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MOLECULAR DIAGNOSIS OF SOME TYPES OF *FUSARIUM* ISOLATED FROM THE SOIL OF KARBALA GOVERNORATE, IRAQ

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Abstract

The results of isolating the *Fusarium* fungi from the soil samples collected from Karbala Governorate showed that the fungi were present in all the samples included in the survey. Exposing it to a chain polymerase reaction to multiply DNA products with sizes ranging from 500-560 nitrogenous base pairs using the front primers (ITS1) and (ITS4) background The results of analyzing the sequences of nitrogenous bases of the double DNA products from the isolated fungal isolates in this study and using the BLAST program proved that some fungal isolates in this study are due to *F. solani* which are 1, 21 and 34, while 39 was dependent on *F. proliferatum* and 36 isolation of *F. oxysporum* f.sp. *ciceris*. The isolates of *F. solani* fungi isolates (1, 21 and 34) isolated in this study showed clear differences in the sequence of nitrogenous bases in some regions of DNA multiplied by the chain polymerase reaction. The isolate 21 of the fungus *F. solani* was the most different in the sequence of nitrogenous bases with different proportions. It was 2% compared to isolates 1 and 34 of the same fungus. It was also revealed through the tree of genetic analysis that the isolation of the fungus *F. solani* (21) appeared in a separate branch from the branch in which isolates 1 and 34 of the same fungus appeared. The results also showed that isolation 36 of the fungus *F. oxysporum* f.sp.*ciceris.* The isolated ciceris in this study showed a clear difference of 2% of the nitrogen base sequences of other isolates belonging to the same fungus and previously registered in the Gen Bank Database. Genetic tree analysis showed the emergence of isolate 63 of *F. oxysporum* f. sp. *ciceris* in a separate branch from the branch in which the other isolates of the fungus appeared and previously registered at the National Center for Biotechnology Information (NCBI), which indicates that this isolation is of high genetic variation compared to other internationally diagnosed isolates. It follows from the results of this study that the two isolates 21 for *F. solani* and isolate 36 for fungus *F. oxysporum* f.sp. *ciceris* were among the genetically different isolates and were not previously registered with the National Biotechnology Information Center (NCBI), so they were registered under the entry numbers MH520067 and MH520068, respectively and their registration is the first in the world.

***Keywords*:** Molecular diagnosis, *Fusarium*, soil

Introduction

The PCR (Polymerase Chain Reaction) technique that was discovered by the scientist Kary Mullis is one of the main important and advanced techniques in Molecular Biology. The discoverer of this technique was awarded the 1993 Nobel Prize (Shampo and Kyle, 2002), that the principle The main in PCR technology is the enlargement and amplification of the DNA chain (Deoxyribo Nucleic Acid) and recognition of variation in the sequence and order of genes on DNA, which varies according to species and strains, and thus an inevitable result and determination of the extent of convergence and spacing between them (Harrow *et al*., 2010). Molecular classification achieved great success in most developed countries and later became widely used and applied worldwide by most researchers. Arif and others (2011) used ITS-Fu-f and its-Fu-r in diagnosing *Fusarium* isolates and analyzing the genes responsible for Tissue deformation in the mango plant growing in the northern belt of India, Toda *et al*. (2020) proved through primers specializing in the identification of fungi isolates *F. oxysporum* as pathogens that infect lily in Japan's Fukushima, and Arif *et al*. (2012) were able to diagnose *F. solani* using the ITS initiator and TEF-1a elongation factor causing rot and wilt disease Many plant families, Najihah and others (2017) explained that the most important pathogens that affect tomato and banana are *F. proliferatum* and *F. virticillioides* through molecular diagnosis using a chain polymerase reaction and detection of genetic diversity with small satellite signals, so the study aimed to isolate Some types of *Fusarium* in some Iraqi soil Molecular diagnosis

