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Phenotypic and molecular diagnosis of *Fusarium oxysporum* and *Macrophomina phaseolina* isolated from cucumis melon roots

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ABSTRACT

This study aimed to isolate and diagnose some fungi escorted with the Cucumis melon roots plants both morphologically and molecularly. The results of isolation and diagnosis revealed 57 isolates from the sampling areas, which included Anbar, Abu Ghraib and Al-Yusufiya, which were represented by *Macrophomina phaseolina*, *Fusarium* spp., and *Rhizoctoinia solani*. The fungus *Macrophomina phaseolina* recorded the most significant values by giving the highest frequent reached 27.08%, followed by *Fusarium* spp. (20.83%), and *Rhizoctoinia solani* (11.45%). The pathogenicity results of the purified isolates of *Fusarium* spp., and *Macrophomina phaseolina* revealed that all isolates were significantly reduced the germination rate. However, the isolate F18 was the most intense isolate in reducing the germination rate (%), which were recorded zero germination rate on Cucumis melon and radish seeds. The isolate F12 revealed a reduction in the germination rate of Cucumis melon and radish seeds (0.67%), followed by the isolate of F. *solani* (F16; 10%) on Cucumis melon seeds. The isolates of *M. phaseolina* revealed a decrement in the germination rate, so that the isolate M1 recorded a zero-germination rate on Cucumis melon and radish seeds, followed by M15 with a germination rate of 3.33% on Cucumis melon seeds, while M7 exhibited a germination rate of 3.33% on radish seeds. The DNA electrophoresis results of the pathogenicity tested isolates of radish seeds and Cucumis melon seeds were the most pathogenic using the specialized initiator ITS4/ITS1, as it recorded the bundles of molecular weight reached 519 for *Fusarium oxyspoum* and 560 for *Macrophomina phaseolina*. These were compared with the gene bank for the presence of a high corresponding for the pathogenic fungi, which were deposited in the gene bank by accession numbers of OK560451, OK560452 and also OK560453 and OK560454, indicating by the codes of MP-Iraq1, MP-Iraq2 and also FS- Iraq1 and FS-Iraq2.

Keywords: Cucumis melon, *Fusarium oxyspoum*, *Macrophomina phaseolina.*
Article type: Research Article.

INTRODUCTION

Cucumis melon is an important vegetable crop belonging to the Cucurbitaceae family. The fruits contain high nutritional elements, vitamins, and mineral elements, which makes it largely cultivated around the world. China is one of the largest Cucumis melon producers, reaching 350,000 hectares and producing 8 million tons annually (Yang *et al.* 2007). Iraq occupies the 12th place in the Cucumis melon cultivation in the world and the second place in the Arab world. Morocco, Iraq, and Egypt are among the Arab countries that cultivate the most Cucumis melon with a planted area reached 16.57 thousand hectares, the production 704 thousand tons, and the production 10,269 tons per hectares (AOAD 2016). Each 100 g of fruit flesh contains protein (0.7 g), carbohydrates (7.5 g), as well as calcium, potassium, phosphorous, vitamins A and C, and folic acid. It also has a medical importance, for eczema treatment. Additionally, the seeds contain volatile oils and fatty acids (Hassan 2001). Cucumis melon can be infected by many fungal, bacterial, nematode, and viral pathogens, which cause great losses of production

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(Singh *et al.* 2006; Al-Abbasi *et al.* 2021; Maslienko *et al.* 2021; Al- Ethawi & Al-Taae 2022; Haider & Hussein 2022). One of the important fungi that infects Cucumis melon is *Macrophomina phaseolina* which was first recorded on Cucumis melon in East Texas in 1933 (Taubenhaus & Ezekiel 1933), and India in 1970 (Punithalingem & Holliday 1970) and its first recorded in Iraq was in 2009 (Ismail *et al.* 2009). This fungus is characterized by its wide familial range. It also infects plants in all growth stages and causes various diseases on crops, whether cultivated in greenhouses or open fields. It also infected by the *Fusarium oxysporum* f. sp. *melonis*, which infects the Cucumis melon crop at any stage of growth from planting the seeds to the fruits ripening and is more affective to the yield, where the plant dries completely, in term of branches, leaves, and the fruits become inedible (Resser 1976; Mas *et al.* 1981). There are no statistics indicating the losses caused by these two pathogens in Iraq, but there are some studies that indicated the dangerous of Cucumis melon wilt disease around the world. In 2004, it was considered as one of the most important problems facing Cucumis melon production in France (Villeneuve & Maignien 2008). It was also considered as one of the most important diseases that affect the Cucumis melon crop in America, as it has been recorded in several states (Pivonia *et al.* 1997; Aegerter *et al.* 2000). Additionally, in Arizona in 2006, this fungus was considered as one of the most important soil-borne pathogens that spread widely in the state, so that, it was found that 80% of dead Cucumis melon plants were caused by *M. phaseolina* (Kubota *et al.* 2007).

