L. plant, which showed symptoms of wilting and root rot, represented by pallor of lower leaves, yellowing and drooping of large leaves as a result of curvature of the petioles of leaves and completely plants wilting, were collected from a number of important nurseries and some home gardens in Baghdad governorate (Al-Gurayat, Al-Sidiyah, Al-Khadhra, Al-Ghazaliya and Al-Dura) in the season of 2021, then placed in polyethylene bags, and the date of sampling and collection site were recorded. As for the seeds, they were obtained from commercial sales offices of two cultivars (shrub and dwarf). The samples were brought to the Plant Diseases Laboratory of the Plant Protection Department - College of Agricultural Engineering Sciences - University of Baghdad to isolation and identification.

Table 1. The location of soil and infected *C. roseus* L. plants sampling

Sampling No.	Location
1	Al-Gurayat
2	Al-Sidiyah
3	Al-Khadhra
4	Al-Dura
5	Al-Ghazaliya

Isolation and identification

Infected plants were washed with water, roots and stems were cut into pieces of 0.5-1 cm in length, then superficially sterilized with sodium hypochlorite solution (1% free chlorine) for 2 minutes.The pieces were transferred to 9 cm diameter Petri dishes

containing sterile PDA (Potato Dextrose Agar) mediaby autoclaveat a 121°C and at a pressure of 5.1 Kg cm^{-2} for 15 minutes, then cooled the nutrient medium and the tetracycline antibiotic was addedat a concentration of 200 mgL^{-1} to prevent bacterial growth.The dishes were grown as 4 plant pieces per dish and incubated in the incubator at a 25 ± 2 °C, and the growth of fungi was monitored for each dish after 3 days. The companion fungi to the *C. roseus* L. seeds were also isolated, as 200 seeds were taken from the seeds of the two cultivars, 100 seeds of the shrub cultivar (local cultivar) and 100 seeds of the dwarf cultivar (imported cultivar), and superficially sterilized by soaking in 1% of sodium hypochlorite solution for 2 minutes, then washed with sterile water and soaked in sterile water for 30 minutes, to facilitate the germination, after which the seeds were dried on sterile filter paper and transferred by sterile forceps to petri dishes with a diameter of 9 cm containing a nutrient medium (PDA) by 4 seeds per petri dish. It was also isolated from the soil taken from the area surrounding the roots of the affected plants using the leaves of the rose (white) as plant traps. The petal leaves of the flower were washed with running water, then superficially sterilized by soaking in 1% of sodium hypochlorite solution for 2 minutes, then washed with sterile water, and placed in the Petri dishes with a diameter of 9 mm and poured on it from the previously prepared soil solution of 10 g of soil 100 ml^{-1} water. This process was carried out inside the isolation room to ensure that no external contamination occurred; the dishes were tightly closed, and placed in the incubator at a 25 ± 2 °C. After 4-7 days a part of the edge of the paper that showed symptoms of infection was taken and the affected part was transferred to the middle of a petri dish containing a pre-prepared PDA nutrient medium, placed in the incubator and the dishes growth was monitored after 3 days. After the appearance of fungal colonies around the infected parts, the fungus was purified by taking part of the edge of the colony and replanted in other dishes and incubated for 5 days. Microscopic examination was carried out to determine the types of the companion fungi, and then the common genera was identified under the micro-power of the compound microscope based on the spores and sexual and asexual structures formed by the fungus.Then the fungi were morphologically identified to the level of genus and specie by Assist. Prof. Dr. Bushra Suber Abdul-Sada Al-Maliki using the approved taxonomic keys (12;20;25; 29). The percentage of the appearance and frequency of the fungus in the samples was calculated according to the following equation: Frequency of fungus in the sample = (Number of plant pieces infected with the fungus/ the total number of pieces used for each sample) x 100.

Purification and preservation of fungi isolates: Six isolates of pathogenic fungi, three isolates of *F. equiseti*, one isolate of *F. oxysporum*, and two isolates of *F. solani*, were purified using single spore technique to obtain pure and homogeneous cultures of the fungus isolates, by adding 3-4 drops of sterile distilled water to the fungus colony growing on the PDA nutrient medium, then a portion of the fungus suspension was taken by the previously sterilized flame needle and a simple outline was made on the surface of the 2% water agar in Petri dishes with a diameter of 9 cm and the dishes were incubated at a 25 ±2 °C for 24 hours, then the germinating macro-conidia spores were individually transferred with the help of light microscopy to the PDA nutrient medium in Petri dishes in the isolation chamber and the dishes were incubated at 25 ±2 °C for 5 days until the growth and sprouting of the fungus (27). The purified isolates were kept in test tubes containing sterilized loam soil by autoclave at a 121 °C and a pressure of 5.1 Kg cm^{-2} for an hour twice in two consecutive days. The sterilized soil was polluted by adding 3 pieces of 0.5 cm diameter taken from near the edges of the colonies of fungus isolates aged 5-7 days grown on PDA culture medium at three replicates for each isolate. The test tubes were placed in the incubator at a 25 ± 2° C for 15 days, after which they were placed in a refrigerator at a 4° C until performing the subsequent tests.