Materials and Methods

Isolation and purification

I took several samples from agricultural soils for three fields, including Al-Husayniya, the western table, and desert farms in Karbala governorate. The fields ranged between 5-10 dunums. The samples were taken randomly and put in paper bags and transferred to the Postgraduate Laboratory (Department of Plant Protection College of Agriculture University of Kufa). The samples were mixed. For each area well, it was rid of impurities and then took 1 g of them and put in test tubes container on 9 ml of sterile distilled water. Shake the tubes well to homogeneous, then the dilution is 10^{-1} Transfer by sterile pipette 1 ml of the previous dilution to a test tube containing (9) sterile water, then dilution became 10^{-2}. Repeat the process until dilution 10^{-4}, then take 1 ml of this dilution and place it in a Petri dish and pour 15 ml from the medium WA food prepared in advance, the dishes were incubated at a temperature of 25 °C. for 48 hours after which they were examined after the end of the incubation period. The method (Nelson *et al*., 1983 and Leslie and Summerell, 2006) was adopted in the phenotypic diagnosis of growing *Fusarium* isolates and these fungal isolates were purified separately by transferring a fungal thread from the edges of the growing colony to the middle of a petri dish containing the food medium P.D.A. The same thing was repeated with all isolates and the dishes were incubated in the dark at a temperature of 25 °C. for 5 days, after which the fungal farms were ostensibly examined to confirm the type of growth, colony color, and pigments produced by the fungus in the food medium.

Extraction of DNA from isolates of the *Fusarium* species isolated in this study.

DNA was extracted from the isolates of *Fusarium* spp using the kit (Cat. No: FAPGK100) supplied by Favorgen, Taiwan, China, and by following these steps:

1- Take about 100 mg of growth of the fungus to be diagnosed and grown on the medium of the 6-day PDA Dextrose Acar and put it in an Eppendorf tube for the purpose of crushing it with a micro pestle after adding 200 micro liters of buffer solution. FATG, then incubated for five minutes at room temperature. 2- Add 200 ml. of buffer solution FABG to the sample and shake for five minutes using the Vortex and incubate it for 10 minutes at a temperature of 70 ° C and using a water bath with shaking the tube every 3 minutes during the incubation period. 3- Add 200 micro liters of 95% ethanol to the sample, then shake for 10 seconds. 4- Transfer the mixture thereafter to the FAPG tube and perform the centrifugation process at a speed of 14000 rpm for five minutes, after which the filtrate was disposed and the container tube (FABG Column) was kept on the DNA and linked to the membrane inside the FAPG tube which was transferred To a new collection tube. 5- Add 400 ml. of W1 buffer solution and centrifuge at 14,000 rpm / minute for 30 seconds to remove the leaky solution and keep the FABG tube. 6- The FABG tube was returned to the collecting tube, adding 600 اتر of wash buffer and centrifugation for 30 seconds to remove the leaching solution with a centrifugation process for three minutes to get rid of the residue of the wash solution. 7- The FAPG tube was placed in a new test tube (Eppendorf tube)) and 100 ml. of Elution buffer was added at the center of the DNA-DNA membrane while left vertically for three minutes at room temperature. The centrifugation process was performed at a speed of 13000 rpm for one minute, then the resulting DNA concentration and purity were measured and kept at -20 °C until use.

Estimating the concentration and purity of the DNA extract.

The DNA concentration was estimated using a Spectrophotometer under 260nm wavelength, and the DNA concentration was determined by the following equation:

DNA concentration (µg / ml) = optical absorption value at a wavelength of 260 nm x 50 x Dillution factor

As the DNA purity was defined by applying the following equation described by William and others (1997):

$$\text{DNA purity} = \frac{\text{amount absorption on length waveforms 260 nm}}{\text{amount absorption on length waveforms 280 nm}}$$

The DNA extracted from the Fusarium spp isolates was then kept at -20 °C until tested using PCR technology.

Use of polymerase chain reaction (PCR) technique

For the purpose of diagnosing isolated fungi in this study, a serial polymerase reaction test was conducted using the kit (Maxime PCR PreMix (i-Taq), Cat. No. 25026) supplied by the Korean company iNtRoN of origin. Carry out a chain polymerase reaction with a total volume of 20 microliter containing 1 microliter of each front initiator (TCCGTA GGTGAACCTGCGG: ITS1) and posterior (TCCTCCGCTTATTGATATGC: TS4) (White *et al*., 1990) and 1 microliter of the extracted DNA. All of the above components are placed in the manufacturer-supplied tube and complete the volume with Nuclease-free water to 20 microliter. The DNA of *Fusarium* spp. isolates was doubled using the following steps and conditions of chain polymerase reaction (PCR): Initial denaturation of the DNA for 5 minutes at a temperature of 98 °C followed by 35 cycles consisting of a final metamorphosis process. denaturation) for 40 seconds at a temperature of 94 ºC, primer annealing for 40 seconds at a temperature of 55 ºC and then an initial elongation of the PCR-amplified product for 1 minute at a temperature of 72 Finally, the final polymerase reaction (PCR) was terminated by a final elongation step at a temperature of 72 C (Zhang *et al*., 2012).