MATERIALS AND METHODS

Sample collection

Samples were collected in fields of Cucumis melon in Baghdad Belt area (Abu Ghraib, Al-Yousifi, Anbar) by 90 samples of Cucumis melon exhibiting symptoms of infection (root rot and dead or wilt plants) during the summer season 2020 approximately one week before the harvest. The samples were brought to the laboratory after being placed inside polyethylene bags titled by all related information, including the sample source and the date (Table 1).

Table 1. The sources of infected Cucumis melon samples collected.

No.	Area	Samples Number	Isolates Number	Isolate F. spp.	Isolate M. spp.	Isolate R. spp.	Date of collection
1	Anbar	35	22	10	10	2	2020 /6 /19
2	Abu Ghraib	20	13	9	1	3	2020 /6 /25
3	Al-Yousifi	35	22	1	15	6	2020 /6 /29

Isolation and diagnosis

The samples were brought to the laboratory; the roots and the crown area were washed by water for 30 min, then dried on blotting papers, separated into small pieces (0.5 cm) from the sphenoid root area, capillary roots, and the crown area and from the stem area connected to the root (2 cm from the crown area), and sterilized using the sodium hypochlorite solution at a concentration of 3% for a period of 2-4 min. Afterward, the roots were placed and sterilized by the sodium hypochlorite solution, and washed with distilled water to remove residual sterilization solution and then placed on filter paper. The sterilized and dried roots were planted in petri dish (9 cm in diameter) containing PDA (Potato Dextrose Agar), sterilized by an oscillator including antibacterial (amoxicillin) at a concentration of 100 mg L^{-1} at 4 plant pieces per dish and incubated at 25 ± 2 ºC for 3 days. The growing fungi from the infected plant pieces were purified by taking a small part of the fungal growths edge and placed in the centre of another petri dish containing the nutrient medium (PDA) for the purpose of purification. The dishes were incubated at 25 ± 2 °C, then the fungi were diagnosed to the level of genus and species depending on the characteristics of the fungal colony, the nature of the mycelium, spores, and structures by Dr. Tareq Abdul-Sadah Karim using the approved taxonomic keys (Booth 1977; Holliday & Punithalinem 1970; Leslie & Summerell 2006). The relative percent of fungi repetition in the samples was calculated using the following equation:

$$\text{Fungi repetition rate in the sample}(\%) = \frac{\text{Plant's parts number infected by funguses in the dishes}}{\text{total plant's parts in the sample}} \times 100$$

Pathogenicity test of isolated fungi

Ten isolates of *Fusarium* spp. and 6 isolates of *Macrophomina phaseolina* obtained through the isolation process of radish and local Cucumis melon seeds were selected from the var. karear according to the method of Bolkan &

Butler (1974). The germination rate of radish and Cucumis melon seeds were 98% and 80% respectively. The seeds were sterilized with sodium hypochlorite solution (3% free chlorine), washed with distilled water, and planted in petri dishes (9 cm in diameter) containing 15-20 mL Potato Dextrose Agar medium mixed with antibiotic (amoxicillin) at a concentration of 100 g L^{-1}. The dishes were inoculated with a 0.5 cm diameter disc taken from the edge of the colonies of *Fusarium* spp. and *M. phaseolina* (Bhatia *et al.* 2013). Ten seeds were placed for each dish at a distance of 1 cm from the edge of the dish after the growth of fungal colonies reached half of the dish and with three replications with the control treatment and then incubated at 25 ± 2 m for three days, afterward, the number of germinated and non-germinating seeds as well as the seeds germination rate (%) were calculated (Singh *et al.* 2012).

