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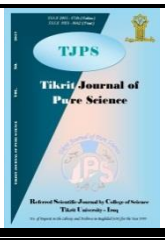

Molecular characterization by PCR-ITS technique of *Fusarium oxysporum* isolated from tomato in Baghdad city

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ABSTRACT

The present study aimed to estimate the pathogenesis of *Fusarium oxysporum* isolates and identify them by molecular technique Polymerase Chain Reaction-Internal Transcript Spacer (PCR-ITS). A total of thirty *Fusarium oxysporum* isolates were isolated from infected tomato plants and diagnosed depending on morphological characteristics. The pathogenicity test was performed for thirty *Fusarium oxysporum* isolates. So it was procedure steps that Genomic DNA was extracted from seven *F. oxysporum* isolates according to pathogenicity test by using ZR Fungal/Bacterial DNA MiniPrep™ kit. Seven *F. oxysporum* isolates showed high pathogenicity. The concentration and purity of the DNA extracted from the seven *F. oxysporum* isolates was (150 – 201) ng/µl and (1.4 -1.9) respectively. ITS gene was amplified by using PCR-ITS The sequencing results of amplified product of ITS gene from *Fusarium oxysporum* isolates indicated that all the seventh sequenced isolates where from one *Formae speciales* of *Fusarium oxysporum* as *Fusarium oxysporum f. sp. Lycopersici*. These results have been devoted to study genetic variability among the species involved in study. Molecular markers are highly important to focus on *F. oxysporum* isolates below the species level therefore, the DNA array containing "genus, species and forma specialis specific" detector for the detection and identification of *F. oxysporum.* Also, Cluster analysis and phylogenetic tree depending on genetic distance revealed the genetic relationship between the thirteen *F. oxysporum* isolates and confirmed sequencing analysis.

Introduction

Growth and productivity of tomato plants (*Lycopersicon esculentum* Mill.) are facing different challenges due to the outbreak of pest and diseases [1] Wilt disease caused by Fusarium *oxysporum f. sp. lycopersici* is highly destructive in both greenhouse and field causes economically loss for this crop [2,3]. Controlling of tomato wilt disease give significant defies because of the ability of this fungus to remain dormant in the soil in the form of spores [4]. In Iraq, fungal diseases are the main reason for the reduction of tomato production, fungal diseases cause significant economic losses, especially wilt diseases, root rot and dapping off caused by *Fusarium oxysporium* [5,6].

Fusarium oxysorum is one of the most important pathogenic fungi that infects plants through roots in all the plant's growth stages. Pathogen causes the symptoms of necrosis and vascular wilt diseases in a wide variety of economically important crops [7]. *Fusarium* classified under the Fungi kingdom, Division of Ascomycota, Class of Sordariomycetes, Hypocreales Order, Family of Nectriaceae, Genus of Fusarium and Species of *Fusarium oxysporum* [8].

Fusarium wilt pathogens show a high grade of host specificity and based on the plant species and plant cultivars that infect by this fungus. *F. oxysporum* are classified into more than 100 formae specials and strains [9,10]. *F. oxysporum* that attack Tomato is *F. oxysorum* f.*sp. Lycoperesici*, which is one of the most important diseases of Tomato in Iraq [1] and it is an economically important disease that exists where a crop is grown causing the destruction of plants that are cultivated in the fields and protected houses, especially in warm areas of the world, where

the rate of loss in the result of the incidence of this fungi is between 10 - 50% per year [11,12]. *F. oxysporum* that attack *Solanum melongena* is *F. oxysporum* f.sp. *melongenae*, and it's considered the most pathogenic fungus on eggplant. *F. oxysporum* f.sp. *melongenae* was initially described in 1958 from *S. melongena* plant suffering from a vascular wilt disease [13] and other cases of financially imperative formae speciales incorporate melonis, tulipae (on tulips), cubense (on banana) and asparagi (on asparagus plants).

In general, morphological characterization of plant pathogenic fungi is achieved by traditional pathogenicity bioassays under greenhouse conditions and this method takes a long time and high costs, also this identification does not give the specific classification of the isolates because it depends only on the morphological characters under microscope and this is not enough to identify the under-species levels because of the high similarity of this species isolates. So, wilt disease in the fields of tomato, middle of Iraq was identified using molecular approaches [14]. Also, characterization and molecular identification of *F. oxysporum* isolates were investigated [15]. At present, molecular biology technologies are being used to determine the specific formae specilas and strains of *F. oxysporum*, since these technologies are usually quick, accurate, and low-cost [16]

The aim of the study was to concentrate on the molecular Identification of *Fusarium oxysporum* which are isolated by PCR-ITSgene.