Electrophoresis using Agarose gel electrophoresis

The Agarose gel layer was prepared after taking 1 g of agarose powder and dissolving it in 100 ml of buffer solution 1 x TBE (Tris boric acid EDTA buffer) until the mixture was converted into a clear solution. 5 microliter of the Ethidium bromide dye was added after the solution was reduced to 45-40 °C. Prepare the mold for pouring the agarose and containing the comb at one of its ends to make holes inside the gel layer, then pour the dissolved agarose containing the dye of Ethidium bromide and leave it to solidify at room temperature. When the agarose layer is hardened, carefully lift the comb and return the mold to its place in the relay device, then add 1 x TBE solution to the Electrophoresis tank, covering the agarose layer at a height of approximately 1 cm. 10 microliter of the DNA was added by polymerase chain reaction (PCR) to each Well (hole) of the previously prepared agarose gel layer. Also, 5 microliter of the DNA ladder was added to the hole on the left side of the added samples for the purpose of determining the size of the double DNA. The power supply electrodes connected to the electrical current and ran at 150 mAh for one hour. After completing the deportation of the samples, I examined the layer of the agarose gel containing the DNA bundles (PCR products) under UV radiation and took pictures of them.

DNA sequence analysis of *Fusarium* spp.

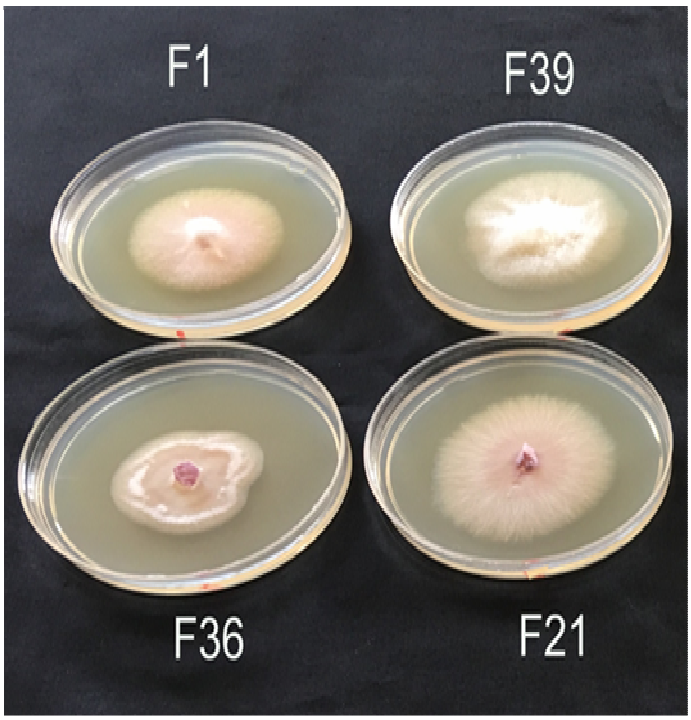
For the purpose of diagnosing isolated fungi, multiplexed PCR amplicons were isolated from *Fusarium* spp. By using the polymerase chain reaction (PCR) with the initiators ITS1 and ITS4 to the Korean company Macrogen for the purpose of determining the Nucleotide sequence and the front and back directions of the double DNA products. All the nitrogen base sequences were analyzed using the BLAST (Basic Local Alignment Search program) Tool) to compare it with the data available at the National Center for Biotechnology Information, NCBI) of *Fusarium* spp and universally diagnosed.

Results and Discussion

Isolation

The results of isolating the *Fusarium* fungi from soil samples collected from Karbala Governorate showed that the fungi were present in all the samples included in the survey, and this is due to the large spread of the fungus and due to its voluntary intrusion and its wide family range in addition to the large production of its reproductive units and its diversity in addition to its long-term restoration. On plant waste, and its reproductive units are also resistant to inappropriate environmental conditions (Romberg and Davis, 2007 and Di *et al*., 2016), as the fungi of the genus *Fusarium* were