Fungi identification: Morphological identification: After purification of 6 isolates of pathogenic fungi, they were morphologically identified to the level of genus and species using the single spore

method after the appearance of the fungal growths, depending on the shape of the conidia spore, conidial carrier and structures they formed using the taxonomic key (29) by Assist. Prof. Dr. Bushra Suber Abdul-Sada Al-Maliki / Plant Protection Department / College of Agricultural Engineering Sciences / University of Baghdad by preparing an examination slide on which two pieces of the nutrient medium (PDA) were placed in the middle and after its solidification, a prick was taken by the needle from the edge of the pathogenic fungus dish and spread it in perpendicular lines for the purpose of spreading the fungus spores on the PDA, and the slides were placed in the closed petri dish and incubated at a 25 ± 2 ° C. After 24 hours, it was identified under a light microscope at a magnification of 40 X, then it was kept in test tubes containing a sterile PDA culture medium supplemented with the Tetracycline antibiotic at a rate of 100 mgL^{-1} to prevent bacterial growth, then the tubes were placed at an angle until solidification, and then the tubes were inoculated by adding a 0.5 cm diameter disc taken from the edges of the colony at the age of 5 days. The tubes were placed in the incubator at a 25 ± 2 °C for 5 days, after which it was kept in the refrigerator at a 4°C until use in subsequent experiments.

Molecular identification of isolated *Fusarium:* Molecular identificationwas carried out to confirm the morphological identification of pathogenic fungi isolates, which gave the highest infection rate and infection severity in the pathogenicity experiment, and single spore method was used to obtain a pure and genetically homogeneous culture of pathogenic fungi isolates. This study was carried out in the laboratory of the Musayyib Bridge Company - Baghdad - Iraq. The process of extracting and purifying DNA from conidia spores and fungal mycelium was carried out using a standard kit (ZR Fungal/Bacterial DNA MiniPrep™) produced by ZYMO-USA Company, which including ZR Bashing Bead™Lysis Tubes (0.1 & 0.5 mm)[1]No. 50, 40 ml Lysis Solution, 100 ml Fungal DNA Binding Buffer[2], 15 ml DNA Pre-Wash Buffer, 50 ml Fungal DNA Wash Buffer, 10ml DNA Elution Buffer, Zymo-spin™ IV Spin Filters (Orange Tops) No. 50, Zymo-Spin™ IIC Columns No. 50 and Collection Tubes, No. 150. The fungal mycelium was taken and placed in an Eppendorf Vials tube, 750 µl of Lysis's solution was added to it and the extraction process was completed according to the manual supplied by the company. To perform the PER amplification, the reaction mixture was prepared with a final volume of 25 µl for each sample. ITS1 Primer (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 Primer (5′-TCCTCCGCTTATTGATATGC-3′) were used. The PCR reaction mixture contained 1 µl of fugus DNA extracted, 2.0 µl primers (1.0 µlof ITS1 primer and 0.1 µl ITS4 primer), µl5.0 of Taq PCR premix mixed with $MgCl_2$ were all placed in 16.5 µl of ddH_2O for the PCR reaction. The tubes were placed in the MULTIGENE OptiMax Gradient Thermal Cycle-USA and follow the following program:

95℃ for 3 min followed by 35 cycles of 94℃ for 1 min,

55℃ for 1min and 72℃ for 1min, followed by a 72℃ cycle for 5min

Then the results of the PCR reaction were tested using the electrophoresis technique on 1.8% agarose gel using T.B.E, then the agarose gel was soaked in T.B.E. The relay was carried out for 30 minutes and 80 volts with a UV-trans illuminator under ultraviolet rays, after which the PCR reaction products were purified and sent to the Korean Macrogen Corporation. The SConES were read using a Sanger Sequencing ABI3730XL device at two directions using the ITS1 and ITS4 primers, then the SConES were submitted to the WebSite NCBI GenBank (http://www.ncbi.nlm.nih.gov), and the SConES were compared in the Genbank database using BLAST software