Molecular diagnosis of *M. phaseolina* and *Fusarium* spp. according to polymerase chain reaction (PCR) technique

Single spore method was applied to activate isolates of *M. phaseolina* and *Fusarium* sp. in petri dishes containing PDA at a temperature of 25 ± 2 °C for 7 days. After completing the growth of the fungal colonies, they were taken to the Musayyib Bridge Company in order to conduct the extraction stages of DNA (Al-Khafajy *et al.* 2020).

DNA extraction of *M. Phaseolina* and *Fusarium* spp.

The DNA extraction process was carried out according to the methodology of the Korean Company Bioneer, as the following process:

1- 500-100 mg mycelium was crushed in a liquid nitrogen using a ceramic manual grinder.

2- 100 mg sample was taken, which was crushed using a small spoon and placed in 2 mL plastic tubes in order to extract the DNA.

3- 180 µL Universal Digestion Buffer + 20 µL Proteinase K was applied to the samples and placed into a water bath at 56 °C for 30 min to break down the cell wall.

4- 100 mL Universal Buffer PF was applied, and the materials were mixed using a vortex device followed by placing in a water bath at 56 - 60 ºC for 5 min in order to break down the cell wall.

5- It was then placed in a centrifuge at a speed of 12000 cycles for 5 min at the room temperature, afterward, the samples were transferred to some clean, sterile micro-tubes, at a volume of 1.5 mL, and the precipitate was neglected.

6- 200 µL Universal Buffer BD was applied and mix by a vortex device in order to isolate each of the protein, genetic material, and other objects and contribute in the adhesion of silica to the genetic material (DNA), as the tubes contain silica.

7- 200 µL Ethanol (96-100%) was applied and shaked by a vortex shaker.

8- The precipitate was transferred to some fine, clean, and sterile tubes and placed in a centrifuge for a minute without any addition.

9- 500 µL Universal Wash Solution was applied and placed in the centrifuge at 9000-12000 rpm.

10- The samples were transferred to some clean, sterile micro-tubes and placed in a centrifuge for two min, then transferred to new clean and sterile micro-tubes to get rid of precipitates.

11- 50-100 µL of Buffer TE solution were applied to the centre of the tubes, as they did not touch the walls, and then placed in the centrifuge for a minute to obtain the genetic material DNA.

The replication DNA extracted

DNA replication was carried out by mixing 5 µL extracted DNA with 4 µL primer (ITS4; 2 µL Forward and 2 µL Reverse) and the sequence of nitrogenous bases from (3-5; Table 2). Thereafter, the volume was completed to 20 µL by applying 11 µL Sterilized Ionic water into small tubes containing 5 µL Master Mix and then the materials were mixed by vortex. The tubes were placed in the thermocycler for the PCR reaction, in order to perform the DNA amplification process according to the optimal conditions of the cycles (Table 3).

Table 2. The sequence of nitrogenous bases for the ITS4/ITS1 primer applied in the diagnosis of *Macrophomina phaseolina* and *Fusarium oxysporum.*

Primer Name	The sequence of nitrogenous bases
ITS1	3-TCCGTAGGTGAACCTGCGG-5
ITS4	3-TCCTCCGCTTATTGATATGC-5

Table 3. The DNA replication program of *Macrophomina phaseolina* and *Fusarium oxysporum.*

Phases	Temperature (C°)	Duration	rotations number
Initial Denaturation	95C°	5 min.	1
Denaturation	94 C°	30 sec.	
Annealing	60 C°	30 sec.	35
Extension	72 C°	45 sec.	
Final extension	72C°	5 min.	1

Electrophoresis phase

Agarose Gel was prepared by dissolving 1 g agarose in 90 mL sterile distilled water and 10 mL 10× TEB buffer solution. Afterward, the mixture was heated to boiling using a convector, then the mixture was cooled to 65 °C with the application of 5 µL ethidium bromide. Thereafter the solution was mixed and poured into the designated place in the electric electrophoresis device, and a sterilized comb with ultraviolet ray was applied in the mixture in order to make holes in the gel and kept for 30 - 45 min at room temperature for hardness. Then the comb was removed and 5 µL Ladder was poured to the first hole (containing standard DNA pieces) and the same amount of extracted DNA was poured to the rest of the holes. Subsequently, the device was covered, and the electrophoresis was carried out at 70 volts for 60 min in order to detect the extracted and amplified DNA bundles, which represent the outputs of the PCR and compared with the standard ladder. Finally, the sample was sent to the Korean Biogene Company in order to obtain the sequence of nitrogen bases.