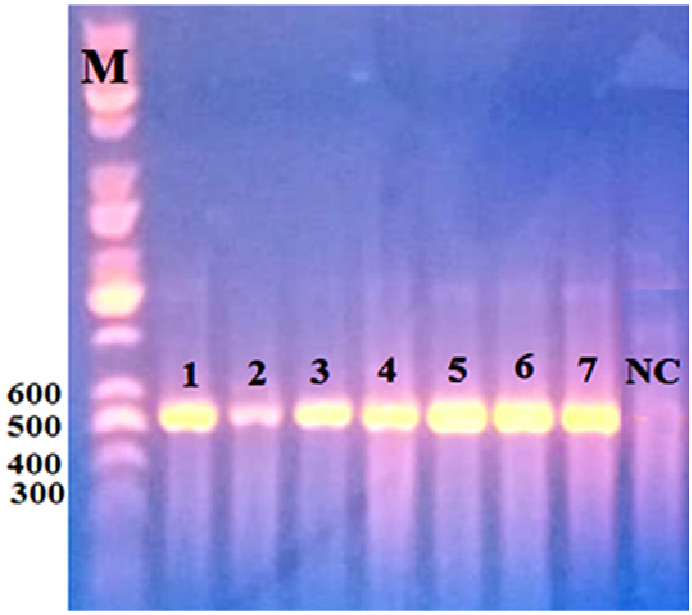
classified phenotypically and microscopically depending on the phenotypic characteristics mentioned by Leslie and Summerell (2006) and the fungi were diagnosed Li It is a subsidiary of the fungus *Fusarium* which F1, F21, and F34, and F36 and F39 as shown in the picture (1)



Picture 1 : Demonstrates the phenotypic properties of fungal isolates

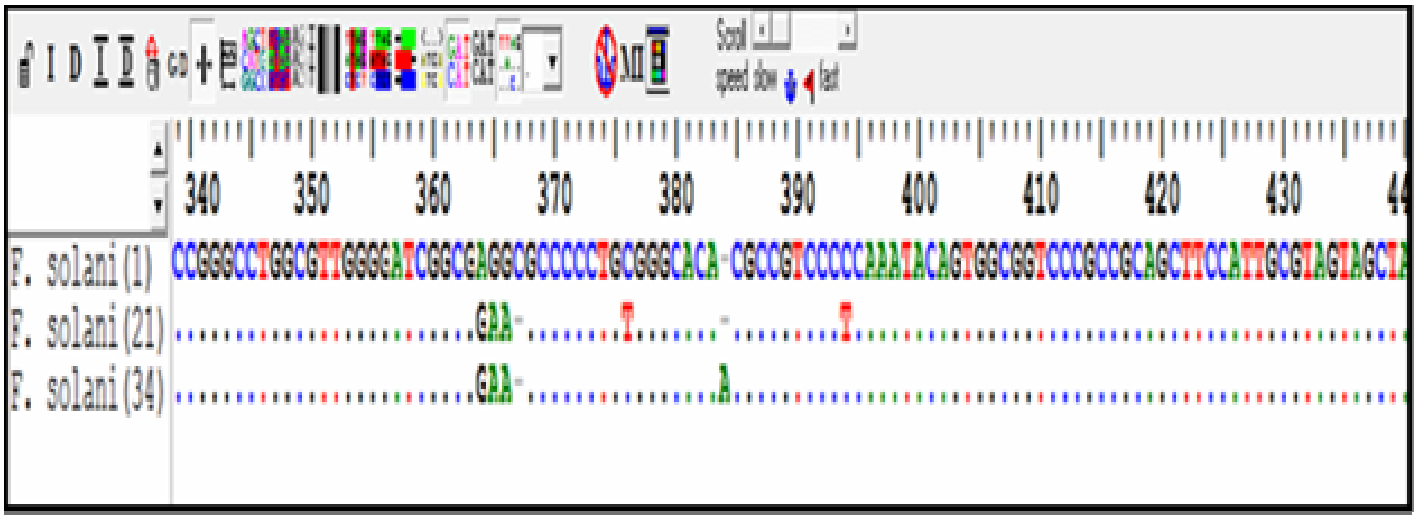
- **Molecular diagnosis**
- **Molecular Diagnosis of *Fusarium* spp. Using polymerase chain reaction (PCR) technology**

The types F1, F21, F34, F36, and F39 that showed importance in this study were confirmed molecular diagnosis using the PCR technique, the results of DNA extraction showed some of the isolates of the *Fusarium* species to be diagnosed and subjected to a series polymerase reaction (PCR) to multiply the products of DNA (PCR-amplified products) with sizes ranging from 500-560 nitrogenous base pairs (Base pairs, bp) and using forward primers (ITS1) and ITS4 background) (Picture 6).