Pathogenicity capability test on *C. roseus* plant in pots: All isolates were prepared by growing them on seeds of *Panicum miliaceum* after washing and sterilizing in a flasksby autoclave at a 121°C and a pressure of 1.5 $Kgcm^{-2}$ for 15 minutes for two consecutive times, then the flasks were inoculated with separately five discs with a diameter of 0.5 cm taken from the edge of the fungal cultures on the PDA culture medium at the age of 7 days, and then the flasks were incubated at a 2 ± 25 ° C for two weeks with shaking to distribute

the inoculum to all seeds. After that the loam soil and pitmoss were sterilized at a of 2:1 (w: :w) ratio by autoclave and re-sterilized twice, then the soil was distributed in plastic pots of 1 Kg capacity, and the inoculum of each of the isolates was added to the potting soil at a ratio of 2% (weight : weight) and each treatment was replicated four times with the control treatment (only sterilized seeds were added), then the pots were covered with polyethylene bags and the bags were punctured for ventilation, and after 3 days, two cultivars (Shrub and Dwarf) of *C. roseus* were planted.The infection rate was calculated after 30 days of planting and the infection severity was calculated according to the following scale:

0 = Healthy plant

1 = 25% of the roots are infected (brown pollution)

2 = 50% of the roots are infected (50% pollution without infecting the crown area)

3 = 75% of the roots are infected (75% pollution with infection of the crown area)

4 = 100% of the roots are infected (dead plant)=

The infection severity was calculated according to Mickenny's equation (1923).

RESULTS AND DISCUSSION

Isolation and identification: The results of sample collection in the Table (2) showed the spread of wilt disease and root rot on *C. roseus* plants in all regions which samples were collected of 2021-2022 season in Baghdad Governorate (Al-Gurayat, Al-Sidiyah, Al-Khadhra, Al-Ghazaliya and Al-Dura) and the infection rate ranged between 15-85%, the Al-Gurayat nurseries recorded a highest infection rate (85%), followed by Al-Sidiyah nurseries (65%), then Al-Khadhra nurseries (40%) while Al-Dura and Al-Ghazaliya recorded the lowest infection rate (15 and 25%) respectively. These rates of infection cannot be ignored, as they caused great economic losses to the owners of the nurseries and greatly reduced the number of *C. roseus* plants. Also, the spread of the disease led to the reluctance of some nursery owners to grow this plant (personal communication). The reason of the widespread of root rot and wilt disease in the nurseries of the Al-Gurayat, Al-Sidiya and Al-Khadhra regions may be attributed to the repeated cultivation in the same nurseries in the spring and autumn season, which led to the accumulation of a number of pathogenic fungi pollen, as these regions specialize in the cultivation of *C. roseus*, in addition to their use of large amounts of organic fertilizers.Also, the sensitivity of the cultivated cultivars may have a role in the spread of the disease in the Al-Ghazaliya and Al-Dura regions, which may be due to the fact that these nurseries are newly established, in addition, it is not excluded that the use of chemical pesticides has a role in the varying rates of infection between the nurseries that grown the *C. roseus* plants.

Table 2. the infection rate in some nurseries of Baghdad regions for the season of 2021-2022

Region	Infection rate (%)
Al-Gurayat	85
Al-Sidiyah	65
Al-Khadhra	40
Al-Dura	15
Al-Ghazaliya	25

The results of isolation and identification of the pathogen of the infected plants collected from different nurseries in Baghdad governorate showed different types of fungi (Table 3), the most frequent species in most samples were the species belonging to the *Fusarium* genus such as *F. solani*, *F. equiset*, *F. oxysporum* at a percentages that reached 35, 22.75 and 20.13 respectively. Also, the isolation results showed the presence of other fungi such as *Pythium* sp., *Rhizoctonia solani*, *Rhizoctonia* sp., *Aspergillus* sp. and *Penicillium* sp. at a frequency of 20.09. These results are in agreement with (23); (30) as a number of genera of fungi that cause root and wilt diseases were isolated from a number of ornamental plants, including the *Fusarium* genus. Also, These results are in agreement with (11), where the *F. solani* fungus was isolated from the roots of *C. roseus* plants infected with root rot from the northern regions of Malaysia. In addition to, the results of this study are in agreement with another study that showed that the *R. solani* and *F. oxysporum* fungi were isolated from a number of nurseries in Karbala and Babylon governorates (18). These fungi were identified based on morphological and microscopic characteristics, as the species belonging to the

Fusarium genus formed Macroconidia and Microconidia, as well as the length of the conidia carrier and the cell forming it Phialides. Also, microscopic examination showed that the fungal mycelium is divided. The small conidia of *F. solani* consist of one or two cells oval or spherical in shape, and the larger spores are of crescent shape. Chlamydospores are also rough-walled spherical in shape (12; 20). As for the *F. oxysporum* fungus, it forms colonies of different colors on the PDA medium, beginning from white cotton or transparent white, as well as may appear in pink or purple.The colonies of the *F. equiset* fungus (Fig.1) were also distinguished by the fact that the colonies of this type didn't give any color or pigments on the media cultureand were white in color, the conidia carriers are short and the large conidia divided from 2-5 cells. The apical cell is pointed, elongated and transparent, the foot cell is prominent and distinct and the chlamydial spores are translucent and spherical produced at the end of fungal mycelium (15).