Materials and Methods

Isolation, Purification and Identification of Pathogens

The infected tomato plants were collected during the month of December 2017 from two greenhouses in Al-Rashidiya's farm (Baghdad province) and a number of plants in each field was 750. The twenty infected tomato plants were collected by crossing the diameters from each greenhouse, then they were put in polyethylene bags individually and the name of the area and the date of collection were written on each bag. After that the samples were taken to the laboratory to isolate and identify the pathogens.

The infected plants were identified based on the symptoms that appeared on the shoot and root, which included yellowing and drooping of leaves, death of some branches, reddish-brown streaks were visible in the vascular tissues when cut with a knife and finally death of plants.

The stems of infected plants were cut at a height of 15 cm above the crown area. After that, the roots were washed by tap water for two hours to remove of the mud suspended. The stems and roots were cut into small pieces (0.5-1 cm) and sterile by immersing them for 3 minutes in the sodium hypochlorite solution (1% free chlorine), then washed by immersing them in sterilized distilled water for three times, after that dried with sterile filter paper. Four pieces (one for the root and three for the stem) were cultured for each infected plant in petri dishes containing autoclaved PDA and incubated for 4 days at 28±2 ˚C [1]

The isolates fungi were purified by taking mycelial plugs with a five mm diameter from the growing margin and put overhead onto fresh PDA in the center of the petri dish and incubated for five days at 27±2 ˚C. *F. oxysporum* were identified based on the morphology of the colony, conidiophores and spores shapes according to [17,18] by Assistant Professor Dr. Alaa Mohsen Al-Aaraji. All fungal isolates were cultured and maintained on autoclaved PDA and incubated for 5 days at 28±2 ˚C. Slant cultures were also prepared, and all stored in a refrigerator at 4 ˚C [19].

Fusarium oxysporum pathogenicity test:

This test was carried out to detect the pathogenicity of *F. oxysporum* isolates. Local cress seeds untreated with fungicide were surface sterilized by immersion in sodium hypochlorite (1 % free chlorine) for two min, rinsed three times with sterile distilled water, dried by sterile filter paper. Ten seeds for three replicates were placed on the sterile filter paper inside a Petri dish containing moisture by putting drops of sterile distilled water, then leaves for 7 days, then the percentage of seeds germination account was counted according to the following formula.

$$P = \frac{\text{Number of non germinating seeds}}{\text{Total number of seeds}} \text{ X } 100$$

P = the percentage of non-germination seeds

Genomic DNA extraction

Genomic DNA was extracted from seven Fusarium *oxysporum* isolates infected tomato plants were selected according to pathogenicity test (named Fox1-7) by using ZR Fungal/Bacterial DNA MiniPrep™ (USA, the epigenetic company). This kit was designed for the simple and rapid isolation of DNA from tough-to-lyse fungi and bacteria in minutes. In the beginning all the isolates used in the DNA extraction were purified several times till getting rid of any contamination (other fungus spores or bacteria) by culturing the isolates on the PDA medium for activation. The step of activation is very necessary for extraction to get pure culture and good yield of genomic DNA.

Agarose gel electrophoresis of DNA:

Agarose gel Electrophoresis was done to determine DNA pieces after the process of extraction PCR reaction to detect bands by using standard DNA to distinguish the band size on the agarose gel.

Primers of gene *ITS*

The primer pair of *ITS* gene that shown in the table (1) was purchased at lyophilized form, they dissolved in the free H_2O (dd) to give a final concentration of 100 pmol/µl as stock solution and keep a stock at -20 to prepare 10 pmol/µl concentration as work primer suspended, 10 µl of the stock solution in 90 µl of the free H2O (dd) water to reach a final volume 100µl, was investigated by IDT (Integrated DNA Technologies company, USA).