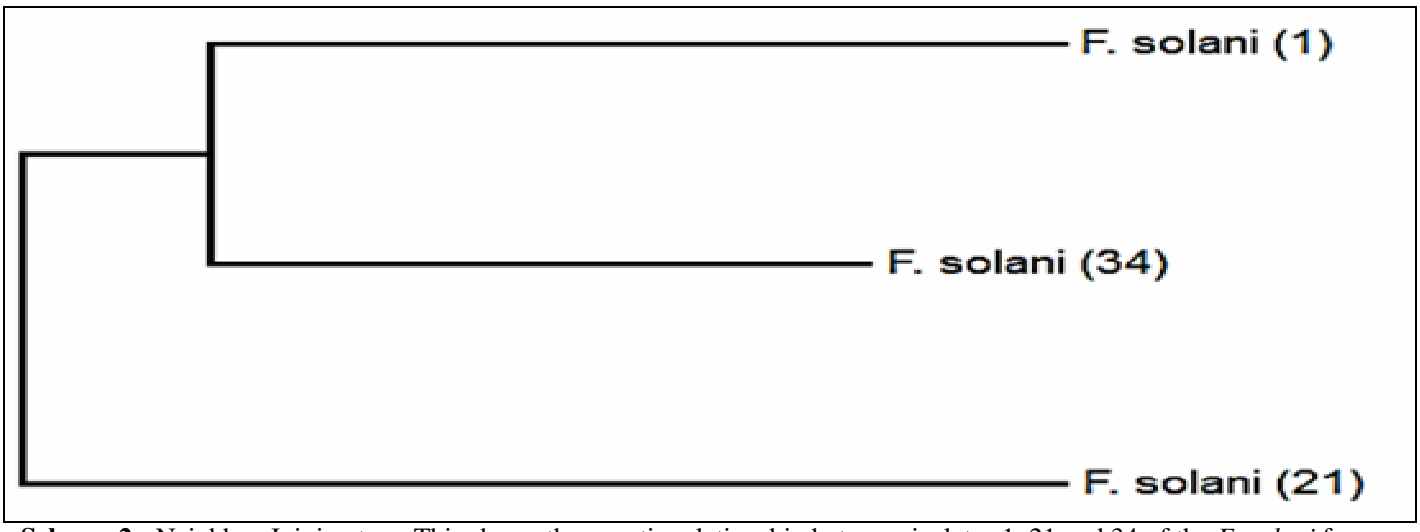


Picture 2 : Multiplex DNA products using chain polymerase reaction (PCR) from isolates of *Fusarium* species using the ITS1 and ITS4 primer pair. M = 1Kbp DNA ladder marker. NC: Comparative treatment (without adding DNA to the rest of the PCR components.

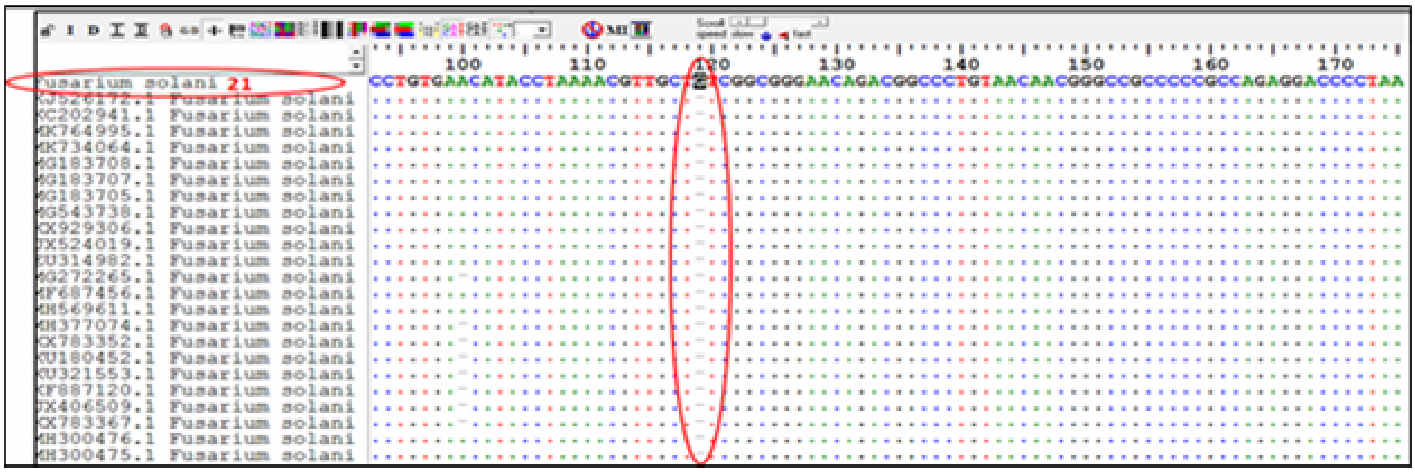
The results of the Nucleotide sequence analysis of DNA products multiplied by fungal isolates isolated in this study and using the BLAST program demonstrated that some fungal isolates isolated in this study are attributed to *F. solani* which are 1, 21 and 34, while Isolation 39 was dependent on *F. proliferatum* and 36 isolate *F. oxysporium* f.sp. *ciceris*. Isolated *F. solani* (1, 21, and 34) isolated in this study are clear differences in the sequence of nitrogenous bases in some regions of DNA multiplied by chain polymerase reaction (PCR) and isolation 21 for *F. solani* was the most different in the sequence of nitrogenous bases with a percentage difference of up to 2% compared to isolates 1 and 34 of the same fungus As in the picture (6). It was also clear from the Neighbor-Joining tree that the isolation of *F. solani* (21) appeared in a separate branch (Clade) from the branch in which isolates 1 and 34 of the same fungus appeared (Scheme 1 and 2).