RESULTS AND DISCUSSION

Samples collection

The results of the collection of 90 samples from the fields of Cucumis melon exhibiting the infection such as wilting, yellowing of leaves, discoloration and rotting of roots and some dead plants from the Baghdad Belt areas (Abu Ghraib, Anbar, and Yusufiya). Fifty-seven isolates were isolated, including 22, 13 and 22 isolates from Anbar Governorate, Abu Ghraib, and Yusufiya respectively that were varied among M. *phaseolina*, *Fusaruium* spp. and *Rhizoctoinia solani*. This is consistent with Hamza *et al.* (2007) and Ben *et al.* (2013).

Isolation and diagnosis

The results in Table 4 revealed the infection of *M. phaseolina*, *Fusarium* spp. and *R. solani* in the roots of Cucumis melon. *M. phaseolina* was the most frequent by recording a recurrence rate of 27.08%, followed by *Fusarium* spp. (20.83%), in all areas of sample collection, including Anbar, Abu Ghraib and Yusufiya, then *R. solani* exhibited a recurrence rate of 11.45%. These results are consistent with previous studies, reporting the importance of these pathogens in infecting the roots of Cucumis melon, which cause root rot and deterioration of the vegetative system (Gordon *et al.* 1989).

Table 4. Fungi escorted Cucumis melon roots.

Fungi names	Sample's number	Repetition (%)
Fusarium spp.	3, 2, 1	20.83
M. phaseolina	3, 2, 1	27.08
R. solani	3, 2, 1	11.45

Notably, *M. phaseolina* is characterized by rapid and white growth in the first three days of being placed in the incubator, then it turns dark brown or black starting from the centre of the colony until it includes the entire colony as a result of the formation of stone bodies. In addition, some isolates are fungal yarn that sticks to cover the dish from the inside, and all isolates formed irregularly shaped stone bodies between elliptical and oval (Jaleel Abdul *et al.* 2016). *Fusarium* spp. isolates revealed a clear variation in their growth on the culture medium with differences in the colours of the fungal colonies ranging between white, orange, red, and purple, while *F. oxysporum* was distinguished by the difference in the colonies colour between white to transparent and pink with a variation in growth speed on the same culture media, exhibiting the presence of genetic variations between the fungal isolates (Hirano & Arie 2006). Macroconidia was also observed, appearing in a crescent shape and divided into 3-5 septa and consisted of a single generative cell (Monophile) on a branched conidial holder that united to form a sporodochia pad. They are single or pairs with small lateral branches at their end, and these traits are identical to those of *F. oxysporium* f. sp. *melonis* (Holliday & Punithalinem 1970; Booth 1977; Leslie & Summerell 2006; Hibbett *et al.* 2007; Fig. 1).