Table 1. The specific primer of gene *ITS*

Primer	Sequence
Forward	5´TATTGTTGCTGTGGGACCTGAG3´
Reverse	5´CCTGAGAATCTGAGTAAATCCACT3´

PCR amplification

PCR premix kit (Intron/Korea) was used for PCR amplification, which contained 2.5U I-Taq DNA Polymerase, 2.5mM DNTPs, 1X Reaction buffer (10X), and 1X Gel loading buffer. The PCR amplification mixture of the specific reaction for diagnosis gene was performed in a total volume of 20µl containing 1.5µl DNA, 1 µl Taq PCRPreMix, 1µl of each primer (10 pmol) then 16.5 µl of distilled water was added into tube to a total volume of 20µl.

Amplification of *ITS* region:

The universal primers (*ITS-1*& *ITS-4*) were used for the amplification of rRNA gene and *ITS* regions which is found in all eukaryote as a conserved region [23]. The thermal cycling conditions were done as follows: Initial Denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 40s, 56°C for 35sec and 72 °C for 35sec with final extension 2 at 72 °C for 10 min using a thermal Cycler (Gene Amp, PCR system 9700; Applied Biosystem). All PCR products were separated by 1.5% agarose gel electrophoresis and visualized by exposure to ultraviolet light (302nm) after staining by red safe stain (Intron Korea).

Sequencing and sequence alignment

All PCR product isolates were sent for Sanger sequencing using ABI3730XL, automated DNA sequences, by Macrogen Corporation – Korea. Homology search was leaded using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov) and BioEdit program to detect polymorphism. The results are received by email then analyzed using genious software.

Results and Discussion

Isolation and identification of *Fusarium oxysporum* isolates

Thirty *F. oxysporum* isolates were obtained from infected tomato plants from two greenhouses in Al-Rashidiya's farm (Baghdad Governorate). The pathogenic fungus *F. oxysporum* isolates were purified and identified depending on morphological and microscopically characters. The morphological characteristics on PDA medium were observed (Figure 1) cottony mycelium and variable color according to age, from cream-colored colony to pinkish violet. Microscopically characters of *F. oxysporum* showed that macroconidia have three- to five-septate, sickle-shaped, with foot-shaped basal cells. Microconidia without septa, oval and borne in false heads, and chlamydospores were visible in culture [17,18,21,22]

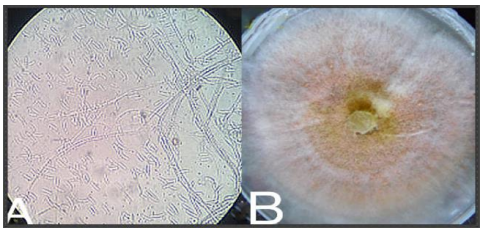

Figure 1. *Fusarium oxysporum* (A) under microscope (B) morphology on PDA medium.

In Iraq, many researchers have pointed to the isolation of *F. oxysporum* from tomato plants infected with it and root rot disease [14,15] Also in study of [23] seven *Fusarium* isolates isolated from Serbia were examined macroscopically and microscopically. During morphological observation, aerial mycelia of all isolates were white at the initial stage, while the underside of colonies became pale pink, violet, purple and brown in the later stages on PDA. Based on their morphological characteristics (shape, form, separation, size, microconidia, macroconidia, phialides, chlamidospores, sclerotia), the isolates were identified as *Fusarium oxysporum.*

Pathogenicity Test of *Fusarium oxysporum*

Results of pathogenicity test showed that all *F. oxysporum* isolates were high pathogenic for local cress seeds, and seven *F. oxysporum* isolates (1, 11, 12, 13, 19, 29 and 30) showed high pathogenicity more than other isolates, which recorded 100 % inhibition growth of local cress seeds (table 2). These seven *F. oxysporum* isolates were selected to complete this study and named (Fox1- 7). These results were consistent with [24], who noted that all isolates of *F. oxysporum* were highly capable of infecting eggplant plants.

The results of this study were in agreement with a study done by [23] who demonstrated that the results of the pathogenicity test revealed that all seven isolates of *Fusarium* species were pathogenic to the seedlings. They showed symptoms like crown rot and root necrosis or root rot. These symptoms were observed within 30 days after inoculation as necrosis and brown discoloration of the root, lower part of the stem necrosis, and a complete rottenness and decomposition.

Table 2. Pathogenicity test of *Fusarium oxysporum* on local cress seeds plants.