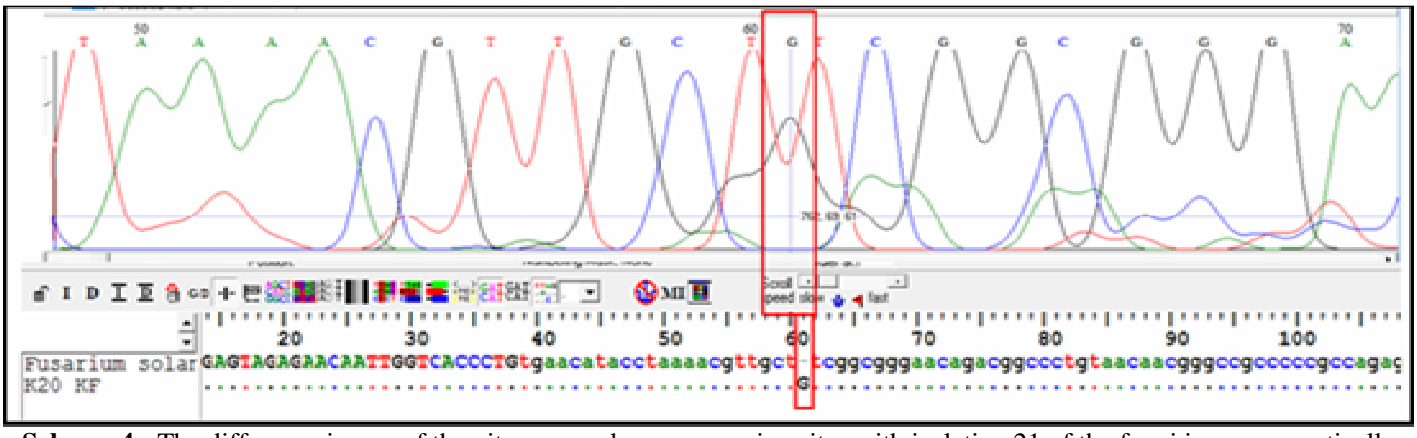
Scheme 1 : Similarities and differences in some regions of the sequence alignments consisting of nucleic acid products (PCR product). Multiply of isolates 1, 21 and 34 of *F. solani*.



Scheme 2 : Neighbor-Joining tree. This shows the genetic relationship between isolates 1, 21 and 34 of the *F. solani* fungus isolated in this study from Karbala.



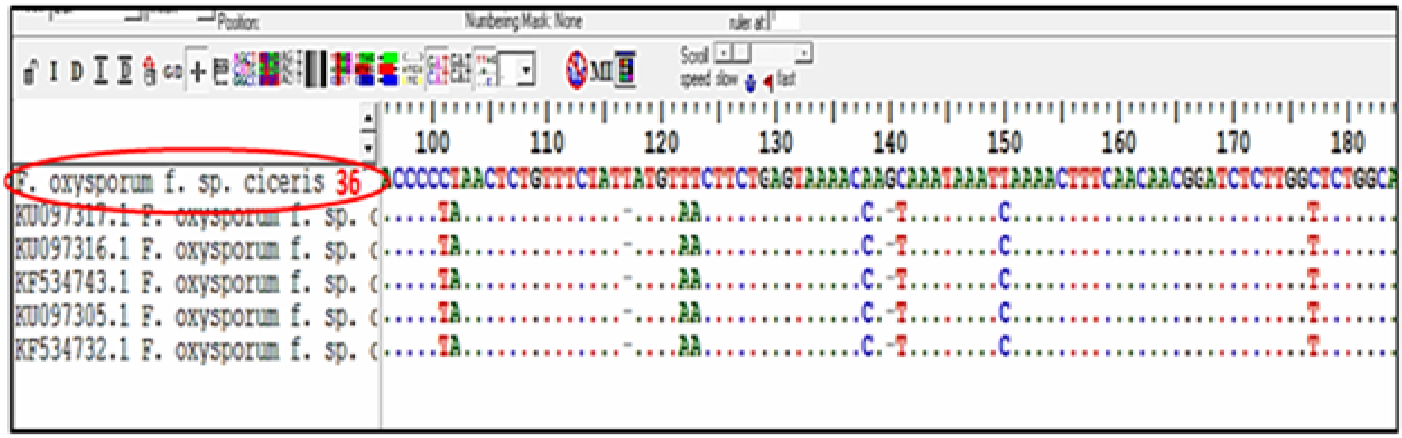
Scheme 3 : Similarities and differences in some regions of the sequence alignments consisting of the DNA product (PCR product). The multiplier of isolate 21 of the fungus *F. solani* isolated in this study and other isolates of the same fungus previously registered at the National Center for Biotechnology Information (NCBI).