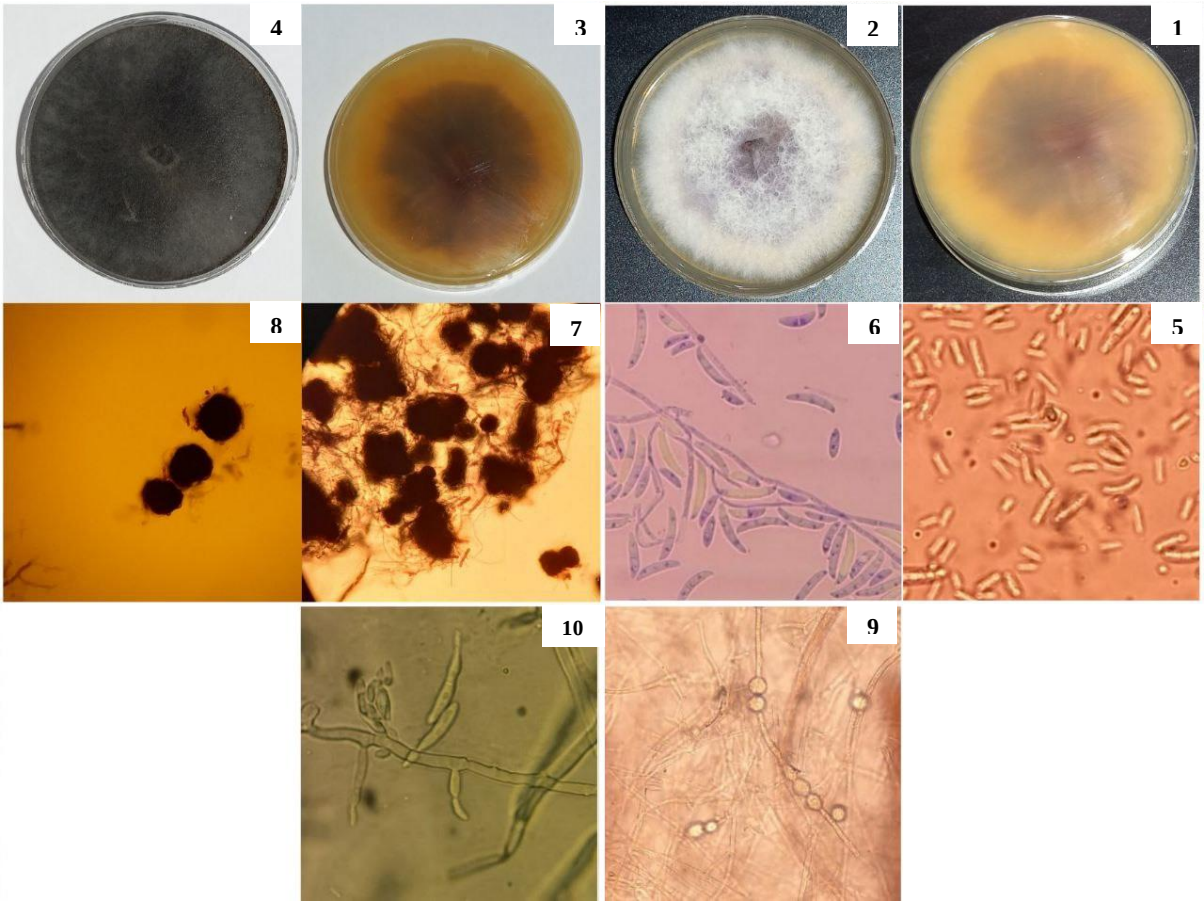

Fig. 1. The morphological characteristics of *M. phaseolina* and *F. oxysporum;* (i) *F. oxysporum* farm on the PDA medium from the back side; (ii) *F. oxysporum* farm on the PDA medium from the front side; (iii) *M. phaseolina* farm on the PDA medium from the back side; (iv) *M. phaseolina* farm on the PDA medium from the side frontal; (v-vi) Microconidia of *F. oxysporum* at a power of ×40 and Macroconidia of *F. oxysporum* at a power of ×40; (vii-viii) The stone bodies of *M. phaseolina* on the strength ×40; (ix) Chlamydospores with a strength of ×40; (x) Monophialde cell of the *F. oxysporum* at ×40 power.

The laboratory test of the isolated fungi pathogenicity

The results revealed that most of the isolates in the laboratory caused a significant decrement in the germination rate (%) compared to the control treatments, reaching 100% germination rate for all isolates of *Fusarium* spp. and *M. phaseolina* on Cucumis melon and radish seeds (Table 5). The isolate of *F. oxysporum* (F18) reduced the germination rate, so that, the germination rate was zero on Cucumis melon and radish seeds, while isolate F12 exhibited a reduced germination rate on Cucumis melon seeds by 0.67%, followed by isolates of F. *solani* (F16) recording 10% germination rate on Cucumis melon and radish seeds, while the two isolates F10 and F4 displayed a reduced germination rate on radish seeds by 6.67% compared to the control treatment, while the germination rate in the rest of isolates ranged between 13.33 and 26.27% on Cucumis melon seeds and between 10 and 20% on radish seeds. The isolates of *M. phaseoline* also displayed a reduction in the germination rate compared to the control treatment in which it was 100%. Isolate M1 recorded a zero-germination rate on both Cucumis melon and radish seeds, followed by isolate M15, recording 3.33% on Cucumis melon seeds. In addition, isolate M7 exhibited a reduction (3.33%) on radish seeds, while in the rest of the isolates ranged between 13.33 and 20% on Cucumis melon seeds, and also between 6.67 and 13.33% on radish seeds. This difference between the isolates of the *Fusarium* spp. may be due to the virulence of the isolates, which is due to the difference in the ability to excrete toxins as well as the secretion of metabolic compounds and enzymes degrading the cell wall of the host such as the peroxidase and ligninase, since these enzymes are of great importance in causing injury and the spread of fungus toxins in cells (Bruce & West 1989; Lozovaya *et al.* 2006; Zhenq *et al.* 2018; Toghneo 2019). The ability of *M. phaseoline* to cause infection may be due to its multiple mechanisms, including the production of

