



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

Noor Saad Nahi Al-Adhami , Alaa Mohsen Al-Araji

Department of Biology, College of Sciences, Baghdad University, Baghdad, Iraq

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Corresponding Author:

Name:

Noor Saad Nahi Al-Adhami

E-mail:

Noraya.saad94@gmail.com

Tel:

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 oxysporum* [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 specialis and strains [9,10]. *F. oxysporum* that attack Tomato is *F. oxysorum* f.sp. *Lycopersici*, 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 speciales 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-ITS gene.

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.

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

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 H₂O (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]

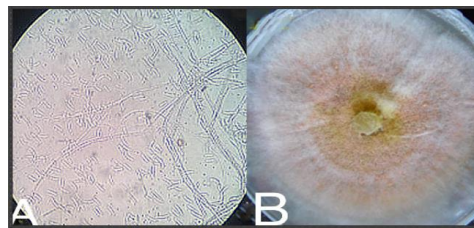


Figure 1. *Fusarium oxysporum* (A) under microscope (B) morphology on PDA medium.

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, chlamydospores, 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.

<i>F. oxysporum</i> 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).

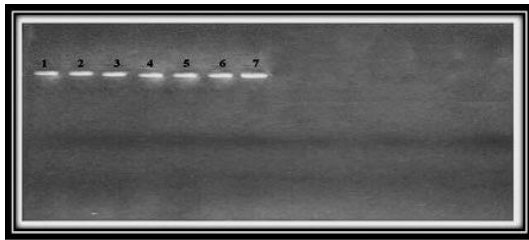


Figure 2. 1 % Agarose gel Electrophoresis of total genomic DNA for *Fusarium oxysporum* isolates, (70 Volt/30 minute).

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.

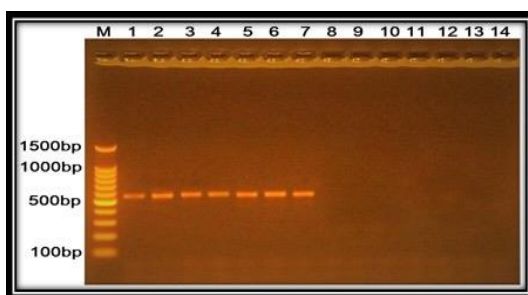


Figure 3. shown PCR product of ITS gene band size 565bp that was electrophoresis on 2% agarose gel ITS gene.

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 were 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).

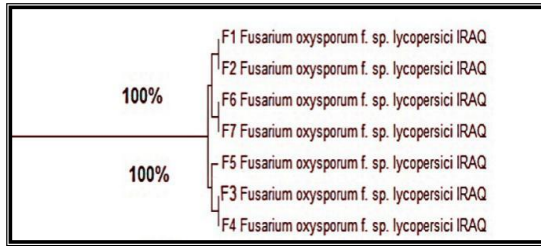


Figure 4. Dendrogram showing genetic diversity among seven of *Fusarium oxysporum* isolates

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* (*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  CCAACCCCTGTGACATACCTATAACGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACG 60
      |||
Sbjct 17 CCAACCCCTGTGACATACCTATAACGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACG 76

Query 61  GGACGGCCCGCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACCTCTGAGTAAAACAAA 120
      |||
Sbjct 77  GGACGGCCCGCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACCTCTGAGTAAAACAAA 136

Query 121 CAAATAAATCAAACCTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAG 180
      |||
Sbjct 137 CAAATAAATCAAACCTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAG 196

Query 181 CAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA 240
      |||
Sbjct 197 CAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA 256

Query 241 CATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAG 300
      |||
Sbjct 257 CATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAG 316

Query 301 CTCAGCTTGGTGTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGT 360
      |||
Sbjct 317 CTCAGCTTGGTGTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGT 376