F. oxysporum isolates	Pathogenicity %
1.	100.0
2.	90.00
3.	93.33
4	93.33
5	90.00
6	90.00
7	93.33
8	93.33
9	90.00
10	90.00
11	100.0
12	100.0
13	100.0
14	90.00
15	93.33
16	90.00
17	90.00
18	93.33
19	100.0
20	90.00
21	90.00
22	93.33
23	90.00
24	90.00
25	90.00
26	90.00
27	93.33
28	90.00
29	100.0
30	100.0
L.S.D	P=0.05
Between isolates	4.554

*each number represents three replicates.

DNA Extraction:

The DNA was extracted efficiently from seven *Fusarium oxysporum* isolates by using wizard ZR Fungal/Bacterial DNA MiniPrep™ Kit. Purity and concentration of DNA were measured using Nanodrop [25]. The yield of the DNA extracted from the *F.oxysorum* isolates was in range of (150 – 201) ng/µl with purity ranged from (1.4 -1.9). Then the product of DNA was confirmed by agarose gel electrophores (Figure 2).

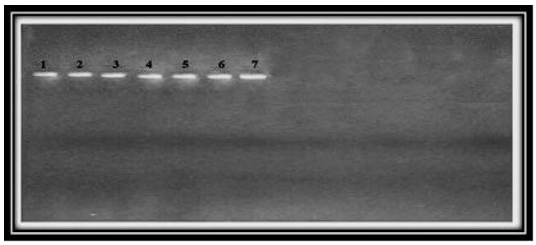

Figure 2. 1 % Agarose gel Electrophoresis of total genomic DNA for *Fusarium oxysporum* isolates, (70 Volt/30 minute).

Genomic DNA was successfully extracted from *F. oxysporum* isolates using ZR fungal DNA kit for the specific PCR and ITS-PCR analysis [26]

***Fusarium oxysporum* identification by gene sequence:**

The identification of the seven *F. oxysporum* isolates was based on the ITS region. The intergenic spacer region was successfully amplified from all *F. oxysporum* isolates. All isolates yielded a unique product size of approximately 550-600bp as shown in (Figure 3).

This result is similar to those obtained in previous studies on identification, characterization and genetic variability of the *Fusarium* isolates where the amplification of the rDNA region of *Fusarium* isolates yielded amplicons in the size range of 600pb. The *Fusarium* species specific PCR primers, ITS1 and ITS4 can provide a fast and accurate tool for the identification and characterization of *Fusarium* species.

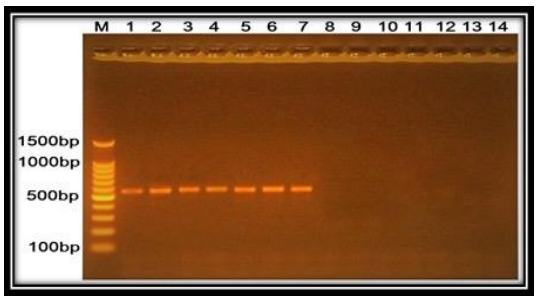

Figure 3. shown PCR product of ITS gene band size 565bp that was electrophoresis on 2% agarose gel ITS gene.

Gene Bank identification of *F. oxysporum* isolates:

Seven DNA samples amplified PCR-products for ITS gene from *Fusarium* species were sequenced for the detecting SNPs within these sequences. Our sequences were compared with reference sequence of National Center Biotechnology Information's (NCBI) Gene Bank. The samples were treated with AB13730XL APPLIED BIOSYSTEMS machine in National Instrumentation Center for Environmental Management NICM/USA company online at (http://nicem.snu.ac.kr /main/?en_skin=index.html).

The result of the sequence analysis has become clear in terms of BLAST search in the National Centre Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and BioEdit program to detect polymorphism. After sequencing analysis on blast website, the acquired results allowed to determine Fusarium species level.

The sequencing results of amplified product of ITS gene from *Fusarium oxysporum* isolates indicated that all the seventh sequenced isolates where from one *Formae speciales* of *Fusarium oxysporum* as *Fusarium oxysporum f. sp. Lycopersici* as shown in Appendix.

The result of this research is consistent with the research of Hussien *et al.,* (2013) [27] who studied sixteen isolates of *Fusarium oxysporum* which were collected from the root and stem fragments of guava plants growing in six districts of Bangladesh. Species identity was based on the colony character, nature of conidiogenous cell, morphology of microconidia, macroconidia and chlamydospores. Eleven isolates were confirmed as *F. oxysporum* through polymerase chain reaction (PCR) using species specific primers designed from the conserved regions of 18S rRNA gene.