Phaseolion toxins that degrade the fungal cell wall (EL-Deeb *et al.* 1987; Hassan 1996). In addition to the enzymes degrading the cell wall, the difference in the germination rate between the different cultivars may be due to the pathogenic fungus attacking the seeds of a particular cultivar to a more severe degree than the seeds of the other variety. This case may be due to the difference in the amount of substances in the seeds of these cultivars, which have a direct effect on the germination of the stone bodies of *M. phaseolina* (Al-Ani 1988).

Table 5. The pathogenicity test of isolated fungi using Cucumis melon and radish seeds.

Fungi's name	Isolate Symbol	Cucumis melon germination rate (%)	Radish seeds germination rate (%)
Fusarium spp.	F16	10.00	10.00
Fusarium spp.	F11	26.27	16.67
Fusarium spp.	F5	16.67	20.00
F. oxysporum	F18	0.00	0.00
Fusarium spp.	F10	20.00	6.67
Fusarium spp.	F25	13.33	20.00
Fusarium spp.	F9	13.33	16.67
Fusarium spp.	F4	10.00	6.67
Fusarium spp.	F7	20.00	10.00
Fusarium spp.	F12	0.67	20.00
control		100.00	100.00
LSD = 5 %		14.52**%	14.37**
M. phaseolina	M1	0.00	0.00
M. phaseolina	M2	13.33	6.67
M. phaseolina	M28	20.00	13.33
M. phaseolina	M7	16.67	3.33
M. phaseolina	M22	13.33	10.00
M. phaseolina	M15	3.33	6.67
control		100.00	100.00
LSD = 5 %		10.63**	10.75**