Query 361 CGAGCTTCCATAGCGTAGTAATCATAACCTCGTACTGGTAATCGTCGCGGCCACGCCG 420
      |||
Sbjct 377 CGAGCTTCCATAGCGTAGTAATCATAACCTCGTACTGGTAATCGTCGCGGCCACGCCG 436

Query 421 TAAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAG 480
      |||
Sbjct 437 TAAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAG 496

Query 481 CATATCAAAGCCGGAGGAA 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
GGACGGCCCGCCCGAGGACCCCTAAACTCTGTTTTAGTGGAAGTTCTGAGTAAAACAAA 120
|||||
Sbjct 77 GGACGGCCCGCCCGAGGACCCCTAAACTCTGTTTTAGTGGAAGTTCTGAGTAAAACAAA
136

Query 121
CAAATAAATCAAACTTTCAACAACGGATCTCTGGTTCTGGCATCGATGAAGAACGCAG 180
|||||
Sbjct 137 CAAATAAATCAAACTTTCAACAACGGATCTCTGGTTCTGGCATCGATGAAGAACGCAG
196

Query 181
CAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA 240
|||||
Sbjct 197 CAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA
256

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

Query 301
CTCAGCTTGGTGTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGT 360
|||||
Sbjct 317 CTCAGCTTGGTGTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGT
376

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

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

Query 481 CATATCAAAAAGCCGGAGGAA 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
CCAACCCCTGTGACATACCAATTGTTGCCTCGGCCGATCAGCCCGCTCCCGGTAAAACGG 76
|||||
Sbjct 3 CCAACCCCTGTGACATACCAATTGTTGCCTCGGCCGATCAGCCCGCTCCCGGTAAAACGG
62

Query 77
AACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATATGTAACCTTCTGAGTAAAACCAT 136
|||||
Sbjct 63 AACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATATGTAACCTTCTGAGTAAAACCAT
122

Query 137
AAATAAATCAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC 196
|||||
Sbjct 123
AAATAAATCAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC 182

Query 197
AAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC 256
|||||
Sbjct 183
AAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC 242

Query 257
ATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTTCGAGCGTCATTTCAACCCTCAAGC 316
|||||
Sbjct 243 ATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTTCGAGCGTCATTTCAACCCTCAAGC
302

Query 317
CCCCGGGTTTGGTGTGGGGATCGGCGAGCCTCACGGCAAGCCGGCCCCGAAATACAGTG 376
|||||
Sbjct 303
CCCCGGGTTTGGTGTGGGGATCGGCGAGCCTCACGGCAAGCCGGCCCCGAAATACAGTG 362

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

Query 437
GGCCAAGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCG 496
|||||
Sbjct 423
GGCCAAGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCG 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 AACCCCTGTGACATACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCGTAAAACGGG
78

Query 61
ACGGCCCGCCCGAGGACCCCTAAACTCTGTTTTTAGTGGAAGTTCTGAGTAAAACAAACA 120
|||||
Sbjct 79 ACGGCCCGCCCGAGGACCCCTAAACTCTGTTTTTAGTGGAAGTTCTGAGTAAAACAAACA
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
CAGCTTGGTGTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGTCG 360
|||||
Sbjct 319 CAGCTTGGTGTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGTCG
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
CGGCCCCGCCGAGGACCCCTAAACTCTGTTTTAGTGGAACCTTCTGAGTAAAACAAACAA 122
|||||
Sbjct 80 CGGCCCCGCCGAGGACCCCTAAACTCTGTTTTAGTGGAACCTTCTGAGTAAAACAAACAA
139

Query 123
ATAAATCAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAA 182
|||||
Sbjct 140
ATAAATCAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAA 199

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

Query 243
TGCGCCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTC 302
|||||
Sbjct 260 TGCGCCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTC
319

Query 303
AGCTTGGTGTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGTCGA 362
|||||
Sbjct 320
AGCTTGGTGTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGTCGA 379