Phylogenetic tree structuring

The phylogenetic tree diagrammatic by Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0. Sequences that showed the highest identity (>95%) and maximum coverage (100%) as shown in (Figure 4). The genetic distance between Iraq and the isolates of the world is detailed according to the Phylogenetic tree and the comparison.

Cluster analysis and phylogenetic tree depending on genetic distance in order to reveal the genetic relationship between the thirteen *F. oxysporum* isolates. Analysis of this dendrogram, the overall result indicated that all *F. oxysporum* isolates were classified into two groups. The first major group was further divided into two subgroups. The first subgroup is divided into two divisions, the first division include the first two isolates which are (F1 and F2), while the second division include the second two isolates which are (F6 and F7). The second subgroup is separated into two divisions, the first division include the first isolate which is (F5), while the second division include the second two isolates which are (F3 and F4).

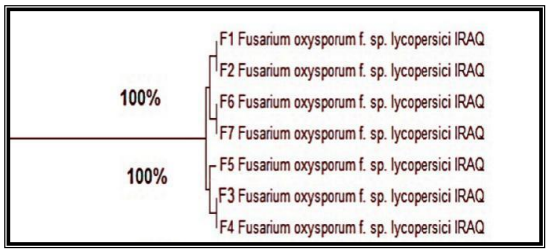

Figure 4. Dendrogram showing genetic diversity among seven of *Fusarium oxysporum* isolates

Conclusion

Plant pathogenic fungi *Fusarium oxysporum* "which causes wilt is a serious pathogen "which economically important "because many members are considered" the causal agents of vascular wilt" or root rot diseases in agricultural" of Iraq. The DNA array containing "genus, species and forma specialis specific" detector for the detection and identification of *F. oxysporum.* And The sequence of *ITS* gene product (565bp) were appeared presence of one forma specialis *F. oxysporum (7 Fusarium oxysporum f. sp. Lycopersici).* Also, Pathogenicity tests cannot determine" the specific species and subspecies of *F. oxysporum.*

Appendix

Appendix 1. Alignment of nucleotide sequencing of isolate (F1) gene bank of NCBI, and appeared compatibility of gene under sequence ID: MG136705.1

After alignment the product amplification of ITS gene for the (F1) isolate that shown to have one transition A>G, one transversion T>A from the Gene Bank, found that part of ITS gene having 99% compatibility with standard in Gene Bank ITS gene as it shown in Figure (appendix1).

```
Query  1    CCAACCCCTGTGACATACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  61   GGACGGCCCGCCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAACAAA  120
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Query  121  CAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  181  CAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA  240
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Query  241  CATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAG  300
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Query  301  CTCAGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGT  360
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Query  361  CGAGCTTCCATAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACGCCG  420
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Query  421  TAAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAG  480
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Query  481  CATATCAAAAGGCCGGAGGAA  501
            |||||||||||||||||||
Sbjct  497  CATATCAATAAGCCGGAGGAA  517
```

Figure (appendix1). Sequence analysis of ITS gene.

Appendix 2. Alignment of nucleotide sequencing of isolate (F2) gene bank of NCBI, and appeared compatibility of gene under sequence ID: MG136705.1

After alignment the product amplification of ITS gene for the (F2) isolate that shown to have one transition A>G, and two transversion T>A, and G>C from the Gene Bank, found that part of ITS gene having 99% compatibility with standard in Gene Bank ITS gene as it shown in Figure (appendix 2).

```
Query                                                                 1
CCAACCCCTGTGACATACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACG  60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 17  CCAACCCCTGTGACATACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACG
76

Query                                                                61
GGACGGCCCGCCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAACAAA  120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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136

Query                                                               121
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Query                                                               181
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Sbjct                                                               197
CAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA  256

Query                                                               241
CATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAG  300
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 257  CATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAG
316

Query                                                               301
CTCAGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGT  360
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 317  CTCAGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGT
376

Query                                                               361
CGAGCTTCCATAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACGCCG  420
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 377  CGAGCTTCCATAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACGCCG
436

Query                                                               421
TAAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAG  480
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                               437
TAAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAG  496

Query  481  CATATCAAAAGGCCGGAGGAA  501
||||||||||||||||||||
Sbjct  497  CATATCAATAAGCCGGAGGAA  517
```

Figure (appendix 2). Sequence analysis of ITS gene.