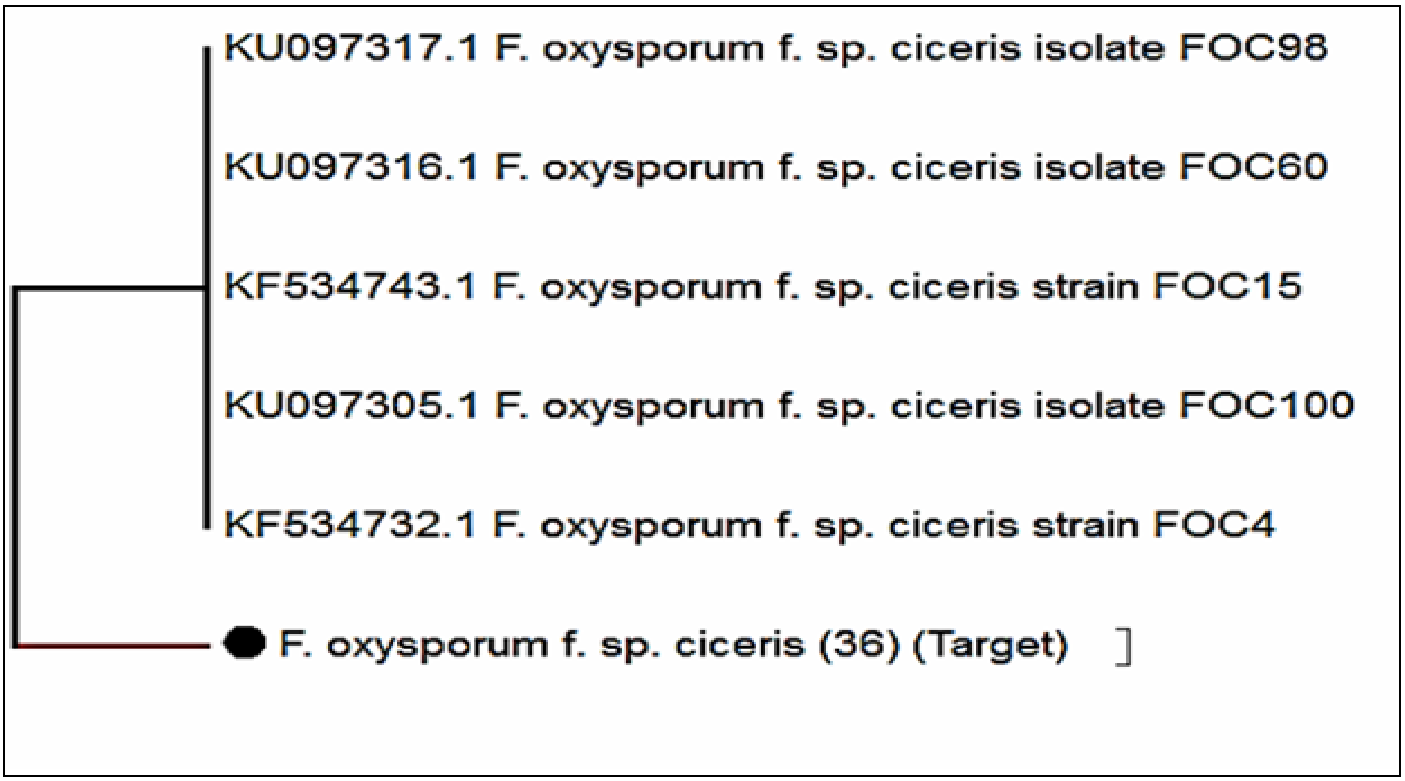
Scheme 4 : The difference in one of the nitrogenous base sequencing sites with isolation 21 of the fungi is more genetically closely related to *F. solani* and was previously registered at the National Center for Biotechnology Information (NCBI).