Table 3. most important species of fungi that isolated from the roots of *C. roseus* plants

Fungus	No. of Isolation	Frequency
Fusarium solani	**10**	**35**
Fusarium equiseti	**9**	**22.75**
Fusarium oxysporum	**7**	**20.13**
Other fungi	**-**	**2.09**

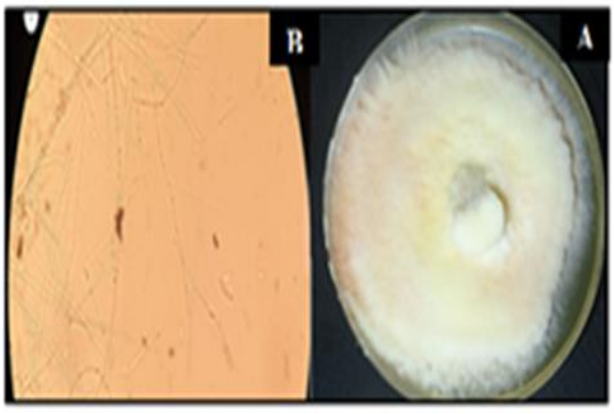

Figure 1. Morphological characteristics of the *Fusarium equiseti* fungus
A- *Fusarium equiseti* colonies on PDA media culture
B- Colonies of *F. equiseti* under the microscope at a magnification of 40X

Pathogenicity capability test on *C. roseus* plant in pots for the most frequent causative agent: The results of testing the pathogenicitycapability of four isolates of the most frequently studied fungi showed that the isolates of fungi differed in the pathogenicitycapability on the*C. roseus* plants for both cultivars (dwarf and shrub), and the differences were highly significant in the rate and severity of infection, (Table 4 and Fig. 2), the FeL1 isolate recorded ahighest infection rate reached 100% for both cultivars (dwarf and shrub) of *C. roseus* plants respectively, and this isolate FeL1 also recorded a highest infection severity reaching 75% for both cultivars (dwarf and shrub) of *C. roseus* plants respectively, i.e. that there are non-significant differences between cultivars. However, the severity of infection in the shrub cultivar was equal in this isolate with that of isolate FeL2 and (75%), while in the control treatment, the infection rate and the infection severity was 0%.The reason of pathogenicity capability may be attributed to genetic differences between fungal isolates collected from different geographical regions (3), or may be due to variation in the amounts of degrading enzymes and secreted toxins (1).Also, It is noted that the FsL8, FeL2 and FoL7 isolates on *C. roseus* plants of the shrub cultivar recorded the highest infection rate (100, 80 and 60%) respectively and infection severity (65, 75 and 52.5%) respectively, while the dwarf cultivar of *C. roseus* plants gave the lowest infection rate (90, 70 and 50%) respectively and infection severity (65, 50 and 40%) respectively. These differences in the degree of sensitivity or resistance of varieties to infection with fungi or microorganisms in general may be due to the difference in the composition of plant cell walls in their containment of active ingredient in stimulating plant defenses, such as containing different percentages of cellulose, callose, lignin and subrin, which play an important role in preventing or reducing the penetration of plant cells from the microorganisms, so as to form cell walls rich in callus that aggregate at the sites of contact with the attacking fungal mycelium, and the rapid aggregate of large chemical molecules, including B-1,3-glucans and lignin-like compounds in the outer shell

that isn't endemic to the pathogenic fungus, and thus it works As secondary mechanical barriers to prevent the penetration of the fungus and fill the spaces between most epidermal cells with phenolic compounds. Also, plants differ in their ability to produce many important enzymes in plant defenses at the sites of entry of the fungus into the epidermis and cortex and collect metabolites in the outer tissues to prevent the fungus from advancing towards the woody vessels and produce Peroxidase, Polyphenol oxidase enzymes and Phenols, which are involved in a number of important physiological and biochemical processes in the plant (4).

Table 4. pathogenicity capability test of the most frequent causative agent on *C. roseus* plant in pots

Treatment	Dwarf cultivar				Shrub cultivar			
	Infection rate		Infection severity		Infection rate		Infection severity	
FoL7	50	d	40	e	60	C	52.5	c
FeL2	70	c	50	d	80	B	75	a
FsL8	90	b	65	c	100	A	65	b
FeL1	100	a	75	b	100	A	75	a
Control	0	e	0	f	0	D	0	d
LSD	8.89		7.26		8.12		9.01	

Figure 2. Testing the pathogenicity of the most frequent causative agent according to the severity of the infection on the *C. roseus* plant

Molecular identification of the *Fusarium* genus isolates using the polymerase chain reaction (PCR) technique

Electrophoresis" The results of electrophoresis on agarose gel of 1.5% of the polymerase reaction products showed the appearance of DNA bound at a weight ranging between 516-588bp from six isolates (Fig. 3). All isolates were recorded in the gene bank and Accession number was given for all isolates (Table 5).