Appendix 3. Alignment of nucleotide sequencing of isolate (F3) gene bank of NCBI, and appeared compatibility of gene under sequence ID: KF914451.1

Amplified product of ITS gene from *Fusarium* species of isolate (F3) appeared 100% compatibility for ITS gene as it shown in Figure (appendix 3).

```
Query                                                                 17
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62

Query                                                                 77
AACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCAT  136
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122

Query                                                                 137
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AAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC  242

Query                                                                 257
ATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGC  316
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  243  ATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGC
302

Query                                                                 317
CCCCGGGTTTGGTGTTGGGGATCGGCGAGCCTCACGGCAAGCCGGCCCCGAAATACAGTG  376
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                 303
CCCCGGGTTTGGTGTTGGGGATCGGCGAGCCTCACGGCAAGCCGGCCCCGAAATACAGTG  362

Query                                                                 377
GCGGTCTCGCTGCAGCTTCCATTGCGTAGTAGTAAAACCCTCGCAACTGGTACGCGGCGC  436
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                 363
GCGGTCTCGCTGCAGCTTCCATTGCGTAGTAGTAAAACCCTCGCAACTGGTACGCGGCGC  422

Query                                                                 437
GGCCAAGCCGTTAAACCCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCG  496
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                 423
GGCCAAGCCGTTAAACCCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCG  482

Query  497  CTGAACTTAAGC  508
             ||||||||||||
Sbjct  483  CTGAACTTAAGC  494
```

Figure (appendix 3). Sequence analysis of ITS gene.

Appendix 4. Alignment of nucleotide sequencing of isolate (F4) gene bank of NCBI, and appeared compatibility of gene under sequence ID: MG136705.1

After alignment the product amplification of ITS gene for the (F4) isolate that shown to have one transition C>T from the Gene Bank, found that part of ITS gene having 99% compatibility with standard in Gene Bank ITS gene as it shown in Figure (appendix4).

```
Query                                                                   1
AACCCCTGTGACATACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCTGTAAAACGGG  60
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19  AACCCCTGTGACATACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACGGG
78

Query                                                                  61
ACGGCCCGCCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAACAAACA  120
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79  ACGGCCCGCCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAACAAACA
138

Query                                                                 121
AATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCA  180
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                 139
AATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCA  198

Query                                                                 181
AAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACA  240
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                 199
AAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACA  258

Query                                                                 241
TTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCT  300
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  259  TTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCT
318

Query                                                                 301
CAGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGTCG  360
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  319  CAGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGTCG
378

Query                                                                 361
AGCTTCCATAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTA  420
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  379  AGCTTCCATAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTA
438

Query                                                                 421
AAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCA  480
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                 439
AAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCA  498

Query  481  TATC  484
       ||||
Sbjct  499  TATC  502
```

Figure (appendix 4). Sequence analysis of ITS gene.

Appendix 5. Alignment of nucleotide sequencing of isolate (F5) gene bank of NCBI, and appeared compatibility of gene under sequence ID: MG136705.1

After alignment the product amplification of ITS gene for the (F5) isolate that shown to have three transversion A>T, T>A and C>G from the Gene Bank, found that part of ITS gene having 99% compatibility with standard in Gene Bank ITS gene as it shown in Figure (appendix 5).

```
Query                                                                    3
ACCCCTGTGACATACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACGGGA  62
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20  ACCCCTGTGACATACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACGGGA
79

Query                                                                   63
CGGCCCGCCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAACAAACAA  122
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  80  CGGCCCGCCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAACAAACAA
139

Query                                                                  123
ATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAA  182
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                  140
ATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAA  199

Query                                                                  183
AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT  242
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                  200
AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT  259

Query                                                                  243
TGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTC  302
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  260  TGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTC
319

Query                                                                  303
AGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGTCGA  362
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                  320
AGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGTCGA  379

Query                                                                  363
GCTTCCATAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTAA  422
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  380  GCTTCCATAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTAA
439

Query                                                                  423
AACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCAT  482
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                  440
AACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCAT  499

Query  483  ATCATAAAGGCGGAGGAA  500
       ||||||||||||||||
Sbjct  500  ATCAATAAGCCGGAGGAA  517
```

Figure (appendix 5). Sequence analysis of ITS gene.