The results also showed that the isolate 36 of *F. oxysporum* f. sp. *ciceris* The isolated in this study showed a clear difference of 2% of the sequences of nitrogenous bases of other isolates belonging to the same fungus and previously registered in the GenBank Database as in the chart (5). Genetic tree analysis showed the emergence of isolate 63 of *F. oxysporum* f. sp. *ciceris* is in a separate branch from the branch in which the other isolates of the fungus appeared and previously registered at the National Center for Biotechnology Information (NCBI), which indicates that this isolation is of high genetic variation compared to other diagnoses globally diagnosed as shown in chart (6).

As noted when comparing the sequence of double nitrogenous bases of isolate 21 from fungus *F. solani* isolated in this study with other isolates of fungus *F. solani* recorded at the National Center for Biotechnology Information (NCBI) there was a difference in some regions of the nitrogenous baseline sequences as in Schemes 3 and 4.



Scheme 5 : Similarities and differences in some regions of the sequence alignments resulting from the DNA product (PCR product) multiplied by the isolate 36 of the fungus *F. oxysporum* f. sp. *ciceris* isolated in this study from Al-Hussainia area in holy Karbala



Scheme 6 : Neighbor-Joining tree shows the genetic relationship between isolates *F. oxysporum* f.sp. *ciceris* Previously isolated and registered at the National Biotechnology Information Center (NCBI) and 36 isolated in this study were from Karbala, Husayniyah region.

From the results of this study, it is concluded that isolates 21 for *F. solani* and 36 isolate *F. oxysporum* f. sp. *ciceris* were among the genetically different isolates and were not previously registered with the National Biotechnology Information Center (NCBI), so they were registered under the entry numbers MH520067 and MH520068, respectively and are registered for the first time in the world.

It was noted in previous studies that the diagnosis of fungal species has been dependent on the morphological and microscopic characters may sometimes give wrong results for the diagnosis process, so this study used the technology of polymerase chain reaction (PCR) in the diagnosis of some isolates of the fungi *F. proliferatum F. solani* and *F. oxysporum* f. *Ciceris* are hormonal and toxicological significance.

PCR technology has been used in previous studies of its high accuracy in diagnosing many organisms including fungi such as *Fusarium* and *Aspergillus* and others (Abdullah *et al*., 2019; Aljuaifari *et al*., 2019 and Torrance *et al*., 2020) to get rid of Diagnostic problems dependent on phenotypic and microscopic characteristics. Despite the usefulness of phenotypic diagnosis in confining the fungi under investigation in smaller groups before proceeding to using other methods of diagnosis, so there are many problems that accompany the phenotypic diagnosis of fungi, including the need for the person in the diagnostic process to have high experience, especially in fungi species close to each other, such as some types of fungi. *Fusarium*, As well as requiring significant time and effort (Yang *et al*., 2007; Hsuan *et al*., 2011; Alhussaini *et al*., 2016). There are also some other factors that affect these phenotypic characteristics, including the type and nature of growth medium, heat, and lighting that can affect the color, shapes, and sizes of spores and the

growth of fungal colonies developing. Some researchers found that there was an error in the phenotypic classification of many fungi diagnosed in previous studies, including species belonging to the *Fusarium* spp. Such as *Fusarium verticillioides* and *Fusarium subglutinans* when re-diagnosed again using a polymerase chain reaction (PCR) (Yang *et al*., 2007; Hsuan *et al*., 2011).

The method of diagnosis of fungi, based on the differences in the ITS (Internal transcribed spacer) DNA, contributed to its high efficacy in diagnosing many fungi such as *F. proliferatum* and *Cladosporium* and *Fusarium verticillioides* (AL-Abedy *et al*., 2018 and Al-Fadhal *et al*., 2018). Dewan *et al*., 2019; Al-Sharmani *et al*. 2019).

Conclusion

The results of this study showed the isolation of some types of *Fusarium* from some Iraqi soil (Karbala Governorate), determining the extent of its distribution and distribution, phenotypic and molecular diagnosis of it, determining the extent of genetic convergence and distance between them and comparing them with the global species and strains recorded in the data bank and recording what is new and not registered.

All isolates of fungi were diagnosed and new types of fungi were detected that showed genetic differences and spacing with the isolates registered in the world These isolates were registered for the first time in Iraq.

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