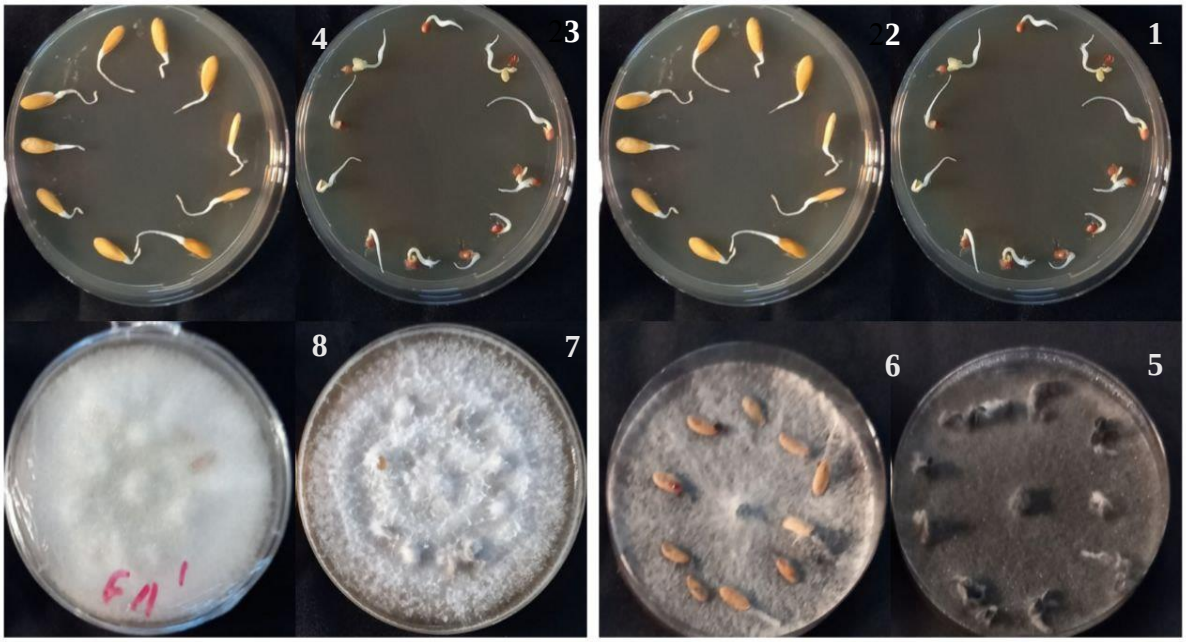

Fig. 2. The pathogenicity test of *F. oxysporum* and *M. phaseolina* isolates on Cucumis melon and radish seeds; (i) Control radish seeds; (ii) Control Cucumis melon seeds; (iii) Control radish seeds; (iv) Control Cucumis melon seeds; (v) *M. phaseolina* & radish seeds; (vi) *M. phaseolina* & Cucumis melon seeds; (vii) *F. oxysporum* & radish seeds; (viii) *F. oxysporum* & Cucumis melon seeds.

The molecular diagnosis Results of *Machrophomina phaseolina* and *Fusarium oxysporum.*

The results revealed the success of extracting the total DNA of the two studied isolates. The results of the electrical migration of the ITS rDNA amplified gene showed the appearance of two bands (Fig. 3) with a molecular weight of 560 and 519 base pairs for the two isolates, *Macrophomina Phaseolina* and *Fusarium oxysporum*, respectively.

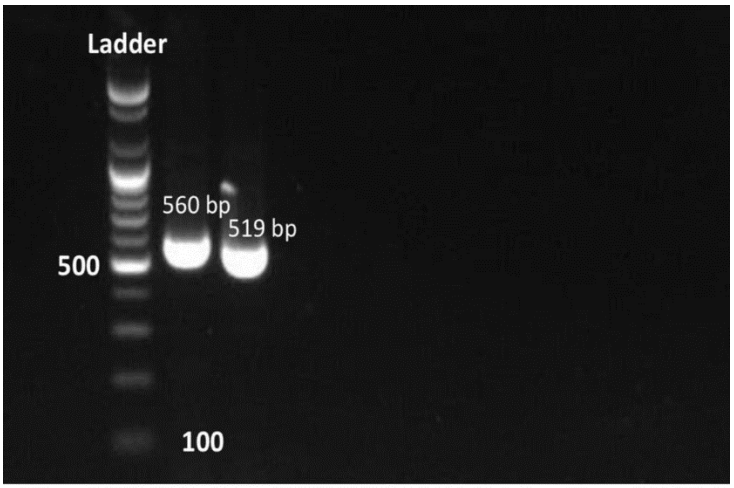

Fig. 3. Electrophoresis of the genetic material of the two fungi: A = *F. oxysporum*; B = *M. phaseolina.*

Determination of the nucleotide sequences reading of the pathogenic fungi DNA

The nucleotide sequences obtained by duplicating the ITS of pathogenic fungi isolates using the ITS1/ITS4 primer after analysis by Dr. Nawras Abdulilah, was compared with the gene bank, revealing a high corresponding rate with *M. phaseolina* and *F. oxysporum*, attaining 99% for both isolates, which have been deposited in the Gene Bank under Accession Number OK560451, OK560452, OK560453, and OK560454, respectively. Phylogenetic tree drawn by SDT v1.2. and by the method of joining the vicinity of the ITS revealed the aforementioned pathogenic fungi isolated from the Cucumis melon in Iraq with their equivalent counterpart from the gene bank, which recorded the highest corresponding rate (99%), and the Iraqi isolates were lined up within one group with the global isolates of *R. solani* as a fungus outside the group (Fig. 4). The diagnosing rate (%) for the Iraqi isolates of *M. phaseolina* and *F. oxysporum* reached 99% comparing with their Indian isolates (MT119461, MZ768560, MK883457 and JX535007) and also with that of Saudi Arabia, i.e., HG798747 regarding *M. phaseolina*, while the *F. oxysporum* corresponded with Indian: KU68728453, Kenyan: KU680359, and Egyptian: MT300173. Depending on the molecular diagnosis and sequencing of the nitrogenous bases of the Iraqi isolates, it is possible to adopt the classification of *M. phaseolina* and *F. oxysporum* isolated from Cucumis melon exhibiting symptoms of infection with pathogenic fungi.