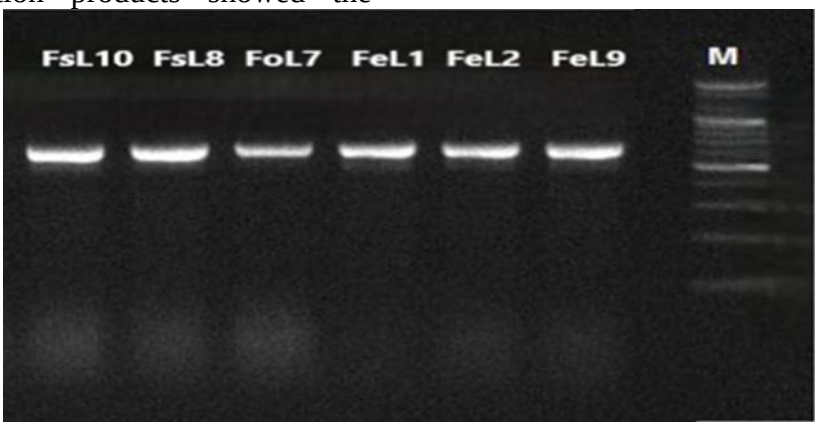

Figure 3. Electrophoresis products on agarose gel for DNA segments with molecular weight 516 to 580 bp (the symbols above the figure represent isolates belonging to the *Fusarium* spp. Genus)

Table 5. bank codes of Iraqi isolates obtained after depositing them in the gene bank

Isolate name	Accession number	Molecular weight (bp)
FoL7	OP349107	516
FsL8	OP349105	539
FsL10	OP349106	537
FeL1	OP349102	518
FeL2	OP349103	522
FeL9	OP349104	521

Nucleotide sequence analysis of the pathogen isolates of the genus *Fusarium* spp: The results of the nucleotide sequence analysis of the pathogens that proved their pathogenicity confirmed that they are three species belonging to the genus *Fusarium* spp. Table (6). The results showed that the sequences of isolates (FeL1, FeL2 and FeL9) that were doubled are isolates of type *F. equiseti* after it showed a nucleotide match ratio of 99.60, 99.39 and 95.97 respectively with the intergenic region located between the 18s ribosomal RNA gene and 28s ribosomal RNA gene when compared with the equivalent nucleotide sequences retrieved from the GenBank from (Brazil JQ936262, Ghana OL58). The results also confirmed that the sequences belonging to the two isolates (FsL8 and FsL10) gave a matching percentage of 99.62 and 99.63 respectively when compared with the sequences of isolates retrieved from the GenBank KU528858 from Tunis and OM936032 from Pakistan and confirmed their diagnosis as belonging to the fungus *F. solani*, while the results of the isolation FoL7, its diagnosis was confirmed to be due to the fungus *F. oxysporum* after it showed a match percentage of 99.98 with the isolate from China with number MN96002.

Table 6. Match ratios of the nucleotide sequences of isolates of the genus *Fusarium* spp and their counterparts in the genebank

Specie	Match ratios	Country	Bank codes for identical isolates in the genebank	Iraqi isolates
Fusarium oxysporum	99.98	China	MN96002	FoL7
Fusarium solani	99.62	Tunis	KU528858	FsL8
Fusarium solani	99.63	Pakistan	OM936032	FsL10
Fusarium equiseti	99.60	Brazil	JQ936262	FeL1
Fusarium equiseti	99.39	Ghana	OL998428	FeL2
Fusarium equiseti	95.97	China	GU528858	FeL9