Query 363
GCTTCCATAGCGTAGTAATCATAACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTAA 422
|||||
Sbjct 380 GCTTCCATAGCGTAGTAATCATAACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTAA
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 TACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACGGGACGGCCCCGCCGA 60
      |||
Sbjct 33 TACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACGGGACGGCCCCGCCGA 92

Query 61 GGACCCCTAAACTCTGTTTTAGTGGAACTTCTGAGTAAAACAAACAAATAAATCAAAC 120
      |||
Sbjct 93 GGACCCCTAAACTCTGTTTTAGTGGAACTTCTGAGTAAAACAAACAAATAAATCAAAC 152

Query 121 TTTCAACAACGGATCTCTTGGTCTGGCATCGATGAAGAACGCAGCAAATGCGATAAGT 180
      |||
Sbjct 153 TTTCAACAACGGATCTCTTGGTCTGGCATCGATGAAGAACGCAGCAAATGCGATAAGT 212

Query 181 AATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAG 240
      |||
Sbjct 213 AATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAG 272

Query 241 TATTCTGGCGGGCATGCCTGTTTCGAGCGTCATTTCAACCCTCAAGCTCAGCTGGTGTG 300
      |||
Sbjct 273 TATTCTGGCGGGCATGCCTGTTTCGAGCGTCATTTCAACCCTCAAGCTCAGCTGGTGTG 332

Query 301 GGACTCGCGGTAACCCGCGTTCGCCAAATCGATTGGCGGTACGTCGAGCTTCCATAGCG 360
      |||
Sbjct 333 GGACTCGCGGTAACCCGCGTTCGCCAAATCGATTGGCGGTACGTCGAGCTTCCATAGCG 392

Query 361 TAGTAATCATAACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTAAAACCCCAACTTC 420
      |||
Sbjct 393 TAGTAATCATAACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTAAAACCCCAACTTC 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
CCCGAGGACCCCTAAACTCTGTTTTTAGTGGAAGTTCTGAGTAAAACAAACAAATAAATC 120
|||||
Sbjct 87 CCCGAGGACCCCTAAACTCTGTTTTTAGTGGAAGTTCTGAGTAAAACAAACAAATAAATC
146

Query 121
AAAACCTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAATGCGA 180
|||||
Sbjct 147
AAAACCTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAATGCGA 206

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

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

Query 301
TGTTGGGACTCGCGGTAACCCGCGTTCCCAAATCGATTGGCGGTCACGTCGAGCTTCCA 360
|||||
Sbjct 327 TGTTGGGACTCGCGGTAACCCGCGTTCCCAAATCGATTGGCGGTCACGTCGAGCTTCCA
386

Query 361
TAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTAACCCCA 420
|||||
Sbjct 387
TAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTAACCCCA 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

نور سعد ناهي الاعظمي ، علاء محسن الاعرجي

قسم علوم الحياة ، كلية العلوم ، جامعة بغداد ، بغداد ، العراق

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 عينة لفطر *Fusarium oxysporum* واستخلاص الحمض النووي من سبع عزلات وفقا لاختبار الامراضية باستخدام ZR Fungal/Bacterial DNA" MiniPrep™ kit واطهرت سبع عزلات لفطر *Fusarium oxysporum* ارتفاع القدرة الامراضية. حيث كان تركيز ونقاء الحمض النووي المستخرج من عزلات *F. oxysporum* السبعة $150 - 201$ ng / μ l و(1.9- 1.4) على التوالي. تم تضخيم عينة الحمض النووي مع كل من البادئات العالمية ITS-1 و ITS-4 وتم تحديد وجود الجين ITS بحجم 565 bp و اشارت نتائج (sequencing) لعزلات *Fusarium oxysporum f. sp. Lycopersici* الى ان جميع العزلات السبعة تعود لنفس *Formae speciales* وهو *Fusarium oxysporum* وقد ساعدت هذه النتائج في دراسة التباين الوراثي بين الانواع المستعملة في الدراسة.