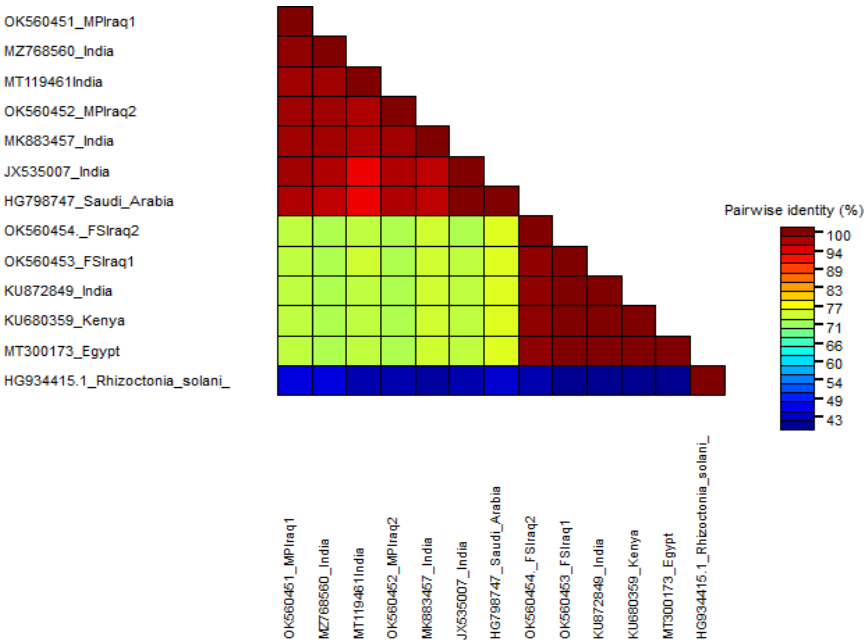

Fig. 4. Corresponding rates of the nucleotide sequences of *M. phaseolina* and *F. oxysporum.*

The colour matrix representing the nucleotide match ratios of the ITS sub-region partial sequences of *M. phaseolina* and *F. oxysporum* isolated from Cucumis melon in Baghdad Governorate and Baghdad Belt regions of Iraq indicated by the codes, i.e., MP-Iraq1 and MP-Iraq2 as well as FS-Iraq1 and FS-Iraq2 respectively with their equivalent counterparts retrieved from the gene bank. The nucleotide sequence of the *R. solani* was included for comparison purpose. Nucleotide analyses were performed using SDT v1.2.

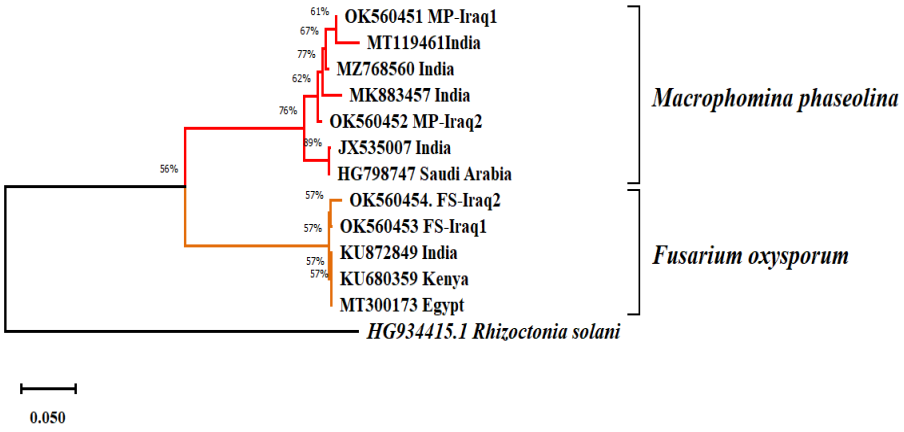

Fig. 5. Genetic relationships of *Macrophomina phaseolina* and *Fusarium oxysporum*.

Genotype tree (Neighbour Joining) was constructed from the partial nucleotide sequences of the ITS of two isolates of *M. phaseolina* and *F. oxysporum* isolated from a Cucumis melon in Baghdad Governorate and Baghdad Belt regions in Iraq, i.e., MP-Iraq1 and MP- Iraq2 as well as FS-Iraq1 and FS-Iraq2 with their equivalent counterparts retrieved from the gene bank. The numeric values attached to each branch represent the boots trap values. The nucleotide sequence of the *R. solani* was included for comparison purpose. Nucleotide analyses were performed using MEGAX software (Muhire 2014; Kumar 2018).

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