Phylogenetic tree from type neighbor joining (Fig. 2) and Table (7) included species of the genus *Fusarium* spp, which were built from molecular nucleotide sequences of the pathogens isolated from the three isolates infected with the three isolates (FeL1, FeL2 and FeL9). With its counterparts (OM372809, ON495951, JQ936262 and OL998428) from China, Brazil and Ghana, respectively, retrieved from the GenBank and belonging to the pathogen *F. equiseti*, as they joined in one main and separate group from the branch of *F. solani* and *F. oxysporum* retrieved from the GenBank. The genes that were included with the nucleotide analyzes as well as separate from the type *Colletotricum acutatum* that was put as a comparison (Out Group), But the Iraqi isolates were different from the isolates within the main group, as isolate FeL1 lined up alone between the isolates of Brazil and China, while the two isolates Fel2 and FeL9 joined within one branch sub-branch within the main branch. As for the nucleotide sequences of the isolates (FeL8 and FeL10), they lined up in one group with their counterparts retrieved from the gene bank and belonging to the causative agent *F. solani* from Pakistan (OM372809 and MN960003) sequentially in one group within a major branch, but the Iraqi isolates joined in one branch with each other . The results also showed that the isolate FoL7 of *F. oxysporum* joined with isolate MN960002 from China in one branch and was also separate from the control fungus recovered from the GenBank. The Iraqi isolates also showed clear genetic differences between them, and they were also completely different from the other isolates retrieved from the GenBank. From this it is clear that there is a heterogeneity between the isolates of the causative *F. equiseti*. This difference may be due to geographical variations in their source, as well as the different isolates among themselves in their ability Pathogenicity this is the first record of

F. equiseti in Iraq and in the world on *Catharanthus roseus* plant. Also, the deposit of nucleotide sequences for 6 isolates of the genus *Fusarium* (3 isolates of *F. equiseti*, 2 isolates of *F. solani* and one isolate of *F. oxysporum*) is the first deposit of isolates An Iraqi isolated from infected *Catharanthus roseus* plants in a GenBank.

Table 7. the bank code and number of nitrogenous bases for the types and isolates that were used in the analysis and drawing the genome tree

Specie	Bank code	Country	Number of nitrogenous bases	Reference
F. oxysporum	**MG136705**	**Pakistan**	**545**	**Naqvi et al., 2017**
F. oxysporum	**MN960002**	**China**	**516**	**Uwaremwe, 2020**
F. solani	**MN960003**	**Pakistan**	**539**	**Uwaremwe, 2020**
F. solani	**OM936032**	**Pakistan**	**541**	**Waqas and Jamal, 2022**
F. equiseti	**AB470890**	**China**	**546**	**Tian, 2009**
F. equiseti	**JO936262**	**Brazil**	**525**	**Leiteey al., 2016**
F. equiseti	**ON495951**	**China**	**513**	**Li, 2022**
F. equiseti	**OL998428**	**Ghana**	**499**	**Appiah-Kubi, 2021**
Colletotricum acutatum	**JQ958570**	**China**	**545**	**Deng et al., 2013**

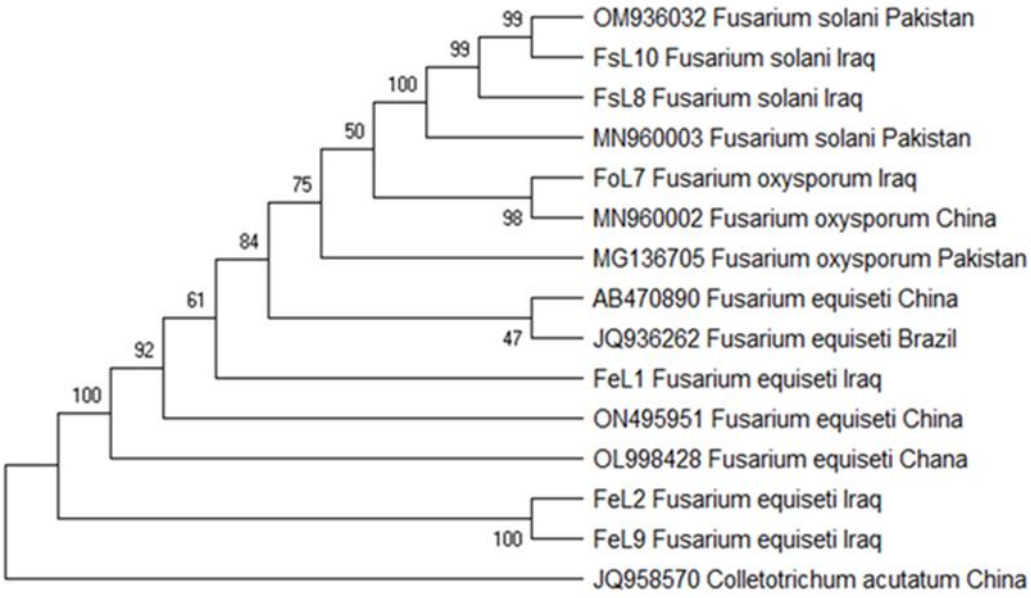

Figure 2. Phylogenetic tree of Iraqi isolates from *Fusarium* genus

CONCLUSIONS

The causes of sudden wilt and root rot on *C. roseus* are *F. oxsysporum*, *F. solani*, *F. equiseti* which appeared at high frequency ratios and the most frequent of which was *F. equiseti*. Also, the results of the molecular identification by PCR technique for the *F. solani*, *F. oxsysporum*, *F. equiseti* fungi is identical to the microscopic description and to the global isolates, and the *F. equiseti* fungus on the *C. roseus* plant is the first recorded in Iraq and global.