Appendix 6. Alignment of nucleotide sequencing of isolate (F6) gene bank of NCBI, and appeared compatibility of gene under sequence ID: MG136706.1

After alignment the product amplification of ITS gene for the (F6) isolate that shown to have one transversion T>A and one transition A>G from the Gene Bank, found that part of ITS gene having 99% compatibility with standard in Gene Bank ITS gene as it shown in Figure (appendix 6).

```
Query  1    TACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACGGGACGGCCCGCCCGA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  33   TACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACGGGACGGCCCGCCCGA  92

Query  61   GGACCCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAACAAACAAATAAATCAAAAC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93   GGACCCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAACAAACAAATAAATCAAAAC  152

Query  121  TTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  153  TTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGT  212

Query  181  AATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  213  AATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAG  272

Query  241  TATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCAGCTTGGTGTTG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  273  TATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCAGCTTGGTGTTG  332

Query  301  GGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGTCGAGCTTCCATAGCG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  333  GGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGTCGAGCTTCCATAGCG  392

Query  361  TAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTAAAACCCCAACTTC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  393  TAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACTTC  452

Query  421  TGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAGGCGG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  453  TGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGG  512

Query  481  AGGAA  485
            |||||
Sbjct  513  AGGAA  517
```

Figure (appendix 6). Sequence analysis of ITS gene.

Appendix 7. Alignment of nucleotide sequencing of isolate (F7) gene bank of NCBI, and appeared compatibility of gene under sequence ID: MG136705.1

Amplified product of ITS gene from *Fusarium* species of isolate (F7) appeared 100% compatibility for ITS gene as it shown in Figure (appendix 7).

```
Query                                                                    1
TGACATACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACGGGACGGCCCG  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                   27
TGACATACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACGGGACGGCCCG  86

Query                                                                   61
CCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAACAAACAAATAAATC  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  87  CCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAACAAACAAATAAATC
146

Query                                                                  121
AAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGA  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                  147
AAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGA  206

Query                                                                  181
TAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCC  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  207 TAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCC
266

Query                                                                  241
GCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCAGCTTGG  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  267 GCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCAGCTTGG
326

Query                                                                  301
TGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGTCGAGCTTCCA  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  327 TGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGTCGAGCTTCCA
386

Query                                                                  361
TAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTAAAACCCCA  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                  387
TAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTAAAACCCCA  446

Query                                                                  421
ACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATA  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                  447
ACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATA  506

Query  481  AG  482
              ||
Sbjct  507  AG  508
```

Figure (appendix 7). Sequence analysis of ITS gene.

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التشخيص الجزيئي بتقنية (PCR-ITS) للفطر *Fusarium oxysporum* المعزول من الطماطم في مدينة بغداد

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الملخص

تهدف هذه الدراسة لتحديد الامراضية للعزلات التي تعود لفطر *Fusarium oxysporum* والمسبب لمرض الذبول الوعائي لنبات الطماطم ودراسة التشخيص الجزيئي للفطر بتقنية PCR-ITS. حيث تم عزل ثلاثون عينة لفطر *Fusarium oxysporum* من نباتات الطماطم المصابة وتشخيصها بالاعتماد على الخصائص المظهرية. حيث تم اجراء اختبار الامراضية لثلاثين عزلة لفطر *Fusarium oxysporum* واستخلاص الحمض النووي من سبع عزلات وفقا لاختبار الامراضية باستخدام ZR Fungal/Bacterial DNA" MiniPrep™ kit واظهرت سبع عزلات لفطر *Fusarium oxysporum* ارتفاع القدرة الامراضية .حيث كان تركيز ونقاء الحمض النووي المستخرج من عزلات *F. oxysporum* السبعة (150 - 201) ng / µl و(1.4 −1.9) على التوالي .تم تضخيم عينة الحمض النووي مع كل من البادئات العالمية (ITS-1 و ITS-4) وتم تحديد وجود الجين ITS بحجم bp565. واشارت نتائج (sequencing) لعزلات *Fusarium oxysporum f. sp. Lycopersici* الى ان جميع العزلات السبعة تعود لنفس Formae specialesوهو *Fusarium oxysporum* وقد ساعدت هذه النتائج في دراسة التباين الوراثي بين الانواع المستعملة في الدراسة.