REFERENCES

1. Aboud, H. M., S. A. Said, H.N. Saleh and M.M. Dewan. 2001. studies for isolate of *Thielaviopsis paradoxa*. the scientific Journal of Iraq Atomic Energy Commission, 3: 150-155. http://dx.doi.org/10.21931/RB/xxx

2. Ahmad, Y.; Hmeed, A. and Ghaffar, A. 2006. Enzymatic activity of fungal pathogens in corn. Pak. J. Bot., 38(4): 1305-1316. https://www.pakbs.org/pjbot/PDFs/38(4)/PJB38(4)1305.pdf

3. Al-Baldawi, A. A.; M. H. Al-Hashimi and N. N. Askar. 1983. Capability of different isolates of *Rhizoctonia solani* Kuhn to infecting different plant families. Crop Protection Research Yearbook. 3(2):263-274.

4. Al-Hugazy, A. F. M. 2018. Diagnostic and Ecological Study of the *Fusarium oxysporum* f. sp. Cucumerinum Fungus That Causes Wilt Disease in Cucumber Plants and Methods of Controlling It under Greenhouse Conditions. PhD.Dissertation, College of Agricultural Engineering Sciences, University of Baghdad. pp. 147.

5. Al-Humairi, Y. N. H. 2013. Integration in the Control of Tomato Fusarium Wilt Disease Caused by *Fusarium oxysporum* f. sp. Lycopersici Fungus and Its Strains Diagnosis.PhD.Dissertation, College of Agriculture, University of Baghdad.pp. 142.

6. Al-Kaif, M. A. I. 2015. Isolation and Identification of the Causes of Rotting Roots and Stems Bases of Broad Bean in Babylon Governorate and Their Resistance to Some Biological Factors and Plant Extracts. M.Sc Thesis, Al-Furat Al-Awsat University - Technical College / Al-Musayyib. pp.166.

7. Al-Maamari, A. M. S., B. Z. K. Bashi and I. G. Al-Daoudi. 2018. Determination of some alkaloids in the callus of *Catharanthus roseus* L. plant in vivo. Syrian J. of Agric. Res. 5 (3):21-36. https://agri-research-journal.net/SjarEn/?p=1521

8. Al-Mayahi, S. H. A. 2020. Isolation and Identification of the Fungus Causing the Soybean (*Glycine max* L. Merrill)Seedlings Death Disease and Induction of Its Resistance. MSc. Thesis, College of Agricultural Engineering Sciences. University of Baghdad.pp. 123.

9. Al-Zargani, N. J. K. 2003. Anatomical Study of Some Species of Apocynaceae Family. M.Sc. Thesis, College of Science, University of Kufa.

10. Anand, T., R. Bhaskaran, T. Raguchander, G. Karthi-keyan; M. Rajesh and G. Senthilraja. 2008. Production of cell wall degrading enzymes and toxins by *Colletotrichum capsici* and *Alternaria alternate* causing fruit rot of Chillies. J. Plan. Protec. Res. 48: 437-451. DOI: 10.2478/v10045-008-0053-2

11. Ayob, F. and W., K. Simarani. 2016. Endophytic filamentous fungi from a *Catharanthus roseus*: Identification and its hydrolytic enzymes. Saudi Pharmaceutical Journal. 24, 273–278. https://doi.org/10.1016/j.jsps.2016.04.019

12. Booth, C. 1977. Fusarium laboratory guide to the identification of the major species.Commonwealth Mycological Institute Kew, Surrey, England. pp:58.

13. Carling, D. E., R. E. Baird, R. D. Gitaitis, K. A. Brainard and S. Kuninaga. 2002. Characterization of AG-13, a newly Reported anastomosis group of *Rhizoctonia solani*. Phytopathology. 92:893-899. doi: 10.1094/PHYTO.2002.92.8.893.

14. Gupta, P. M. R., P. Sharma., P. K. Rai., H. S. Meena and S. K. Singh. 2019. Isolation and characterization of a new fungal species, *Fusarium equiseti* (Corda) Sacc., from Brassica juncea in Bharatpur. Journal of Oilseed Brassica, 10(2): 130-133. https://www.cabidigitallibrary.org/doi/pdf/10.5555/20203507453

15. Hami, A., R. S. Rasool, N. A. Khan , S. Mansoor, M. A. Mir, N. Ahmed and K. Z. Masoodi, 2021. Morpho molecular identifcation and frst report of *Fusarium equiseti* in causing chilli wilt from Kashmir (Northern Himalayas). Scientifc Reports , pp.14. doi: 10.1038/s41598-021-82854-5.

16. Hirano, Y. and T. Arie, 2006.PCR-based differentiation of *Fusarium oxysporum* f. sp. *lycopersici* and radicis-lycopersici and races of *F. oxysporum* f. sp. *lycopersici*. J Gen Plant Pathol 72:273–283. DOI: 10.1007/s10327-006-0287-7

17. Holcomb, G. E. and D. E. Carling. 2002. First report of web blight caused by *Rhizoctonia solani* on *Catharanthus roseus* in louisiana. Plant Dis., 86(11). DOI: 10.1094/PDIS.2002.86.11.1272C

18. Jubeir, N. A. 2021. Molecular diagnosis of the Fungus That Causes Diseases of the Roots and Stems Bases of the *Cartharanthus roseas* Plant in Some Nurseries in Karbala and Babylon Governorates and Its Biological and Chemical Control.MSc. Thesis, Plant Protection Department, College of Agriculture, University of Karbala.

19. Kharwar R. N, V. C Verma, G Strobel and Ezra, D. 2008. The endophytic fungal complex of *Catharanthus roseus* (L.) G. Don. CurrSci 95: 228–233. Corpus ID: 88897133

20. Leslie, J.F. and B.A. Summerell. 2006. The Fusarium laboratory manual. UK: Blackwell Publishing Ltd.pp388. https://doi.org/10.1002/9780470278376

21. Li, P. L., Y. X, Shi ., M. Y , Guo ., X. W, Xie ., A. L, Chai and B. J, Li . 2017. Fusarium wilt of cauliflower caused by *Fusarium equiseti* in China. Canadian Journal of Plant Pathology, 39(1) : 77-82 . DOI: 10.1080/07060661.2017.1301998

22. McMillan, R. T.; and J. F. Garofalo, 2004. *Phytophthora parasitica* wilt of new cultivars of *Catharanthus roseus* . Proceedings of the

Florida State Horticultural Society, 117: 316–317.

23. Nejat, N., A. Valdiani, D .Cahill, Y. M. M.Tan and R. Abiri. 2015. Ornamental exterior versus therapeutic interior of Madagascar periwinkle (*Catharanthus roseus*): the two faces of a versatile herb. Sci World J. https://doi.org/10.1155/2015/982412

24. O'Neill, T.M. 2009.Protected ornamentals .Detection, prevalence and control of seed-borne disease.Agriculture and Horticulture Dev. Board. 252:1-52.

25. Parmeter , J. and H.S. Whitney .1970. Taxonomy and nomencleature of the imperfect stage in: *Rhizoctonia solani* Biology and pathology. Journal R. University of California. 7-19. Pp566.

26. Raheem, S. S., M. A. Al-Dossary and H. T.AL-Saad.2021. Ability of some fungi to biogegrade carbendazim fungicide. Iraqi Journal of Agricultural Sciences:52(1):259-267. https://doi.org/10.36103/ijas.v52i1.1256

27. Scott, J. B. and S. Chakrabortey. 2010. Genotypic diversity in *Fusarium pseudograminearum* populations in Australian wheat fields. Plant Pathology.59:338-377. https://doi.org/10.1111/j.1365-3059.2009.02219.

28. Shaikh, S. G.; H. C.; Pathak, V. S. Kumawat, and S. R. Kothule, 2020. Phytochemical Analysis of *Catharanthus roseus L*. (G.) DON. International Journal For Research In Applied Sciences And Biotechnology, 5: 1–8. https://www.ijsdr.org/papers/IJSDR2005050.pdf

29. Sneh, B., S. Jabaji- Hare, S. Neate and G. Dijst. 1996. Rhizoctonia Species: Taxonomy, Molecular Biology, Ecology, Pathology And Disease Control. Kluwer Academic Publishers, London. 578pp. DOI:10.1007/978-94-017-2901-7Corpus ID: 37459985

30. Thomas, P. A., J. Woodward, F. E. Stegelin, and S. V. Pennisi, 2009. A Guide for CommercialProductionofVinca.Bulletin1219. https://extension.uga.edu/publications/detail.html?number=B1219&title=a-guide-for-commercial-production-of-vinca

31. Verpoorte, R., R. Van Der Heijden and P. R. H. Moreno, 1997. Biosynthesis of TerpenoidIndole Alkaloids in Cataranthus roseus Cells. In: The Alkaloids. Cordell, G. A. (Ed), Academic, San Diego, 49: 221-229. https://doi.org/10.1016/S1099-4831(08)60050-4

32. Verpoorte, R.; A. Contin, and J. Memelink, (2002). Biotechnlogy for theproduction of plant secondary metabolites.*Phytochem. Rev.*, 1(1): 13-25. https://doi.org/10.1023/A:1015871916833